TIGHT JUNCTIONS – THE LINK BETWEEN HIV-ASSOCIATED INTESTINAL BARRIER DYSFUNCTION AND LOSS OF IMMUNE HOMEOSTASIS

by

CHARLOTTE YUK-YAN CHUNG

Submitted in partial fulfillment of the requirements
For the degree of Doctor of Philosophy

Dissertation Advisor: Alan D. Levine, PhD

Department of Pharmacology
CASE WESTERN RESERVE UNIVERSITY

January, 2015
CASE WESTERN RESERVE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

We hereby approve the thesis/dissertation of

CHARLOTTE YUK-YAN CHUNG

candidate for the degree of Doctor of Philosophy

Committee Chair
Derek J. Taylor

Committee Member
Alan D. Levine

Committee Member
Noa Noy

Committee Member
Alex Y. Huang

Committee Member
Donald D. Anthony

Date of Defense
July 18, 2014

*We also certify that written approval has been obtained
for any proprietary material contained therein.
Table of Contents

Table of Contents 1
List of Tables 6
List of Figures 7
Acknowledgements 10
Abstract 11

CHAPTER 1: INTRODUCTION 13

1.1 Human Immunodeficiency Virus (HIV) infection: Clinical features and Pathogenesis 14
   Emergence of the AIDS epidemic 14
   Molecular Biology of HIV-1, the causative agent of HIV infection 15
   HIV-1 Replication Cycle 20
   Natural course and loss of immune homeostasis in HIV infection 24
   Pathogenesis of HIV-mediated CD4 T cell depletion 26
   The development of Antiretroviral therapy (ART) 28
   Highly active combination antiretroviral therapy 32
   HIV infection as a chronic infection in the post-ART era 32
   Systemic Inflammation plays a role in non-AIDS morbidities and mortality 34
   Causes of systemic inflammation in the ART-treated HIV+ patient 35

1.2 Intestinal mucosal barrier in HIV infection 37
   Constituents and function of the intestinal mucosal barrier 37
   Loss of gut mucosal immune homeostasis in the HIV-infected individual 41
   Intestinal structural barrier loss in the HIV-infected individual 43

1.3 Intestinal epithelial tight junctions: structure, function and regulation 46
   Tight junctions govern intestinal epithelial paracellular permeability 46
   Involvement of Tight Junction Dysregulation in HIV Infection 49
1.4 Overall objective of the thesis

CHAPTER 2: PROGRESSIVE PROXIMAL-TO-DISTAL REDUCTION IN EXPRESSION OF THE TIGHT JUNCTION COMPLEX IN COLONIC EPITHELIUM OF VIRALLY-SUPPRESSED HIV+ INDIVIDUALS

2.1 Abstract

2.2 Introduction

2.3 Materials and Methods

2.4 Results

Study population
Relative abundance of epithelial cells is not decreased in the intestine of HIV-infected individuals
No microscopic change in the structure and subcellular localization of intestinal epithelial tight junction components in HIV-infected individuals
Transcripts for colonic, but not terminal ileal, tight junction proteins are down-regulated in HIV-infected individuals
Expression of tight junction mRNA continuously decreases along the proximal-to-distal axis in the HIV+ colon
Tight junction protein expression decreases in the descending colon of HIV+ Individuals
Tight junction mRNA down-regulation in HIV infection is a result of an overall change in intestinal epithelial cell transcriptional regulation
Microbial translocation and immune activation correlated with a decrease in colonic TJ transcript expression

2.5 Discussion

CHAPTER 3: MECHANISMS FOR TIGHT JUNCTION DYSREGULATION IN THE ART-TREATED HIV-INFECTED PATIENT

3.1 Preface
3.2 Contribution of Protease Inhibitors to Progressive Colonic Epithelial TJ Downregulation
3.3 Mechanisms for Immune-mediated TJ Regulation

Mechanisms of TJ Regulation
Immune-mediated Epithelial TJ Regulation

CHAPTER 4: ACTIVATED T CELLS INDUCE A BIPHASIC PERTURBATION IN INTESTINAL EPITHELIAL PERMEABILITY VIA ELEVATED CLAUDIN 2 AND 4

4.1 Abstract
4.2 Introduction
4.3 Materials and Methods
Caco-2 monolayer Preparation and Transepithelial Resistance Measurements
Peripheral Blood Mononuclear cell Isolation and T cell Stimulation

In vitro Caco-2 cell – T cell Co-culture System
4.4 Results

Design of an in vitro intestinal epithelial cell – T cell co-culture system to recapitulate the architecture of the intestinal mucosa

Co-culture with T cells initiates a biphasic perturbation in Caco-2 monolayer permeability

PBTs must be activated and present on the basolateral surface of Caco-2 monolayer to initiate biphasic TER response

Activated T cell-induced biphasic perturbations in intestinal epithelial permeability is mediated by factors accessible only in a local environment

Activated T cells stimulate an increase in claudin-2 and -4 protein expression in Caco-2 cells

Early increase in monolayer resistance is initiated by the presence of stimulated T cells, while prolonged exposure causes barrier dysfunction

Increases in claudin-2 and claudin-4 protein level are maintained after T cell removal

4.5 Discussion

CHAPTER 5: FUTURE PERSPECTIVES

5.1 Conclusions

5.2 Significance – TJ as a mediator between HIV-associated intestinal barrier dysfunction and loss of immune homeostasis

5.3 Unresolved questions and future studies

5.4 Implications for intestinal barrier dysfunction in other intestinal inflammatory diseases
List of Tables

Table 2-1. Primers used for Real Time qPCR 110
Table 2-2. Demographics and Clinical Parameters of Study Cohorts 111
Table 2-3. Average Fluorescence Intensity for Occludin and ZO-1 staining of colonic surface epithelium sections 112
Table 2-4. Average Fluorescence Intensity for Occludin and ZO-1 staining of colonic crypt epithelium sections 113
Table 3-1. ART Regimen of ART-treated HIV+ Individuals 129
List of Figures

Figure 1-1. Structure and genome of the HIV-1 virus 52
Figure 1-2  Overview of the HIV-1 Replication Cycle 54
Figure 1-3. The healthy intestinal mucosa 55
Figure 1-4. HIV-associated damage to the intestinal mucosa 56
Figure 1-5. Tight junction structure 57
Figure 2-1. Cohort Diagram illustrating the assignment of intestinal biopsy and plasma samples to each analytical method 93
Figure 2-2. Relative abundance of epithelial cells in intestinal biopsies is not decreased in HIV+ individuals 94
Figure 2-3. Subcellular localization of tight junctional proteins, occludin and ZO-1, is unaltered in the HIV colon 96
Figure 2-4. Tight junctional transcripts are decreased in the colon, not the terminal ileum, of HIV+ individuals 98
Figure 2-5. Tight junctional transcript levels in HIV+ individuals decrease progressively from proximal-to-distal colon 99
Figure 2-6. Tight junctional protein levels are decreased in the descending colon of virally-suppressed HIV+ individuals 100
Figure 2-7. Human β defensin-3 and E-cadherin expression varies differentially from proximal-to-distal HIV+ intestine 101
Figure 2-8. Microbial translocation and systemic immune activation marker levels inversely correlate with colonic TJ transcript levels 103
Figure 2-S1. Tight junctional transcripts are decreased in the colon, not the terminal ileum, of male HIV+ individuals 104
Figure 2-S2. Tight junctional transcripts are decreased in the colon, not the terminal ileum, of virally-suppressed HIV+ individuals 105
Figure 2-S3. Tight junctional protein levels are decreased in the descending colon of HIV+ individuals 106
Figure 2-S4. Tight junctional protein levels are decreased in the descending colon of male HIV+ individuals 107
Figure 2-S5. Human β defensin-3 and E-cadherin expression varies differentially in the colon of HIV+ males  

Figure 2-S6. Human β defensin-3 and E-cadherin expression varies differentially in the colon of virally-suppressed HIV+ individuals  

Figure 3-1. Expression of tight junctional transcripts from proximal-to-distal gut of individual ART-treated HIV+ patient  

Figure 3-2. No significant differences in descending colonic tight junctional transcript levels were observed between treated HIV+ individuals on PI-based and non-PI based ART regimens  

Figure 3-3. Mechanisms of TJ regulation  

Figure 4-1. Co-culture with activated T cells initiates a biphasic perturbation in Caco-2 monolayer permeability  

Figure 4-2. Activated PBTs on the basolateral side of Caco-2 monolayers initiate biphasic permeability alterations  

Figure 4-3. Activated PBTs induce biphasic epithelial permeability changes via factors accessible only in the local environment  

Figure 4-4. Activated T cells induce claudin-4 protein in Caco-2 monolayer  

Figure 4-5. Early phase TER increase is maintained after removal of activated PBTs, while prolonged exposure to activated PBTs causes barrier dysfunction  

Figure 4-6. Increases in claudin-2 and claudin-4 protein level are maintained after T cell removal  

Figure 4-S1. Schematics of the in vitro Caco-2 – T cell co-culture system  

Figure 4-S2. Caco-2 monolayers differentiate into stable, intact monolayers within 14 days of culture  

Figure 4-S3. Caco-2 – T cell co-culture in Configuration A models the architecture of the intestinal mucosa
Figure 4-S4. Decreased TER and increased inulin flux in the late phase of the T cell-induced response are not due to Caco-2 cell death

Figure 4-S5. Transcript levels of tight junctional proteins are not altered at the peak and trough of biphasic monolayer permeability response

Movie 4-1. Caco-2 – T cell co-culture in Configuration A models the architecture of the intestinal mucosa

Figure 5-1. Intestinal epithelial barrier breakdown in the HIV-infected individual

Figure 5-2. Outcomes of activated T cells-induced intestinal epithelial barrier regulation, dependent on duration of T cell contact, play a role in intestinal diseases
ACKNOWLEDGEMENTS

My heartfelt gratitude goes to my thesis advisor, Dr. Levine, for inspiring me to mature as an independent scientist, to always approach questions with an inquisitive mind, and to persevere in face of challenges. I would also like to acknowledge my committee members, whose invaluable feedback and guidance were instrumental towards the development of my thesis. Special thanks goes to Dr. Eric Arts, for his valuable perspectives and continual enthusiastic support.

A huge thank you to my colleagues in the Levine Lab: Tejpal Gill, Wendy Goodman, Jeffrey Meisch, Samantha Stubblefield, Lopa Das, Stephanie Alden, Shannon Ohlemacher, Maria Torres-Castillo, Nicholas Battaglia, Ryan Vogel, and Alex Aylyarov, for their support, encouragement and input.

To my family – thank you for your unfailing love, support and affirmation towards my decision to pursue a combined MD-PhD degree. To my mentors and teachers throughout the years – you have all played a part in shaping me into who I am today. To my dear friend, brother, and mentor, Ferdinand, who inspires me to grow both professionally and personally – thank you, from the bottom of my heart.

To the ever elusive ‘Erik’, thank you for being there through the ups and downs, for believing in me, and for motivating me to always better myself. To each and every one of my friends here, thank you for letting me play a part in your lives, because it is you who have made Cleveland feel like home to me. Last but not least, I give thanks to God. All things are possible only through Him.
TIGHT JUNCTIONS – THE LINK BETWEEN HIV-ASSOCIATED INTESTINAL BARRIER DYSFUNCTION AND LOSS OF IMMUNE HOMEOSTASIS

Abstract

by

CHARLOTTE YUK-YAN CHUNG

Systemic inflammation in the HIV-infected patient plays a crucial role in the pathogenesis of CD4+ T cell depletion, and is recently postulated to also drive the development of non-AIDS related morbidities, including cardiovascular, liver, and kidney diseases, in patients on suppressive antiretroviral therapy (ART) who are largely healthy otherwise. Systemic inflammation results from elevated circulating levels of microbial products, which, in untreated HIV infection and other disease processes, was shown to originate from the gut, secondary to increased intestinal permeability. Indeed, HIV drastically disrupts the intestinal mucosal immune and structural barrier. Recent efforts have characterized the mechanisms causing intestinal epithelial dysfunction in untreated HIV infection. Persistently increased intestinal permeability in ART-treated HIV-infected patients points to a similar origin for circulating microbial products in this population; however, a mechanism for the intestinal epithelial dysfunction involved is lacking.

We analyzed intestinal biopsy tissues for changes in the epithelium on the cellular and molecular levels, and demonstrated the first direct molecular evidence of
intestinal epithelial dysregulation in the suppressive ART-treated HIV-infected population. The colonic epithelium was grossly intact, but progressive transcriptional downregulation of TJ components was observed along the proximal-to-distal colon. Our results highlight the importance of TJ dysregulation in HIV-associated intestinal epithelial barrier breakdown. How HIV-mediated intestinal mucosal dysfunction contributes to epithelial TJ dysregulation is currently unknown. To investigate the modulatory effects of immune cells on intestinal epithelial permeability, I developed an in vitro intestinal epithelium – immune cell co-culture system. Caco-2 cell, a human colorectal adenocarcinoma cell line, monolayer showed biphasic permeability perturbations, evaluated using transepithelial resistance and paracellular inulin flux, when co-cultured with activated T cells on the basolateral side of the monolayer. Early strengthening of the barrier was accompanied by a transient increase in claudin-2 and a sustained increase in claudin-4 protein levels. Prolonged T cell contact resulted in weakening of the epithelial barrier. Our results reveal that activated T cells both protect and disrupt the intestinal epithelium via modulating intestinal TJ protein levels. We propose that HIV-associated loss of intestinal mucosal immune homeostasis impacts activated T cell-induced intestinal epithelial TJ regulation, thereby promoting intestinal barrier dysfunction.
CHAPTER 1

INTRODUCTION
1.1 Human Immunodeficiency Virus (HIV) Infection: Clinical Features and Pathogenesis

Emergence of the AIDS epidemic
In June 1981, the United States Center for Disease Control (CDC) first reported the unprecedented occurrence of concurrent Pneumocystis pneumonia, cytomegalovirus (CMV) infection, and candida mucosal infection in five previously healthy homosexual men (1). Rapidly escalating number of reports of this new deadly syndrome of acquired immunodeficiency surfaced over the next year, describing manifestations of multiple rare life-threatening opportunistic infections and cancers in association with a “prodromal syndrome” of generalized lymphadenopathy and chronic fatigue. The syndrome was recognized in intravenous drug users, Haitian immigrants, and individuals with a history of hemophilia, in addition to homosexual males (2). This epidemic quickly gained national and international attention, and in September 1982, the CDC started referring to it as ‘acquired immune deficiency syndrome’ or ‘AIDS’.

In 1983, within two years since the emergence of AIDS, the human immunodeficiency virus-1 (HIV-1) was isolated and identified as the etiological agent of AIDS (3, 4) – an accomplishment recognized by the 2008 Nobel Prize for Medicine. By 1985, an ELISA-based screening test for anti-HIV antibodies was developed (5); and in 1987, the first antiretroviral agent, zidovudine, was approved by the FDA. These remarkable early advances in understanding the basic
epidemiology, clinical features, and viral pathogenesis of HIV, as well as the rapid public health responses in surveillance and prevention, are testimonies to the concerted international efforts of clinicians, basic scientists, and government authorities against the defining public-health crisis of modern times.

Molecular Biology of HIV-1, the causative agent of HIV infection

The causative agents of AIDS, HIV-1 and HIV-2, are lentiviruses closely related to, and believed to have originated from, their counterparts in primates known as simian immunodeficiency viruses (SIV), via multiple cross-species transmission from chimpanzees and gorillas (HIV-1), and sooty mangabey monkeys (HIV-2) in Africa in the early 20th century (6, 7). The HIV-1 virus causes the majority of HIV infection worldwide, while HIV-2, with lower transmissibility, is primarily confined to West Africa. Slower progression to AIDS is observed with following HIV-2 infection, with long-term non-progression being a more frequently observed phenotype than in HIV-1 infection.

HIV-1 is a spherical virion with a diameter of about 100 nm, coated by the 4nm thick viral envelope, a lipid bilayer that contains both virally encoded envelope glycoprotein (Env) appearing as envelope spikes, as well as cell derived membrane proteins (8). The inner surface of the membrane is lined with matrix protein (MA). The cone-shaped viral core, enclosed by a shell of capsid (CA) protein arranged in a hexameric-ring lattice structure, contains two copies of the viral RNA genome associated with nucleocapsid (NC) proteins, viral enzymes
reverse transcriptase (RT), integrase (IN), and protease (PR), and viral accessory proteins (Nef, vpr, vif) (Figure 1-1A).

The HIV provirus genome, around 9.8 kilobases in length, is flanked by long terminal repeats (LTRs) on both ends (Figure 1-1B). The promoter that initiates transcription of the proviral genome is located in the 5’ LTR. LTRs contain three subregions: i) the U3 (unique 3’ end) region contains binding sites for cis-acting DNA elements, i.e. cellular transcription factors such as NF-kappa B, which induce HIV transcription by cellular RNA polymerase II following activation of the T cell receptor; ii) the R (repeated) region demarcates the boundaries of the resultant transcript; iii) the U5 (unique 5’ end) region contains the Tat binding site. As such, transcription of the HIV-1 provirus is initiated by inducible and constitutive cellular transcription factors, and further regulated by the viral transcription transactivator Tat. The primary HIV-1 transcript has multiple splice sites, allowing for the production of i) the fully spliced mRNA that translates into early HIV proteins; as well as ii) partially spliced mRNA and iii) unspliced mRNA, both translating into late viral proteins required for virion production and assembly. The unspliced mRNA is also packaged into virions as genomic RNA. The HIV provirus encodes for fifteen mature viral proteins consisting of structural, enzymatic, regulatory, and accessory proteins (9-11).

1) Structural HIV Proteins
The structural proteins are contained within the three polyproteins Gag, Pol, and Env. The gag gene encodes for the p55 Gag precursor protein that triggers viral particle budding from the surface of an infected cell. After budding, the virally encoded PR cleaves p55 into four proteins in order from the N- to C-terminus: MA, CA, NC, and p6. MA lines the inner surface of the virus envelope membrane, and is also part of the complex escorting viral DNA to the nucleus, allowing HIV-1 to infect non-dividing cells. CA encases the core of the virus particle, and interacts with cellular component cyclophilin A to facilitate viral uncoating (12). NC coats the genomic RNA inside the core, mediating the incorporation of the RNA into assembling virions via recognition of specific packaging signals, while also aiding in reverse transcription. P6 aids the incorporation of vpr into the assembling virion, and is required also for the efficient release of budding virions.

The env gene encodes for the HIV envelope glycoprotein, which is initially synthesized as a gp160 precursor. Cellular protease cleaves gp160 into the outer subunit gp120 and transmembrane subunit gp41, both which reside on the viral envelope membrane and are crucially responsible for viral entry and fusion to target cells.

2) Enzymatic HIV Proteins

The pol polypeptide is synthesized as part of the Gag-pol fusion protein, which is cleaved away from the gag polypeptide by PR during viral maturation and then further cleaved into the enzymes PR, RT, and IN that reside within the viral capsid.
PR functions as a dimer to cleave polyproteins into individual mature proteins during the viral maturation process. RT is a RNA- and DNA-dependent DNA polymerase that synthesizes a complimentary double stranded (ds) DNA from the single stranded (ss) viral RNA genome. RNase H, considered part of RT, digests the original RNA template to allow for synthesis of the complimentary DNA strand. IN mediates the integration of the double-stranded HIV proviral DNA into the host cell genome.

3) Regulatory HIV Proteins

The HIV genome also encodes for regulatory proteins tat and rev. Tat is a transcription transactivator that binds to the transactivation response element (TAR) region, at the 5’ end of the HIV-1 RNA transcript. Tat binding drastically increases the efficiency of the elongation phase during transcription, allowing for the generation of full-length transcripts instead of primarily short transcripts, and is thus critical for HIV-1 replication. Rev is an RNA binding protein produced by the fully spliced HIV transcript, which binds to the Rev response element (RRE) located in the env coding region (13). Rev binding induces the switching from early to late phase of HIV gene expression (49) by facilitating the nuclear export of unspliced or partially spliced HIV-1 transcripts. As a result, structural and accessory proteins required for the assembly of infectious virions can be translated.

4) Accessory HIV Proteins
Accessory HIV-1 proteins Nef, vif, vpr, and vpu are not necessary for virus replication, but instead perform multiple functions that underlie their importance as virulence factors that promote virus spread and disease induction.

1) Nef downregulates CD4 and MHC Class I molecules from the cell surface via ubiquitination and proteosomal degradation, resulting in the promotion of virion budding and the reduction of cytotoxic T cell-mediated killing of HIV infected cells. Nef also inhibit apoptosis of the host infected cell (14), thereby promoting virion replication.

2) Vif is a polypeptide that is packaged into HIV virions, promoting viral infectivity by enabling efficient synthesis of the proviral DNA (15). Vif stabilizes the reverse transcription complex by binding to and causing the degradation of host restriction factor APOBEC3G (16).

3) The vpr protein mediates the nuclear localization of the preintegration complex following virus uncoating (17), thus allowing HIV-1 to infect non-dividing cells.

4) Vpu is a membrane protein located on the internal side of the host cell membrane. It promotes the degradation of CD4 via the host ubiquitin/proteasome pathway (18), thus freeing the viral env proteins from forming complexes with CD4 within the cellular endoplasmic reticulum and promoting their transport to the cell surface for virion assembly. Vpu also enhances virion release from the host cell surface.
HIV-1 Replication Cycle

HIV-1, belonging to the retrovirodae family, possesses a ss RNA genome. Following infection of the target cells, CD4+ T cells and macrophages, and reverse transcription, it integrates its newly-synthesized ds DNA genome into the host cell genome and hijacks the host cell transcription mechanism, transforming the cell into a virus producer (19). The HIV-1 replication cycle can be viewed as a 13-step process, as depicted in Figure 1-2, and are detailed as below.

1) Viral entry: Attachment and fusion
Gp120 and gp41, subunits of the HIV envelope protein, non-covalently interact and aggregate into trimers of heterodimers on the virus surface. These HIV envelope spikes are the key mediators for HIV entry into target cells. Virions bind to host cell surface via interactions between Env to host cell attachment factors including heparin sulfate, DC-SIGN on dendritic cells (20) and LFA-1 on lymphocytes. Fusion is triggered by the binding of gp120 to CD4 receptors on target cell surface. This causes a conformation change in gp120 that enables further interactions with the co-receptor, either CCR5 or CXCR4 (Figure 1-2, Step 1). Co-receptor binding triggers exposure of gp41, which inserts its fusion peptide into the target cell membrane, thus destabilizing the cell membrane. Refolding of gp41 into a 6-helix bundle brings the viral membrane and target cell membrane into close proximity, initiating membrane fusion and pore formation (21, 22) (Figure 1-2, Step 2). Currently, the intricate details of how the pore forms and
expands are still unknown, but the end result is the delivery of the viral core through the pore into the host cell cytoplasm.

2) Post-entry Events: Uncoating, Reverse Transcription, Nuclear Import, and Integration

The viral core delivered into the cytoplasm undergoes an uncoating process (Figure 1-2, Step 3), during which the virus partially sheds its CA protein lattice to release the reverse transcription complex – viral genomic RNA, lysine t-RNA which functions as the primer, replication enzymes RT and IN, MA and NC proteins, vpr, and other host cell proteins. Premature uncoating induced by the host HIV restriction factor TRIM5α prevents subsequent reverse transcription, highlighting the importance of optimal timing for uncoating.

Subsequent reverse transcription results in the synthesis of complementary ds DNA from the ss viral RNA genome. This process is catalyzed by the HIV-1 RT, which is a heterodimer composed of the p66 and p51 subunits. The RNA- and DNA-dependent DNA polymerase functions to extend the DNA strand, while the RNase H digests the RNA template, allowing the formation of the second DNA strand. The cellular restriction factor APOBEC3G inhibits reverse transcription by mutating cytidines on the viral DNA to uracils (23), while HIV-1 produces the accessory protein Vif, which binds to and degrades APOBEC3G as a countermeasure.
The newly synthesized ds proviral DNA associates with IN into a nucleoprotein complex called the preintegration complex, which moves into the nucleus. Integrase performs two distinct steps: 1) 3'-processing of LTR ends of the ds DNA, performed in the cytoplasm, and 2) DNA strand transfer, in which the host genome integration site is cleaved and the proviral DNA is inserted. Single-strand gaps are repaired with the help of cellular repair enzymes, resulting in the stable integration of the HIV provirus into the genomic DNA of the infected cell (Figure 1-2, Step 6), which, from this point onwards, will behave essentially like a cellular gene.

3) Viral Gene Transcription and Nuclear Export

Whether integration leads to latent or transcriptionally active forms of infection depends on the chromosomal environment and the availability of cellular activators. Viral gene transcription from the integrated provirus is initiated from the U3 promoter in the 5' LTR, following binding of cellular transcription factors. Transcription is performed by the cellular RNA polymerase II, which initially synthesizes short nonpolyadenylated transcripts. Tat, the viral transactivator protein, binds to the TAR element on these short transcripts, and the resultant Tat-TAR complex recruits the cellular protein positive transcription elongation factor b (p-TEFb) complex to the HIV LTR. Cdk9 within the p-TEFb phosphorylates the C-terminus domain of RNA polymerase II to stimulate transcription elongation, such that full-length transcripts can be generated (24) (Figure 1-2, Step 7).
HIV transcripts are cotranscriptionally spliced before transport to the cytoplasm. Rev, a viral regulatory protein, promotes nuclear export of incompletely spliced or unspliced viral transcripts by simultaneously binding to the RRE element in the these transcripts to form and Rev-RNA multimer, and the host nuclear export factor CRM1/exportin (via a leucine-rich nuclear export sequence) (25) (Figure 1-2, Step 8). Translation of partially spliced transcripts produces the structural, enzymatic, and accessory proteins needed to assemble infectious virions, while the unspliced transcripts are packaged as genomic RNA of progeny virions.

4) Viral assembly, virion budding and release

New virus particles contain Gag and Gag-pol polyproteins, two copies of the viral RNA genome, Vif, Nef, and Vpr. These components are assembled at the plasma membrane (Figure 1-2, Step 10). Myristylation of the MA portion of Gag polyproteins allow Gag to associate with the plasma membrane, after which the late (L) domains on the p6 portion of Gag interact with cellular class E vacuolar protein sorting (VPS) proteins that promote viral budding and severing of the viral particle from the plasma membrane (26). This process hijacks the physiological pathway of multivesicular bodies formation from late endosomes, mediated by endosomal sorting complex required for transport (ESCRT) complexes (Figure 1-2, Step 11-12). The host cell membrane protein tetherin acts as a restriction factor by binding to and inhibiting budding of virions, an action counteracted by the direct binding of viral accessory protein Vpu to tetherin.
5) Virion maturation

Immature virion particles are converted to infectious virions during or shortly after release (Figure 1-2, Step 13). Proteolysis of Gag and Gag-Pol polyproteins by PR yields the mature structural proteins MA, CA, NC, and functional enzymes PR, RT, and IN. This process results in structural rearrangement that forms the viral core shell. The resultant mature virion proceeds to infect another target cell, repeating the HIV-1 life cycle.

Natural course and loss of immune homeostasis in HIV infection

HIV is transmitted sexually and parenterally. Within the first few months of acquisition, primary HIV infection, if symptomatic, presents with an acute retroviral syndrome with findings of fever, generalized malaise, lymphadenopathy, rash, sore throat, and diarrhea. After resolution of these symptoms, the patient enters a clinically asymptomatic latent period that can persist for years, until AIDS-defining symptoms of immunodeficiency (such as Pneumocystis pneumonia and Kaposi’s sarcoma) emerge, at which point the patient is mortally susceptible to opportunistic infections. Effective antiretroviral therapy (ART) prevents progression to AIDS.

Even at the infancy of AIDS discovery, it was already noted that patients suffer from a characteristic “profound disorder of immunoregulation” (2), with the hallmark being a gradual but progressive depletion of CD4+ T cells, in terms of both quantity and quality. Clinically, peripheral blood (PB) CD4+ T cell count is
used as a measure to define disease progression to AIDS (severe immunosuppression is defined as < 200/mm$^3$), as well as to guide the timing of ART initiation (27). Of note, a small percentage of ART-naïve infected patients, known as long-term non-progressors, can maintain clinical stability and CD4+ T cell count for many years, although most ultimately progress to AIDS. An even smaller subset of treatment-naïve infected patients naturally control their infection with no evidence of viremia, and are known as elite controllers.

During primary HIV infection, active viral replication in lymphoid tissues results in a peak in viremia as indicated by high plasma concentrations HIV RNA, mirroring a drop in PB CD4+ T cell count that eventually re-stabilizes at a mildly depressed level during the clinically latent period. Destruction of naïve and memory CD4+ T cells results in a gradual drop PB CD4+ T cell count, paralleling a rising plasma viremia, towards the development of AIDS. In striking contrast, massive infection of activated and memory CD4+ T cells in the gut-associated lymphoid tissue (GALT) during primary HIV infection results in a pronounced and perhaps permanent depletion of gut lamina propria CD4+ T cell population, despite years of ART (28-30). Concurrent with the immunodeficiency, the systemic immune system shows aberrant activation. This paradoxical situation, initiated soon after HIV acquisition and persisting into chronic and ART-treated HIV infection, manifests as increased frequencies of activated B and T cells (31, 32), elevated levels of circulating proinflammatory cytokines and chemokines (33, 34), and increased immune cell turnover (35).
Pathogenesis of HIV-mediated CD4 T cell depletion

HIV gains entry and infects CD4+ helper T cells and macrophages, via binding of its envelope proteins, gp120 and gp41, to the CD4 receptor and a chemokine receptor, either the β-chemokine receptor CCR5 or α-chemokine receptor CXCR4, on target cells. Based on the co-receptor usage for entry, HIV-1 viruses can be classified into three groups: i) Macrophage-tropic (M-tropic), or R5 strains utilizing CCR5 as the coreceptor, ii) T-cell line tropic (T-tropic), or X4 strains utilizing CXCR4 as the coreceptor, and iii) dual-tropic, or R5X4 strains (10). Coreceptor usage can significantly affect the course of disease progression. Around 20% of Caucasians express the CCRdelta32 mutation, which lead to non-functional CCR5 receptors. Individuals heterozygous for this mutation demonstrate slower progression to AIDS, and this mutation was shown to be more common among long-term non-progressors (36). The error prone reverse transcription process can cause a gradual shift in the prevalent tropism of the HIV quasispecies in an HIV-infected individual over time. R5 viruses infect monocytes, macrophages, and T cells, and are present throughout all clinical stages of the HIV infection. X4 viruses infect T cells but not monocyte and macrophages, and emerge in the later stages of an HIV infection, in around 40% of patients. The emergence of X4 viruses correlates with rapid CD4+ T count decline and progression to AIDS (37). Given the dominance of the R5 virus in the initial stages of the disease and its persistence throughout the duration of the infection, the focus of this thesis is on the pathogenesis involving R5 HIV-1 strains.
Activated as well as effector memory CD4+ T cells are the major target cells of HIV, as a result of their high CCR5 expression. Infected CD4+ T cells home to lymphoid tissues, resulting in wide dissemination of the virus and establishment of a chronic and persistent infection in such tissues (38). While the field has unraveled much of the intricacies on the molecular virology end, the mechanism through which HIV induces CD4+ T cell loss remains under debate.

The observation that ART, by effectively inhibiting viral replication, attenuates disease progression to AIDS establishes that virus replication plays an important role in pathogenicity. The implication is that HIV mediates destruction of activated CD4+ T cells, while the immune system fails to compensate via homeostatic proliferation. However, several observations have cast doubts on the importance of cytopathic effects of the virus as the sole cause of CD4+ T cell depletion: i) the low degree of productive infection in PB CD4+ T cell (0.01-1%) (39) cannot account for the massive degree of cell death; ii) initiation of ART does not cause immediate changes in CD4+ T cell death rates (40), suggesting that the virus is not directly responsible for T cell death; iii) natural primate hosts of SIV do not show disease progression even in the presence of high viral loads; and iv) elevated turnover rates for uninfected CD8+ T cells (41) suggest that a reason other than homeostatic proliferation is simultaneously causing CD4+ and CD8+ T cell depletion.
Instead, chronic systemic inflammation is proposed to drive the HIV-mediated immune dysfunction and disease progression, via i) increasing the number of HIV-susceptible CD4 target cells and ii) uninfected T cell exhaustion through the physiological response of activation-induced cell death. Notably, chronic systemic inflammation has been shown to be a better prediction of disease progression than plasma viral load (32, 42, 43). It is also a critical factor distinguishing pathogenic from nonpathogenic SIV infection in nonhuman primates – in nonpathogenic SIV infections, systemic immune activation is rapidly attenuated in the chronic phase (44). In elite controllers, T cell activation level was shown to be inversely associated with CD4+ T cell counts (45), strongly indicating that, independent of HIV replication, immune activation is an important contributor to CD4+ T cell depletion. These evidences highlight the importance of addressing the excessive systemic inflammation when considering strategies to treat an HIV infection and potentially eradicate the virus.

The development of Antiretroviral therapy (ART)

In the early days of the HIV epidemic, the only therapies available attempted to control the opportunistic infections, but such strategies were minimally effective, and HIV remained a fatal disease that, by 1993, became the number one killer of young adults in the US. As the field made strides in understanding HIV viral factors and host immune responses, antiretroviral (ARV) agents were rapidly developed to target specific replicative steps of the HIV life cycle: viral binding (entry inhibitors) and fusion (fusion inhibitors), RNA replication
(nucleoside/nucleotide and non-nucleoside reverse transcriptase inhibitors), integration (IN inhibitors), and maturation (PR inhibitors).

The process of HIV entry and fusion is currently targeted by two FDA-approved ARVs. Maraviroc is a CCR5 antagonist that, by binding to the transmembrane domain of CCR5, allosterically prevents gp120 binding, while the fusion inhibitor enfuvirtide, a peptide designed based on the gp41 C-terminal sequence, disrupts formation of the gp41 six-helix bundle that is required for membrane fusion. Several other compounds are currently being developed to target the HIV entry process via three major directions (21): 1) blocking CD4 binding with inhibitors targeting the gp120 CD4 binding site and compounds that downregulate CD4, 2) inhibiting coreceptor binding with CCR5 blocking antibodies and CXCR4 inhibitors, and 3) fusion inhibitors that are orally bioavailable, with D-peptides against gp41 being an attractive possibility (46).

RT was the first viral protein targeted by antiretroviral therapeutics, with the development of zidovudine (AZT) in 1987. Currently, eight nucleoside/nucleotide analogs (NRTIs) and five non-nucleoside inhibitors (NNRTIs) are approved. NRTIs mimic natural dNTPs and are catalytically incorporated by RT into the growing DNA strand, but prevent the addition of the next dNTP. Resistance occurs via exclusion or excision mechanisms. NNRTIs inhibit RT by allosterically inducing the formation of and subsequently binding to a hydrophobic binding pocket. Resistance to NNRTIs, which weakens drug binding, arises quicker than
that to NRTIs, since the catalytic activity of RT is not dependent on a highly-conserved NNRTI-binding pocket (47).

IN is the viral enzyme responsible for the integration of ds DNA into the host cell genome. Functional IN exists as a tetramer bound to the viral ds DNA in a complex called the intasome, with two divalent metal ions playing important roles in the strand transfer process (48). IN inhibitors bind to the intasome, preferentially inhibiting the DNA strand transfer activity by chelating the metal ions (49) and displacing the 3’-end of the ds DNA from the active site, thus they are called IN strand transfer inhibitors (INSTIs). There are currently three FDA-approved INSTIs: Raltegravir (RAL), Elvitegravir (EVG), and Dalutegravir (DTG). A new class of IN inhibitors, called LEDGINs, are currently under development. LEDGINs are designed to bind to the IN binding pocket for LEDGF/p75, a host-derived IN cofactor that tethers IN to a host chromosome and stimulates IN activity. LEDGINs would act both as allosteric inhibitors of the IN catalytic activity, and competitive inhibitors for the LEDGF/p75-IN interaction (50).

PR is critically responsible for cleavage of the nine peptide sequences within Gag and Gag-pol polyproteins to generate mature infectious virions following their release. PR functions as a homodimer that recognizes the asymmetric shape of the peptide substrate instead of a particular amino acid sequence (51). Currently 9 FDA-approved PR inhibitors (PIs) on the market; all of them are competitive inhibitors binding to the PR active site (52). PI usage is linked to the development
of metabolic complications, including insulin resistance (53) and dyslipidemia (54), and frequent side effects such as gastrointestinal symptoms, which limit their usage (55). Rapid metabolism of PI by cytochrome P450 (CYP) 3A in the intestines and liver has led to problems of low systemic exposure and thus resistance development. It was recognized that ritonavir, a PI itself, is an inhibitor or CYP3A, thus leading to the development of ritonavir-boosted PI-based ART regimens. More recently, Cobicistat, a mechanism-based CYP3A inhibitor, was approved for use as a pharmacokinetic enhancer of PIs atazanavir and darunavir in Europe, following its use in enhancing the effectiveness of IN inhibitor elvitegravir as part of a fixed-dose tablet in combination with NRTIs emtricitabine and tenofovir (56).

Several new strategies for ARV development have focused on mimicking or enhancing the functions of cellular restriction factors. Small molecule inhibitors, such as PF-3450074, binding to viral CA protein stimulate premature viral core uncoating, replicating the function of restriction factor TRIM5α. Another cellular restriction factor, APOBEC3G, which inhibits reverse transcription, was discovered to be degraded by HIV accessory protein Vif, sparking interest in the development of Vif-mediated APOBEC3G degradation inhibitors. Other novel anti-HIV strategies involve targeting the transcription elongation process induced by Tat-mediated recruitment of the p-TEFb complex, as well as the virion budding process involving host ESCRT complexes (26).
Highly Active Combination Antiretroviral Therapy

The discovery of the first NRTIs in the late 1980s created much excitement, but it was realized early on that monotherapy directly targeting HIV was brief and minimally effective, due to the rapid emergence of drug resistance. The early availability of protease inhibitors in 1995 allowed for the development of dual class triple combination therapy. Landmark studies in 1996 first reported the efficacy of such triple-therapy combination regimen, now known as highly active antiretroviral therapy (HAART) or simply ART, in reducing mortality and deterring development of drug resistance (57, 58). Combination ART of three to four ARVs is now the mainstay of antiretroviral treatment. Currently, around 40 FDA-approved single or combination ARV drugs are on the market, with the most recent recommended combination regimen consisting of two nucleoside reverse transcriptase inhibitors plus one non-nucleoside reverse transcriptase inhibitor, protease inhibitor, or integrase inhibitor (59). Another widely used regimen consist of combinations of an IN inhibitor or entry inhibitor with RT inhibitors and PR inhibitors. Factors affecting the selection of the right combinations depend on availability of drugs and socioeconomic considerations, ease of administration and adherence potential, compatibilities between the various agents, potential adverse drug effects, comorbidities, and drug resistance profile of the individual patient (47).

HIV infection as a chronic infection in the post-ART era

The development of combination ART is a notable success – while this strategy cannot eradicate the virus and is thus not curative, its effectiveness in improving
health, prolonging life, and reducing the risk of HIV transmission has transformed the infection from an almost universally fatal disease to a treatable chronic illness. At the end of 2012, 9.7 million HIV-infected individuals worldwide are receiving ART (60), with the majority of such patients showing suppressed viral load (to undetectable levels) and recovery of PB T cell counts to levels considered normal in HIV-negative individuals. Thus, it is certainly fitting to ask the question posed by an appropriately titled article in the Nov 2013 issue of The Lancet: “Are antiretrovirals enough for people living with HIV?” (61)

Indeed, advances in new drugs, drug classes, treatment strategies, and the increased usage of ART have continually improved the long-term success of ART. With low rates of virological rebound in ART-adherent HIV+ individuals and the slow development of extensive virological failure of multiple drug classes (62, 63), it is conceivable for patients to achieve long-term virological suppression i.e. treatment success that is likely to be maintained throughout their lifetime (64, 65). Yet, while HIV can now be considered a potentially well-controlled chronic disease, ART does not restore full health, with recent studies revealing that ART-treated HIV-infected individuals have a shortened life expectancy of around 10 years and increased mortality rates compared to uninfected individuals, even if we only consider only patients within the last decade, in countries where ARVs are widely accessible (66, 67). Multiple studies have demonstrated that patients on suppressive ART have an excessive risk of non-AIDS events (68, 69) including cardiovascular (70), liver and kidney diseases (33, 71), non-AIDS associated
cancers, and neurocognitive decline (72). Many of these symptoms are associated with aging, thus raising the possibility that HIV causes premature aging (73).

**Systemic Inflammation plays a role in non-AIDS morbidities and mortality**

These observations can be partly attributed to excessive traditional risk factors such as smoking and substance use (74), as well as known side effects of long-term ART, such as metabolic complications including hyperglycemia, insulin resistance, dyslipidemia, which would contribute to the risk for cardiovascular disease (75-77). However, a seminal paper on the SMART study group demonstrated higher rates of non-AIDS related mortality and cardiovascular events in HIV+ patients on intermittent ART compared to those on continuous ART, suggesting that the virus contributed more towards non-AIDS related mortality and morbidities than ART (78). Thus there is another etiological factor other than ART and contributed to by the infection process, which leads to the development on non-AIDS morbidities.

Chronic immune activation is not corrected by ART, with HIV+ patients showing elevated levels of inflammatory markers including IL-6, D-dimers, and C-reactive protein despite years of suppressive ART (79). Importantly, levels of inflammatory markers strongly associated with increased mortality in infected patients with optimal T cell recovery (80), suggesting that increased mortality is associated with the residual systemic inflammation in the ART-treated patient. Accumulating
evidence demonstrate that non-AIDS comorbidities are consequences of the chronic inflammation (71, 72, 81-84), further confirming the pivotal role of systemic inflammation in premature aging of ART-treated HIV patients.

Causes of systemic inflammation in the ART-treated HIV+ patient

In recent years, much effort has been focused on delineating the potential causes for persistent systemic inflammation under the setting of effective ART-mediated viral suppression. Low levels of viremia persist in some patients who are on suppressive ART (85, 86), suggestive of residual ongoing HIV replication, which, in some patients, can be suppressed by intensification of therapy with an additional antiretroviral drug Raltegravir (87-89). Reduced T cell activation and levels of D-dimer was observed in tandem. It thus appears that residual HIV replication contributes to the persistent inflammation in some patients. Treatment of chronic viral infections including cytomegalovirus and hepatitis C virus was shown also to reduce immune activation in ART-treated HIV+ patients (90, 91), indicating that excessive burden of co-infections partly contributes to the residual systemic inflammation.

In 2006, it was demonstrated for the first time that circulating levels of microbial products lipopolysaccharide (LPS), a component of the outer cell membrane of Gram-negative bacteria, are elevated in chronically HIV-infected individuals as compared to uninfected individuals (92). The same was observed in rhesus macaques with pathogenic SIV infection. In contrast, LPS levels remained
unchanged in SIV-infected sooty mangabeys, which are natural hosts of SIV that harbor nonpathogenic infections despite high levels of viremia. Importantly, plasma LPS level was shown to correlate with the frequency of activated memory CD8+ T cells as well as the plasma level of the pro-inflammatory cytokine IFN-α. An association between plasma LPS level, T cell activation levels, and degree of CD4+ T cell depletion under the setting of undetectable viral loads in elite controllers (45) further confirms circulating microbial products as a cause of immune activation in HIV infection independent of the virus, ultimately leading to CD4+ T cell depletion. ART suppresses but does not fully normalize circulating levels of microbial products, which continues to correlate with T cell activation and soluble markers of immune activation (92-94), highlighting the contribution of elevated circulating microbial products to systemic inflammation in the ART-treated patient. *In vitro* studies demonstrating activation and death of T cells following exposure to various bacterial toll-like receptor (TLR) ligands (95) reinforcing the causal relationship between circulating microbial products and immune activation. Moreover, bacterial TLR ligands were shown to induce monocyte expression of the coagulation-promoting tissue factor, the increase of which parallels the change in circulating levels of microbial product in chronically infected and ART-treated HIV-infected patients (96). These findings provide a potential mechanistic link between microbial products and the excess risk of coagulation-mediated cardiovascular disease and other non-AIDS events in the ART-treated HIV-infected population (97).
This phenomenon of translocation of microbes and/or microbial products into systemic circulation without causing overt bacteremia, termed ‘microbial translocation’, has been observed in association with systemic inflammation in other disease processes including inflammatory bowel disease (IBD) (98, 99), after laparoscopic surgeries (100), graft-versus-host disease (GVHD) (101, 102), excessive alcohol consumption, obesity, and diabetes (69). In such instances, microbial translocation occurs as a result of gastrointestinal (GI) tract damage. The GI tract mucosa, uniquely positioned in close proximity to the large luminal bacterial load, serves as a structural and immunological barrier against microorganism invasion. The association between gut epithelial structural damage, local and systemic microbial translocation, and systemic inflammation, demonstrated in a pigtail macaque primate model (103), implicates microbial translocation and systemic inflammation as direct consequences of damage to the GI tract. In light of evidence demonstrating reversal of elevated LPS levels in SIV-infected rhesus macaques following an extended “bowel-sterilizing” antibiotic regimen (92), the origin of microbial translocation in the SIV/HIV infections is also believed to be the gut, as a consequence following SIV/HIV-mediated damage to the gut mucosa.

1.2 **Intestinal Mucosal Barrier In HIV Infection**

*Constituents and function of the intestinal mucosal barrier*

The intestinal mucosa is the largest mucosal surface of the human body, acting as a barrier between host tissues and the external environment that covers an area
of approximately 100 m² (104). In face of the massive antigenic challenge from the up to $10^{14}$ commensal bacteria (the microbiota) residing in the adult human intestine lumen, in addition to dietary and environmental antigens, the intestine has evolved to function as a unique structural and immunological barrier, via which strong immune responses can be mounted against pathogenic insults, and immunological tolerance is induced in response to harmless antigens. The normal GI function requires balanced interactions between the microbiota, the local innate and adaptive immune system, and the intestinal epithelial cells (IECs).

Architecturally, the intestinal surface forms invaginations (crypts); in addition, protusions (villi) are also found in the small intestine. The only cellular border separating the intestinal lumen from the subepithelial tissues is a single layer of polarized IECs (Figure 1-3) comprising of specialized cell types: absorptive enterocytes, mucin-secreting goblet cells, hormone-producing enteroendocrine cells, and antimicrobial peptide-secreting Paneth cells, which originate from the intestinal stem cells at crypt bases (105). This physical barrier is protected by a layer of mucus on the luminal surface – a gel-like mixture of mucin, phospholipids, electrolytes, and water that physically separates the intestinal epithelium from the luminal bacteria.

The immunological arm of the intestinal barrier is accomplished by the GALT, which is divided into organized and diffuse lymphoid tissues (Figure 1-3). Organized tissues located in the mucosa and extending into the submucosa of the
intestinal wall, including the mesenteric lymph nodes, Peyer’s patches (aggregated lymphoid follicles), and other smaller isolated lymphoid follicles, are induction sites for mucosal immune response. In addition, numerous immune cells are diffusely scattered in the lamina propria and epithelium of the intestinal mucosa, in close proximity to the epithelial monolayer at the intestinal surface. Such diffuse lymphoid tissue consists of lymphocytes, macrophages, and dendritic cells, and is responsible for the effector functions of the mucosal immune response.

At sites of induction (Peyer’s patches and isolated lymphoid follicles), specialized IECs (M cells) in the follicle-associated epithelium (FAE), directly overlaying the follicles, sample and deliver luminal antigens to dendritic cells in the subepithelial dome area immediately below the FAE. These professional antigen-presenting cells interact with and present the antigens to the lymphocytes in the T cell and/or B cell areas within the site of induction. Following priming, activated lymphocytes drain into mesenteric lymph nodes, undergo further differentiation, migrate into systemic circulation, eventually home back to the GALT following expression of gut-homing α4β7 integrin, and redistribute to the diffuse effector sites of the GALT (106). Effector cells residing in between IECs (intraepithelial lymphocytes) are mostly CD3+ T cells, the majority of which is CD8+, while the lamina propria contains CD4+ and CD8+ cells at a ratio similar to that in PB. Due to constant exposure of antigens, the majority of the T cells in the intestinal mucosa are CCR5+, with an effector memory phenotype (107-109).
Immunological tolerance of the intestinal environment to harmless antigens is maintained by immune cells that have distinct phenotypes and functions compared to their PB counterparts, which collectively contribute to the uniquely tolerogenic GALT immune response. There is an appreciable population of Treg cells in the lamina propria – a specialized T cell subset that promote an anti-inflammatory environment. Equally important are the mucosal T cell subsets that produce IL-17 and/or IL-22 – cytokines that are important for the maintenance and repair of the intestinal epithelial barrier, as well as for production of antimicrobial peptides (6). More than 80% of the body’s activated B cells (plasma cells) reside in the intestinal mucosa, secreting IgA antibodies that bind to luminal antigens and shield the intestinal epithelium via immune exclusion (110). Multiple subsets of dendritic cells in the GALT primarily induce differentiation of suppressive and protective T and B-cell subsets: regulatory CD4+ and CD8+ T cells, Th17 cells, and IgA-secreting plasma cells (111, 112). The GALT is also the major reservoir of macrophages in the body (113, 114), with specialized resident macrophages lacking co-receptors to bacterial ligands and cytokines but retaining pattern-recognition receptors such as TLR, thus conferring phagocytic and bacteriocidal activity while maintaining inflammatory anergy in the gut. Such non-inflammatory macrophages prevent microbes and microbial products that traverse the epithelial barrier from accessing systemic circulation (115).
**Loss of gut mucosal immune homeostasis in the HIV-infected individual**

The GALT contains up to 80% of the body’s lymphocytes (116), among which is the majority of the body’s T cells (117). Given that greater than 90% of intestinal lamina propria CD4+ T cells are CCR5+ (29), the gut mucosa contains the body’s largest pool of CCR5+ CD4+ T cells, i.e. the preferential target cells of HIV. HIV therefore significantly alters gut mucosal immune homeostasis via targeting mucosal T cells (Figure 1-2). In acute HIV/SIV, massive depletion of CD4+ T cells occurs rapidly, and to an extent that is much more profound than that observed in PB. In SIV-infected rhesus macaques, a majority of intestinal CD4+ T cells were depleted by day 21 post SIV infection (118-120), and similarly early and massive loss of gut mucosal T cells is seen in HIV-infected individuals (29, 30). Notably, this severe loss in gut CD4+ T cells persists without recovery into the chronic phase of the disease (28, 29, 121). Multiples studies have examined the effects of ART on intestinal CD4+ T cell recovery, demonstrating wide variations in the degree of CD4+ T cell reconstitution (116). Despite several years of ART, gut mucosal T cell reconstitution is typically incomplete (28, 122), with evidence suggesting more significant recovery with earlier ART initiation (28, 30, 123, 124). HIV/SIV induces alterations in the balance of various T cell subsets, with a preferential loss of Th17 and Th22 subsets (125-127), shifting perhaps to a Treg (128) or Th1 phenotype (126). The functional impairment of this mucosal Th17 loss was demonstrated through increased S. typhimurium dissemination from the gut in SIV-infected primates (129). Importantly, while gut CD4+ T cell depletion occurs acutely in nonpathogenic SIV infections of sooty mangabey and African
green monkeys (130, 131), the phenomenon does not progress or is reversed in some cases (132), and gut Th17 populations are preserved (128). It is thus suggested that the altered balance of T cell subsets involving preferential mucosal Th17 cell loss may be critical to HIV-induced gut mucosal damage and microbial translocation.

While HIV induces persistent intestinal mucosal immunodeficiency with CD4+ T cell depletion, it paradoxically induces, simultaneously, a local inflammatory environment in the gut mucosa, with elevated lamina propria levels of proinflammatory mediators β-chemokines, TNF-α, IL-6, IL-10 and IFN-γ. This phenomenon is initiated early in the infection, and persists into the chronic phase, even when patients are treated with ART (133-137). Persistent GALT CD4+ and CD8+ T cell activation, shown not to be significantly reduced following ART (138), may partly explain the ongoing inflammation in the gut mucosa. Another plausible explanation is the significant infiltration and accumulation of CD8+ T cells in the lamina propria. Influx of mucosal CD8+ T cells occurs during acute HIV/SIV infection (139, 140), although they fail to clear the HIV infection. Elevated gut mucosal CD8+ T cell level is maintained through chronic infection, as measured using absolute number of mucosal CD8+ T cells as opposed to CD4/CD8 ratios (140-142). Robust and polyfunctional mucosal CD8+ cells responses are maintained in chronic HIV infection, such as multifunctional HIV-specific CD8+ T cells with the ability to degranulate and produce multiple cytokines (143), perhaps contributing to the mucosal inflammation. Following ART, intestinal mucosal CD8+
T cells levels may be reduced, but remain significantly elevated compared to healthy controls (135, 141, 142) while displaying attenuated responses to HIV antigens with a “monofunctional” profile (143, 144).

*Intestinal structural barrier loss in the HIV-infected individual*

HIV-mediated damage to the gut mucosa is not limited to the GALT, but involves also abnormalities in the structure and function of the physical barrier provided by the intestinal epithelium (Figure 1-4). With the destruction of the GALT, it is possible that the alterations in local epitheliotrophic factors from the immune compartment would negatively impact IEC homeostasis. Indeed, intestinal epithelial dysregulation begins in acute HIV infection and persists into the chronic phrase, with down-regulation of genes involved in IEC maintenance, growth and differentiation, as well as metabolic and digestive functions (145, 146). Upregulation of genes with intestinal mucosal protective and regenerative activity in elite controllers (147) confirms the pivotal role intestinal mucosal integrity may play in controlling disease progression. Epithelial barrier breakdown was recently confirmed in chronically SIV-infected rhesus macaques, with damage to colonic tissues ranging from multifocal epithelial disruptions to epithelial loss and overt ulceration. Resultant LPS infiltration into the lamina propria confirms the hypothesis that microbial translocation is a consequence of SIV-induced intestinal structural barrier damage (148).
In humans, intestinal barrier loss manifests clinically, primarily as diarrhea and weight loss in acute HIV infection, while chronically HIV-infected patients often show symptoms of pathogen-negative diarrhea, malnutrition and wasting, termed ‘HIV enteropathy’, although the incidence has dropped since the introduction of ART (149). Histologically, the enteropathy involves damage to the epithelial layer including small intestinal villous atrophy and villous blunting with increased epithelial apoptosis at villus tips (150), which is thought to cause increased epithelial proliferation and crypt hyperplasia (151). Indirect assessments of intestinal permeability, through measuring urinary excretion of orally consumed oligosaccharides, demonstrate increased small intestinal permeability in AIDS patients and some chronic HIV patients, regardless of therapy status (152, 153). In another study, through in vitro impedance spectroscopy and flux analysis of duodenal biopsies, increased small intestinal permeability was suggested to be caused by a leak flux mechanism (154), alluding to an intestinal paracellular barrier defect. Structural defects and increased permeability in the small intestine appear to be critically involved in HIV enteropathy, particular in the pre-ART era.

More recently, plasma measures of intestinal fatty acid binding protein (I-FABP), a marker of small intestinal epithelial cell apoptosis (155), have been associated with HIV progression (97, 156, 157), eluding to IEC apoptosis as a mechanism for HIV-induced small intestinal barrier loss. Indeed, during early SIV infection, epithelial apoptosis resulted from direct virotoxic effects of HIV gp120 binding to IEC surface orphan G-protein coupled receptor GPR15/Bob (158, 159), which
leads to tubulin depolymerization and loss of enterocyte ionic balance in vitro (160).

To further confirm and define the HIV-mediated permeability increases of the GI tract in a site-specific manner, our lab conducted a clinical study assessing urinary excretion of oligosaccharide probes sucrose, mannitol, lactulose, and sucralose (161). Dependent upon the GI site at which each probe is metabolized, regional permeability of various segments of the GI tract can be precisely determined (162, 163). Our study demonstrated increased small intestinal and colonic permeability in HIV-infected patients, which was not corrected by ART. We identified epithelial damage to be the cause for elevated small intestinal permeability, a conclusion that is consistent with data suggesting the involvement of IEC apoptosis in HIV-mediated small intestinal barrier loss. In contrast, our results revealed increased colonic permeability in HIV-infected individuals in the absence of epithelial damage, suggesting that barrier function between intact epithelial cells was lost – implicating increased intestinal epithelial paracellular permeability, primarily governed by intercellular tight junctions, as a critical component of HIV-induced colonic barrier dysfunction.
1.3 Intestinal Epithelial Tight Junctions: Structure and Function

Tight junctions govern intestinal epithelial paracellular permeability

The majority of the intestinal barrier is provided by the epithelial cell membranes that are impermeable to hydrophilic solutes except at locations where specific transporters, on the apical and basolateral cellular membranes, allow regulated passage of nutrients, ions and water (164), creating a transcellular pathway for the permeation of such molecules. The space between adjacent cells, the paracellular space, provides an alternate route for solute traffic that is largely sealed by intercellular junctions – tight junctions (TJ), adherens junctions, and desmosomes, which comprise the apical junctional complex. TJs, located closest to the luminal surface in the most apical portion of the lateral intercellular space, seal the paracellular space and is the rate-limiting determinate of the overall barrier function in an intact intestinal epithelium (164). In contrast, the more basolateral adherens junctions and desmosomes maintain cellular proximity while allowing for intercellular communication, epithelial polarization, and tight junction assembly.

TJs form barriers with permeability and size and/or charge selectivity that differs depending on tissue type (165) – small intestinal epithelium has leakier TJs than more distal portions of the intestine, to allow for water and nutrient absorption; while the urinary bladder and gallbladder epitheliums have essentially impermeable TJs to prevent urine and concentrated bile acids from entering
circulation. The basal permeability and selectivity of a TJ is determined by its precise molecular composition; in addition, barrier properties can be acutely regulated.

TJs are multi-protein complexes (Figure 1.5) visualized on electron microscopy as intercellular kissing points, comprising of anastomosing strands that interact with similar strands on adjacent cells. The number of TJ strands typically positively correlates with the tightness of the epithelium (166). Such strands consist of more than 30 different transmembrane proteins. The claudin family of tetra-span TJ proteins comprises of 27 members, each with differing barrier-forming or charge-selective channel-forming functions and expression profile. The TJ-associated Marvel proteins (TAMP), including tetra-spanning proteins occludin, tricellulin, and MarvelD3, are found in all TJs throughout the body. Other strand-forming proteins have one or three transmembrane domains, such as the junctional adhesion molecule (JAM) family, Crb3, CAR and Bves, and are thought to influence the strand formation process instead of TJ permeability properties. Strand-forming proteins interact with cytoplasmic plaques via binding to the PDZ domains of intracellular TJ adaptor proteins including zona occludens-1 (ZO-1), ZO-2, ZO-3, Par3, Par6 etc., which in turn are anchored to the perijunctional actomyosin ring that encircles epithelial cells, thus allowing for cytoskeletal regulation of TJ permeability.
The overall transepithelial resistance (TER) of the TJ barrier, a measurement of ion conductance across the TJ, was found not to be directly proportional to strand number, but is instead a logarithmic function of the number of TJ strands (167), leading to the model that the strands form absolute barriers populated by pores that can open and close to allow for ion movements. TJs display size selectivity, with two functionally distinct components for solute passage: a high-capacity, charge-selective “pore” pathway permeable to small ions and uncharged molecules of ~ 4Å or less, and a low-capacity “leak” pathway for flux of larger solutes and macromolecules, regardless of charge (168, 169). These two pathways are thought to be differentially regulated, with members of the claudin family governing the leakiness or tightness, as well as the charge selectivity, of the ion-selective ‘pore’ pathway (170, 171), while TAMP proteins are important for ‘leak’ pathway regulation (169, 172, 173). The precise selectivity and barrier properties of tetra-spanning TJ proteins are determined by their first extracellular loop, thus resulting in the varying sealing or channel-forming properties of different claudin proteins. For example, claudin-2 has been described as a cation-selective channel-forming protein based on the observation that overexpression reduces TER via increasing permeability to cations (174, 175). Other channel-forming claudins include claudins-10a, -10b, -15, and -17, while claudins-1, -3, -5, -11, -14, and -19 are classified as sealing claudins. Other claudins have been shown to be both sealing and pore forming, dependent on the cellular system studied. One example is claudin-4, for which the majority of data supports a sealing function (170, 176, 177), but was reported to form anion channels when expressed with
claudin-8 (178). Strand-forming TJ proteins can form homotypical and heterotypical interactions with other TJ proteins in the same strand and in the plasma membrane of the neighboring cells (171), and more than one claudin is expressed in a single cell. The resultant combinations and permutations can form a broad range of pores with unique properties, which ultimately cooperate to determine the overall barrier function of the TJ and the epithelium. It is important to appreciate that the background of other strand-forming proteins in a TJ modify the functional consequences of each individual component, thus studies demonstrating inconsistencies in the function of a particular claudin protein may reflect various consequences of altering the interactions with or causing the displacement of other claudins from the TJ.

Involvement of Tight Junction Dysregulation in HIV Infection

In the context of HIV infection, the presence of intestinal barrier dysfunction suggests the involvement of intestinal TJ dysregulation. Such intestinal TJ dysregulation would be maintained throughout the course of an untreated HIV infection, and is also predicted to persist in the patient on suppressive ART, following the pattern as observed for increased intestinal permeability (161). Such intestinal TJ dysregulation would play a crucial role in the pathogenesis of CD4+ T cell death, allowing the passage of microbial products that drives systemic inflammation, and more importantly, would implicate a mechanism that accounts for the sustained systemic inflammation in the ART-treated HIV-infected patient, mediating the immunopathogenesis of non-AIDS morbidities.
Indeed, intestinal epithelial breakdown has been confirmed in the acute and chronic pathogenic SIV infection (148), but was characterized by severe phenotypes involving epithelial disruptions, although the study was not designed to probe for TJ dysregulation on the molecular level. In HIV-infected patient, a previous study characterized the ileal and rectal epithelium of untreated chronically-infected patients, and indeed revealed barrier defects with increases in claudin-2 protein expression (179). Another study demonstrated duodenal TJ dysregulation via decreased claudin-1 and increased claudin-2 protein expression during untreated HIV infection, which were reversed in ART-treated patients (150), consistent with our clinical study suggesting epithelial damage, not increased paracellular TJ permeability, as the mechanism for elevated small intestinal permeability.
1.4 **OVERALL OBJECTIVE OF THE THESIS**

While mechanisms for intestinal epithelial dysfunction and the involvement of TJ dysregulation have been investigated in acute HIV/SIV infection and in untreated chronic disease, direct molecular characterization of sustained increase in intestinal permeability is lacking in the ART-treated HIV-infected population. We hypothesize that increased colonic permeability in this population is a result of intestinal epithelial TJ dysregulation that remains uncorrected following ART. We believe this phenomenon crucially propagates the residual systemic inflammation that creates excessive risk of non-AIDS morbidities in this population. Moreover, the drivers for intestinal epithelial dysfunction and TJ dysregulation in the setting of HIV infection are currently unknown. We propose that interactions between the intestinal mucosal immune and epithelial compartments modulate IEC TJs, and that HIV-associated loss of intestinal mucosal immune homeostasis alters such IEC-immune cell cross-talk, thereby prompting the increase in intestinal permeability.

To this end the studies presented here aim to:

1. Characterize the intestinal epithelial dysfunction and TJ disruption in the intestinal epithelium of the ART-treated HIV-infected population
2. Investigate the modulatory effects of immune cells, in particular T cells, on intestinal epithelial permeability
Figure 1-1. Structure and genome of the HIV-1 virus. (A) A mature HIV-1 virion. (B) The HIV-1 proviral genome where the coding sequences for gag, pol, vif, vpr, vpu, env, tat, rev, and nef are indicated by gray rectangles. The 5’ and 3’ LTR regions are shown flanking the provirus. The major domains for the gag, pol, and env genes are also indicated. Abbreviations: LTR, long terminal repeat; MA,
Matrix; CA, Capsid; NC, Nucleocapsid; PR, protease; RT, Reverse Transcriptase; IN, Integrase; gp, glycoprotein.
**Figure 1-2 Overview of the HIV-1 Replication Cycle.** The thirteen-step HIV-1 replication cycle is depicted, where each step of the cycle is a potential drug target. Major host proteins playing a role in the cycle are listed. White boxes indicate currently available antiretroviral drug classes and their sites of actions. Blue boxes indicate cellular restriction factors. Abbreviations: INSTI, Integrase strand transfer inhibitor; LTR, long terminal repeat; NRTI, nucleoside/nucleotide reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor.
Figure 1-3. The healthy intestinal mucosa.
Figure 1-4. HIV-associated damage to the intestinal mucosa. HIV-induced damage includes: 1) villi blunting, 2) crypt hyperplasia, 3) IEC apoptosis, 4) CD4+ T cell depletion, 5) CD8+ T cell influx, 6) microbial translocation, 7) mucosal inflammation, 8) HIV virions in the lamina propria, 9) macrophage impairment, and 10) loss of defensins.
**Figure 1-5. Tight junction structure.** TJs, the most apical intercellular junction on the lateral membrane of IECs, consist of strand-forming claudins, TAMPs, and other proteins (JAM, CAR, Bves etc.), anchored to the actomyosin ring via adaptor proteins such as ZO-1.
CHAPTER 2

PROGRESSIVE PROXIMAL-TO-DISTAL REDUCTION IN EXPRESSION OF THE TIGHT JUNCTION COMPLEX IN COLONIC EPITHELIUM OF VIRALLY-SUPPRESSED HIV+ INDIVIDUALS

Acknowledgments

I would like to thank co-authors Stephanie L Alden, Nicholas T. Funderburg, and Pingfu Fu for performing the experiments, analyzing the data, and help with the manuscript. We would like to thank the physicians and personnel of the Cleveland Special Immunology Unit and the University Hospitals Digestive Health Institute, particularly Michele Gallagher, Beth Bednarchik, and Wendy Brock, and Dr. Wendy Liu for help in patient recruitment. We would also like to thank Drs. Richard Jurevic, Scott Howell, Alex Huang, Kevin Cooper, and Maria Hatzoglou for their continual collaboration and support, and Jeff Meisch and Dr. Tejpal Gill for helpful discussions and technical support. We thank the Cleveland Immunopathogenesis Consortium (BBC/CLIC) for valuable discussions and advice. We acknowledge all volunteers who participated in the study.

---

1 This chapter is published in PLoS Pathog. 2014. 10(6)
2.1 Abstract

Effective antiretroviral therapy (ART) dramatically reduces AIDS-related complications, yet the life expectancy of long-term ART-treated HIV-infected patients remains shortened compared to that of uninfected controls, due to increased risk of non-AIDS related morbidities. Many propose that these complications result from translocated microbial products from the gut that stimulate systemic inflammation – a consequence of increased intestinal paracellular permeability that persists in this population. Concurrent intestinal immunodeficiency and structural barrier deterioration are postulated to drive microbial translocation, and direct evidence of intestinal epithelial breakdown has been reported in untreated pathogenic SIV infection of rhesus macaques. To assess and characterize the extent of epithelial cell damage in virally-suppressed HIV-infected patients, we analyzed intestinal biopsy tissues for changes in the epithelium at the cellular and molecular level. The intestinal epithelium in the HIV gut is grossly intact, exhibiting no decreases in the relative abundance and packing of intestinal epithelial cells. We found no evidence for structural and subcellular localization changes in intestinal epithelial tight junctions (TJ), but observed significant decreases in the colonic, but not terminal ileal, transcript levels of TJ components in the HIV+ cohort. This result is confirmed by a reduction in TJ proteins in the descending colon of HIV+ patients. In the HIV+ cohort, colonic TJ transcript levels progressively decreased along the proximal-to-distal axis. In contrast, expression levels of the same TJ transcripts stayed unchanged, or progressively increased, from the proximal-to-distal gut in the
healthy controls. Non-TJ intestinal epithelial cell-specific mRNAs reveal differing patterns of HIV-associated transcriptional alteration, arguing for an overall change in intestinal epithelial transcriptional regulation in the HIV colon. These findings suggest that persistent intestinal epithelial dysregulation involving a reduction in TJ expression is a mechanism driving increases in colonic permeability and microbial translocation in the ART-treated HIV-infected patient, and a possible immunopathogenic factor for non-AIDS related complications.
2.2 Introduction

Chronic systemic inflammation, characterized by increased frequencies of activated B and T cells (31), elevated levels of circulating proinflammatory cytokines and chemokines (33), and more rapid immune cell turnover (35), is a hallmark of HIV/SIV infection and a better predictor of disease progression than plasma viral load (32, 42). Accumulating evidence suggests that this systemic inflammation plays a role in non-AIDS related comorbidities including cardiovascular diseases (31, 81-83), liver diseases (33, 71, 84, 180), and neurocognitive decline (35, 72), resulting in shortened life expectancy and premature aging in patients treated with long term antiretroviral therapy (ART) (32, 42, 67, 181). In addition, plasma levels of microbial products, such as lipopolysaccharides (LPS) and bacterial 16s rDNA, are elevated in chronically HIV-infected individuals and associated with markers of immune activation (92, 93, 182), implicating circulating microbial products, due to microbial translocation, as a major cause of HIV-associated systemic inflammation (183). An association between circulating microbial products and systemic inflammation has been observed in other disease processes such as inflammatory bowel disease (98, 99) and after laparoscopic surgeries (100, 184). Moreover, conditioning regimens for stem cell therapy cause gastrointestinal (GI) tract injury that facilitates the translocation of microbial products from the intestinal lumen to systemic circulation, ultimately stimulating the immune system and exacerbating graft-versus-host disease (101, 102). Klatt et. al. highlight the association between gut epithelial structural damage, local and systemic microbial translocation, and systemic
inflammation, in SIV-naïve pigtail macaques (103), suggesting microbial translocation and systemic inflammation as direct consequences of damage to the GI tract in the absence of chronic viral infection.

The GI tract is a major target site for HIV infection, as the mucosal immune system contains the majority of the body’s T cells (117). In addition, greater than 90% of intestinal CD4+ T cells are CCR5+ (29), providing a large pool of target cells that are preferentially depleted by HIV. Independent of route of transmission, within weeks of HIV or SIV infection, rapid and severe depletion of intestinal lamina propria CD4+ T cells occurs and persists into the chronic phase of the disease (28, 29, 121), with preferential depletion of the Th17 and Th22 subsets (126, 127). Significant accumulation of mucosal CD8+ T cells during HIV infection has also been shown (141, 142); both effects drastically alter mucosal immune homeostasis. Coincident with early mucosal CD4+ T cell loss, gene expression profiling reveals intestinal barrier dysfunction in primary HIV and SIV infection, as exemplified by down-regulation of genes associated with epithelial maintenance and digestive functions (147, 185). Upregulation of genes with intestinal mucosal protective and regenerative activity in elite controllers (147) confirms the pivotal role intestinal mucosal integrity may play in limiting systemic inflammation and controlling disease progression.

Intestinal barrier dysfunction, long recognized in HIV patients with advanced disease, includes manifestations of pathogen-negative diarrhea and
malabsorption (151). Indirect assessments of intestinal permeability, through measuring urinary excretion of orally consumed oligosaccharides, demonstrate increased small intestinal permeability in symptomatic AIDS patients and some asymptomatic chronic HIV patients, regardless of therapy status (152, 153). Notably, increased small intestinal permeability did not correlate with intestinal structural change (152), and, through in vitro impedance spectroscopy and flux analysis of duodenal biopsies, was suggested to be due to a leak flux mechanism (154), alluding to an intestinal barrier defect as a result of tight junction (TJ) down-regulation. Our recent clinical report demonstrated increased small intestinal and colonic permeability in HIV-infected patients, which was not corrected by ART, further implicating intestinal barrier dysfunction as an ongoing pathophysiological change in ART-treated patients (161). Our current study, using human intestinal biopsies, extends evidence for intestinal damage in SIV infection of non-human primates (148) and explores the molecular mechanisms behind increased intestinal permeability in ART-treated HIV+ patients. We hypothesize that HIV-associated dysregulation in intestinal epithelial cells will lead to TJ down-regulation, resulting in persistent intestinal barrier dysfunction in the ART-treated patients, contributing to microbial translocation and systemic inflammation.

2.3 Materials and Methods

Ethics Statement

The University Hospitals Institutional Review Board (IRB) has reviewed the following submission:
Principal Investigator: Dr. Alan D Levine, Ph.D.

Protocol Title: Loss of Intestinal Barrier Function in HIV Infection

UHCMC IRB number: 06-07-31

Submission Type: Continuing Review

Review Type: Full Board

Date of Committee Review: 04/16/2013

As such, the UHCMC IRB has determined that with respect to the rights and welfare of the individuals, the appropriateness of the methods used to obtain informed consent and the risks and potential medical benefits of the investigation, the current submission is acceptable under Federal Human Subject Protection regulations promulgated under 45 CFR 46 and 21 CFR 50 and 56.

The current expiration date for this study is: 04/26/2014

Patient Recruitment

Subjects undergoing routine screening colonoscopies were recruited from the Digestive Health Institute and the Special Immunology Unit of the University Hospitals Case Medical Center, Cleveland, OH, with the exclusion criteria of any known or suspected gastrointestinal disease. After written informed consent was obtained, eight pinch biopsies, two each from the terminal ileum, ascending colon, transverse colon, and descending colon, were obtained from thirty-one patients with HIV (median age 51 years, interquartile range [IQR] 50 – 55 years) and thirty-five healthy controls (median age 56 years, IQR 50 - 61 years). Peripheral blood was collected immediately following the colonoscopy procedure into EDTA-
containing tubes to obtain plasma samples, which were stored at -80°C until assay. Apart from three HIV+ subjects who were simultaneously evaluated for chronic diarrhea, in whom no significant terminal ileal or colonic histopathological findings were identified, there were no reports of diarrhea prior to the colonoscopy preparation regimen for other HIV+ subjects and all controls subjects. HIV+ patients were enrolled notwithstanding their CD4 count and viral load, and all were under ART treatment. Controls were not specifically tested for HIV, but had no reported history of HIV infection. All study protocols were approved by the Institutional Review Board at University Hospitals Case Medical Center.

Relative Cell Abundance Determined by Nuclear Staining
Formalin-fixed, paraffin-embedded, 5-µm biopsy sections from the ascending, transverse, and descending colon were deparaffinized, rehydrated, mounted in Fluoroshield mounting medium with DAPI (AbCam). Two biopsies per donor from each location were analyzed in a total of five HIV+ patients and five healthy controls. Individual images were obtained on a Leica DMI 6000 B inverted microscope using a 20x objective connected to a Retiga EXI camera (Q-imaging), and composite images of each section were generated through stitching. A threshold intensity for excluding the background was established to specifically analyze nuclear staining. Epithelial cell nuclei and lamina propria cell nuclei were identified manually, after which measurements for the total area they occupy were enumerated using Metamorph Imaging Software (Molecular Devices). The built-in count nuclei application module was used to determine epithelial cell numbers by
setting an approximate nuclear width at 3-8 µm. Relative epithelial cell abundance (compared to lamina propria cells) was determined by the ratio of epithelial nuclei area to lamina propria nuclei area. Luminal barrier coverage, defined as the length of the intact epithelial/luminal border relative to lamina propria cell abundance, was designated as the ratio of border length to lamina propria nuclei area. Epithelial cell packing density was calculated as the number of epithelial cells per 100 µm of intact luminal border.

Confocal Microscopy
Paraformaldehyde-fixed, frozen, 5-µm biopsy sections from the ascending, transverse, and descending colon were blocked with 10% normal goat serum, incubated with rabbit anti-occludin or anti-ZO1 antibody, and detected with chicken or goat Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen Life Technologies). Sections were mounted in Fluoroshield mounting medium stained with DAPI (AbCam), and visualized with the Perkin Elmer Ultraview VoX confocal microscope, using an oil-immersion 100x magnification objective lens connected to a Leica DMI 6000 B inverted microscope. En face and transverse Z-stack images (0.3 µm thickness) were obtained using Volocity 6.2 (Perkin Elmer). After applying a threshold to eliminate non-specific staining, 3D reconstruction of tight junctions was performed and analyzed using Imaris 3.0 (Bitplane Scientific Software). An average of 7 fields of view on the intestinal surface and 5 fields of view in the crypts were imaged and analyzed for each biopsy obtained, two biopsies per location, from a total of three HIV+ patients and
three healthy controls. Average fluorescence intensity for occludin or ZO-1 staining was analyzed for each field of view.

**Real-time qPCR**

Snap-frozen biopsy specimens stored at -80°C were homogenized with a bead beater (Retsch) for 3 min at a frequency of 30 Hz/second to ensure complete homogenization. Total RNA was extracted using the PureLink RNA Mini Kit (Invitrogen Life Technologies, Carlsbad, CA) and quantified with the Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE). cDNA was transcribed from 1 µg of total RNA using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies). Transcript levels of human beta-defensin 3 (hBD-3), E-cadherin, and tight junctional proteins occludin, zona occludens 1 (ZO-1), claudin-2, and claudin-4 were determined by SybrGreen-based real-time PCR using CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). After an evaluation of eight commonly used housekeeping transcripts for genetic stability based on geNormPlus analysis (Biogazelle), β-actin and eukaryotic translation elongation factor 1-alpha 1 (eef1A1) were identified and used as references. Primers used are summarized in Table 2-1. Calibrated normalized relative quantities (CNRQ) of target genes were determined with qBasePlus (Biogazelle) analysis (186).

**Immunoblotting**

Total protein was extracted in 60 µl of SDS-RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.3% SDS, 1% Triton X, 1 mM EDTA, 1:100 protease inhibitors) from
snap-frozen descending colonic biopsies using a bead beater (Retsch) for 3 min at a frequency of 30 Hz/second, followed by constant agitation for 2 h at 4°C. Proteins were separated by 10% polyacrylamide gel electrophoresis (Invitrogen Life Technologies) and electro-transferred onto nitrocellulose membrane (Invitrogen Life Technologies). After blocking with 5% non-fat milk solution, membranes were probed with rabbit antibodies against glyceraldehyde 3-phosphoate dehydrogenase (GAPDH), cytokeratin-18, occludin (AbCam), claudin-2, and mouse antibodies against claudin-4 (Invitrogen Life Technologies) and β-actin (AbCam). After incubation with HRP-conjugated goat anti-rabbit (Thermo Fisher Scientific) or anti-mouse (AbCam) secondary antibody, signals were visualized with enhanced chemiluminescence, using West Pico Supersignal (Pierce). Chemiluminescence for all membranes was detected using Hyblot CL Autoradiography Film (Denville Scientific).

Densitometric Analysis

The amount of protein in each band was quantified by densitometry using ImageJ (National Institutes of Health). Dilution series were electrophoresed to determine optimal loading amounts for each target protein, to guarantee that bands fell within the linear range of detection. Extracts yielding bands that were too underexposed or overexposed were re-electrophoresed after adjusting the loading volume to obtain bands that were accurately quantified. An inter-gel control sample used to normalize intensity variations between gels was electrophoresed on all gels. The densitometric intensity of each target protein occludin, claudin-2, and claudin-4
was normalized to the intensity of cytokeratin-18 in each extract, to determine epithelial-specific TJ protein levels. Analysis was performed on samples from thirteen HIV+ patients and thirteen healthy controls.

**Measurement of soluble inflammatory markers**

Plasma was prepared by centrifugation of EDTA-treated whole blood for 10 min at 1610 g and then frozen at -80°C until assay. Soluble CD14 (sCD14) levels were measured using the Quantikine kit (R&D Systems). Samples were thawed on ice and analyzed in batches in duplicate, background was subtracted, and mean values were reported.

**Measurement of LPS**

Plasma samples were diluted to 10% with endotoxin-free water and then heated to 85°C for 15 min to denature plasma proteins. Plasma levels of LPS were quantified with a commercially available Limulus Amoebocyte Lysate assay (QCL-1000, Lonza) according to the manufacturer’s protocol. Samples were analyzed in triplicate; backgrounds were subtracted, and mean values were reported.

**Statistical Analysis**

For evaluation of the histological samples, statistical analysis was performed utilizing a mixed-effects model that took the repeated measurements from the same individual into account, and unstructured covariance structure was used for the inference. Analyses were performed using SAS (Statistical Analysis System, version 9.2). All other analyses were performed using Prism 5.0 (GraphPad
Relative cell abundance, transcript levels in the terminal ileum and colon, as well as protein levels in the descending colon are represented using box-and-whisker plots constructed using Tukey’s method, where outliers are noted as distinct data points. Statistical analysis for mRNA, protein levels, and plasma sCD14 levels was performed on all data points, including outliers, via Mann Whitney U test. To analyze for TJ transcript levels versus gut location (proximal-to-distal), the results were analyzed using the Kruskal-Wallis test, a non-parametric version of the one-way ANOVA, with a post test adjustment for multiple comparisons to evaluate linear trend. Spearman’s rank correlation was calculated for immune activation markers versus colonic TJ transcript levels. For samples that were analyzed with multiple comparisons, a False Discovery Rate (FDR) analysis, using the Benjamini and Hochberg’s approach(187), was implemented with the SAS procedure PROC MULTTEST. P-values adjusted for FDR are reported. All tests were two-sided and p-values less than 0.07 were considered significant.

2.4 Results

Study population

Our clinical study on intestinal barrier integrity in an HIV+ population, in which we measured urinary excretion of orally consumed oligosaccharides (161), revealed increased permeability in the small intestine and colon. These results demonstrated that increased small intestinal permeability is a result of epithelial damage, while colonic paracellular permeability increased without epithelial
damage, suggesting a loss in barrier function between intact epithelial cells. Importantly, increases in intestinal permeability were uncorrected in the ART-treated HIV+ patient population. To investigate the mechanism behind the persistent increase in intestinal permeability in ART-treated patients, we obtained intestinal pinch biopsy and plasma samples from thirty-one ART-treated HIV+ patients and thirty-five healthy controls undergoing screening colonoscopies at the Digestive Health Institute at University Hospitals Case Medical Center. Age range was similar between the control and HIV+ cohorts. More males participated than females in both cohorts, with the HIV+ cohort showing a higher percentage of males. HIV+ subjects have been infected for a median of 13.6 years, and reached a median peripheral blood CD4+ T-cell nadir of 176 cells/µl at a median of 6.5 years before the time of biopsy. At the time of biopsy, the median viral load and CD4+ T-cell count of the HIV+ cohort were 48 copies/ml and 569 cells/µl respectively. All but four HIV+ patients had undetectable viral load. All HIV+ patients studied have been under treatment with antiretroviral therapy for a median of 11.2 years, with uninterrupted treatment for a median of 4.1 years prior to study entry. All patients were on a minimum of three antiretroviral drugs, including at least two reverse transcriptase inhibitors and a combination of protease inhibitors and integrase inhibitors, at the time of biopsy. Demographics and clinical parameters of the cohorts are summarized in Table 2-2. Analysis of the plasma levels of immune activation marker sCD14 and LPS, as an indicator of microbial translocation, was performed on twenty-one HIV+ patients and twenty-one healthy controls within our cohorts. Since a limited number of small biopsies
were obtained per study subject, we were restricted to undertaking a single molecular or histological analysis on a sample obtained from each subject, with concurrent quantitative PCR and immunoblotting performed on biopsies from three HIV+ and seven control subjects. Subjects in each cohort were randomly assigned to various analyses. Figure 2-1 details the breakdown of biopsy and plasma samples from our cohorts into each analytical method.

Relative abundance of epithelial cells is not decreased in the intestine of HIV-infected individuals

The intestine is comprised of a one-cell layer thick epithelium, lining the interface with the gut lumen and separating the outside environment from the plethora of immune cells in the lamina propria. To assess an overall loss of epithelial cells as a potential mechanism for increased colonic permeability, we compared the abundance of epithelial cells relative to lamina propria cells in intestinal biopsy samples from HIV+ patients and healthy controls (Figure 2-2). The epithelium in the HIV+ gut is grossly intact and continuous, without areas of focal epithelial cell loss, crypt bifurcation, neutrophil-induced injury, flattened epithelium, or ulceration, either on the luminal surface or in the crypts (Figure 2-2A). Relative abundance of epithelial cells in the HIV+ gut, reflected by the ratio of epithelial cell nuclei to lamina propria cell nuclei, shows no changes in the ascending, transverse, and descending colon (Figure 2-2B). Similarly, considering the extent of intact epithelial-luminal border as a measure of barrier coverage, the relative length of the epithelium in the HIV gut is not altered at all three colonic sites (Figure 2-2B).
This evidence for no loss in relative epithelial cell abundance and tissue length in the HIV-infected population is confirmed and extended by finding no change in cell density or packing in the HIV epithelium, as assessed by the number of epithelial cells per 100 µm of epithelium (Figure 2-2C).

These results directly indicate no changes in the abundance of epithelial cells relative to lamina propria cells, not the absolute number of epithelial cells. A previous report demonstrating the restoration of CD4+ T cells and increase in CD8+ T cells, both measured in absolute numbers, in the HIV+ gut mucosa after prolonged ART (141) enables us to conclude that the absolute number of intestinal epithelial cells is not decreased in the colon of HIV-infected individuals. No change in epithelial cell packing in the HIV+ intestinal epithelium also indicates that permeability in the HIV gut is not manifest at the cellular level, suggesting an alternative mechanism, at the molecular level, for the increase in colonic permeability in the ART-treated HIV+ individual.

*No microscopic change in the structure and subcellular localization of intestinal epithelial tight junction components in HIV-infected individuals*

Since non-absorbable saccharide probes were utilized in our clinical study, the results reflect an increase in paracellular permeability (extracellular, within the intercellular spaces between epithelial cells), as opposed to solute movement through the transcellular pathway (163). Paracellular intestinal barrier function is primarily mediated by apically located transmembrane tight junctions (TJs) that
seal the intercellular space between adjacent epithelial cells. One potential molecular mechanism for increased colonic intestinal paracellular permeability is the disruption of intercellular TJs. In an intact epithelial cell layer, the space in between individual epithelial cells is sealed by the apical junctional complex composed of the TJ and the subjacent adherens junction, with passage through the TJ being the rate-limiting step of the paracellular transport pathway, and for overall transepithelial solute transport (188). The TJ is a multi-protein complex composed of transmembrane proteins, including members of the claudin family and TJ-associated marvel proteins such as occludin, which form the intramembranous TJ strands, and intracellular scaffold proteins such as ZO-1, which connect the strand-forming proteins to the cytoskeleton and are important for TJ assembly and regulation (188). Tissue-specific expression governing the combination of sealing and channel-forming claudin proteins in TJ complexes is the major determinant of distinct barrier properties and selectivity (171, 189).

To examine the subcellular localization and organization of the TJ complex in the intestinal epithelium, we studied the distribution of TJ components occludin and ZO-1 in the HIV+ intestine, through high magnification (100x) confocal microscopy. The TJ is located in the apical region of the lateral epithelial cell membrane, close to the luminal surface and away from the basolaterally-situated nucleus and the lamina propria. When the healthy intestinal epithelium is viewed in cross-section, both occludin and ZO-1 appear as distinct dot-like or line-forming structures on the luminal border (Figure 2-3A). When viewed en face, TJs, assembled as a ring-like
structure in association with the intracellular perijunctional actinomyosin ring, form a ‘chicken wire’ appearance in the crypts of control colonic tissue (Figure 2-3B). Intact TJs are seen in the healthy control population throughout the colon spanning the ascending, transverse, and descending segments. In the HIV+ intestine, no obvious changes are seen in occludin and ZO-1 distribution, both on the intestinal surface and in the colonic crypts (Figure 2-3A and 2-3B), indicating that epithelial TJ intracellular localization to the lateral plasma membrane is maintained, and that TJs are structurally intact, aligned between epithelial cells, in the ART-treated HIV+ population.

The composition of TJs was also examined in these colonic biopsies. The abundance of occludin and ZO-1 were determined and represented by the average fluorescence intensity in stained ascending, transverse, and descending colonic sections. Average fluorescence intensity is shown in Tables 2-3 and 2-4. No significant difference in occludin intensity in HIV+ samples is seen at the intestinal surface or crypt for all three colonic sites examined. Similarly, no significant difference in ZO-1 abundance is seen on the intestinal surface. In the crypt, significant increases in ZO-1 intensity are observed in the transverse HIV+ colon.

*Transcripts for colonic, but not terminal ileal, tight junction proteins are down-regulated in HIV-infected individuals*
To investigate whether the expression of the intestinal epithelial tight junctional complex is regulated in HIV+ patients at the transcriptional level, mRNA concentrations for a panel of TJ proteins were determined in intestinal biopsies through quantitative real-time PCR (Figure 2-4). mRNA levels were quantified using qBasePLUS software, which expresses transcript levels as calibrated normalized relative quantities, calculated based on endogenous levels of β-actin and eef1A1 as controls, selected based on geNorm analysis.

While changes in TJ structure and subcellular localization were undetectable in our system using microscopic immunofluorescence, significant decreases in ZO-1 (p < 0.01) and occludin (p < 0.05) mRNA expression in the colon (Figure 2-4A) are observed in HIV+ individuals when compared to healthy control subjects. A significant decrease (p < 0.01) in the transcript expression level of claudin-2, a cation-selective channel-forming protein, is also observed in the colon of HIV+ subjects, when compared to the expression level in healthy controls. Similarly, a significant decrease (p < 0.01) in colonic mRNA expression in the HIV+ patient is detected for another claudin family member, claudin-4 (Figure 2-4A), which functions predominantly as a sealing protein with controversial anion-channel-forming activity. The decrease in TJ mRNA varies from 1.4-fold for claudin-4 to 2.7-fold for claudin-2. We propose that these modest changes in mRNA expression, normalized across a wide stretch of tissue, explain why focal changes in protein expression were undetectable via confocal microscopy examining only a limited number of fields of view. Strikingly, the expression levels of all four TJ
mRNAs studied are not changed in the terminal ileum of HIV+ patients as compared to their expression in healthy controls (Figure 2-4B), suggesting an HIV-associated tissue-specific down-regulation of TJ transcripts in the GI tract, seen only in the colon and absent in the terminal ileum. This decrease in TJ mRNA only in the colon is consistent with our clinical study that revealed HIV-associated increased paracellular permeability in the colon and tissue damage in the small intestine (161).

Expression of tight junction mRNA continuously decreases along the proximal-to-distal axis in the HIV+ colon

Since small intestinal and colonic biopsies were obtained from four different locations along the GI tract, namely terminal ileum, ascending colon, transverse colon, and descending colon, we are uniquely positioned to investigate whether decreased colonic epithelial TJ mRNA expression in the HIV+ population is differentially distributed relative to anatomical location (Figure 2-5). Using a Kruskal-Wallis analysis with a post test adjustment for linear trend, ZO-1 gene expression shows a significant direct linear increase in transcript level from proximal-to-distal intestine in the healthy control population (p = 0.045). This expression pattern is dramatically reversed in the HIV+ population, where ZO-1 transcript levels demonstrate a significant inverse trend between expression and gut location (p = 0.049) (Figure 2-5A). Comparing ZO-1 transcript levels at each specific intestinal site, we observe a significant decrease in HIV+ individuals as compared to the healthy control population in the more distal portions of the GI
tract, namely the transverse (p = 0.05) and descending (p < 0.01) colon, consistent with the concept that there is a continual decrease in ZO-1 mRNA expression in the HIV+ population as one travels distally in the colon (Figure 2-5A).

In contrast to ZO-1, occludin, claudin-2, and claudin-4 transcript levels remain relatively constant from proximal-to-distal gut in the healthy control population (Figure 2-5B – 2-5D). While we do not observe a significant linear trend toward reduction in occludin expression toward the distal colon in the HIV+ population, similar to ZO-1 we do find that occludin transcript level is significantly reduced only in the distal, namely the transverse and descending colon in the HIV+ cohort (p < 0.05; Figure 2-5B). In congruence with ZO-1 expression in the HIV+ population, claudin-2 and claudin-4 mRNA expression show a significant linear trend toward reduction from the terminal ileum to descending colon (p = 0.044 and p = 0.059; Figure 2-5C and 2-5D). Examining each colonic location individually, significant decreases in claudin-2 expression are observed in the transverse and descending colon (p < 0.05; Figure 2-5C). A similar pattern holds for claudin-4 (Figure 2-5D), with the HIV+ population showing significantly decreased claudin-4 transcript expression in the transverse (p < 0.05) and a trend toward a decrease in the descending (p < 0.08) colon, while claudin-2 and -4 transcript levels remain unchanged in the terminal ileum and ascending colon (Figure 2-5C and 2-5D). Overall, HIV infection is associated with a significant modification in the intestinal
TJ complex’s anatomic expression profile, delineated by a proximal-to-distal decreasing gradient in mRNA expression.

To eliminate the potential effects of differences between the percentages of male versus female subjects in the control and HIV+ cohorts, we reanalyzed the expression of intestinal epithelial tight junction transcripts only for the male subjects, which represent the overwhelming majority of HIV+ volunteers. Significant decreases in all four TJ mRNA levels are again observed in the colon of HIV+ males (Figure 2-S1A), while transcript levels are not altered in the terminal ileum (Figure 2-S1B), replicating the observation for the entire HIV+ and healthy control cohorts. Upon examination of TJ expression levels along the proximal-to-distal axis of the gut for male subjects, we reconfirmed our conclusions on HIV-associated modifications of intestinal TJ complex transcript expression profile, reflected by progressive decreases in transcript levels toward the distal HIV+ intestine. We see an increase in mRNA level as the location varies from proximal-to-distal for ZO-1 and claudin-2 in healthy males, which is reversed in HIV+ males. For occludin and claudin-4, transcript levels are relatively constant from the terminal ileum to the descending colon of healthy males, while claudin-4 shows a progressive decrease toward the distal colon in HIV+ males. ZO-1, occludin, claudin-2, and claudin-4 median transcript levels are all decreased 1.5 to 2.9-fold in the descending colon of HIV+ males (data not shown).
We also recognize that HIV is a highly heterogeneous disease. While all of our patients are on ART, some of the patients did display a detectable viral load, which may influence intestinal permeability or GI function. With only four patients showing detectable viral load, this study was not powered to compare the fully virally-suppressed group to these four subjects, yet we could eliminate the impact of detectable viral load on TJ transcript expression by removing them from the analysis. Decreases with identical degrees of significance in all four TJ mRNA levels are observed in the colon of virally-suppressed HIV+ subjects (Figure 2-S2A), while transcript levels are not altered in the terminal ileum (Figure 2-S2B), replicating the observation for the entire HIV+ cohort. Upon examination of TJ expression levels along the proximal-to-distal axis of the colon in fully HIV-suppressed individuals, we found additional decreases in TJ mRNA expression in the ascending colon, which was not seen in the full HIV+ cohort (1.8-fold for ZO-1; 1.7-fold for occludin; 2.3-fold for claudin-2; 1.6-fold for claudin-4).

*Tight junction protein expression decreases in the descending colon of HIV+ Individuals*

To verify the HIV-associated decrease in TJ transcript levels, we measured the protein levels of TJ components occludin, claudin-2, and claudin-4 in the distal colon, the location that showed the greatest reduction in transcript levels in the HIV patient. Total protein lysates from the descending colon of virally-suppressed HIV+ individuals and healthy controls were subjected to immunoblotting (Figure 2-6A) followed by densitometric analysis. Equal loading of samples was verified
using GAPDH and β-actin. To accurately quantify the density of each band, samples were electrophoresed twice, varying the loading amounts if needed to obtain a band intensity that fell within the linear range of detection. To examine the TJ protein levels in epithelial cells specifically, levels of each target protein were normalized to the epithelial cell-specific cytokeratin-18 protein, a major cytoplasmic intermediate filament protein expressed in one-layered internal epithelial tissue (190). Normalized protein levels of occludin, claudin-2, and claudin-4 all show a significant 3.1 to 3.2-fold decrease in the descending colon of the virally-suppressed HIV+ cohort (Figure 2-6B) compared to levels in controls, in agreement with the observed decrease in occludin, claudin-2, and claudin-4 transcript levels in the distal colon of the HIV+ gut. As we described for TJ mRNA, median protein expression levels for occludin, claudin-2, and claudin-4 for the entire HIV+ cohort, as well as in the male HIV+ subjects, are similarly reduced 2.6 to 3-fold in the HIV+ descending colon (Figures 2-S3 and 2-S4). Eliminating the limited number of HIV+ patients with diarrhea did not alter these results (data not shown).

Tight junction mRNA down-regulation in HIV infection is a result of an overall change in intestinal epithelial cell transcriptional regulation

To investigate whether the transcriptional modification observed in the ART-treated HIV+ population is specific to TJ components, we measured the transcript levels of two epithelial cell-specific proteins that are not part of the TJ complex, namely human beta defensin-3 (hBD-3) and E-cadherin, along the proximal-to-
distal gut. hBD-3 is an inducible, broad-spectrum anti-microbial peptide, produced by colonic enterocytes as an innate immune effector to protect against luminal pathogens (191). E-cadherin is a transmembrane protein component of adherens junctions mediating cell-cell adhesion (192).

Analysis of non-TJ cellular components revealed differing patterns of HIV-associated transcription alteration (Figure 2-7). Similar to that observed for TJ transcripts, transcription of hBD-3 shows a significant down-regulation in the colon of HIV+ individuals (p <0.05), but examination of hBD-3 transcription at specific colonic locations revealed a distinct pattern (Figure 2-7A). Transcription is relatively constant in the healthy control population along the ascending, transverse, and descending colon, while in the HIV+ population, although a significant hBD-3 transcriptional down-regulation is observed only in the distal descending colon (p < 0.05), there is no significant linear trend between hBD-3 transcript level and anatomical location (Figure 2-7A). E-cadherin, on the other hand, shows transcriptional up-regulation in the HIV+ colon (p < 0.01), compared to levels in the uninfected colon, in stark contrast to other transcripts studied (Figure 2-7B). Comparing between the healthy controls and the HIV+ population at each gut location, we observed significant upregulation of E-cadherin transcript levels at the transverse (p < 0.06) and descending (p < 0.05) HIV+ colon. In the terminal ileum and ascending colon, E-cadherin transcript levels are unchanged. E-cadherin transcript levels do not vary with location in the healthy or HIV+ gut. (Figure 2-7B). Identical results for hBD-3 and E-cadherin transcript levels in the
colon are observed when only male donors or virally-suppressed populations were examined separately (Figures 2-S5 and 2-S6).

Microbial translocation and immune activation correlated with a decrease in colonic TJ transcript expression

It has been previously reported that circulating levels of the microbial cell wall constituent, LPS, is elevated in both ART-treated and untreated HIV+ patients (92, 93, 182), and we hypothesize that the accessibility of translocated luminal microbial products is mediated by a decrease in intestinal TJ expression. To confirm that an inflammatory mediator indicative of LPS exposure is elevated in our HIV+ cohort, we measured plasma levels of soluble CD14 (sCD14), an LPS co-receptor that promotes its binding to Toll-like receptor-4 and is shed from activated monocytes. Levels of sCD14 were elevated in samples from HIV+ patients compared to healthy controls (Figure 2-8A). Furthermore, there are inverse correlations that trend toward significance between levels of LPS or sCD14 and claudin transcript expression in the descending colon of both HIV+ and healthy control subjects (r = -0.79, p = 0.059 for claudin-4 vs. sCD14; and r = -0.76, p = 0.073 for claudin-2 vs. LPS; Figures 2-8B and 2-8C), demonstrating a direct link between TJ gene expression in the distal colon and immune activation in HIV infection.

2.5 Discussion

Microbial translocation from the gut, originating from the enormous quantity of intestinal commensal bacteria, is implicated as a major driver of the chronic
systemic inflammation that not only predicts pathogenic HIV disease progression and poor response to ART, but, more importantly, may mediate the immunopathogenesis of non-AIDS morbidities, including cardiovascular, liver, and neurocognitive diseases, that shorten the life expectancy of long-term ART-treated HIV-infected individuals (69, 193). Elevated microbial translocation is attributed to the simultaneous effects of intestinal mucosal immunodeficiency and disruption to the epithelial barrier, a hypothesis confirmed in pathogenic SIV infection (148). Structural damage to the intestinal epithelium has been demonstrated in ART-naïve HIV-infected population (150, 179), and we now provide the first direct molecular evidence of gut barrier breakdown in virally-suppressed HIV+ patients, corroborating our clinical data demonstrating persistence of increased intestinal permeability in the ART-treated population (161). Intestinal epithelial disruption is restricted to the colon and manifests at the molecular level as a down-regulation of the TJ components ZO-1, occludin, claudin-2, and claudin-4, via transcriptional control. The colonic epithelium remains grossly intact, and the packing and relative abundance of epithelial cells are maintained. Moreover, we observed a progressive decline in TJ expression along the proximal-to-distal axis of the HIV+ colon, in contrast to the relatively flat or increasing gradients observed in the healthy intestine. Finally, concurrent alterations in the transcriptional pattern of non-TJ epithelial-specific genes suggest that tight junctional down-regulation in the HIV+ gut occurs as part of an overall intestinal epithelial disruption through modified regulation of transcription.
The dramatic and rapid depletion of CD4+ T cells from the intestinal mucosa during HIV and SIV infection led to the speculation that injury to the immune component of the intestinal lamina propria is permissive for increased translocation of microbial products into systemic circulation (183). Indeed, mucosal immunodeficiency begins in the early phase of HIV or SIV infection and is characterized by a profound and selective depletion of CD4+ T cells within days of infection (118) and preferential loss of IL-22 and IL-17 producing T cells (125, 127), impaired neutrophil recruitment and macrophage phagocytic function (148), and local mucosal inflammation (135, 136, 193). Recent literature indicates that mucosal immunodeficiency and structural epithelial deterioration concurrently drive microbial translocation and HIV progression (194). Intestinal epithelial disruption occurs in early SIV infection through epithelial cell apoptosis, secondary to interactions with the intestinal epithelial cell-associated alternative SIV coreceptor GPR15/Bob (159). In humans, epithelial dysregulation begins in primary HIV infection and persists into the chronic phase, with down-regulation of genes involved in epithelial maintenance, growth and differentiation, as well as metabolic and digestive functions (146, 147). Our results, while highlighting the significant decrease in TJ mRNA and protein expression in chronic HIV infection, reveal a broader change in intestinal epithelial cell transcriptional regulation, even in the setting of viremic control. Our findings are in agreement with the notion of overall epithelial dysregulation in chronic HIV infection, rather than a specific disturbance in TJs, leading to structural deterioration of the epithelial barrier on the molecular level.
Evidence for epithelial barrier disruption contributing to SIV pathogenesis has been demonstrated in the non-human primate model. A comprehensive survey of the entire colonic tissue revealed epithelial barrier breakdown in chronic (non-AIDS and AIDS), untreated SIV-infected rhesus macaques, varying from multifocal colonic epithelial disruptions to epithelial loss and overt ulceration (148). This breakdown in the epithelium is associated with in situ LPS infiltration into the lamina propria and local immune activation (148). Our results demonstrating an intact intestinal epithelium at the endoscopic and light microscopic level with no decrease in epithelial barrier coverage in the HIV+ colon is more in agreement with a recent study showing microbial translocation in situ with little morphological evidence of human intestinal epithelial breaches (179). Together these reports suggest distinct mechanisms between humans and primates for intestinal barrier loss. However, we acknowledge that the studies in humans are limited by a random sampling of the colonic mucosa via a restricted number of biopsy samples. Discrete sites of epithelial barrier loss or ulceration, not visible to the clinician, may have been missed in our study. Thus, we do not dismiss the possible contribution of epithelial barrier breakdown, at the cellular level, to HIV-associated microbial translocation, in addition to epithelial TJ down-regulation.

While global intestinal epithelial cell function is compromised during HIV infection, in this report we focus on decreased TJ expression in the HIV+ intestinal epithelium as promoting the translocation of microbial products to the lamina
propria and systemically, extending in vitro evidence that demonstrated TJ downregulation as a response of genital and intestinal mucosal epithelium to direct HIV-1 exposure (195). In contrast, Smith et al. (179) reported increased levels of claudin-2 protein in the ileum and rectum during chronic HIV infection. Similarly, Epple et al. (150) demonstrated mucosal barrier defects in the duodenum of HIV-infected individuals including increased mannitol permeability, decreased claudin-1, and increased claudin-2 protein expression. These earlier results were obtained from untreated viremic patients. Our findings are complementary, except for claudin-2, and expand the current understanding of HIV-associated TJ disruptions in patients with effective viral suppression. While Epple et al. showed that duodenal mucosal barrier changes are reversed in the small intestine of antiretroviral-treated patients, we systematically probed for TJ disruptions along the length of the colon, highlighting the greatest down-regulation of TJs, both at the transcriptional and translational levels, in the most distal, descending portion of the colon. These distinct differences in response of the small and large intestines to HIV infection and ART highlight the contributions of apoptosis in the former and paracellular permeability in the latter (103, 159, 161), suggesting unique pathologies of HIV damage along the alimentary canal, and must always be tempered by the sample size and heterogeneity of the populations studied.

As noted above, our previous clinical study indicates that the increase in small intestinal permeability seen in virally-suppressed, HIV-infected individuals is
primarily a result of epithelial cell damage. Consistent with these clinical results, in this report we find that the TJ complex is down-regulated only in the colon, not the small intestine. In the pre-antiretroviral era, advanced HIV disease was associated with small intestinal structural defects, including villous atrophy and crypt hyperplasia (196). More recently, multiple studies have shown correlation between disease progression, circulating microbial products due to translocation, and plasma levels of intestinal fatty acid binding protein (I-FABP) (97, 156, 157), which is a marker of small intestinal epithelial cell apoptosis (155). These results collectively stress the importance of epithelial cell damage through apoptosis as the predominant mechanism for loss of small intestinal barrier integrity.

We also present results pertaining to perturbations in the expression pattern of various epithelial-specific transcripts along the proximal-to-distal axis of the HIV and healthy gut, from the terminal ileum to the descending colon. The distally increasing pattern observed for ZO-1, for instance, in the healthy population is consistent with findings from gene expression mapping along the normal colon identifying transcripts that are differentially expressed from proximal-to-distal segments, a subset of which demonstrates a gradual monotonic change in expression levels, characteristically an increase toward the distal colon (197). The other subset identified consisted of transcripts with a dichotomous proximal versus distal colon expression pattern. We postulate that such gene expression profiles along the longitudinal axis of the gut is programmed by embryonic development and established by interactions with the external environment. The small and large
intestines are distinct organs in the alimentary canal, with unique functions of digestion and absorption of nutrients, and reabsorption of water and electrolytes, respectively. Consecutive anatomical regions within the colon also perform distinct functions, with the proximal portion relatively more involved in solidification of fecal contents as compared to the distal portion, which is responsible for the transient storage of feces (198). Indeed, such functional differences mirror and are determined by the various intrinsic differences between the terminal ileum, proximal, and distal colon in terms of embryologic origin, morphology, and proliferative capacity (199). Interactions with luminal content, dependent on diet, and microbiota further modify the epithelium, the degree to which is dependent on colonic transit time, shown to be slowest in the proximal colon (200). In addition, luminal microbiota shifts along the length of the colon in quantity and diversity (201), and metaproteome analysis of the colonic mucosal-luminal interface demonstrate significant anatomic region-related differences in host-microbial interactions (202). Such variations have pathological consequences, with the classic “two-colon concept” of colorectal carcinoma describing striking differences in clinical, molecular, and epidemiological features of tumors in the proximal and distal colon (203), and more recent data revealing a gradual increase in the frequency of the CpG island methylator phenotype, microsatellite instability, LINE-1 methylation, as well as BRAF, KRAS, and PIK3CA mutations in tumors along the bowel at different colonic subsites (204).
We acknowledge that our observed HIV-associated intestinal TJ downregulation and gene expression pattern alterations are likely a result of complex interactions between the gut environment and intestinal epithelial cells. While we cannot fully address all of the sources of heterogeneity with this limited sample size, we sought to minimize the potential impact of identifiable confounders, while acknowledging that our methodology involves an appreciable number of multiple comparisons, thus reducing our statistical power. While there can be small differences in intestinal transcript expression patterns in females versus males (198), our conclusions are maintained in age-matched male subjects in our cohorts, eliminating age and gender as confounding factors for the observed HIV-associated changes. Using a similar strategy, we were able to eliminate detectable viral load as a potential confounder. Gene expression profile along the colonic mucosa is modulated after colectomy surgery to correspond to the new (proximal or distal) location (198), providing evidence that colonic transcript expression levels along the proximal-to-distal axis are responsive to pathophysiological perturbations or insults. In light of the importance of the specific microbiome composition on maintaining the gut’s structural barrier, as well as local and systemic immunity (115), alterations of the luminal microbiome (dysbiosis) in association with HIV infection (193, 205, 206) in a colonic subsite-specific manner would influence local epithelial function and transcriptional activity, resulting in differential proximal-to-distal TJ component expression patterns between the HIV+ and healthy colon. It is important to recognize that luminal and mucosa-associated enteric bacteria represent two distinct populations, with the
mucosa-associated population displaying local heterogeneity (201, 207). Studies on stool microbiome reveal increased diversity and altered composition of microbiota in the HIV gut – changes that persist in the ART-treated patient (138, 206). Similarly, dysbiotic mucosal-adherent microbiota are observed in the ART-treated HIV-infected patient (208), paving the way to a systematic study of dysbiosis along the longitudinal axis of the HIV gut, that may directly influence intestinal epithelial cell metabolism and function. In addition, expansion of the enteric virome, associated with a previously undescribed set of viruses, in pathogenic SIV infection (205) raises the possibility of viral contributions to HIV progression and intestinal pathology.

HIV-associated enteric dysbiosis may also have consequences on the lamina propria T cell populations. Total bacterial load in the stool of HIV-infected subjects negatively correlates with duodenal T cell activation, while the proportions of *Enterobacteriales* and *Bacteriodales* are associated with duodenal CD4+ T cell loss (138). Enrichment of gut bacteria that catabolize tryptophan via the kynurenine pathway (208) may inhibit the differentiation of Th17 cells, a T cell subset important in maintaining mucosal immunity shown to be depleted in the HIV lamina propria. Recent data from our laboratory demonstrate that T cell activation can modulate intestinal epithelial barrier permeability, suggesting possible contributions of lamina propria T cells on intestinal epithelial TJ regulation.
In the ART era, non-AIDS associated complications are now a major clinical focus and concern. Systemic inflammation, possibly initiated by circulating microbial products from the gut lumen, is a likely contributing factor to HIV morbidity. Our demonstration of inverse correlations between markers of immune activation and TJ transcript levels and a proximal-to-distal gradient of decreased TJ expression in the HIV colon provide a needed mechanism for increased intestinal permeability in the well-controlled, ART-treated HIV-infected patient, which contributes to microbial translocation and systemic inflammation.
Figure 2-1. Cohort Diagram illustrating the assignment of intestinal biopsy and plasma samples to each analytical method.
Figure 2-2. Relative abundance of epithelial cells in intestinal biopsies is not decreased in HIV+ individuals. Fixed paraffin-embedded intestinal biopsy sections were stained with DAPI and imaged. (A) Representative images of biopsy sections from a healthy control (left panel) and an HIV+ individual (right panel) are shown with nuclei staining in blue. Abundance of epithelial (EPI) cells, represented by either the area occupied by epithelial cell nuclei (arrow) or the length (Length) of epithelial-luminal border (white line, inset), was compared to the abundance of lamina propria (LP) cells indicated by area occupied by LP cell nuclei (arrowhead). (B) Relative abundance of epithelial cells expressed as the ratio between EPI/LP nuclei area or Length/LP nuclei area, and (C) epithelial cell packing density denoted by number of epithelial nuclei per 100 µm of epithelial-luminal border, were determined in the ascending, transverse, and descending colon and compared between healthy controls and HIV+ individuals. Box-and-whisker plots were constructed using Tukey’s method, where black dots identify the outliers (n = 5 for both cohorts).
Figure 2-3. Subcellular localization of tight junctional proteins, occludin and ZO-1, is unaltered in the HIV colon. Fixed, frozen biopsy sections of the ascending, transverse, and descending colon in HIV+ individuals and healthy controls were stained for occludin or ZO-1 (green), and counterstained with DAPI (blue). Representative images at the luminal surface (A) and in the crypt (B) of the ascending, transverse, and descending colon are shown.
Figure 2-4. Tight junctional transcripts are decreased in the colon, not the terminal ileum, of HIV+ individuals. Calibrated normalized relative quantities (CNRQ) of ZO-1, occludin, claudin-2, and claudin-4 transcripts were determined in total RNA isolated from (A) colonic (n = 9 for HIV+, n = 13 for healthy controls) and (B) terminal ileal (n = 10 for HIV+, n = 8 for healthy controls) biopsies of HIV+ individuals and healthy controls. Levels are normalized to β-actin and eef1α1 expression. Box-and-whisker plots were constructed using Tukey’s method, where black dots identify the outliers. Statistical analysis was performed on all data points, including the outliers (* p<0.05, ** p<0.01 between HIV+ and healthy controls).
Figure 2-5. Tight junctional transcript levels in HIV+ individuals decrease progressively from proximal-to-distal colon. Total RNA was isolated from biopsies obtained from the terminal ileum, as well as the ascending, transverse, and descending colon, of HIV+ individuals (red square) and healthy controls (blue circle). CNRQ of (A) ZO-1, (B) occludin, (C) claudin-2, and (D) claudin-4 transcripts were compared at each location. The Kruskal-Wallis test, a non-parametric version of the one-way ANOVA, was performed, with p-values for the HIV+ (HIV) and control (HC) cohorts shown in the top left of each panel, after which $r^2$ and p-value were determined when appropriate using a post-test for linear trend for each transcript across location in the two cohorts separately. Linear regression is shown as a representation (For HIV+ $n = 9$ at all colonic locations and $n = 10$ at the terminal ileum; for healthy controls $n = 8$ for terminal ileum, $n = 7$ for ascending colon, and $n = 9$ at transverse and descending colon; # $p<0.08$, * $p≤0.05$, and ** $p<0.01$ between HIV+ and healthy controls).
Figure 2-6. Tight junctional protein levels are decreased in the descending colon of virally-suppressed HIV+ individuals. Total protein lysate extracted from descending colonic biopsies of HIV+ individuals and healthy controls were immunoblotted for occludin, claudin-2, claudin-4, cytokeratin-18, GAPDH, and β-actin. (A) A representative blot is shown, with H denoting an HIV+ sample, C denoting a healthy control sample, and N denoting the inter-gel normalizing control sample. (B) Specific bands within the linear density range for occludin, claudin-2, and claudin-4 were quantitated by densitometric analysis, and compared between virally-suppressed HIV+ individuals and healthy controls. Target protein levels were normalized against cytokeratin-18 protein levels. Box-and-whisker plots were constructed using Tukey’s method, where black dots identify the outliers. Statistical analysis was performed on all data points, including the outliers (For HIV+ n = 10 for all proteins; For healthy controls n = 13 for occludin and claudin-4, n = 12 for claudin-2; # p<0.07, * p<0.05).
**Figure 2-7. Human β defensin-3 and E-cadherin expression varies differentially from proximal-to-distal HIV+ intestine.** Total RNA was isolated from intestinal biopsies, and CNRQ of transcripts for (A) human β defensin-3 \( (n = 11 \text{ for HIV+}, n = 12 \text{ for control}) \) and (B) E-cadherin \( (n = 9 \text{ for HIV+}, n = 13 \text{ for control}) \) were measured in the colon of HIV+ individuals and healthy controls (upper panel). Box-and-whisker plots were constructed using Tukey’s method, where black dots identify the outliers. Statistical analysis was performed on all data points, including the outliers. Transcript levels were also compared between HIV+ individuals (red square) and healthy controls (blue circle) at each location: terminal ileum, ascending, transverse, and descending colon (lower panel). The Kruskal-Wallis test, a non-parametric version of the one-way ANOVA, was performed for each transcript in the HIV+ (HIV) and control (HC) cohorts separately, with \( p \)-values shown in the top left of each panel. Linear regression is
shown as a representation (hBD3: For HIV+ n = 8 for transverse colon, and n = 9 for ascending and descending colon; for healthy controls n = 6 for ascending colon, and n = 7 for transverse and descending colon; E-cadherin: For HIV+ n = 9 for the terminal ileum and ascending colon, n = 7 for transverse colon, and n = 8 for descending colon; for healthy controls n = 9 for the terminal ileum and descending colon, and n = 7 for ascending and transverse colon; # p<0.06, * p<0.05, and ** p<0.01 between HIV+ and healthy controls).
Figure 2-8. Microbial translocation and systemic immune activation marker levels inversely correlate with colonic TJ transcript levels. (A) Plasma samples from HIV+ individuals and healthy controls were thawed and analyzed by ELISA to measure levels of soluble CD14 (sCD14). Medians are denoted, with bars representing the interquartile range (n = 21 for both cohorts; # p<0.07). Plasma levels of (B) the immune activation marker sCD14 and (C) the microbial product LPS inversely correlate with transcript levels of claudin-4 and claudin-2, respectively, in the descending colon of subjects from both the HIV+ and healthy control cohorts. Spearman’s rank test was used to determine correlations. The black lines represent the linear regression estimate.
Figure 2-S1. Tight junctional transcripts are decreased in the colon, not the terminal ileum, of male HIV+ individuals. Calibrated normalized relative quantities (CNRQ) of ZO-1, occludin, claudin-2, and claudin-4 transcripts were determined in total RNA isolated from (A) colonic (n = 8 for HIV+, n = 7 for healthy controls) and (B) terminal ileal (n = 8 for HIV+, n = 6 for healthy controls) biopsies of male HIV+ individuals and healthy controls. Levels are normalized to β-actin and eef1α1 expression. Box-and-whisker plots were constructed using Tukey’s method, where black dots identify the outliers. Statistical analysis was performed on all data points, including the outliers (* p<0.05, ** p<0.01 between HIV+ and healthy controls).
Figure 2-S2. Tight junctional transcripts are decreased in the colon, not the terminal ileum, of virally-suppressed HIV+ individuals. Calibrated normalized relative quantities (CNRQ) of ZO-1, occludin, claudin-2, and claudin-4 transcripts were determined in total RNA isolated from (A) colonic (n = 8 for HIV+, n = 13 for healthy controls) and (B) terminal ileal (n = 7 for HIV+, n = 8 for healthy controls) biopsies of virally-suppressed HIV+ individuals and healthy controls. Levels are normalized to β-actin and eef1α1 expression. Box-and-whisker plots were constructed using Tukey’s method, where black dots identify the outliers. Statistical analysis was performed on all data points, including the outliers (* p<0.05, ** p<0.01 between HIV+ and healthy controls).
Figure 2-S3. Tight junctional protein levels are decreased in the descending colon of HIV+ individuals. Total protein lysate extracted from descending colonic biopsies of HIV+ individuals and healthy controls were immunoblotted for occludin, claudin-2, claudin-4, cytokeratin-18, GAPDH, and β-actin. Specific bands within the linear density range for occludin, claudin-2, and claudin-4 were quantitated by densitometric analysis, and compared between HIV+ individuals (including those with detectable viral load) and healthy controls. Target protein levels were normalized against cytokeratin-18 protein levels. Box-and-whisker plots were constructed using Tukey’s method, where black dots identify the outliers. Statistical analysis was performed on all data points, including the outliers ($n = 13$ for both cohorts, except $n = 12$ for healthy controls in claudin-2; # $p \leq 0.07$).
Figure 2-S4. Tight junctional protein levels are decreased in the descending colon of male HIV+ individuals. Total protein lysate extracted from descending colonic biopsies of male HIV+ individuals and healthy controls were immunoblotted for occludin, claudin-2, claudin-4, cytokeratin-18, GAPDH, and β-actin. Specific bands within the linear density range for occludin, claudin-2, and claudin-4 were quantitated by densitometric analysis, and compared between male HIV+ individuals and healthy controls. Target protein levels were normalized against cytokeratin-18 protein levels. Box-and-whisker plots were constructed using Tukey’s method, where black dots identify the outliers. Statistical analysis was performed on all data points, including the outliers (n = 11 for HIV+, n = 7 for healthy controls; # p<0.09).
Figure 2-S5. Human β defensin-3 and E-cadherin expression varies differentially in the colon of HIV+ males. Total RNA was isolated from intestinal biopsies, and CNRQ of transcripts for (A) human β defensin-3 (n = 9 for HIV+, n = 7 for healthy controls) and (B) E-cadherin (n = 8 for HIV+, n = 7 for healthy controls) were measured in the colon of male HIV+ individuals and healthy controls. Box-and-whisker plots were constructed using Tukey’s method, where black dots identify the outliers. Statistical analysis was performed on all data points, including the outliers. (** p<0.01 between HIV+ and healthy controls).
Figure 2-S6. Human β defensin-3 and E-cadherin expression varies differentially in the colon of virally-suppressed HIV+ individuals. Total RNA was isolated from intestinal biopsies, and CNRQ of transcripts for (A) human β defensin-3 ($n = 9$ for HIV+, $n = 12$ for healthy controls) and (B) E-cadherin ($n = 8$ for HIV+, $n = 13$ for healthy controls) were measured in the colon of virally-suppressed HIV+ individuals and healthy controls. Box-and-whisker plots were constructed using Tukey’s method, where black dots identify the outliers. Statistical analysis was performed on all data points, including the outliers (* $p<0.05$ between HIV+ and healthy controls).
Table 2-1. Primers used for Real Time qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZO-1</td>
<td>Forward: AGGGCCCAAGCCTGCAGAGT</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGAGGGACAGCTGCAGCACC</td>
</tr>
<tr>
<td>Occludin</td>
<td>Forward: CCACGCCGGTTCTGAAGTGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCACAGGACTCGCCGCACTG</td>
</tr>
<tr>
<td>Claudin-2</td>
<td>Forward: GGGCACAAGTGGTTGCATGCT</td>
</tr>
<tr>
<td></td>
<td>Reverse: ATGGCCTGGGCAGCCTGAT</td>
</tr>
<tr>
<td>Claudin-4</td>
<td>Forward: CTCTGTCCTGGCTCACTGCT</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGGAGGCCACCACGCGATTG</td>
</tr>
<tr>
<td>hBD-3</td>
<td>Forward: ATCTTCTGTATTCTCTCTCTCTG</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCACTTGGCCGATCTCTCTCTC</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Forward: CGACCCAACCCAAAGATCTATC</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGGTGTCTACTTTGTCTATTG</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: CAGGCACCAGGGCGTGATGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGATGCTCGCGCTCGCATGGG</td>
</tr>
<tr>
<td>eef1A1</td>
<td>Forward: CTGTGGCTCGATTCTCTGTT</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCGTTCTTACCACACTGATT</td>
</tr>
<tr>
<td>Demographic/Clinical Parameter</td>
<td>Control (n = 35)</td>
</tr>
<tr>
<td>------------------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Age, median years (IQR)</td>
<td>56 (50 - 61)</td>
</tr>
<tr>
<td>Female sex, %</td>
<td>40</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
</tr>
<tr>
<td>White/Other, %</td>
<td>31.4</td>
</tr>
<tr>
<td>Black, %</td>
<td>60</td>
</tr>
<tr>
<td>Hispanic, %</td>
<td>---</td>
</tr>
<tr>
<td>Unknown, %</td>
<td>8.6</td>
</tr>
<tr>
<td>Duration of Disease, median years (IQR)</td>
<td>---</td>
</tr>
<tr>
<td>CD4 nadir, median cells/µl, (IQR)</td>
<td>---</td>
</tr>
<tr>
<td>Duration since CD4 nadir, median years (IQR)</td>
<td>---</td>
</tr>
<tr>
<td>Viral load, median copies/ml (IQR)</td>
<td>---</td>
</tr>
<tr>
<td>CD4 count, median cells/ µl (IQR)</td>
<td>---</td>
</tr>
<tr>
<td>Duration of ART, median years (IQR)</td>
<td>---</td>
</tr>
<tr>
<td>Duration of continuous ART, median years (IQR)</td>
<td>---</td>
</tr>
</tbody>
</table>
**Table 2-3.** Average Fluorescence Intensity for Occludin and ZO-1 staining of colonic surface epithelium sections

<table>
<thead>
<tr>
<th>Colonic Surface</th>
<th>Occludin</th>
<th>ZO-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>HIV</td>
</tr>
<tr>
<td>Ascending</td>
<td>1085.58</td>
<td>1186.83</td>
</tr>
<tr>
<td>Transverse</td>
<td>815.1</td>
<td>674</td>
</tr>
<tr>
<td>Descending</td>
<td>753.4</td>
<td>1079.6</td>
</tr>
</tbody>
</table>

*SD refers to the standard error of the difference in average fluorescence intensity between the control and HIV cohorts using the mixed-effects model.*
Table 2-4. Average Fluorescence Intensity for Occludin and ZO-1 staining of colonic crypt epithelium sections

| Colonic Crypt | Occludin | | ZO-1 | |
|---------------|----------|---|-----|---|---|---|---|
|               | Control  | HIV| SD* | p  | Control | HIV| SD* | p  |
| Ascending     | 1366.18  | 1616.44| 217.4 | 0.402 | 756.8  | 768.7| 120.3 | 0.958 |
| Transverse    | 849.6    | 1031.9| 162.9 | 0.402 | 1177.6 | 1913.2| 132.3 | <0.0012 |
| Descending    | 1102.5   | 1348.7| 166.9 | 0.402 | 896.1  | 1250.4| 143.2 | 0.102 |

* SD refers to the standard error of the difference in average fluorescence intensity between the control and HIV cohorts using the mixed-effects model.
CHAPTER 3

MECHANISMS FOR TIGHT JUNCTION DYSREGULATION IN THE ART-
TREATED HIV-INFECTED PATIENT
3.1 PREFACE

In Chapter 2, I demonstrated molecular evidence of epithelial dysfunction in the intestinal biopsies of ART-treated HIV+ individuals, and further characterized the associated TJ disruption as a progressive transcriptional downregulation from proximal-to-distal colon in this patient population. We turn now to the mechanisms via which TJ dysregulation can occur in the ART-treated HIV+ patient, which will be the focus of this chapter and Chapter 4.

We re-examined our data to determine whether the trend for progressive proximal-to-distal colonic decrease of TJ transcript levels exist in each individual patient included in the HIV+ cohort (Figure 3-1). While we observe such a trend for all four TJ transcripts studied (ZO-1, occludin, claudin-2, claudin-4) in the HIV cohort as a whole, clear progressive proximal-to-distal decreasing trends along the intestines are only apparent in a select few patients (Figure 3-1A-D). Such results are not surprising given the heterogeneity inherent to the HIV+ population.

In comparing between the ART-treated HIV+ cohort and the uninfected healthy cohorts, it is evident that subjects in the two cohorts differ in multiple ways, all of which may contribute to the development of TJ dysregulation. We raise the possibility that the epithelial dysfunction and TJ downregulation observed in the colon of ART-treated HIV+ individuals can be driven by one or more of the following: 1) side effects of the long-term ART regimen, 2) the persistent presence of HIV virions and pools of latently-infected cells, and 3) the loss of intestinal
mucosal immune homeostasis that persists in the virally-suppressed HIV+ individual.

3.2 CONTRIBUTION OF PROTEASE INHIBITORS TO COLONIC EPITHELIAL TJ DOWNREGULATION

Gastrointestinal symptoms including diarrhea, nausea, vomiting are frequently reported as side effects following ART, and have been cited as the most frequent reason for discontinuation of ART (55). Comparing between the various ARV classes, PIs are generally the least well-tolerated, with diarrhea reported to be the most common adverse effect. Around 20% of patients on PIs experience diarrhea compared to around 5% of patients on other ARVs such as NRTIs and NNRTIs (209). We speculate that PI use may adversely affect intestinal barrier function. PI usage is also associated with metabolic complications including insulin resistance and dyslipidemia – symptoms that are closely associated with, and may perhaps contribute to, the development of non-AIDS complications. As such, we tested the hypothesis that ART, in particular PI usage, contributes to the development of intestinal epithelial TJ downregulation in the ART-treated HIV+ cohort.

As summarized in Table 3-1, all patients included in our ART-treated HIV+ cohort are on combination ART consisting of at least three different ARVs at the time of study, with all patients except one taking two or more NRTIs. 16/31 (52%) of patients are on an NNRTI-based regimen, and out of the 15/31 (48%) of patients
on a PI-based regimen, all but one is taking a PI boosted with ritonavir. Three patients are also on an additional IN inhibitor.

We compared the levels of TJ transcripts in the descending colon, the intestinal location demonstrating the greatest drop in TJ transcript levels, of treated patients on a PI-based regimen, patients on a non-PI-based regimen, and uninfected healthy controls (Figure 3-2). Compared to healthy controls, patients on a non-PI-based regimen show significantly decreased ZO-1, occludin, and claudin-2 transcript levels in the descending colon. No significant differences in transcript levels of ZO-1, occludin, claudin-2, or claudin-4 are observed between treated patients on a PI-based regimen versus a non-PI-based regimen, suggesting that PIs do not further alter TJ transcript levels in the descending colon.

Given the limited size of each cohort, we present the above analysis as a preliminary examination of whether ART, in particular PI usage, is associated with intestinal epithelial dysfunction. We do not find evidence that PI usage alters intestinal epithelial TJ transcript levels. This finding prompts us to further consider other mechanisms for intestinal epithelial dysfunction and TJ downregulation in the ART-treated HIV+ patient.

### 3.3 MECHANISMS FOR IMMUNE-MEDIATED TJ REGULATION

Mechanisms of TJ Regulation
TJs are dynamic structures that are continuously being assembled or disassembled via addition of newly produced protein components and removal of components by endocytosis. Different components have different stability with TJ strand: ZO-1 and occludin are highly mobile between the cell membrane and the cytosol, while claudins are stably integrated (168, 210). It thus follows that the TJ is a highly plastic structure that can be acutely regulated via several mechanisms (Figure 3-3).

Anchoring of TJs to the perijunctional actomyosin ring allows for TJ regulation via cytoskeleton changes. Microfilament depolymerization results in condensation of the actomyosin ring, which reduces epithelial barrier function in cultured monolayers. Occludin endocytosis is also shown to play a role in barrier dysfunction following microfilament depolymerization (168). Myosin light chain kinase (MLCK) was shown to be a key regulator of TJ, via phosphorylation of myosin light chain (MLC) that triggers actomyosin contraction, resulting in increased TJ permeability (165). This mechanism plays a role in the physiological process of Na⁺-nutrient transport induced intestinal paracellular permeability (211), as well as following pathophysiological stimuli including enteropathogenic *Escherichia coli* infection (212, 213), TNF-α induced barrier loss in IBD and GVHD (214, 215), and T cell-induced acute diarrhea (216).

Endocytosis trafficking of TJ proteins is another common mechanism for TJ remodeling. Concurrent with perijunctional actomyosin ring contraction, T cell
activation or TNF-α also induced occludin endocytosis via caveolar endocytosis (217, 218). Internalization of occludin and other TJ components has also been reported to occur via other pathways of endocytosis, following a wide range of stimuli (165, 219), which may further initiate endocytosis of interacting partners on neighboring cells (220).

Post-translational modifications influence the turnover of various transmembrane strand-forming and intracellular TJ proteins, leading to alterations in TJ assembly, as well as pore density and characteristics (171). Phosphorylation/Dephosphorylation of C-terminal domains of occludin and claudins were shown to alter interactions with TJ adaptor proteins, an example being EphA2 phosphorylation of claudin-4 disrupting interactions with ZO-1 (221), resulting in increased protein internalization and turnover (222). Other modifications such as palmitoylation (223) and SUMOylation (224) have also been shown to modulate claudin localization to TJs.

Finally, TJ composition can be altered by transcriptional and translational modulation, although the exact molecular mechanisms for such increases are not thoroughly understood. Transcription factor GATA-4 and regulator symplekin were found to regulate claudin-2 and ZO-2 expression (225-227). Food component quercetin increases claudin-4 expression (228). Claudin-2 expression is increased in colonic biopsies of ulcerative colitis patients (229); the effect is attributed to IL-13 induced selective increase in claudin-2 expression (230).
Immune-mediated Epithelial TJ Regulation

Situated at the lateral membranes of epithelial cells, TJs are exposed to a multitude of extracellular stimuli that have the potential for TJ regulation via the mechanisms described above (231). Such interactions can occur through: 1) direct interactions of TJ proteins to other cellular proteins: lymphocyte function-associated antigen 1 (LFA-1) in leukocytes binds to JAM-1 in endothelial cells and facilitates leukocyte transepithelial migration (232, 233); 2) direct interactions with external antigens: *Clostridium perfringens* enterotoxin directly binds to claudin-3 and -4 and causes their removal from TJs (234, 235); 3) indirect effects of soluble factors: the physiological TJ regulator zonulin, the secretion of which is upregulated by the zonula occludens toxin secreted by *Vibrio cholera* (163), activates epithelial growth factor receptor (EGFR) to cause TJ protein phosphorylation and TJ disassembly (236); multiple cytokines and growth factors also enhance or disrupt TJs; 4) cellular stresses (237).

In the GI tract, the close proximity of the GALT and the epithelial barrier permits complex cross-talk between IEC and mucosal immune cells that mutually regulate cellular homeostasis and function. Intestinal immune cell – IEC regulation has been shown to promote differentiation and activation of the immune cells, as well as differentiation and repair of the epithelial barrier (104, 238). Presence of the gut microbiome and dietary antigens engage both compartments' response to foreign antigens, which promote further cross-talk (104). Modification of the intestinal
barrier, both immunologically and structurally, can be a consequence of such IEC-immune cell interactions, with epithelial TJS playing a role in mediating these effects.

In general, pathogenic bacteria disrupt the intestinal barrier, while most dietary nutrients restore the barrier. In delineating how intestinal immune cells may modulate intestinal TJ permeability, much investigation has focused on the TJ regulatory effects of individual cytokines and other secreted factors of immune cells. According to a recent survey of cytokines and growth factors that modulate intestinal epithelial TJ, TNF-α, IFN-γ, Interleukin (IL)-1β, IL-4, IL-6 and IL-13 increase TJ permeability, while TGF-β, IL-10, IL-17, and epithelial growth factor (EGF) decrease intestinal TJ permeability. Barrier dysfunction results from MLCK-induced actomyosin contraction, TJ protein internalization, or increased expression of claudin-2, while restoration of TJ barrier is achieved either through increasing expression of TJ components, or inhibiting barrier dysfunction from other stimuli (239). In particular, it should be noted that the TJ pore and leak pathways are differentially regulated. While TNF-α causes occludin internalization, resulting in an increase in permeability of the leak pathway, IL-13 up-regulates claudin-2 and decreases TER by increasing permeability of the pore pathway to cations, without affecting the flux of macromolecules (169).

In considering the pathogenesis of systemic and intestinal autoimmune and inflammatory diseases critically mediated by known inflammatory cytokines, the
cytokine’s effects on intestinal TJ permeability are often exacerbated, and are implicated to play roles in both the initiation and development of the disease. In IBD, intestinal barrier impairment has been demonstrated in the patient’s non-involved intestinal segments (240) and in his/her healthy first-degree relatives (241), suggesting that the barrier impairment may initiate the disease process. Increased intestinal levels of TNF-α was shown to be clinically significant in the intestinal inflammatory process, and accordingly, increased intestinal permeability and intestinal TJ alterations resembling that caused by TNF-α were demonstrated, with internalization of multiple TJ components, and increased claudin-2 expression (171). Similarly, in patients with Type I diabetes involving T cell-mediated autoimmune attack on pancreatic β cells, increased intestinal levels of TNF-α and IFN-γ are implicated to play a role in causing the observed increase in intestinal permeability.

There exist, however, a paucity of studies employing a less reductionist approach in examining immune-mediated epithelial TJ regulation. Given that cytokines are rarely secreted in isolation by immune cells after stimulation, it is perhaps more physiologically relevant to investigate the aggregate effects of multiple cytokines on TJ regulation, and taking one step further, to examine TJ regulation mediated by immune cells – a phenomenon that would involve effects downstream of 1) released factors following immune cell – epithelial cell interaction, and 2) signaling pathways activated by direct contact between the two cell types.
In the HIV gut, loss of immune homeostasis is multifaceted, as discussed in Chapter 1, involving multiple cell types and resulting in a general inflammatory mucosal microenvironment. In particular, the quantity and composition of the mucosal T cell compartment is drastically altered. We propose that such HIV-associated loss of intestinal mucosal immune homeostasis disrupts epithelial TJ permeability maintained by homeostatic intestinal immune cell – IEC interactions in the healthy gut. To examine this hypothesis, we must first gain an understanding of how intestinal immune cells regulate IEC TJs and paracellular permeability in the disease-free setting. Accordingly, we examined the epithelial permeability and TJ consequences following the co-culture of IEC monolayers with activated T cells, in an in vitro model mimicking the healthy gut mucosa. We hereby present our findings in Chapter 4.
Figure 3-2. No significant differences in descending colonic tight junctional transcript levels were observed between treated HIV+ individuals on PI-based and non-PI based ART regimens. Total RNA was isolated from descending colonic biopsies of healthy controls and HIV+ cohorts, and CNRQ of (A) ZO-1, (B) occludin, (C) claudin-2, and (D) claudin-4 were measured. CNRQ of each transcript are shown for healthy controls (n = 9), ART-treated HIV+ individuals on PI-based regimen (n = 4), and ART-treated HIV+ individuals on non-PI-based regimen (n = 5). The Kruskal-Wallis test, a non-parametric version of the one-way ANOVA, was performed with the Dunn’s post-hoc multiple comparison test, for comparisons between all three patient groups (* p < 0.5).
**Figure 3-3 Mechanisms of TJ regulation.** TJ regulation is achieved by: 1) Actomyosin contraction, 2) Endocytosis, 3) Post-translational modification, and 4) Transcription/translational control.
Table 3-1. ART Regimen of ART-treated HIV+ Individuals.
ART regimen for each patient at the time of biopsy are listed and further classified into their corresponding drug classes.

<table>
<thead>
<tr>
<th>Patient</th>
<th>ART Regimen</th>
<th>NRTI</th>
<th>NNRTI</th>
<th>PI</th>
<th>IN Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>71</td>
<td>EFV/FTC/TDF</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>EFV/FTC/TDF</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>76</td>
<td>ATV/r, TDF/FTC</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>101</td>
<td>EFV, FTC/TDF</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>113</td>
<td>ATV/r, TDF/FTC</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>117</td>
<td>FPV/r, TDF/FTC</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>152</td>
<td>ATV, ABC/3TC</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>154</td>
<td>EFV/FTC/TDF</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>156</td>
<td>ATV/r, TDF/FTC, RAL</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>158</td>
<td>ATV/r, TDF/FTC</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>167</td>
<td>ETR, RAL, ABC, ddl</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>170</td>
<td>EFV/FTC/TDF</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>79</td>
<td>LPV/r, ABC, TDF</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>82</td>
<td>ABC, EFV/FTC/TDF</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>EFV/FTC/TDF</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>99</td>
<td>EFV/FTC/TDF</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>ATV/r, TDF/FTC</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>131</td>
<td>ATV/r, ddl, TDF/FTC</td>
<td>3</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>133</td>
<td>EFV/FTC/TDF</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>EFV/FTC/TDF</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>153</td>
<td>ATV/r, TDF,RAL</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>176</td>
<td>ATV/r, ABC, TDF</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>177</td>
<td>ATV/r, 3TC, TDF</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>188</td>
<td>DRV/r, ABC/3TC, EFV</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>211</td>
<td>ATV/r, FTC, TDF</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>217</td>
<td>ATV/r, ABC/3TC</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>221</td>
<td>NVP, TDF/FTC</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>226</td>
<td>EFV/FTC/TDF</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>227</td>
<td>EFV/FTC/TDF</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S13-14435</td>
<td>DRV/r, TDF/FTC</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>179</td>
<td>EFV, ABC/3TC/ZDV</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: NRTI, Nucleoside/nucleotide reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; IN, integrase; EFV, efavirenz; FTC, emtricitabine; TDF, tenofovir; ATV, atazanavir; /r, ritonavir-boosted; FPV, fosamprenavir; ATV, atazanavir; ABC; abacavir; 3TC, lamivudine;
RAL, raltegravir; ETR, etravirine; ddl, didanosine; LPV, lopinavir; DRV, darunavir; NVP: nevirapine; ZDV, zidovudine
CHAPTER 4

ACTIVATED T CELLS INDUCE A BIPHASIC PERTURBATION IN INTESTINAL EPITHELIAL PERMEABILITY VIA ELEVATED CLAUDIN 2 AND 4

Acknowledgements

I would like to thank co-author David McDonald for his contribution towards performing experiments and collecting data. We would like to thank Drs. Calvin Cotton, Jim Dennis, Richard Jurevic, Scott Howell, Kevin Cooper, and Maria Hatzoglou for their continual collaboration and support, and Jeff Meisch and Dr. Tejpal Gill for helpful discussions and technical support.

This work was supported by grants from the National Institutes of Health (AI 076174 to ADL; T32 GM007250, TL1 TR000441, T32 GM-008803 supported CYC) and the Center for AIDS Research at Case Western Reserve University (AI 036219).
4.1 Abstract

Many immunodeficiency (e.g., HIV infection) and inflammatory diseases (e.g., Crohn’s disease) target the intestinal mucosa, causing severe and persistent T cell depletion, activation, and imbalance. We hypothesize that alterations in the gut immune microenvironment disrupts intestinal epithelial cell – mucosal T cell interactions, leading to epithelial dysregulation and altered intestinal permeability. To explore the effects of T cells on epithelial cell function, Caco-2 cells, a human colorectal adenocarcinoma cell line, were grown on transwell supports to form an intact, polarized epithelial monolayer. Purified T cells were transferred to the apical or basolateral surface of the monolayer. Integrity of the monolayer was evaluated using transepithelial resistance and paracellular inulin flux. T cell receptor and CD28 activated, but not resting, T cells on the basolateral, but not apical, surface of the monolayer induced a biphasic change in epithelial integrity, with an early strengthening of the barrier followed by a late increase in permeability, not due to epithelial cell death. Early increase in barrier function is associated with elevated claudin 2 and 4 levels, while continued T cell contact reversed claudin 2 increase.
4.2 Introduction

The single cell layer of epithelium within the gastrointestinal tract functions as a primary partition between the underlying gut-associated lymphoid tissue (GALT) of the host and the vast array of microbiota of the external world, and thus represents a crucial site of innate and adaptive immune regulation. Columnar epithelial cells, the predominant phenotype in the lower GI tract, establish and maintain a selectively permeable structural barrier, enabling fluid exchange and ion transport, while discriminating and hence restricting the passage of larger molecules. Critically, intestinal permeability is regulated by environmental, cellular, and immunological stimuli that include dietary nutrients, luminal bacteria, and secreted proteins including cytokines and growth factors (188, 242).

Although the epithelial cell plasma membrane is impermeable to hydrophilic molecules except at sites of specific transporters, there exists a means for transepithelial transport via the passive movement of material through the space between adjacent cells, known as the paracellular pathway, which is sealed by the apical junctional complex, composed of the tight junction (TJ), adherens junction, and desmosome (164, 243-245). Both desmosomes and the adherens junction, composed of cadherins, provide strong adhesive bonds that maintain cellular proximity, sustain epithelial cell polarization, maintain intercellular communication, and prevent apoptosis (246, 247). In contrast, the TJs seal the paracellular space and are the rate-limiting determinate of paracellular flux (165, 248). TJs are multi-protein organelle composed of the transmembrane claudin family of proteins and
TJ-associated Marvel proteins (occludin, tricellulin, and MarvelD3) that together define the specific characteristics of intestinal TJ permeability, along with a variety of intracellular membrane proteins and regulatory molecules that link the TJ to the actin cytoskeleton, controlling their assembly and maintenance (188). Transport across the TJ is achieved and regulated independently by the low-capacity leak and high-capacity pore pathways (168), which allow paracellular passage of large solutes, such as proteins or lipopolysaccharide, but not whole bacteria (249), and selected charged molecules smaller than 4Å (175, 250), respectively. This size and charge selectivity of the tight junction is regulated by the expression and composition of specific claudin proteins (230) and can be modified by physiological and pathophysiological stimuli, including cytokines and other immune mediators (251-253).

In the healthy individual the mucosa prevents or minimizes the translocation of non-pathogenic, commensal and pathogenic, non-commensal microflora via the combined efforts of the physical barrier established by the epithelium and immunologic protection mediated by the GALT, comprising of the macrophages, dendritic cells, IgA-secreting plasma cells, intraepithelial lymphocytes, and antigen-specific T cells that inhabit the underlying lamina propria (110). Regulation of the tight junction barrier by individual cytokines has been described in cell culture, animal models, and human disease. Among the inflammatory cytokines implicated in modifying intestinal tight junction function are TNF-α (252), LIGHT (218), IFN-γ (251), IL-1β (254), IL-13 (230), and IL-17 (255), many of which are
critical mediators of intestinal diseases that associate with a loss in barrier function. Both forms of human inflammatory bowel disease (IBD) – Crohn’s disease and ulcerative colitis, celiac disease, graft-versus-host disease, and a wide variety of animal models of colitis and ileitis are associated with striking changes in cytokine production that correlate with intestinal barrier dysfunction (188). Similarly, we and others have reported an increase in intestinal permeability and corresponding microbial translocation in SIV and HIV infections (92, 161), which involves extensive loss in intestinal immune homeostasis characterized by depletion of mucosal CD4+ CCR5+ T cells (183), infiltration of CD8+ T cells (140-142), and sustained mucosal inflammation (135, 136).

Our current understanding of immune control of intestinal permeability and regulation of TJ leak and pore pathways is limited to the study of single cytokine exposure or depletion in isolation. While such studies are absolutely critical to elucidate the details of intestinal epithelial cell – immune cell cross-talk and its role in TJ regulation, inflammatory changes in the gut mucosa are generally complex, involving dysfunction in multiple immune cell types and dysregulation of multiple cytokines, such as that recognized in IBD and HIV/SIV infection. Resultant disruption of the intestinal epithelial barrier is also not limited to isolated TJ changes, but involves gross cellular dysfunction and morphological changes.

Indeed, genetic disruption of tight junction regulatory mechanisms is insufficient to cause a change in host growth patterns, intestinal morphology, enterocyte
structure, brush border architecture, epithelial cell proliferation, migration and apoptosis, or chronic intestinal disease (256), which are pathophysiological changes accompanying intestinal TJ disruption, particularly relevant in IBD. These observations are consistent with the presence of barrier disruption in healthy relatives of patients with Crohn’s disease (241), suggesting that increased intestinal permeability in the absence of epithelial cell dysfunction is insufficient to cause intestinal disease. Conversely, an increase in intestinal permeability did induce mucosal immune cell activation, as evidenced by increased numbers of lamina propria T cells, enhanced mucosal IFN-γ, TNF-α, and IL-10 gene expression, and reorientation of dendritic cells within the lamina propria (256). This increase in mucosal immune activation may enhance barrier loss that, in turn, enables further translocation of microbial products and perpetuates the inflammatory cycle.

The observations that loss of tight junction barrier function leads to marked increases in mucosal immune activation, suggestive of a potential feed forward amplification, indicate that a more global, less focused investigation is needed. We therefore developed and validated an in vitro co-culture cellular model of the intestinal mucosa, in which we characterized the capacity of activated T cells to perturb the permeability of an intestinal epithelial cell monolayer. Our results indicate that activated T cells in close proximity to the epithelial basolateral membrane initiate a biphasic perturbation in transepithelial resistance, by inducing
both a prolonged increase in claudin 4 and a transient increase in claudin-2 expression.

4.3 Materials and Methods

Caco-2 monolayer Preparation and Transepithelial Resistance Measurements

Caco-2 cells, a human colorectal adenocarcinoma cell line, were maintained in standard cell culture conditions at 37°C and 5% CO₂ in Eagle’s Minimum Essential Medium (EMEM) (ATCC, Manassas, VA) supplemented with 20% fetal bovine serum (FBS, Hyclone, Thermo Scientific, Logan, Utah). Cells (65,000 cells/transwell) were seeded on 0.33 cm² polycarbonate membrane transwell filters (Corning, Cambridge, MA) with 0.4-µm pores, either on the top side or underside of the transwell filter, depending on experimental conditions, and allowed to grow and differentiate to form an intact, well-differentiated, polarized intestinal epithelial monolayer. Cells seeded on the underside of the transwell filter were allowed to attach for two days before the transwell filter was flipped over and cultured in the normal configuration. Media was replenished every two to three days. The integrity of the monolayer was determined via transepithelial resistance (TER) measurements obtained with an epithelial voltohmmeter under open-circuit conditions (World Precision Instruments, Sarasota, FL). TER values were expressed as the resistance multiplied by the area of the transwell filter (Ω·cm²), and were corrected by subtracting a blank reading that included the empty transwell filter and fluid resistances (around 30-50 Ω·cm²). Monolayers were
studied 20-25 days after confluence, when baseline TER was stabilized to at least 270 Ω•cm².

**Peripheral Blood Mononuclear cell Isolation and T cell Stimulation**

All study protocols were approved by the Institutional Review Board at University Hospitals Case Medical Center. After written informed consent, peripheral blood mononuclear cells (PBMCs) were isolated via Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO) density separation from venous blood of healthy donors drawn into heparin-coated tubes. T lymphoblasts were prepared by 0.5% phytohaemagglutinin (Invitrogen Life Technologies, Carlsbad, CA) stimulation for 48 h in the presence of 5 ng/ml IL-2 (R&D Systems, Minneapolis, MN), and subsequently expanded in complete media containing RPMI 1640 (Thermo Fisher Scientific, Willington, DE), 10% FBS supplemented with 5 ng/ml IL-2 for 7 d to obtain a population of ~95% pure CD3+CD45RO+ T lymphoblasts. Fresh CD3+ peripheral blood T cells (PBTs) (>90% purity by flow cytometry) were obtained by pan-T cell magnetic bead negative selection (Miltenyi Biotec, Cambridge, MA) after PBMC isolation and an adherence step for removal of monocytes and some dendritic cells.

T lymphoblasts or fresh PBTs were rested for 24 h in media without IL-2 prior to stimulation with plate-bound anti-CD3 (Ortho Diagnostics Systems, Raritan, NJ) and soluble anti-CD28 (Ancell, Bayport, MN) in 96 round-bottom plates at a
concentration of $1 \times 10^6$ cells per milliliter. T lymphoblasts were co-stimulated for 24 h, while fresh PBTs were co-stimulated for 48-72 h.

In vitro Caco-2 cell – T cell Co-culture System

Stimulated T cells were harvested, resuspended in RPMI 1640 supplemented with 10% FBS and 2.5 mM HEPES, and added to Caco-2 monolayers at a concentration of $1 \times 10^6$ cells per transwell. Depending on experimental conditions, T lymphoblasts or PBTs were added to either the upper or lower chamber resulting in three distinct configurations (Figure 4-S1). The other chamber was replenished with EMEM supplemented with 20% FBS. In experiments where conditioned media was added instead of T cells, conditioned media was collected either after fresh PBTs were co-stimulated via anti-CD3/anti-CD28 for 72 h, or from parallel co-cultures where co-stimulated PBTs were added to the Caco-2 monolayer as in Configuration A (Figure 4-S1). For experiments where T cells were removed during the co-culture, the top and bottom chambers were replenished with the existing media after T cells were pelleted and removed.

Paracellular Inulin Flux

Transwell cell culture inserts seeded with Caco-2 monolayers were incubated in the presence of fluorescein isothiocyanate-labeled inulin (0.25 mg/ml, Sigma-Aldrich) added to the lower chamber. After one h 50 µl from the upper and lower chambers was withdrawn, and fluorescence was measured using the GENios Pro fluorescence plate reader (Tecan, Männedorf, Switzerland). Flux was calculated
as the percentage of total fluorescence found in the upper well/h/cm², and normalized to the flux observed in an empty transwell filter without cells.

Confocal Microscopy

Caco-2 monolayers were washed twice in HBSS and fixed with 1:1 methanol:acetone. Monolayers were blocked with 10% normal goat serum (Invitrogen Life Technologies), incubated with rabbit anti-occludin or anti-ZO1 antibody, goat Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen Life Technologies), and the nuclear stain DRAQ5 (BioStatus, Leicestershire, UK). The filter membrane was cut from the transwell insert and mounted in Fluoroshield mounting medium (AbCam). Images were captured with a Perkin Elmer Ultraview VoX confocal microscope, using an oil-immersion 100x magnification objective lens connected to a Leica DMI 6000 B inverted microscope. Z-stack images (0.3 µm thickness) were obtained using Volocity 6.2 (Perkin Elmer).

Immunoblotting

Caco-2 monolayers were washed once in ice-cold HBSS and scraped into 25 µl of SDS-RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.3% SDS, 1% Triton X-100, 1 mM EDTA, 1:100 protease inhibitors (Sigma-Aldrich, St Louis, MO). After homogenization, incubation for 30 min at 4°C, and centrifugation at 1500 g for 15 min, concentrations of whole cell protein lysates were determined (Bio-Rad Laboratories, Hercules, CA). Lysates were diluted in Laemmli buffer (62.5 mM
Tris-HCl, pH 6.8, 2% SDS, 10% (w/v) glycerol, 0.005% bromophenol blue), boiled for 10 min, separated by 10% polyacrylamide gel electrophoresis (Invitrogen Life Technologies) and electro-transferred onto a nitrocellulose membrane (Invitrogen Life Technologies). After blocking with 5% non-fat milk solution (Carnation, Wilkes-Barre, PA), membranes were probed with rabbit antibodies against GAPDH (Trevigen, Gaithersburg, MD), occludin (AbCam, Cambridge, MA), claudin-2, and mouse antibodies against claudin-4 (Invitrogen Life Technologies). After incubation with HRP-conjugated goat anti-rabbit (Thermo Fisher Scientific) or anti-mouse (AbCam) secondary antibody, signals were visualized with enhanced chemiluminescence, using West Pico Supersignal (Pierce, Rockford, IL). Chemiluminescence for all membranes was detected using Hyblot CL Autoradiography Film (Denville Scientific, South Plainfield, NJ). The amount of protein in each band was quantified by densitometry using ImageJ (National Institutes of Health). The densitometric intensity of each target protein occludin, claudin-2, and claudin-4 was normalized to the intensity of the loading control GAPDH.

Statistical Analysis

Values are given as mean ± s.d.. All statistical analysis was done using Prism 5.0 (GraphPad Software, San Diego, CA). TJ transcript levels and all transepithelial resistance (TER) time courses were analyzed with repeated measures two-way analysis of variance (ANOVA) with a Bonferroni post test corrected for multiple comparisons to the control, except for time courses with T cell removal, in which
the post test was applied for multiple comparisons of all data pairs. Inulin flux measurements were analyzed using one-way ANOVA with Dunnett’s post test. Statistical analysis for protein quantification of the scanned immunoblots was performed via a one-way ANOVA with Bonferroni correction for multiple comparisons. P-values less than 0.05 were considered significant.

4.4 Results

Design of an in vitro intestinal epithelial cell – T cell co-culture system to recapitulate the architecture of the intestinal mucosa

The intestinal mucosa is comprised of a single layer of columnar epithelial cells resting on a basement membrane, with its basolateral surface in close proximity to the underlying lamina propria. The loose connective tissue of the lamina propria is populated by lymphocytes, mast cells, macrophages, and polymorphonuclear cells (257), suggesting bilateral cross-talk between the innate and adaptive arms of the mucosal immune system at the intestinal border. In order to examine the interactions between intestinal epithelial cells and lamina propria immune cells, we developed an in vitro co-culture model mimicking the in vivo intestinal mucosal architecture, in which Caco-2 cells, a human colorectal adenocarcinoma cell line, are seeded on the underside of a transwell filter, followed by incubation with immune cells on top of the transwell filter (Figure 4-S1 Configuration A). Once confluency is reached, Caco-2 cells mature into a polarized epithelial monolayer resembling the in vivo orientation, with expression of the apical junctional complex component occludin closer to the apical cell membrane (away from the transwell
filter), and the nucleus closer to the basolateral surface (closer to the transwell filter). As Caco-2 cells are grown to confluence and allowed to mature, transepithelial electrical resistance (TER) was quantified. We found a progressive increase in TER over time (Figure 4-S2), indicating that these cells form a functional barrier. The transwell filter mimics the basement membrane, separating the basolateral membrane of the epithelial cells from the immune cells on the top side of the filter (Figure 4-S3).

Co-culture with T cells initiates a biphasic perturbation in Caco-2 monolayer permeability

The lamina propria is primarily an effector lymphoid tissue with a vast pool of activated CCR5+ CD4+ T cells (258), thus we sought to examine the role for T cells on the barrier function of the intestinal epithelium. We measured TER of the Caco-2 monolayer co-cultured with T lymphoblasts that had been stimulated via crosslinking CD3 and activating CD28 for 24 h prior to co-culture. Stimulated T lymphoblasts induced a biphasic change in TER in the Caco-2 monolayer, compared with an untreated control monolayer. The Caco-2 monolayer exhibited an initial significant increase in TER observed 20-40 h after co-culture, followed by a decline in TER commencing two days after co-culture, resulting in a TER value that is significantly decreased compared with a control monolayer (Figure 4-1A). To more accurately represent the in vivo environment, the experiment was replicated using freshly isolated peripheral blood T cells (PBTs) co-stimulated for three days instead of cultured T lymphoblasts. Similar to that observed for co-
culture with T lymphoblasts, co-culture with activated, freshly isolated PBTs induced an initial increase in TER followed by a progressive decrease (Figure 4-1B). All subsequent experiments were performed using freshly isolated PBTs.

_PBTs must be activated and present on the basolateral surface of Caco-2 monolayer to initiate biphasic TER response_

Having determined that anti-CD3/anti-CD28-stimulated T cells induced a biphasic perturbation in electrical resistance of the Caco-2 monolayer, we next examined whether T cell activation prior to co-culture is required for the response. Caco-2 monolayers were co-cultured with either stimulated or resting PBTs. As shown earlier, stimulated T cells initiated the biphasic TER response characterized by a short-term increase followed by a progressive decrease. In contrast, co-culture with resting PBTs had no effect on TER and monolayer barrier function (Figure 4-2A). Since the differentiated Caco-2 monolayer displays distinct apical versus basolateral polarity, we also investigated whether the orientation of contact was a determining factor for the response. Caco-2 cells were grown on the top side of the transwell filter (Figure 4-S1, Configuration C), allowing for the addition of stimulated PBTs in contact with the apical surface of the Caco-2 monolayer, a configuration that did not alter TER (Figure 4-2A). These findings indicate that interaction with activated T cells on the basolateral surface is required for a T cell-induced biphasic TER response in intestinal epithelial cells.
To begin to address whether the TJ-mediated leak or pore pathways are targeted by activated T cell contact, alterations in Caco-2 monolayer permeability to non-ionic macromolecules was investigated by measuring the flux of inulin (M.W. = 2,000 to 5,000) across the monolayer. Since the monolayer is essentially impermeable to inulin at baseline, further decreases in monolayer permeability at the peak of the biphasic TER response did not alter inulin flux (data not shown). During the second phase of the TER response, the decrease in TER in Caco-2 monolayers co-cultured with stimulated T cell was accompanied by a concurrent significant increase in inulin flux (Figure 4-2B), confirming that stimulated T cells on the basolateral side of the Caco-2 monolayer induced a loss of barrier function that increased the permeability to both ionic and non-ionic molecules. To investigate if the activated T cell-induced decrease in TER and increase in inulin flux in the second phase of the response is due to Caco-2 cell death, we assessed Caco-2 cell viability. Caco-2 monolayers exhibiting a decreased TER after coculture with activated T cells did not show appreciable changes in the proportion of propidium iodide-permeable cells as determined via flow cytometry (Figure 4-S4), indicating that loss of permeability in phase 2 is likely due to modulation of its barrier function.

*Activated T cell-induced biphasic perturbations in intestinal epithelial permeability is mediated by factors accessible only in a local environment*

Having confirmed that activated T cells must be present at the basolateral surface of the Caco-2 monolayer to modulate epithelial permeability, we sought to
characterize the nature of the T cell-epithelial cell interaction. TER was not altered in Caco-2 monolayers grown on the top side of transwell filters and co-cultured in a non-contact, basolateral configuration with activated T cells settled to the bottom of the lower chamber (Figure 4-3A, Figure 4-S1 Configuration B), while Caco-2 monolayers treated as shown earlier, separated from activated PBTs by only the 10 micron thickness of the transwell filter membrane, demonstrated the expected biphasic TER perturbations. These results indicate that activated T cells need to be in close proximity to the basolateral surface of the Caco-2 monolayer to mediate the response.

To determine whether monolayer permeability changes are mediated by soluble factors produced by activated T cells, we examined whether conditioned media from activated PBTs was sufficient to induce epithelial TER modulations. When Caco-2 monolayers were cultured with conditioned media from the 3-day PBT anti-CD3/anti-CD28 co-stimulation process, TER remained unchanged when compared to control monolayers (Figure 4-3B). Conversely, monolayers treated with conditioned media replenished every day from activated PBT – Caco-2 co-cultured in parallel wells demonstrated a marginal increase in TER, similar to the early TER increase of the biphasic response, although not to the same magnitude as in the monolayers cultured with activated PBTs (Figure 4-3C). Together these findings indicate that soluble factors produced by co-stimulated PBTs are insufficient to induce TER responses.
The requirement that activated T cells be in close proximity to the basolateral surface of the Caco-2 monolayer, along with results demonstrating that conditioned medium was insufficient to induce the biphasic TER response, suggest that epithelial permeability alterations may be mediated by direct T cell-epithelial cell contact. While activated T cells do extend plasma-membrane projections that can fit through 0.4 µm transwell filter membrane pores (259), the 10 µm-thick filter membrane presents a significant distance that the projections must reach to contact the basolateral surface of the Caco-2 cells. Imaging with a DeltaVision deconvolution fluorescent microscope did not reveal evidence that T cell extensions traversed the entire thickness of the filter membrane (data not shown). The most plausible explanation of our results is that the T cell-epithelial cell interaction is mediated by the local production of soluble factors by activated PBTs, a scenario that could not be effectively replicated by daily replenishment of conditioned media.

Activated T cells stimulate an increase in claudin-2 and -4 protein expression in Caco-2 cells

Since a perturbation in TER of intact monolayers is often attributed to a change in paracellular permeability mediated by tight junctions, we assessed the morphological distribution of the tight junction proteins occludin and zona occludens-1 (ZO-1) in Caco-2 monolayers via confocal microscopy at the peak and the trough of the biphasic response after co-culture with activated T cells (Figure 4-4A). In both control and treated monolayers, occludin and ZO-1 are
localized to the lateral cellular membranes, forming the classic “chicken wire” appearance encircling each cell.

Since no structural changes in tight junctions (TJ) are visible at light microscopic resolution, we investigated the ability of activated T cells to modulate barrier function by regulating TJ protein expression (Figure 4B-D). Total cell lysates prepared from Caco-2 monolayers at the peak and trough of the TER response after co-culture with activated T cells (Figure 4B) were immunoblotted for occludin, claudin-2, and claudin-4. At peak TER, i.e. when monolayer permeability is the lowest, activated PBTs induced a 1.6-fold increase in claudin-4 protein in Caco-2 monolayers when compared to untreated control monolayers (Figure 4C). Elevated levels of claudin-4, which predominantly functions as a sealing component of tight junctions (170), would strengthen the paracellular barrier, in line with the observed increase in TER. As expected, we did not observe changes in claudin-4 levels in Caco-2 monolayers co-cultured with resting PBTs. No noticeable changes in the levels of occludin and claudin-2 are observed. RNA transcript levels, determined by RT-PCR, for occludin, ZO-1, claudin-2, and claudin-4 were unchanged (Figure 4-S5), suggesting that claudin-4 translation or mRNA/protein stability may be affected.

The increase in claudin-4 protein expression persisted into the second phase of the Caco-2 response, when TER has fallen to values lower than that observed in control monolayers and monolayers co-cultured with resting PBTs (Figure 4-4D).
Moreover, our results suggest that claudin-4 protein upregulation (1.7-fold) observed at the trough of the TER response at 90 h may be more pronounced than that seen at the peak at 43 h, eluding to the possibility that claudin-4 protein upregulation is a process that is induced via interaction with activated T cells and strengthened over time. The persistent increase in claudin-4 protein is paradoxical to the observed decrease in TER indicative of loss of barrier function, suggesting that while claudin-4 upregulation may be the mechanism causing the initial strengthening of the barrier, the eventual barrier dysfunction results from a distinct mechanism that weakened the Caco-2 barrier even in the presence of increased claudin-4 protein. In the second phase of the response, activated PBTs also induce a 2-fold increase in claudin-2 protein levels, when compared to that of untreated control monolayers (Figure 4-4D), indicating a different kinetics for induction of claudin-2 versus claudin-4. An increase in claudin-2 levels at a time that the barrier function is destabilized is consistent with its known role in TJ pore formation, which would increase TJ permeability to cations and thus decrease TER (170, 171). Occludin levels are unchanged in Caco-2 monolayers co-cultured with activated or resting PBTs. Again, tight junctional mRNA levels are unchanged at the trough (Figure 4-S5).

*Early increase in monolayer resistance is initiated by the presence of stimulated T cells, while prolonged exposure causes barrier dysfunction*

To shed light on the mechanism leading to prolonged increased in claudin-4 protein, we investigated whether sustained T cell-epithelial cell contact is
necessary for permeability perturbations. Caco-2 monolayers were co-cultured with activated PBTs, after which T cells were removed at 22, 48, 91, and 120 h (Figure 4-5). When T cells were removed during the first phase of the response, i.e. when TER was increased compared to that of control monolayers, the TER increase was maintained for the duration of the culture, while monolayers continually co-cultured with activated T cells showed the characteristic biphasic response that resulted in a subsequent drop in TER (Figure 4-5A). When stimulated PBTs were removed at 22 h, TER remained stable and elevated, and by 48 h TER was significantly different from that of monolayers continually co-cultured with activated PBTs and control monolayers. Similarly, for monolayers from which stimulated PBTs were removed at 48 h, TER was maintained at an elevated level that, by 91 h, was significantly different from monolayers cultured with stimulated PBTs. Of note, the effects of T cell removal showed a graded response. When activated PBTs were removed at 22 h, before TER reached its maximum, the monolayer maintained its increased TER at that sub-maximal level, while monolayers that achieved maximal TER before T cell removal at 91 hours remained at the highest TER value, an almost three-fold increase compared to control monolayers.

After the epithelial monolayer entered the second phase of the response, during which TER was on a decline, removal of activated T cells resulted in the recovery of barrier function within 24 h, while the TER of monolayers continually co-cultured with activated PBTs remained depressed and reached a trough (Figure 4-5B). As
shown earlier, by 91 h of co-culture, a significant decrease in TER of the Caco-2 monolayer was observed and reached a level that was 55% of the untreated control monolayer and equivalent to 31% of its peak. Removal of activated PBTs at this time allowed the monolayer to recover from the barrier dysfunction. TER returned to 140% of that measured in untreated control monolayers – a value that, by 120 h, was significantly higher compared to monolayers continually co-cultured with stimulated PBTs. In fact, the monolayer persisted at this elevated TER for the remainder of the culture. We did not observe a significant rebound of TER following removal of activated PBTs at 120 h, when TER response has reached its trough, but since the experiment was not continued beyond 146 h, we do not rule out the possibility that the monolayers might still recover.

Our results indicate that while interaction with activated T cells is needed for initiation of the early phase increase in epithelial monolayer TER, the continual presence of T cells is not required for its maintenance. On the other hand, the late phase increase in permeability resulting in a decrease in TER and barrier dysfunction is a transient consequence of prolonged and sustained exposure to activated T cells, which can be reversed once the stimulus is removed.

*Increases in claudin-2 and claudin-4 protein level are maintained after T cell removal*

Since we demonstrated prolonged increases in claudin-2 and claudin-4 protein level in Caco-2 monolayers co-cultured with activated PBTs, we investigated
whether this response would be maintained following T cell removal. Total protein lysates were prepared at 146 h from monolayers where activated PBTs had been removed at 22, 48, 91, and 120 h, and immunoblotted for occludin, claudin-2, and claudin-4 (Figure 4-6). In agreement with our previous observations, Caco-2 monolayers co-cultured with activated PBTs for the duration of the experiment showed the characteristic biphasic permeability response (Figure 4-5) and exhibited increased levels of claudin-4 protein at the end of the co-culture, as compared to untreated control monolayers. Caco-2 monolayers from which PBTs were removed at various time points also showed increased levels of claudin-2 and claudin-4 protein compared to untreated control monolayers. No noticeable change in protein levels of occludin was observed (Figure 4-6A).

Claudin-4 protein levels of all monolayers that interacted with activated PBTs were significantly increased compared to untreated control monolayers (Figure 4-6B). The increase in claudin-4 levels models the increase in TER, where monolayers with PBTs removed at sub-maximal TER showed a significant, sub-maximal 2.1-fold increase in claudin-4 protein level compared to untreated control monolayers, while claudin-4 levels for monolayers with PBTs removed at peak TER, or after peak TER was reached, achieved a maximal increase of 2.7 to 3.1-fold. Claudin-4 protein levels of monolayers that reached peak TER was significantly different from that of both monolayers that reached sub-maximal TER and untreated control monolayers. Our results indicate that claudin-4 protein levels in Caco-2 monolayers co-cultured with activated PBTs vary together with the early phase
TER increase, with a graded increase in claudin-4 protein level. As observed for the TER response, activated PBTs are required for the initiation of claudin-4 protein upregulation, but are not necessary for the maintenance of elevated claudin-4 protein levels.

Levels of claudin-2 were also increased in Caco-2 monolayers where activated PBTs had been removed at 22, 48, 91, and 120 h. This increase in claudin-2 was previously observed at an early time point (i.e. 90 h) within the trough of the biphasic response, as demonstrated in Fig. 4D. We propose that an increase in claudin-2 protein levels may be initiated by contact with activated PBTs, similar to the response seen for claudin-4, although with slower kinetics. Sustained contact with activated PBTs eventually causes a gradual decrease in claudin-2 protein level to baseline at 146 h after the start of co-culture, as shown in Fig. 6A. Removal of T cells would prevent such gradual decrease, thus Caco-2 monolayers from which PBTs were removed at various time points retained the significant, increased levels of claudin-2, compared to untreated control monolayers and monolayers co-cultured with activated PBTs without T cell depletion (Figure 4-6C).

4.5 Discussion
Loss of intestinal barrier function and resultant microbial translocation have been demonstrated in various disease states, including IBD (99, 260), HIV (92, 150, 161), pancreatitis (261), graft-versus-host disease (101, 102), excessive alcohol
consumption (262), and diabetes (263). Microbial translocation in these diseases has been attributed to GALT disruption that results in a general inflammatory environment in the intestinal mucosa (115, 264, 265). Mechanistically, intestinal TJ dysregulation has been demonstrated in such diseases (148, 266) and is thought to contribute to the weakened epithelial barrier. Understanding how dysregulation of the immune compartment would negatively impact epithelial permeability requires delineating the role of immune cells and mediators in TJ regulation. Much effort has been focused on the isolated effects of single cytokines on intestinal epithelial permeability (222), but the overall impact of immune cells on intestinal epithelial barrier dysfunction likely involves multiple cytokines as well as possible direct interactions between the two cellular compartments. We provide evidence of the complex alterations in epithelial paracellular permeability in response to IEC – T cell interaction, which is defined by a biphasic response with an initial strengthening of the barrier and a late-phase barrier disruption not due to epithelial cell death. The response requires activated T cells in close proximity, on the basolateral surface of the epithelial monolayer, and is dependent on local secretion of soluble factors at the epithelial cell – T cell interface. Short-term interaction with activated T cells initiates an increase in claudin-2 and claudin-4 protein levels, with claudin-2 changes displaying slower kinetics. Elevated TJ protein levels are not dependent on continual T cell exposure. Prolonged exposure to activated T cells reverses the increase in claudin-2 protein levels to baseline, while claudin-4 levels remain elevated.
The close proximity of the intestinal epithelium and the GALT distinctively enables two-way communication between the two compartments, which serves to shape and maintain mucosal immune homeostasis. IEC-immune cell interactions influence the function of antigen-presenting cells and lymphocytes. IECs secrete the soluble factor, thymic stromal lymphopoietin, activating B cells and dendritic cells to promote Treg differentiation as well as limit a proinflammatory cytokine response (104). Direct IEC-intraepithelial lymphocyte (IEL) interaction leads to the development of a highly effective mucosal T cell population (267). Conversely, the immune compartment also influences IEC differentiation and proliferation. IELs secrete keratinocyte growth factor, which is important for epithelial repair, while epithelial contact with both lymphocytes and macrophages support IEC differentiation. Furthermore, T cells activated by epithelial cells, but not macrophages, promote epithelial expression of non-classical HLA class I molecules (238), highlighting the complexity and specificity of IEC-immune cell regulation. Our findings extend this paradigm of IEC-immune cell communication in the intestinal mucosa and provide evidence that interactions with activated T cells regulate the barrier function of the intestinal epithelium, in addition to its differentiation and proliferation. The complexity of the interaction is underscored by the observation that the epithelial response undergoes two phases, involving increased levels of multiple TJ proteins with different kinetics and distinct requirements for continual T cell exposure. Our result that, unlike conditioned media from the Caco-2/activated T cell co-culture, conditioned media from anti-CD3/anti-CD28 co-stimulated PBTs alone did not alter monolayer permeability
highlights the requirement for both epithelial and T cells to be present and interacting to induce an epithelial permeability response. In aggregate these results indicate that immune control of the intestinal epithelium implicated in disease states should be considered as the conglomerate effects of multiple direct and indirect epithelial cell-immune cell interactions.

Inflammatory diseases associated with intestinal barrier dysfunction are known to trigger distinct changes in TJ protein expression. Ulcerative colitis is accompanied by downregulation of claudin-1, while claudin-4 and claudin-2 are upregulated (230). Similarly, decreased claudin-1 and increased claudin-2 expression are demonstrated in the small intestine of untreated HIV infection (150), while in HIV patients under effective antiretroviral therapy, downregulation of claudin-2, claudin-4, occludin, and ZO-1 is most evident in the distal colon (266). In this in vitro study, epithelial cell interaction with activated T cells similarly triggered differential regulation of TJ proteins, with no effect on occludin expression, but increased levels of claudin-2 and claudin-4 showing different kinetics and distinctive requirements for continual T cell presence.

Cytokine regulation of TJ permeability has been mainly attributed to two mechanisms: (i) turnover of specific claudin/occludin proteins at the TJ, secondary to transcriptional regulation or post-translational modifications, and (ii) contraction of the actomyosin cytoskeletal anchoring the TJ and the concurrent endosome-mediated internalization of TJ proteins, often occludin (222). Our results suggest a
third mechanism. Co-culturing Caco-2 cells in vitro with anti-CD3/anti-CD28 activated T cells results in increased protein levels of claudin-4 and claudin-2, in the absence of mRNA upregulation. We do not observe any changes in occludin or ZO-1 subcellular localization, advocating against the involvement of TJ protein endocytosis. We propose that activated T cells modulate Caco-2 TJ permeability through the novel mechanism of altering translation and/or degradation of claudin-2 and claudin-4 proteins.

The working model of the TJ consists of anastomosing strands forming an absolute barrier, populated by pores that allow the passage of small ions and uncharged molecules (169). Precise barrier and selectivity of the pores are dependent on the combination of the claudin proteins expressed, which influence the homotypic and heterotypic interactions between strand forming TJ proteins within the same strand or on neighboring cells (171). Claudin-2 is a channel-forming TJ protein allowing passage of cations and water, and its presence is proposed to lower TER and increase paracellular permeability. In intestinal epithelial monolayers (170), claudin-2 is up-regulated by multiple stimuli including cytokines IL-6 (268) and IL-13 (230). Claudin-4 is primarily considered a sealing protein, increasing TER of epithelial monolayers following overexpression (176, 177). However, it was demonstrated to act as an anion channel when expressed together with claudin-8 (178), suggesting that the accompanying claudin family expression profile determines claudin-4 function. The biphasic Caco-2 permeability response induced by activated T cells would thus represent the
summation of the individual effects of increased expression of claudin-4 and claudin-2 on TJ strand formation, as well as the consequences of altering complex interactions among the binding partners, which may include displacing other proteins from the TJ strands.

The intestinal mucosa is architecturally divided into two compartments: the epithelial monolayer covering the intestinal wall with its apical surface facing the gut lumen and its basolateral surface contacting the basement membrane, directly opposing the underlying lamina propria, where GALT immune cells reside. In the intact intestinal mucosa, the majority of T cells are diffusely scattered in the lamina propria, along with a comparatively smaller population of IELs. IECs are structurally and functionally polarized, with differential expression of membrane proteins on either the apical or basolateral surface, such as pathogen-sensing toll-like receptors that are mostly located on the basolateral surface, engaged only if the epithelial barrier is breached (269), and basolateral expression of HLA class II antigens for mucosal T cell stimulation (238). Under the same logic, IECs would express receptors that respond to immune-mediated signals or immune cells only on the basolateral side closer to the lamina propria. Thus it is physiologically relevant that an in vitro epithelial monolayer only responds to basolateral, not apical, T cells. In the healthy individual, while constant sampling of luminal antigens via specialized epithelial cells and dendritic cells induces a memory phenotype in the majority of lamina propria T cells, the generally non-responsiveness of the mucosal T cell population, along with specialized immune
cell populations such as regulatory T cells and unique intestinal dendritic cell subsets, maintains immunological tolerance toward harmless antigens (106). In the setting of intestinal inflammatory diseases, the balance between tolerance and immunity is lost, with general mucosal inflammation resulting from epithelial breaches that allow translocation of microbial products or even whole microbes. Our study reveals that the intestinal epithelium can respond appropriately to mucosal inflammation by acutely initiating a more protective paracellular barrier following signals elicited by activated T cells. However, prolonged exposure to activated T cells eventually reverses the initial epithelial protective response, perhaps foreshadowing the lingering effects of T cell activation in propagating barrier dysfunction and microbial translocation, as observed in chronic intestinal inflammatory diseases such as IBD.

We have provided evidence, at a mechanistic level, that IEC-T cell interaction modulates epithelial paracellular permeability. The change in permeability is achieved by both a transient increase in claudin-2 and a prolonged increase in claudin-4 protein levels, proposed to be secondary to alterations in protein translation or stability. While the exact molecular mechanisms mediating activated T cell-induced epithelial barrier perturbations and TJ regulation are yet to be defined, our results highlight the dual roles of the activated T cell - as a sentinel alerting the intestinal epithelium to respond to injuries and insults, and as a harbinger of pathology contributing to intestinal epithelial disruption and microbial translocation in the setting of chronic intestinal inflammation.
Figure 4-1. Co-culture with activated T cells initiates a biphasic perturbation in Caco-2 monolayer permeability. Caco-2 monolayers grown on the underside of transwell filters were co-cultured with anti-CD3/anti-CD28-stimulated (A) T lymphoblasts or (B) freshly isolated PBTs added to the upper chamber of the transwell, as in Configuration A (Figure 4-S1). TER measurements of untreated Caco-2 monolayers and Caco-2 monolayers co-cultured with T cells were followed over time ($n = 3$; * $p<0.0001$, # $p<0.01$).
Figure 4-2. Activated PBTs on the basolateral side of Caco-2 monolayers initiate biphasic permeability alterations. (A) Caco-2 monolayers were co-cultured with fresh PBTs and TER measurements were recorded over time. Anti-CD3/anti-CD28-stimulated or resting PBTs were added to the basolateral side of the Caco-2 monolayer grown on the underside of transwell filters as in Configuration A. Alternatively, anti-CD3/anti-CD28-stimulated PBTs were added to the apical surface of Caco-2 monolayers grown on the top side of transwell filters as in Configuration C. TER measurements of untreated Caco-2 monolayers are shown (n = 3; * p<0.0001). (B) Caco-2 monolayers at the end of the co-culture period in Panel A were washed in HBSS, after which inulin flux was measured for 1 h (n = 3; # p<0.001).
Figure 4-3. Activated PBTs induce biphasic epithelial permeability changes via factors accessible only in the local environment. (A) Activated or resting PBT were added to the bottom chamber on the basolateral side of Caco-2 monolayers grown on the top side of transwell filters, with epithelial and T cells not in close proximity (Configuration B). TER of such co-cultures with no T cell-epithelial cell contact was followed over time. TER of Caco-2 monolayers in close proximity to activated PBTs on the basolateral side (Configuration A) and untreated control monolayers were recorded in parallel. (B) Conditioned medium, obtained from PBTs stimulated with plate-bound anti-CD3 and soluble anti-CD28 for three d in round-bottomed 96 well plates, was added to the basolateral side of Caco-2 monolayers grown on the underside of transwell filters as in Configuration A. TER was followed over time. TER of Caco-2 monolayers co-cultured with
activated PBTs on the basolateral side and untreated control monolayers was recorded in parallel. (C) Caco-2 monolayers were co-cultured with conditioned medium, added to the basolateral side, transferred from parallel Caco-2 cell – stimulated PBTs co-cultures in Configuration A. Conditioned media from both the upper and lower chambers were replaced daily with conditioned media from a different parallel co-culture replicate. TER of such Caco-2 monolayers co-cultured with conditioned media, as well as Caco-2 monolayers co-cultured with activated PBTs on the basolateral side and untreated control monolayers were followed over time ($n = 3$ for all conditions; * $p<0.0001$, # $p<0.001$).
Figure 4-4. Activated T cells induce claudin-4 protein in Caco-2 monolayer.

(A) Caco-2 monolayers, co-cultured with anti-CD3/anti-CD28-stimulated PBTs in Configuration A (Stim) and untreated (Control), were fixed at the peak and the trough of the biphasic permeability response and stained for occludin or ZO-1. Representative images are shown. (B) TER of Caco-2 monolayers co-cultured with anti-CD3/anti-CD28-stimulated or resting PBTs (Configuration A) and untreated control monolayers was followed over time. (At \( t = 0, 28, \) and \( 43 \) h: \( n = 12 \) for stimulated T cells and control, \( n = 11 \) for resting T cells; at \( t = 48, 73, \) and \( 90 \) h: \( n = 6 \) for stimulated T cells and control, \( n = 5 \) for resting T cells; * \( p<0.0001, \)
At 43 h (TER at peak) and 90 h (TER at trough) (indicated with arrows), Caco-2 monolayers were harvested and protein lysates were obtained by pooling two transwell filters into each lysate sample. Total protein lysates from Caco-2 monolayers co-cultured with resting PBTs (Unstim), stimulated PBTs (Stim), and untreated monolayer (Control), harvested when TER measurements were at (C) the peak and (D) the trough of the biphasic permeability response, were immunoblotted for tight junctional proteins claudin-2, claudin-4, and occludin. GAPDH was used as a loading control.
Figure 4-5. Early phase TER increase is maintained after removal of activated PBTs, while prolonged exposure to activated PBTs causes barrier dysfunction. Caco-2 monolayers were co-cultured with activated PBTs on the basolateral side (Configuration A). At 22, 48, 91, and 120 h, culture medium was removed from the upper chamber of the transwell filters, centrifuged to remove T cells, and cell-free medium was returned to the upper chamber. TER of monolayers from which activated PBTs were removed during (A) the early TER increase (22 and 48 h), and (B) the subsequent TER decrease (91 and 120 h) was followed over time. TER of Caco-2 monolayers continuously co-cultured with activated PBTs on the basolateral side and untreated control monolayers was recorded in parallel (n = 3 for all conditions; Panel A: * Stimulated T cells compared to control: p < 0.01 22 h onward; § Remove T cells at 22 h compared to stimulated T cells: p < 0.01 48 h onward; # Remove T cells at 48 h compared to stimulated T cells: p < 0.0001 91 h onward; Panel B: * Stimulated T cells compared to control: p < 0.05 41.5 h onward; § Remove T cells at 91 h compared to stimulated T cells: p < 0.0001 120 h onward).
Figure 4-6. Increases in claudin-2 and claudin-4 protein level are maintained after T cell removal. Caco-2 monolayers were co-cultured with activated PBTs on the basolateral side (Configuration A). At 22, 48, 91, and 120 h, culture medium was removed from the upper chamber of the transwell filters and cell-free conditioned medium was returned to the upper chamber. At the end of the co-culture period, Caco-2 cells were harvested and protein lysates were obtained. (A) Total protein lysates were immunoblotted for tight junctional proteins occludin, claudin-2, and claudin-4. Samples were prepared from duplicate wells of Caco-2 monolayers from which activated PBTs were removed at 22 h, 48 h, 91 h, and 120 h, monolayers continuously cultured with activated PBTs (Stim), and untreated monolayer (Ctrl). GAPDH was used as a loading control. (B) Densitometric analysis of claudin-4 protein levels normalized to GAPDH levels, relative to untreated monolayers (* p < 0.05, ** p < 0.01, *** p < 0.001 versus control). (C) Densitometric analysis of claudin-2 protein levels normalized to GAPDH levels,
of monolayers from which activated PBTs were removed (T cell removal), monolayers continuously co-cultured with activated PBTs (No T cell removal), and untreated monolayers (Ctrl) \((n = 2\) for control and No T cell removal, \(n = 8\) for T cell removal; ** \(p < 0.01\)).
Caco-2 cells, a human colorectal adenocarcinoma cell line, were seeded on a transwell filter and within 3 weeks differentiated into an intact epithelial monolayer. In **Configuration A**, Caco-2 cells grown on the underside of the transwell filter enables the addition of T cells to the upper chamber in close proximity to the basolateral surface of the cells, a configuration that models physiological conditions. When Caco-2 cells are grown on the top side of the transwell filter, T cells can be added to the bottom chamber, resulting in **Configuration B** where T cells are on the basolateral side of the epithelial cells but not in close proximity to the monolayer. Alternatively, T cells can be added to the upper chamber of the transwell as in **Configuration C**, where T cells are in contact with the apical surface of the epithelial monolayer.
Figure 4-S2. Caco-2 monolayers differentiate into stable, intact monolayers within 14 days of culture. Caco-2 cells, seeded onto the underside of transwell filters at a concentration of 65,000 cells/transwell, were allowed to grow and differentiate over time, with replenishment of media every two to three days. TER measurements of differentiating monolayers were followed ($n = 4$). Caco-2 cells differentiate into stable, intact monolayers within 14 days of culture.
Figure 4-S3. Caco-2 – T cell co-culture in Configuration A models the architecture of the intestinal mucosa. T cells pre-stained with CFSE (Green) were added to the upper chamber of a transwell filter with Caco-2 cells seeded on the underside, as in Configuration A. Two days after initiation of co-culture, the transwell filter was fixed and stained for occludin (Red) and nuclei (Blue Hoechst stain), as described for Movie 1. Note that the filter membrane pores bind the Cy5-labeled anti-rabbit secondary antibody non-specifically (Red). Images were captured in successive 1 µm z-sections from the bottom of the sample to the top. Z frames were transposed so that the bottom frame is #1, and top frame is #50. Representative images from Z = 10, 15, 38, and 50 are shown, demonstrating the progression from bottom to top, modeling the intestinal mucosal architecture of occludin staining near the apical surface of the Caco-2 monolayer, Caco-2 nuclei
near the basolateral side of the monolayer, pores in the filter membrane, and finally, T cells on the upper side of the filter membrane.
Figure 4-S4. Decreased TER and increased inulin flux in the late phase of the T cell-induced response are not due to Caco-2 cell death. (A) Caco-2 monolayers co-cultured with anti-CD3/anti-CD28-stimulated PBTs (red) and untreated control monolayers (blue) in Configuration A (Fig. S1A in supplementary material) were trypsinized at $t = 72$ h and harvested along with the media in the bottom chamber. Cells were stained for 10 min in 1.25 µg/ml with propidium iodide (PI), resuspended in PBS with 1% FBS and 0.1% sodium azide, and analyzed by flow cytometry using the MACSQuant flow cytometer and MACS Quantify software. Caco-2 cells were identified by size and complexity (forward and side scatter), and the level of PI staining was assessed. Data is represented in a histogram where the y-axis denotes % of total cells, and the x-axis shows PI intensity. (B) As a positive control, Caco-2 monolayers treated with 0.2 µg/ml doxorubicin (DOX) for 24 h (red) before harvest show increased PI staining compared to untreated control monolayers, indicative of Caco-2 cell death.
Figure 4-S5. Transcript levels of tight junctional proteins are not altered at the peak and trough of biphasic monolayer permeability response. (A) TER of Caco-2 monolayers co-cultured with anti-CD3/anti-CD28-stimulated or resting PBTs (Configuration A, Fig. S1A in supplementary material) and untreated control monolayers was followed over time. (At t = 0, 22.5, 31.5, 42.5 h: n = 6; at t = 88.5 and 101 h: n = 3; * p<0.0001). At 42.5 h (TER at peak) and 101 h (TER at trough) (indicated with arrows), Caco-2 monolayers were harvested into 300 µl of lysis buffer. Lysate was homogenized by passing through a 21-gauge needle 5 times. Total RNA was extracted and qPCR was performed as described previously (266), using reference genes eukaryotic translation elongation factor 1-alpha 1 (eef1A1) and hypoxanthine phosphoribosyltransferase 1 (HPRT1). Calibrated normalized relative quantities (CNRQ) of ZO-1, occludin, claudin-2, and claudin-4 transcripts were determined with qBasePlus analysis from 400 ng of total RNA from
monolayers co-cultured with stimulated or resting PBTs, or untreated control monolayers, isolated at (B) the peak and (C) the trough of the biphasic response (n = 3).
Movie 4-1. Caco-2 – T cell co-culture in Configuration A models the architecture of the intestinal mucosa. Stimulated T cells, pre-stained with 10 µM CFSE for 10 min (Green), were added to the upper chamber of a transwell filter with Caco-2 cells seeded on the underside, as in Configuration A (Fig. S1A in supplementary material). Two days after initiation of the co-culture, the transwell filter was fixed in 4% paraformaldehyde, stained with rabbit anti-occludin, then incubated with Cy5-labeled anti-rabbit antibody (Red) and Hoechst stain (Blue, nuclei). Note that the filter membrane pores bind the Cy5-labeled anti-rabbit secondary antibody non-specifically (Red). Images were captured with a 60X objective in successive 1 µm z-sections using a Deltavision Core fluorescence imaging system. Z-sections were deconvolved using the Applied Precision Softworx analysis software and displayed in a z-frame movie at 5 frames per second, from the bottom of the sample to the top.
CHAPTER 5

FUTURE PERSPECTIVES
5.1 CONCLUSIONS

ART-treated HIV-infected patients have a shortened life expectancy compared to the healthy uninfected. Increased mortality is thought to be secondary to microbial products originating from the gut lumen, which stimulate systemic inflammation and downstream non-AIDS morbidities. This thesis aims to characterize the cause of increased intestinal permeability in the ART-treated HIV-infected population. My work demonstrates:

1) The first direct molecular evidence of intestinal epithelial dysfunction in the ART-treated HIV+ population. While the intestinal epithelium is grossly intact, progressive proximal-to-distal colonic TJ downregulation is observed. TJ transcript levels inversely correlate with systemic level of microbial products and markers of systemic inflammation, suggesting that colonic TJ transcriptional downregulation contributes to premature aging, despite effective viral suppression, in this population.

2) The novel report of decreased intestinal epithelial permeability in response to an inflammatory stimulus. In a non-disease related in vitro co-culture system, activated T cells in close proximity to the basolateral surface of an intestinal epithelial monolayer regulate intestinal epithelial permeability and TJ protein levels – tightening intestinal epithelium in the short term, while increasing epithelial leakiness following prolonged exposure. Such results highlight the contribution of inflammatory signals and T cells to the homeostatic maintenance of intestinal epithelial barrier strength, and
extend implications to HIV-associated loss of intestinal mucosal immune homeostasis as a driver for concurrent intestinal barrier dysfunction and epithelial TJ dysregulation.

5.2 **SIGNIFICANCE – TJ AS A MEDIATOR BETWEEN HIV-ASSOCIATED INTESTINAL BARRIER DYSFUNCTION AND LOSS OF IMMUNE HOMEOSTASIS**

Microbial translocation and systemic inflammation play critical roles in the pathogenesis of HIV, and thus have been extensively studied in the untreated HIV infection, both in the acute and chronic (non-AIDS and AIDS) phases. Recently, it was recognized that systemic inflammation remains uncorrected even in patients effectively treated with ART, who suffer from excessive risk of non-AIDS associated morbidities such as cardiovascular and liver diseases despite long-term treatment success. The focus of the field has thus shifted to elucidating and potentially preventing the drivers for the persistent systemic inflammation in this patient population, which is associated with the incidence of multiple non-AIDS associated events.

In untreated patients, systemic inflammation is due to translocated bacterial products that originate from the gut, secondary to increased intestinal permeability involving epithelial breakdown. Previous work from our laboratory demonstrated persistently increased intestinal permeability in the ART-treated HIV-infected
population as well (161), suggesting the gut is indeed the source of microbial translocation and systemic inflammation in this patient population. I demonstrate in this thesis the first direct molecular evidence that epithelial dysregulation indeed persists in the colon of suppressive ART-treated HIV-infected patients. Such epithelial dysregulation does not involve gross loss of the intestinal barrier, as in untreated SIV/HIV infection, but involves progressive transcriptional down-regulation of TJ components along the proximal-to-distal colon, implying intestinal epithelial TJ and paracellular permeability as key mediators of increased colonic intestinal permeability in the ART-treated HIV+ patient.

My data extends the field’s understanding of HIV-associated intestinal epithelial barrier breakdown to the largely unexplored ART-treated HIV+ population (Figure 5-1). Much investigation in this area has focused on the untreated HIV+ population. In the acute SIV infection, enterocyte apoptosis resulting from direct SIV binding (159) causes IECs loss and epithelial disruption, as confirmed in acute and chronic SIV-infected macaques (148). Similar virotoxic effects are implicated in the small intestine of untreated HIV+ patients (158, 160). Regulation of selected TJ proteins, in particular upregulation of claudin-2 and downregulation of claudin-1, also plays a role in weakening the intestinal epithelial paracellular barrier (150, 179). Overall, evidence supports epithelial barrier breakdown via both apoptosis and selective TJ regulation in the untreated HIV infection, with the small intestinal mucosa as a primary target. In the ART-treated HIV-infected population, isolated studies demonstrated that this small intestinal TJ
dysregulation previously evident in the untreated HIV+ individual is reversed by suppressive ART (150). My work suggests that, unlike in untreated HIV infection, the colonic mucosa, not the small intestinal mucosa, is the relevant site of epithelial barrier dysfunction in the ART-treated population. Furthermore, instead of targeted regulation of particular TJ components, as demonstrated in untreated HIV infection, my data suggests a non-selective downregulation of multiple TJ constituents, secondary to overall epithelial transcriptional dysregulation, allowing passage of microbial products through the weakened inter-epithelial TJs. In both settings, the resultant microbial translocation drives systemic inflammation in the HIV-infected individual.

The above paradigm serves to characterize the epithelial barrier breakdown involved in the HIV gut, but the unresolved question remains in the molecular mechanism behind the observed intestinal epithelial TJ dysregulation. To address this issue, I developed an in vitro intestinal epithelium – T cell co-culture model system. In the HIV-infected patient, untreated or ART-treated, insults to the intestinal mucosa are multi-faceted. The most widely recognized is the severe and prolonged depletion of lamina propria CD4+ T cells, which drastically impacts gut mucosal immune homeostasis. My in vitro results demonstrate that cross-talk between the intestinal epithelium and stimulated T cells actively regulates epithelial permeability and modulates protein levels of TJ components claudin-2 and claudin-4, thereby confirming that activated T cells influence intestinal epithelial barrier maintenance via TJ regulation. Extending these observations to
the HIV gut, where drastic depletion of activated CD4+ mucosal T cells persists, I hypothesize that the resultant loss of intestinal epithelial cell – mucosal T cell interactions would potentially dampen the homeostatic signals from the T cell compartment, therefore contributing to HIV-associated intestinal epithelial barrier breakdown. Another corollary stems from our observation that prolonged presence of activated T cells results in epithelial barrier disruption. Despite CD4+ T cell depletion, HIV causes a paradoxical inflammatory intestinal mucosal environment, leading us to postulate that prolonged exposure to inflammatory mediators may contribute to intestinal TJ dysregulation.

While the exact consequences of HIV-induced mucosal CD4+ T cell depletion on activated T cell – epithelial cell cross-talk remain to be dissected, my thesis work demonstrates that activated T cells regulate epithelial TJ, thereby identifying intestinal epithelial TJ dysregulation as a potential sequela of loss of mucosal immune homeostasis, which feeds forward to bring about intestinal barrier dysfunction in the HIV-infected individual.

5.3 **UNRESOLVED QUESTIONS AND FUTURE STUDIES**

This thesis presents an *in vitro* epithelial cell – immune cell co-culture model system, which can be used to explore intestinal epithelial permeability perturbations following interactions with any immune cell population of interest. I focused on the role of activated T cells, isolated via pan-T cell negative selection,
on intestinal epithelial barrier maintenance and dysfunction. It is simplistic to only consider the effects of the aggregate population of all PB T cells on intestinal epithelial permeability regulation, given the complexity of HIV-induced loss of mucosal immune homeostasis. HIV induces preferential depletion of the Th17 and Th22 subsets (125, 127), thus altering mucosal T cell subset balance. The activated PBTs utilized in our studies show a CD4/CD8 ratio consistent with that found in PB (data not shown), and the assumption is that the T cell subset balance would also be reflective of that found in venous blood of healthy individuals; further studies will be needed to address the intestinal epithelial consequences resulting from this aspect of the HIV-induced mucosal immune disruption.

My assay and hypothesis focus on aggregate T cell effects on epithelial permeability and the potential implications in the setting of mucosal CD4+ T cell depletion. Importantly, CD4+ T cell depletion is not the only feature of HIV-associated mucosal immune defect. Delineating the epithelial consequences of CD8+ T cell would help identify potential barrier-disrupting consequences of HIV-induced mucosal CD8+ T cell accumulation. Furthermore, impaired macrophage phagocytic function in the HIV gut (148) may also impact the epithelial barrier. The in vitro co-culture model system can be utilized to define the epithelial permeability effects of each of the aforementioned T cell subset and immune cell populations, obtained from healthy individuals, through which we can begin to postulate the
potential intestinal epithelial permeability consequences following HIV-associated GALT changes.

To directly address the intestinal epithelial consequences of HIV-associated immune compartment disruption via our in vitro co-culture model system, it would be worthwhile to investigate epithelial monolayer permeability effects of immune cells, either following infection with HIV ex vivo, or isolated from HIV+ patients. It is, however, pertinent to recognize that ex vivo HIV infection of immune cells from healthy controls would not entirely recapitulate the in vivo situation, given the existence of latently infected pools of immune cells and the low proportion of PB T cells (0.01-1%) that are productively infected in the HIV-infected individual (39). Alternatively, to probe for the overall consequences of HIV-associated loss in mucosal immune homeostasis on the intestinal epithelial barrier, we can perhaps co-culture Caco-2 monolayers in vitro with an artificially recreated immune cell cocktail that resembles that found in the HIV gut lamina propria, with corresponding proportions of immune cell subsets.

My studies demonstrated only the role of activated PBTs in intestinal epithelial barrier regulation. It is well appreciated that intestinal mucosal T cells display a unique phenotype that significantly differs from that of circulating T cells, such as different co-stimulatory requirements that predispose mucosal T cells to the development of anergy and immune tolerance. A more accurate depiction of the epithelial cell - T cell cross-talk in the intestinal mucosa would require repeating
the studies with activated lamina propria T cells, freshly isolated from intestinal samples obtained from bowel resection surgeries.

While we observed increased claudin-2 and claudin-4 protein levels in Caco-2 cells following co-culture with activated T cells, the mechanism through which activated T cells effect TJ modifications remains largely unexplored. Our data indicating increased TJ protein levels in the absence of transcript level alterations suggests the involvement of changes in protein translation and/or stability, in contrast to the majority of data demonstrating stimuli-induced acute TJ regulation via transcriptional control. To elucidate the molecular mechanism, I can follow a two-fold approach: identification of the immune mediator(s) from the activated T cells, and determination of the epithelial signaling pathway that results in the regulation of claudin-2 and -4, on the protein level.

Taking the first approach, I considered the Notch-1 pathway and TNF-α as potential candidates for mediating the IEC-T cell interaction. Activation of Notch-1 in Caco-2 cells was previously suggested to be involved in lymphocyte-mediated intestinal mucosal barrier function (270). However, blocking the Notch pathway with a γ-secretase inhibitor in our co-culture system did not alter the Caco-2 biphasic permeability response (data not shown). TNF-α, a proinflammatory cytokine secreted by activated T cells, is known to increase epithelial permeability via MLCK-mediated actomyosin ring contraction (271). Nonetheless, blocking TNF-α with an anti TNF-α antibody in my system was ineffective in ameliorating
the biphasic permeability response (data not shown). A starting point for identifying other possible pathways is to analyze existing proteome or transcriptome data on activated T cells to identify pathways and specific proteins/genes upregulated or otherwise altered upon activation (272-274). In particular, I have identified the Wnt signaling pathway to be a candidate pathway for further studies, given its relevance in T cells during development and following activation (275), and its known role in TJ formation and apicobasal polarity in epithelial and endothelial cells (276, 277). To investigate the relevance of identified candidates in activated T cell-induced intestinal permeability alterations, pharmacological blockage of the associated pathway can be performed in vitro. I can alternatively employ a genetic approach by knocking down the expression of said candidate in the immortalized Jurkat T cell line or primary T cell line, before co-culture with Caco-2 monolayers in the in vitro model system.

To investigate the epithelial signaling pathway involved in elevated claudin-2 and claudin-4 protein levels, it would be useful to perform an RNA-seq or proteomic analysis on Caco-2 cells following co-culture with activated T cells, at the peak and trough of the TER response, to identify key epithelial regulators that may be responsible for both tightening and weakening of the barrier. A particular focus should be on pathways and/or molecules that may be involved in regulation of protein stability or translation, given our data pointing against transcriptional regulation of involved TJ components.
The success of ART has turned HIV into a treatable chronic disease, but the need for developing new strategies to combat the residual systemic inflammation in ART-treated patients will be crucial in normalizing life expectancy for the ART-treated HIV+ individuals. I discovered and identified persistent colonic epithelial alteration in the ART-treated HIV-infected individuals, eluding to increased colonic permeability as a driver for microbial translocation and the residual systemic inflammation. Given the exploratory nature of the study, it provides a starting point from which further investigations can be launched to confirm our results in a larger cohort of ART-treated HIV+ patients. Importantly, a well-designed follow-up study should control for potential confounders including but not limited to age, sex, ethnicity, viral load, CD4 count, and existing intestinal symptoms, given the inherent heterogeneity of the HIV+ population. The study I presented was not designed to delineate the respective contributions of HIV, side effects of ART, and GALT disruptions to the colonic epithelial dysregulation – information that would be crucial to guide the development of novel therapeutic strategies to correct for the persistent colonic epithelial dysregulation. To answer these questions, future studies should involve additional cohorts of ART-naïve HIV-infected patients (ART-naïve, viremic, elevated systemic inflammation), ART-treated viremic patients (ART-treated, viremic, elevated systemic inflammation), ART-treated immune failures (ART-treated, not viremic, elevated systemic inflammation), long-term non-progressors (ART-naïve, viremic), and elite controllers (ART-naïve, not viremic).
My work highlights TJ disruption as a driver for intestinal barrier dysfunction in the setting of an HIV infection, suggesting that therapeutic interventions that strengthen TJs to reverse intestinal epithelial barrier disruption, when used in combination with ART, would be beneficial for attenuating microbial translocation and systemic inflammation that persists in such patients who are otherwise virally suppressed and immunologically competent. TJ-strengthening agents that can be considered are: i) zinc, shown to stabilize epithelial barrier function in Crohn’s disease (278); ii) quercetin, a flavonoid shown to upregulate claudin-4 expression (228), and iii) berberine, a traditional remedy shown to prevent claudin-2 upregulation (279). Given the inflammation-modulating effects of commensal bacteria, recent investigations have focused on the potential beneficial role of probiotics in the SIV-infected macaques (280), which have previously been shown to cause TJ remodeling (281) and improve the chance of maintaining remission of IBD (282). However, much progress still needs to be made in further defining TJ structure, regulation, and function, before specific TJ-targeting pharmacological agents can be developed.

Another issue currently under much scrutiny in the clinical management of HIV-infected patients involves the timing of ART initiation, as we attempt to balance between the cost and toxic effects associated with ART, and the potential benefits of early ART initiation. Systemic inflammation can be attenuated in patients who initiate ART early, but remains persistently abnormal when ART is initiated during chronic infection (283, 284). Early ART initiation is also correlated to fewer non-
AIDS events (97, 285); interestingly, reversal of intestinal mucosal immune homeostasis loss and restoration of gut mucosal CD4+ T cells may be more significant with early ART initiation, although recovery is rarely complete (116). Normalization of the GALT in this setting may ameliorate intestinal epithelial dysfunction and TJ downregulation; it would thus be interesting to characterize the intestinal epithelial TJ composition and barrier disruption in this patient population, so as to investigate the potential benefits of early ART initiation on the level of intestinal barrier dysfunction.

**5.4 IMPLICATIONS FOR INTESTINAL BARRIER DYSFUNCTION IN OTHER INTESTINAL INFLAMMATORY DISEASES**

A major thrust of my thesis work is to understand the mechanism of intestinal epithelial barrier disruption in the HIV-infected individual. To that end, I propose that HIV-associated loss of intestinal immune homeostasis alters the activated T cell-mediated regulation of intestinal epithelial TJ protein levels. The paradigm of activated T cell-mediated biphasic TJ modulation provides a framework from which we can explain how activated intestinal mucosal T cells can both protect the intestinal barrier and promote epithelial breaches, depending on the duration of T cell contact (Figure 5-2). This is of particular importance in disease processes involving dysregulation of both the intestinal epithelial barrier and the GALT. In the healthy gut, the largely immunologically tolerant intestinal mucosa maintains a baseline epithelial permeability set point. During a transient infection, when
pathogens invade the intestinal epithelium and reach the lamina propria, activation of mucosal T cells initiates a decrease in epithelial permeability, tightening the epithelial TJs to prevent further entry of pathogens. T cell activation is only short lived – as the infection is resolved, epithelial permeability re-equilibrates to baseline. However, in the cases of chronic inflammatory diseases of the intestine, such as IBD and celiac disease, or disease processes involving prolonged intestinal mucosal immune dysregulation, including GVHD, HIV infection, and diabetes, the presence or imbalance of activated mucosal T cells is long-lasting. Our findings indicate that the intestinal epithelium will respond to the prolonged contact with activated mucosal T cells with an eventual loss in barrier function, exacerbating the ongoing decline in mucosal immune homeostasis.

Indeed, this proposition is supported by studies demonstrating that TNF-α, potentially secreted by activated mucosal T cells, is the major mediator of intestinal barrier dysfunction in IBD (164), which is also implicated in causing intestinal barrier disruption in GVHD (286). However, my results indicate that the Caco-2 biphasic permeability response induced by activated T cell was not mediated by TNF-α, thus pointing us to reconsider whether other immune regulatory pathways exist to cause intestinal barrier dysfunction in these disease processes. Furthermore, this model proposes that TJ would be a key mediator in intestinal barrier dysfunction in intestinal inflammatory diseases. As previously mentioned for HIV infection, the concept that TJ-targeting therapies may
potentially correct the intestinal barrier defect in these diseases thus warrants further investigation.

As we further dissect the contributions of the intestinal mucosal immune compartment on epithelial permeability and barrier function, as it pertains to HIV infection, we should also consider how similar mechanisms might be involved in the pathogenesis of other diseases involving the disruption of both intestinal structural and immunological barriers.
Figure 5-1. Intestinal epithelial barrier breakdown in the HIV-infected individual
Figure 5-2. Outcomes of activated T cells-induced intestinal epithelial barrier regulation, dependent on duration of T cell contact, play a role in intestinal diseases.
NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

Jun 19, 2014

This is a License Agreement between Charlotte Chung ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

<table>
<thead>
<tr>
<th>License Number</th>
<th>3412630496322</th>
</tr>
</thead>
<tbody>
<tr>
<td>License date</td>
<td>Jun 19, 2014</td>
</tr>
<tr>
<td>Licensed content publisher</td>
<td>Nature Publishing Group</td>
</tr>
<tr>
<td>Licensed content publication</td>
<td>Nature Reviews Microbiology</td>
</tr>
<tr>
<td>Licensed content title</td>
<td>The structural biology of HIV-1: mechanistic and therapeutic insights</td>
</tr>
<tr>
<td>Licensed content author</td>
<td>Alan Engelman and Peter Cherepanov</td>
</tr>
<tr>
<td>Licensed content date</td>
<td>Mar 16, 2012</td>
</tr>
<tr>
<td>Volume number</td>
<td>10</td>
</tr>
<tr>
<td>Issue number</td>
<td>4</td>
</tr>
<tr>
<td>Type of Use</td>
<td>reuse in a dissertation / thesis</td>
</tr>
<tr>
<td>Requestor type</td>
<td>academic/educational</td>
</tr>
<tr>
<td>Format</td>
<td>print and electronic</td>
</tr>
<tr>
<td>Portion</td>
<td>figures/tables/illustrations</td>
</tr>
<tr>
<td>Number of figures/tables/illustrations</td>
<td>1</td>
</tr>
<tr>
<td>High-res required</td>
<td>no</td>
</tr>
<tr>
<td>Figures</td>
<td>Figure 1 Schematic Overview of the HIV-1 Replication Cycle</td>
</tr>
<tr>
<td>Author of this NPG article</td>
<td>no</td>
</tr>
<tr>
<td>Your reference number</td>
<td>None</td>
</tr>
<tr>
<td>Title of your thesis / dissertation</td>
<td>Tight Junctions – The Link Between HIV-Associated Intestinal Barrier Dysfunction And Loss Of Immune Homeostasis</td>
</tr>
<tr>
<td>Expected completion date</td>
<td>Jul 2014</td>
</tr>
<tr>
<td>Estimated size (number of pages)</td>
<td>220</td>
</tr>
<tr>
<td>Total</td>
<td>0.00 USD</td>
</tr>
<tr>
<td>Terms and Conditions</td>
<td></td>
</tr>
</tbody>
</table>
Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

5. The credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

   **Note:** For republication from the *British Journal of Cancer*, the following credit lines apply.
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)
   For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:
   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   **Note:** For adaptation from the *British Journal of Cancer*, the following credit line applies.
   Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)
7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

Note: For translation from the British Journal of Cancer, the following credit line applies.
Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication).

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Special Terms:

v1.1

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number 501332506. Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact RightsLink Customer Support: customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.


28. Guadalupe, M., Reay, E., Sankaran, S., Prindiville, T., Flamm, J., McNeil,


Drugs 74, 195–206


antiretroviral-naive subjects randomized to nelfinavir or efavirenz plus dual nucleosides. *AIDS* 19, 1807–1818


98. Caradonna, L., Amati, L., Magrone, T., Pellegrino, N. M., Jirillo, E., and


123. Mehandru, S., Poles, M. A., Tenner-Racz, K., Jean-Pierre, P., Manuelli, V.,


215


infection is associated with significant mucosal inflammation characterized by increased expression of CCR5, CXCR4, and beta-chemokines. *J. Infect. Dis.* **182**, 1625–1635


141. Ciccone, E. J., Read, S. W., Mannon, P. J., Yao, M. D., Hodge, J. N.,


homosexual men. AIDS 9, 1009–1016


Physiol 73, 283–309


213. Shifflett, D. E., Clayburgh, D. R., Koutsouris, A., Turner, J. R., and Hecht,


236. Fasano, A. (2012) Zonulin, regulation of tight junctions, and autoimmune


Ammori, B. J., Leeder, P. C., King, R. F., Barclay, G. R., Martin, I. G.,


