SIMIAN IMMUNODEFICIENCY VIRUS PATHOGENESIS AND THE GASTROINTESTINAL TRACT

by

Jessica Malzahn

B.S., University of Wisconsin – La Crosse, 2005

Submitted to the Graduate Faculty of

the Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2011

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This thesis was presented

by

Jessica M. Malzahn

It was defended on

April 20, 2011

and approved by

Thesis Advisor: Yue Chen, MD, PhD, Assistant Professor, Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh

Phalguni Gupta, PhD, Professor, Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh

Anthony Bauer, PhD, Associate Professor of Medicine, Department of Medicine/Gastroenterology, School of Medicine, University of Pittsburgh

SIMIAN IMMUNODEFICIENCY VIRUS PATHOGENESIS AND THE GASTROINTESTINAL TRACT

Jessica Malzahn, M.S.

University of Pittsburgh, 2011

It has been shown that HIV/SIV preferentially replicates in the gut associated lymphoid tissue (GALT) which leads to immune activation and disease progression. Different sections of the gastrointestinal (GI) tract have unique biological functions with specialized lymphoid tissue composition and distribution, which may contribute differently to viral pathogenesis. We hypothesize that the GALT serves as an active viral replication site during the acute and AIDS stages of HIV/SIV infection and as a viral reservoir in long-term non-progressors (LTNP) and antiretroviral therapy (ART) treated individuals; this viral replication results in local immune activation which contributes to disease progression. The RNA and DNA were isolated from tissues along the GI tract of uninfected and SIV infected rhesus macaques in different disease stages with or without ART. Real-time PCR was utilized to measure viral RNA and DNA, and mRNAs of CD4, TNF-α, IL-6, IL-1β, and MyD88. Our results showed that SIV DNA and RNA were detected in all GI tissues from infected monkeys regardless of the disease stage and drug intervention. However, the viral load distribution profile in the GI tract varied from monkey to monkey. Despite the undetectable viral load in peripheral blood, both viral RNA and DNA were detected in GI tissues of ART treated monkeys. Compared to the uninfected monkeys, low levels of CD4 mRNA were detected in SIV infected monkeys, particularly LTNP. There is a positive association between viral load and mRNA levels of TNF-α, IL-6, and MyD88 in the stomach and duodenum. However, no association was observed between viral loads and IL-1β mRNA levels

in any of the GI tissues examined. Data from this study indicates that the entire GI tract serves as a SIV replication site in all stages of infection, which leads to pro-inflammatory cytokine production and local inflammation. This study reveals the importance of the entire GI tract in HIV/SIV pathogenesis. Especially, in ART treated individuals with undetectable viral loads in blood, active viral replication in gut tissue may lead to development of drug resistant variants and faster progression to AIDS. This will have a profound impact on clinical intervention and public health as a whole.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

There is no way that I can fully express my extreme gratitude to and respect for Dr. Yue Chen. Her mentorship and ability to lead by example would put some of the greatest military leaders to shame. I have never had a mentor care so much not only about my performance, but also about how much I have truly learned. Thank you for creating an environment where I felt comfortable asking questions and admitting when I didn't know answers. Thank you for your confidence in me which helped grow my confidence in myself. I would also like to thank my committee members, Dr. Phalguni Gupta and Dr. Anthony Bauer whose suggestions and insight greatly helped to build this project. Additionally, great thanks are deserved for my classmates and friends, Maria Hillenbrand and Matthew Nicholaou. Maria befriended me from the beginning and guided me through the hardest parts of this program. She calmed me when classes were overwhelming and always found a way to help me through. Without her, this grad school experience would have been more of a chore instead of the exhilarating experience it has been. Matt has been a constant source of support. Whether I needed encouragement when it felt like I was failing, direction when I lost my focus, or an ear to listen as I talked things out; Matt was always supportive. Lastly, I'd like to thank my family, who has never doubted my ability to succeed. To my sisters Stephanie and Alison: thank you for all of the grad school commiserations and for the relationships that we're continuing to build. I love that my sisters are becoming my best friends. To my parents, Paul and Jan: your support and encouragement have helped me more than you know. I truly wouldn't have attempted this if I didn't know that you were fully behind me. Thank you.

1.0 INTRODUCTION

For the last 20 years, tremendous progress has been made for understanding HIV pathogenesis, developing effective treatment for HIV infected people and searching for an effective vaccine and other prophylactic methods to prevent people from contracting HIV. However, there is currently still no effective method (other than condom use and abstinence from high risk transmission activities) to prevent HIV infection or eradicate HIV from infected patients. According to the UNAIDS 2010 Global Report, there are approximately 33.3 million people currently living with HIV (Fig 1). Due to the effective prevention strategy and effort, new infections have decreased by more than 25% in 33 countries (22 of which are in sub-Saharan Africa) and a 19% decrease in AIDS-related deaths among children globally since 2001 (Fig 2). Due to ART treatment available worldwide, 20% fewer people died of AIDS related causes in 2009 alone However, there are some countries where HIV incidence has increased by more than 25% since 2001. An estimated 2.6 million new HIV infected cases were reported in 2009 alone with approximately 370,000 children contracted during the perinatal or breastfeeding period. Figure 1 shows the global prevalence of HIV in 2009, with the highest prevalence areas remaining sub-Saharan Africa and Russia. Figure 2 shows the global incidence rate or rate of new infections from 2001 to 2009.

Source: UNAIDS.

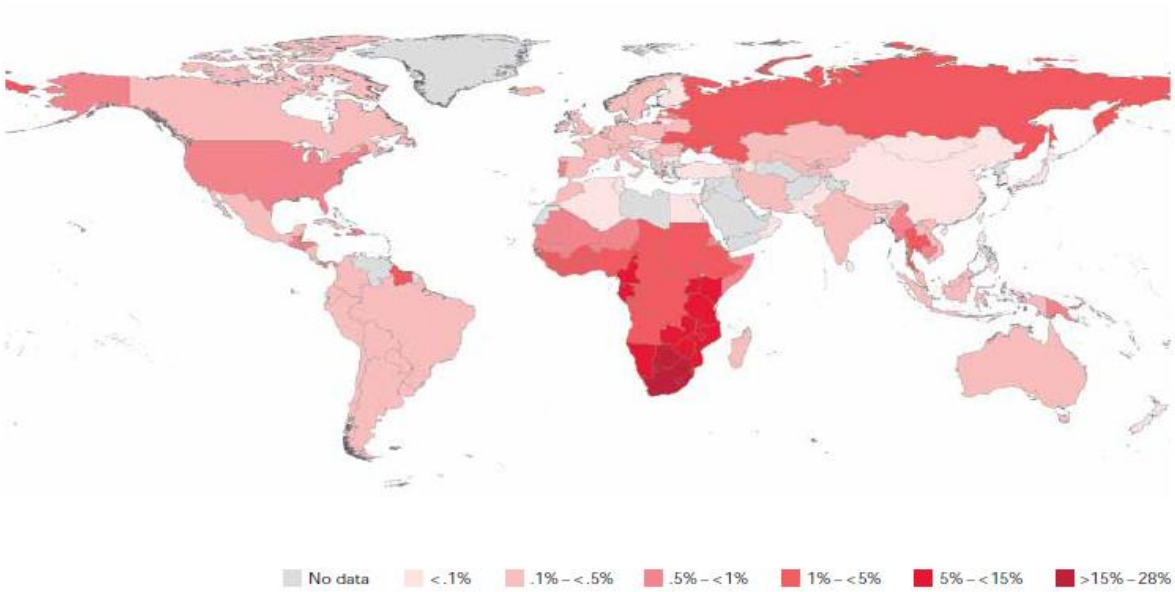

Figure 1 Global prevalence of HIV infection in 2009.

Source: UNAIDS.

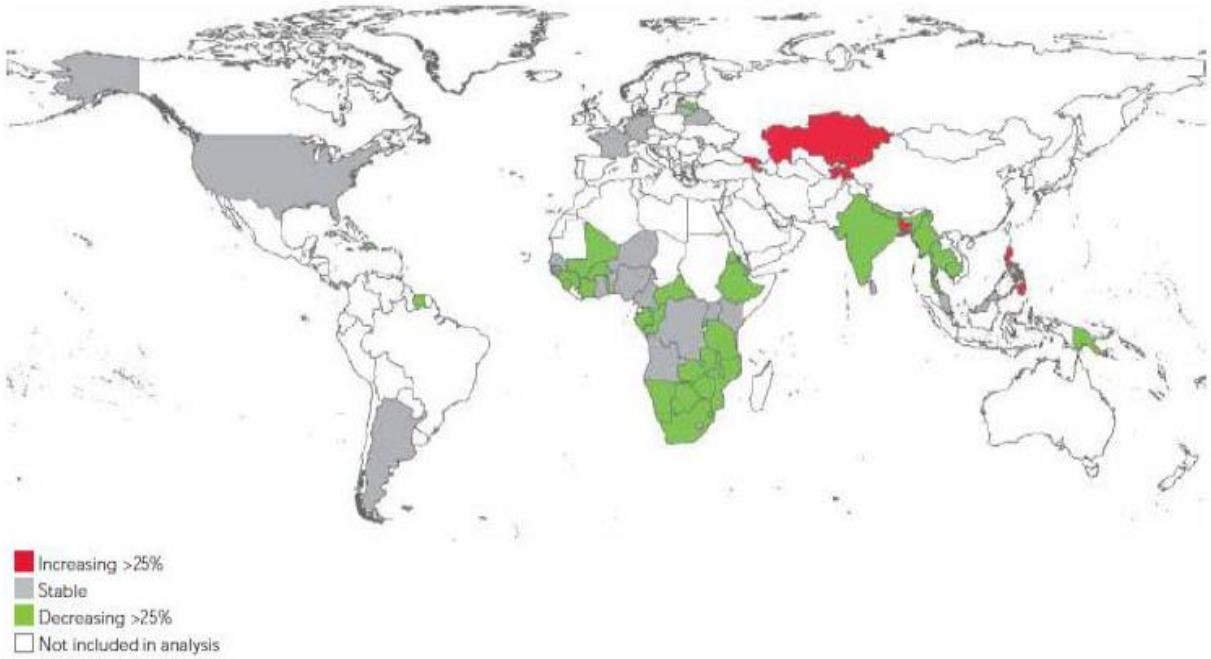

Figure 2 Changes in the global incidence rates of HIV from 2001 to 2009.

1.1 HUMAN IMMUNODEFICIENCY VIRUS

1.1.1 Virus Life Cycle

HIV belongs to the retroviridae class of viruses and is further classified with the lentiviruses, or "slow" viruses. Retroviruses are characterized by the need to use reverse transcriptase to convert their RNA genome to DNA for incorporation into the host genome. Lentiviruses are characterized by a long period of time between initial infection and the onset of serious symptoms [1]. HIV attaches to and enters a cell by binding to CD4 and a co-receptor (CCR5 or CXCR4) on the surface of the cell, and fusing with the cellular membrane (Fig 3). On the way into the cell, the virus is uncoated to liberate the viral RNA genome and the reverse transcriptase that are carried in the virion. In the cytoplasm, reverse transcriptase converts the viral RNA genome to DNA, and with the help of viral protein R (vpr), the cDNA enters the nucleus. Once inside the nucleus the viral DNA is inserted into the host genome with the help of viral protein integrase. No new virus is produced from this cell until it is activated, and once it is, transcription occurs and mRNA is produced from the viral (and host) genome. This mRNA leaves the nucleus for translation in the cytoplasm, and viral proteins are made. Assembly occurs at the membrane, and a new virus buds off of the host cell. Viral protein protease further matures the virion and it is completely released from the cell [1].

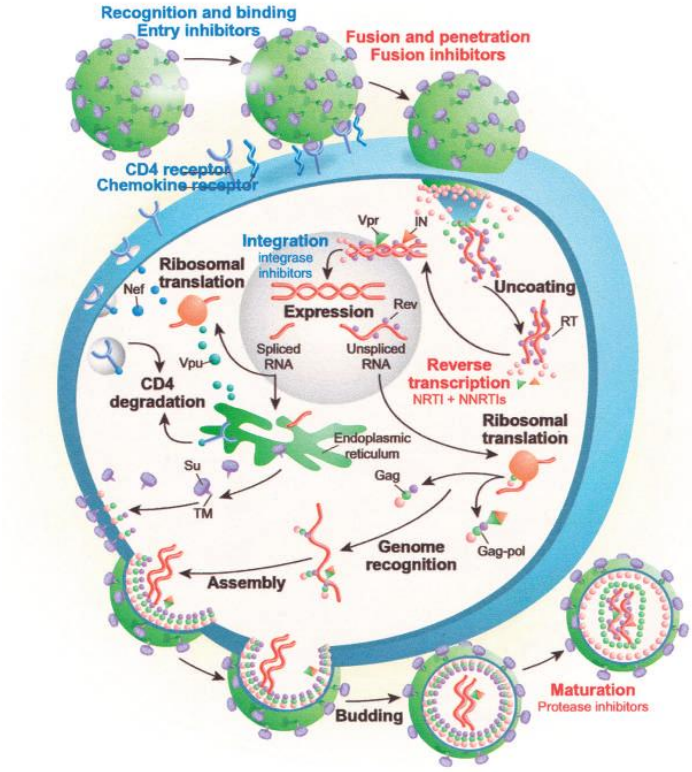

Figure 3 HIV Life Cycle [1] (Permission granted from rightsholder, Nature Publishing Group)

1.1.2 HIV Infection and Disease Progression

The majority of HIV infections are acquired through sexual activity, blood transfusion or mother-to-child transmission. During the first few weeks of infection (acute phase), a human often suffers from flu-like symptoms with a rash. This initial batch of symptoms is due to the innate immune response to the initial viral load peak around 10 days post infection. After the acute phase, the human is without symptoms (chronic or latent phase). During this time HIV infects $CD4^+$ T cells or T-helper cells, which signal other immune cells such as $CD8^+$ T cells, or cytotoxic T cells, and B cells to perform their functions. Quickly, these $CD4^+$ T cells are killed

by the virus faster than the body can produce them, and the $CD4^+$ T cell count begins to drop. A normal $CD4^+$ T cell count is 800 to 1200 per mm^3 of blood. When the $CD4^+$ T cell count falls below 200 cells per mm^3, the person becomes vulnerable to infections that a person with a normally functioning immune system would not be infected by, or "opportunistic infections." It is these opportunistic infections and some rare cancers that define the onset of AIDS, which is the last stage of HIV infection before death [1,2,3].

Disease does not progress at the same rate for all people that are infected. The chronic phase of HIV infection can last from 3 to 20 years, depending on disease progression (Fig 4). There are some factors that have been shown to have an effect on the rate of disease progression and severity of HIV infection such as: type of transmission, amount of virus at the time of transmission, co-infection with other microorganisms, age, genetic disposition, and host immune response [4,5]. As a result, infected individuals have been categorized in many different ways by various groups, but generally they are categorized as rapid progressors, intermediate progressors, and long-term non-progressors (LTNP). Rapid progressors typically progress to AIDS within 3-5 years without therapy, while LTNP remain asymptomatic for 10-20 years with $CD4^+$ cell counts above 500 cells/mm^3 [4]. Unsurprisingly, these LTNP are of particular interest to researchers because of their apparent natural ability to control the virus.

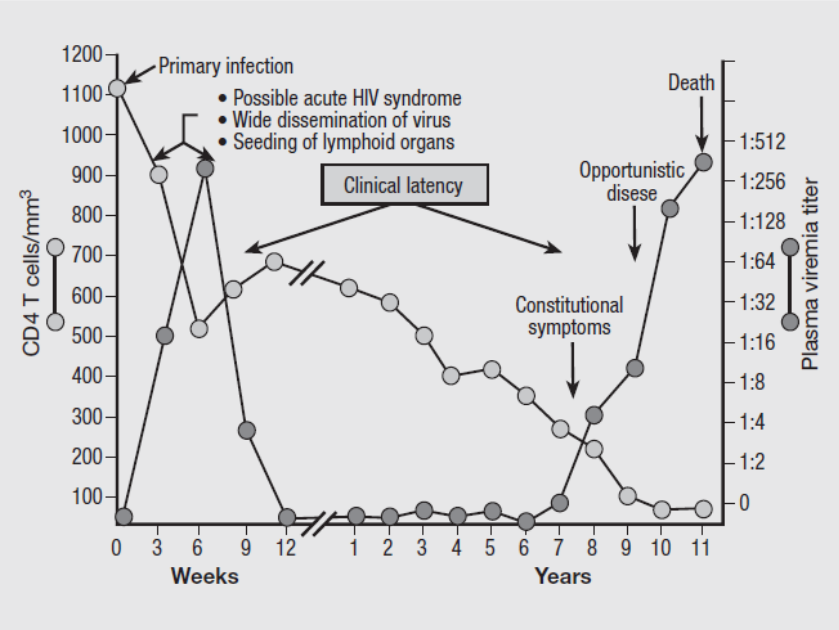

Figure 4 Natural disease progression relating CD4$^+$ T cell count and plasma viral load without treatment. (Permission granted from rightsholder, New England Journal of Medicine)

1.1.3 Long-Term Non-Progressors/Elite Controllers

As previously mentioned, within the population of HIV-infected individuals, there is a subpopulation (2-5%) considered "long-term non-progressors" (LTNP) [4]. For still undetermined reasons, this population does not progress to AIDS despite lack of therapy. A study in 2010 by Julg, et al, [6] determined that not only do LTNP have decreased levels of plasma viral replication, but they also have decreased numbers of CD4 T cells with integrated HIV provirus compared to rapid progressors (Fig 5). This would suggest that either these individuals have a unique ability to control viral replication, they are infected with viruses that do not replicate, or they have CD4 T cells that are resistant to HIV [6].

Some studies have shown that long-term non-progression is due to infection by an attenuated strain (due to mutated proteins produced by that strain), or due to mutation that causes

an attenuated strain post-infection. Additionally, studies have shown genetic factors involved in long-term non-progression such as heterozygosity for the CCR5-Δ32 polymorphism which produces modified CCR5 receptors that HIV cannot bind to, or host mutations that result in up-regulation of the production of chemokines that competitively inhibit HIV-1 binding to CCR5 or CXCR4. Lastly, studies have shown that many LTNP carry the HLA class I B57 allele that enhances presentation of antigenic peptides on the surface of infected CD4 T cells to CD8 T cells, causing more HIV infected cells to be killed before more virus can be produced [4].

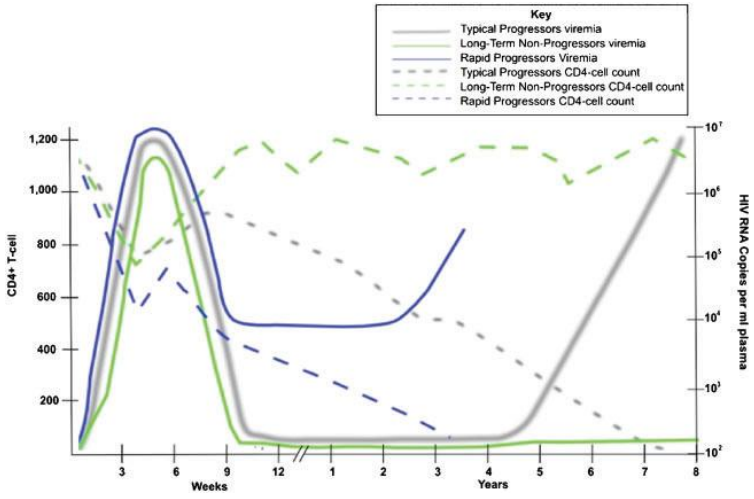

Figure 5 Comparison of virus levels to CD4$^+$ T cells in various progressor types [4] (Permission granted from rightsholder, Society for General Microbiology, LTD)

1.1.4 Antiretroviral Therapy

While LTNP are unique in their ability to control HIV, rapid and intermediate progressors also have the ability to control HIV with the help of antiretroviral therapy (ART). When the first anti-HIV drug (AZT) was used in 1987, the life expectancy with HIV was less than a year. In 1996, a new class of drugs (protease inhibitors) was introduced along with the idea of combining 3 types of drugs to make highly-active antiretroviral therapy (HAART), a concept that is still

used today and allows HIV patients to survive for decades [3,7]. While there is no question that HAART is effective at controlling virus replication, controversy lies over whether early treatment with ART (or HAART) leads to mucosal $CD4^+$ T cell restoration [8] or not [9,10].

There are currently eight classes of HIV drugs, that each target a part of the viral life cycle. Nucleoside reverse transcriptase inhibitors are nucleoside analogs that bind to reverse transcriptase and result in chain termination of the DNA strand. Non-nucleoside reverse transcriptase inhibitors also bind to reverse transcriptase, but they cause a conformational change in the protein such that nucleotide incorporation is decreased. Protease inhibitors block the enzyme protease which is needed for virion maturation and release from the host cell. Integrase inhibitors prevent the viral DNA from incorporating into the host genome. Entry inhibitors block the proteins needed for the virus to bind to the host cell, CD4, CCR5, or CXCR4. The class "Entry inhibitors" are actually further broken down according to the stage of entry that is prevented: Fusion inhibitors target the proteins of the virus that are used to bind to the host cell and Co-receptor antagonists use chemokine receptors to block HIV from binding. Lastly, Maturation inhibitors block a step in late Gag processing that converts the pre-capsid protein to the mature capsid protein. [7].

The success behind the concept of HAART is that targeting multiple steps of the viral life cycle decreases the chances of viral resistance, in that most viral replication is stopped before it has a chance to produce mutated progeny. There has been controversy over when in the disease stage to start HAART. Because the drugs cannot completely eliminate the virus, residual replication occurs, and eventually enough mutant viruses are allowed to replicate and a resistant strain emerges. The rate at which viruses become resistant to drugs is mainly based on medication compliance. If an individual takes their medication when he/she are supposed to then

resistance does not occur as quickly as when medication is taken inconsistently or when a patient goes on a "drug holiday" where they take a break from medication. Because of the eventual viral resistance and the difficulty of patient compliance, some argue that administering HAART early in infection is unwise, and instead believe that HAART should be prescribed when the patient begins to show signs of immune deterioration, mainly when the $CD4^+$ T cell count gets below a certain level. Others argue that beginning HAART immediately upon knowledge of infection keeps the viral load low, and decreases the amount of virus that can be transmitted [3,7,11]. Current guidelines issued in 2009 by the US DHHS and in 2010 by the International AIDS Society-USA recommend treatment for asymptomatic patients with cD4 cell counts $\leq$ 500 cells/mm3 and recommend consideration for treatment for those with counts > 500 cells/mm3 [12].

1.2 SIMIAN IMMUNODEFICIENCY VIRUS

Since HIV infects only humans, SIV infected monkeys have been extensively used as a suitable animal model for HIV/AIDS related researches. Although SIV infected monkeys have a similar disease course like HIV-infected patients, there are some difference in clinical symptoms and characteristics of disease progression between HIV infected humans and SIV infected monkeys, which should be considered when extrapolating the experimental date derived from SIV infected monkey to HIV infected humans.

1.3 CLINICAL DISEASE PROGRESSION

1.3.1 Clinical Disease in Humans

In humans, the previous discussed symptoms (flu-like symptoms with potential rash) generally occur 2 to 6 weeks after infection, and can last from a few days to 10 weeks, but usually symptoms last less than 14 days [13,14]. The viral load peaks for most infected humans at 4 weeks. The viral set-point is established at different times post-infection depending on the progression status. Human rapid progressors establish a viral set point around 8 weeks and progress to AIDS in only a few years without treatment. Moderate or typical progressors' set point is lower and is established around 16 weeks with progression to AIDS occurring in 7 to 10 years, and slow progressors take longer to establish a set point, around 24 weeks, have a significantly lower set point than rapid or moderate progressors and can live for decades without progressing to AIDS [14].

1.3.2 Clinical Disease in Macaques

The survival time for SIV-infected Rhesus macaques is around 290 days (depending on dose, route of infection, and strain virulence). The viral load for most infected Rhesus macaques peaks at 14 days post infection. Rapid progressors will reach the AIDS stage in less than a year, and will have detectable SIV by 3-5 days post-infection, with the viral load set point only slightly decreasing following the high peak at 14 days. Moderate progressors reach AIDS in 1-3 years, have a high peak like rapid progressors, but have a much lowered set point by 1-3 months post infection. Rhesus macaques considered slow progressors survive longer than 3 years post

infection, have a lower viremic peak than rapid or moderate progressors, and have a much lowered set point that is reached around a year post infection [15].

1.3.3 Natural Controllers

Somewhat like long-term non-progressors in humans, there are some primates that exist that are considered natural controllers of SIV. They are not considered LTNP because they simply never progress. Sooty manages, African green monkeys, and mandrills have all been found to be natural SIV hosts [16]. Rhesus macaques and Cynomolgus macaques however, progress quite rapidly, and are a more suitable animal model for SIV/HIV. Much like LTNP in humans, it is of value to study these natural controllers, because perhaps within them lies the secret of SIV/HIV control that could lead to a curative drug.

Some differences between natural controllers and natural progressors have been found that may shed light on this. A study in 2010 by Brenchley [16] revealed that a quantitative and qualitative differences exists in T cell depletion between natural and non-natural hosts. Particularly, several studies have found that natural hosts express very low amounts of CCR5 on CD4 T cells, and yet their CD8 T cell expression of CCR5 is comparable to rhesus macaques and even humans. Additionally, it has been found that African green monkeys down-regulate CD4 expression as naïve CD4 T cells are activated to memory T cell status, such that the function of memory T cells is carried out by CD4 negative T cells, which protects those cells from SIV infection.

1.4 HIV AND THE GASTROINTESTINAL TRACT

1.4.1 HIV Pathogenesis in the GI tract

Regardless the route of HIV-1 transmission, HIV quickly invades gastrointestinal tract directly or via blood circulation after infection. Due to the special environment of GI tract, HIV rapidly replicates in the gut associated lymphoid tissue and establishes the largest virus reservoirs in the body.

Acute HIV infection results in a rapid decrease in blood $CD4^+$ T cells, however, as soon as the immune system responds, the $CD4^+$ T cell count increases. As disease progresses the blood $CD4^+$ T cell count will slowly drop until AIDS sets in, however ART can make that decline less severe. The $CD4^+$ T cell dynamic change along the GI tract post HIV infection is quite different. It has been reported that $CD4^+$ T cells in the lamina propria of GI tract were severely depleted within the first few *days* of infection, and their numbers stayed low throughout the entire course of disease [17] . Primary HIV-1 infection is associated with preferential loss of $CD4^+$ T lymphocytes in patients infected with HIV [18,19]. Furthermore, following ART, $CD4^+$ T cells fail to reconstitute in the GI tract despite the increased $CD4^+$ T cell count in peripheral blood. A recent study showed that HIV proviral DNA persisted in $CD4^+$ T cells in the terminal ileum after almost 10 years of effective therapy [20].

HIV disease progression has been linked to immune activation as a result of gut-associated lymphoid tissue (GALT) disruption [21]. During acute infection, the virus has a ready supply of activated CD4 T cells in the GALT. Rapid HIV replication results in CD4 T cell decline and inflammation in GALT, which lead to mucosal barrier dysfunction. During the chronic stage, loss of GALT integrity may play a major role in HIV pathogenesis [22].

Researchers have found higher levels of microbial products in the systemic circulation of HIV infected patients than in healthy subjects, with a similar outcome observed in the pathogenic SIV rhesus macaque model [21]. Marchetti et al found that microbial translocation is associated with sustained failure in $CD4^+$ T cell reconstitution in AIDS patients and conducted a study including patients on HAART and patients that did not receive treatment, and they found that in patients with suppressed viral loads due to HAART, low-level microbial translocation still occurs and is still associated with a lower $CD4^+$ T cell count [23].

It is not clear whether immune activation causes the immune system to break down so that it can no longer control microbial translocation or if the translocated bacteria activate the immune system so much that it drives the immunodeficiency seen in HIV. The reality is that most likely both occur, causing a cycle where one situation triggers the other. Regardless, the sustained immune activation is detrimental to T cell function and survival because the large pools of target cell are activated and prone to HIV infection [24]. Even though there is extreme $CD4^+$ T cell depletion at the gut, and microbial translocation has been shown to cause immune activation, there has still been no positive association between plasma viral load and LPS [21,25].

In addition to severe $CD4^+$ T cell depletion, HIV/SIV has also been shown to interfere with stromal and epithelial cells of the intestinal mucosa in the form of apoptosis during acute infection [25]. It has been found that as pro-inflammatory genes are up-regulated due to infection, genes that are responsible for epithelial regeneration are down-regulated [10]. Of interest, it has been noted that during chronic infection, IL-6 and STAT3 are up-regulated [26], and mucosal IL-2, IL-6, and TNF-α are increased. All of this up-regulation results in increased epithelial permeability [27].

1.4.2 Anatomy of the GI tract

The purpose of the gastrointestinal tract is to break down food, absorb the nutrients, and remove unneeded waste. The GI tact begins with the esophagus, a muscular tube, which moves food from the mouth to the stomach. Food travels through the esophagus using peristalsis and gravity, and movement is aided by mucus. The stomach is made up of a mucosal layer and 3 muscle layers at different angles. In the stomach gastric juices break down food and the muscles churn the food to mix it with the gastric juices. At the exit of the stomach is the pyloric sphincter that releases digested food (now in liquid form) to the beginning of the small intestine. The small intestine is made up of a mucosal layer, a sub mucosa (a loose layer covering vessels and nerves), muscularis (muscle layer with outer and inner circular muscle fibers), and outer serosa (an outer protective membrane). The small intestine is made up of the duodenum, jejunum, and ileum. The inner lining of the small intestine has millions of projections called villi. Every villus contains a lacteal (lymph vessel) and a network of tiny blood vessels which provide nourishment. Each villus is also covered by epithelium that absorbs nutrients. The epithelial cells themselves have numerous projections called microvilli. Together, the villi and microvilli increase the total surface area of the small intestine for efficient and optimum absorption of nutrients. After the food exits the stomach it enters the duodenum which contains circular ridges that help with the propulsion of food. The duodenum receives secretions from the liver and pancreas to aid in digestion. After the duodenum the food travels through the long and coiled jejunum and ileum. After the ileum, the food enters the cecum, the first part of the large intestine. Similar to the small intestine, the large intestine has a mucosal layer, sub mucosa, and muscularis layer. The large intestine is made up of the cecum, colon, and rectum, and is the location in the body where stools are formed. The cecum is the short pouch that connects the

small intestine to the colon. The colon is the major part of the large intestine; its main function is to convert the parts of food that were not absorbed by the small intestine into stools. The colon draws the water out of the food and reabsorbs it into the body. It also absorbs vitamins that are produced by bacteria in the colon. To do this, the colon has muscles that propel stool along the colon, with an inner lining that secrets mucus to lubricate the inside. Stools are collected in the rectum before passage through the anus [28].

1.4.3 What is known about how HIV affects the GI tract

1.4.3.1 HIV is attracted to the GI tract

The GI tract has been found to be particularly permissive to replication by both HIV-1 and SIV in human and non-human primates [29]. This is mainly due to the fact that the gastrointestinal (GI) mucosa contains 50-80% of the body's total CD4 T cells [30]; due to GALT specific biological function majority of the CD4 T cells are activated. It is therefore an important site of viral replication and CD4 T cell depletion during acute HIV infection [31,32,33,34,35] . In contrast to the systemic immune system, the $CD4^+$ T cells of the mucosal immune system are mostly $CCR5^+$ activated memory T cells [36] which demonstrates why the GI tract is considered to be the most important site of viral replication [34]. Additionally, studies have shown on average 5 times more HIV DNA in CD4 T cells of the gut than in the peripheral blood [20,30].

1.4.3.2 Influx of cytokines to the GI tract upon infection

The innate immune system has two important responses to viral infection; phagocytosis to take up the pathogen, and elimination of infected cells by Natural Killer cells (NK cells). Upon recognition of a foreign pathogen or infected cells, macrophages that conduct phagocytosis and

NK cells that kill the infected cell produce a variety of cytokines that will cause more innate immune cells to infiltrate the area in an attempt to eliminate the pathogen [37]. As these activated dendritic cells (DC's), additional macrophages, and NK cells are brought to the area, and as cytokines cause vascular dilation, damage is done to the tissues surrounding infected area. When this area is along the GI tract, damage is done to the epithelial wall and the barrier with the gut lumen is breached.

1.4.3.3 Microbial Translocation occurs due to cytokines

Because certain areas of the GI tract use bacteria help process its contents, a breach in the barrier of the lumen of the GI tract can allow this helpful bacteria to cross the epithelial barrier and enter the tissue beneath, this is called microbial translocation. When bacteria enter this forbidden area, the innate immune system responds by activating more immune cells, causing a larger inflammatory reaction. One job that is shared by many of the cytokines produced is lymphocyte activation. So as this innate response occurs, more activated T cells are brought to the area.

1.4.3.4 Additional inflammatory response due to microbial translocation

Because more target cells are brought to the area, more of them become infected by HIV, this perpetuates the cycle causing more immune activation, microbial translocation, target cell activation, and infection. If this cycle is allowed to continue, disease progression can be increased as the CD4 T cells are literally being drawn in by the immune activation due to microbial translocation and then ambushed by HIV when they arrive. This depletion of CD4 T cells at the gut has been repeatedly shown [10,16,19,21,26,30]

This immune activation is so important that it has been shown that the fundamental difference between progressive or HIV infection and non-progressive SIV infection is the

absence of immune activation during the chronic phase of infection in natural hosts. A study by Brenchley in 2010 showed that microbial products were present at very low amounts in infected natural controllers (African green monkeys and sooty managabeys) even in the presence of high viral loads [16]. This suggests that the lack of microbial translocation plays a part in the lack of systemic immune activation [16].

1.4.4 Cytokines involved in innate immune activation at the GI tract

Immune depletion is due to immune activation [21,31] , particularly IL-1β, TNF-α, and IL-6 production; increased production of which have all been associated with higher levels of viral replication [38]. Interleukin-1β is a 153 amino acid, 17.5 kDa protein that is produced by macrophages and epithelial cells. It is a prototypical pro-inflammatory cytokine whose production results in T cell and macrophage activation. It is processed and released from cells by a mechanism involving caspase-1. Interestingly, activation of NFκB inhibits IL-1β release by macrophages [39]. Tumor necrosis factor–α is a 157 amino acid, 17 kDa protein that functions when configured as a trimer. It is produced by macrophages, NK cells, and T cells. TNF-α promotes inflammation as well as DC and endothelial cell activation (which results in cell to cell junction changes and increased fluid loss). Importantly, IL-1β and TNF-α are the main cytokines that draw in more immune cells to the area of infection. Interleukin-6 is a 184 amino acid, 25 kDa protein that is produced by T cells, macrophages, and endothelial cells. Production of IL-6 results in T and B cell growth and differentiation, as well as acute phase protein production [37] and inhibition of cellular apoptosis [40].

1.4.5 What is unknown about how HIV affects the GI tract

Although much research has been done to elucidate the role of GALT in SIV/HIV infection, there is still controversy regarding the role of GI pathogenesis in disease progression. Furthermore, the exact role of each of the GI components in the SIV/HIV pathogenesis is still unclear. To date, no comprehensive studies have been performed in every part of GI tract to elucidate which GI part is most important in HIV infection and viral reservoir formation, which part contains the most detrimental immune activation which propels disease progression, or what effect HAART has on the those pathogenic changes. Elucidating the pathogenic change in different parts of GI tissue will assist us in designing an effective therapeutic intervention for infected patients.

1.5 HYPOTHESIS

We hypothesize that gut associated lymphoid tissue serves as a main viral replication site during acute SIV/HIV-1 infection and as a viral reservoir in long-term non-progressors (LTNP) and in antiretroviral therapy (ART) treated individuals; this viral replication results in local immune activation which contributes to disease progression.

1.6 SPECIFIC AIMS

1. Optimize multiplex real-time PCR methodologies for monkey GI tissue.
2. Assess the SIV and CD4 distribution in various anatomic locations throughout the GI tract in various stages of SIV infection.
3. Elucidate the cytokine profile in various anatomic locations of the GI tract and its association with viral load.

2.0 MATERIALS AND METHODS

2.1.1 Animals and Tissue Samples

The snap-frozen GI tissues from the Rhesus monkeys listed in the upper portion of Table 1 were received from Dr. Murphey-Corb and Dr. Reinhart's Labs. Those monkeys were part of previous studies for SIV pathogenesis. The snap-frozen GI tissues from Rhesus monkeys in the lower part of Table 1 were received from Dr. Barratt-Boyes's lab. Those monkeys were used in an ART study in 2008/2009. Monkeys that received ART as part of the ART study received: PMPA, a nucleoside phosponate analog, at 30mg/kg daily, subcutaneously from days 7 to 20, this was reduced to 20 mg/kg once daily, subcutaneously from day 21 to 35, FTC, a nucleoside reverse transcriptase inhibitor at 30 mg/kg once daily subcutaneously from day 7 to 35, and an experimental oral integrase inhibitor at 10 mg/kg twice daily by mouth (in kool-aid, a primate cookie, or similar). Additionally, the CD4+ T cell count from the blood, and the plasma viral load were provided for the ART study monkeys and are included in Table 1.

Table 1 Monkey Subjects and Tissue Samples

Monkey ID	Disease Stage	Inoculation Strain	Route of Infection	Plasma Viral Load (mRNA copies/mL)	CD4+ T cells/μL	Monkey Species	Lab Origination
M6600	uninfected	NA	NA	NA	NA	Rhesus macaque	Dr Reinhart
M5600	uninfected	NA	NA	NA	NA	Rhesus macaque	Dr Reinhart
M5299	Acute	SIV/DeltaB670	IV	NA	NA	Rhesus macaque	Dr. Murphey-Corb
M5699	Acute	SIV/DeltaB670	IV	NA	NA	Rhesus macaque	Dr. Murphey-Corb
M9797	Long term non-progressor	SIV/DeltaB670	IV	NA	NA	Rhesus macaque	Dr. Murphey-Corb
M9497	Long term non-progressor	SIV/DeltaB670	IV	NA	NA	Rhesus macaque	Dr. Murphey-Corb
M9697	Long term non-progressor	SIV/DeltaB670	IV	NA	NA	Rhesus macaque	Dr. Murphey-Corb
M9597	Long term non-progressor	SIV/DeltaB670	IV	NA	NA	Rhesus macaque	Dr. Murphey-Corb
M6199	AIDS	SIV/DeltaB670	IV	NA	NA	Rhesus macaque	Dr. Murphey-Corb
M5199	AIDS	SIV/DeltaB670	IV	NA	NA	Rhesus macaque	Dr. Murphey-Corb
M1799	AIDS	SIV/DeltaB670	IV	NA	NA	Rhesus macaque	Dr. Murphey-Corb
M126-08	Acute +ART	SIVmac251	IV	<200	749.71	Rhesus macaque	Dr. Barratt-Boyes
M127-08	Acute +ART	SIVmac251	IV	<200	829.85	Rhesus macaque	Dr. Barratt-Boyes
M129-08	Acute +ART	SIVmac251	IV	<200	679.98	Rhesus macaque	Dr. Barratt-Boyes
M132-08	Acute +ART	SIVmac251	IV	<200	1682	Rhesus macaque	Dr. Barratt-Boyes
M134-08	Acute +ART	SIVmac251	IV	<200	332.01	Rhesus macaque	Dr. Barratt-Boyes
M128-08	Acute	SIVmac251	IV	7343	273.03	Rhesus macaque	Dr. Barratt-Boyes
M130-08	Acute	SIVmac251	IV	52361	422.78	Rhesus macaque	Dr. Barratt-Boyes
M133-08	Acute	SIVmac251	IV	4200	714.23	Rhesus macaque	Dr. Barratt-Boyes
M139-08	Acute	SIVmac251	IV	6716	427.62	Rhesus macaque	Dr. Barratt-Boyes
M140-08	Acute	SIVmac251	IV	21974	367.99	Rhesus macaque	Dr. Barratt-Boyes
R222	uninfected	NA	NA	NA	1365.1	Rhesus macaque	Dr. Barratt-Boyes

2.1.2 Isolation of RNA and DNA from all GI tissue samples

2.1.2.1 Isolation Method

The RNA and DNA were isolated from the snap-frozen tissues (Table 1) by homogenization followed by use of the Qiagen AllPrep DNA/RNA Mini ® kit. Briefly, an approximately 20 mg piece of tissue was placed into a pre-cooled thick plastic bag. The bag was placed on a metal

pedestal half submerged in dry ice (so that the metal pedestal became very cold), and the tissue was smashed with a hammer until it became a fine powder. Then 600 μL of the lysis buffer (with β-mercaptoethanol added) was added and the solution was mixed until it turned viscous. Then the solution was transferred to a 2mL centrifuge tube and further homogenized using an OMNO International TH-01 Homogenizer and OMNI International Non-sterile Hard Tissue disposable rotor stator generator probes until no tissue chunks were visible and the entire solution was homogenous. The subsequent DNA/RNA isolation procedures were based on the instruction in the AllPrep DNA/RNA mini® Handbook (Nov 2005) with the Simultaneous Purification of Genomic DNA and Total RNA from Animal Tissues. To avoid residual DNA contamination in RNA preparation, samples were treated with DNase during the RNA isolation procedure. All DNA samples were eluted twice with 40 μL of Elution buffer, and all RNA samples were double eluted with 40 μL of RNAse-free water (40 μL was added to the column, it was spun, and then the flow through was added back to the column to pick up any residual RNA).

2.1.2.2 Nucleic Acid Quantitation

Following the DNA/RNA isolation from the tissues, the nucleic acid concentration for each sample was measured using a Bio-Rad Smart Spec 3000. The purity of RNA or DNA samples has been confirmed to be near 100% by RT real time PCR amplifying only cDNA target, real time PCR only amplifying DNA target and amplifying both cDNA/DNA targets. All samples were diluted to 80 ng/μL and stored in -80ºC freezer for the subsequent procedures.

2.1.3 PCR

2.1.3.1 Reverse Transcriptase PCR

Reverse transcription was performed to convert the isolated RNA into cDNA. For every RNA sample, the reverse transcription (RT) mixture was prepared using the reagents found in Table 2.

Table 2 Reverse Transcriptase PCR Reagents

Reagents	RT mixture for one reaction (µL)
1. 10X PCR BUFFER	5
2. 25Mm MgCl2	11
3. dNTPs (25mM each)	1
4. RTssII (200u/µL)	0.3125
5. RNAsin (40u/µL)	0.5
6. Random Hexamers (100uM)	1.25
7. NF-water	28.44
Total Volume (µl)	47.5

To a tube with 47.5µL of RT mix, 2.5 µL of 80ng/µL RNA (200 ng total) was added. The tube was placed in an ABI GeneAmp PCR system 9700 thermocycler and run at 25ºC for 10 min, 48ºC for 30 min, 95ºC for 5 min, and then cooled to 4ºC indefinitely. Upon completion of the reverse transcription, the cDNA samples were stored at -80ºC until use.

2.1.3.2 Real-Tim PCR Procedure

Depending on the targets measured in the PCR, uniplex or duplex real time PCR was performed. The real-time PCR mixture for uniplex was prepared according to the reagent list in Table 3. The real-time PCR mixture for duplex was prepared according to the reagent list found in Table 4.

Table 3 Real-time Uniplex PCR Reagents

Reagents	Mixture for one reaction (μL)
2X Universal Taqman Mastermix	12.5
20X Primer and Probe	1.25
Nuclease-Free Water	6.75
Total	20

Table 4 Real-Time Multiplex PCR Reagents

Reagents	Mixture for one reaction (μL)
2X Universal Taqman Mastermix	12.5
20X Primer and Probe (18S)	0.625
20X Primer and Probe (LTR or CD4)	1.875
Nuclease-Free Water	6.75
Total	20

The RNA and DNA from each tissue sample were analyzed via real-time PCR targeting LTR of SIV and 18S as an endogenous/loading control in multiplex. Furthermore, the RNA (cDNA)2.1.3.3 was analyzed targeting CD4 mRNA (using 18S as an endogenous/loading control in multiplex), and then in uniplex the RNA (cDNA) was analyzed targeting IL-6, IL-1β, TNF-α, and MyD88 mRNA using their respective primer/probe (Table 5, 6).

Table 5 SIV LTR and Gag primer/probe sequences

Target	Purpose	Sequence (5'→ 3')
LTR	Forward	TGG GAG GTT CTC TCC AGC AC
	Reverse	AAT GGC AGC TTT ATT GAA GAG G
	Probe	6FAM TTC CCT GCT AGA CTC TCA CCA GCA CTT GG TAMRA
Gag	Forward	CAG GGA A/ideoxyl/ideoxyl/AAG CAGATGAATTAG
	Reverse	GTT TCA CTT TCT CTT CTG CGT G
	Probe	6FAM ATT TGG ATT AGC AGA AAG CCT GTT GGA G TAMRA

Table 6 Primer/Probe references

Target	Lot number	Distributer
CD3	Rh02826503_m1	ABI
TNF-α	Rh02789783_m1	ABI
IL-6	Rh02789322_m1	ABI
IL-1β	Rh02789775_m1	ABI
CD4	Rh02621720_m1	ABI
MyD88	Rh01573837_m1	ABI
18S	4319413E-1002047- Vic-mgb	ABI

Regardless of the complexity of the PCR reaction, the rest of the PCR procedure was the same. After the real-time PCR mix was made, 20 μL was aliquoted into the appropriate wells of a 96-well plate. Subsequently, 5 μL of sample DNA (20ng) or cDNA (20ng) was added to the appropriate well. The plate was placed in a ABI Biosystems 7000 quantitative PCR machine using the SDS 7000 program, and was run at 50ºC for 2 min, 95ºC for 10 min, then through 50 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. The amplification plot for every individual sample was carefully scrutinized to ensure the real amplification recorded. Once the run was complete the data was exported to an excel spread sheet and analyzed (see Section 2.1.3.3).

2.1.3.3 Real-Time PCR Analysis

Quantitation Using a Standard Curve

To minimize the effect of DNA/RNA input variation on target quantitation in the samples, the 18S Ct from all of the samples in the same plate was averaged (one average for RNA and one average for DNA if they were run on the same plate). Then the "normalized Ct" for a particular target was calculated using the following equation:

Normalized Ct = (the target Ct value for that sample * average 18S Ct)/18S Ct value for that sample.

Next, two replicates of each standard curve point were averaged and graphed on a line graph, including a trendline with an R^2 value and the slope of the line equation. The slope equation was used to calculate the quantity of each sample. The slope equation in y=mx+b form was switched it x=(y-b)/m form to calculate the quantity of each sample, where y was the normalized LTR Ct for each sample, and the other values remained the same from the equation. The quantity of each sample was the log copies/reaction. Next, replicate log copies/reaction for each sample were averaged, and that was the reported value.

Relative Quantitation without a Standard Curve

All of the relative quantitations were compared to the sample with the least amount of replication, or the highest Ct value. Analysis began with the identification of the sample with the highest average Ct. The Ct values and the Sample name were then Copy and pasted (with the highest value Ct sample at the top as the reference sample) into the Macro. Next, the 18S values were copy and pasted into the last column in the same row as the corresponding sample. From the Ct value of the test target and the 18S Ct, the macro calculated the mean of the target Ct, the

Standard deviation, the ΔCt (Target Ct – 18S ct) for each replicate, the mean ΔCt of the multiple replicates for each sample, 2- ΔCt which is 2 raised to the power of the negative of whatever the value of the mean ΔCt is, ΔΔCt which is the mean ΔCt – the mean ΔCt of the first sample (or lowest replicating sample), 2- ΔΔCt which is 2 raised to the power of the negative of the ΔΔCt for that sample, this was the value reported as the relative value or "relative difference."

2.1.3.4 Statistical Analysis

In order to determine if ART significantly has an impact on the viral and host parameters that were measured, a two-sample, unequal variance student's t-test with two distribution tails was conducted. The students t-test was used to analyze the difference in relative CD4 mRNA distribution, viral load (both un-normalized and CD4-normalized), the viral RNA/DNA ratio, relative TNF-α, IL-6, IL-1β, and MyD88 distribution. Samples of the same tissue type were compared between ART and no ART. The uninfected monkey was not included in the analysis because there was only one subject. The analysis was conducted using Excel 2007 ®.

3.0 RESULTS

3.1 AIM 1: OPTIMIZATION OF MULTIPLEX REAL-TIME PCR METHODOLOGIES FOR MONKEY GI TISSUE

3.1.1 Optimization of Sample Input and SIV target sequence for Real-Time PCR

The amount of input DNA in a real time PCR has an important effect on the outcome. As a result, troubleshooting is necessary to determine the optimal amount of cDNA or DNA to be included in a PCR. In addition, in the search for the most sensitive and reproducible PCR results for SIV quantitation, two well conserved SIV targets, LTR and Gag (Fig 6) were also compared using their respective primers/probe in real time PCR. For this purpose, three tissue samples were chosen based on a previous screening result via real-time PCR. A high viral load sample (M5699 duodenum, from an acute monkey), moderate viral load sample (M1799 colon, from an AIDS monkey), and low viral load sample (C643 mesenteric lymph node, an acute monkey that was not used for further study) were used in this assay. From each sample, cDNA input equivalent to 10, 20, or 40 ng of RNA was loaded into a real-time PCR, and the Ct values from PCR results were subsequently compared. In the high viral load sample, the 20 ng cDNA input had relative detection sensitivity that was 1.2 times higher than 10 ng and 1.5 times higher than 40 ng cDNA input when LTR was targeted to detect SIV (Fig 7A). When Gag was used as the

SIV target there was low level detection in the 10 ng input, moderate detection in the 40 ng , and the best detection results in the 20 ng cDNA input . However, the detection was much more efficient in all reactions when LTR was the PCR target. In the moderate viral load, there was almost equal detection among all three input amounts with the LTR target (Fig 7B). When Gag was targeted in the moderate viral load sample there is slightly lower detection with 20 and 40 ng of input , and much lower detection with 10 ng of input . Again, the detection efficiency is lower for Gag than LTR in the moderate viral load sample. In the low viral load reaction the best detection is seen with 20 ng of input, followed by 40 ng and 10 ng when LTR is the SIV target (Fig 7C). However, virtually no virus is detected in any of the reactions when Gag is used for the PCR target. Because of the more sensitive detection results in the 20 ng of input for the high and low viral load samples, it was determined that 20 ng is the optimal amount of cDNA/DNA for use in this assay. Additionally, the results showed that in our experimental conditions, LTR primers/probe are more sensitive than the Gag primer/probe set in measuring viral load. Therefore, in subsequent experiments, the LTR target was used with 20 ng of input cDNA or DNA to detect SIV loads in the samples.

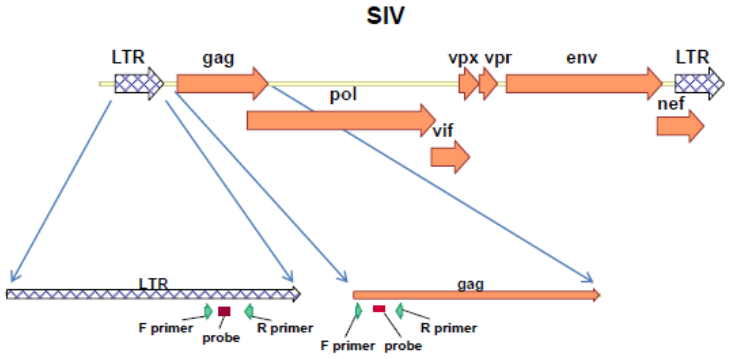

Figure 6 Location of LTR and gag primers and probes.

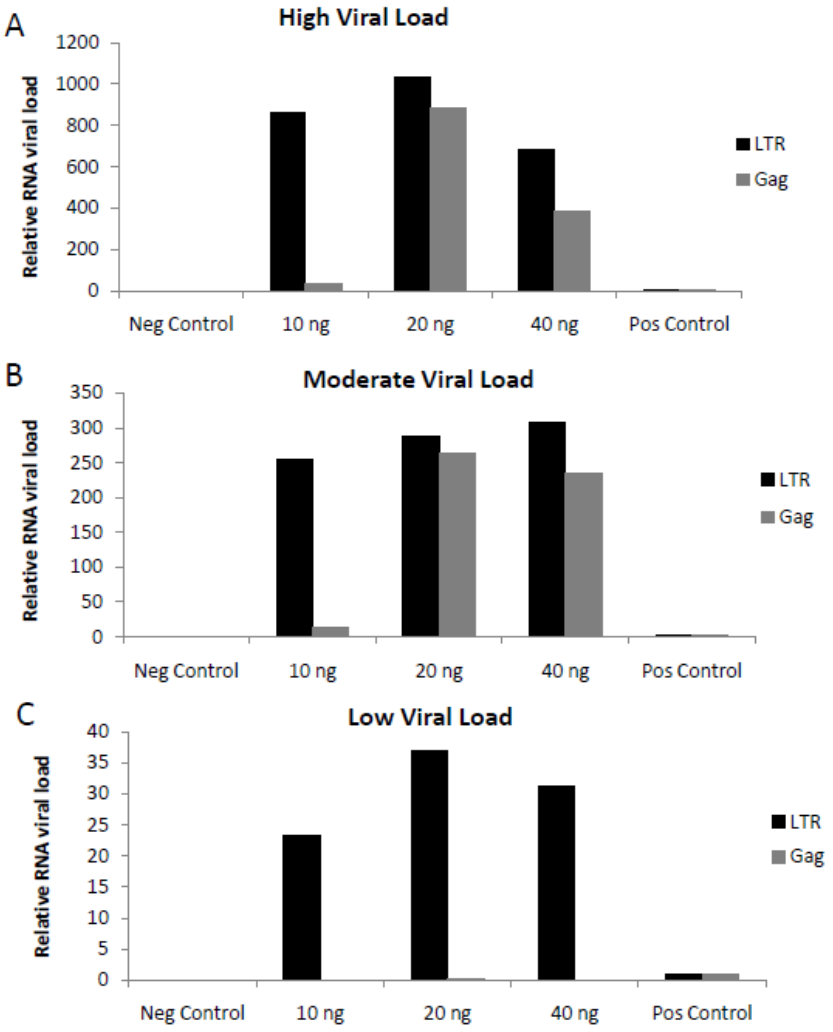

Figure 7 Optimization of cDNA amount in real-time PCR targeting LTR and Gag of SIV.

3.1.2 Multiplex PCR Optimization

In order to save time, reagents, sample, and money, multiplex PCR was chosen as the detection method for this study. 18s ribosomal RNA is produced in all eukaryotic cells. Due to the limited effect of its expression under various physiological and pathological conditions, 18s rRNA is an ideal target for an endogenous control. Because of the overwhelming abundance of 18S copies in each sample, equal amounts of primers/probes targeting 18S and LTR (or CD4) may not be ideal for co-amplification via multiplex real-time PCR due to the substrate exhaustion. To find

the optimal primer ratio between LTR (CD4) and 18S for efficient co-amplification of SIV and 18S targets, various primer concentration ratios (18S:LTR) consisting of 1:1, 0.5:1, 1:2, and 0.5:1.5 were tested in real time PCR. Each primer ratio was used to detect SIV in a high viral load sample (M1799 Stomach, this monkey had AIDS) and low viral load sample (M9697 Duodenum, this monkey was a LTNP). This experiment was run twice to ensure that the results were reproducible. The results showed that the primer ratio of 0.5 (18S):1.5 (LTR) provides the best detection results for 18S and LTR. Furthermore, similar results were acquired when the 0.5:1.5 primer ratio was tested in a tissue sample with 18S and CD4 primers (data not shown).

In a follow up experiment, the same samples were tested using the same primers/probes to narrow down the primer concentration further. The ratios tested were 0.5:1.5, 0.25:1.5, and 0.125:1.5. Again, the results showed that the 0.5:1.5 ratio had the best detection for LTR and CD4 as evidenced by the lower Ct values (data not shown). As a result, for all subsequent multiplex assays, the PCR master mix was prepared with a 0.5:1.5 18S:LTR/CD4 ratio.

3.1.3 Multiplex Primer Interference

In a multiplex PCR, more than one set of primers and probes co-amplify and detect their respective targets simultaneously in the same tube. Because of the multiple PCR reactions, interference between the two reactions can occur, resulting in inefficient amplification of one or both targets. This interference could occur when the primer/probe sequences of one target are similar to the primer/probe sequences for the other target, or if the targets are too close, primer binding to one target could cause blockage of primer or probe binding to the other target. To ensure that the primer/probe sets did not interfere with each other, an initial experiment was performed to test the compatibility of the primers and probes. For this purpose, the Qiagen

AllPrep DNA/RNA Mini kit® was used to isolate the RNA. RNA from two different GI tissue locations from the same monkey (the esophagus with a high viral load and the ileum with a low viral load) were isolated using the Qiagen AllPrep DNA/RNA Mini kit® and were analyzed via real-time PCR using 20 ng of nucleic acid with primer ratio of 0.5:1.50. Each sample was tested in multiplex PCR targeting 18S:LTR and 18S:CD4, as well as in uniplex targeting each target alone as control. The results showed the same detection efficiency for both samples in multiplex as well as uniplex (Fig 8) As a result, the primers and probes were considered compatible for further testing in multiplex PCR.

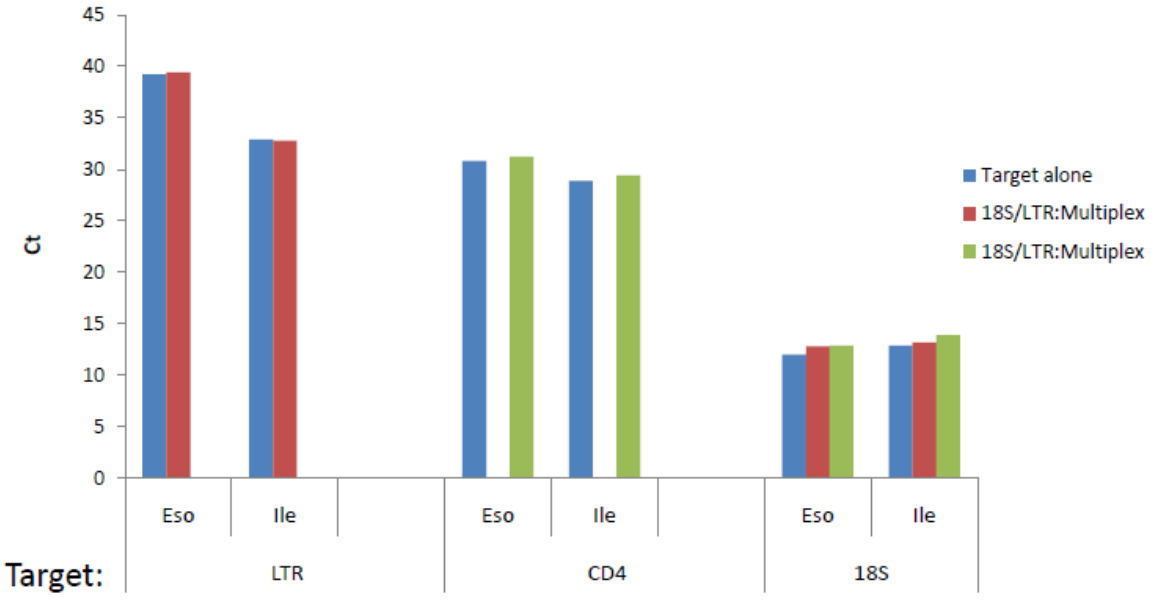

Figure 8 Multiplex/uniplex compatibility comparison in Real-Time PCR.

To further assess the compatibility of the primers/probes of 18S and LTR (or CD4) in multiplex PCR to detect within a wide viral load range, serially diluted samples with known high or low viral loads were tested targeting 18S and LTR both in multiplex and uniplex real time PCR. The results show that nearly identical Ct values were detected in multiplex and uniplex in each dilution when targeting 18S (Fig 9A) as well as targeting SIV LTR (Fig 9B). The same

experiment was performed targeting 18S and CD4 (data not shown) with similar results. These results indicate that there are no interferences of the primers/probes used in our multiplex PCR.

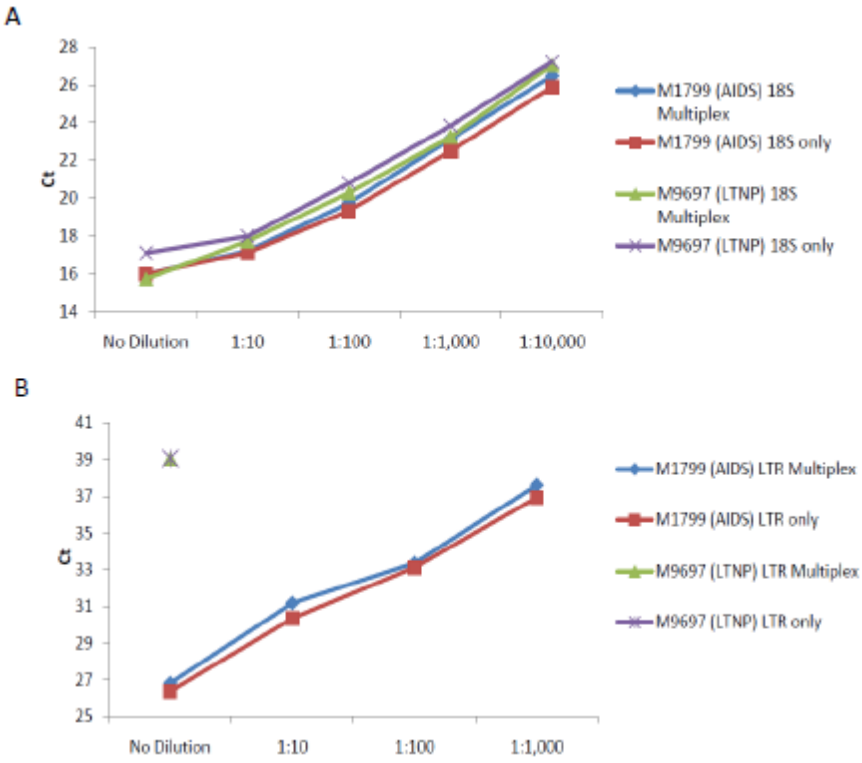

Figure 9 Test for multiplex primer interference targeting 18S (A) and LTR (B).

3.1.4 Standard Curve

3.1.4.1 Preparation of Standard Curve

A 10 fold dilution series was generated from a SIV RNA stock prepared from an SIV culture supernatant containing SIV strain 17E and was quantitated using a well established SIV standard curve from Dr. Reinhart's lab. The standard curve dilution series from $1x10^7$ copies to 10^1 were included in each RT real time PCR run to quantify SIV copy number in each sample.

3.1.4.2 Standard Curve in multiplex PCR vs. uniplex PCR

To quantify SIV in each sample, the SIV standard series were included in each PCR run. To make this quantification more accurate, the standard curve series were also run in multiplex PCR conditions. To validate the amplification efficiency in the entire range of the standard curve in multiplex, the standard curve series was tested by targeting LTR in multiplex and uniplex conditions. Since nearly the same Ct was detected for each dilution sample in the range of $1x10^7$ to 10^1 copies/reaction in multiplex and uniplex (Fig 10), it is suggested that there is no primer/probe interference in multiplex PCR in the detection range of the standard curve.

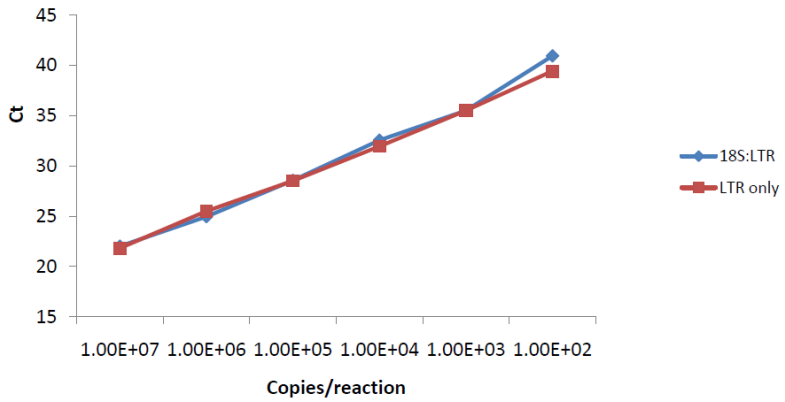

Figure 10 Detection of standard curve targeting LTR in multiplex and uniplex.

3.2 AIM 2: ASSESS THE SIV AND CD4 DISTRIBUTION IN VARIOUS ANATOMIC LOCATIONS THROUGHOUT THE GI TRACT IN VARIOUS STAGES OF SIV INFECTION

3.2.1 CD4 mRNA distribution in the GI tract

It has been previously shown that higher levels of $CD4^+$ T cell depletion occur in the GI tissues than in the blood [19]. Because of this phenomenon, an important part of this study was determining the $CD4^+$ T cell levels in different parts of GI tissues of the infected monkeys. To achieve this goal, all tissues were analyzed for the relative amount of CD4 mRNA in multiplex real time PCR using 18S as the endogenous control. While this method does not quantitate the actual number of CD4 T cells, it provides information about the presence of $CD4^+$ cells that can be used to compare between tissues and between monkeys in various disease stages or in those receiving treatment. Detectable CD4 mRNA was found in all tested tissues of the GI tract (Fig 11). In the monkeys from the ART study, all three sets of monkeys (uninfected, ART, No ART) had the most relative CD4 mRNA in the mesenteric lymph node (Fig 12). However, there is no significant difference in the relative CD4 mRNA between SIV infected monkeys that received ART and those that did not receive ART, nor do these two groups differ from the uninfected monkeys.

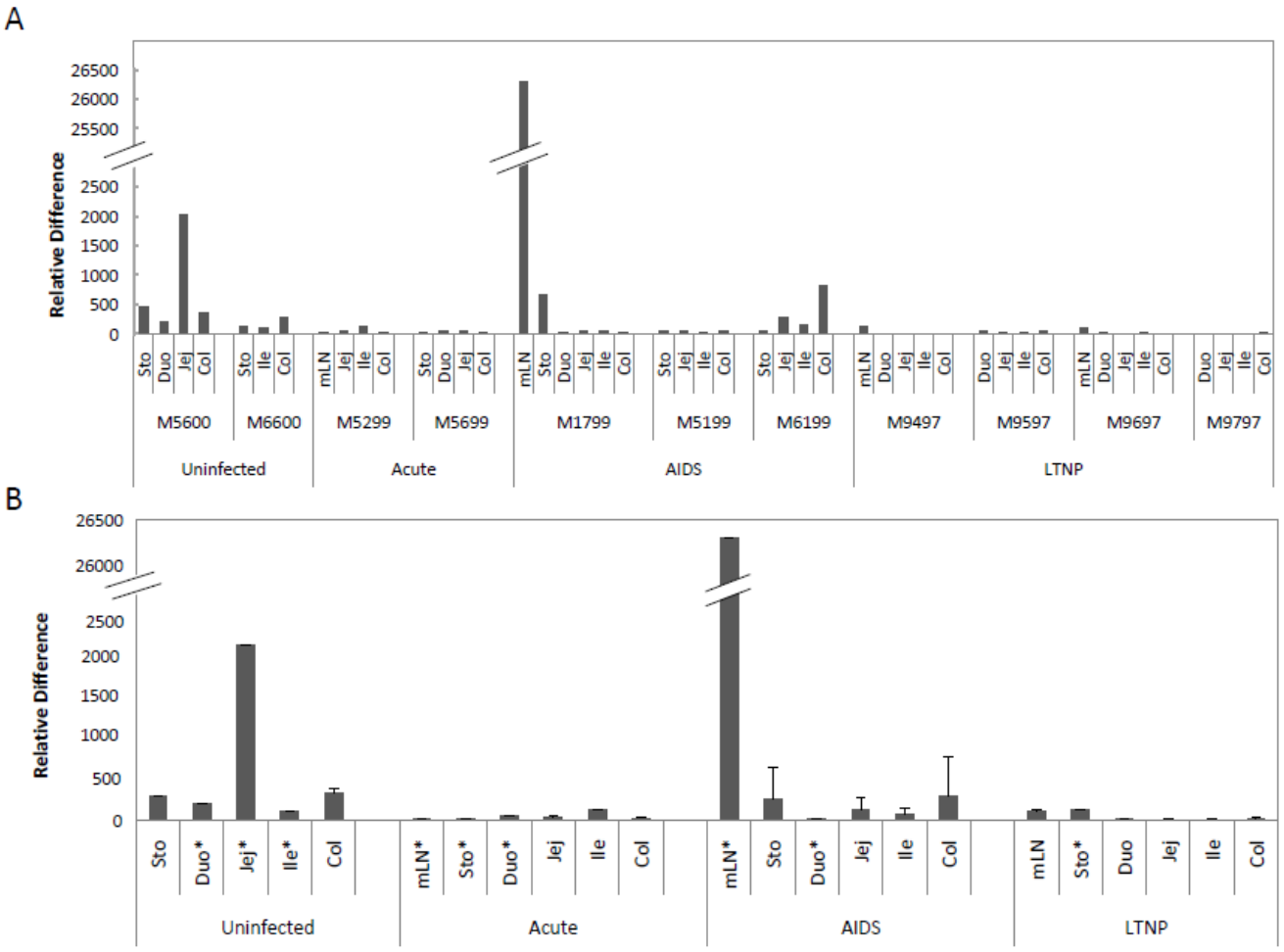

Figure 11 Comparison of CD4 mRNA in Uninfected, acute, AIDS, and LTNP monkeys by individual monkey and tissue (A), and averaged by tissue (B). *Only one tissue is available in that disease stage.

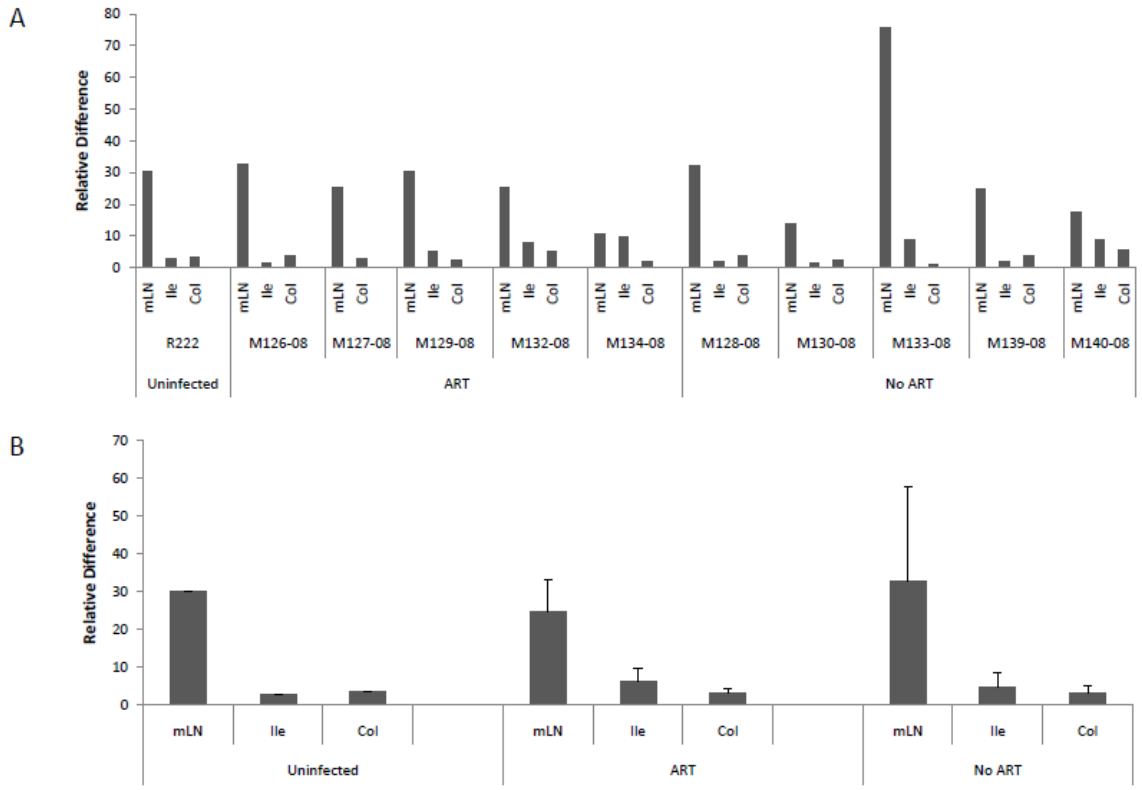

Figure 12 CD4 mRNA comparison in uninfected, SIV-infected ART treated, and SIV-infected untreated monkeys. Comparison by individual tissue and sample (A) and averaged by tissue (B).

3.2.2 Viral Load

3.2.2.1 Viral Load in GI tissues

Clinically, viral load is routinely determined by measuring virus copy number in the blood. However, because the viral replication kinetics differ between the blood and the GI tissue, it is important to determine the viral load in the GI tissue from infected monkeys. For this purpose, all monkey samples were analyzed for viral RNA and DNA targeting the LTR of SIV, and this was replicated five times.

As expected there is overall, more viral nucleic acid present in the tissues of acute and AIDS monkeys than in the tissues of LTNP monkeys (Fig 13). Slightly more viral RNA was detected in the tissues of the Acute and AIDS monkeys, whereas more viral DNA was present in the tissues of the monkeys considered LTNP (Fig 14). Additionally, no clear pattern has been detected in terms of virus distribution from one end of the GI tract to the other.

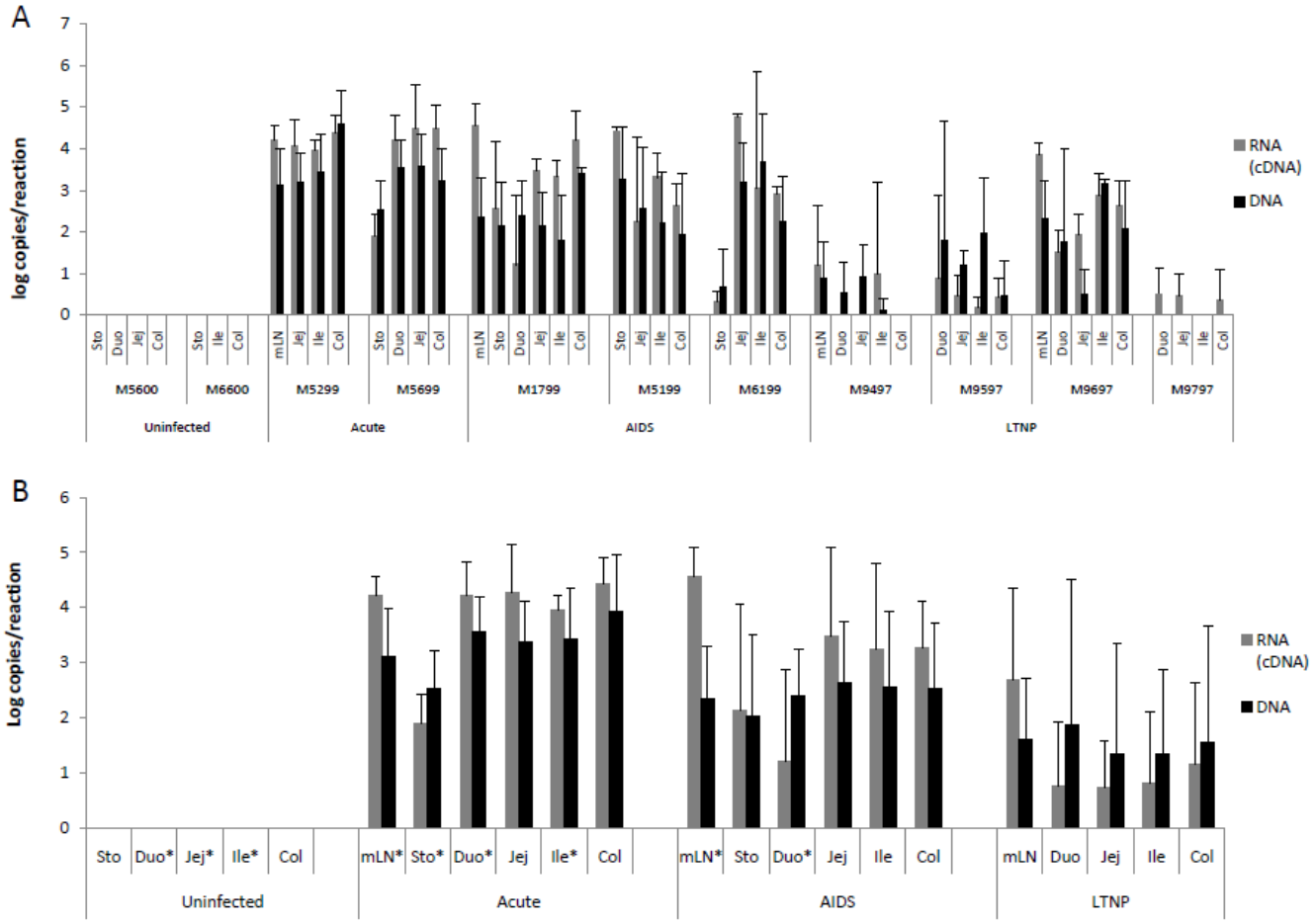

Figure 13 Comparison of SIV RNA and DNA in uninfected, acute, AIDS, and LTNP monkeys. Analysis shown by individual tissue (A) and by averaged tissue (B). Error bars represent standard deviation.

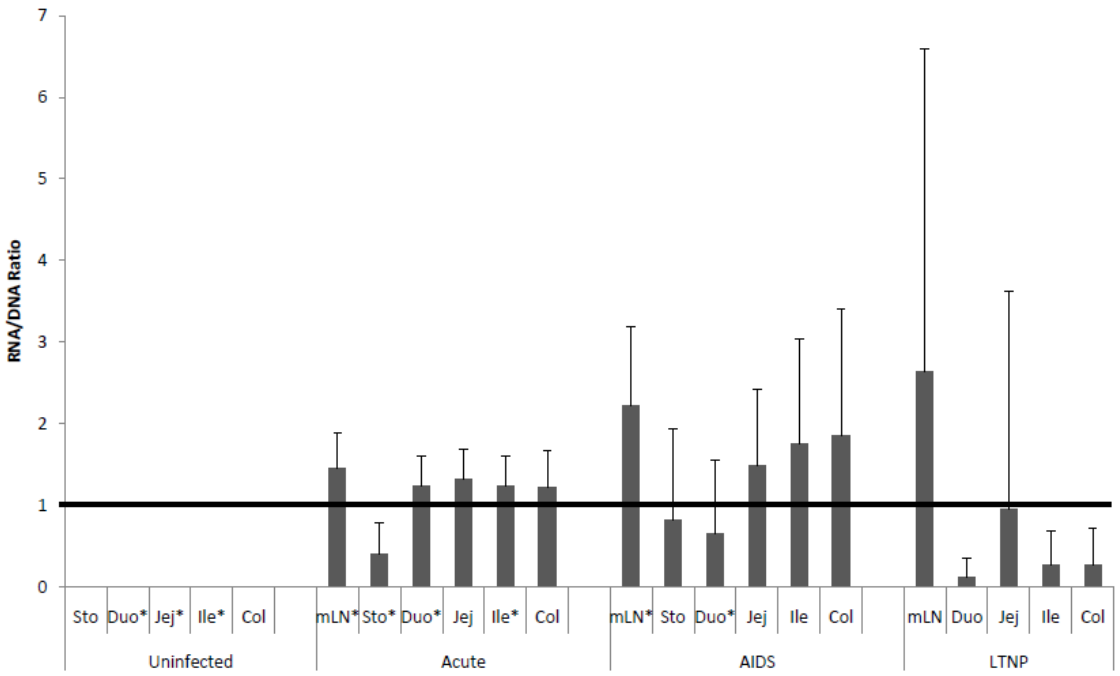

Figure 14 Viral RNA/DNA ratio in uninfected, acute, AIDS, and LTNP monkey samples averaged by tissue. Error bars represent standard deviation.

Comparing the tissue viral loads of the monkeys in the ART study, the treatment significantly decreases the tissue viral load with all p-values <0.01 when compared with the same nucleic acid type in the same tissue in monkeys that did not receive ART (Fig 15). The plasma viral load for all of the monkeys on ART was below the detection limit (<200 viral RNA copies/mL) (Table 1), however, there is still detectable viral RNA (and DNA) in the tissues of the GI tract. There is significantly more viral DNA present in the GI tissues of the monkeys that received ART compared to those that did not receive therapy (Fig 16). It is possible that this viral load could increase if ART is discontinued.

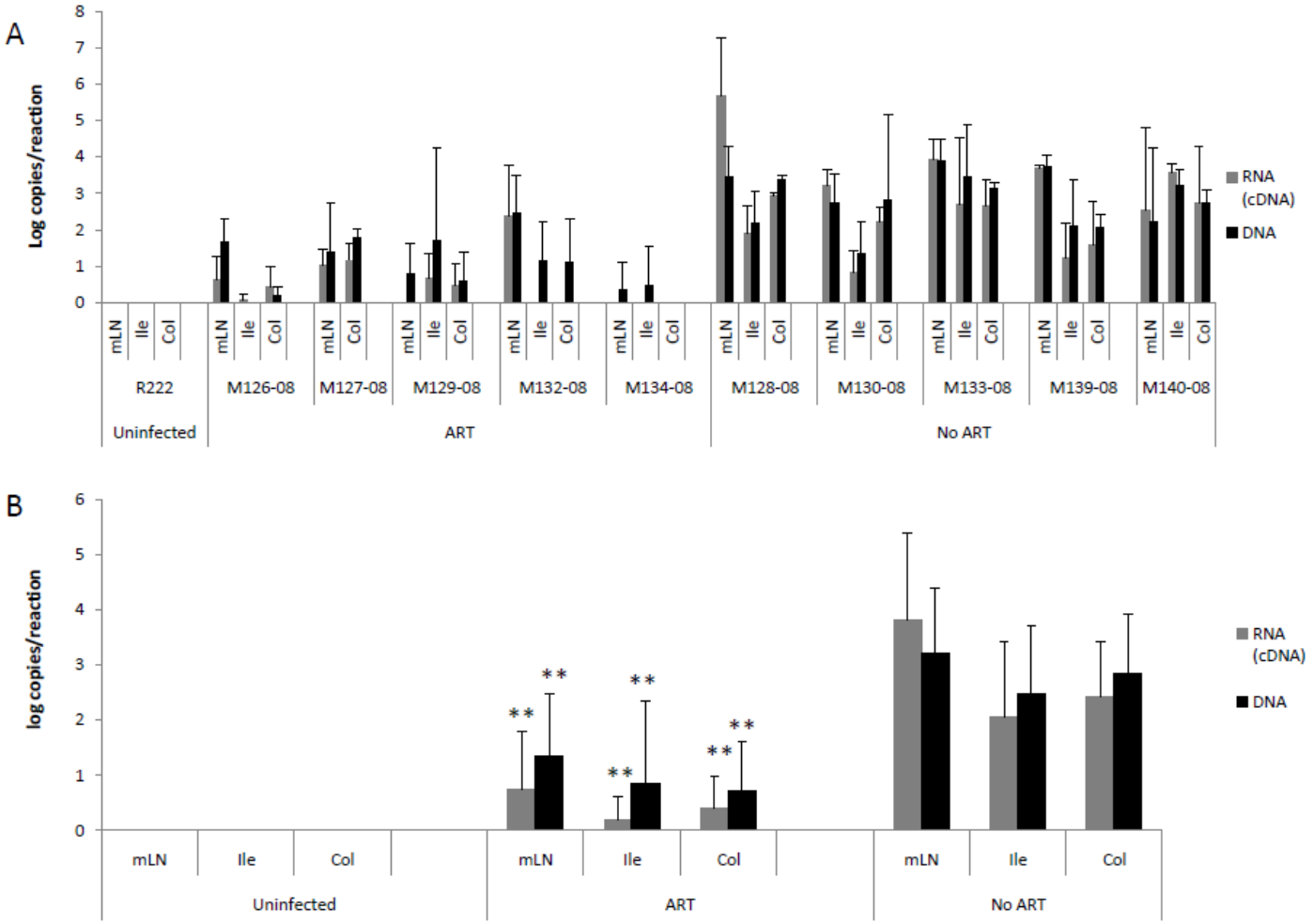

Figure 15 Comparison of SIV RNA and DNA in uninfected, ART, and untreated monkeys. Analysis shown by individual tissue (A) and by averaged tissue (B). Error bars represent standard deviation. ** p<0.01 per student's t-test.

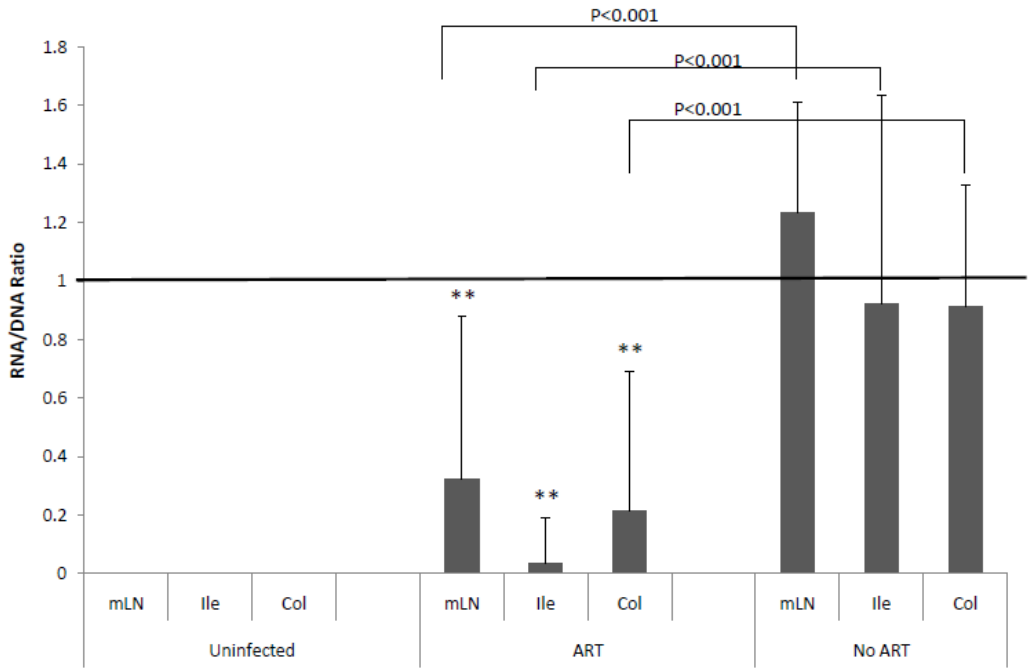

Figure 16 Comparison of viral RNA/DNA ratio in uninfected, ART, and untreated monkeys. Error bars represent standard deviation.

3.2.2.2 Viral Load in GI tissues normalized by CD4 mRNA

In order to account for the tissue location and the potential for inadvertent testing of patches of lymphoid tissue or patches of tissue that did not have many $CD4^+$ T cells, the viral load has been normalized by relative CD4 mRNA presence in the same tissue. This depiction of the viral load corrects for the fact that a viral load may appear high in a tissue as a result of a patch of lymphocyte-rich tissue being tested.

When the viral load is normalized by CD4, the acute and AIDS stages of disease monkeys have more viral RNA and DNA present in the tissue (Fig 17). Upon normalization, the RNA to DNA ratio changes such that almost all tissues (in all three disease stages) have more viral RNA than viral DNA (Fig 18).

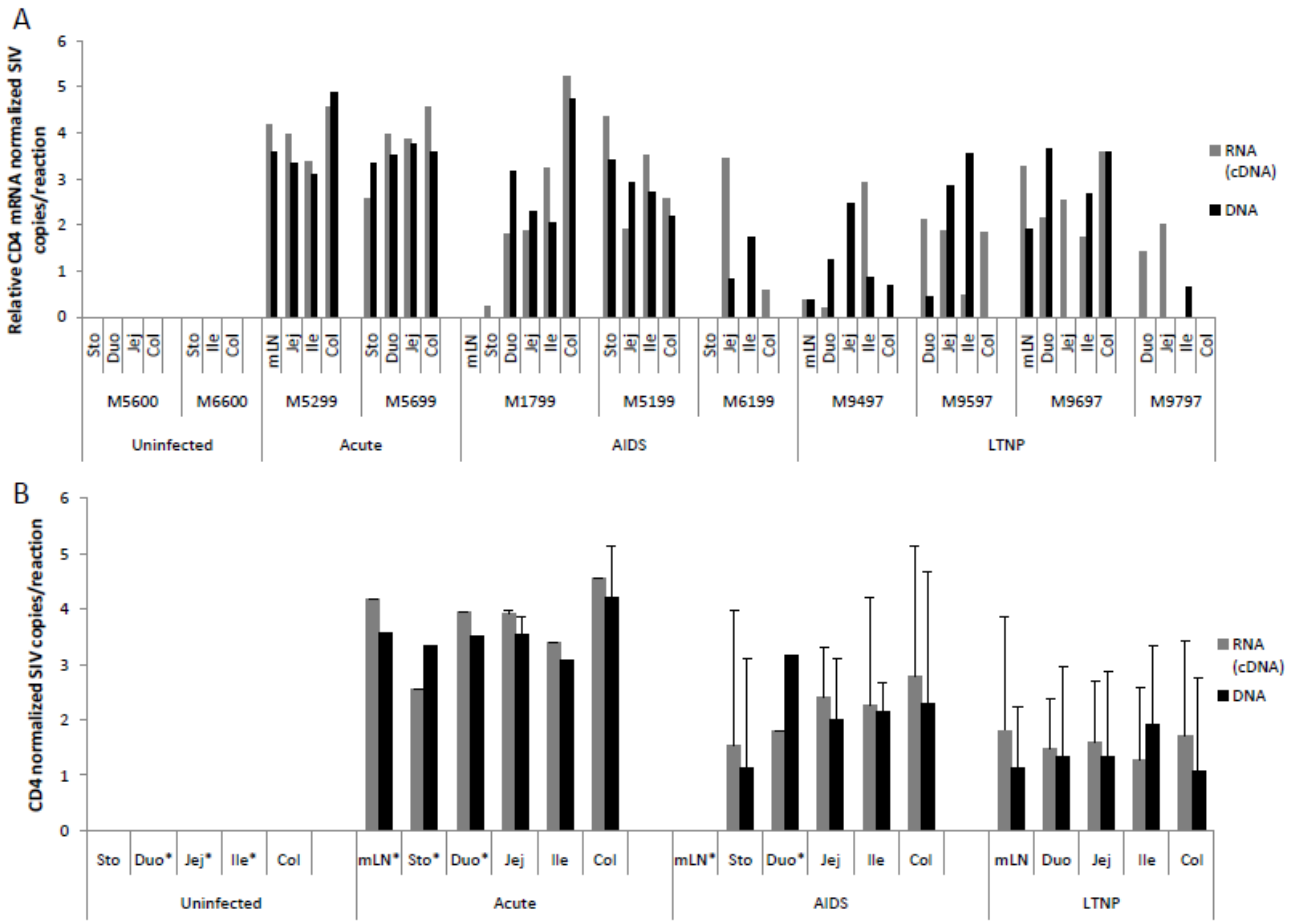

Figure 17 CD4 normalized viral load in uninfected, acute, AIDS, and LTNP monkeys. Analysis shown by individual tissue (A) and by averaged tissue (B). Error bars represent standard deviation.

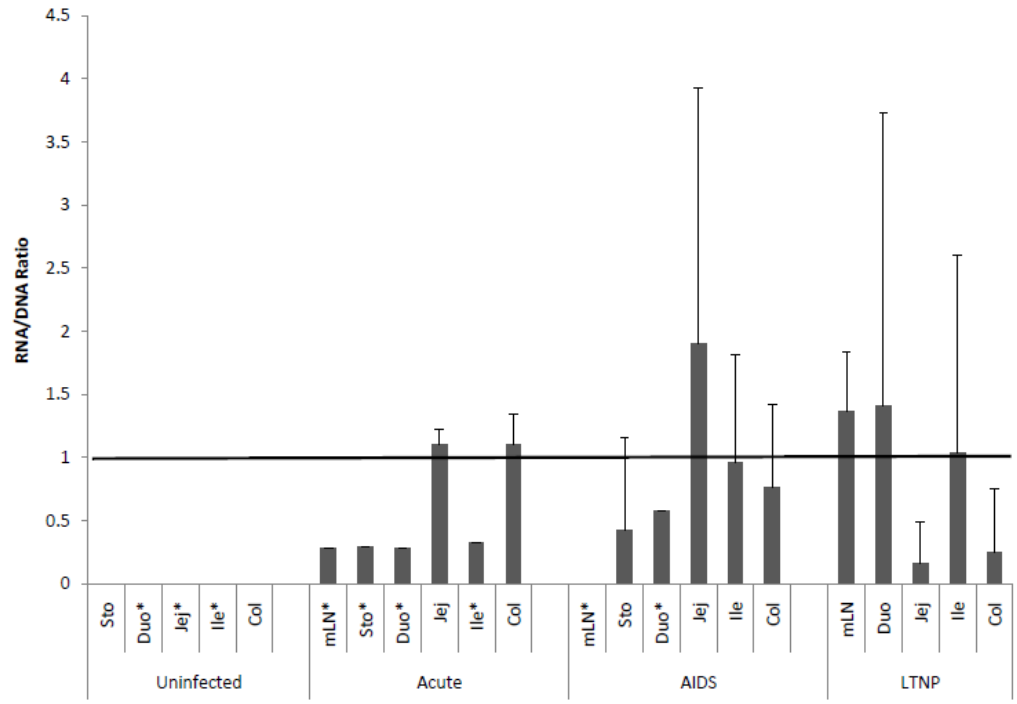

Figure 18 CD4 normalized SIV RNA/DNA ratio of uninfected, acute, AIDS and LTNP monkeys. Error bars represent standard deviation.

When the viral load for the ART study monkeys is normalized by CD4, again there is significantly more virus present in the monkeys that did not receive therapy than the monkeys that did receive ART (Fig 19). In the mesenteric lymph node there is significantly less viral RNA and DNA in ART monkey compared to the therapy naive monkeys. In the ileum there is significantly less viral RNA and in the colon there is significantly less viral DNA in the monkeys that received ART compared to those that did not (Fig 20). Before the viral load was normalized by CD4, the monkeys that did not receive therapy had more viral RNA present than viral DNA and the monkeys that received ART had more viral DNA present than viral RNA. Particularly in the ileum, after CD4 normalization of the viral load, there is a significant

difference in the viral RNA to DNA ratio. The monkeys that received ART had significantly more DNA than RNA present in the ileum than the monkeys that did not receive ART.

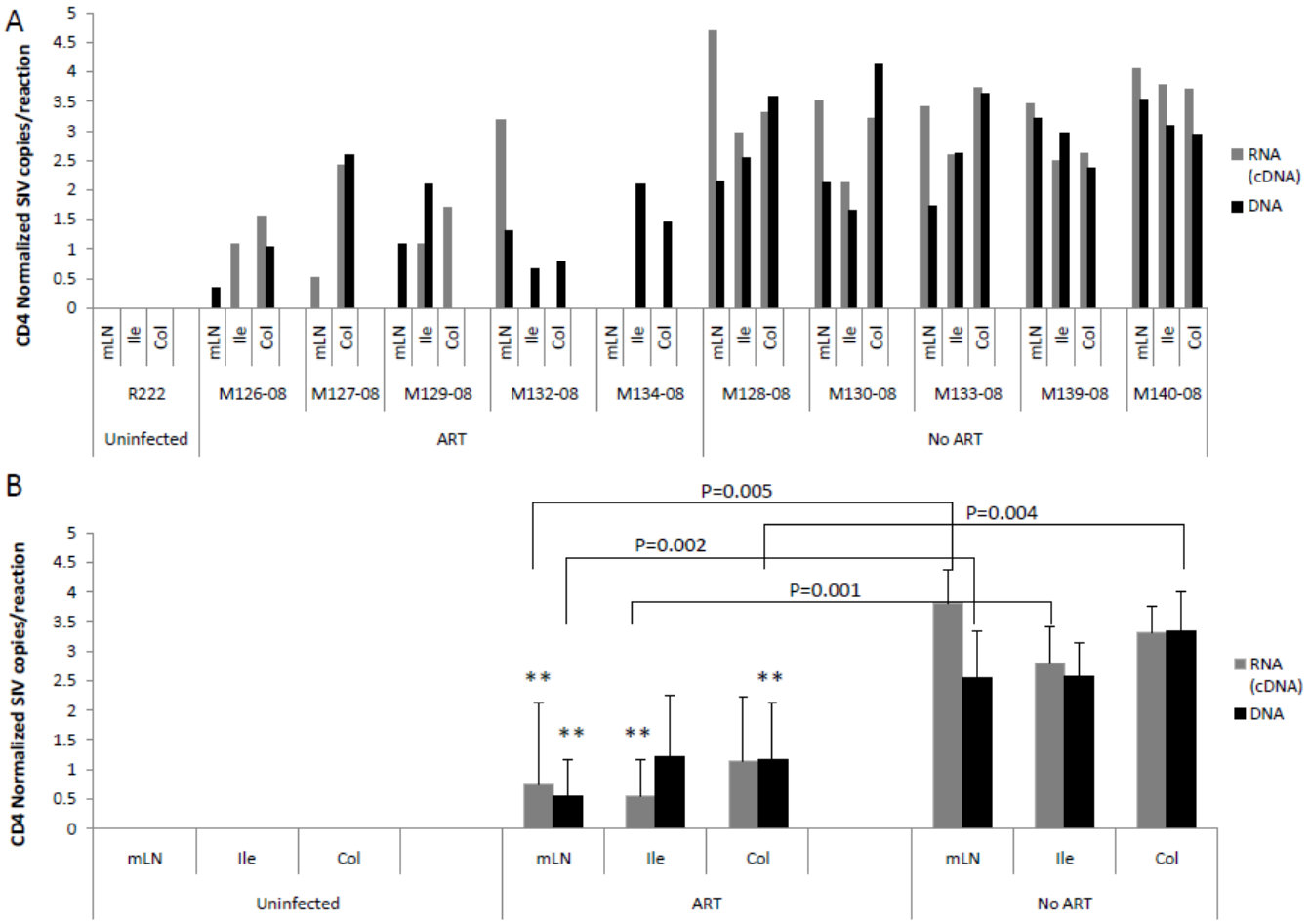

Figure 19 CD4 mRNA normalized viral load in uninfected, ART, and untreated monkeys. Analysis shown by individual tissue (A) and by averaged tissue (B). Error bars represent standard deviation.

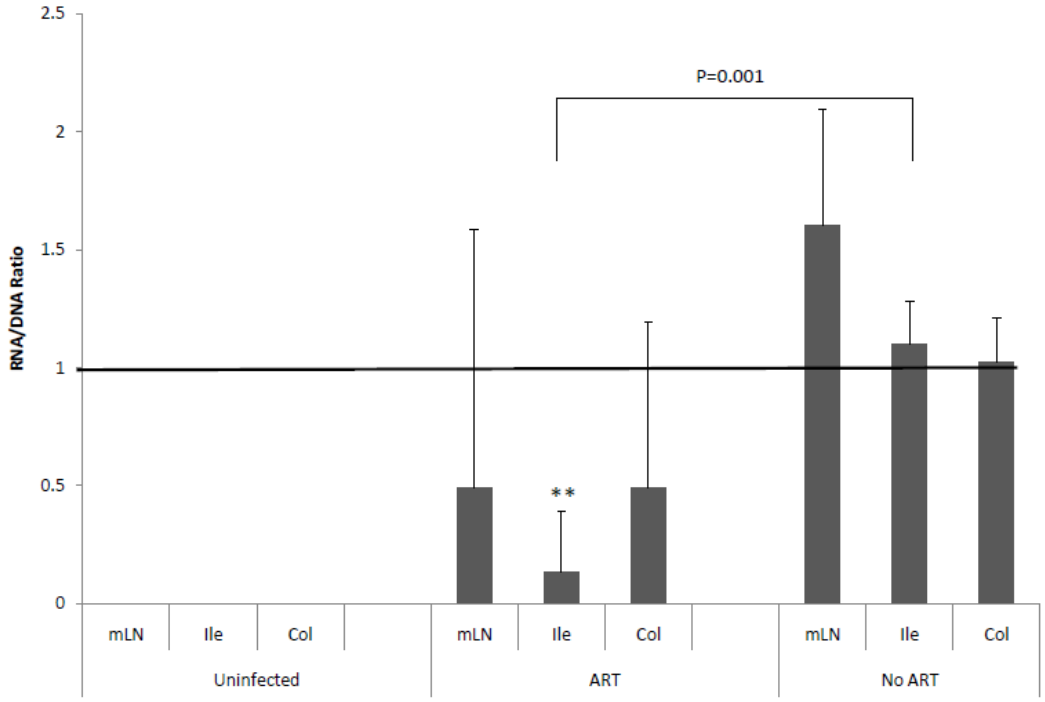

Figure 20 SIV RNA/DNA ratio in uninfected, ART treated, and untreated monkeys. Error bars represent standard deviation.

3.3 AIM 3: ELUCIDATE THE CYTOKINE PROFILE IN VARIOUS ANATOMIC LOCATIONS OF THE GI TRACT AND IT'S ASSOCIATION WITH VIRAL LOAD

3.3.1 TNF-α in GI tissues

TNF-α is produced by immune cells in response to various infection. To explore the immune status in SIV infected GI tissues, TNF-α mRNA was targeted for real time PCR amplification in the RNA isolated from GI tissues, and the relative 18S quantity from the same RNA sample derived from the CD4 multiplex PCR were used for normalization of the TNF-a mRNA level. The tissue with the highest relative TNF-α mRNA is again the mesenteric lymph node from

monkey M1799 with AIDS (Fig 21), which is six times greater than the stomach from monkeys 5199 with AIDS, the next highest. More relative TNF-α mRNA is seen in monkeys in the AIDS stage of infection, followed by acute, uninfected monkeys, and the LTNP had the least TNF-α mRNA.

In all 11 of the ART study monkeys, relative TNF-α mRNA is higher in the mesenteric lymph node (Fig 22). Additionally, the monkeys that received ART had slightly more TNF-α mRNA than those that did not receive treatment and the uninfected monkeys have the least amount of TNF-α mRNA.

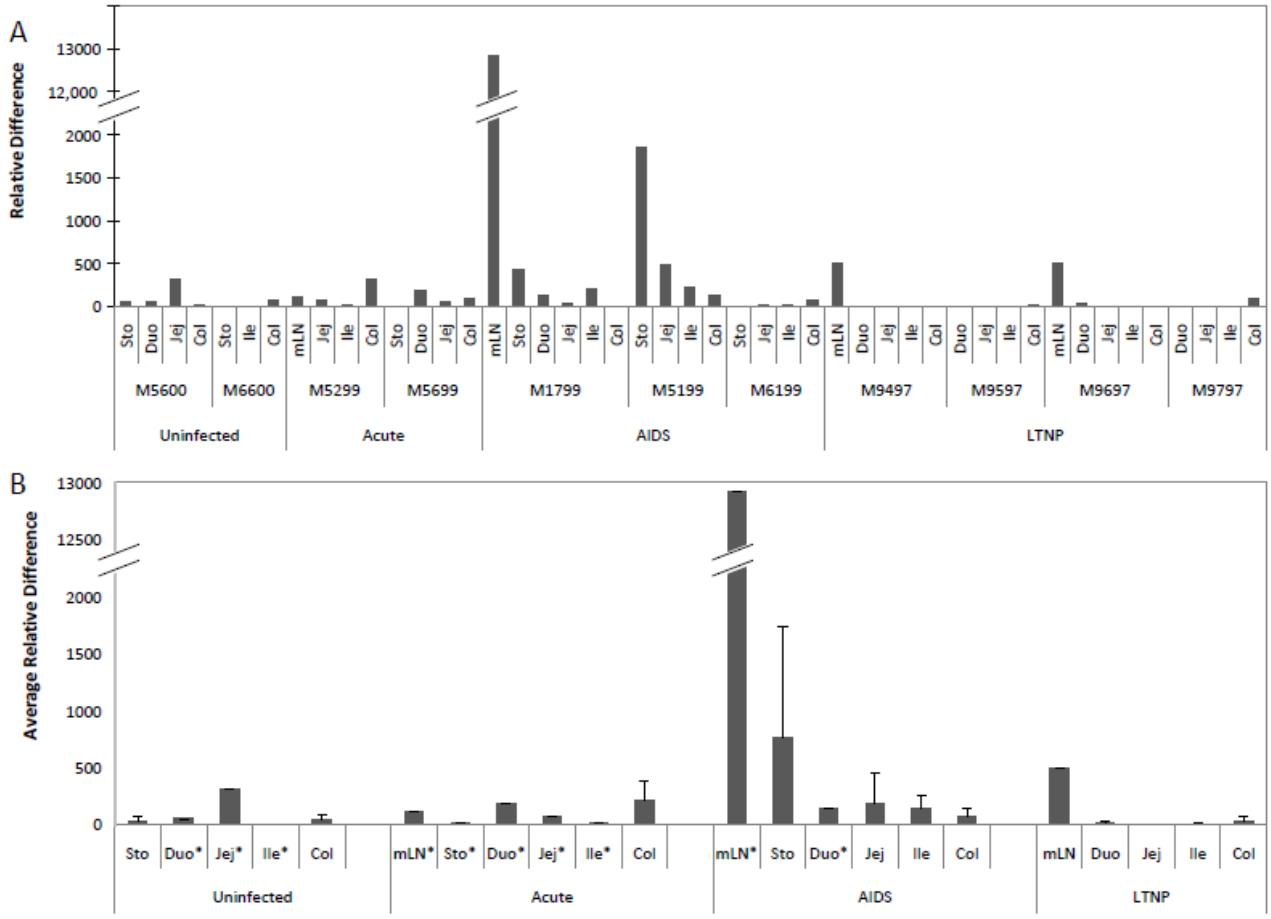

Figure 21 Relative TNF-α difference in uninfected, acute, AIDS, and LTNP monkeys. Analysis shown by individual tissue (A) and by averaged tissue (B).

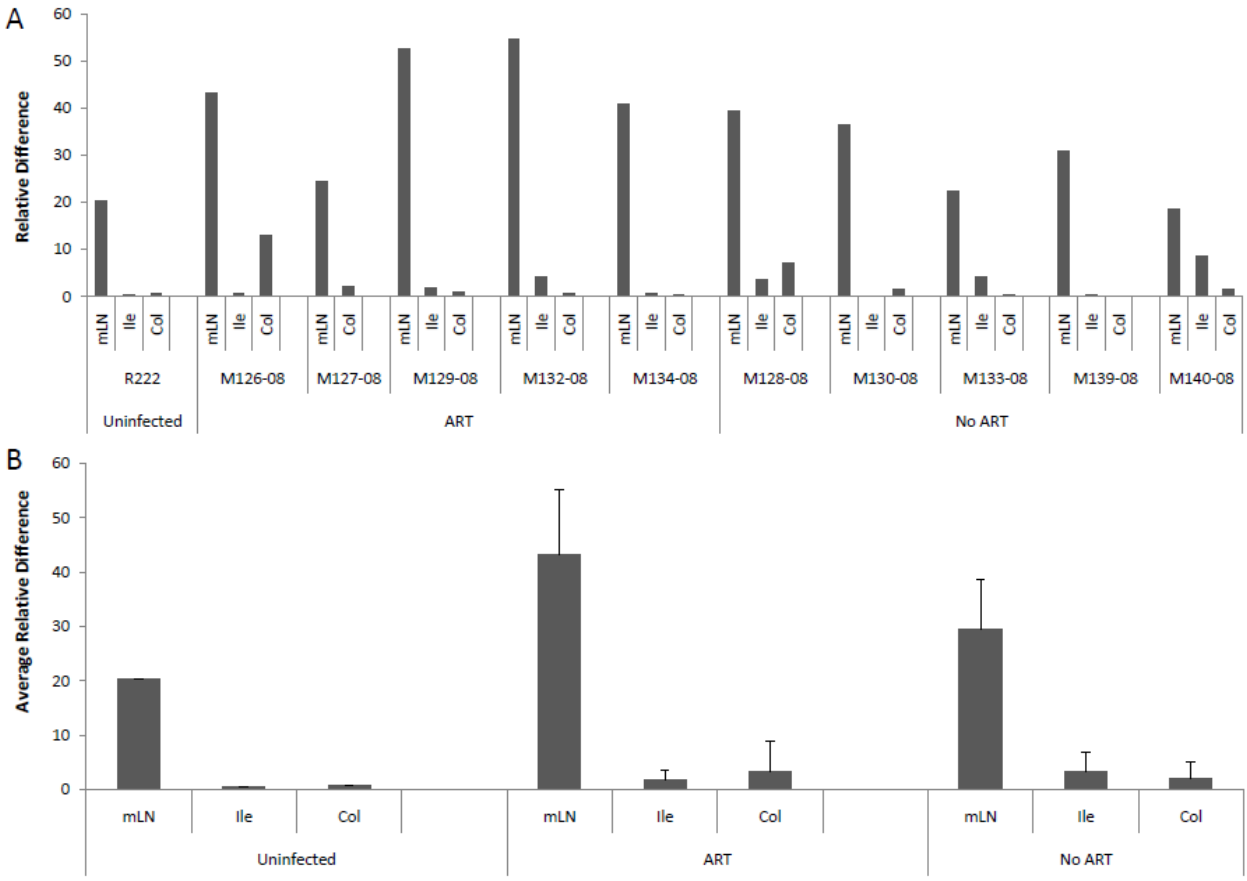

Figure 22 Relative TNF-α difference in uninfected, ART, and untreated monkeys. Analysis shown by individual tissue (A) and by averaged tissue (B).

3.3.2 IL-6 in GI tissues

Interleukin-6 is produced by T cells in response to antigen. Because its function is similar to TNF-α, it is unsurprising to see a similar pattern of IL-6 mRNA presence as TNF-α in the tissues. The mesenteric lymph node from AIDS monkey M1799 had the highest relative IL-6 mRNA levels followed by similar amounts seen in the stomach of M5199 (AIDS) and the mesenteric lymph node in M9697 (LTNP), who also had decently high relative TNF-α mRNA

presence (Fig 23). Like the TNF-α, the most IL-6 mRNA is seen in the monkeys with AIDS, followed by acute. The uninfected monkeys and LTNP had very low expression (except for in the mesenteric lymph node of the LTNP).

Much like TNF-α, the monkeys in the ART study all had the highest levels of relative IL-6 in the mesenteric lymph node, except for monkey M140-08 who did not receive treatment and had almost identical levels between the mesenteric lymph node and the ileum (Fig 24). No real difference is seen in IL-6 mRNA levels between the infected monkeys that received ART and those that did not. The uninfected monkeys however, had low levels of IL-6.

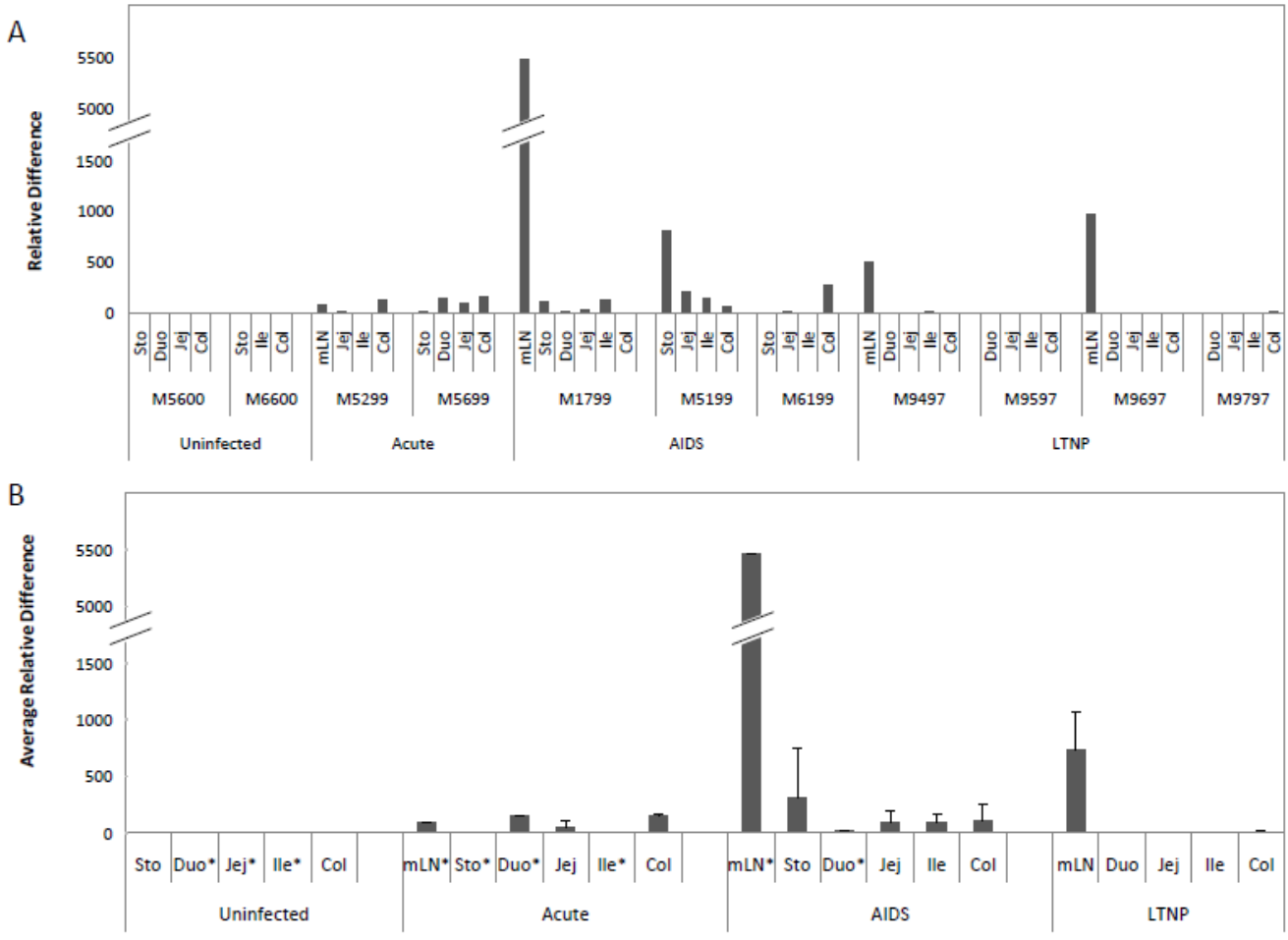

Figure 23 Relative IL-6 mRNA expression in uninfected, acute, AIDS, and LTNP monkeys. Analysis shown by individual tissue (A) and by averaged tissue (B).

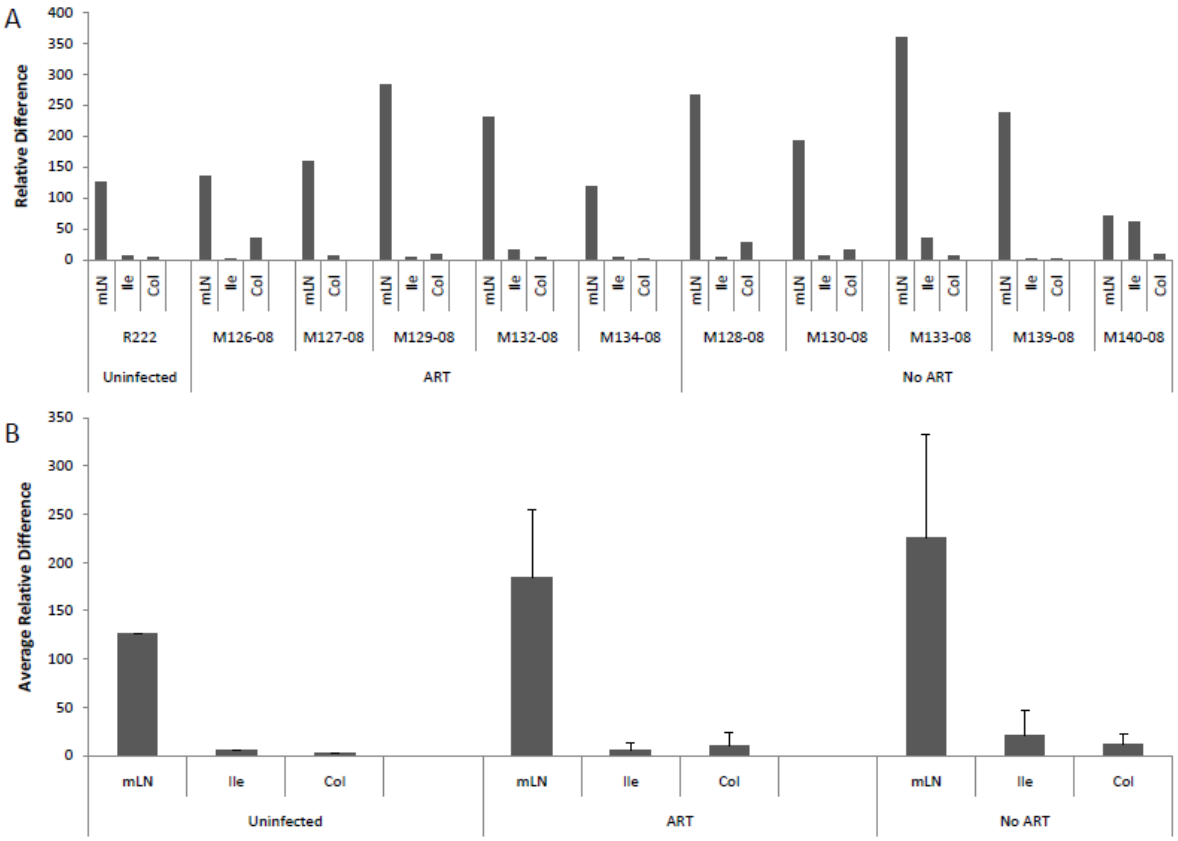

Figure 24 Relative IL-6 mRNA expression in uninfected, ART treated and untreated monkeys. Analysis shown by individual tissue (A) and by averaged tissue (B).

3.3.3 IL-1β in GI tissues

Interleukin-1β is considered a mediator of inflammation. The uninfected monkeys had the most consistently high IL-1β mRNA expression among tissues, while the stomach of AIDS monkey M1799 and the mesenteric lymph node of LTNP monkey M9697 had the highest relative expression (Fig 25). Followed by the uninfected monkeys, the LTNP had the next highest IL-1β

mRNA, with the AIDS monkeys having the next highest, and the monkeys in the acute stage had the least amount ofIL-1β.

In the ART study monkeys the mesenteric lymph node had the highest relative IL-1β mRNA expression among all of the samples and, no real difference in IL-1β levels was seen between the uninfected, ART treated, or untreated monkeys (Fig 26).

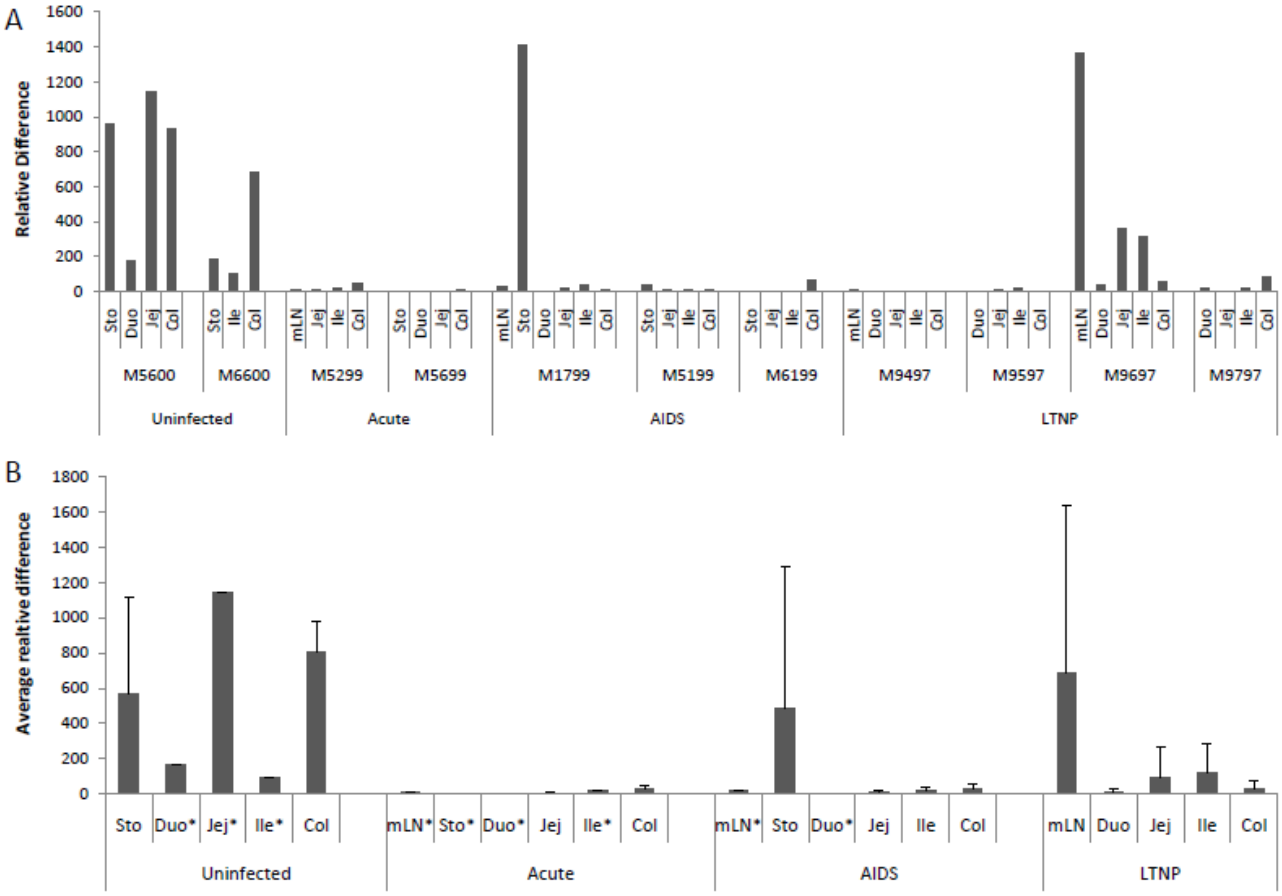

Figure 25 Relative IL-1β mRNA expression in uninfected, acute, AIDS, and LTNP monkeys. Analysis shown by individual tissue (A) and by averaged tissue (B).

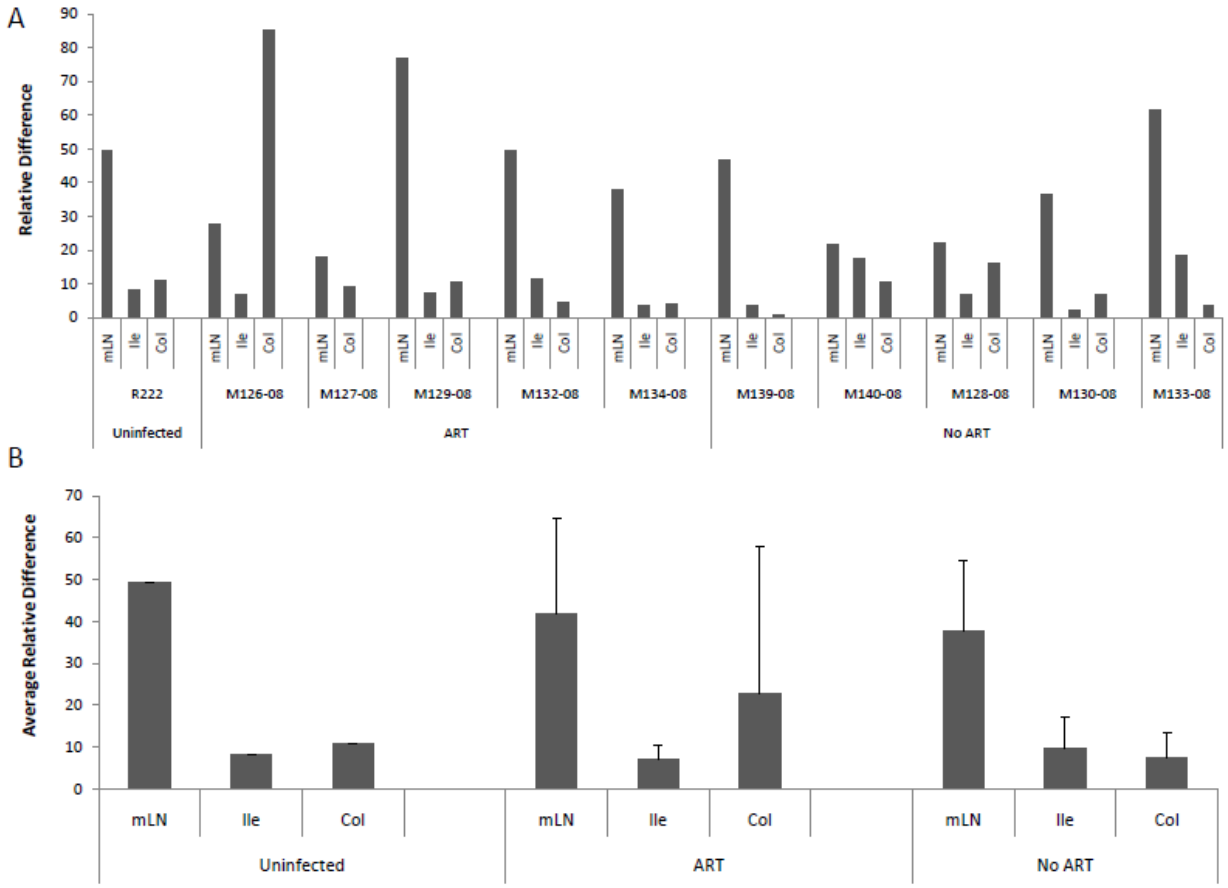

Figure 26 Relative IL-1β mRNA expression in uninfected, ART treated, and untreated monkeys. Analysis shown by individual tissue (A) and by averaged tissue (B).

3.3.4 MyD88 in GI tissues

MyD88 is a toll-like receptor signaling molecule. It participates in the signaling of TLR 1-11 (except TLR3) but particularly in TLR1, TLR2, and TLR4 recognizing gram positive bacteria (TLR 1 and 2), and lipopolysaccharide from gram negative bacteria (TLR4). Targeting MyD88 is used in the study as a marker to indicate an immune response via TLRs to the presence of bacteria, possibly present as a result of barrier dysfunction in the GI tract. Comparison to uninfected samples, abnormally high relative MyD88 mRNA expression could be identified,

which could be a result of barrier dysfunction. For this target, two samples from monkeys with AIDS had the highest expression, the mesenteric lymph node from M1799 and the stomach from M5199 (Fig 27). Overall, the monkeys with AIDS had the most MyD88 mRNA, followed by the acute monkeys. Additionally, the uninfected monkeys had more MyD88 mRNA than LTNP monkeys.

In the ART study monkeys, there is fluctuation in the tissue location that contains the most MyD88 mRNA, in the majority of the monkeys the mesenteric lymph node has the most, but there are a few where the ileum or the colon have the most (Fig 28). No real difference was seen between the uninfected, ART treated, or untreated monkeys.

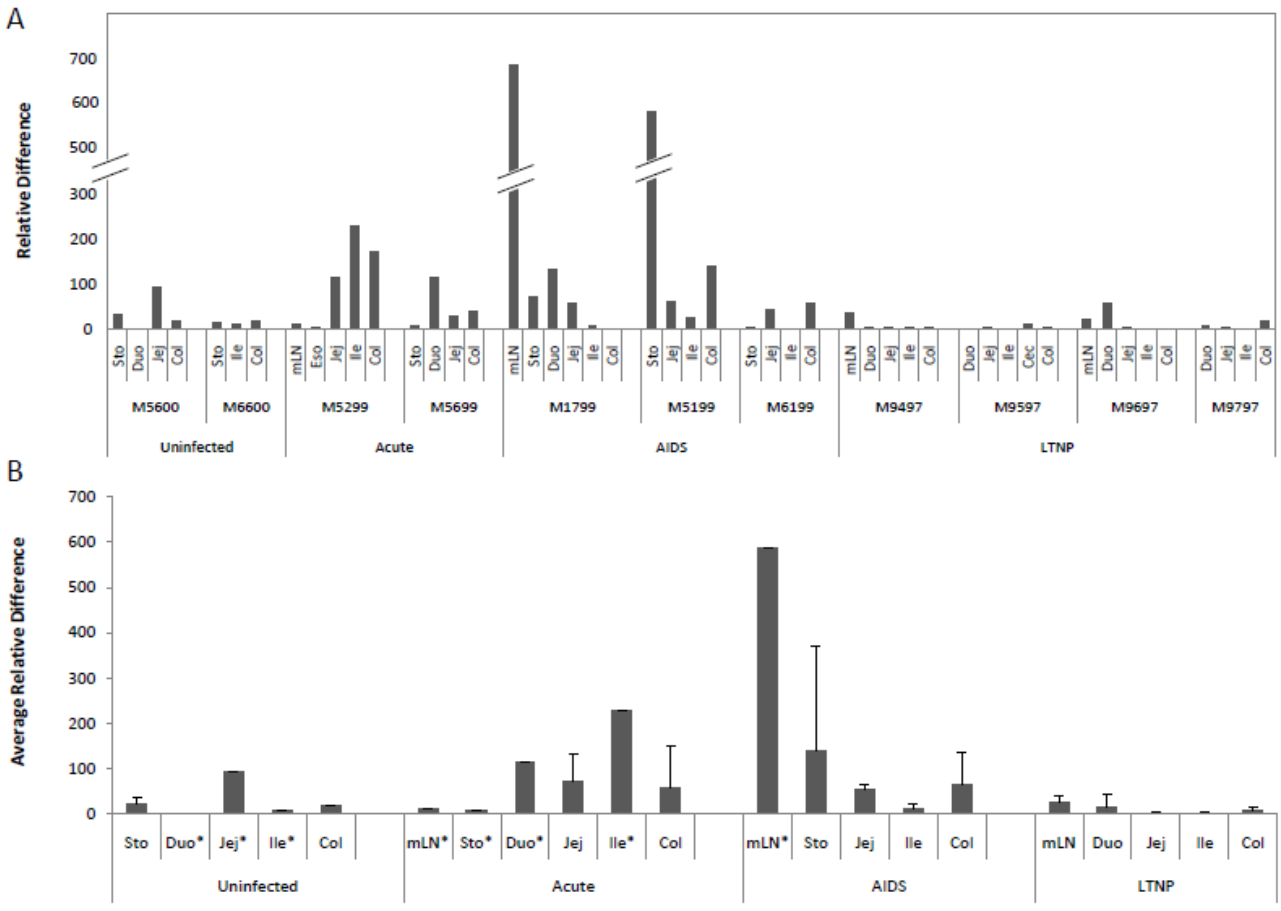

Figure 27 Relative MyD88 mRNA expression in uninfected, acute, AIDS, and LTNP monkeys. Analysis shown by individual tissue (A) and by averaged tissue (B).

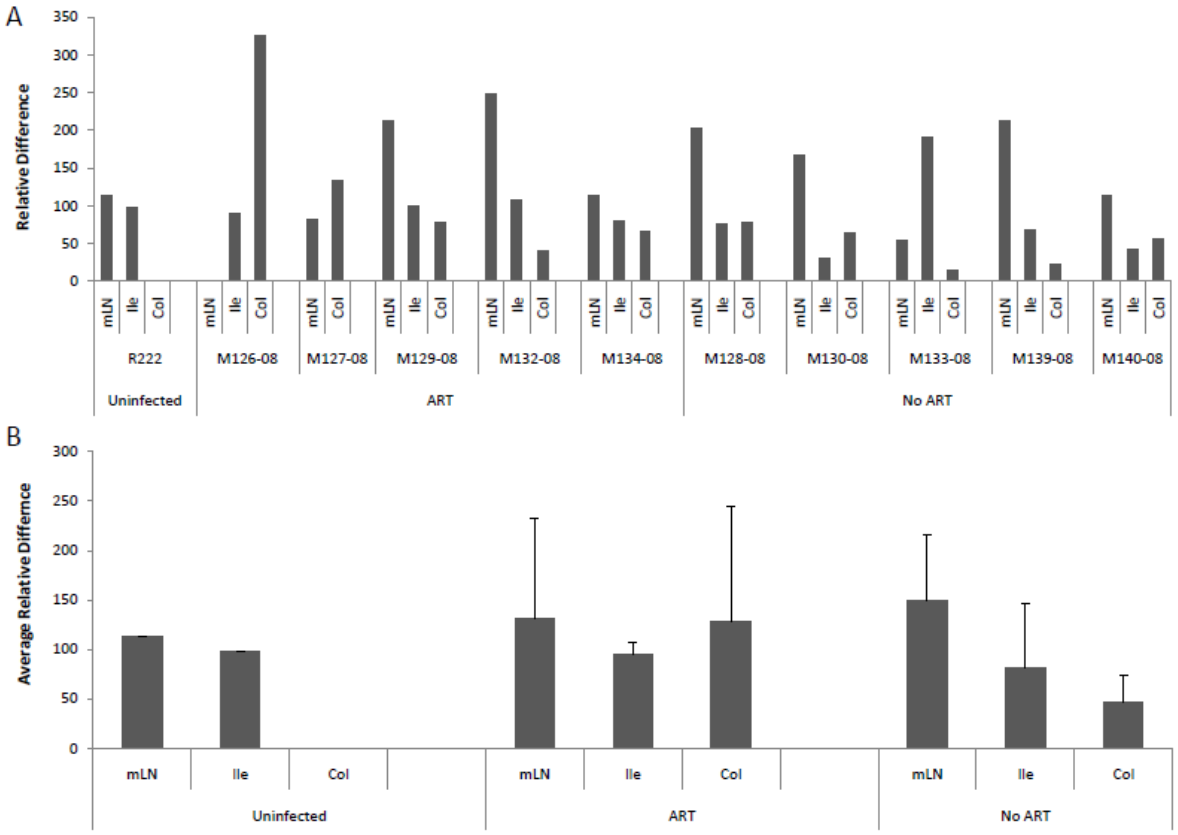

Figure 28 Relative MyD88 mRNA expression in uninfected, ART treated, and untreated monkeys. Analysis shown by individual tissue (A) and by averaged tissue (B).

3.3.5 Relative cytokine expression compared to viral load

Inflammation plays an important role in disease progression. Because inflammation is driven by cytokines, it is important to determine the relationship between viral RNA load and relative cytokine expression. In order to visualize this relationship, relative cytokine levels were plotted against viral load for each of the tissues. Because of the low number of replicate tissues for the acute, AIDS, and LTNP monkeys, all of the viral load and cytokine data for each tissue location

are on the same graph (Fig 29, 30) with no differentiation of disease stage except for color and shape of each data point. Similarly, due to the low number of monkeys for each disease stage, it was not possible to include a trend description to compare the acute, AIDS and LTNP groups separately. In the ART study there are five monkeys that received ART and 5 that did not, because of the higher number of replicates, the two groups could be compared with each other.

In the mesenteric lymph node, while positive associations with viral load were seen for TNF-α, IL-6, and MyD88, none of them were strong relationships with an R^2 value over 0.22 for the acute, AIDS and LTNP monkeys (Fig 29). For the ART study monkeys, R^2 values for TNF-α and IL-6 increased slightly for monkeys without ART (Fig 31). These associations became nonexistent for the monkeys with ART. Interestingly, a strong negative association is seen in the mLN with IL-1β with an R^2 value of 0.9664 in the acute, AIDS and LTNP monkeys; however this association is not apparent in the mLN of ART study monkeys that did not receive ART. Similar to the other cytokines, no association is seen between viral load and IL-1β in the ART study monkeys that received ART. In the stomach, strong associations are seen between viral load and TNF-α, IL-6, and MyD88 with R^2 values over 0.75 for each, while no association of the viral load is seen with IL-1β. In the duodenum a moderately strong positive relationships are seen with TNF-α and MyD88, with the strongest relationship between viral load and IL-6 with an R^2 value of 0.8908. Again, no relationship is seen in the duodenum between viral load and IL-1β. In the jejunum, very weakly positive associations are seen with TNF-α and IL-6 (Fig 29). A moderately positive relationship is present between viral load and MyD88 with an R^2 value of 0.4293, while no association exists for IL-1β. In the ileum of the acute, AIDS, and LTNP monkeys, positive associations are seen with TNF-α, IL-6, and MyD88 however, none of these associations are strong, with all of the R^2 values below 0.24 (Fig 30). In the ART study monkeys

that did not receive ART, a positive association is seen with TNF-α with an R^2 value of 0.9555, a more positive, moderately strong association is seen with IL-6, and interestingly, a moderately strong, slightly positive association is seen with IL-1β at an R^2 value of 0.5833. In these monkeys, no association is seen with MyD88. In the ART study monkeys that received ART, no association is seen with any of the cytokines. Lastly, in the colon of the acute, AIDS, and LTNP monkeys, positive but weak associations are seen with TNF-α, IL-6, and MyD88 with no association seen with IL-1β. The positive associations with TNF-α, IL-6, and MyD88 are slightly stronger in the ART study monkeys that did not receive ART, with again, no association with IL-1β. Like the previous tissues, there is no association between viral load and any of the cytokines in the ART study monkeys that did not receive ART. Table 7 contains all of the slope and R^2 values for Figures 29, 30, and 31

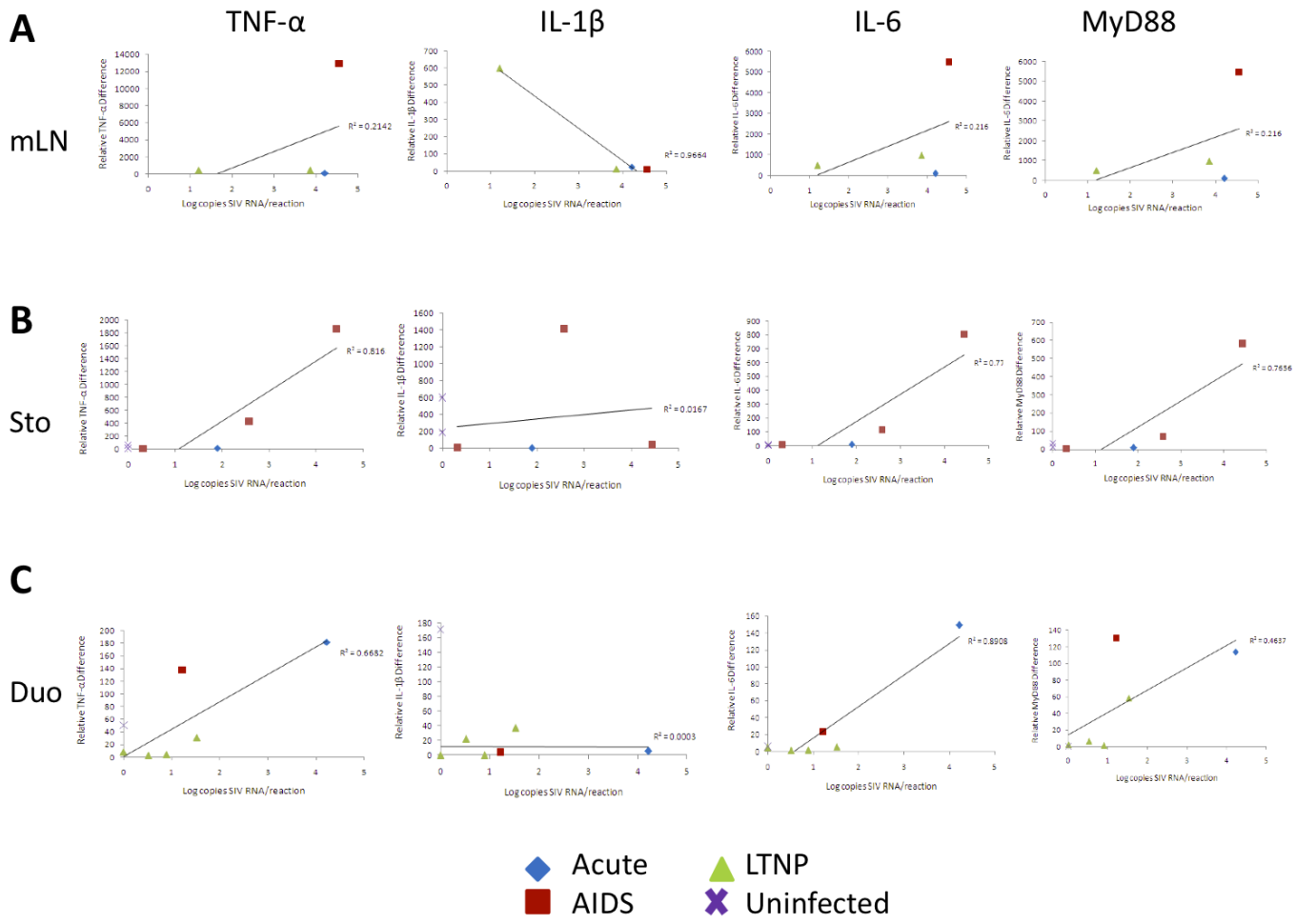

Figure 29 Cytokine expression compared to SIV RNA viral load in the mesenteric lymph node (A), stomach (B), and duodenum (C) of Uninfected, acute, AIDS, and LTNP monkeys

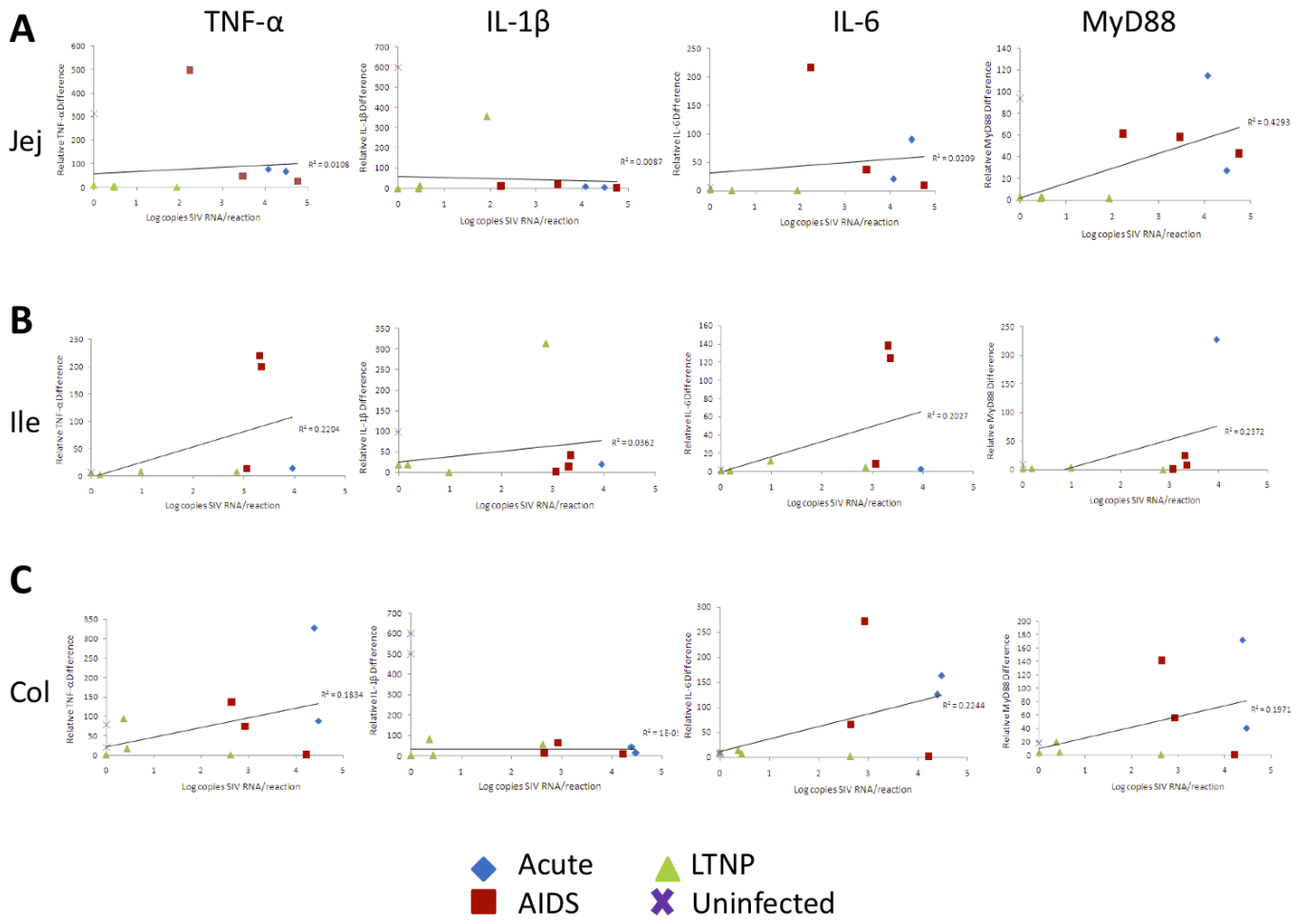

Figure 30 Cytokine expression compared to SIV RNA viral load in the jejunum (A), ileum (B), and colon (C) of Uninfected, acute, AIDS, and LTNP monkeys.

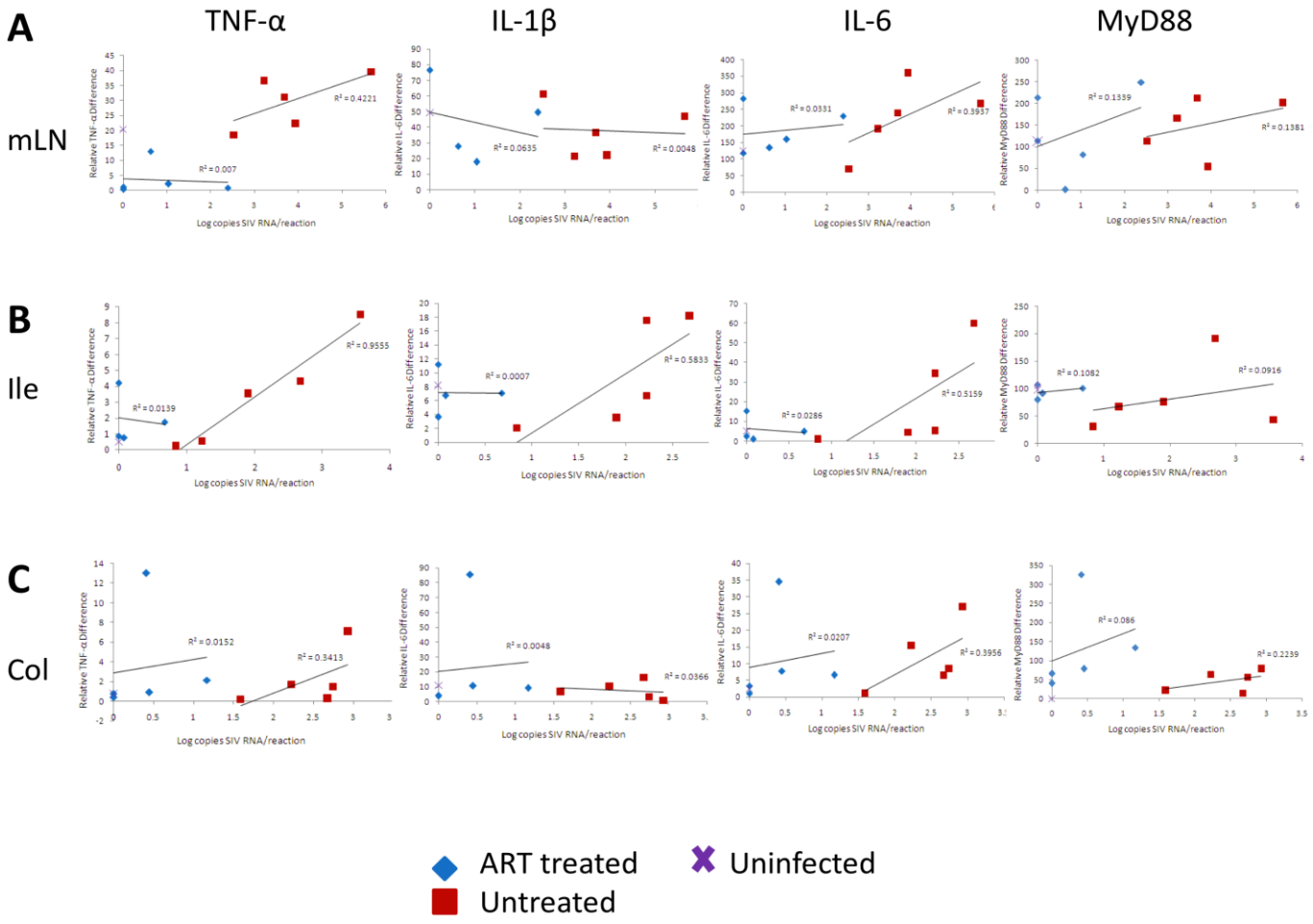

Figure 31 Cytokine expression compared to SIV RNA viral load in the mesenteric lymph node (A), ileum (B), and colon (C) of Uninfected, SIV-infected ART treated, and SIV-infected untreated monkeys.

Table 7 Slope of the line from cytokine vs. viral load graphs

	TNF-α	IL-6	IL-1β	MyD88
mLN	1903 (0.2142)	761.18 (0.216)*	-188.3 (0.9664)	98.304 (0.2045)
mLN - ART	-0.456 (0.007)	12.812 (0.0331)	-6.5017 (0.0635)	37.201 (0.1339)
mLN - No ART	5.0241 (0.4221)	57.169 (0.3937)	-1.0027 (0.0048)	20.98 (0.1381)
Sto	465.69 (0.8163)	198.09 (0.773)	52.827 (0.0167)	143.11 (0.7656)
Duo	43.309 (0.6682)	37.322 (0.8908)	-0.1713 (0.0003)	26.921 (0.4637)
Jej	8.9407 (0.0108)	6.0023 (0.0209)	-5.8851 (0.0087)	13.687 (0.4293)
Ile	28.014 (0.2204))	16.869 (0.2027)	12.86 (0.0362)	24.519 (0.2372)
Ile - ART	-0.5772 (0.0139)	-3.3286 (0.0286)	-0.2572 (0.0007)	11.988 (0.1082)
Ile - No ART	2.985 (0.9555)	26.51 (0.5159)	8.5251 (0.5833)	17.593 (0.0916)
Col	24.936 (0.1834	25.119 (0.2244)	-0.0611 (0.00001)	16.029 (0.1971)
Col - ART	1.389 (0.0152)	4.1 (0.0207)	5.0989 (0.0048)	70.811 (0.086)
Col - No ART	3.1007 (0.3956)	11.694 (0.3956)	-2.1419 (0.0366)	24.368 (0.2239)

*** (R^2 value) for all**

4.0 DISCUSSION

4.1 BRIEF OVERVIEW OF CURRENT STUDY

Samples from the GI tract of rhesus macaques were analyzed via real-time PCR for the presence of SIV, CD4 mRNA, and for a small panel of cytokine mRNA, in an effort to better understand the role of the GI tract in SIV infection, inflammation and disease progression. To assess the effect of ART interference on SIV induced pathogenesis in the GI tract, GI tissues from monkeys that were part of an ART study were used in this study. In order to determine the dynamic changes of viral load and immune status in the GI tract changed throughout the course of SIV infection, GI tissues were obtained from uninfected monkeys, monkeys in the acute and AIDS stage, as well as monkeys considered long-term non-progressors.

It was found that SIV RNA and DNA can be detected in all tissues (that were tested) of the GI tract from SIV infected monkeys, even when the viral load is undetectable in the blood (as was the case for the monkeys that received ART). In a relative comparison of CD4 mRNA in each of the tissues, it was found that uninfected monkeys had the most consistent amplification, followed by monkeys with AIDS. The monkeys in the acute stage had the least relative CD4 mRNA. In the ART study monkeys, no difference was seen between monkeys that received ART and those that did not.

4.2 SIGNIFICANCE OF RESULTS

4.2.1 Optimization of Nucleic Acid Isolation and Multiplex Real-Time PCR

4.2.1.1 Optimal sample input for real-time PCR

ABI and other PCR troubleshooting resources do not give a recommendation for the actual amount of DNA (or cDNA) to include in a PCR, it is only recommended that no more than 100 ng be used per reaction and that the sample amplification cross the threshold between 20 and 30 cycles. Because detection efficiencies in real time PCR are based on the specific primer and probe characteristics, different amounts of DNA input were tested in this study in order to obtain the most sensitive detection results.

The results of the experiment conducted to determine the amount of nucleic acid to add to the PCR were quite close. That is there was no significant difference between the input DNA when SIV LTR was used as target. There was however, a significant difference in sensitivity of detection when SIV Gag was used as target. Detection was determined to be better when the samples amplified at an earlier Ct. Because this detection was initially done based on relative difference, and because we hypothesized that we would be detecting a low amount of virus in some of the tissues (particularly in LTNP), the target and primers that provided the best detection were important to have.

4.2.1.2 Multiplex PCR optimization

Multiplex PCR was first used in 1988 to simultaneously amplify multiple loci in the human dystrophin gene [41]. Since then the procedure has become widely used in areas such as pathogen identification, gender screening, linkage analysis, forensic studies, template

quantitation, and genetic disease diagnosis [42]. One of the main benefits of multiplex PCR is that an endogenous control can be used to ensure the quality of the template (it is in a detectable state) and the quantity (there are enough copies to be detected) are such that if the sample truly is positive, it will be analyzed as positive. Additional benefits are that multiplex PCR saves resources and time, which added together save money. Some disadvantages to multiplex PCR are that the primers can interfere with each other, such that if they have similar sequences they could inadvertently bind to the opposite target, not enough to amplify, but enough to prevent the correct primer from binding. Additionally, both targets compete for the PCR components; the polymerase, the dNTP's, etc. if not enough of these components are added to the reaction, then one target could be amplified significantly more than the other, resulting in a falsely low amplifying sample. For an appropriate assay, the two targets must amplify at somewhat similar efficiency. Finally, primers for both targets must be compatible in their melting temperature and contain different detection probes so that both are allowed to amplify, but detection can be differentiated. The results from Figure 8 show the target from a sample will be amplified at the same rate when tested in uniplex as well as multiplex. This was found to be true for both a high viral load and a low viral load sample. Figure 9 shows that when these high and low samples are diluted, they still amplify at the same rate in uniplex as well as multiplex indicating that multiplex is effectively and accurately detecting the target sequences across a variety of target concentrations.

4.2.1.3 Use of the Qiagen AllPrep DNA/RNA mini kit ® for RNA and DNA isolation

For an ideal comparison in the viral RNA and DNA of a sample, both nucleic acid types must come from the same tissue. Previous studies however, have performed analyses using either just RNA, with the most common isolation method being the RNAeasy mini kit® made by Qiagen

[10,30,43] (additionally the Mag Attract virus mini M48 kit® also by Qiagen has been used to isolate RNA from plasma [44]) or DNA with the most common isolation method being the DNA blood mini kit® by Qiagen [30]. In one study both the RNA and DNA were tested from a pool of separated and re-suspended gut biopsy cells. For this study, in order to get a true comparison, a kit was chosen that can isolate the RNA and the DNA from the same piece of tissue.

In order to prevent contamination of the RNA with DNA, the RNA isolation procedure included DNase treatment. Additionally, during the reverse-transcriptase PCR, some of the RNA samples were tested for residual, contaminating DNA by being added to a reverse-transcriptase PCR mix that contained everything except the reverse transcriptase itself. If DNA were present, it would amplify in the real-time PCR that was conducted subsequently (because it would not need the reverse transcriptase to convert it to DNA, because it is already DNA). These two measures ensured the purity of the RNA samples. Additionally, the commercial kit allowed for the same level of purity for each of the samples for better, more accurate comparison.

The samples used in Figure 8 were the first where the Qiagen kit was used to isolate both RNA and DNA from the same tissue. Because the samples were amplified both in uniplex as well as multiplex, it was determined that this Qiagen kit was appropriate for use in our assay.

4.2.1.4 Standard curve optimization in multiplex PCR

In order to effectively quantitate the amount of virus in a sample, a standard curve containing a known quantity of the target must be used. In the case of quantitation SIV in GI tissues, one option for creating a standard curve is cloning the SIV target sequence into a plasmid and using the quantitated recombined plasmid to generate a standard curve via a serial dilution. An additional option is to use purified and quantitated SIV to generate a standard curve via a serial

dilution. It was decided to use the latter option, and so SIV (strain 17E) was grown in tissue culture, purified, and quantitated using a well-established standard curve generously donated by Dr. Reinhart's lab. Because virus without eukaryotic cells was used for the standard curve, it does not contain any more than residual 18S levels from the culture cells it was grown in. However, to ensure that the primers do not interfere with each other, the standard curve was run in a real-time PCR assay both in multiplex mix (containing primers/probes of both 18S and LTR, but targeting the LTR of SIV) and uniplex mix (only containing LTR primers/probe, targeting the LTR of SIV). Because the replication kinetics were the same in multiplex and uniplex, the standard curve is fit for use in multiplex.

4.2.2 CD4 mRNA distribution

It has been previously shown that higher levels of $CD4^+$ T cell depletion occur in the GI tissues than is detected in the blood [19]. Because of this phenomenon, an important part of this study was determining the $CD4^+$ T cell level in the GI tissues. In most studies, $CD4^+$ T cell counts are quantitated by flow cytometry from blood and tissue samples. Because it was difficult to prepare a single cell suspension from snap-frozen tissue for quantifying $CD4^+$ T cells by flow cytometry, CD4 mRNA was instead measured by RT-real time PCR as a surrogate for $CD4^+$ T cells in this study. Limitations to this method include: a single $CD4^+$ cell may produce dissimilar amounts of CD4 mRNA and multiple cell types other than $CD4^+$ T cells produce CD4 mRNA as well. CD4 is most commonly expressed on αβ T cells, (the familiar $CD4^+$ T helper cell that is the target of HIV), somewhat frequently on macrophages, less frequently on monocytes, DC's and NK cells, and rarely on γβ T cells [45]. An ideal study would have been able to use flow cytometry to

identify $CD4^+$ T cells present in the tissues, however, due to the limitations of the tissue samples, real-time PCR was utilized as the detection method for CD4 cells in current study.

As shown in Figure 11, low levels of CD4 mRNA were detected in all GI tissues from acute infected monkeys and in most of the GI tissues of AIDS monkeys compared to that of uninfected monkeys. These results are consistent with previous reports that $CD4^+$ T cells in GI tract were depleted in acute infected and AIDS individuals. However, in some GI tissues from AIDS monkeys, particularly the stomach and colon, approximately the same levels of CD4 mRNA were detected as that of the uninfected monkeys in those tissues. The reason of these results is unclear. Also of interest, is the extreme low level of CD4 mRNA that was observed in the LTNP monkeys. Because low levels of virus and high levels of $CD4^+$ T cells are generally associated with long-term non-progression, this result is unexpected. This severe depletion could be a result of the length of the infection; while the exact length of survival is unknown for the LTNP, it can be assumed that they probably survived for at least two to three years of SIV infection without developing AIDS.

In figure 12, where the ART study monkeys were sacrificed at 36 days post infection, no difference in CD4 mRNA levels was observed in the mesenteric lymph nodes, ileum and colon between the monkeys that were uninfected or infected with or without ART. While again these are slightly surprising results, it was expected that the monkeys that were infected without therapy would have the lowest amount of CD4 mRNA in GI tissue, followed by infected monkeys that received ART, with the highest amount of CD4 mRNA found in the monkeys that were uninfected. This discrepancy could be due to only one uninfected control monkey available. Furthermore, ART treatment in these monkeys might not prevent CD4 T cell depletion in the GI tissues.

The analysis of CD4 mRNA in this study differs from previous studies in that $CD4^+$ cells were measured and compared in multiple tissues throughout the GI tract with and without ART intervention. Studies have shown that $CD4^+$ T cell counts in blood decreases during HIV/SIV infection [44,46]. Additionally, there are some reports that the amount of $CD4^+$ T cells was decreased in a tissue biopsy such as the intestine [10], colon [47], or jejunum [8], and in multiple lymph nodes [48,49]. One study measured $CD4^+$ T cells in the blood, duodenum, terminal ileum, colon, and rectum and found decreased levels in all [30]. However, amidst all of this research, none covers the GI tract from stomach to colon in infected individuals.

The restoration of $CD4^+$ T cells upon HAART/ART administration remains controversial. Some groups have conducted studies that show $CD4^+$ T cell depletion remains at around 50-60% of the pre-infection level [47], others have shown that initiating ART during the primary phase of infection leads to 80-95% $CD4^+$ T cell restoration [8]. In terms of the interesting results for the relative $CD4^+$ mRNA level for the LTNP, a study by Ling [46] showed that early destruction of $CD4^+$ T cells was similar between LTNP and progressors, but that around day 20-25 post infection, the LTNP had better T cell restoration that remained higher than the progressors for the length of infection.

4.2.3 Viral Load in GI tissues

Real time PCR is a common technique for quantitating HIV/SIV viral load in both the blood and in tissues. Previously, *env* [20], *pol, tat, pol/tat* exon [30], *gag* [34,44] and the LTR [50] have been used as PCR targets for SIV. In current study, the SIV loads from the tissues were measured using LTR as a PCR target. As shown in Figure 13, more viruses were detected in all tested GI tissues from acute and AIDS monkeys compared to LTNP monkeys, with the

additional pattern of generally more viral RNA present in the GI tissues of monkeys in the acute and AIDS stage, with more viral DNA in the tissues of the monkeys considered LTNP. It is expected to observe more viral RNA during the acute and AIDS stage of infection because at these stages the virus is actively replicating. During long-term non-progression, we do not expect to see a large amount of replicating virus; instead we expect to see more integrated proviral DNA. This viral DNA, while not indicative of replicating virus, could be expressed if the cell becomes activated and begins translation and transcription, resulting in active virus particles. During ART intervention the viral replication is stopped by the treatment, and as such, significantly more viral DNA is present than viral RNA, even though these monkeys were in the acute stage of infection (Fig 15). When the viral load is normalized by CD4 the viral RNA and viral DNA pattern cannot be seen and on average an equal amount of viral RNA and viral DNA are present in the tissues, except in the ileum where there is significantly more viral DNA than RNA in the monkeys treated with ART. There is still however, significantly more virus present in the monkeys that did not receive ART compared to those that did.

This study spans the GI tract from the stomach to the colon, and includes ART intervention. In a very similar study, Yukl et al [30] measured the levels of HIV DNA, vRNA, and T cell activation in the duodenum, ileum, ascending colon and rectum in 8 HIV-1 positive patients that received ART with T cell counts >200 cells/μL. They found that HIV DNA levels increased from the duodenum to the rectum, with the median HIV RNA level peaking in the ileum. In their viral RNA to DNA comparison, they saw more viral RNA than DNA in the PBMC's compared to the large bowel, and more viral RNA than DNA in the duodenum compared to the large bowel. Our study however, did not consistently see the same pattern, even in the CD4 normalized viral load. Perhaps a trend of increasing viral RNA (but not DNA) from

the stomach to the Colon was seen in the AIDS monkeys (Fig 17), but for the rest of the stages and ART monkeys the vial loads in the tissues were quite comparable in the amount of viral RNA or DNA such that a single tissue did not stand out as having consistently more virus (either vRNA or vDNA) than others.

4.2.4 Cytokine mRNA in GI tissues

The presence of cytokines can be tested via ELISA, PCR, or other specialized cytokine detection assays. In this study, real-time PCR was utilized to measure and compare the relative cytokine mRNA in each tissue. All of the monkey tissue samples were analyzed for the mRNA of TNF-α, IL-6, IL-1β, and MyD88 via reverse transcriptase real-time PCR and tested in duplicate.

Monkeys in the AIDS stage had the highest levels of TNF-α mRNA present in the tissues, followed by the acute stage, uninfected monkeys, and LTNP monkeys had the least (Fig 21). No change was seen in the TNF-α levels in the tissues of the ART study monkeys upon ART intervention. While the infected monkeys had slightly more TNF-α than the uninfected monkeys, no difference was seen between the infected monkeys that received ART and those that did not (Fig 22). When analyzing the relationship of viral loads and TNF-α levels, positive associations are revealed in the tissues from the stomach to the colon in all tissues except the jejunum where the TNF-α level stays the same regardless of the viral load. A similar effect is seen in the ART study monkeys where the amount of TNF-α increases with viral load in the tissues of infected ATR naive monkeys, but in the monkeys that did receive ART, no association is present. While positive associations are seen in all of the tissues, these associations are strongest in the stomach and jejunum, as well as in the ileum of ART naïve monkeys. It has largely been agreed upon that cytokines in general, and TNF-α in particular, increase as HIV

infection progresses [38,51,52,53]. TNF-α is a cytokine that initiates intracellular signaling cascades, most of which are NFκB-dependent and result in a wide array of biological effects [38]. There exists, however, debate on whether TNF-α levels are altered by ART. A study by Zara et al measuring the viral load in cervico-vaginal lavage, revealed a decrease in TNF-α with ART administration [54]. Alternatively, a study by Giron-Gonzalez in 2000 showed increased levels of TNF-α in HIV infected subjects compared to uninfected, but HAART administration did not significantly reduce TNF-α levels [55]. Our results agree with current findings that the monkeys with AIDS had higher levels of TNF-α than acute or uninfected, and with LTNP having the least. This indicates that lack of TNF-α activation during HIV/SIV infection could be a factor of non-progression. Additionally, our TNF-α ART intervention results agree with those of Giron-Gonzalez in 2000 in that no difference was seen upon ART administration.

Similar to TNF-α, the monkeys with AIDS were found to have the highest levels of IL-6 mRNA, followed by the acute monkeys, LTNP, and the uninfected monkeys had the lowest IL-6 mRNA levels (Fig 23). There is no significant difference in IL-6 levels between infected monkeys that received ART and those that did not (Fig 24). When the IL-6 mRNA levels were analyzed with viral loads from the same tissues, a positive association is observed in all of the tissues except the jejunum with the strongest associations again in the stomach and duodenum. In the ART study monkeys, no positive association is observed between IL-6 levels and viral loads in any tissues of ART treated monkeys, whereas a positive association is observed in the ileum of the ART naïve monkeys.

Many studies have shown that IL-6 increases with HIV infection [38,56]. In a study by Mohan a significant increase in IL-6 expression was shown in the colon and jejunum of SIV infected macaques, particularly in infected monkeys with chronic diarrhea [26]. It was

previously thought that HIV directly increased IL-6 secretion by monocytes and macrophages, but more recently it has been shown that rather than HIV having a direct effect on IL-6 receptor expression, the nonspecific stimulation of macrophages results in increased cytokine production, which enhances HIV replication [38]. A previous study measuring IL-6 and TNF-α levels in children before and after HAART showed a decrease in both IL-6 and TNF-α upon HAART intervention. Additionally, for every 10% increase of $CD4^+$ T cell counts, the IL-6 level decreased by 31% [57]. However in our current study, low viral loads in GI tissues of ART treated monkeys did not coincide with decreased IL-6 levels in the same tissues. This discrepancy may be due to the low level of infection in these ART study monkeys.

Relatively less information is available concerning MyD88 expression and HIV infection. In this study, MyD88 served as an indicator of immune activation; an active participant in the toll-like receptor response to bacterial pathogens in the GI tissues resulting from barrier dysfunction and microbial translocation. MyD88 is associated with TLRs 1-11 (except 3) [58], and has recently been shown to play a critical role in host defense against enteric pathogens, not only through the induction of an inflammatory response, but also by maintaining intestinal epithelial cell homeostasis [59].

Much like the pro-inflammatory cytokines, higher MyD88 mRNA levels were detected in GI tissues of AIDS monkeys, followed by the acute monkeys, the uninfected monkeys with lowest levels detected in the LTNP (Fig 27). When the relative MyD88 mRNA is analyzed with tissue viral load, a positive association is observed in all of the tissues from the stomach to the colon, including the mesenteric lymph node; although none of these associations are strong except for the association seen in the stomach. The infected ART naive monkeys had slightly less MyD88 mRNA in GI tissues compared to the ART treated monkeys (Fig 28). The lowest

MyD88 levels were detected in the uninfected monkeys. There is no association between MyD88 expression levels and viral loads in gut tissues of ART treated and ART naive monkeys.

Currently the role of IL-1β in HIV/SIV pathogenesis is unclear. The presence of IL-1β in serum of HIV infected patients has previously shown to be both decreased [60,61] and increased [38,62,63]. In an interesting study by He et al in 2000, HIV positive hemophiliacs were shown to have decreased IL-1β compared to HIV negative hemophiliacs [64]. However, in the cervico-vaginal tissue study by Zara et al, increased IL-1β was seen with increased viral DNA in cervico-vaginal lavage samples. Additionally it has been shown that PBMC's and DC's infected with HIV were able to produce more IL-1β in response to stimuli such as LPS with higher viral load [38,63]. In our current study, the uninfected monkeys had the most IL-1β mRNA in GI tissues followed by the LTNP monkeys (at least 2 of them), and less IL-1β mRNA in GI tissues was detected in the AIDS and acute monkeys (Fig 25). There was a slight increase in IL-1β mRNA levels in ART treated monkeys (Fig 26). When relative IL-1β mRNA levels are analyzed with viral load in the same tissues, there is no association between viral loads and IL-1β mRNA levels. Interestingly, there is a strong negative association between viral loads and IL-1β mRNA levels in the mesenteric lymph node of SIV infected monkeys. Our results indicate that IL-1β may not significantly contribute to SIV induced pathogenesis in the GI tract.

In general, higher levels of pro-inflammatory cytokines. TNF-α and IL-6 mRNA were detected in monkey GI tissue during the acute and AIDS stages of infection. MyD88 mRNA levels, an indicator of potential microbial translocation due to barrier dysfunction, were also higher in the acute and AIDS monkeys. Oppositely, higher levels of IL-1β were detected in long-term non-progressors and uninfected monkeys. It is possible that the higher TNF-α expression in the acute and AIDS monkeys may contribute to decreased IL-1β expression in

those same monkeys. The initiation of NFκB dependent signaling cascades by TNF-α may cause inhibition of IL-1β, because NFκB inhibits IL-1β release from macrophages [39].

In summary, our current study showed that SIV infection depletes CD4 T cells from the GI tissues in infected monkeys. Of all the disease stages, long-term non-progressors had particularly low CD4 mRNA levels in gut tissues. Active replicating viral RNA was detected in all GI tissues regardless of disease stage and therapeutic intervention. SIV load distribution profile in the GI tract varied from monkey to monkey. Positive associations were observed between viral load and pro-inflammatory cytokines in the GI tract, particularly in the upper GI tissues, whereas no association was observed between viral loads and IL-1β levels. In ART study monkeys, treatment significantly decreased the viral loads in GI tissues. However, there were no significant changes in the levels of CD4 and pro-inflammatory cytokines. Furthermore, no associations were observed between the viral loads and cytokine levels in either ART treated or ART naive monkeys. Our study indicates that the GI tract significantly contributes to SIV/HIV pathogenesis and harbors active replicating virus in ART treated and LTNP individuals. This information will have a significant impact on therapeutic strategies and vaccine development.

4.3 RELEVANCE OF STUDY FOR HIV TREATMENT

The GI tract is clearly a location of viral residence. Even when virus cannot be detected in the blood it can be detected in various locations along the GI tract. Because of this, the GI tract may be an important location for drug focus. Additionally with the connection seen between pro-inflammatory cytokines and disease progression, perhaps successful drugs will not only target the virus, but will also down-regulate the inflammatory immune response. Another route of HIV

drug success may involve ensuring penetration of the drug to the GI tract. In a study in 2010 by Van Merle et al, HIV drug resistance to zidovudien was tested (via sequencing and targeting known drug resistance sequences) in esophagus, stomach, duodenum, and colon biopsy samples [65]. It was found the highest levels of drug resistance were in the colon. This could be a result of low levels of drug reaching the GI tract.

In an attempt to use the knowledge of the importance of the GI tract in HIV infection, recent studies in nonhuman primates have indicated that vaccination at mucosal sites can elicit a strong immune response in the periphery and at mucosal sites, however, disease course after challenge did not differ by route of vaccination [66].

4.4 FUTURE DIRECTIONS

While the evidence for the association between HIV/SIV infection and microbial translocation is quite strong [10,43,44,67], it is still a relatively new topic that remains a bit controversial. A study by Leinert et al. actually concluded that microbial translocation is not the major driving force of immune activation in HIV infection [44]. Additionally, a study by Lay et al concluded that while the GI tract is a major site of infection, it is not the source of virus that is detected in the blood, and therefore not a major source of virus production in the systemic infection of the host [49]. Future studies will solidify the evidence for the association between HIV/SIV infection and microbial translocation and will look at the in-depth role of microbial translocation on disease progression.

Studies in the future will also look into the exact cytokines and factors that drive microbial translocation. If the specific factors that perpetuate microbial translocation can be

identified, subsequent studies can look into drugs to block these factors and prevent disease progression through microbial translocation.

4.5 PUBLIC HEALTH IMPLICATIONS

Treatment for HIV has come a long way since AZT was first used as a therapy for AIDS in 1987 [3]. However, even with the development of protease inhibitors and the use of HAART, drug resistance still arises in the virus, and new drug combinations are needed to keep HIV in check. If drugs can be developed that target the most active sites of infection, HIV replication and the resulting drug resistance will decrease. Turning the attention of researchers and physicians to the GI tract could prove vital in the disease progression and treatment of HIV infected patients.

Drug therapy efficiently targeting the GI tract could prevent disease progression and ultimately lead to HIV/SIV eradication. Because replicating virus is harbored in the GI tissue of ART treated individuals, it is speculated that currently available drugs do not efficiently penetrate the GI tract to prevent viral replication as observed in the blood. This active viral replication in gut tissue may lead to development of drug resistant variants and faster progression to AIDS. Development of drugs that are able to efficiently penetrate the GI tissue will prevent viral replication in this anatomic location, slowing disease progression in SIV/HIV-infected individuals.

Opportunistic infections of the GI tract in HIV-infected individuals range from the esophagus to the rectum [68,69]. In view of the importance of the GI tract in HIV infection, early identification and treatment of these opportunistic infections by physicians is vital to

preventing the long-term inflammation that leads to the microbial translocation/disease progression cycle.

With additional research and early therapeutic intervention, the reservoir of HIV could be better targeted by drugs and disease progression slowed to a snail's pace. We just have to keep moving forward.

BIBLIOGRAPHY

1. Klimas N, Koneru AO, Fletcher MA (2008) Overview of HIV. Psychosom Med 70: 523-530.

2. Levy JA (2006) HIV pathogenesis: knowledge gained after two decades of research. Adv Dent Res 19: 10-16.

3. Palmisano L, Vella S (2011) A brief history of antiretroviral therapy of HIV infection: success and challenges. Ann Ist Super Sanita 47: 44-48.

4. Poropatich K, Sullivan DJ, Jr. (2011) Human immunodeficiency virus type 1 long-term non-progressors: the viral, genetic and immunological basis for disease non-progression. J Gen Virol 92: 247-268.

5. Liu J, Keele BF, Li H, Keating S, Norris PJ, et al. (2010) Low-dose mucosal simian immunodeficiency virus infection restricts early replication kinetics and transmitted virus variants in rhesus monkeys. J Virol 84: 10406-10412.

6. Julg B, Pereyra F, Buzon MJ, Piechocka-Trocha A, Clark MJ, et al. (2010) Infrequent recovery of HIV from but robust exogenous infection of activated CD4(+) T cells in HIV elite controllers. Clin Infect Dis 51: 233-238.

7. Ghosh RK, Ghosh SM, Chawla S (2011) Recent advances in antiretroviral drugs. Expert Opin Pharmacother 12: 31-46.

8. George MD, Reay E, Sankaran S, Dandekar S (2005) Early antiretroviral therapy for simian immunodeficiency virus infection leads to mucosal CD4+ T-cell restoration and enhanced gene expression regulating mucosal repair and regeneration. J Virol 79: 2709-2719.

9. Guadalupe M, Sankaran S, George MD, Reay E, Verhoeven D, et al. (2006) Viral suppression and immune restoration in the gastrointestinal mucosa of human immunodeficiency virus type 1-infected patients initiating therapy during primary or chronic infection. J Virol 80: 8236-8247.

10. Sankaran S, George MD, Reay E, Guadalupe M, Flamm J, et al. (2008) Rapid onset of intestinal epithelial barrier dysfunction in primary human immunodeficiency virus infection is driven by an imbalance between immune response and mucosal repair and regeneration. J Virol 82: 538-545.

11. Mayer KH, Venkatesh KK (2010) Antiretroviral therapy as HIV prevention: status and prospects. Am J Public Health 100: 1867-1876.

12. Kitahata MM (2010) When to start antiretroviral therapy. Top HIV Med 18: 121-126.

13. Kahn JO, Walker BD (1998) Acute human immunodeficiency virus type 1 infection. N Engl J Med 339: 33-39.

14. Kassutto S, Rosenberg ES (2004) Primary HIV type 1 infection. Clin Infect Dis 38: 1447-1453.

15. Trichel AM, Rajakumar PA, Murphey-Corb M (2002) Species-specific variation in SIV disease progression between Chinese and Indian subspecies of rhesus macaque. J Med Primatol 31: 171-178.

16. Brenchley JM, Silvestri G, Douek DC (2010) Nonprogressive and progressive primate immunodeficiency lentivirus infections. Immunity 32: 737-742.

17. Mehandru S, Poles MA, Tenner-Racz K, Horowitz A, Hurley A, et al. (2004) Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. J Exp Med 200: 761-770.

18. Schneider T, Jahn HU, Schmidt W, Riecken EO, Zeitz M, et al. (1995) Loss of CD4 T lymphocytes in patients infected with human immunodeficiency virus type 1 is more pronounced in the duodenal mucosa than in the peripheral blood. Berlin Diarrhea/Wasting Syndrome Study Group. Gut 37: 524-529.

19. Veazey RS, DeMaria M, Chalifoux LV, Shvetz DE, Pauley DR, et al. (1998) Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. Science 280: 427-431.

20. Chun TW, Nickle DC, Justement JS, Meyers JH, Roby G, et al. (2008) Persistence of HIV in gut-associated lymphoid tissue despite long-term antiretroviral therapy. J Infect Dis 197: 714-720.

21. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, et al. (2006) Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med 12: 1365-1371.

22. Paiardini M, Frank I, Pandrea I, Apetrei C, Silvestri G (2008) Mucosal immune dysfunction in AIDS pathogenesis. AIDS Rev 10: 36-46.

23. Marchetti G, Bellistri GM, Borghi E, Tincati C, Ferramosca S, et al. (2008) Microbial translocation is associated with sustained failure in CD4+ T-cell reconstitution in HIV-infected patients on long-term highly active antiretroviral therapy. AIDS 22: 2035-2038.

24. Cullen BR, Greene WC (1989) Regulatory pathways governing HIV-1 replication. Cell 58: 423-426.

25. Li Q, Estes JD, Duan L, Jessurun J, Pambuccian S, et al. (2008) Simian immunodeficiency virus-induced intestinal cell apoptosis is the underlying mechanism of the regenerative enteropathy of early infection. J Infect Dis 197: 420-429.

26. Mohan M, Aye PP, Borda JT, Alvarez X, Lackner AA (2007) Gastrointestinal disease in simian immunodeficiency virus-infected rhesus macaques is characterized by proinflammatory dysregulation of the interleukin-6-Janus kinase/signal transducer and activator of transcription3 pathway. Am J Pathol 171: 1952-1965.

27. Epple HJ, Schneider T, Troeger H, Kunkel D, Allers K, et al. (2009) Impairment of the intestinal barrier is evident in untreated but absent in suppressively treated HIV-infected patients. Gut 58: 220-227.

28. Silverthorn DU (2007) Human physiology : an integrated approach. San Francisco: Pearson/Benjamin Cummings. 1 v. (various pagings) p.

29. Yukl S, Wong JK (2008) Blood and guts and HIV: preferential HIV persistence in GI mucosa. J Infect Dis 197: 640-642.

30. Yukl SA, Gianella S, Sinclair E, Epling L, Li Q, et al. (2010) Differences in HIV burden and immune activation within the gut of HIV-positive patients receiving suppressive antiretroviral therapy. J Infect Dis 202: 1553-1561.

31. Epple HJ, Allers K, Troger H, Kuhl A, Erben U, et al. (2010) Acute HIV infection induces mucosal infiltration with CD4+ and CD8+ T cells, epithelial apoptosis, and a mucosal barrier defect. Gastroenterology 139: 1289-1300.

32. Lackner AA, Mohan M, Veazey RS (2009) The gastrointestinal tract and AIDS pathogenesis. Gastroenterology 136: 1965-1978.

33. Kewenig S, Schneider T, Hohloch K, Lampe-Dreyer K, Ullrich R, et al. (1999) Rapid mucosal CD4(+) T-cell depletion and enteropathy in simian immunodeficiency virus-infected rhesus macaques. Gastroenterology 116: 1115-1123.

34. Mattapallil JJ, Douek DC, Hill B, Nishimura Y, Martin M, et al. (2005) Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. Nature 434: 1093-1097.

35. Veazey RS, Tham IC, Mansfield KG, DeMaria M, Forand AE, et al. (2000) Identifying the target cell in primary simian immunodeficiency virus (SIV) infection: highly activated memory CD4(+) T cells are rapidly eliminated in early SIV infection in vivo. J Virol 74: 57-64.

36. Sharkey ME, Teo I, Greenough T, Sharova N, Luzuriaga K, et al. (2000) Persistence of episomal HIV-1 infection intermediates in patients on highly active anti-retroviral therapy. Nat Med 6: 76-81.

37. Murphy KP, Travers P, Walport M, Janeway C (2008) Janeway's immunobiology. New York: Garland Science. xxi, 887 p. p.

38. Connolly NC, Riddler SA, Rinaldo CR (2005) Proinflammatory cytokines in HIV disease-a review and rationale for new therapeutic approaches. AIDS Rev 7: 168-180.

39. Gabay C, Lamacchia C, Palmer G (2010) IL-1 pathways in inflammation and human diseases. Nat Rev Rheumatol 6: 232-241.

40. Mitsuyama K, Sata M, Rose-John S (2006) Interleukin-6 trans-signaling in inflammatory bowel disease. Cytokine Growth Factor Rev 17: 451-461.

41. Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT (1988) Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 16: 11141-11156.

42. Edwards MC, Gibbs RA (1994) Multiplex PCR: advantages, development, and applications. PCR Methods Appl 3: S65-75.

43. Nazli A, Chan O, Dobson-Belaire WN, Ouellet M, Tremblay MJ, et al. (2010) Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. PLoS Pathog 6: e1000852.

44. Leinert C, Stahl-Hennig C, Ecker A, Schneider T, Fuchs D, et al. (2010) Microbial translocation in simian immunodeficiency virus (SIV)-infected rhesus monkeys (Macaca mulatta). J Med Primatol 39: 243-251.

45. Gibbings D, Befus AD (2009) CD4 and CD8: an inside-out coreceptor model for innate immune cells. J Leukoc Biol 86: 251-259.

46. Ling B, Veazey RS, Hart M, Lackner AA, Kuroda M, et al. (2007) Early restoration of mucosal CD4 memory CCR5 T cells in the gut of SIV-infected rhesus predicts long term non-progression. AIDS 21: 2377-2385.

47. Mehandru S, Poles MA, Tenner-Racz K, Jean-Pierre P, Manuelli V, et al. (2006) Lack of mucosal immune reconstitution during prolonged treatment of acute and early HIV-1 infection. PLoS Med 3: e484.

48. Dinoso JB, Rabi SA, Blankson JN, Gama L, Mankowski JL, et al. (2009) A simian immunodeficiency virus-infected macaque model to study viral reservoirs that persist during highly active antiretroviral therapy. J Virol 83: 9247-9257.

49. Lay MD, Petravic J, Gordon SN, Engram J, Silvestri G, et al. (2009) Is the gut the major source of virus in early simian immunodeficiency virus infection? J Virol 83: 7517-7523.

50. Fuller DH, Rajakumar PA, Wilson LA, Trichel AM, Fuller JT, et al. (2002) Induction of mucosal protection against primary, heterologous simian immunodeficiency virus by a DNA vaccine. J Virol 76: 3309-3317.

51. Valdez H, Lederman MM (1997) Cytokines and cytokine therapies in HIV infection. AIDS Clin Rev: 187-228.

52. Aukrust P, Liabakk NB, Muller F, Lien E, Espevik T, et al. (1994) Serum levels of tumor necrosis factor-alpha (TNF alpha) and soluble TNF receptors in human immunodeficiency virus type 1 infection--correlations to clinical, immunologic, and virologic parameters. J Infect Dis 169: 420-424.

53. Godfried MH, van der Poll T, Weverling GJ, Mulder JW, Jansen J, et al. (1994) Soluble receptors for tumor necrosis factor as predictors of progression to AIDS in asymptomatic human immunodeficiency virus type 1 infection. J Infect Dis 169: 739-745.

54. Zara F, Nappi RE, Brerra R, Migliavacca R, Maserati R, et al. (2004) Markers of local immunity in cervico-vaginal secretions of HIV infected women: implications for HIV shedding. Sex Transm Infect 80: 108-112.

55. Giron-Gonzalez JA, Lopez-Sanchez A, Elvira J, Perez E, Fernandez-Gutierrez C (2000) Effect of patient adherence to antiretroviral therapy on CD4+ cell count, HIV-1 RNA, and serum concentrations of tumor necrosis factor and its soluble receptors. Eur J Clin Microbiol Infect Dis 19: 852-858.

56. Breen EC, Rezai AR, Nakajima K, Beall GN, Mitsuyasu RT, et al. (1990) Infection with HIV is associated with elevated IL-6 levels and production. J Immunol 144: 480-484.

57. Cervia JS, Chantry CJ, Hughes MD, Alvero C, Meyer WA, 3rd, et al. (2010) Associations of proinflammatory cytokine levels with lipid profiles, growth, and body composition in HIV-infected children initiating or changing antiretroviral therapy. Pediatr Infect Dis J 29: 1118-1122.

58. Kawai T, Akira S (2006) TLR signaling. Cell Death Differ 13: 816-825.

59. Gibson DL, Ma C, Bergstrom KS, Huang JT, Man C, et al. (2008) MyD88 signalling plays a critical role in host defence by controlling pathogen burden and promoting epithelial cell homeostasis during Citrobacter rodentium-induced colitis. Cell Microbiol 10: 618-631.

60. Lathey JL, Kanangat S, Rouse BT (1994) Differential expression of tumor necrosis factor alpha and interleukin 1 beta compared with interleukin 6 in monocytes from human immunodeficiency virus-positive individuals measured by polymerase chain reaction. J Acquir Immune Defic Syndr 7: 109-115.

61. Reddy MM, Grieco MH (1989) Neopterin and alpha and beta interleukin-1 levels in sera of patients with human immunodeficiency virus infection. J Clin Microbiol 27: 1919-1923.

62. Alonso K, Pontiggia P, Medenica R, Rizzo S (1997) Cytokine patterns in adults with AIDS. Immunol Invest 26: 341-350.

63. Berman MA, Sandborg CI, Calabia BS, Andrews BS, Friou GJ (1987) Interleukin 1 inhibitor masks high interleukin 1 production in acquired immunodeficiency syndrome (AIDS). Clin Immunol Immunopathol 42: 133-140.

64. He L, Terunuma H, Hanabusa H, Iwamoto A, Oka S, et al. (2000) Interleukin 18 and interleukin 1beta production is decreased in HIV type 1-seropositive hemophiliacs but not in HIV type 1-seropositive nonhemophiliacs. AIDS Res Hum Retroviruses 16: 345-353.

65. van Marle G, Church DL, Nunweiler KD, Cannon K, Wainberg MA, et al. (2010) Higher levels of Zidovudine resistant HIV in the colon compared to blood and other gastrointestinal compartments in HIV infection. Retrovirology 7: 74.

66. Duerr A (2010) Update on mucosal HIV vaccine vectors. Curr Opin HIV AIDS 5: 397-403.

67. Estes JD, Harris LD, Klatt NR, Tabb B, Pittaluga S, et al. (2010) Damaged intestinal epithelial integrity linked to microbial translocation in pathogenic simian immunodeficiency virus infections. PLoS Pathog 6.

68. Al Anazi AR (2009) Gastrointestinal opportunistic infections in human immunodeficiency virus disease. Saudi J Gastroenterol 15: 95-99.

69. Huppmann AR, Orenstein JM (2010) Opportunistic disorders of the gastrointestinal tract in the age of highly active antiretroviral therapy. Hum Pathol 41: 1777-1787.