

**EFFECTS OF SIV INFECTION ON LYMPHOID CHEMOKINE CXCL13,
PANETH CELL PHENOTYPE AND INTESTINAL ANTIMICROBIAL PEPTIDE
EXPRESSION**

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ABSTRACT

To date, the complex nature of the host's response to the human immunodeficiency virus still intensely studied. Amongst the contributing factors to the host responses are chemokines. Attention to the role of homeostatic chemokines in HIV-1 infection has been concentrated on changes in their constitutive expression. There is evidence that demonstrates the expression levels of CXCL9 and CXCL13 are increased during infection. CXCL13 is a homeostatic, constitutively expressed chemokine responsible for the migration of lymphocytes into the germinal centers of lymphoid tissues. I examined the levels and locations of CXCL13, amongst other homeostatic chemokines, and local cell populations by detailed in situ methods in macaque lymphoid and intestinal tissues. My results indicate that there were distinct localization patterns of CXCL13 mRNA compared to protein in germinal centers. These patterns shifted during the course of SIV infection, with increased mRNA expression within and around follicles during AIDS compared to uninfected or acutely infected animals. Unexpectedly, CXCL13 expression was also found in abundance in Paneth cells in crypts throughout the small intestine. In the gastrointestinal tract, Paneth cells produce antimicrobial peptides as an innate immune mechanism to protect the epithelium from pathogenic infection. Therefore, I expanded my analyses to include Paneth cells, and chemokines and antimicrobial peptides not previously demonstrated to be expressed by Paneth cells in intestinal tissues. I examined the expression

patterns of multiple chemokines including CCL25, as well as antimicrobial peptides including α -defensins 6, β -defensin 2, rhesus θ -defensin 1, and Reg3 γ . Additionally, I evaluated other Paneth cell-associated factors in situ in cynomolgous macaque intestinal tissues. Results showed that no other chemokines were expressed by Paneth cells, making CXCL13 unique among the group. DEFA1, BDEF2, RTD-1, and Reg3 γ expression was also clearly in Paneth cells and localized to the same cellular compartment as CXCL13 and DEFA6. These peptides have not been shown previously to be expressed by Paneth cells. I also found that during SIV infection there are differences in the expression levels of several antimicrobial peptides. These findings are of public health importance because they expand our understanding of mucosal immunology, innate antimicrobial defenses, homeostatic chemokine function, and host protective mechanisms against microbial translocation.

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PREFACE

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I dedicate this dissertation to my daughter, Olivia. This body of work is yours to have as proof that you can do anything you put your mind to. In your life, may you dream big and reach for the stars...

1.0 INTRODUCTION

Chemokines are critical contributors to immune function and development. They are small, secreted proteins that mediate the trafficking of cells during normal and inflammatory conditions. The functional activities of chemokines are diverse. They play critical roles in the migration of immune cells as part of the homeostatic balance of the immune system and in drawing the appropriate responding cell populations to sites of inflammation. Chemokines are also widely known to possess apoptotic, anti-apoptotic and/or chemorepulsive properties as well as antimicrobial activity against bacterial and viral pathogens. Manipulating the chemokine/chemokine receptor network could be a reasonable target for prevention or treatment of pathogenic infections. This very idea has been illustrated and highly instrumental in treating HIV-1 infection using a strategy that is not directly antiretroviral. To gain entry into a macrophage or T cell, HIV-1 requires the usage of two receptors: CD4 and either CCR5 or CXCR4. CCR5 is the primary coreceptor used during HIV transmission and infection. Maraviroc, a CCR5 chemokine receptor antagonist that prevents this viral entry, is the first of its kind to be approved by the Federal Drug Administration (FDA) for treatment of R5-tropic viral infection in humans. This exemplifies the value in studying chemokine and chemokine receptor expression and function during infections, which is the focus of this dissertation.

1.1 CHEMOKINES

Chemokines are small proteins that signal through seven transmembrane domain G protein coupled receptors (GPCRs).¹ They are chemotactic cytokines that direct the trafficking of leukocytes through the blood, tissues and lymphatics. Chemokines are classified based on their structure and the configuration of the cysteine residues at their N-termini. As such, there are approximately 50 C, CC, CXC, and CX3C chemokines. Although they are commonly known for their migratory function, chemokines have additional functions. A few of these functions include protection from apoptosis, angiogenesis, anti-angiogenesis, T cell polarization, and DC licensing and each of these mechanisms is suspected to be modified during SIV infection.²

Chemokines can be grouped as either inflammatory or homeostatic. Inflammatory chemokines have been described as such because of their ability to draw leukocytes to sites of inflammation. These chemokines are produced under pathological conditions by infiltrating or endogenous cells to the affected tissue. Alternatively, there are also chemokines involved in normal housekeeping functions, including the maturation of leukocytes in the bone marrow and the regeneration of circulating leukocytes.³ These chemokines are homeostatic chemokines. They are constitutively expressed and they maintain the normal trafficking of immune cells throughout all tissues in the body.⁴ Amongst the group of homeostatic chemokines are CCL19, CCL21, CCL25, CCL28, CXCL12, and CXCL13, which direct constitutive trafficking to lymph nodes (LNs), gut-associated lymphoid tissues (GALT), and germinal centers, respectively.

Chemokines bind to a superfamily of seven-transmembrane domain GPCRs. CXC-chemokines bind to their corresponding CXC-receptors, whereas CC-chemokines bind to CC-receptors. There are approximately 20 chemokine receptors that have been identified.¹ When a

chemokine binds to its cell receptor signal transduction occurs and cells migrate up a chemokine gradient. The chemokine receptor then undergoes internalization ensuing in a cell refractory period.¹

A common function of chemokines and chemokine receptors is control of migration of cells to sites of infection and inflammation. Tissue damage and inflammatory diseases result when there is excessive recruitment of cells by chemokines and their receptors. Examples of this have been demonstrated in individuals with chronic obstruction pulmonary disease (COPD) and multiple sclerosis (MS).^{5,6} AIDS is another disease where chronic inflammation is mediated by chemokines and their receptors.⁷

1.2 HIV/SIV/AIDS

HIV/AIDS is one of the largest pandemics to impinge on man, affecting over 60 million people worldwide.⁸ This global public health issue recently reached its 30th anniversary and despite tremendous advancements and effort by the scientific community in understanding the virus and consequences of infection, disease eradication and prevention, the virus maintains the upper hand in this battle. According to the 2012 UNAIDS report, globally, there are 34.2 million people living with HIV-1. In 2011, there were 2.5 million people newly infected with HIV-1. This amounts to over 7,000 new cases of HIV-1 infection per day. In 2011, 1.7 million people suffered AIDS-related deaths, a reduction from the 2.3 million deaths reported in 2005 (UNAIDS), but nevertheless the epidemic continues.

Acquired immunodeficiency syndrome (AIDS) was first described in 1981.⁹ It was two years before human immunodeficiency virus (HIV-1), was isolated as the etiological agent of

this disease.¹⁰ Originally termed lymphadenopathy-associated virus (LAV)¹⁰, human T lymphotropic virus (HTLV)-III,¹¹ or AIDS-associated retrovirus (ARV)¹² individuals infected with HIV-1 suffer from suppression of their immune system that renders the body vulnerable to opportunistic infections and multiple forms of cancer.¹³ AIDS is a slow progressing disease that is spread through sexual contact, through contaminated blood and blood products, and by mother-to-child transmission.¹³

HIV-1 is a lentivirus and a member of the Retroviridae family of viruses. It primarily infects CD4+ T cells and macrophages, gaining entry to a cell via fusion to a target cell by binding of the viral envelope glycoprotein gp120 to the CD4 target cell surface receptor. Though the process of binding to CD4 is essential for viral entry, the virus must also bind to a co-receptor to gain full entry to a target cell. Chemokine receptors CXCR4 and CCR5 are the required co-receptors needed by the virus. gp120 on the viral envelope binds to CD4 on the target cell and leads to a conformational change that exposes the structural elements on the V3 loop of gp120. The V3 loop and the co-receptor CCR5 or CXCR4 on the cell surface interact and result in a second conformational change allowing viral glycoprotein, gp41, to be revealed. gp41 then binds with the CD4+ cell membrane, leading to fusion of the membranes, and ultimately allowing the viral core access into the CD4+ cell cytoplasm.¹⁴⁻¹⁶ Expression levels of CXCR4 and CCR5 have been evaluated in HIV-1 CD4+ cells and it has been shown that advanced disease is associated with lower expression of CXCR4 and higher levels of CCR5 on those cells.¹⁷

Cross-species transmission of SIV is the major source of pathogenic infections in humans.¹⁸ HIV-1 and HIV-2 are two types of human AIDS viruses. HIV-1 is responsible for the global AIDS pandemic, and HIV-2 causes AIDS in regions of West Africa. These two types of

human AIDS viruses are distinguished from one another based on their evolutionary relationship with other primate lentiviruses. HIV-1 originated from the simian immunodeficiency virus (SIV) found in African chimpanzees and gorillas. More specifically, HIV-1 was confirmed by sequence and phylogenetic analysis to be a result of cross-species transmission of SIVs from chimpanzees (SIVcpz).¹⁸⁻²⁰ HIV-1 consists of three groups: M, N, and O. Group M makes up the largest group with 11 clades denoted subtypes A through K. The majority of HIV-1 group M viruses arose from one cross-species event. HIV-1 and SIVcpz were first linked as originating from the same viral lineage based on the presence of the *vpu* gene in their genomic structure.^{19,21} The roots of HIV-1 have been traced to the western region of Africa that includes Gabon, Equatorial Guinea, Cameroon, and the Republic of Congo. In this region HIV-1 subtypes M, N, and O coexist within human populations where *Pan troglodytes troglodytes* chimpanzees infected with the closely related SIVcpz also reside.^{19,20,22} HIV-2 consists of six phylogenetic subtypes termed A through F.¹⁸ HIV-2 arose from at least eight cross-species events by SIV from sooty mangabeys (SIVsm). Both viruses code for the accessory protein VPX encoded in their genomic structure and are within the same phylogenetic lineage.^{23,24}

There are multiple and complex events that define the course of HIV-1 and progression to AIDS. These include the acute phase, partial immune restoration, clinical latency, and AIDS. The acute phase of infection is characterized by HIV-1 infection of a target cell, which quickly spreads amongst the CD4+ T cell population, and leads to the onset of generalized chronic immune activation. During this time polyclonal B-cell activation occurs, along with increases in the activated phenotypes of T-cells, and increased production of pro-inflammatory cytokines and chemokines.^{25,26} The blood contains high levels of viremia. Viral integration into the host cell DNA occurs and allows the virus to evade the host immune system. The virus then lies dormant

for an extended period of time without being expressed. Two to four weeks post-infection CD8+ T cells mount an immune response that lowers viral replication and restores CD4+ T cell levels. Subsequently, the virus undergoes a latency phase, during which the host remains symptom-free for several years despite ongoing viral replication. AIDS occurs when the host's overly stimulated immune system is no longer capable of combating viral replication and becomes susceptible to opportunistic infections. An individual is clinically defined with AIDS once their CD4+ T cell count is below 200 CD4+ T cells per cubic millimeters of blood and they are diagnosed with one or more opportunistic infections.²⁷

Despite isolation of HIV in 1983, it was approximately two more years until the first form of treatment was developed. Azidothymidine (AZT) was the first commercially available anti HIV-1 drug approved for the treatment of patients with advanced disease. AZT, a dideoxynucleoside reverse transcriptase inhibitor (RTI)²⁸ acts directly to block reverse transcription, the copying of the viral RNA genome to DNA. Successive anti HIV-1 therapeutics included non-nucleoside RT inhibitors (NRTI), protease inhibitors (PI), fusion/entry inhibitors, integrase inhibitors, maturation inhibitors, and highly active antiretroviral treatment (HAART), which is the combination of two or more antiretroviral drugs. The use of HAART delays disease progression and slows viral replication, therefore, reducing viral loads.²⁹

Compared to antiretroviral therapy, the development of an HIV-1 vaccine has proven much more difficult for HIV/AIDS researchers. Vaccine studies have concentrated on the development of two types of immune responses for ideal prophylactic vaccine candidates: 1) a humoral or B cell response that can generate neutralizing antibodies to inhibit viral entry; or 2) a T-cell based vaccine that stimulates a specific T-cell responses to curb viral propagation. The very nature of the virus has presented many obstacles in vaccine development.

AIDS research and vaccine trials rely on the use of animal models that emulate HIV-1 infection in humans. Mimicking HIV-1 infection routes in humans has been difficult without a model system that completely recapitulates the process that occurs in humans. HIV-1 developed from cross-species transmission of SIVcpz from chimpanzees and gorilla. However, the use of chimpanzees as an animal model for human infection has not proven to be the best model given these animals do not show disease development in captivity.^{30,31}

In contrast, there are other non-human primates that provide useful models to study HIV/SIV pathogenesis. These NHPs harbor similar cell types that are susceptible to viral infection, suffer from the severe depletion of CD4+ T cells in the GALT and are vulnerable to opportunistic infections.³² In addition, they exhibit virological and immunological characteristics of acute infection that are very similar to those observed in humans.³³ Asian macaques are non-natural hosts of SIV and develop disease similarly to HIV-1 in humans although at a rapid rate. Disease progression to AIDS occurs within 1-2 years compared to 8-10 years in humans. The three most-commonly used macaque species for HIV-1 animal studies are the rhesus macaque (*Macaca mulatta*), the pig-tailed macaque (*Macaca nemestrina*) and the cynomolgus macaque (*Macaca fascicularis*). These macaques differ in their usage in HIV studies due to the lack of availability of some Indian and Chinese species and in the severity of the pathogenesis of the infected viral strain.

HIV-2 developed from cross-species transmission of SIVsmm from sooty mangabeys.²³ Sooty mangabeys and African green monkeys are natural SIV hosts that are used as a model of non-pathogenic SIV. These animals do not develop disease and serve for comparison between non-pathogenic and pathogenic SIV infection. In these natural hosts they exhibit induction of viral replication, severe depletion of mucosal CD4+ T cells and the activation of innate and

adaptive immunity.³⁴ In contrast from non-natural SIV hosts, the viruses circulated among these animals do not cause chronic immune activation, depletion of mucosal or peripheral CD4+ T cells, or destruction of lymph node architecture.^{32,34}

1.3 CHEMOKINE RESPONSES TO HIV/SIV INFECTION

In the late 90's investigators deemed the chemokine and chemokine receptor network as essential to understanding AIDS.³⁵ Today, the roles chemokines play in SIV pathogenesis are still extensively studied. The chemokines CCL3, CCL4, and CCL5, also known as MIP-1 α , MIP-1 β , and RANTES respectively, are considered potential suppressive factors for HIV-1 and SIV.³⁶ Cocchi *et al.*, first provided evidence that CD8+ T cell production of these chemokines suppressed HIV-1 activity. Subsequently, it was determined that the receptors for these chemokines served as viral entry co-receptors for HIV-1, HIV-2 and SIV.³⁷⁻⁴⁰ Initial studies showed that the cellular determinants of viral entry were not only the presence of CD4, but also a fusion cofactor on the CD4+ target cell. CXCR4, also known as fusin, and CCR5, originally termed CC CKR5 (CC chemokine receptor 5), were the primary cofactors found to permit HIV-1 infection.³⁷⁻³⁹ HIV-1 isolates that preferentially infect CD4+ T cell with CXCR4 on their surface are termed T-tropic or X4 viruses. For viral entry to occur, HIV-1 adheres to a target cell by gp120 interacting with both CD4 and CXCR4. This induces conformational changes in the gp120/gp41 complex that allow membrane fusion by gp41.

Unlike the specific expression of CXCR4 on T cells, CCR5 expression is found mostly on macrophages with some expression by CD4+ T cells. HIV-1 isolates that infect CCR5+ cells are termed M-tropic or R5 viruses.³⁸ In cases of R5 viral infection, HIV-1 gp120 binding to

CCR5 is CD4-dependent. Antibody inhibition of CCR5 provides reduction of binding of HIV gp120 to CCR5 by 87%.⁴¹ There are individuals who possess a 32 nucleotide deletion (Δ 32) homozygous genotype that results in the absence of surface-expressed CCR5, rendering them resistant to HIV-1 infection.^{39,42-44} The Δ 32 mutation does not result in any evident effects detrimental to the host. Reduction in cell surface expression of CCR5 in individuals that contain the Δ 32 mutation is associated with a slower rate of disease progression after HIV-1 infection.^{45,46} In 1998, Moriuchi *et al.*, provided evidence that showed upregulation of CXCR4 expression on macrophages allows infection by X4 viruses.⁴⁷ Over the course of infection, coreceptor usage transfers from predominantly CCR5 over to CXCR4 usage. Approximately, 50% of individuals infected with HIV-1 display a switch to CXCR4 preference upon disease progression and the decline in CD4+ T cell levels.^{35,46,48} This coreceptor shift is caused by mutations that occur in the viral life cycle and allow the virus to infect a larger population of cells.

The use of anti-retrovirals, such as AZT or HAART, in combating HIV infection can be efficacious at inhibiting disease progression. However, adverse side effects of the drugs can result in poor adherence by HIV-1 infected patients. The development of novel antiretrovirals has been driven largely by the emergence of drug resistant viral strains but also by efforts to reduce toxicity. Given that the Δ 32 CCR5 mutation is not harmful to the immune system, CCR5 is a prime candidate for drug and vaccine approaches. Maraviroc was the first early entry inhibitor approved by the FDA. It acts by preventing binding of HIV-1 gp120 to CCR5 and inhibiting the conformational changes necessary for viral entry into target cells.⁴⁹

There have been efforts to examine modifications to host chemokine gene expression after SIV infection. A study has identified changes in CXCL9 expression during SIV infection.⁷

They showed upregulation of CXCL9 in rhesus macaque lymphoid tissues during SIV infection and developed a basic model that proposed a possible mechanism in which IFN- γ -inducible chemokines are upregulated, causing a positive-feedback loop that could serve as a source chronic inflammation during SIV infection.⁷ They found that CXCL13 was among the genes that were upregulated in spleen tissues during SIV infection. Another interesting aspect about this chemokine is that it has also been described as an agonist for the CXCR3 chemokine receptor.⁵⁰ It has been described how during infection there is an induction of IFN- γ that results in an increase in CXCR3 ligand secretion which then causes an increase in recruitment of CXCR3+ T-lymphocytes.⁷ This could signify that CXCL13 could contribute to the positive-feedback loops driven by IFN- γ .

1.4 CXCL13

CXCL13 is a member of the homeostatic functional group of chemokines and signals through its receptor CXCR5. CXCL13 was originally termed B-lymphocyte chemoattractant (BLC) and was found in the germinal centers (GC) of lymphoid follicles in the lymph nodes, spleen and Peyer's patches.^{51,52} Subsequently, it was also detected on high endothelial venules (HEV) of the secondary lymphoid organs.⁵³ CXCL13 is secreted by stromal cells located in the follicles of lymphoid tissues, although other cells might also produce it. CXCL13 drives migration of B cells, follicular helper T cells (Tfh), and a subset of dendritic cells (DCs) to the follicles of lymphoid organ.⁵¹

CXCL13 also has been implicated in the formation of ectopic lymphoid tissue in chronic inflammation. Examples of this can be seen in cases of multiple sclerosis (MS) and rheumatoid

arthritis (RA).^{54,55} In MS, CXCL13 is expressed in active MS lesions.⁵⁴ MS patients show induced expression of this chemokine in cerebral spinal fluid (CSF) and these levels correlate with the number of B cells present in the CSF of patients with clinically isolated syndrome (CIS) and relapsing-remitting MS (RRMS).^{54,56} Of late, CXCL13 is suggested to be a prognostic marker for MS, and increased concentrations of CXCL13 in CSF are positively correlated with increased MS disease activity.⁵⁷

CXCL13 in CSF also can be found in other infections and inflammatory conditions of the central nervous system (CNS).^{58,59} It has been observed at high levels in Lyme neuroborreliosis⁶⁰ (NB) and is a key factor in Lyme disease of skeletal muscle in NHPs.⁶¹ In humans, elevated levels of CXCL13 are found in the CSF of European patients with Lyme NB.⁵⁸ Multiple studies have evaluated and confirmed the induction of CXCL13 during Lyme disease and have proposed CXCL13 as both a diagnostic and therapy-response marker in Lyme NB.⁶²⁻⁶⁴

Helicobacter pylori is a Gram(-) bacterium that resides in the upper GI tract and has been linked to cases of gastritis and gastric ulcers. Chronic inflammation is one of the pathophysiologic characteristics of this disease and includes the formation of mucosa-associated lymphoid tissue (MALT).^{65,66} CXCL13 has been implicated as the driving force in the development of lymphoid aggregates and gastric lymphomas in a normally MALT-deficient region of the GI tract.⁶⁷ In a similar fashion, in 2002, Carlsen *et al.* examined a possible role for CXCL13 and CXCR5 in normal gut associated lymphoid tissues (GALT) by comparing its expression in normal and diseased colonic tissues.⁶⁸ These authors described the secretion of CXCL13 by aberrant lymphoid aggregates in the colonic tissue of patients with ulcerative colitis

.⁶⁸

Although this homeostatic chemokine is predominantly involved in chemotaxis of lymphocytes, it also has antimicrobial properties. In 2002, Yang *et al.* found that CXCL13 was among a group of chemokines, including CCL20, with the ability to kill microbes.⁶⁹ In an antimicrobial assay, CXCL13 showed antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. In this manner, CXCL13 is acting similarly to defensins.⁷⁰ Defensins are cationic antimicrobial peptides (AMPs) that are secreted by polymorphonuclear leukocytes and other epithelial cells, such as Paneth cells, in response to bacterial, fungal and viral pathogens.⁷¹ The functions of defensins also seem to overlap the functions chemokines as at least some of them are both chemotactic for CD4, CD8 and DCs and they stimulate degranulation of leukocytes.⁷² The structures of chemokines and defensins are also similar in that they both contain highly conserved cysteine residues. Defensins are produced and secreted by many cell types. One specialized cell that is unique to the small intestine and is a major source for intestinal defensins is the Paneth cell.

1.5 CXCL13 & HIV

Few studies have evaluated changes in CXCL13 expression and function during HIV-1 or SIV infection. In 2005, Widney *et al.* evaluated serum samples from HIV-1⁺ subjects who were healthy or in advanced disease and compared them to healthy uninfected control subjects and they determined that the mean serum CXCL13 levels are three-fold higher in those individuals that are infected with HIV-1 and the levels of CXCL13 correlated with levels of CXCL10 in advanced disease.⁷³ They showed a 21% decrease in serum levels of CXCL13 after a year of

HAART. In a follow-up study, Regidor *et al.* reevaluated the effect of highly active antiretroviral therapy (HAART) on serum levels of molecules associated with immune activation and inflammation.⁷⁴ It was shown that CXCL13 levels decrease by 40% two to three years after HAART initiation. They also established a correlation between baseline CD4 and CXCL13 levels. Higher levels of CD4⁺ T cells resulted in greater decrease of CXCL13 after HAART. Similarly, Cagigi *et al.* (2008) identified altered CXCL13 production by B cells that are increasingly responding to CXCL13 in HIV-1 infected patients.⁷⁵ They also found CXCL13 protein expression in LNs of HIV-1 infected individuals located within the GC light zone and they showed CXCL13 protein expression in a pattern that resembles the follicular DC network.⁷⁵ Much of the work on CXCL13 and HIV-1 is based on evaluating a possible role for CXCL13 in B-cell abnormalities and dysfunction during HIV-1 infection, which are additional hallmarks of HIV-1 infection. There are significant B cell abnormalities marked by polyclonal activation, hypergammaglobulinemia, and circulating immune complexes.^{13,76} Given that CXCL13 is responsible for trafficking of B cells into the marginal zones of follicles, it is possible that modifications to its expression might contribute to the B cell dysfunction occurring in HIV-1 infection.

1.6 PANETH CELLS AND ANTIMICROBIAL PEPTIDES

The bacterial load of the small intestine is minimal but increases greatly in the colon.⁷⁷ Among the factors that keep the levels of bacteria in the small intestine low, are the antimicrobial constituents that are expressed and secreted by Paneth cells. Paneth cells are specialized epithelial cells located in the crypts of Lieberkühn that are present throughout the small intestine

(Figure 1 and Figure 2) and these cells increase in number as the concentration of bacteria increases to the terminal ileum.⁷⁷

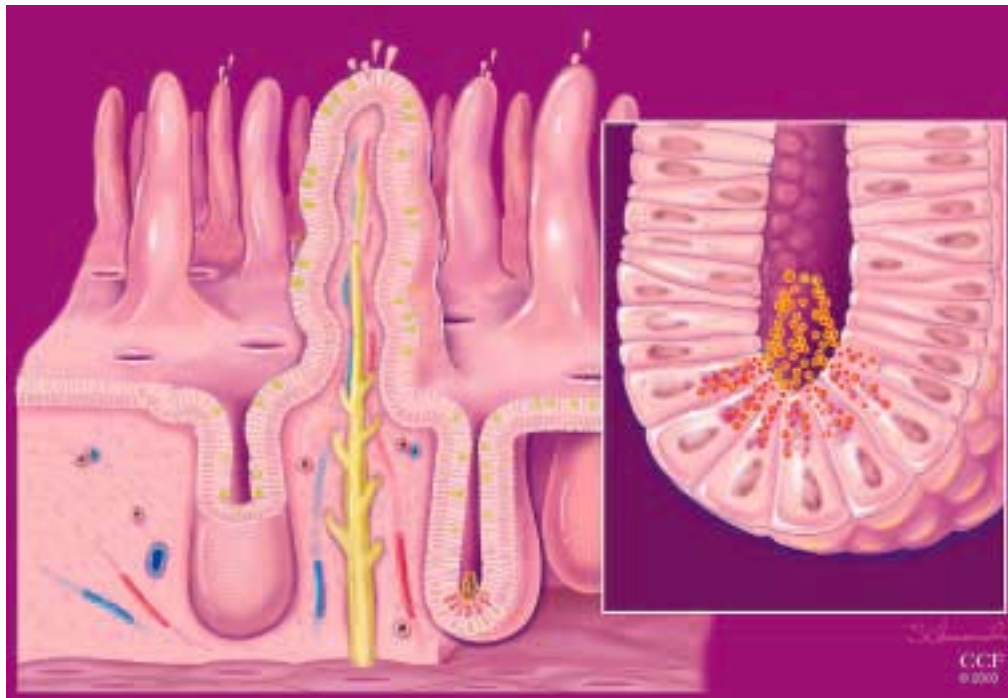


Figure 1. Schematic diagram of the small intestinal villus-crypt architecture.

Intestinal stem cells reside at the neck of the crypt. Cells that migrate toward the crypt base differentiate into Paneth cells. Inset: Paneth cells release their secretory vesicles into the narrow crypt lumen. (Used with permission, from Cellular and Molecular Life Sciences)⁷⁸

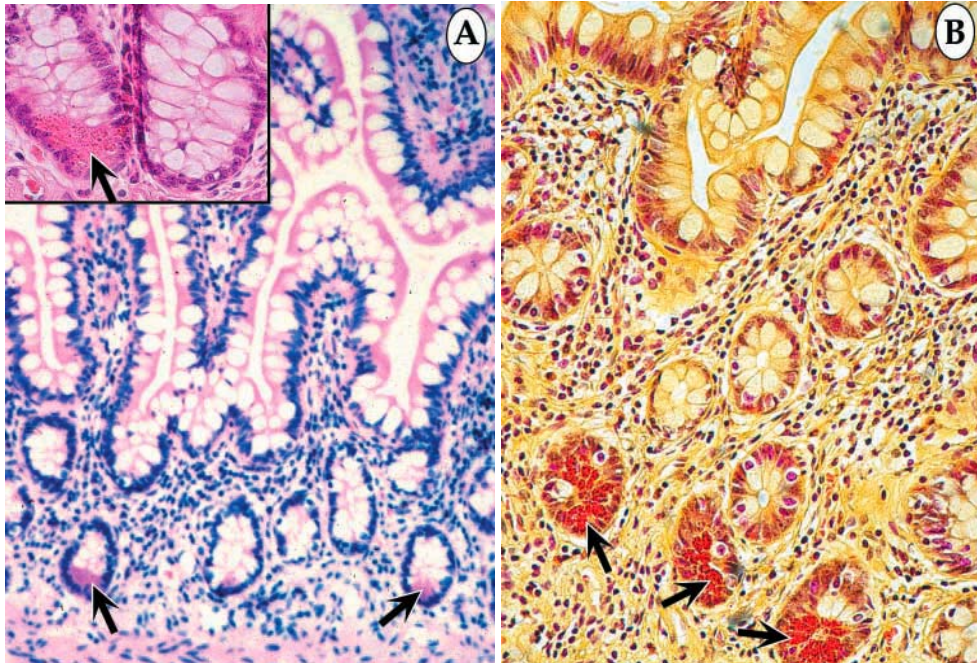


Figure 2. Histology of the human small intestinal crypts.

A. Low-power view of paraffin-embedded section of normal adult ileum stained with hematoxylin and eosin. Arrows denote Paneth cells at the base of the crypts. (Inset: Higher-power view of Paneth cells.) Arrow denotes Paneth cell secretory granules. B. Paraffin-embedded section of adult ileum stained with phloxine-tartrazine. Arrows denote Paneth cells. (Used with permission, from Cellular and Molecular Life Sciences)⁷⁸

Although most of what is secreted by Paneth cells is lysozyme,^{77,79} defensins are also generated and released by these specialized intestinal epithelial cells. These defensins are secreted by and are contained within the secretory granules of Paneth cells.^{77,80,81} Paneth cells also express other antimicrobial agents that aid in host defense.^{77,81-86} These include secretory phospholipase A₂ (sPLA₂), RegIII γ , and tumor necrosis factor (TNF)- α . Antimicrobial peptides (AMPs) secreted by Paneth cells have several physiologically overlapping roles in the intestine.⁷⁷ Those functions include shaping the composition of and limiting the number of commensals populating the small intestine, protecting the intestine and its stem cells from invading pathogens, and acting as paracrine signaling molecules.^{77,85} Ayabe *et al.* have described Paneth cells as responsible for the homeostatic environment of the intestinal villi and crypts by regulating microbe infiltration.⁸² Paneth cells express sensors that trigger responses that protect

the epithelium of the small intestine. These sensors include toll-like receptors (TLRs) and nucleotide-binding domain leucine-rich repeat containing receptors (NLRs), (e.g., NOD2). TLR9 acts by sensing CpG DNA around Paneth cells and stimulating degranulation.⁸⁷ NOD2 senses bacterial peptidoglycan muramyl dipeptide (MDP) around Paneth cells. A deficiency in the *nod2* gene is associated with decreased α -defensin expression and antimicrobial activity.⁸⁸

These pattern recognition receptors (PRRs) in concert with AMPs assist in preventing bacterial translocation by protecting intestinal epithelial cells (IECs), thereby maintaining the gut epithelial barrier and intestinal homeostasis.⁸⁹ IECs and Paneth cells secrete several types of AMPs. As previously mentioned, those include defensins, cathelicidins and other larger AMP molecules.

Defensins are small, cationic, amphipathic peptides. These proteins contain a disulfide bond structure that allows them to protect IECs from invasion by creating pores in the bacterial cell membrane. Similar to chemokines, they contain conserved regions that are rich in cysteine residues. There are three groups of defensins: α , β , and θ -defensins.^{90,91} α -defensins are released by epithelial cells and Paneth cells, as well as, neutrophils and macrophages in humans, non-human primates and rodents.⁷⁷ Humans and non-human primates (NHPs) contain between five and six α -defensins (HNP 1-4 and HD-5 & HD-6 in humans; RED1-6 in NHPs) (refs). HD-5 and HD-6 are processed into their pro-peptide form by trypsin once they are secreted by Paneth cells, whereas HNP1-4 are processed by matrix metalloproteinase 7 (MMP-7) just as cryptidins are processed in mice.⁹² β -defensins (hBDs) are secreted by epithelial cells, including enterocytes and keratinocytes, in all mammalian species. hBD1 is the only β -defensin known to be constitutively expressed in the gut, whereas hBD2, hBD3, and hBD 4 are stimulated upon pathogen recognition.⁹³⁻⁹⁵ θ -defensins differ from α - and β -defensins in their structure.⁷⁷ They

have a cyclic configuration and are active in non-human primates.⁹⁶ In humans, the peptide signal sequence of the θ -defensin gene contains a premature stop codon that inhibits the protein from being produced.⁹⁷ Nonetheless, the repaired version (Retrocyclin) is being explored as a topical microbicide.⁹⁸

In general, defensins have antimicrobial activity at low micromolar concentrations, yet Paneth cells produce effective local concentrations that are minimally needed to be bactericidal.^{82,86} In 2004, Tanabe *et al.* localized the mRNAs of rhesus macaque paneth cell α -defensins, which they termed rhesus enteric α -defensins (REDs) in the small intestine. They found that these defensins had antibacterial activity against *E. coli*, *L. monocytogenes*, *S. aureus*, and *Vibrio cholera* at concentrations lower than 10 μ g/ml.

Antimicrobial peptides have also been shown to possess anti-viral properties. Several studies have investigated the potential for defensins to have anti-HIV capabilities. Wang *et al.* measured the binding capabilities of α and θ defensins to HIV-1. They found that retrocyclin-2 was able to bind to gp120 and CD4 and provided protection from HIV-1 infection *in vitro*.⁹⁹ They also provided evidence that human alpha defensins 1, 2, and 3 also bind to gp120. More recently, retrocyclin RC-101, has been investigated for its potential to provide protection against HIV-1 transmission. Gupta *et al.* provide evidence that RC-101 prevents X4 and R5 HIV-1 variant transmission in a cervical tissue-based organ culture model and is a strong candidate for development of an HIV-1 entry-inhibiting microbicide.⁹⁸ Another study investigating the impact of α defensins on HIV-1 disease progression found that immature DCs produce alpha defensins 1, 2, and 3 that could protect against progression of disease.¹⁰⁰

Another family of AMPs includes the cathelicidins. Cathelicidin structures include a conserved cathelin domain, signal peptide domain and a C-terminal antimicrobial domain that

varies among different species. LL-37 is a member of the cathelicidin group of AMPs, and is the only cathelicidin found in humans.¹⁰¹ LL-37 is known as human cationic antimicrobial protein (hCAP18) in its precursor form. This peptide is converted to its mature form by cleavage from a serine protease.¹⁰² LL-37 is expressed by epithelial cells, neutrophils, and macrophages, and can exert antibacterial, anti-fungal, and anti-viral activity.¹⁰³ Its bactericidal activity can be directed toward Gram(+) or Gram(-) bacteria.¹⁰⁴ LL-37 is another AMP that contains chemokine-like function. It is chemotactic for human peripheral monocytes, neutrophils, and CD4 T lymphocytes.⁸² In chemokine-like fashion, its expression can also be constitutive or induced. Stimulation of LL-37 expression is induced by bacterial byproducts such as butyrate,¹⁰⁵ vitamin D3,⁹⁹ pro-inflammatory cytokines, or injury. In contrast, bacterial infection by *Shigella* or *Neisseria* or bacterial exotoxins¹⁰⁶ can down-modulate LL-37 expression.^{106,107} Inhibition of LL-37 expression has been linked to virulence.¹⁰⁸

Ganguly *et al.* 2009, investigated the role LL-37 plays in inflammatory psoriasis. They and Chamilos *et al.*, 2012 showed that this cathelicidin forms complexes with extra-cellular self-RNA. By forming these complexes they are able to induce TLR7 and TLR8 activation and stimulate the production of IFN- α by pDCs and TNF- α and IL-6 by mDCs.^{109,110}

Regenerating islet-derived protein 3 gamma (Reg3 γ) is an antimicrobial lectin expressed by Paneth cells.⁸⁹ It was revealed in 2006 by Cash *et al.* to specifically target Gram(+) bacteria by binding to their peptidoglycan layer. Reg3 γ belongs to a family of secreted proteins that are made up of conserved sequence motifs in C-type lectin carbohydrate domains. Reg3 γ expression has been found largely in the small intestine,⁸⁹ but also in conducting airways.¹¹¹ Bacteria^{112,113} and mucosal damage¹¹⁴ cause inflammatory responses that increase gastrointestinal expression of mouse RegIII γ .¹¹⁵

There are other AMPs that act to eliminate pathogens in the small intestine, differing in their mechanism compared to defensins. Lipocalin 2 (Lcn2) expression is induced by IL-22¹¹⁶ and IL-17¹¹⁷ during inflammation. Lcn2 acts to inhibit bacterial growth by binding to iron sequestering siderophores.^{69,118} It acts bacteriostatically by binding to and inactivating enterochelin. It was shown that *lcn2* transcription is induced during infection by *Salmonella typhimurium* and is secreted into the intestinal lumen of rhesus macaques.¹¹⁹ Lcn2 secretion is also stimulated by TLRs on immune cells that sense bacteria.

Much in the way chemokines and AMPs have overlapping functions, Chemerin is a protein chemotactic for GPCR-chemokine like receptor 1 (CKLR1),¹²⁰ and also is suspected of displaying antimicrobial properties. The predicted structure of chemerin contains a conserved region of cysteine residues that mimics antibacterial cathelicidins, such as LL-37. Kulig *et al.* have recently shown how chemerin has antimicrobial activity toward *E. coli* and *K. pneumoniae*.¹²¹

Overall, there are several types of AMPs produced as a result of the innate immune defenses in the GI tract. Paneth cells are specialized epithelial cells that express multiple types of AMPs, including defensins and lysozyme, in response to microbes in the small intestine. In addition to defensins there are other categories of AMPs. Cathelicidins and lectins are examples of those AMPs. It has been established that chemokines and AMPs have overlapping antimicrobial and chemotactic functions.

1.7 THE INTESTINE AS A SITE OF VIROLOGIC AND IMMUNOLOGIC ACTIVITY DURING HIV/SIV INFECTION

The gastrointestinal tract is recognized as a site of dramatic T cell loss and immune activation during HIV-1 infection. The importance of the gut in HIV infection is evident with the amount of HIV replication and loss of mucosal CD4+ T cells^{115,122-125} but intestinal enteropathy was first seen in 1984 by Kotler *et al.* Intestinal enteropathy includes inflammation to the intestinal epithelial layer with villous atrophy, crypt hyperplasia, and villous blunting.¹²⁶⁻
¹²⁸ In 1993, it was reported that rhesus macaques with SIV infection also suffered from severe gastrointestinal dysfunction.^{126,129} They also were found to have chronic inflammation, crypt villus blunting, and crypt hyperplasia.^{127,129}

Damage to the epithelial barrier in the GI tract has been suspected to allow microbial translocation to occur, leading to chronic immune activation during infection.^{123,130,131} Microbial translocation constitutes the passage of pathogens across the mucosal layer of the GI tract. When bacterial endotoxin crosses this barrier, it can travel to the LNs and disseminate systemically throughout the body.¹³² In 2006, Brenchley and Douek reported that microbial translocation is a cause of systemic immune activation in chronic HIV-1 infection.¹³³ They used lipopolysaccharide (LPS) as an indicator for microbial translocation and compared levels of plasma LPS in controllers and progressors to uninfected rhesus macaques. More recently, Klatt *et al.* showed that there was increased microbial translocation in pigtailed macaques (PTMs), which correlated with increased immune activation and associated with increased levels of T cells generating IL-17, even in the absence of overt infections. They suggested that there is a relationship between microbial translocation and disease progression in these PTMs.¹³⁰

In 2006, Brenchley and Douek also compared the plasma LPS levels in SIV-infected natural hosts, sooty mangabeys, to uninfected sooty mangabeys and found similar low levels of immune activation.¹³³ They reasoned that the differences in LPS levels in natural hosts could provide a clue into their ability to maintain the epithelial barrier and their inability to develop pathogenic infection. African NHPs such as sooty mangabeys and African green monkeys are natural hosts of SIV and have been found to tolerate SIV infections. The levels of plasma viral RNA and the depletion of CD4+ T cells in the gut in non-pathogenic infection are similar to that of pathogenic SIV, yet these natural hosts do not progress to AIDS. There are several factors that have been suggested to contribute to this lack of disease progression: 1) natural hosts have higher numbers of CD8+ T cells than macaques; 2) there is less CD4+ cell apoptosis; 3) they lack T cell activation in blood, peripheral LNs and in the gut; 4) they also possess an anti-inflammatory immune activation profile whereas pathogenic infection leads to a pro-inflammatory state of immune activation; and 5) they express lower levels of CCR5 on CD4+ T cells in blood, LNs and mucosal tissues than non-natural SIV hosts.¹³⁴⁻¹³⁷

Chronic immune activation in pathogenic SIV infection is associated with progression to AIDS.¹³⁸⁻¹⁴¹ Microbial translocation has been identified as a contributor to immune activation induction. Several factors lead to the translocation of microbes from the lumen into the lamina propria including a reduction in the Th17 population.¹³⁹ Natural hosts of SIV infection serve an ideal model to determine SIV pathogenesis because they display many of the characteristics that non-natural hosts do, however, they do not progress to AIDS.

1.8 SUMMARY

Chemokines and their receptors have an established role in HIV and SIV infection with regards to viral entry into target host cells, yet there are functions these chemoattractant proteins possess that have not been fully defined. The HIV/AIDS epidemic began over three decades ago and through extensive research with both small and large animal models, the pieces to this complex viral infection are emerging. Chemokines and their receptors contribute to the movement of the very cells that the virus targets, yet it is these very proteins that can be engineered to fight the virus. A major anatomic landmark for HIV-1 infection is in the GI tract, a tissue compartment that innately expresses one of the body's best immune barriers enabling it to maintain an almost sterile environment for much of its lengths in the small intestine. Understanding how HIV-1 manipulates the chemokine network and how it counteracts such an extensive defense system is essential to developing therapeutics and vaccines against the virus.

2.0 STUDY PREMISE

2.1 RATIONALE

In 1983, the virus that causes AIDS was identified as human immunodeficiency virus (HIV-1).¹⁰ To date, the complex nature of the host's response to the virus is still intensely studied. Amongst the contributing factors to the host responses are chemokines. Studies of inflammatory chemokines have focused on their ability to draw cells to sites of inflammation, and attention to the role of homeostatic chemokines in HIV-1 infection has been concentrated on changes in their constitutive expression. The chemokines CCL3, CCL4, CCL5 and CXCL12 are the corresponding ligands for a small subset of chemokine receptors that permit HIV-1 infection of susceptible cells. CCR5 and CXCR4 are HIV-1 co-receptors that allow infection by macrophage and T cell-tropic viruses, respectively. There is evidence that demonstrates SIV infection is associated with modifications to the expression levels of CCR5.¹⁷ In addition, studies have also shown that the expression levels of CXCL9 and CXCL13 are increased in the spleens of AIDS-developing macaques.⁷ CXCL13 is a constitutively expressed chemokine responsible for the migration of lymphocytes into the germinal centers of lymphoid tissues. Constitutively expressed homeostatic chemokines have multiple functions in immunity. Chemokines exhibit several functions that overlap those of antimicrobial peptides. In the gastrointestinal tract, Paneth cells produce antimicrobial peptides as an innate immune

mechanism to protect the epithelium from pathogenic infection. Clarifying the functions of chemokines, and determining the effects HIV-1 infection has on chemokine and antimicrobial peptide expression has yet to be fully defined. *The overall hypothesis for these studies is that SIV infection leads to changes in the expression of lymphoid chemokine CXCL13 and antimicrobial peptides in lymphoid and intestinal tissues.*

2.2 SPECIFIC AIMS

Specific Aim 1: Measure the effects of SIV infection on the expression of CXCL13 and other homeostatic chemokines in multiple tissue compartments at different stages of infection. Chemokines play an important role in lymphocyte migration during immune responses. Homeostatic chemokines constitutively expressed during the non-inflammatory state but also can be induced during an infection. During an investigation into the expression levels of inflammatory chemokine, CXCL9, during HIV-1 infection, an induction of homeostatic chemokine CXCL13 was found in the spleen of AIDS-developing macaques. I hypothesized that during SIV infection there are an induction in the expression of homeostatic chemokine CXCL13 and its ability to draw responder cell populations. Histologic staining methods were used to visualize and measure patterns of expression and localization of major homeostatic chemokines (e.g., CXCL13 and CCL21) and local immune cell populations in multiple cynomolgous macaque lymphoid and intestinal tissues across disease stages. This allowed me to characterize the lymphoid and intestinal environments from SIV-infected and AIDS-developing macaques and identify changes in CXCL13 expression.

Specific Aim 2: Determine whether SIV infection induces changes to Paneth cell phenotype and Paneth cell chemokine expression in macaque small intestine. Chemokines have protective roles in the intestine beyond immune cell recruitment. These collective functions could potentially be important during diseases that cause gastrointestinal inflammation such as SIV infection. After discovering that Paneth cells express CXCL13, I sought to determine whether expression levels of factors expressed by Paneth cells were altered during SIV infection. I hypothesized that there is a modification to Paneth cell phenotype during SIV infection. To address this, I examined Paneth cell expression profiles of Paneth cell-associated factors, as well as, antimicrobial peptides and chemokines in tissues from healthy macaques and compared them to tissues from SIV-infected and AIDS developing macaques. Additionally, I examined factors such as Trypsin 2 that could potentially be involved in modulating antimicrobial peptide function in Paneth cells.

Specific Aim 3: Characterize the populations of antimicrobial peptides, including chemokines, produced by Paneth cells. I hypothesized that the repertoire of antimicrobial peptides expressed by Paneth cells is larger than previously described. It has been demonstrated that many chemokines can act as antimicrobial peptides. I have found in my initial studies that CXCL13 and other small intestinal antimicrobial peptides such as α -defensin 6 are expressed in the crypts of the small intestinal villi, but not by the large intestine. It is conceivable that other antimicrobial peptides are expressed in previously unidentified compartments in the small and large intestine during SIV infection. To address this, I examined the locations in which AMPs are expressed in macaque tissues and determine the effects of SIV infection on their expression via immunostaining methodologies and in situ hybridization.

Summary: Altogether, these studies assessed possible modifications to the patterns of expression of homeostatic chemokines and AMPs in multiple tissue compartments during SIV infection. They provide new insight into the expression of antimicrobial agents from Paneth cells. As such they have the potential to impact our understanding of mucosal immunology, homeostatic chemokine function, and host protective mechanisms against microbial translocation.

3.0 MATERIALS AND METHODS

3.1 ANIMALS AND TISSUE PROCESSING

These studies were performed under the approval and guidance of the University of Pittsburgh Institutional Animal Care and Use Committee. Adult cynomolgous macaques (*Macaca fascicularis*) were used in this study and were inoculated intrarectally with the pathogenic SIV/DeltaB670 isolate.¹⁴² These animals and their clinico-virologic states have been described previously¹⁴³ and are included here for immediate referral (Table 1).^{144,145} Fixation and processing of tissues at necropsy were completed as described and were placed in storage at -80°C for approximately 2-5 years prior to sectioning.¹⁴⁶ Frozen tissue sections were cut from fixed, cryopreserved tissues at 10µm, thaw-mounted on microscope slides (SuperFrost Plus; Fisher), and stored at -80°C until use.

Table 1: Study Animals and Clinico-Virologic States.

Animal	Stage of infection	Virus*	Week post-infection	Viral load in plasma [†] (copies/ml)	Clinical findings
M5602	Uninfected	None	NA	ND [§]	Normal
M6202	Uninfected	None	NA	ND	Normal
M6802	Uninfected	None	NA	ND	Normal
M7102	Uninfected	None	NA	ND	Normal
M13402	Uninfected	None	NA	ND	Normal
M13502	Uninfected	None	NA	ND	Normal
M5702 [¶]	Exposed/ uninfected	SIV/DeltaB670	2	ND	Normal
M7402 [¶]	Exposed/ uninfected	SIV/DeltaB670	2	ND	Normal
M6002	Acute	SIV/DeltaB670	2	2,600,000	Normal
M7902	Acute	SIV/DeltaB670	2	3,100,000	Normal
M8002	Acute	SIV/DeltaB670	2	1,450,000	Normal
M13202	Acute	SIV/Delta B670	3	41,300,000	Normal
M19102	Acute	SIV/Delta B670	2	380,000,00	Normal
M13002	Clinical latency	SIV/DeltaB670	8	180	Normal
M13102	Clinical latency	SIV/DeltaB670	8	3,600,000	Normal
M5802	AIDS	SIV/DeltaB670	49	1,950,000	Weight loss, CD4 ⁺ T-lymphocyte decrease
M5902	AIDS	SIV/DeltaB670	14	1,750,000	Weight loss, CD4 ⁺ T-lymphocyte decrease, lymphoma
M6102	AIDS	SIV/DeltaB670	62	1,100,000	Soft stools
M6302	AIDS	SIV/Delta670	42	1	Diarrhea
M7002	AIDS	SIV/DeltaB670	48	640,000	Weight loss, CD4 ⁺ T-lymphocyte decrease, watery diarrhea
M12402	AIDS	SIV/DeltaB670	42	4100	Weight loss, CD4 ⁺ T-lymphocyte decrease, PCP
M12802	AIDS	SIV/DeltaB670	37	286,000	Weight loss, CD4 ⁺ T-lymphocyte decrease, PCP

*Intrarectal infection.

[†]Real-time RT-PCR was used to determine plasma viral loads at necropsy.

[§]ND, not determined.

[¶]SIV was undetectable in plasma

3.2 IN SITU HYBRIDIZATION

In situ hybridization (ISH) for all chemokine (including CXCL13), defensin, and AMP mRNAs were performed as described.¹⁴⁷ Specifically, the protocol included first post-fixing slides in 4% paraformaldehyde (PF) for 20 min, then rinsing them in 70% ethanol for 20 min, followed by 5 min in 85% ethanol, and then 5 min in 95% ethanol. Slides were allowed to dry until tissues were completely free of moisture and then pretreated by microwaving in 0.01 M citrate buffer (Invitrogen; pH 6.0). Tissue sections were acetylated in 0.25% acetic anhydride and 0.1 M triethanolamine twice for 10 min at room temperature and dehydrated in graded ethanols for 5 min each and allowed to completely dry. Hybridization reactions were completed by combining 10% hybridization buffer, 10mM of ATP, CTP, and GTP (In vitro transcription kit), and ³⁵S-labeled UTP. Probes were completed by adding 2M dithiothreitol (DTT) and allowed to hybridize overnight at 50°C. Tissue sections were then placed in a series of washes and lastly subjected to emulsion autoradiography with varying exposure times depending on the target mRNA.

3.3 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) was performed on tissues as previously described (17). Briefly, cut tissue sections were post-fixed in 1% PF for 5 min and then washed in graded ethanols for 5 min each and allowed to dry completely. Slides were then pretreated in a heat source (Table 2), and in a pretreatment buffer (Table 2) for 10-20 min and allowed to cool for 30 min. Slides were then rinsed twice in 1X PBS for 3 min. Tissues were incubated with an

antibody (Table 2) for 1 hour at room temperature in a humid chamber. A parallel tissue on the same slide was incubated with a control antibody, either a concentration matched isotype or Ig negative control. The primary antibodies were then detected with secondary antibody SuperPicture Kit (Zymed Laboratories, South San Francisco, CA) according to the manufacturer's instructions. The kit was matched to the source of the primary antibody, All tissues were subsequently counterstained with hematoxylin (Fisher Chemicals, Fairlawn, NJ), then placed in graded alcohols for 5 min, xylenes for 5 min and mounted with glass coverslips with Permount (Fisher) and allowed to dry overnight.

Table 2: Antibodies used in these studies.

Antibody	Supplier	Catalog No.	Pretreatment Buffer	Heat Source	Dilution
CXCL13	R & D Systems	AF801	Dako Target Retrieval buffer (DTRB)	Microwave 2 min Power 2, 8 min Power 4 (MW)	1:50
CD20	Dako	M0755	NaCitrate	MW	1:100
CD79a	Fisher	MS-357-R7	NaCitrate	MW	1 drop
CD35	Santa Cruz	sc-52644	DTRB	Retriever	1:250
DEFA6	Sigma	HPA019462	NaCitrate	MW	1:1000
E-cadherin	BD Biosciences	61081	NaCitrate	MW	1:1000
Ki67	Dako	M-7240	DTRB	Steamer	1:100
Lysozyme	ThermoFisher	PA1-29680			
<u>Controls</u>					
IgG	Dako	AB108C	*	**	***
Mouse IgG2a	Dako	X0943	*	**	***
Mouse IgG1	Dako	X0931	*	**	***
Rabbit Ig	Dako	X0936	*	**	***
<u>Additional Antibodies Examined In These Studies[#]</u>					
CCL21	R & D Systems	AF366			
CCL28	Santa Cruz	sc-27337			
CXCL12	R & D Systems	AF310-NA			
CXCR5	Santa Cruz	sc-8179			
Defensin	Abd Serotec	MCA1465T			
DEFA5	Abcam	AB62757			
<i>E. coli</i>	Dako	B0357			
Lgr5	Abgent	AP2745d			
TLR9	Santa Cruz	sc-13215			
NOD2	Santa Cruz	sc-71714			

* Control antibody pretreatment buffer was matched to target primary antibody pretreatment buffer

** Control antibody heat source was matched to target primary antibody heat source

*** Control antibody dilution was calculated and matched to target primary antibody concentration and dilution

Multiple pretreatment buffers, heat sources, and antibody dilutions were used in an attempt to optimize a protocol to use these antibodies, however, the optimization was unsuccessful.

Simultaneous detection of CXCL13 protein and either DEFA6 or BDEF2 mRNA was performed by conducting IHC prior to completing the ISH protocol. Tissues were pretreated by microwaving in 1X Dako Target Retrieval buffer (Cat no. S1699, Dako) for 2 min at power setting 2, for 8 min at power setting 4, and then allowed to cool for 30 minutes covered. Slides were rinsed with 1X PBS twice for 3 min, and then blocked with one drop of Dako Protein Block (Cat no. X0909 Dako) for 10 min. Subsequently, 100ul of goat anti-CXCL13 primary antibody (Cat. no. AF801, R&D Systems 1:50) were added to one tissue section on each slide and 100ul of goat anti-IgG antibody (Cat. No AB108C Dako) were added to the second tissue section. They were incubated in a humid chamber for 1 hr. Tissues were then rinsed twice in 1X PBS for 3 min. They were subsequently incubated with the anti-goat secondary HRP-conjugated antibody (Invitrogen; Anti-goat SuperPicture Kit) for 10 min at room temperature in a humid chamber. Slides were rinsed one last time and allowed to incubate in the humid chamber with diaminobenzene (DAB) (Invitrogen; SuperPicture Kit) for 10 min at room temperature. Slides were rinsed then for a few seconds in MilliQ water and then immediately placed in 0.1M triethanolamine (pH) with 500ul acetic anhydride to progress into the acetylation step of the ISH protocol. The remaining ISH steps were followed as described above except that all DTT concentrations were reduced to 10 mM.

3.4 IMMUNOFLUORESCENCE STAINING

Single color immunofluorescence staining of macaque tissues was performed by first post-fixing and pretreating slides as mentioned above. Tissue sections were then incubated in a

humid chamber with a protein block (Dako) for 10 min, then an unconjugated primary antibody diluted in 1X PBS at room temperature for one hour. Each antibody was subsequently detected with a conjugated secondary antibody (i.e., donkey anti-goat antibody conjugated to Alexa Fluor 488 or Alexa Fluor 647). For two-color immunofluorescence staining, CXCL13, and either DEFA6 or CD20 were detected by simultaneously incubating goat anti-CXCL13 detected by donkey anti-goat Alexa Fluor 647 and rabbit anti-DEFA6 (Sigma, Catalog No. HPA019462, 1:1000) detected by donkey anti-rabbit antibody conjugated to Alexa Fluor 488 for 1 hour at room temperature. All tissue sections for immunofluorescence were treated with Autofluorescence Eliminator (Chemicon International) for 15 minutes before mounting with Prolong Gold Antifade with DAPI for nuclear staining (Molecular Probes).

3.5 REAL-TIME RT-PCR

Total RNAs were purified from snap-frozen axillary and mesenteric LN, spleen, ileal and colon samples from SIV-infected and control cynomolgous macaques. CXCL13 mRNA expression was measured using both a real-time RT-PCR SYBR Green assay (Applied Biosciences, Foster City, CA, USA) and a designed set of primers and probe generated by Dr. Shulin Qin using Primer Express software. Primers and probes for DEFA6, TLR9, NOD2, Reg3 γ , RTD-1, CAMP, DEFA5, β -GUS, and E-cadherin mRNA targets were commercially available (Applied Biosystems, Foster City, CA, USA) (Table 3). The comparative cycle threshold (Ct) method was employed to calculate mRNA expression levels relative to an uninfected macaque that served as the calibrator, with β -GUS serving as the endogenous control (Godfrey et al).

Table 3: CXCL13 Real-time RT-PCR primers and probes

Gene	Forward Primer	Reverse Primer	Probe
CXCL13	CCAGACGCTTCATTGATCGA	ATGATTTCCTTTCCTTGGACAACCA	6FAM- TTCAAATCTCGCCTCGTGGG A

Table 4. Real-time RT-PCR primers and probes

Gene	Probe	Catalog No.
DEFA6	Taqman	Rh02913163_m1
TLR9	Taqman	Hs00152973-m1
NOD2	Taqman	Hs002523394_m1
Reg3g	Taqman	Hs01595404_m1
RTD-1	SybrGreen/ Taqman	Rh02790032_m1
CAMP	Taqman	Rh02787757_m1
DEFA5	Taqman	Hs00360716_m1
E-cadherin	Taqman	Hs01023894_m1
β GUS	Taqman	Hs99999908_m1

3.6 CLONING OF PARTIAL CDNAS

To generate partial cDNAs containing the DEFA6, BDEF2, and RTD-1 open reading frames (ORFs), RNA was purified from cryopreserved cynomolgous ileal tissue and cDNA synthesis was performed as described (2). A human Reg3 γ cDNA was purchased from Open Biosystems (ThermoFisher Scientific, Lafayette, CO). Primers used for PCR amplification were designed based on the corresponding human sequences for α -defensin 5 and β -defensin 2 (Genbank accession numbers AY859407 and AF040153). The resultant forward and reverse primers are listed in Table 4. PCR products were ligated into the pGEM-T vector (Promega), screened by colony PCR and DNA sequenced.

Table 5. Cloning primers

Gene	Forward Primer	Reverse Primer
AMPs		
DEFA6	rhDEFA5_F2_CF_CTCACCATCCTTGCTGCCATTC	rhDEFA5_R2_CF_CTAGGAAGCTCGGCGACAGCA
BDEF2*	SS_HBD2_F1_CATCAGCCATGAGGGTCTTG	SS_HBD2_R1: CTCATGGCTTTTGCAGCATT
RTD-1*	SS_RTD1_F1_GGAGACCCGGGACAGAGGAC	SS_RTD1_R1: 5'-AAAGAGAGAAAATCTTGCAACAAGGTA
DEFA1*	SS_GTGACCCCAGCCATGAGG	SS_CTGGGTTGTCTGTTGGAATCTG
Lcn2	BFJ.hu.LCN2.F1_ATG CCC CTA GGT CTC CTG TG	BFJ.hu.LCN2.R15'- TCA GCC GTC GAT ACA CTG GT
Chemerin	BFJ.hu.chemerin.F1_ATGCGACGGC TGCTGATCCC	BFJ.hu.chemerin.R1_TTA GCT GCG GGG CAG GGC CT
Trypsin2	HuTryp2_F_CL_TGC CCC CTT TGA TGA TGA TG	HuTryp2_R1_CL_TTA GCT GTT GGC AGC TAT GG
Reg3γ	SQ_hu.Reg3g_F1_ATG CTG CCT CCC ATG GCC CT-3'	SQ_hu.Reg3g_R1_CTA GTC CTT GAA CTT GCA GAC ATA GGG T
Chemokines		
CXCL13	TRRHBCA1_F2_AGACAGAATGAAGTTCATCT	TRRHBCA-1R2_GTGGAAATATCAGCATCAGGG
CCL19	TRRHMIP3BF1_ATGGCCCTGCTACTGGCCC	TRRHMIP3BR1_TTAAGTCTGCTGCGGCGCTTCATCTT
CCL21	TRRH6CKF1_ATGGCTCAGTCACTGGCTCTGA	TRRH6CKR1_CTATGGCCCTTAGGGGTCTG
CCL25	TRRHTECKF_ATGAACCTGTGGCTCCTG	TRRHTECKR1_TCACAGTCTGAATTAGCTGA
CXCL12	TRRHSDFI AF1_GCCATGAACGCCAAGGTCGTGG	TRRHSDFIAR1_TTACTTGTTTTAAAGCTTTCTCCAG
mCXCL13	BFJ.m.CXCL13_F1_5' GAACTCCACCTCCAGGCAGA 3'	BFJ.m.CXCL13_R1_5' CTTTTGAGATGATAGTGGCT 3'
SIV	AGGGCTCAATCTGCCAGCCTCTCCGCAGAG GGAACACCCAGGCTCTACCTGCTAGTGCTG CACTCTGCCAGCACC GGCCAAGTGCTGGT TGGCAGCTTATTGAAGAGGCTTTAAGCA	CTCTGCGGAGAGGCTGGCAGATTGAGCCCT CAGCACTAGCAGGTAGAGCCTGGGTGTTCC ACCAGCACTTGGCCGGTCTGGGCAGAGTG TGCTTAAAGACCTCTTCAATAAAGCTGCCA

3.7 PHLOXINE-TARTRAZINE STAINING

Tissues used for visualization of Paneth cell granules were sectioned at 10 μm and then mounted on super-frost glass slides. They were post-fixed in 4% paraformaldehyde for 20 min and then dehydrated for 20 minutes in 70% ethanol, and then 5 minutes in 85% and 95% ethanols. These tissues were then stained by a modified protocol of Lendrum's procedure. Specifically, tissues were stained with hematoxylin for 2 minutes, and then washed in MilliQ water for 5 minutes. They were then transferred into the phloxine solution for 20 minutes. Tissues were then rinsed in new milliQ water three times with agitation for 30 seconds and then blot dried for 5-10 seconds. Next, the tissues were placed in the tartrazine solution for 5 hours and 45 minutes and subsequently rinsed in 95% ethanol twice for 5 minutes. Last, the tissues were placed in xylenes for 5 minutes and then mounted with a glass coverslip with Permount and dried overnight at room temperature.

3.8 IMAGE CAPTURE AND ANALYSIS

Images of *in situ* hybridization, immunohistochemistry and immunofluorescence tissue sections were captured on a Nikon (Melville, NY) E600 microscope mounted with a SPOT charged-couple device (CCD) (Diagnostic Instruments, Sterling Heights, MD) camera. The MetaVue software package (Universal Imaging, West Chester, PA) was used to view captured images. Confocal microscopy images were captured on an Olympus Fluoview 500 microscope in the University of Pittsburgh Center for Biological Imaging with kind assistance from microscopy expert Jason Devlin.

3.9 PHYLOGENETIC ANALYSES

Human and rhesus (RED) α defensin sequences were first identified and obtained by queries submitted on the NCBI BLAST server. ClustalX (2.0) software (www.clustal.org) was used to align all human α -defensin, RED, and other rhesus and cynomolgous macaque α defensin sequences. They confirmed by a bootstrapped phylogenetic analysis using SEQBOOT with 1,000 replicates, followed by DNAdist (with Kimura 2-parameter method), Neighbor-joining and consense programs from the PHYLIP 3.69 package (<http://www.evolution.genetics.washington.edu/phylip.html>).

3.10 STATISTICAL ANALYSES

All statistical analyses were performed with the Prism software package. Real-time RT-PCR data were analyzed using the two-sample *t* test and Mann-Whitney nonparametric test. A *p* value <0.05 was considered significant. To quantify correlations between SIV PVLs and fold-change in AMP mRNA, the Pearson and Spearman-ranked correlation coefficients (ρ) were determined. P-values of ≤ 0.05 were considered statistically significant.

3.11 TECHNICAL ACKNOWLEDGEMENTS

The following contributions were important to the execution of portions of this dissertation. Preliminary data regarding CXCL13 mRNA expression was evaluated using real-time RT-PCR by Anthony Cillo and Dr. Yongjun Sui. Dr. Shulin Qin also assessed the expression of several homeostatic chemokines by real-time RT-PCR and made those data available to analyze and compare to data collected on CXCL13. He also was critical in discussing questions or issues with experimental design. Beth Fallert Junecko completed the preliminary ISH for CXCL13 and SIV in LN and spleen tissues. She also cloned the partial AMP cDNAs for use in ISH. Cynthia Klamar optimized the Lendrum's phloxine-tartrazine staining protocol to stain for PC granules. She also conducted the test ISH on subadjacent ileum tissues for BDEF2 and RTD-1. Sonali Songhavi generated plasmids containing AMP partial cDNAs that were used in ISH on small intestinal tissues. Dr. Anil Ohja provided guidance and expertise in antimicrobial assay development as well as provided temporary bench space to

conduct these assays. He also provided the murine tissues for CXCL13 IHC assays. Dr. Islam Mohammad provided his knowledge of the mouse gut anatomy for the dissection of the mouse intestinal tissues. Dr. Berthoni Deslouches provided instruction and optimized the antimicrobial assays that were tested for inclusion in Specific Aim 3. Dr. Robert Montelaro provided the LL-37 peptide that was used in these antimicrobial assays. Dr. Jeremy Martinson and Blair Gleeson contributed analyses they conducted on CXCL13 single nucleotide polymorphisms (SNPs) in NHPs in order to assess the reliability of the CXCL13 real-time RT-PCR assays. Finally, Stella Berendam and ChengLi Shen lent their knowledge of phylogenetic analyses and phylogenetic tree development software to the establishment of the phylogenetic analyses on alpha defensins.

4.0 RESULTS

4.1 CXCL13 MRNA AND PROTEIN ARE LOCALIZED TO ADJACENT MICROANATOMIC COMPARTMENTS IN MACAQUE LYMPHOID TISSUES

To understand better CXCL13 expression in lymphoid tissues and to define potential changes in CXCL13 expression during pathogenic SIV infection, we examined mRNA expression patterns in lymph node (LN) and spleen tissues by ISH. Cells producing CXCL13 mRNA localized to a ring around germinal centers (GCs) in LN tissue sections (Figure 3A). Subjacent tissue sections were immunostained for CXCL13 protein and, unexpectedly, CXCL13 protein signal was intensely localized to the center of the GCs (Figure 3A). In these regions, there were networks of CXCL13 clearly visible after immunofluorescent staining for CXCL13 in LNs (Figure 3A). In addition, in LN paracortices there were individual cells that stained for CXCL13. Parallel staining of subjacent tissue sections with control goat Ig did not yield specific signal (not shown). The mRNA and protein expression patterns of CXCL13 in macaque spleen tissues were similar to those in LNs (Figure 3B). These data indicate that CXCL13 producer cells are localized in a microanatomic compartment distinct from and immediately adjacent to that harboring the highest levels of CXCL13 protein in the centers of GCs.

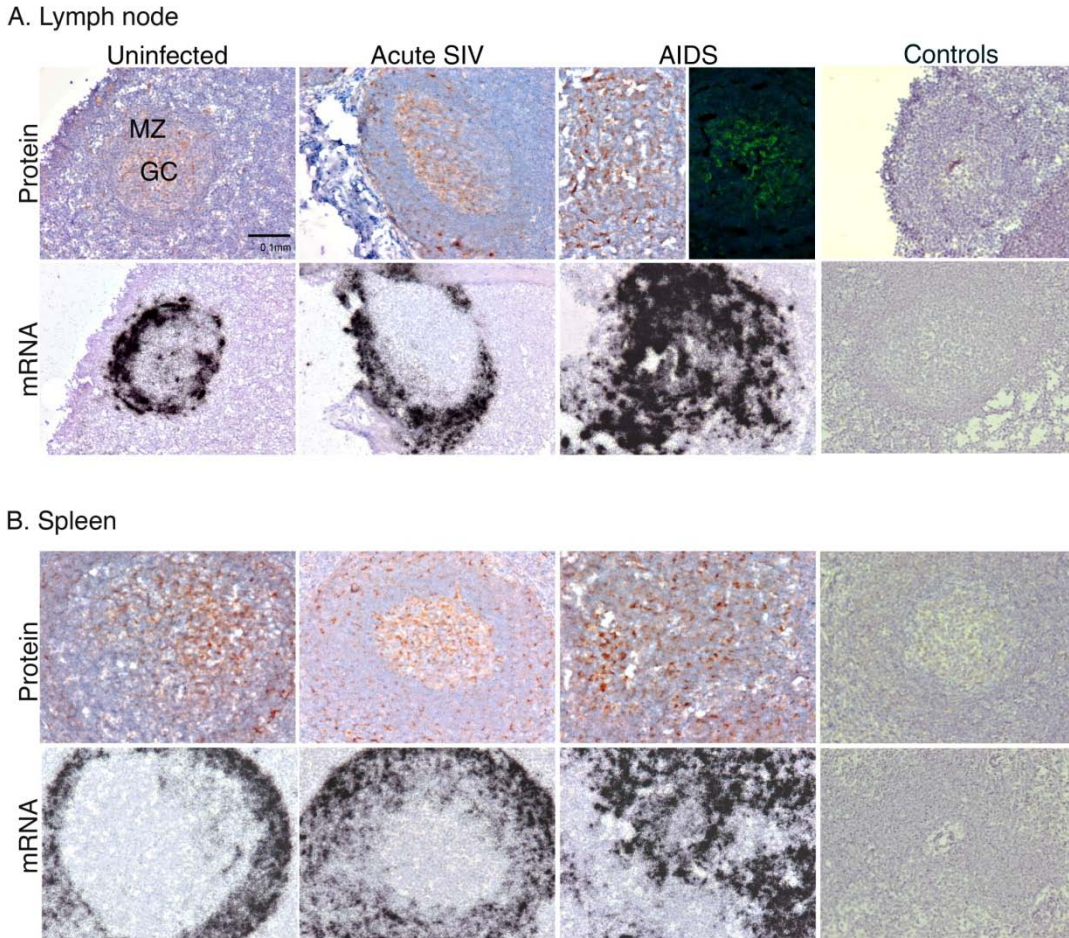


Figure 3. CXCL13 protein and mRNA localize to different microanatomic compartments in lymph node and spleen.

A. Immunohistochemistry (top images), immunofluorescent (top AIDS center-right image) and *in situ* hybridization (bottom images) detection of CXCL13 protein and mRNAs in uninfected, acutely SIV-infected, AIDS-developing macaque LN tissues; B. Immunohistochemistry (top images) and *in situ* hybridization (bottom images) detection of CXCL13 protein and mRNAs in uninfected, acutely SIV-infected, AIDS-developing macaque spleen tissues. Isotype and sense controls were clear and contained no signals (far right images). (MZ: Mantle zone; GC: Germinal center; Original magnification = x200)

CXCL13 expression in LN and spleen tissues was also examined across SIV disease states by staining tissues from SIV/DeltaB670 infected animals that have been previously examined for inflammatory chemokine expression in lymphoid¹⁴⁵ and lung¹⁴⁴ tissues. We compared expression patterns in LNs from uninfected, acutely SIV-infected, and AIDS-developing macaques and found that CXCL13 mRNA expression was upregulated during acute

infection and maintained at high levels during AIDS (Figure 3A and 3B). In contrast, CXCL13 protein expression increased to a limited extent across disease states. The distribution of CXCL13 expression signal appears to expand during SIV infection leading to the presence of CXCL13 mRNA beyond the outer zone of the GC into the center and throughout the GC. This could suggest an increase in the number of CXCL13-producing cells recruited to the region, or an induction of the amount of CXCL13 produced by cells localized to this microanatomic compartment. Parallel analysis of spleen tissues from the same macaques yielded similar results. Altogether these findings suggest that SIV directly or indirectly induces CXCL13 expression levels resulting in modification to CXCL13 expression patterns and, therefore, disparate localization of mRNA and protein.

4.2 LOCALIZATION OF CCL19, CCL21, AND CXCL12 AND RESPONDER CELL POPULATIONS IN MACAQUE LYMPHOD TISSUES

CXCL13 is one of a group of homeostatic chemokines expressed in lymphoid tissues. CCL19 and CCL21 are also homeostatic chemokines abundantly expressed in the thymus, LN and spleen. These chemokines induce migration of CCR7⁺ DCs, B cells, and T cells. Previously, our lab found the expression of homeostatic chemokine CCL21 was reduced during SIV infection in rhesus macaque LN and spleen tissues.¹⁴⁸ In addition, CCL19 expression in macaque LNs was found to be low in the absence of infection.¹⁴⁸ Here, we sought to determine if those same changes to CCL19 and CCL21 occurred in SIV-infected lymphoid tissues of cynomolgous macaques. ISH and IHC for CCL21 in mesenteric LN and spleen tissue sections was carried out to examine changes in expression patterns and levels as a function of SIV

infection. We found a dense pattern of CCL21 expression in the paracortex regions of the LNs surrounding GCs and lymphatic vessels (Figure 4). We did not find any changes to CCL21 expression in the LN of the cynomolgous macaque during SIV infection. However, as in rhesus macaques, there is a decrease in CCL21 expression during acute SIV infection in spleen tissues that continues into AIDS (Figure 6). Unlike CCL21, CCL19 in the spleen increases in acute SIV-infected tissues and that increase is maintained during AIDS (Figure 6). This data was also seen in rhesus macaque lymphoid tissues.

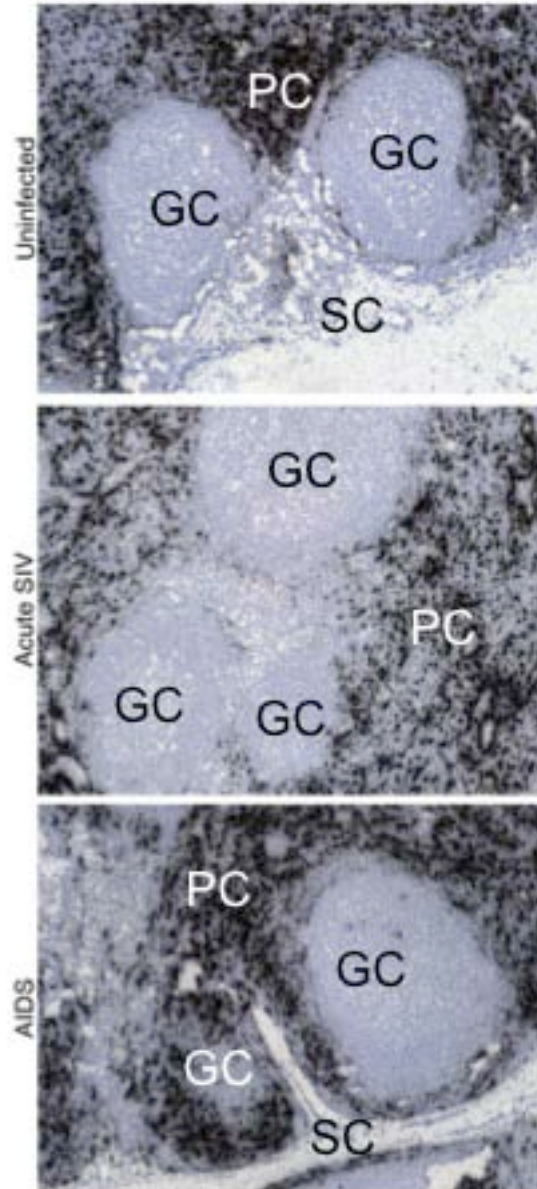


Figure 4. CCL21 mRNA is localized to the paracortex in cynomolgous macaque mesenteric lymph nodes.

In situ hybridization detection of CCL21 mRNA in uninfected, acutely SIV-infected, AIDS-developing macaque spleen tissues; Sense controls were clear and contained no signals. (PC: Paracortex, GC: germinal center, SC: subcapsule; Original magnification = x200)

In addition to identifying changes in homeostatic chemokine expression in lymphoid tissues, we attempted to identify cell populations localizing to the same microanatomic compartment as CXCL13. Therefore, we examined markers CD20, CD79a, and CD35 for

mature B cells, immature B cells, and follicular dendritic cells (FDCs), respectively all cells that can reside in LN GCs (Figure 5) to determine possible CXCL13 producing cells. Mature B cells were found primarily in GCs of uninfected SIV-infected macaques, although there were also regions of the paracortex of the LN that contained appreciable markers of individual CD20+ cells. In comparison to mature B cells, immature B cells expressing CD79a localized mostly to GCs. Staining for CD79a had a distinct pattern from CD20 in that there was a darker ring of CD79a+ cells around the GCs and a lighter zone of staining in the center of the GC. CXCL13 has been described as being generated by stromal cells in lymphoid tissues.⁵³ Therefore, we stained for the FDC specific marker, CD35. CD35 expression in GCs was in a pattern similar to both the mRNA and protein expression of CXCL13, with GCs containing a lighter ring of expression around the GC, like that of CXCL13 mRNA, and a darker center of CD35 expression, like that of CXCL13 protein. Overall, there was an overlap in the expression pattern of CD20+ cells, CD79a+ cells, and CD35+ cells. Not one cell population completely mimicked the expression pattern of CXCL13. Examining changes in each of these cell markers did not reveal striking differences between uninfected and SIV-infected LNs.

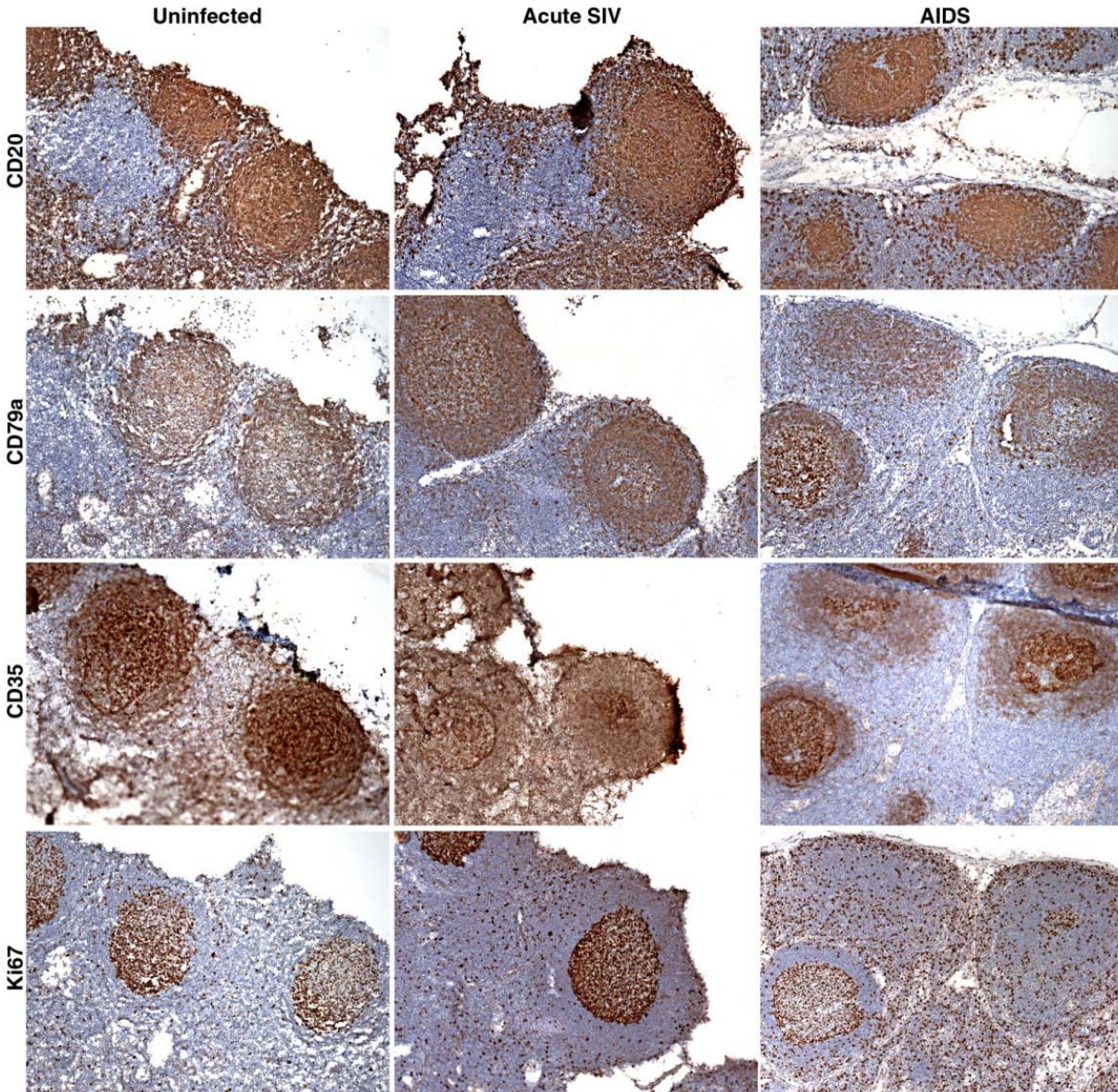


Figure 5. B cells and follicular dendritic cell expression patterns in cynomolgous mesenteric lymph nodes.

Immunohistochemistry, detection of mature B cells (CD20), immature B cells (CD79a), and follicular dendritic cells (CD35) in uninfected, acutely SIV-infected, AIDS-developing macaque LN tissues; Immunohistochemistry detection of proliferation marker Ki67 in uninfected, acutely SIV-infected, AIDS-developing macaque LN tissues. Isotype controls were clear and contained no signals. (Original magnification = x100)

To examine changes in cell proliferation during SIV infection the presence of the marker Ki67 was identified (Figure 5). In uninfected macaques, cell proliferation was mostly within the

GC. Examining nearly subadjacent tissue sections for CD20 and CD79a suggests that many of these cells are B cells. In LNs from acute SIV-infected macaques, Ki67 is mostly concentrated in GCs, with individual cells that are positive for Ki67 scattered throughout the paracortex. The pattern of cell proliferation in AIDS-developing macaques contrasted significantly from uninfected and acutely SIV-infected macaques. The paracortex and GC regions in LNs of AIDS-developing macaques had similar numbers of Ki67+ cells where there was an almost even distribution of cell proliferation in GCs. Comparing CXCL13 expression patterns to Ki67+ cell expression across disease states suggests in uninfected and acutely SIV-infected macaques could contain more proliferating cells that are also localized with CXCL13. This is not found in AIDS-developing macaques, where the pattern of Ki67 is no longer highly concentrated in GCs.

To identify possible modifications to local cell populations in the spleen during SIV infection, and in an attempt to determine colocalization of these cells with CXCL13, CCL19 and CCL21, we examined the expression CD20, CD79a, and CD35 to distinguish between mature and immature B cells and FDCs on subadjacent tissue sections (Figure 6). The pattern of CD20+ cells is adjacent to but not colocalized with the signal for CCL21 and CCL19 whereas the pattern for CD79a positive cells is contained within CCL21 and CCL19 producing cell populations. The expression of CD79a also appears in a similar pattern to CXCL13 protein in the spleen of uninfected and acutely SIV-infected tissues. The pattern of CD79a expression on subadjacent tissues sections of AIDS-developing tissues appear to localize with CCL19 and CCL21 mRNA expression and suggests colocalization. In uninfected tissues, CD35 expression was in periarterial lymphoid sheaths (PALS) and contained three distinct zones of expression. CD35 expression in acutely SIV-infected and AIDS-developing macaques was similar to the expression of both CCL21 and CCL19 (Figure 6). We observed changes in the expression patterns of

CD20+, CD79a+, and CD35+ in the spleen of AIDS-developing tissues. These findings suggest modifications to the presence of these cell populations during SIV infection that could be driven by differences in CCL19 and CCL21 expression in adjacent regions of the lymphoid tissues. Similar to the LN, there is overlap of the expression patterns between each of these cell populations and CXCL13.

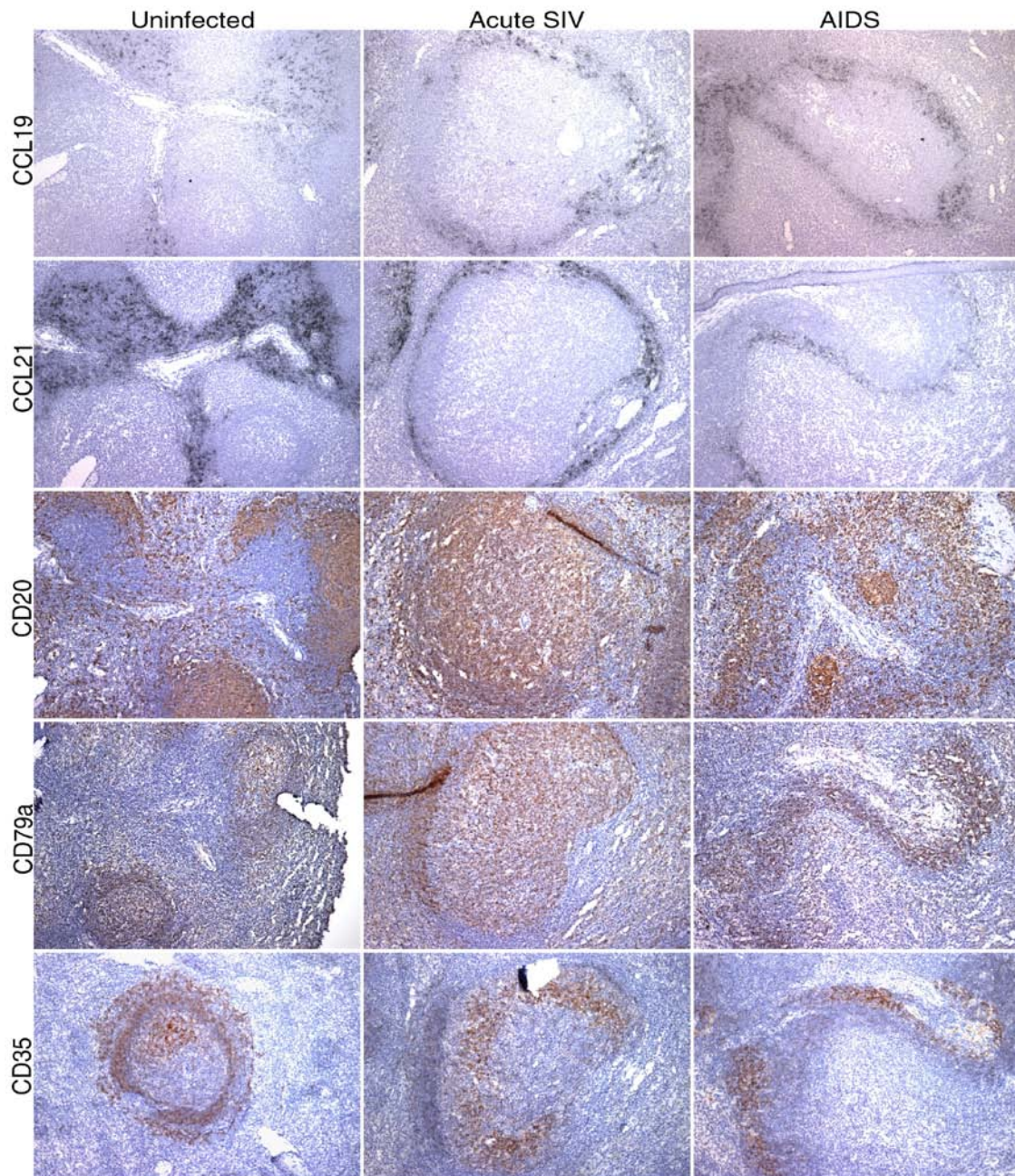


Figure 6. Expression patterns of CCL19, CCL21, and responder cells in spleen.

In situ detection of CCL19 and CCL21 in uninfected, acutely SIV-infected, and AIDS-developing macaque spleen tissues. Immunohistochemistry detection of mature B cells (CD20), immature B cells (CD79a), and follicular dendritic cells (CD35) in uninfected, acutely SIV-infected, AIDS-developing macaque spleen tissues. Isotype controls were clear and contained no signals. (Original magnification = x200)

HIV-1 utilizes primarily two chemokine receptors for entry into cells.³⁸ Those receptors are CCR5 and CXCR4.^{37,38} CXCL12, another member of the homeostatic chemokine family, is the ligand for CXCR4 and is one of the chemokines responsible for the movement of lymphocytes during angiogenesis. To determine if SIV infection altered the expression of this homeostatic chemokine, given the importance of its receptor to viral entry, we utilized ISH to localize the patterns of cells expressing CXCL12 mRNA (Figure 7). In the LNs, CXCL12 expression was largely contained within the medulla and efferent lymphatic system, and in the spleen, its expression is throughout the red pulp sinuses. We found that the levels of CXCL12 in the LN and spleen increased during acute SIV infection as compared to uninfected tissues, whereas AIDS-developing macaques had CXCL12 expression levels similar to uninfected animals. Our results could suggest that cells expressing CXCR4, the CXCL12 chemokine receptor, would be less likely to exit out of lymphoid organs via the efferent lymphatics given the reduction in CXCL12 expression.

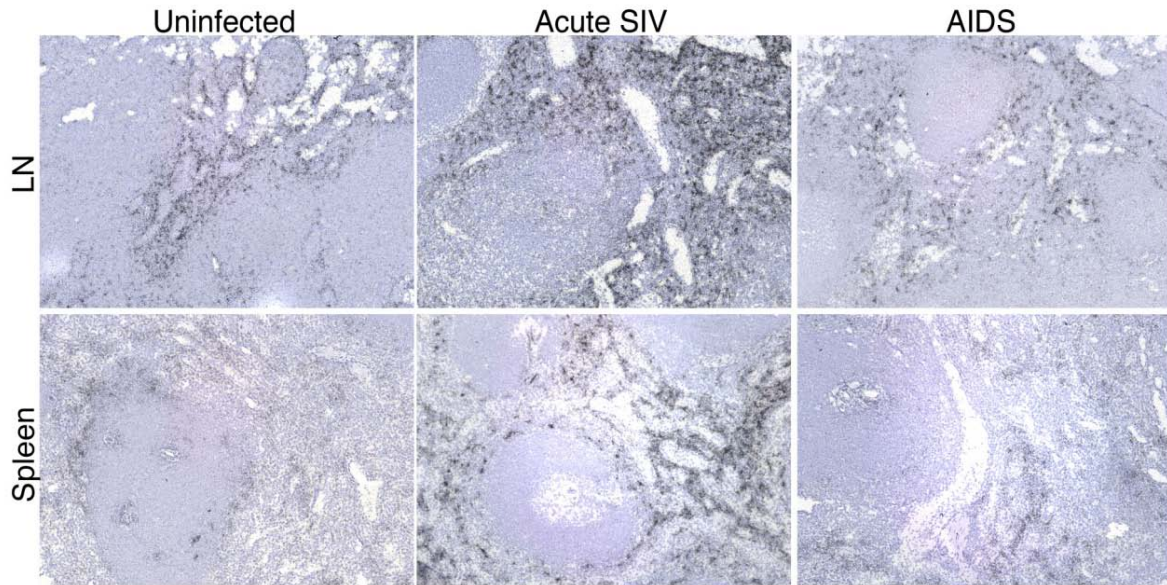


Figure 7. CXCL12 expression increases in the medullary regions of lymph node and spleen during SIV infection.

A. In situ hybridization detection CXCL12 mRNA in uninfected, acutely SIV-infected, AIDS-developing macaque LN (top row) and spleen tissues (bottom row); Sense controls were clear and contained no signals. (Original magnification = x200)

With CXCL13 expression upregulated during SIV infection, we sought to determine if there were relationships between this CXCL13 expression and SIV RNA expression. On subadjacent tissues, we conducted ISH for SIV mRNA and visually compared GCs for CXCL13 and SIV expression levels (Figure 8). There were no apparent associations between changes in SIV expression and CXCL13 expression in the lymph nodes or spleen in uninfected animals or during acute-SIV infection. Where CXCL13 formed a dense ring of mRNA around the follicle, there was a light cloud of SIV RNA contained in the center of the GC. However, during AIDS there was one notable change. Those GCs that contained dramatic increases in CXCL13 expression and had expansion of the GCs also contained what has been described as unspliced SIV RNA. FDCs harbor immune complexes on their surface and contain unspliced viral genomic RNA rather than being productively infected by SIV.¹⁴⁹

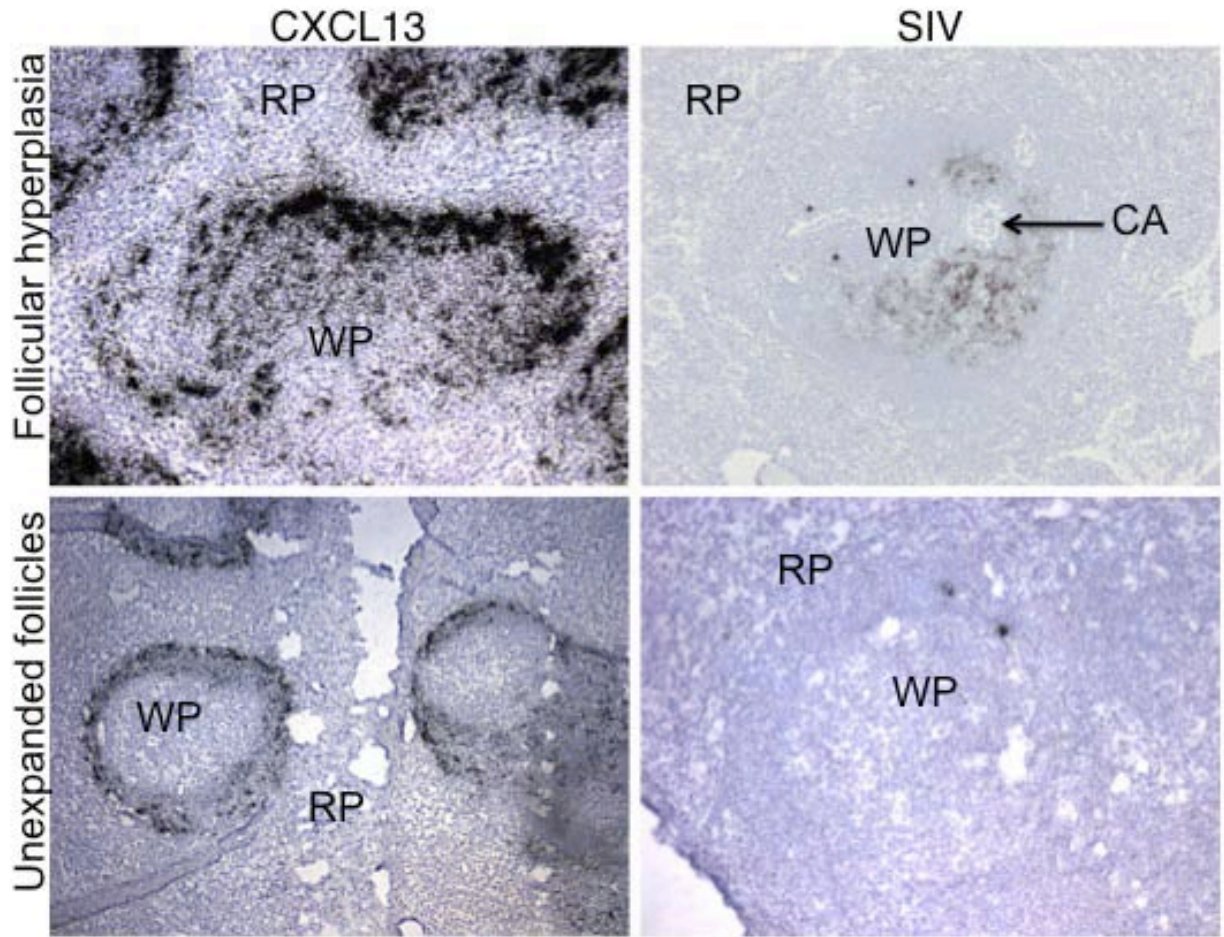


Figure 8. Expansion of follicles and CXCL13 expression in the spleen coincides with changes in expression patterns of SIV RNA.

In situ hybridization detection CXCL13 mRNA in AIDS-developing macaque spleen (left images) and SIV (right images); Sense controls were clear and contained no signals. (RP: Red pulp, WP: White pulp, CA with arrow: Central arteriole. Original magnification = x200)

4.3 CXCL13 IS EXPRESSED IN PANETH CELLS

Intestinal tissues are considered to be one of the largest lymphoid organs and are a site in which many CD4⁺ T lymphocytes are lost during HIV-1 and SIV infection¹⁵⁰. Additionally, CXCL13 expression has been found in GALT in patients with ulcerative colitis.⁶⁸ Therefore, we

examined the microanatomic distribution of CXCL13 in different regions along the gastrointestinal (GI) tract. Using ISH and IHC, we localized CXCL13 mRNA and protein to lymphoid follicles throughout the intestines, including in duodenum, jejunum, ileum, and colon, again with mRNA⁺ producer cells distributed in a three dimensional spherical shell surrounding high levels of CXCL13 protein within GCs (Figure 9 and figure 10).

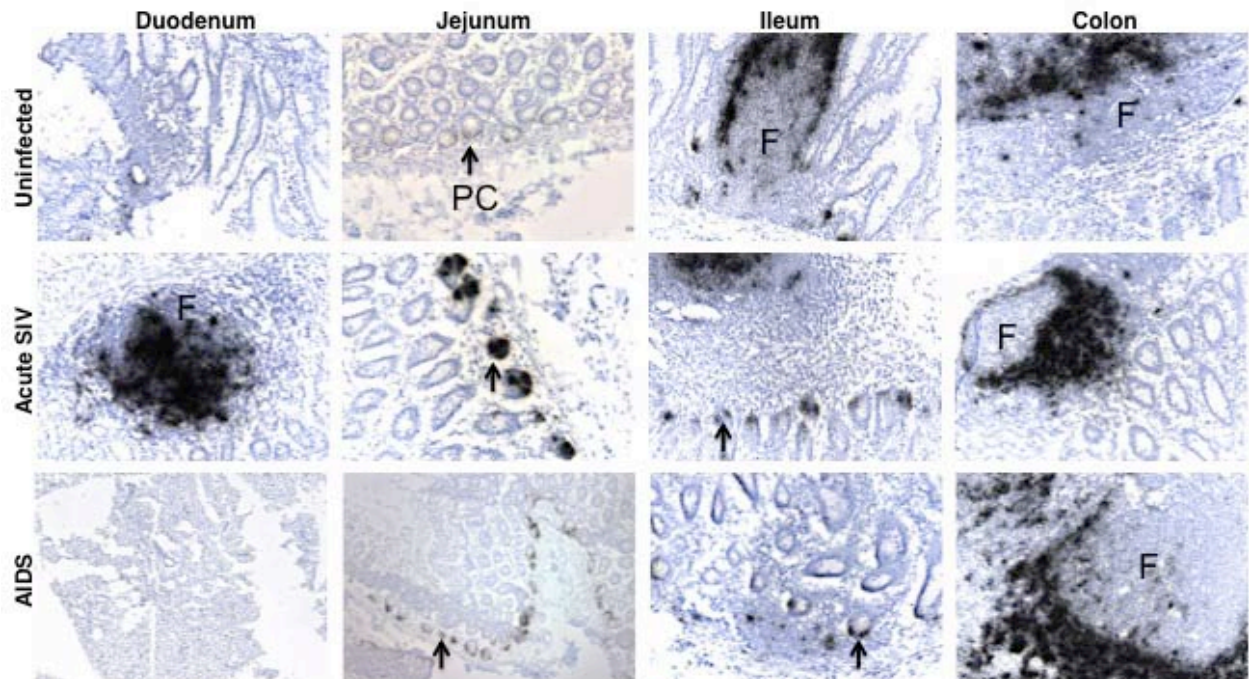


Figure 9. CXCL13 mRNA expression in GALT and Paneth cells in the macaque intestine.

In situ hybridization detection of CXCL13 mRNAs in uninfected, acutely SIV-infected, and AIDS-developing macaque duodenum (left images), jejunum (left center images), ileum (right center images), and colon (right images) tissues. (PC: Paneth cell, F: Follicle. Arrow denotes PC. Original magnification = x200)

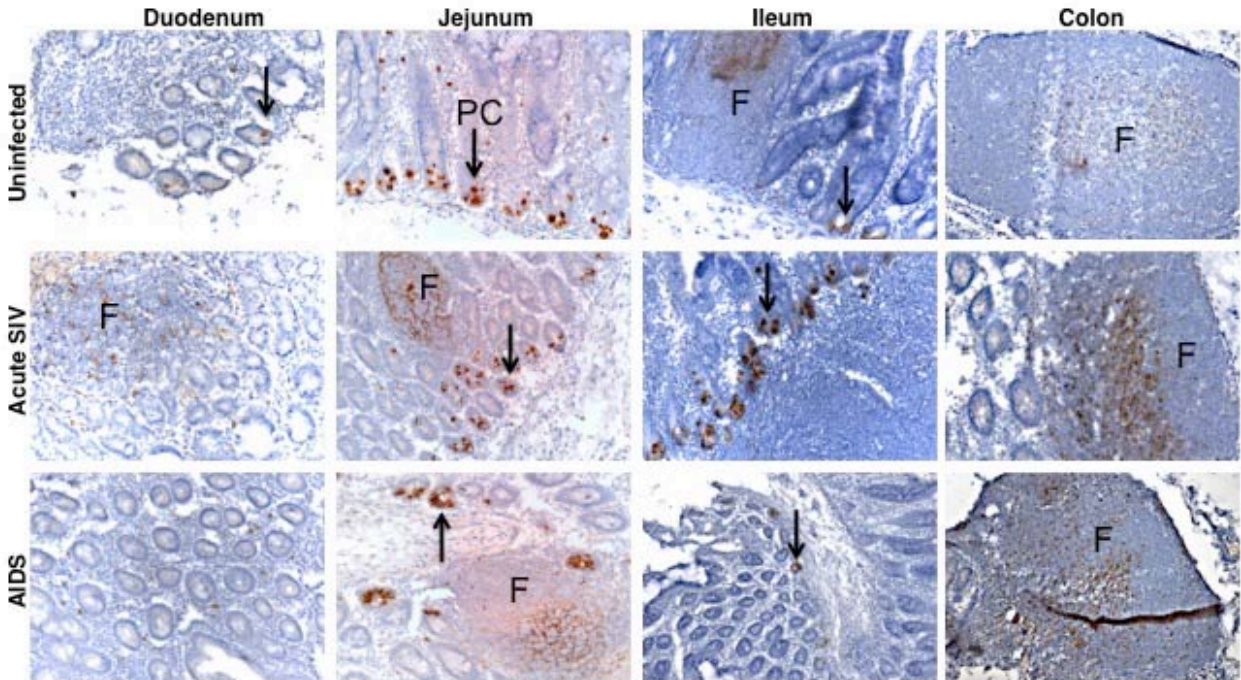


Figure 10. CXCL13 protein expression in the GALT and Paneth cells in the macaque intestine.

Immunohistochemistry detection of CXCL13 protein in uninfected, acutely SIV-infected, and AIDS-developing macaque duodenum (left images), jejunum (left center images), ileum (right center images), and colon (right images) tissues. (PC: Paneth cell, F: Follicle. Arrow denotes PC. Original magnification = x100 and x200)

Interestingly, we observed intense expression of CXCL13 mRNA and protein in a subset of cells in the crypts of Lieberkühn (Figure 9, Figure 10, and Figure 11). Cells expressing CXCL13 in crypts were observed in duodenal, jejunal, and ileal tissues from all 16 macaques studied, but were not observed in colon.

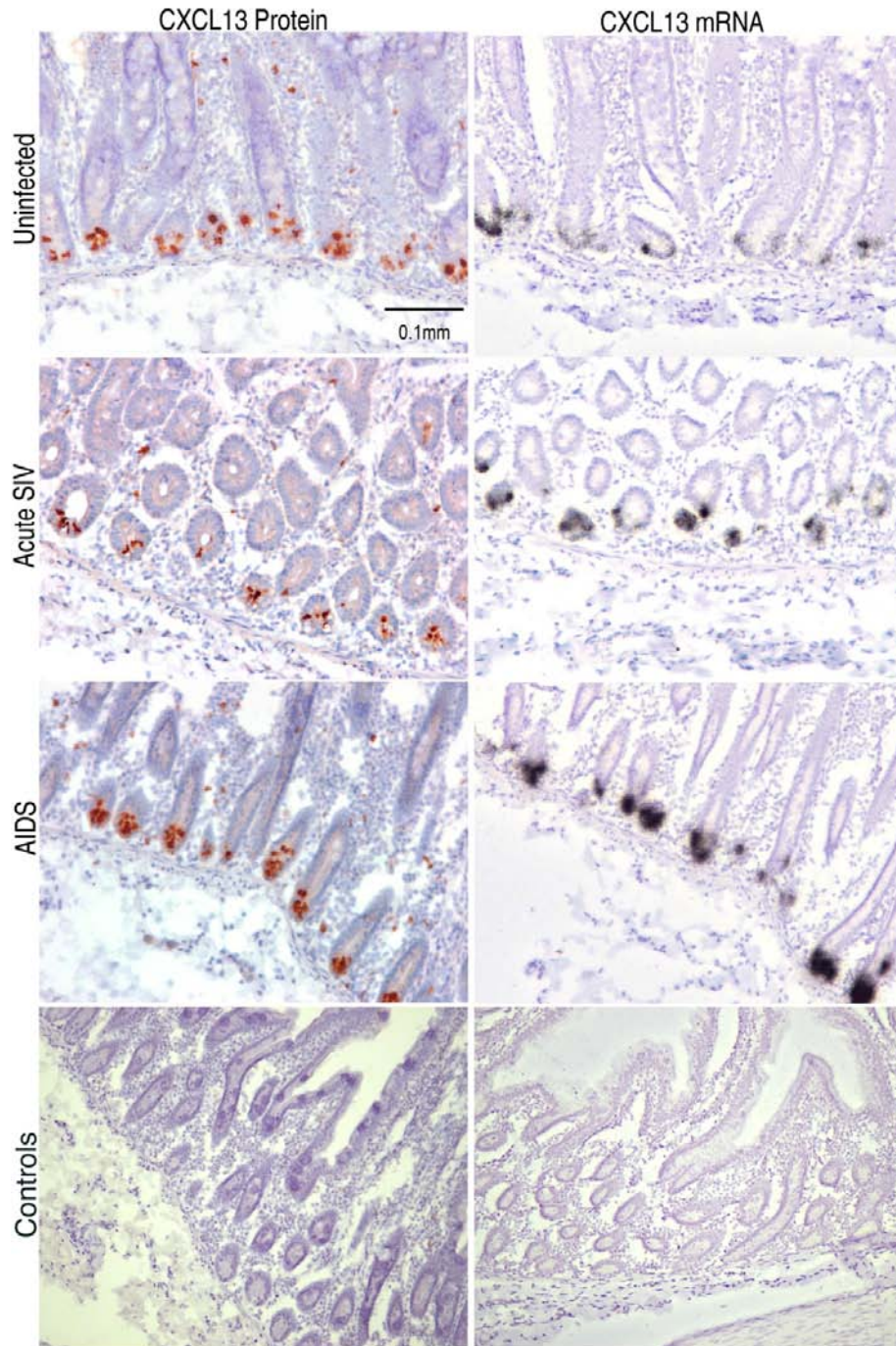


Figure 11. CXCL13 expression in Paneth cells of the small intestine.

Immunohistochemistry detection of CXCL13 protein (left images) and *in situ* hybridization detection of CXCL13 mRNAs (right images) in uninfected, acutely SIV-infected, and AIDS-developing macaque tissues. Sense controls were clear and contained no signals. (Original magnification = x100)

In the crypts of Lieberkuhn, Paneth cells are specialized intestinal epithelial cells that produce AMPs and are found only in the small intestine¹⁵¹. The regional distribution of CXCL13 expression in intestinal crypts we observed could be due to its production by Paneth cells. To determine if Paneth cells were the source of CXCL13 in the intestinal crypts, we individually and simultaneously stained for α -defensin 6 (DEFA6), which is an AMP uniquely expressed by Paneth cells.^{152,153} In macaque small intestine, we found Paneth cell secretory granules, DEFA6 protein, and DEFA6 mRNA localized to intestinal crypts in patterns identical to those obtained for CXCL13 (Figures 12A-12B, and Figures 14A-B), and similar to CXCL13, DEFA6 was expressed in the jejunum and ileum, but not in the colon. Simultaneous IHC for CXCL13 protein and ISH for DEFA6 mRNA (Figure 12C) demonstrated their co-localization to the same cells in crypts. In addition, two-color immunofluorescence staining and confocal microscopy confirmed that CXCL13 and DEFA6 are co-expressed by Paneth cells in intracellular granules, with most CXCL13 signal overlapping a large proportion of the DEFA6 signal (Figure 12H). These findings confirm that that source of CXCL13 in intestinal crypts is the Paneth cell and are to our knowledge the first to show the expression of a lymphoid homeostatic chemokine by this specialized intestinal epithelial cell.

To verify if other AMPs were expressed by the same intracellular granules in Paneth cells we simultaneously conducted IHC for CXCL13 protein and ISH for DEFA1, BDEFA2, RTD-1, and Reg3 γ (Figure 12C-G). Expression of mRNAs for each of these AMPs colocalized with CXCL13 in some granular regions, however, both signals did not always overlap. This could suggest that Paneth cells vary in their ability to produce specific AMPs.

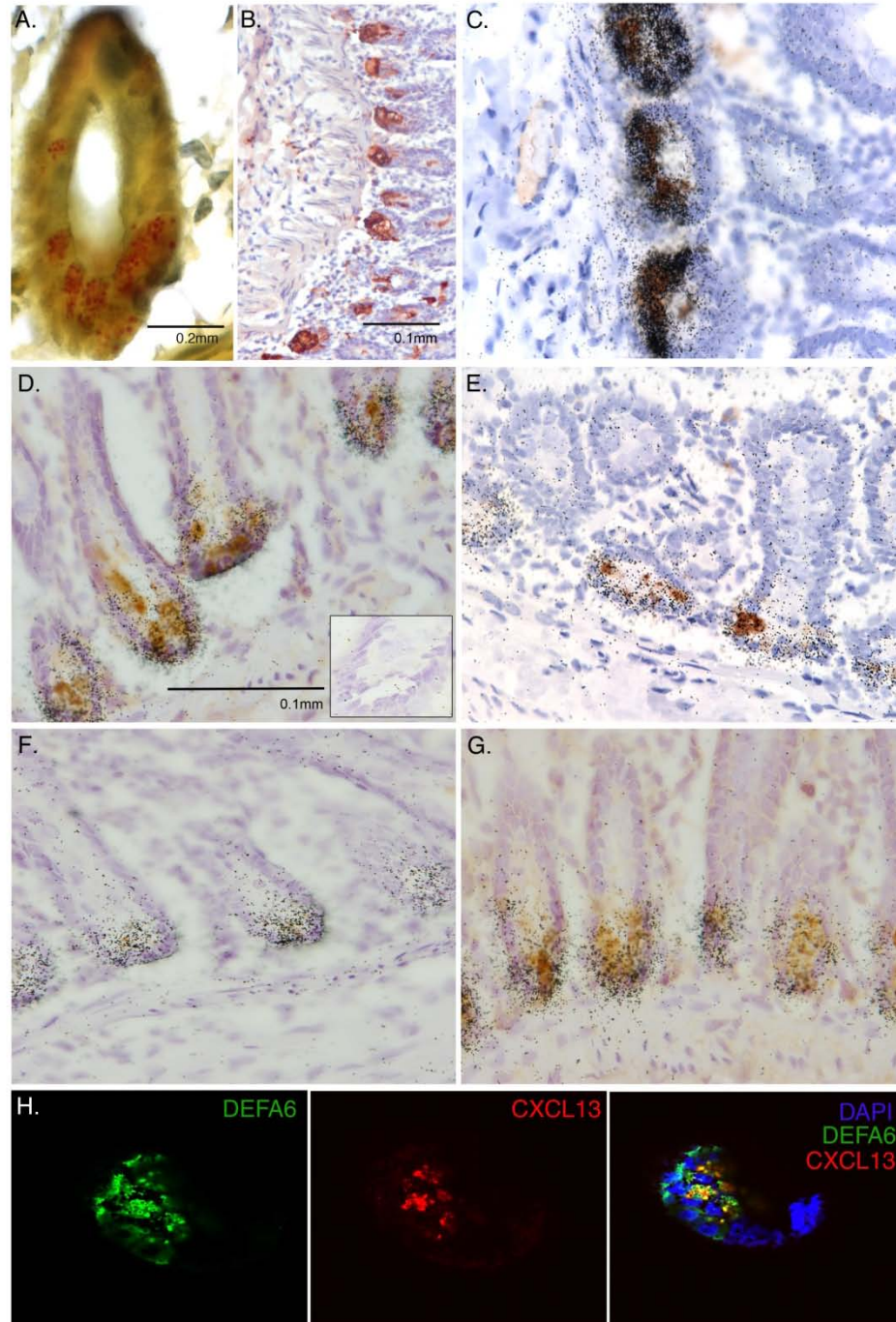


Figure 12. Antimicrobial peptides α -defensin 6, α -defensin 1, β -defensin 2, rhesus θ -defensin 1, and Reg3g colocalize with CXCL13 expression in the ileum.

A. Phloxine-tartrazine staining for Paneth cell granules in the crypts of the Lieberkuhn of the macaque ileum. B. Immunohistochemical detection of cynomolgous DEFA6 protein in macaque ileum tissues (Original magnification = x200). C. Double label immunohistochemical detection of CXCL13 protein and *in situ* hybridization for alpha defensin-6 mRNAs in macaque ileum tissues. D. Double label for CXCL13 protein and DEFA1 mRNAs in the ileum. E. Double label

for CXCL13 protein and BDEF2 mRNAs in the ileum; F. Double label for CXCL13 and RTD-1 mRNAs in the ileum.. G. Double label for CXCL13 protein and Reg3 γ mRNAs in the ileum; H. Immunofluorescent detection of DEFA6 and CXCL13 protein colocalization in macaque ileum tissues. Isotype and sense controls (inset of D.) were clear and contained no signals. (Original magnification = x400; Black silver grains represent mRNA signal; Brown staining represents protein signal)

4.4 CXCL13 IS UNIQUELY EXPRESSED IN PANETH CELL AMONGST A SUBSET OF CC AND CXC CHEMOKINES

To determine whether other chemokines also were expressed strongly in intestinal crypts, or whether CXCL13 was unique in this property, we examined the localization of mRNA producer cells of multiple chemokines along the macaque GI tract. These included CCL25, a ligand for CCR9 that directs the trafficking of intestinal homing T and B lymphocytes (Figure 14), as well as CXCL1, CXCL9, CXCL10, CXCL11, CXCL12, CCL19, CCL20, and CCL21 (Figure 13). Yang *et al.* evaluated a group of chemokines for their antimicrobial potential and found that approximately 50% of the chemokines they evaluated exhibited antimicrobial activity.¹⁵⁴ Based on their data, we chose to determine if Paneth cells also expressed a subgroup of those chemokines that demonstrated some of the greatest antimicrobial activity. We did not find any of these other chemokines, which have been shown to exhibit antimicrobial activity,¹⁵⁴ expressed by Paneth cells making expression of CXCL13 unique.

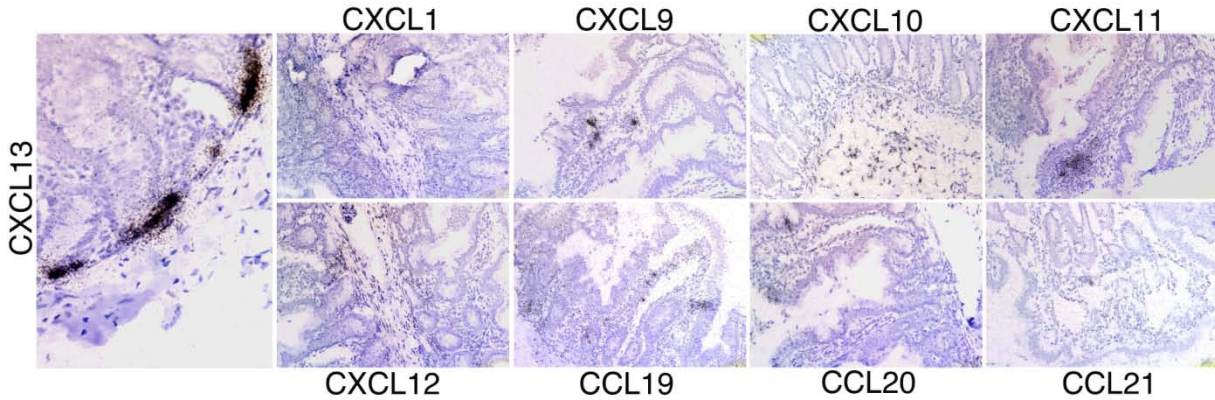


Figure 13. Unique expression of CXCL13 in Paneth cells in the small intestine.

In situ hybridization CXCL13 (far left image), CXCL1, CXCL9, CXCL10, CXCL11 (top row images), CXCL12, CCL19, CCL20, and CCL21 (bottom row images) mRNA in uninfected macaque ileum tissues. Sense controls (not shown) were clear and contained no signals. (Original magnification = x200)

CCL25 is known to be expressed by small intestinal epithelium, and consistent with this, the mRNA expression patterns for CCL25 differed greatly from CXCL13, with CCL25 producer cells found uniformly across the epithelium of the small intestine and not in the GALT, Paneth cell laden crypts, or colon. None of the other chemokine producer cells were localized to intestinal crypts, and therefore not in Paneth cells (Figure 14D-F).

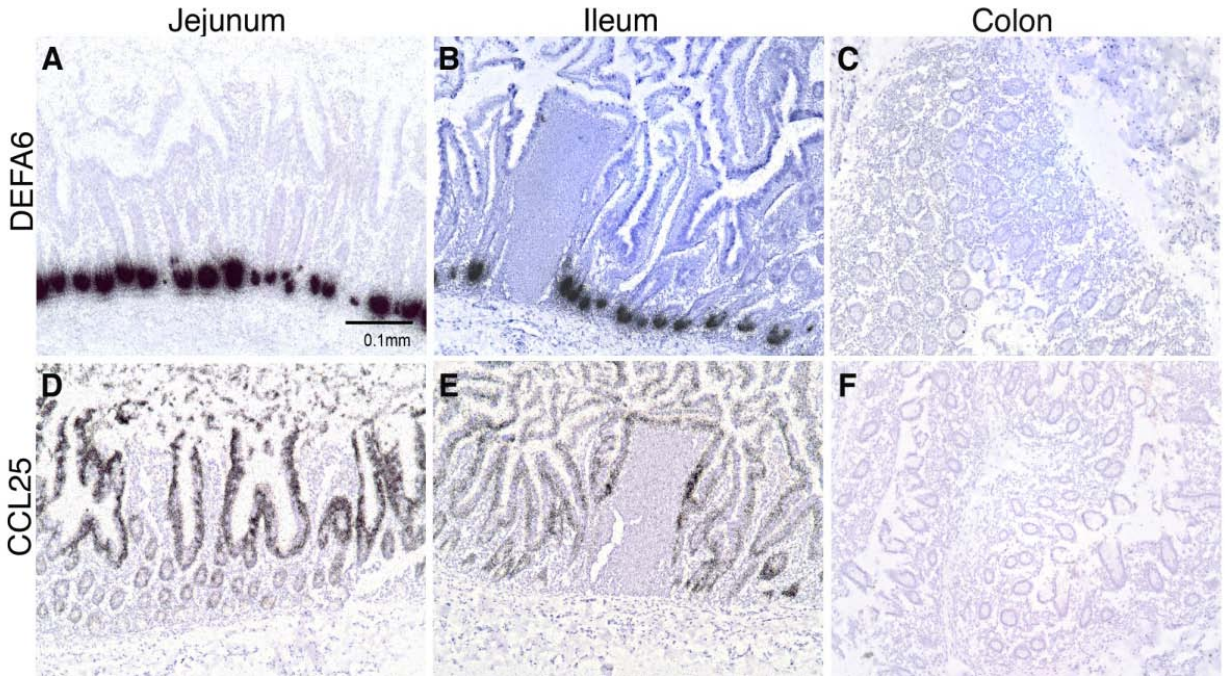


Figure 14. Antimicrobial peptides α -defensin 6 and CCL25 mRNA are present in the cynomolgous macaque small intestine.

In situ hybridization of DEFA6 (A-C) and CCL25 (D-F) mRNA in uninfected, acutely SIV-infected, and AIDS-developing macaque jejunum, ileum, and colon tissues.) Sense controls (not shown) were clear and contained no signals. (Original magnification = 100X)

To determine if cells known to express CXCR5 and with the potential to respond to CXCL13 were also localized near Paneth cells, we identified CD20+ mature B cells by IHC and immunofluorescence (IF) (Figure 15). Our results show that most CD20+ cells are localized to follicular regions (Figure 15A and C). There are also regions with small numbers of individual CD20+ cells (Figure 15B). It is possible that this signal could be consistent with drawing B cells to crypts. However, by performing two-color immunofluorescent staining for CXCL13 and CD20 in the intestine, we found CD20+ cells were not localized immediately adjacent to CXCL13 producing Paneth cells. CXCL13 (displayed in green) appears to be secreted into the lumen and contained within the granules of Paneth cells, whereas the CD20+ (displayed in red) B cells are located in the lamina propria (Figure 15E). CXCL13 and CD20 does appear to colocalize in follicular regions and we see overlapping green and red signals (Figure 15F).

These results provide evidence that support our hypothesis that CXCL13 produced by Paneth cells is functioning as an AMP and not particularly to direct migration of B cells that may contain its receptor CXCR5. T cells also express the CXCL13 receptor CXCR5 and it is possible those cells could be responding to CXCL13 in this microenvironment if it were functioning as a lymphoid chemokine, however, the presence of T cells was not examined in these studies.

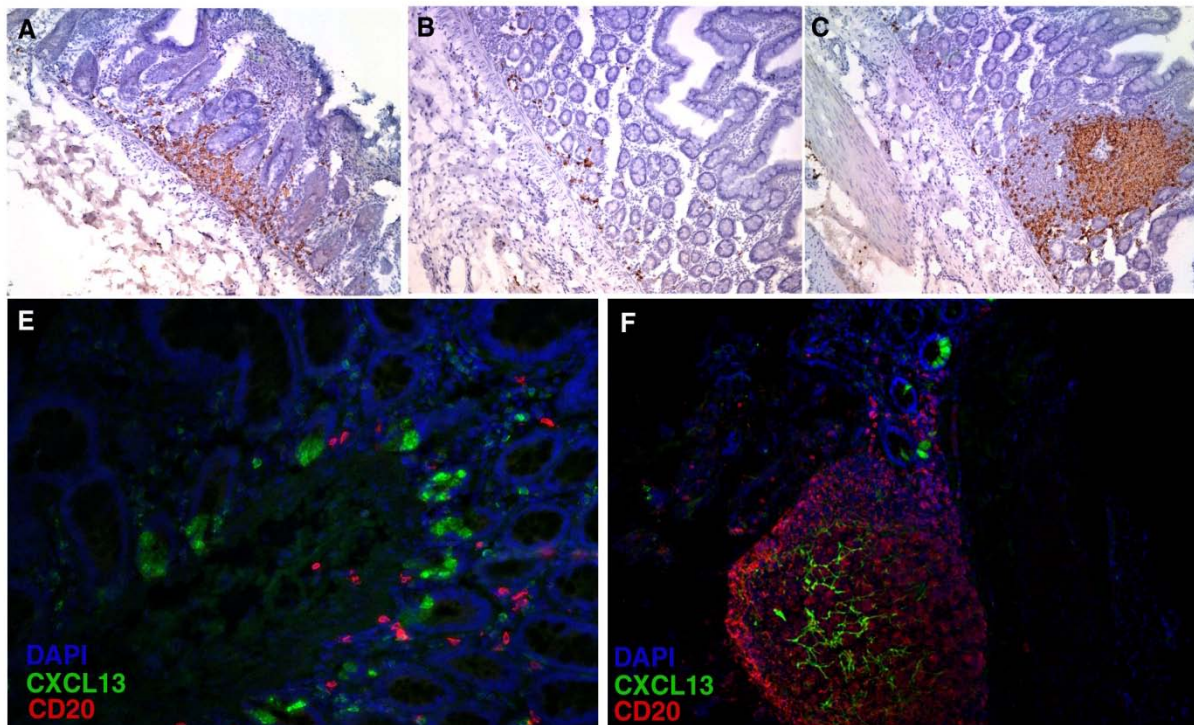


Figure 15. CXCL13 and mature B cells do not colocalize in Paneth cells in the ileum.

A-C. Immunohistochemistry detection of CD20 in the macaque ileum E-F. immunofluorescent detection of CXCL13 and CD20 protein in macaque ileum Paneth cells and ileum follicle. Isotype controls (not shown) were clear and contained no signals. (Original magnification = x200)

To determine if expression of CXCL13 in Paneth cells was unique to cynomolgous macaques, we explored avenues to detect its expression in other species of animals. We utilized IHC and ISH to look for CXCL13 mRNA expression in multiple compartments of uninfected

rhesus macaque and murine intestinal tissues. In the rhesus macaque, CXCL13 expression also was found in both lymphoid follicles and in Paneth cells (Figure 16).

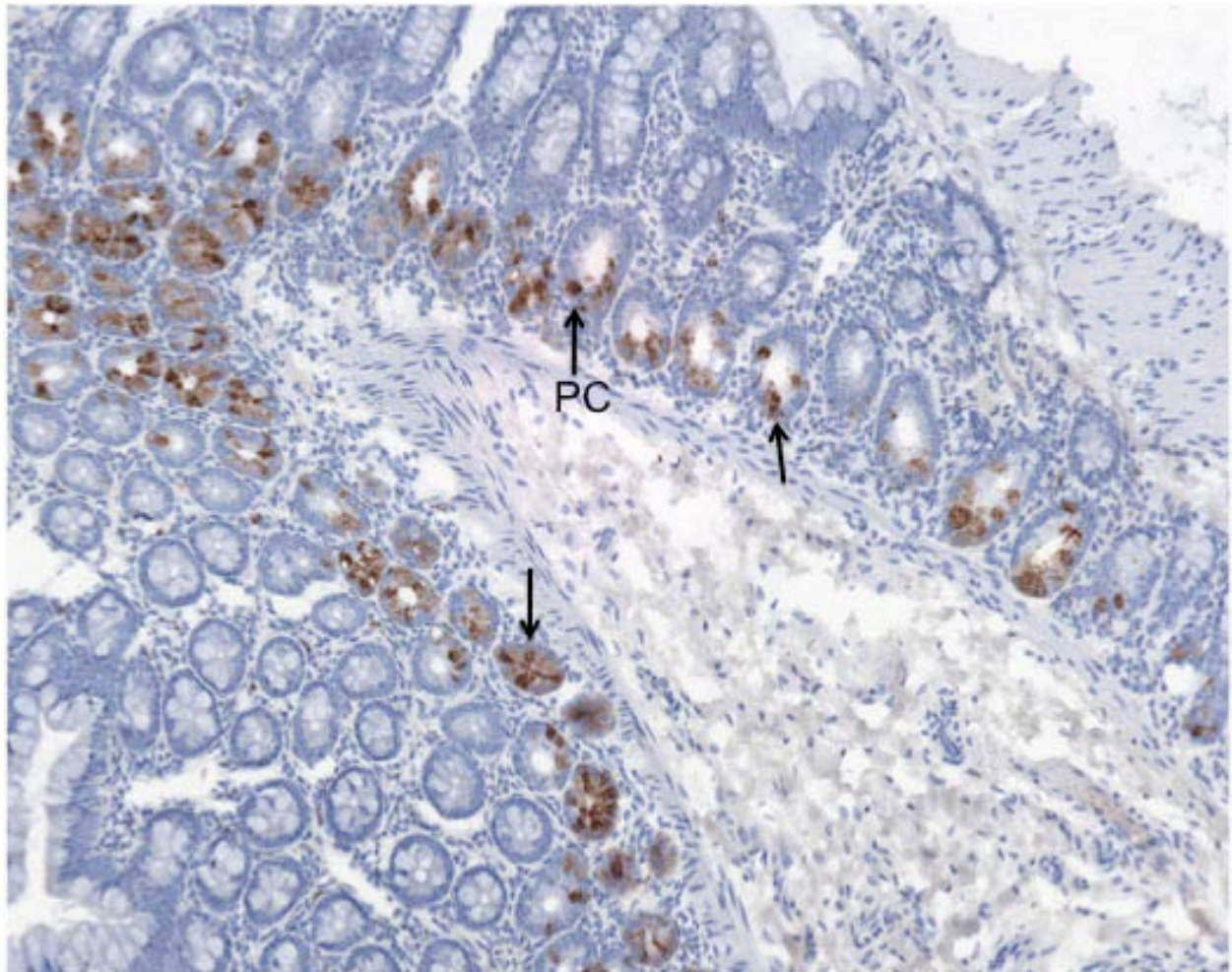


Figure 16. CXCL13 protein expression in the rhesus macaque small intestine.

Immunohistochemistry detection of CXCL13 protein in the rhesus macaque ileum. Isotype controls (not shown) were clear and contained no signals. (Original magnification = x100; PC and arrows denote Paneth cells in crypts)

In mouse intestinal tissues, however, CXCL13 expression was only observed in GALT and not Paneth cells (Figure 17). These findings indicate there are differences in gut immunity and host defenses across species. Several factors, including disparities in the gut microbiome could drive the selective expression of a homeostatic chemokine in Paneth cells of NHPs but not in other

small animal species, although examination of a larger number of species will need to be performed.

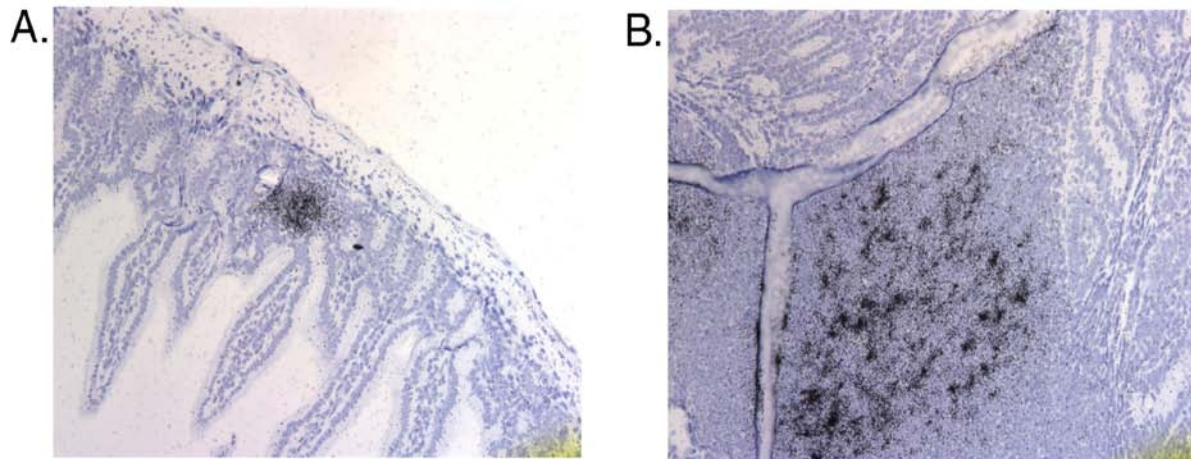


Figure 17. CXCL13 mRNA is not present in Paneth cells in the murine small intestine.

In situ hybridization detection of CXCL13 mRNAs in uninfected mouse A. ileum epithelial and B. ileum lymphoid tissues. Sense controls (not shown) were clear and contained no signals. (Original magnification = x200)

4.5 PANETH CELLS CONTAIN A LARGER REPERTOIRE OF ANTIMICROBIAL PEPTIDES THAN PREVIOUSLY DESCRIBED

Paneth cells produce AMPs in the crypts of Lieberkuhn to protect the intestinal epithelium and local stems cells from infection. There are several classifications of AMPs that have been described, including defensins, cathelicidins, and lectins. Given the clear expression of CXCL13 by Paneth cells, strongly suggesting it has antimicrobial functions in the intestine, we next examined whether other AMPs not previously recognized as expressed by Paneth cells are also produced them. We chose to evaluate a member of several types of AMPs, including a β -defensin (BDEF2), a θ -defensin (RTD-1), a cathelicidin (LL-37), a lectin (Reg3 γ), as well as

other factors that are not typically thought of as antimicrobial peptides, yet have antimicrobial properties. These would include Lcn2 and chemerin. Although both DEFA5 and DEFA6 are AMPs produced by Paneth cells, β -defensins have not been localized to these specialized cells. Unexpectedly, β -defensin 2 (BDEF2) mRNA producing cells were localized to intestinal crypts (Figure 18A) and simultaneous ISH for BDEF2 mRNA and IHC for CXCL13 protein revealed co-localization to the same cells in crypts (Figure 12E). Additionally, we found rhesus θ -defensin 1 (RTD-1) expressed specifically in intestinal crypts, similar to DEFA5 and BDEF2 (Figure 18B). In contrast to what was previously known about BDEF2 and RTD-1, macaques are known to express Reg3 γ as an AMP^{89,155}, and it is expressed by Paneth cells in response to Gram-positive bacteria^{89,155}. By using ISH we found Reg3 γ was indeed expressed in crypts throughout the small intestine but was absent from the colon (Figure 18C). In summary, these findings provide the first evidence that in macaques BDEF2 and RTD-1 are expressed by Paneth cells, and expand our understanding of the repertoire of AMPs used by these cells to protect the crypts and shape the intestinal milieu.

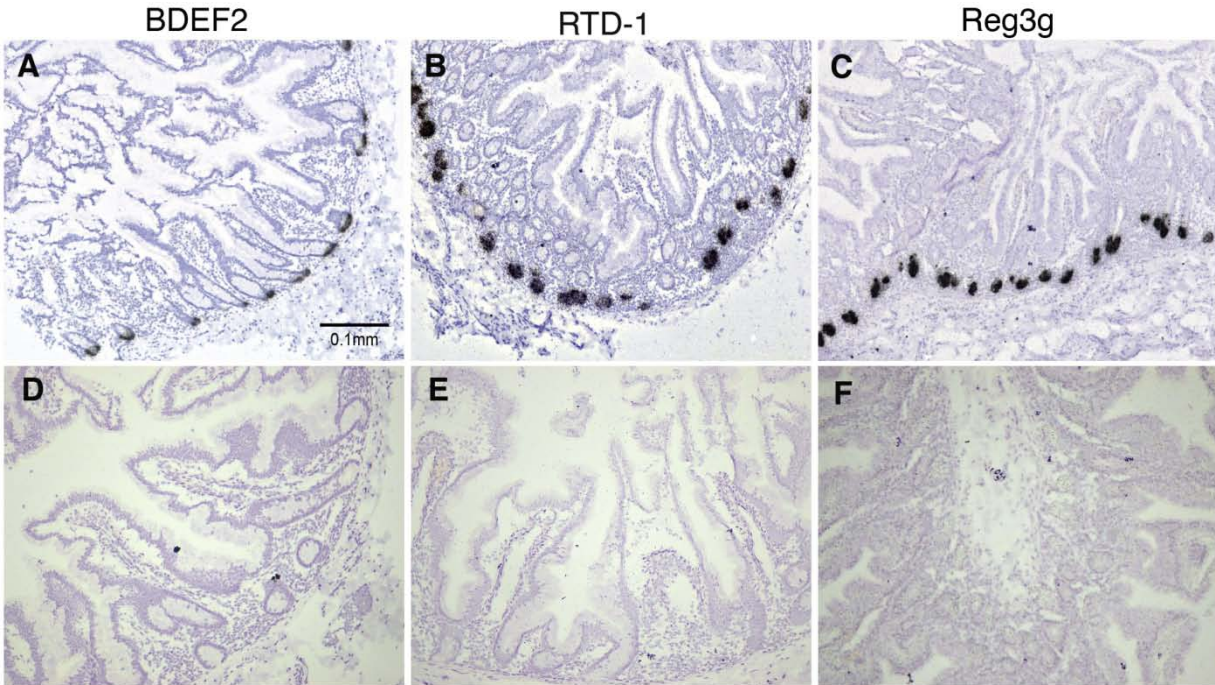


Figure 18. β -defensin 2, θ -defensin 1, and Reg3g are localized to Paneth cells in the cynomolgous macaque ileum.

In situ hybridization detection of A. BDEF2, B. RTD-1 and C. Reg3 γ mRNAs in cynomolgous uninfected macaque ileal tissues. D-F. Sense controls contained no signal. (Original magnification = 100X)

In our efforts to identify AMPs, expressed by Paneth cells, we used ISH to examine expression of DEFA1 in the small and large intestine. We found DEFA1 expression by cells in the crypts of the small intestine (Figure 19). We believe this suggests Paneth cells produce DEFA1. DEFA5 and DEFA6 are the only known α -defensins expressed by Paneth cells and our findings provide evidence that the repertoire of AMPs produced by Paneth cells is much larger than previously described.

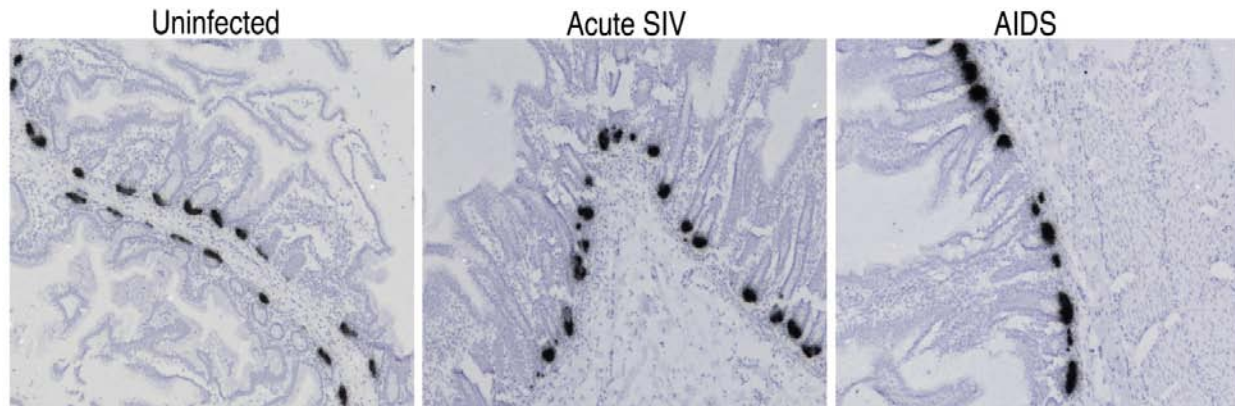


Figure 19. Cynomolgous macaque α -defensin 1 is localized to Paneth cells in the ileum.

In situ hybridization detection of DEFA1 mRNAs in cynomolgous uninfected (left image), acute SIV-infected (center image), and AIDS developing (right image) macaque ileal tissues. Sense controls contained no signal (not shown) (Original magnification = 100X)

The presence of some REDs in the small intestine has been established (Tanabe 2004). Our laboratory cloned and sequenced confirmed cynomolgous macaque DEFA6 and DEFA1. To confirm whether the cynomolgous macaque DEFA6 and DEFA1 sequences we used as probes in our studies were the same or distinct from the previously published macaque RED sequences, we used phylogenetic analyses to compare the published RED and human α -defensin sequences to our cynomolgous macaque α -defensin sequences (ORFs only). Based on these analyses, DEFA6, used here, sequence aligns and phylogenetically groups with the RED6 sequence previously published by Tanabe *et al.* (Figure 20). Interestingly, our DEFA1 cynomolgous/rhesus sequence was not identical to any of the previously published RED sequences. It did, however, align and closely cluster with the human α defensins 1 and 3 (Figure 19). This finding suggests that we were able to identify a novel alpha defensin not previously identified in cynomolgous macaque Paneth cells. These findings again suggest that Paneth cells express a larger repertoire of AMPs than previously predicted and another α -defensin, other than DEFA5 and DEFA6, is expressed by Paneth cells in the cynomolgous macaque.

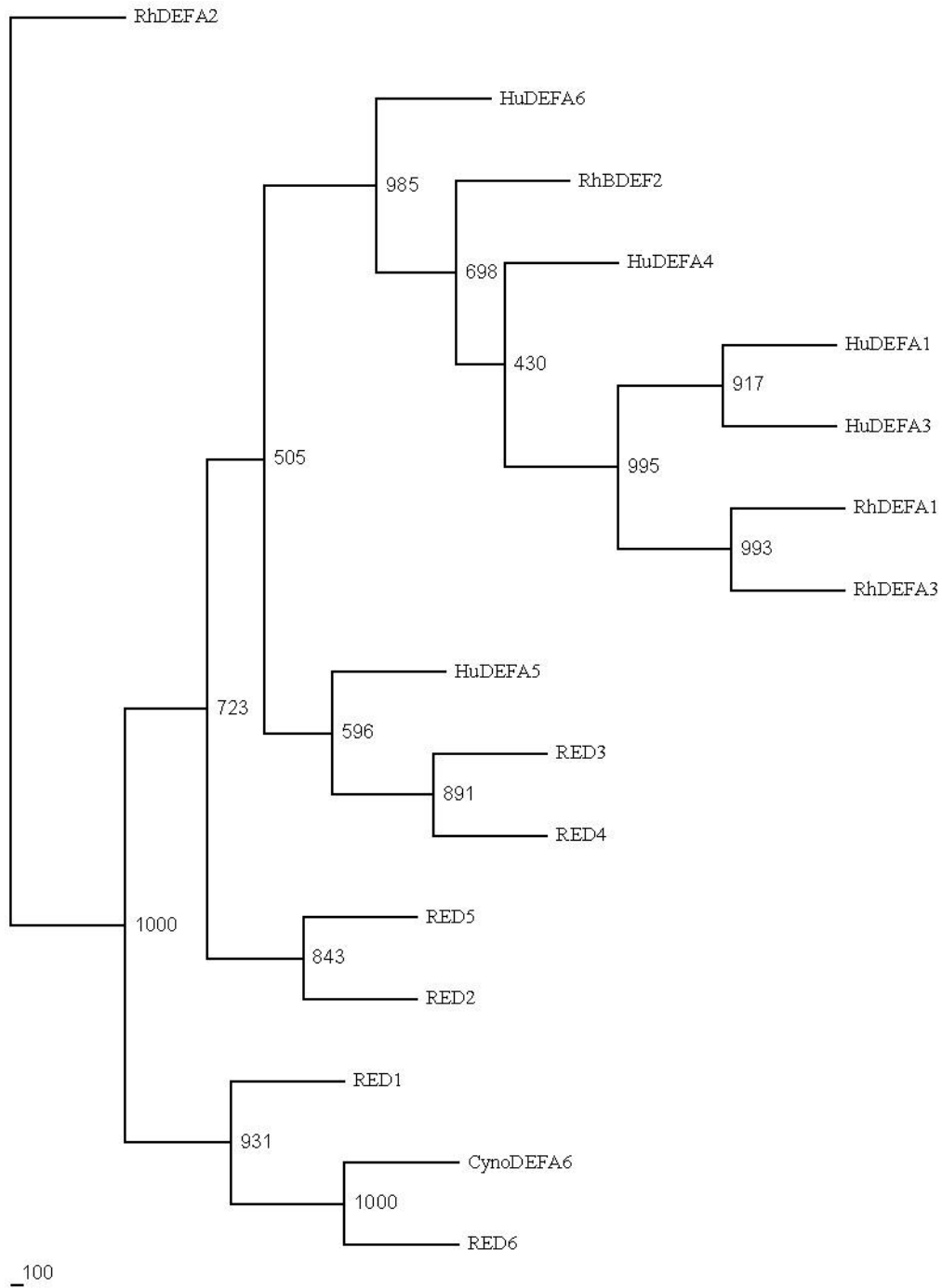
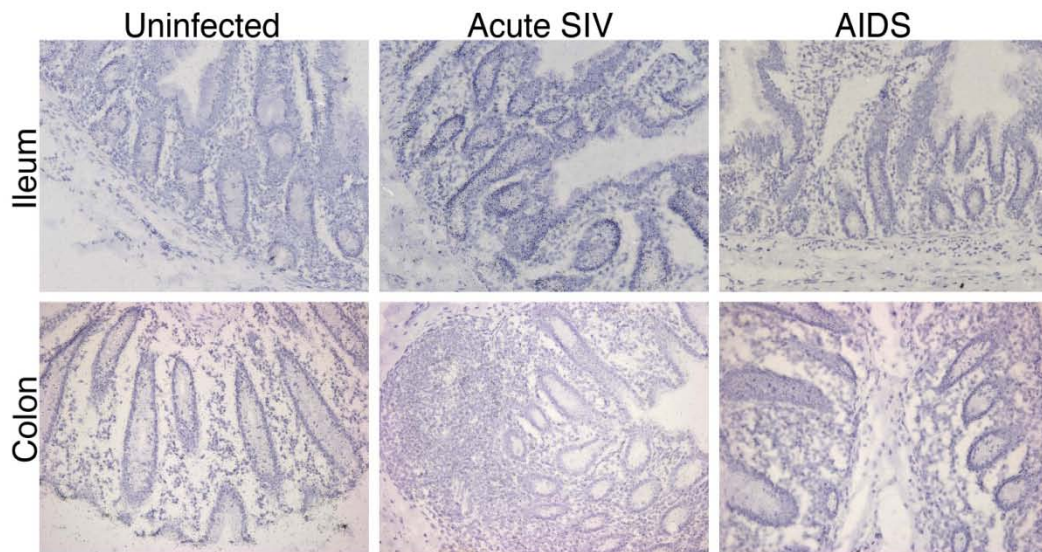


Figure 20. Phylogenetic relationships among human, rhesus macaque, and cynomolgous macaque defensins.

RED, human, rhesus, and cynomolgous α -defensin sequence clusters are shown. Bootstrap values are shown at the corresponding nodes.

Chemerin and Lcn2 are not traditionally described as AMPs but they do possess antimicrobial properties that could aid in host defense mechanisms. In our studies we found AMPs not previously seen expressed by Paneth cells, therefore, we assessed additional targets including chemerin and Lcn2. Chemerin is similar in structure to cathelicidins and has activity against *E. coli* and *K. pneumoniae*. By ISH we determined whether Paneth cells express these peptides and determined if there were changes to mRNA expression levels in uninfected, acutely SIV-infected, and AIDS-developing macaque ileal and colon tissues. ISH did not reveal Paneth cells as a producer cell type for chemerin. There were low levels of intestinal epithelial expression of chemerin in the ileum and colon, although those levels did not appear to change as a function of SIV infection (Figure 21). Lcn2 binds to siderophores and prevents iron sequestration by bacteria, affording it a more direct antimicrobial role compared to chemerin. We also evaluated changes to Lcn2 expression in the ileum and colon of SIV-infected tissues by ISH and compared them to uninfected tissues. Based on the mRNA expression pattern in ileum Lcn2 is expressed by epithelial cells in the crypts, but its distribution is more widespread indicating that expression is not limited to Paneth cells (Figure 21).

A. Chemerin



B. Lipocalin 2

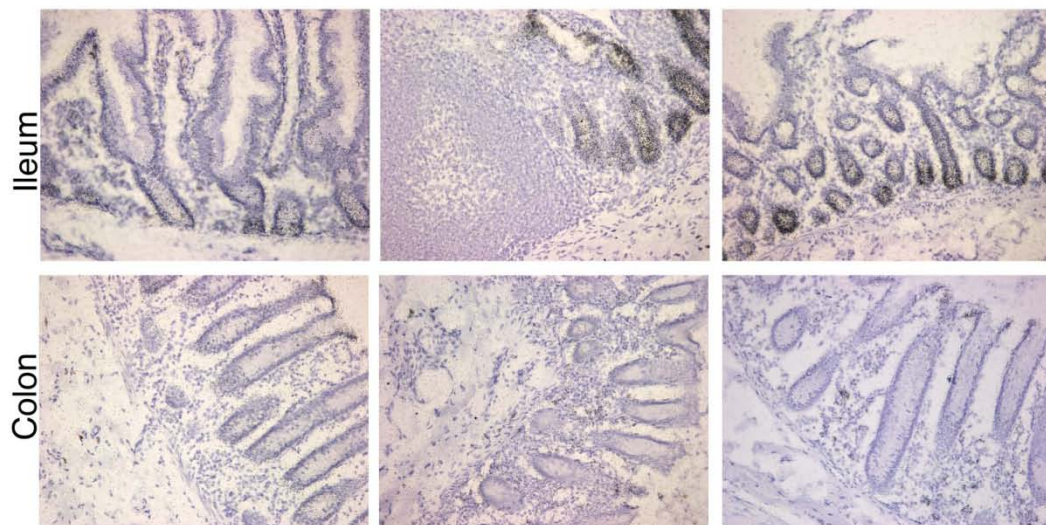


Figure 21. Expression patterns of chemerin and Lcn2 mRNAs in the cynomolgous macaque ileum and colon.

In situ hybridization detection of A. chemerin and B. Lcn2 mRNAs in cynomolgous uninfected, acute SIV-infected, and AIDS developing macaque ileal (top images) and colon (bottom images) tissues. Sense controls contained no signal (not shown) (Original magnification = 100X)

4.6 AMTIMICROBIAL PEPTIDE EXPRESSION DURING SIV INFECTION

To determine whether SIV infection impacted levels of expression of Paneth cell AMPs, including CXCL13, we examined tissues from animals in different stages of SIV infection. Admittedly these intestinal tissues were fixed and cryopreserved without attention at the time to tissue orientation and interest in Paneth cells, so we were not able to reliably obtain a uniform representation of crypts and Paneth cells for quantitative analysis of tissue sections. However, at a qualitative level, there were no obvious differences in the intensities or distributions of signals for CXCL13, DEFA6 and BDEF2 of the ISH or IHC data in intestinal crypts. Uninfected animals showed robust expression of CXCL13, DEFA6, BDEF2, RTD-1, and Reg3 γ mRNAs. ISH data for DEFA1, RTD-1 and Reg3 γ show moderate changes to the levels of signal intensities across disease states (Figure 17 and Figure 22). RTD-1 is decreased in tissues of acutely-infected SIV macaques as compared to uninfected and AIDS-developing macaques. In contrast, DEFA1 and Reg3 γ appears to increase to a small extent during acute-infection increases further during AIDS (Figure 19 and Figure 22).

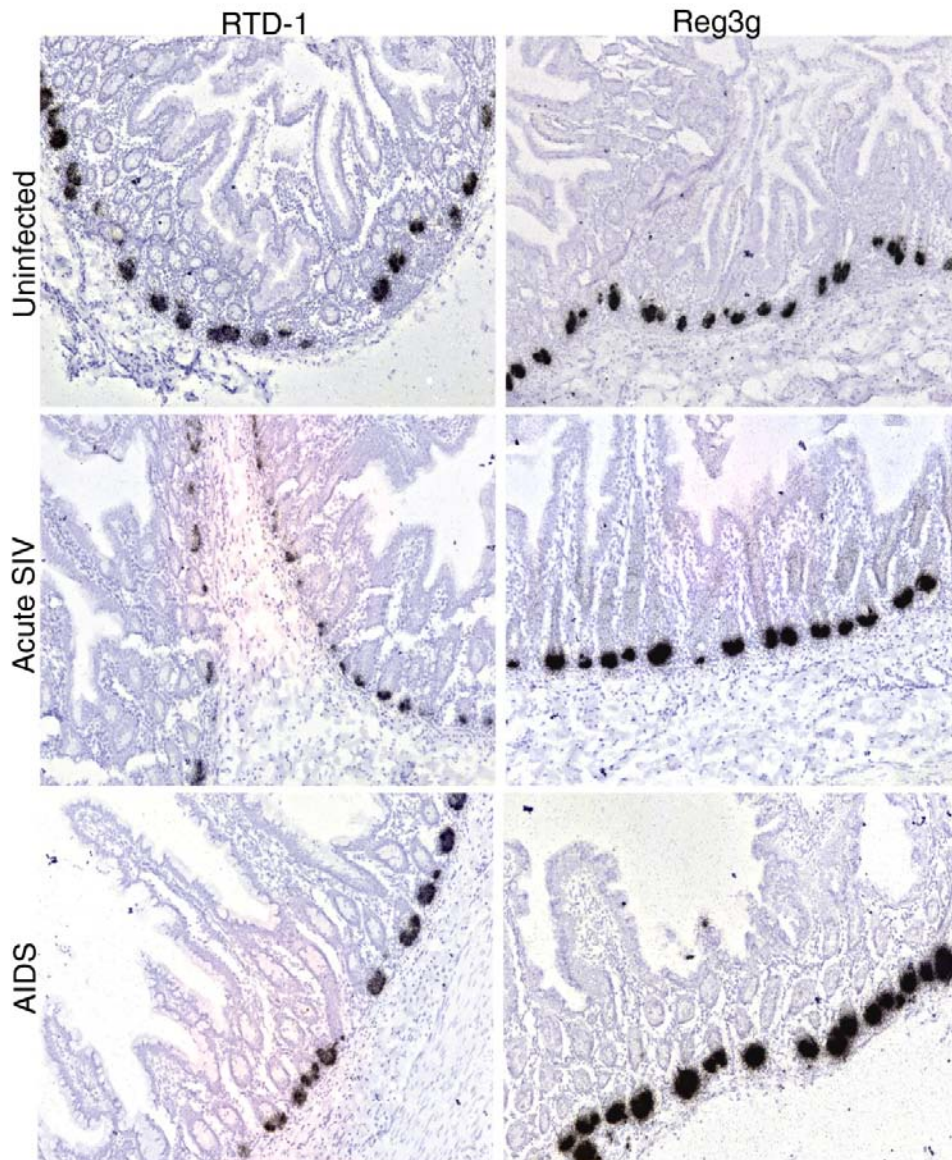


Figure 22. θ -defensin 1 and Reg3g mRNAs are localized to Paneth cells in the cynomolgous macaque ileum.

In situ hybridization detection of RTD-1 (left images) and Reg3 γ (right images) mRNAs in cynomolgous uninfected, acute SIV-infected, and AIDS developing macaque ileal tissues. Sense controls contained no signal (not shown) (Original magnification = 100X)

Multiple attempts to use a commercially available real-time RT-PCR assay for macaque *cxcl13* mRNA, and to develop an assay in-house, failed to meet our quality control expectations, (meaning that the shapes of the amplification plots were different between different animals).

Therefore, accurate measurement of *cxc113* mRNA levels in lymphoid and intestinal tissue RNA preparations was not achieved. Measurements of *cxc113* mRNA levels in RNA preparations from homogenized intestinal tissues additionally were complicated by the inability to distinguish the contributions of signals from lymphoid follicles versus from Paneth cells. In contrast, the real-time RT-PCR assay for DEFA6 was robust and DEFA6 was expressed uniquely by Paneth cells. Using this assay we found that the levels of expression for this AMP increased approximately 10-fold during acute infection and AIDS in the ileum (Figure 23A). This increase in defensin expression is consistent with a recent report that SIV infection leads to induction of rhesus enteric defensin (RED).¹⁵⁰ In support of this, correlation analyses show a positive correlation between SIV viral loads and DEFA6 mRNA ($r^2 = 0.53$, p value = 0.04) (Figure 23B).

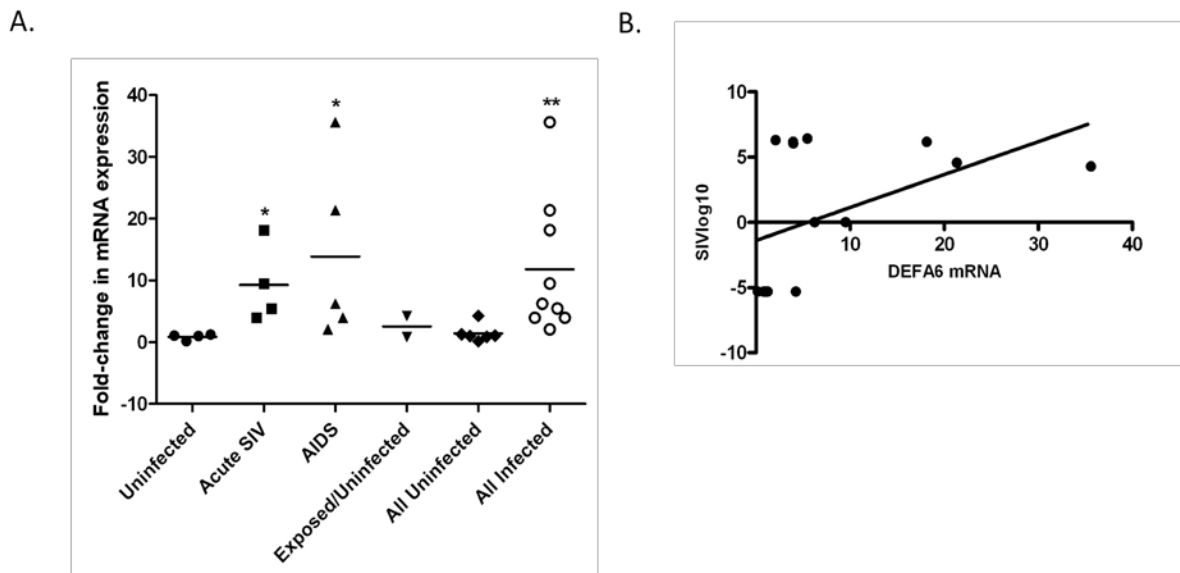


Figure 23. Ileal induction of DEFA6 mRNA during SIV infection is correlated with SIV plasma viral loads.

A. DEFA6 mRNA levels were measured in the indicated tissue by using real-time RT-PCR. B. Correlation analyses of DEFA6 mRNA and plasma SIV viral loads (*) denotes statistical significance (p-value <0.05) relative to uninfected animals (Mann-Whitney non-parametric test). Significant positive correlation was determined by Spearman's non-parametric correlation analyses. Individual animal's expression values were normalized to a β -GUS endogenous control and then normalized to an uninfected control "calibrator" sample.

Using real-time RT-PCR, Lcn2 mRNA levels were also measured and revealed that there were no significant differences in the expression after SIV infection, although the highest expression was observed in a subset of SIV-infected macaques (Figure 24).

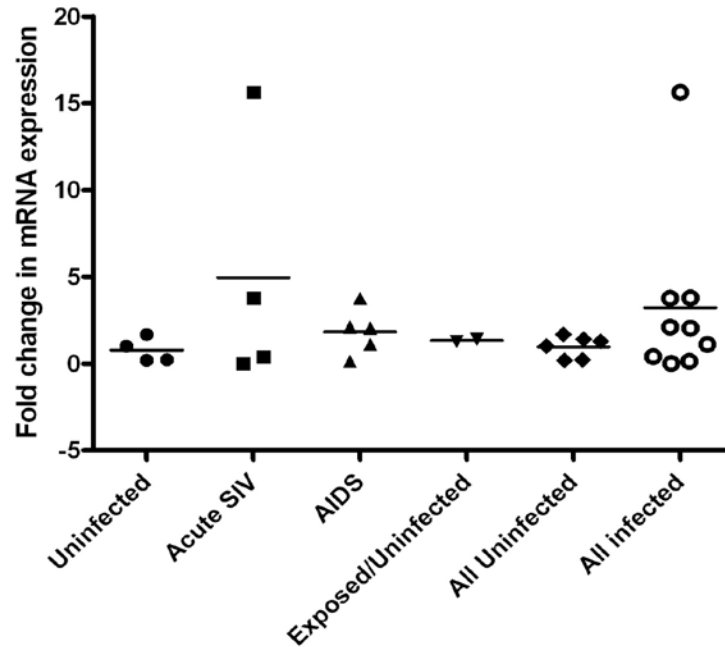


Figure 24. Measurement of Lcn2 mRNA expression in cynomolgous macaque ileum tissues.

Lcn2 mRNA levels were measured in the indicated tissue by using real-time RT-PCR. The mean fold-changes in expression relative to an uninfected calibrator sample are shown. All p-values were > 0.05 by Mann-Whitney non-parametric analyses. Individual animal's expression values were normalized to a β -GUS endogenous control and then normalized to an uninfected control "calibrator" sample.

LL-37 is a well-known human cathelicidin that has a defined antimicrobial function.^{156,157}

It has broad antibacterial activity against both Gram (+) and Gram (-) bacteria. We determined whether Paneth cells express this specific AMP and whether there were changes to its expression levels after SIV infection in SIV-infected animals compared to those that were uninfected. Based on real-time RT-PCR analysis of ileum, there were no significant differences in acute SIV-infected or AIDS-developing macaques compared to their uninfected counterparts (Figure 25).

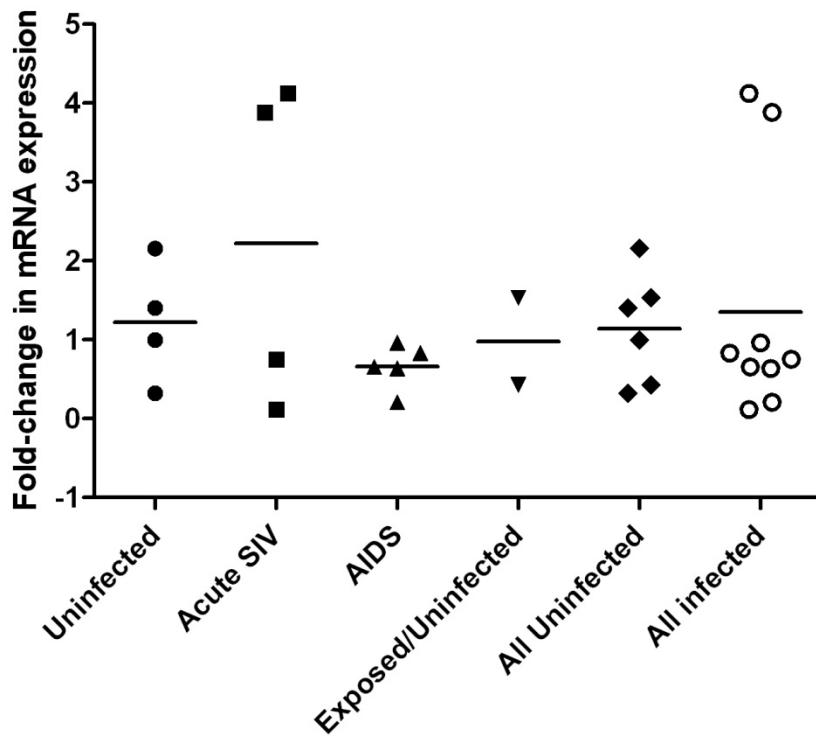


Figure 25. Measurement of LL-37 mRNA expression in cynomolgous macaque ileum tissues.

LL-37 mRNA levels were measured in the indicated tissue by using real-time RT-PCR. The mean fold-changes in expression relative to an uninfected calibrator sample are shown. All p-values were > 0.05 by Mann-Whitney non-parametric analyses. Individual animal's expression values were normalized to a β -GUS endogenous control and then normalized to an uninfected control "calibrator" sample.

4.7 PANETH CELL MARKERS AND SIV INFECTION

Paneth cells aid in host defense and immunity by protecting the gastrointestinal tract from bacterial pathogens. Upon stimulation by potentially invading bacteria, they secrete AMPs into the crypt. After discovering CXCL13 expression in macaque Paneth cells, we asked whether Paneth cell phenotype was affected by SIV infection. To address this, we evaluated changes in protein and/or mRNA expression of multiple commonly known antimicrobial factors generated

by Paneth cells. We also determined whether SIV infection had effects on these factors by evaluating their expression levels across disease states. To accomplish this we measured expression levels of Tryp2, NOD2, TLR9, IL-17, and ROR γ t.

In mice, matrilysin processes AMPs produced by Paneth cells.¹⁵⁸ In humans this function belongs to trypsin 2 (Tryp2).¹⁵⁸ It has not been determined whether macaques also express Tryp2 in Paneth cells, thus attributing this protease the likely function of processing AMPs in macaques. ISH was used to determine if macaques also express Tryp2 in the small intestine. Tryp2 was not only expressed in the ileum of the small intestine, but it was also expressed by Paneth cells (Figure 26). This finding suggests that Tryp2 processes AMPs in macaques similarly to humans.

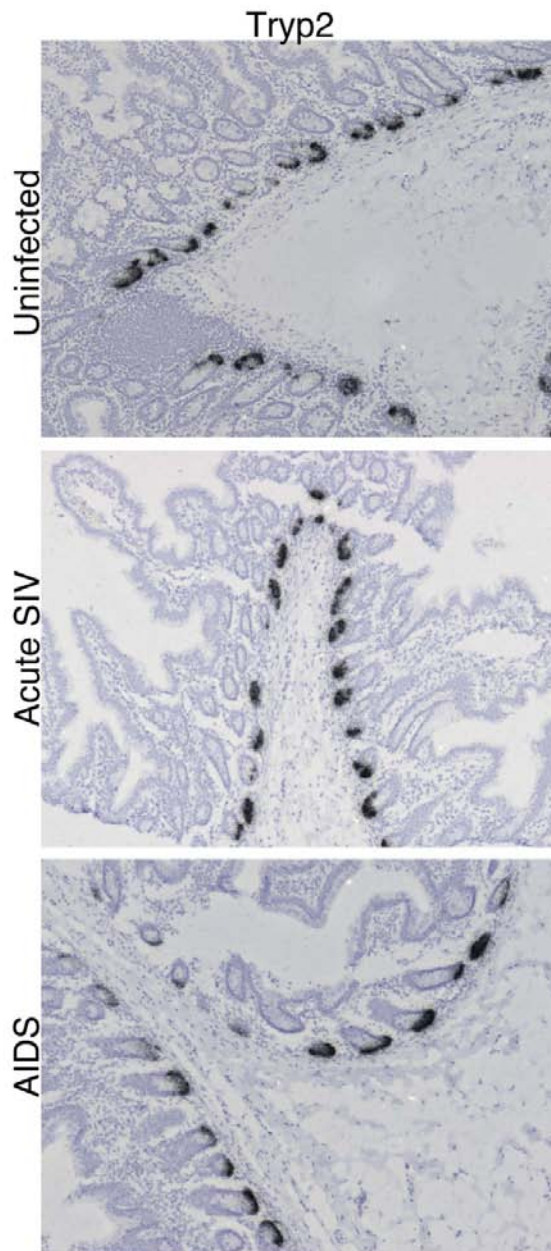


Figure 26. Trypsin 2 is expressed in Paneth cells in the cynomolgous macaque ileum.

In situ hybridization detection of Tryp2 mRNAs in cynomolgous uninfected, acute SIV-infected, and AIDS developing macaque ileal tissues. Sense controls contained no signal (not shown) (Original magnification = 100X)

TLR9 acts as a sensor to CPG DNA and in the small intestine stimulation of this TLR causes degranulation of Paneth cells.⁸⁷ To determine if macaque Paneth cells express TLR9 and whether there are modification to TLR9 expression in Paneth cells after SIV infection, we

attempted to use IHC and real-time RT-PCR to measure possible changes to TLR9 protein or mRNA expression. Immunostaining for TLR9 proved unsuccessful in our tissues, however, in homogenized small intestinal tissue RNA preparations, real-time RT-PCR revealed that there was a significant decrease in TLR9 expression in AIDS-developing macaques compared to uninfected animals (Figure 27), suggesting that SIV infection might influence Paneth cell phenotype. Chronic immune activation in pathogenic SIV infection has been associated with microbial translocation.¹⁴⁰ A reduction in TLR9 during AIDS could indicate a possible contribution to the intestinal enteropathy and microbial translocation that occurs during infection.

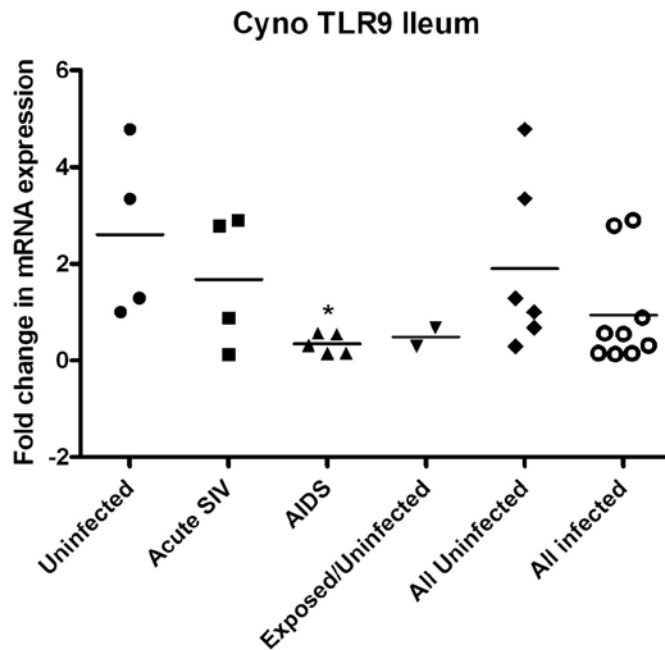


Figure 27. TLR9 mRNA expression is significantly reduced during SIV infection in cynomolgous macaque ileum tissues.

TLR9 mRNA levels were measured in the indicated tissue by using real-time RT-PCR. The mean fold-changes in expression relative to an uninfected calibrator sample are shown. (*) denotes statistical significance (p -value <0.05) relative to uninfected animals (Mann-Whitney non-parametric test). Individual animal's expression values were normalized to a β -GUS endogenous control and then normalized to an uninfected control "calibrator" sample.

Paneth cells likely detect pathogens via multiple PRRs, one of those being NOD2. NOD2 senses bacterial peptidoglycan MDP, LPS, and modifications to the *nod2* gene are associated with chronic inflammatory disease of the small intestine.^{159,160} Given the chronic inflammation in the gut during SIV infection, we sought to determine whether there were changes in the expression levels of NOD2 in SIV-infected macaques compared to uninfected animals. Attempts to determine differences in expression of NOD2 by immunohistochemical analyses were unsuccessful and real-time RT-PCR showed no differences between uninfected, acute SIV-infected or AIDS developing macaques (Figure 28).

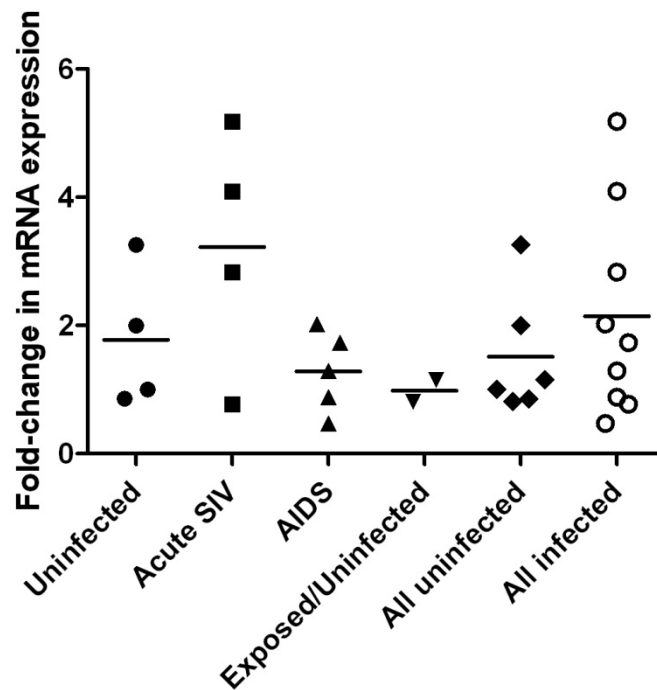


Figure 28. Measurement of NOD2 mRNA expression in cynomolgous macaque ileum tissues.

NOD2 mRNA levels were measured in the indicated tissue by using real-time RT-PCR. The mean fold-changes in expression relative to an uninfected calibrator sample are shown. All p-values were > 0.05 by Mann-Whitney non-parametric analyses. Individual animal's expression values were normalized to a β -GUS endogenous control and then normalized to an uninfected control "calibrator" sample.

We also examined changes in cell proliferation of the gut by using IHC and staining for Ki67 positive cells in the small and large intestine of uninfected, acute SIV-infected, and AIDS-developing macaques (Figure 29). Our results showed no significant changes in cell proliferation across disease states or across regions of the gut.

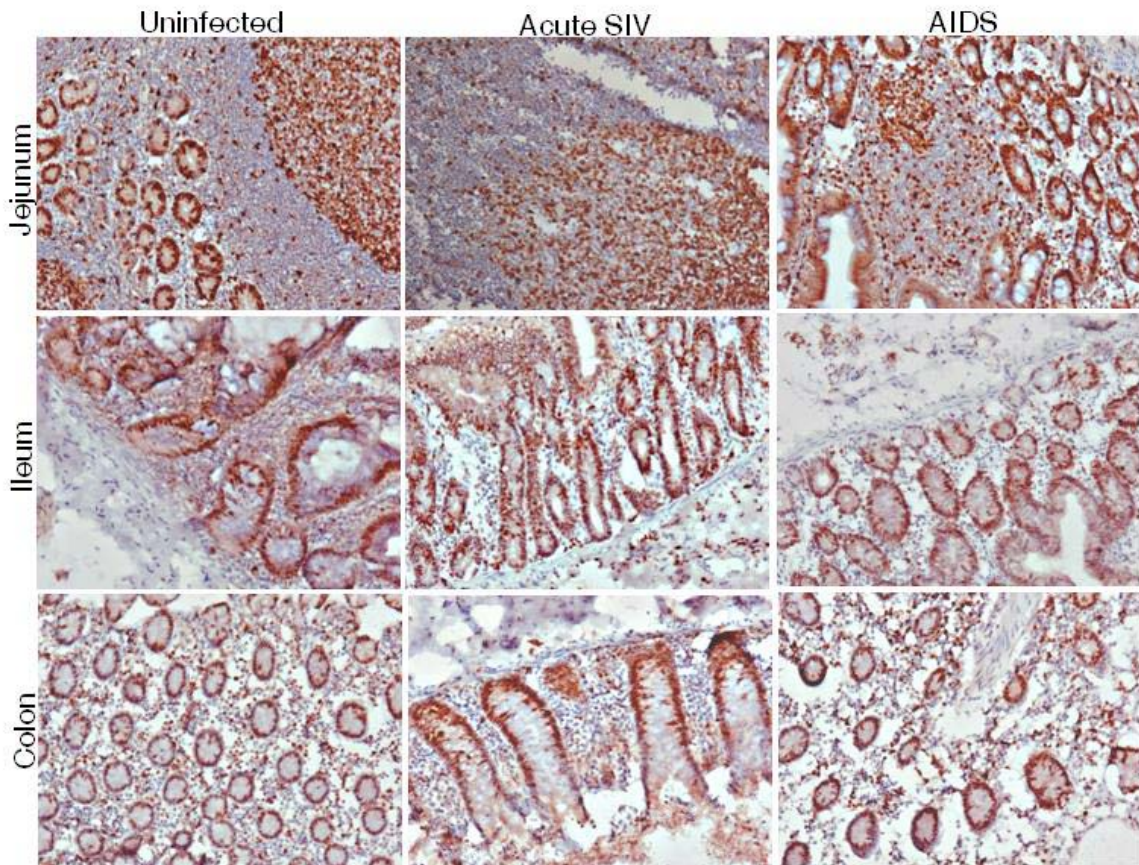


Figure 29. Cell proliferation in the cynomolgous macaque small intestine.

Immunohistochemistry detection of KI67 protein in the macaque jejunum (top row), ileum (center row), and colon (bottom row) in uninfected (left column), acutely SIV-infected (center column), and AIDS-developing tissues (right column). Isotype controls (not shown) were clear and contained no signals. (Original magnification = x100)

To examine the potential for microbial translocation in our study animals, we attempted to identify *E. coli* in the colon of uninfected, acute SIV-infected and AIDS-developing macaques (data not shown). Due to issues with assay optimization we could not determine whether there

were changes in the levels of *E. coli* across disease states, however, we did not find any *E. coli* in any of our tissues that appeared to have translocated from the lumen into the lamina propria.

E-cadherin is a cell-to-cell adhesion glycoprotein present in the small intestine and colon encoded by the *Cdh1* gene. In mice, knocking out this gene results in changes in Paneth cell phenotype. Maturation of Paneth cells is inhibited, AMP production is reduced, and clearance of pathogenic bacteria from the lumen is prevented in *Cdh1*^{-/-} mice.¹⁶¹ Therefore as a measure of intestinal epithelial integrity, we sought to determine changes in macaque e-cadherin expression (Figure 30). IHC analyses of this protein in macaque intestinal tissues showed staining of E-cadherin along the epithelium down into the crypts of the ileum, however it did not show any discernible changes in SIV-infected ileum compared to uninfected controls.

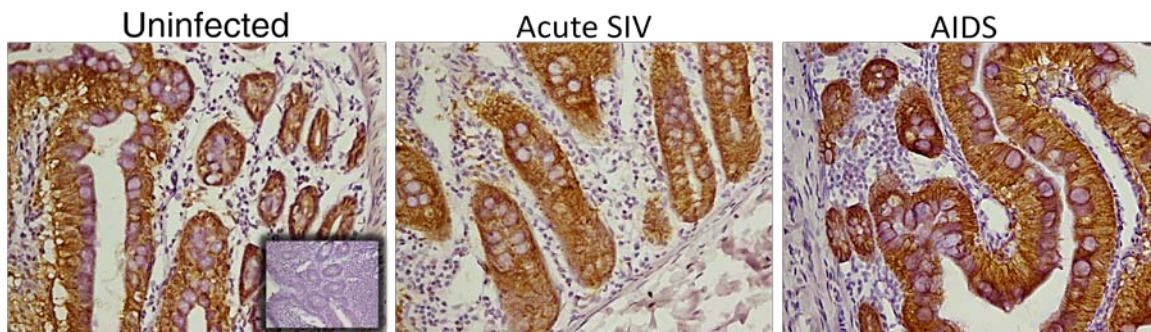


Figure 30. E-cadherin protein expression in the cynomolgus macaque small intestine.

Immunohistochemistry detection of e-cadherin protein in the cynomolgus macaque ileum uninfected, acutely SIV-infected, and AIDS-developing tissues. Isotype controls (not shown) were clear and contained no signals. (Original magnification = x100)

In an investigation of the contributions of IL-17 to the systemic inflammatory response syndrome caused by tumor necrosis factor (TNF), it was discovered, in mice, that Paneth cells produce IL-17.¹⁶² This established a possible new role for IL-17 during an acute inflammatory response, beyond production by Th17/Tc17 cells, by showing TNF stimulation results in the release of IL-17 by Paneth cells. Therefore, we explored whether SIV infection alters IL-17

mRNA levels in the small intestine. Real-time RT-PCR did not show any significant differences in SIV-infected intestinal tissues compared to uninfected controls (Figure 31). However, both the acutely SIV-infected and AIDS-developing macaque groups contained animals that had mRNA expression with a 4-fold increase than any of the uninfected animals. Given that IL-17 secretion is also regulated by the presence of T helper 17 cells (Th17) we simultaneously examined whether there were any changes to the Th17 population by measuring RORgt mRNA levels. Again, we found no significant changes to RORgt levels in the ileum of SIV-infected animals (Figure 32).

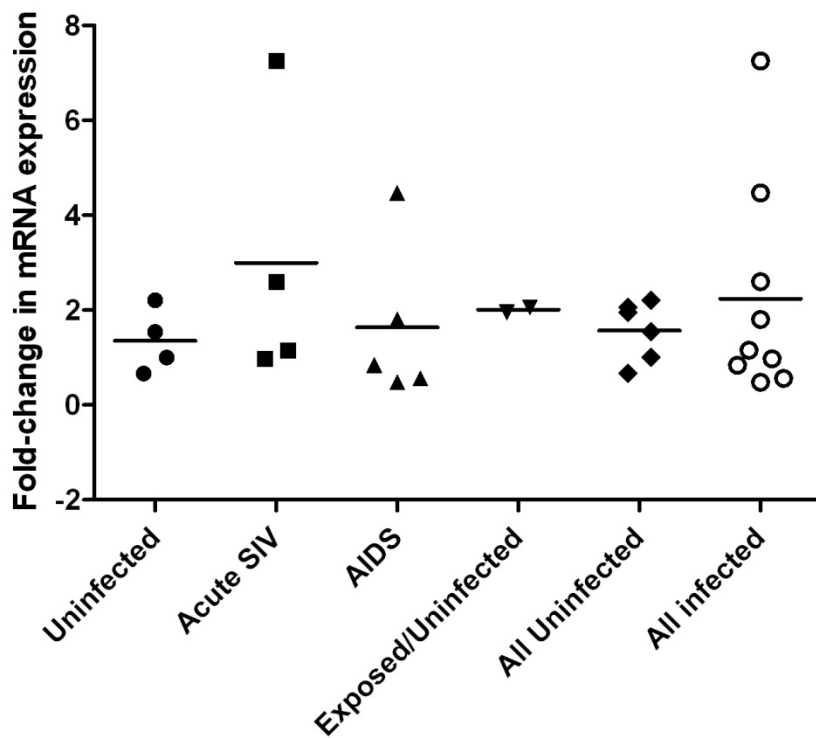


Figure 31. Measurement of IL-17 mRNA expression in cynomolgous macaque ileum tissues.

IL-17 mRNA levels were measured in the indicated tissue by using real-time RT-PCR. The mean fold-changes in expression relative to an uninfected calibrator sample are shown. All p-values were > 0.05 by Mann-Whitney non-parametric test. Individual animal's expression values were normalized to a β -GUS endogenous control and then normalized to an uninfected control "calibrator" sample.

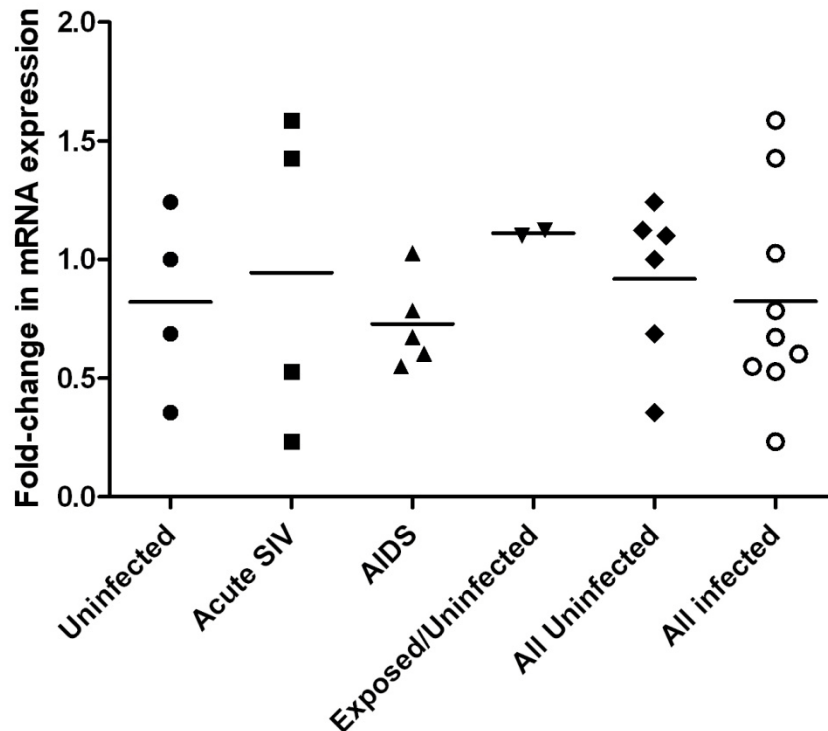


Figure 32. Measurement of RORgt mRNA expression in cynomolgous macaque ileum tissues.

RORgt mRNA levels were measured in the indicated tissue by using real-time RT-PCR. The mean fold-changes in expression relative to an uninfected calibrator sample are shown. All p-values were > 0.05 by Mann-Whitney non-parametric test. Individual animal's expression values were normalized to a β -GUS endogenous control and then normalized to an uninfected control "calibrator" sample.

Finally, we evaluated possible relationships between SIV viral loads and AMPs (Table 6). Using parametric and non-parametric correlation analyses measurements we determined several significant associations. Our results show a significant Pearson's correlation between IL-17 and DEFA6, Lcn2, and NOD2. We also found significant levels of NOD2 with detectable higher levels of Lcn2, LL-37, and TLR9. There were also positive correlations between levels of Lcn2 and LL-37. By measuring correlations via Spearman rank correficient test, we found a positive correlation between SIV PVL and DEFA6 (Figure 23), as well as between TLR9 and

LL-37 and NOD2. IL-17 was once again found correlated with Lcn2 and NOD2. Lastly, we found NOD2 positively correlated with levels of Lcn2.

Table 6. Correlation analyses of the relationships amongst SIV and antimicrobial peptides.

	SIV log10	DEFA6	TLR9	IL-17	RORgt	LL-37	Lcn2	NOD2
SIV log10		0.47	-0.25	0.20	0.04	0.22	0.33	0.28
DEFA6	0.53 [#]		-0.17	0.64 [*]	-0.33	0.12	0.47	0.18
TLR9	-0.29	-0.19		0.27	0.00	0.43	0.22	0.70 [*]
IL-17	-0.18	0.32	0.41		-0.28	0.45	0.91 [*]	0.59 [*]
RORgt	0.01	-0.38	-0.21	-0.13		0.02	-0.36	-0.20
LL-37	0.01	0.12	0.60 [#]	0.31	-0.13		0.52 [*]	0.68 [*]
Lcn2	0.16	0.42	0.19	0.68 [#]	-0.48	0.32		0.65 [*]
NOD2	0.25	0.21	0.67 [#]	0.51 [#]	-0.32	0.31	0.51 [#]	

*Denotes p-value <0.05 by Pearson's correlation analyses (green cells).

[#]Denotes p-value <0.05 by Spearman's non-parametric correlation analyses (blue cells).

4.8 SUMMARY

Studies from our laboratory previously determined that there was an induction in the expression levels of homeostatic chemokine CXCL13 in the spleen of AIDS-developing rhesus macaques. To explore further the expression levels of CXCL13 during SIV infection, we evaluated differences in acutely SIV-infected and AID-developing cynomolgous macaque CXCL13 expression levels and patterns compared to uninfected macaques. We found increased CXCL13 mRNA and protein levels in both acutely-SIV infected and AIDS-developing macaques compared to uninfected macaques. In addition, we found differences in the expression patterns of CXCL13 mRNA and protein. In our efforts to determine CXCL13 expression levels in the GALT, we unexpectedly found CXCL13 expression in Paneth cells in the crypts of Lieberkuhn. We confirmed this finding by demonstrating that DEFA6, a known and uniquely expressed Paneth cell AMP, colocalizes within the granules of Paneth cells with CXCL13. Additionally, we determined that Paneth cells express a larger repertoire of AMPs than previously described. Our results show that DEFA1, BDEF2, RTD-1, Reg3 γ , and Lcn2 also localize to macaque Paneth cells in the small intestine, as well as Tryp2, a human AMP processing peptide. The effects of SIV infection on AMP expression were seen in the differences in expression levels of DEFA6, RTD-1, and Reg3 γ . Both DEFA6 and Reg3 γ had increases in mRNA expression during acute SIV-infection that continued in AIDS-developing macaques. RTD-1 mRNA expression decreased in acutely SIV-infected macaques and then returned to expression levels similar to uninfected macaques in AIDS-developing macaques.

Discovering CXCL13 expression by Paneth cells led us to investigate the effects of SIV-infection on other Paneth cell-associated factors. We found a significant decrease in the expression levels of TLR9 in AIDS-developing macaques compared to uninfected animals.

5.0 OVERALL DISCUSSION

CXCL13 is a homeostatic chemokine that controls, in large part, the migration of B cells, follicular helper T (T_{fh}) cells and DCs into the follicles of secondary lymphoid tissues.⁵¹ Because CXCL13 is responsible for trafficking of B cells and T_{fh} cells into the marginal zone of follicles, it is possible that modifications to its expression might contribute to the B cell dysfunction occurring in HIV-1 infection.¹⁶³ Here we have examined the expression levels and patterns of CXCL13 in lymphoid tissues, including intestine, and found not only increased expression of CXCL13 during infection, but also that SIV infection drives a more disordered distribution of CXCL13 producer cells in LN and spleen and that CXCL13 is expressed by Paneth cells in small intestine.

A limited number of studies have examined CXCL13 in the context of HIV-1 or SIV infection. CXCL13 levels are elevated in plasma during HIV-1 infection and correlate with levels of CXCL10 in advanced disease.⁷³ In addition, CXCL13 production by B cells is increased in HIV-1 infected patients and there is increased migration toward CXCL13 by peripheral blood B from patients with advanced disease compared to uninfected controls.⁷⁵ CXCL13 protein expression during HIV-1 follicular hyperplasia is associated with the GC light zone and the follicular DC (FDC) network. These reports are consistent with our findings of an expansion and redistribution of CXCL13 producer cells in LN and spleen. In GC immunology, CXCL13 drives the migration of B cells and Tfh cells together in the GCs for the generation of

high-affinity antibodies. GCs are also a reservoir for FDC populations that can harbor immune complexes. Therefore, changes in CXCL13 could drive further the movement of these populations together, provide the necessary opportunity for FDCs to capture virus on their surface, and contribute to the B cell dysfunction that occurs during SIV infection.

There is disparate localization of CXCL13 mRNA and protein in lymphoid tissues

During pathogenic SIV infection, we had previously identified CXCL13 as one of the two chemokines with the largest increases in mRNA expression in spleen tissues.¹⁴⁸ We initiated the studies here with the goal of obtaining an improved understanding of the immunobiology of this chemokine in the macaque model for HIV/AIDS. We found CXCL13 protein was centrally located within GCs whereas CXCL13 mRNA was generally localized in cells defining a shell in the mantle and marginal zones of follicles. This disparate microanatomic localization of CXCL13 mRNA and protein was unexpected and underscores the complexity of the *in vivo* immunobiology of chemokines within tissues. The mechanism by which CXCL13 is concentrated within GCs, compared to the spherical shell of producer cells and the more distant paracortices is not clear. There are multiple possible mechanisms, however. For example, the intense staining in the center of the sphere circumscribed by the CXCL13 producer cells could be a consequence of bidirectional diffusion away from the producer cells in both the central follicular and paracortical directions, resulting in increased overall concentration within GCs and a reduced concentration in paracortices. Alternatively, CXCL13 release might be polarized and limited to the follicular surfaces of producer cells. This would lead to lower concentrations of CXCL13 outside the GCs and increased concentrations within the GCs. In addition, there might be an active transport mechanism that maintains the extracellular CXCL13 within the GC,

perhaps piggybacking on cells migrating toward the GC or in microchannels. The latter would be consistent with the thin, cord-like structures we have observed (e.g., Figure 1) in LNs and spleen. Furthermore, there might be differential expression of proteases that process CXCL13 in lymphoid tissue microcompartments, with a higher concentration of proteases in the surrounding paracortices relative to the inside of the GCs. Such compartmentalization of proteases would be consistent with the ability of GCs to harbor immune complexes on FDC surfaces for long periods of time¹⁶⁴⁻¹⁶⁶. Regardless of the mechanism(s), the compartmentalization of CXCL13 within lymphoid tissue GCs is consistent with its role in bringing T_{fh} cells, B cells, and DCs together.

Cagigi et al. demonstrated the expression of CXCL13 by immature CD1a⁺ DCs.⁷⁵ They did not, however, find a large amount of CXCL13 expression by mature DCs and suggested that this is result of a loss of CXCL13 upon DC maturation. In an attempt to identify and distinguish CXCL13 producer cells from responding cells we evaluated expression of B cells and FDCs by immunostaining for CD20, CD79a, and CD35. Identifying a true pattern of producer cells versus responder cells proved difficult by IHC alone because each of their signals contains regions that overlapped amongst one another. The patterns of expression of these cell populations were similar to CXCL13, although, not just one population completely colocalized with CXCL13 mRNA or protein signals. It is possible that there could be overlapping populations of producer and responding cells. In addition, there were no clear differences in expression levels of these cell populations by IHC as a function of SIV infection. Another cell type that also displays a pattern of expression in GCs similar to CXCL13 mRNA but was not evaluated in this study is the metallophilic macrophage cell.¹⁶⁷ This specialized subset of macrophages has been shown to be involved in Ag-trapping in the spleen.¹⁶⁷ They have a distinct pattern of expression in the outer marginal zone of GCs that is comparable to the pattern of CXCL13.¹⁶⁷

CXCL13 expression levels are modified during SIV infection

In this study, we found there were disease-specific changes in CXCL13 expression, with the mRNA⁺ producer cells expanding more broadly into the GCs and surrounding microcompartment as the course of infection progressed. Recently, increases in LN T_{fh} cells in HIV-1 infected individuals were observed despite the overall reduction in the number of CD4⁺ T cells that occurs during infection¹⁶⁸. Given that T_{fh} cells express CXCR5, the CXCL13 receptor, as well as CXCL13 per se, the more distributed pattern of CXCL13 producer cells we observed could be driven by recruitment and/or expansion of T_{fh} cells. In addition, a recent comparison between uninfected and SIV-infected rhesus macaque T_{fh} cells revealed a 5-fold induction of CXCL13 expression in T_{fh} cells that were SIV-infected¹⁶⁹. B cell affinity maturation and isotype switching occurs in GCs¹⁷⁰, and given the known B cell dysfunction that occurs during HIV-1 and SIV infection^{163,171-173}, it is conceivable that a chemokine that orchestrates the migration of these cells into follicles could contribute to humoral immune response abnormalities that arise during infection. Consistent with this notion, it has been argued recently that there is a decrease in memory B cell levels due to the distortion of the B cell population toward GC B cells and plasma cells during chronic HIV-1 infection¹⁶⁸. Alternatively, it was demonstrated that B cells in serum and tissues of HIV-1 infected individual's expressed significantly higher levels of CXCL13, further alluding to a contribution by the chemokine to B cell dysfunction.

Previous studies by our laboratory also have shown modifications to other homeostatic chemokine expression levels in rhesus macaques during SIV infection. In an attempt to examine these changes in the cynomolgous macaque model we evaluated mRNA expression patterns and

levels by ISH in uninfected, acutely SIV-infected, and AIDS-developing macaques. Our findings show changes to the levels of CCL19 and CCL21 mRNA in the LN and spleen, consistent with our previous findings in rhesus macaques.¹⁴⁸ CCL19 and CCL21 guide lymphocytes and DCs to and within lymphoid tissues via their receptor CCR7 and have demonstrated an ability to promote inflammation.¹⁷⁴

CXCL12 is a homeostatic chemokine whose receptor CXCR4 is known for its role as an HIV-1 co-receptor. Mutations in the *cxcl12* gene are associated with resistance to HIV-1 transmission.^{175,176} To study the expression patterns of CXCL12 between uninfected and SIV-infected macaques, we examined expression patterns of CXCL12 mRNA in LN and spleen by ISH. There is a visible induction of CXCL12 mRNA expression in the medulla of LNs and medullary sinuses of the spleen during acute SIV infection. HIV-1 strains utilize CXCR4 as an entry coreceptor occurs late in infection. This suggests the selection of CCR5 as an entry coreceptor is caused by an inhibition in the shift in tropism. Gonzalez et al. provided evidence of DC-mediated enhancement in the propagation of X4 viruses that was inhibited by the production of CXCL12 by mature monocyte-derived DCs (mMDDCs).¹⁷⁷ They suggest that DCs present in the LNs produce CXCL12 preventing the appearance of X4-tropic viruses. The induction of CXCL12 in our studies could support this finding.

CXCL13 is expressed by Paneth cells in the macaque small intestine

It has been demonstrated that subsets of chemokines and AMPs possess both chemotactic and antimicrobial activity. A major finding from these studies was the discovery that CXCL13 is expressed by Paneth cells. Paneth cells are specialized intestinal epithelial cells that express AMPs and proinflammatory cytokines that contribute to innate immunity¹⁵¹. The AMPs

secreted by Paneth cells have several overlapping roles in the intestine⁷⁷, including shaping the composition of and limiting the number of commensals populating the small intestine, protecting the intestine and its stem cells from invading pathogens, and acting as paracrine signaling molecules.⁷⁷ It has been argued that Paneth cells are responsible for the homeostatic environments surrounding the intestinal villi and crypts by regulating microbe infiltration⁸². Given the expression of CXCL13 by Paneth cells, one of its major functions in the small intestine is likely as an AMP, which would not have been anticipated for a LN homeostatic chemokine. The same regional distribution of CXCL13 along the GI tract as other Paneth cell expressed AMPs, and the co-localization of CXCL13 and DEFA6 in Paneth cells supports the interpretation that CXCL13 functions *in vivo* as an AMP. To confirm further that the expression of CXCL13 in Paneth cells is likely due to its ability to function as an AMP and not for its normal chemotactic function, we sought to determine whether mature B cells, which are known to express the CXCL13 receptor CXCR5, were located in the lumen adjacent to Paneth cells expressing CXCL13. Therefore, we evaluated CD20+ mature B cells in the same tissues and did not find colocalization of CXCL13 and CD20 in Paneth cells. CD20 and CXCL13 localization in the GALT showed a similar pattern to the MLNs and spleen supporting the claim that CXCL13 is providing its known function in this region of the intestine. However, its lack of colocalization with CD20 in Paneth cells suggests that it functions as an AMP in this compartment. Furthermore, approximately 50% of chemokines have been shown to possess antimicrobial activity in *in vitro* assays, including CXCL13.¹⁵⁴ Amongst the microbes against which CXCL13 exhibited antimicrobial activity were the intestinal bacteria *E. coli* and *S. aureus*.¹⁵⁴ This antimicrobial activity is consistent with our discovery that CXCL13 is expressed by Paneth cells. To explore further the antimicrobial property of CXCL13, we attempted to have a macaque

CXCL13 peptide synthesized to use in antimicrobial assays with known gut Gram (+) and Gram (-) bacteria. Our lab previously had success with the synthesis of macaque chemokines CXCL11, truncated CXCL11, and CCL20 for use in chemokine inhibition and antimicrobial studies, however, the development of the CXCL13 peptide proved to be a challenge.

To determine if CXCL13 expression in Paneth cells was specific to cynomolgous macaques, we examined the expression of CXCL13 in both the rhesus macaque and in the mouse, and found differences among the species of animals. CXCL13 was found in the GALT, but is not present in Paneth cells of the mouse, unlike both rhesus and cynomolgous macaques, where CXCL13 is present in both GALT and Paneth cells.

The cxcl13 promoter is stimulated by the short chain fatty acid sodium butyrate

Short chained fatty acids such as sodium butyrate are produced by intestinal bacteria and we found that the macaque CXCL13p was induced by this product of bacterial fermentation.¹⁷⁸ The antimicrobial cathelicidin LL-37 is also stimulated by sodium butyrate^{157,179-181}. The upregulation of the CXCL13p by microbial products is consistent with our interpretation that has CXCL13 has AMP function in the intestine.

The AMP repertoire expressed by Paneth cells is larger than previously described

In addition to CXCL13, we show here that DEFA1, BDEF2, RTD-1, and Reg3 γ are also expressed by macaque Paneth cells. DEFA1 is contained within the granules of neutrophils and contributes to human systemic innate immunity. It has not, however, been shown to be expressed by human or macaque Paneth cells. Lower levels of this AMP, along with DEFA2 and DEFA 3, were found in patients with inflammatory bowel disease meaning there could be

modification to the expression levels of these AMPs during HIV-1 infection, which also causes inflammation and intestinal enteropathy.^{127,128} DEFA1 has been shown by a few mechanisms to inhibit HIV-1 infection, including preventing binding of Env to CD4 and co-receptors. β -defensins are expressed by epithelial cells in multiple mucosal tissues, including skin, stomach, and colon.¹⁸² However, they have not been reported to be expressed by Paneth cells. In addition to AMP activity, at least some β -defensins also possess chemotactic activity. For example, BDEF2 is chemotactic for CCR6⁺ cells,¹⁸³ cells that are responsive to the chemokine CCL20. The θ -defensins are cyclic defensins originally isolated from leukocytes and bone marrow of rhesus macaques¹⁸⁴. Rhesus θ -defensins and repaired human retrocyclin have broad antimicrobial properties with inhibitory effects on HIV-1, influenza virus, and a broad swath of bacteria. As with BDEF2, predominant expression in the GI tract by Paneth cells has not been reported. Finally, Reg3 γ is an AMP expressed by murine Paneth cells⁸⁹ and our findings reveal the same for macaque Reg3 γ . Altogether, these findings increase our understanding of the composition of the AMP repertoire produced by Paneth cells, the diversity of which likely reflects the diverse set of microbes targeted as an innate protective mechanism against microbial translocation in the intestinal crypts.

Finding a significant induction of DEFA6 during SIV infection led us toward examining expression levels of other AMPs found to be expressed by macaque Paneth cells during SIV infection. We attempted to utilize ISH and real-time RT-PCR to determine expression level differences among uninfected, acute SIV-infected, and AIDS-developing ileal tissues. Macroscopic changes to mRNA levels were visualized by ISH for RTD-1 and Reg3 γ during acute SIV and AIDS disease states. Fluctuations to the mRNA levels of these AMPs could contribute to the subverted gut microbiome during SIV infection. Attenuated expression of

defensins could suggest a compromise to host immunity or may signal the host is attempting to prevent a shift toward a state of inflammation.

Paneth cell-associated factors during SIV infection

Revealing CXCL13 expression by Paneth cells stimulated us to more broadly investigate the effects of SIV infection on Paneth cell phenotype. It has been demonstrated that microbial translocation is associated with chronic immune activation in SIV infection and it is conceivable that Paneth cells, given their secretion of AMPs in response to invading bacteria, could play an important role in this process. We examined differences in expression of several Paneth cell markers to determine if they were altered during SIV infection. Our findings show most of the Paneth cell markers we evaluated were unchanged. Two exceptions were TLR9 and DEFA6. Expression of TLR9 was significantly decreased in AIDS-developing tissues compared to uninfected animals. TLR9 is responsible for recognition of DNA rich in CpG motifs and decreases in its expression could signify a weakness in the immune defenses that are beneficial for the development of chronic immune activation. A significant increase in DEFA6, in contrast, was found in those same AIDS-developing macaques. This could provide evidence in an attempt by the natural immune processes of the gut to combat any increases in bacterial concentration due to a reduction in TLR9.

Translocation of microbial products into the intestinal lumen stimulates TLRs and triggers the immune system into a state of inflammation, activating memory and effector CD4+ and CD8+ T cells. Funderburg et al. investigated T cell activation of peripheral blood mononuclear cells (PBMCs) from uninfected individuals by TLR ligands¹⁸⁵ and *in vitro* stimulation by microbial TLR ligands induced activation of lymphocyte homing and activation.

In our analyses of changes to TLR9 expression during SIV infection, however, we did not find significant increases in a Paneth cell-associated TLR9 expression. This could be evidence further to support an avenue for increase immune activation. One study suggests an inhibition of TLR9-mediated recognition of bacterial and viral infection may contribute to chronic activation of the immune system. Their results show how interaction between HIV-1 and SIV gp120 with pDCs renders them incapable to responding to TLR9-stimulating pathogens.¹⁸⁶

There is a preferential loss of IL-17 producing CD4+ T cells during the course of SIV infection in the gastrointestinal tract.¹³⁹ Our examination of changes to IL-17 mRNA levels in the ileum revealed no significant differences between SIV-infected and uninfected macaques. Additionally, there was no decrease in the levels of ROR γ t in these same animals. This suggests that there was no reduction in the Th17 population during infection in our group of study animals. It is possible there could be differing levels of Th17 cells in other regions of the small intestine other than the ileum and there could be a preferential loss of these cells in other compartments during SIV infection especially in the colon where damage to the mucosal barrier is observed. Additionally, in our studies we utilized cynomolgous macaques for our animal model, where as other studies evaluating the role of IL-17 in microbial translocation was in a Pigtail macaque (PTM) animal model. Klatt et al. showed differences in microbial translocation in PTM versus rhesus macaques (RM). In their PTM, they found significant more gut epithelial damage and microbial translocation in PTMs than in RMs.¹⁴¹

During microbial translocation, there is substantial structural damage to the gastrointestinal epithelium in PTMs.¹⁴¹ The epithelial tight junction protein, claudin, has been evaluated for its association with microbial translocation. E-cadherin is a cell-cell adhesion protein that that is also involved in maintaining tissue architecture. A loss in e-cadherin has been

shown to attenuate Paneth cell maturation. For our studies, we evaluated alterations to e-cadherin expression during SIV infection and found no changes in its expression in acute SIV-infected or AIDS-developing macaques compared to uninfected macaques.

Trypsin is responsible for processing AMPs in the gastrointestinal tract of humans. In the mouse, this activity is conducted by MMP-7. Results from our studies show that Tryp2 is also expressed by macaque Paneth cells. The use of trypsin in macaques to proteolytically process AMPs is reasonable given their close phylogenetic relationship to humans.

Lastly, we evaluated the relationships between SIV PVL and AMPs by conducting correlation analyses. Where we previously found no significant differences in mRNA levels during SIV infection, we now found significant associations between levels of several AMPs and Paneth cell-associated factors. Interestingly, the single correlation with SIV PVL was with DEFA6.

6.0 SUMMARY

The importance of evaluating changes to chemokine and chemokines receptor expression levels during SIV infection has been demonstrated in studies of not only CCR5 and HIV-1 coreceptor usage, but also in studies of chemokines such as CXCL9 and CXCL13.^{7,148}

In normal germinal center biology, T-cell dependent stimulation of antigen occurs and activates B cells to migrate into GCs.¹⁸⁷ The GCs are divided into distinct zones where cells undergo proliferation, clonal expansion and affinity maturation.¹⁸⁷ FDCs also reside in these regions and have the potential to harbor immune complexes and secrete TNF cytokines. Tfh also migrate into GCs and assist in this development process. Cyster *et al.* describe how the microarchitecture of the GC is influenced by the distribution and interaction of each of these cell types.¹⁸⁸ B cells have been shown to be highly motile between each of the zones in the GC.¹⁸⁹ CXCL13 plays a vital role in the movement of each of these cell types throughout each microcompartment of the GC given their expression of its receptor, CXCR5. A hallmark of HIV-1 infection is disruption to humoral immunity.¹⁶³ Hyperactivation and exhaustion of B cells, production of short-lived plasmablasts and an increase in the production of B-cell stimulatory cytokines¹⁹⁰ are all characteristics of B cell dysfunction during HIV-1 infection. A reduction of the levels of these cytokines is seen after the initiation of HAART.⁷⁴ Similarly, there is a reduction in the serum levels of CXCL13.⁷⁴ Studies evaluating the effects of HAART on biomarkers of B-cell activation, found higher baseline levels of CD4+ T cells were associated

with greater decreases in CXCL13 levels after HAART.⁷⁴ Our findings provide further evidence to support a possible role for CXCL13 in the disruption of B cell maturation and antibody production in GCs during SIV infection. We found a significant increase in the distribution of CXCL13 in follicular regions that coincides with disease progression. Increases in the production of CXCL13 can stimulate the movement of responder cell populations in the GCs and have detrimental effects to the B cell maturation process, therefore, having a downstream effect on the production of high-affinity antibodies.

In a compilation of all changes to chemokines, AMP, and Paneth cell-associated factor expression into one graphic image (Figure 33), we determined that amongst the chemokines we evaluated, CXCL13 exhibits the greatest increase during SIV infection. Evaluating each group separately, we see that there are more fluctuations in the expression of chemokines and AMPs than of the Paneth cell-associated factors across disease states. During HIV-1 infection there is a dramatic decrease in the population of T cells located in the GI tract, making it an important site of investigation. In our efforts to elucidate changes in CXCL13 expression in the GALT, we made a unique finding. We observed CXCL13 expression by Paneth cells in the macaque small intestine. For the first time, we provide evidence to support its possible function as an antimicrobial peptide by demonstrating colocalization with known Paneth cell marker, DEFA6. Therefore, the findings presented here sustain the notion that chemokines function as AMPs. This has been demonstrated for many chemokines *in vitro*, but localization of CXCL13 to Paneth cells, which are specialized for production of AMPs, and transcriptional induction of the CXCL13p by the intestinal bacterial product sodium butyrate, provides strong support that at least a subset of chemokines provide antimicrobial function *in vivo*. That a lymphoid homeostatic chemokine is functioning in these dual capacities is intriguing and it will be

interesting to unravel the possible selective pressures that have led to the multi-functionality of CXCL13 and subsets of chemokines generally.

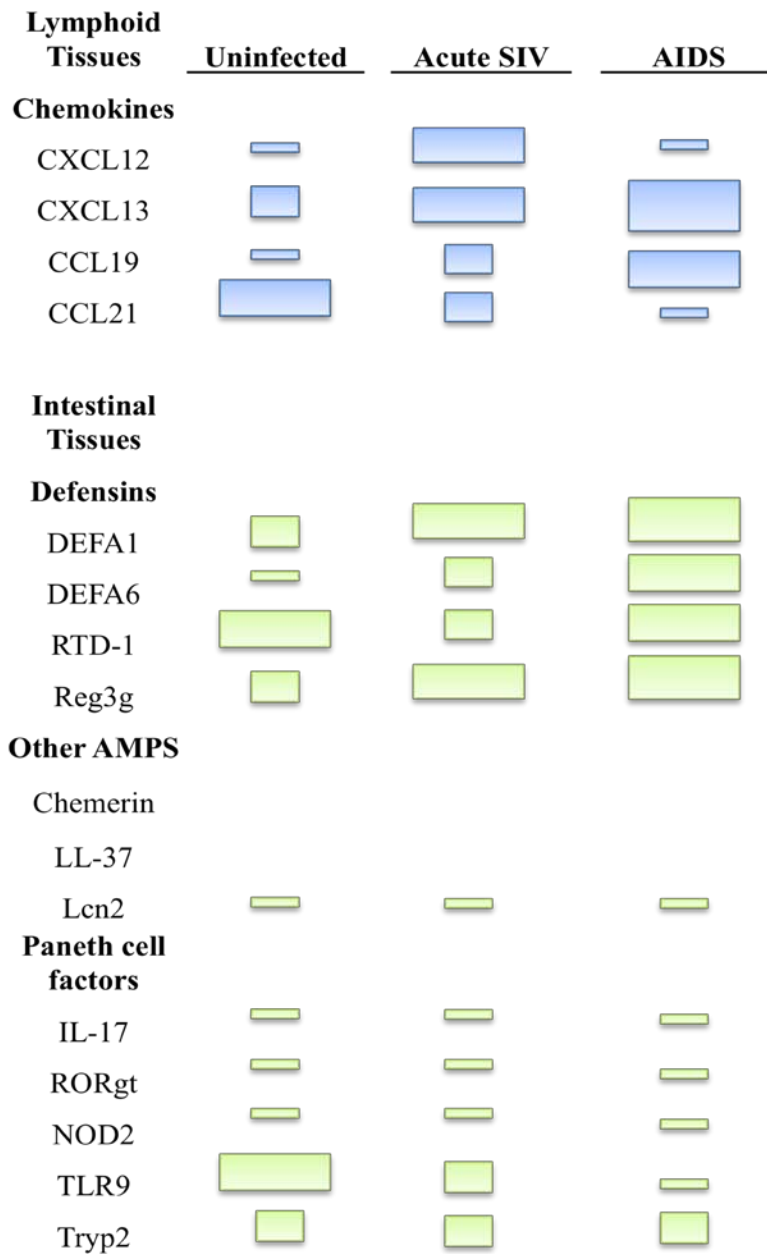


Figure 33. Graphical representation of changes to chemokines and antimicrobial peptide expression levels as a function of SIV infection.

Blue cells represent lymphoid chemokines evaluated in the LN and spleen. Green cells represent AMPs evaluated in the intestine. Varying size cells represent either an increase or decrease the level of expression.

6.1 FUTURE WORK

Over the course of this study, our findings led to the development of many questions that were not addressed due to time constraints. The following are areas of study that warrant further investigation.

The most commonly known function of CXCL13 is to drive the flow of lymphocytes into the GCs. Given the B-cell dysfunction that occurs during HIV-1 infection, it would be beneficial to conduct CXCL13 inhibition studies to determine the effects on B cell maturation and the development of high-affinity antibodies during infection.

Revealing CXCL13 expression by PCs in the small intestine has lead to many other questions regarding this known homeostatic chemokine's function. Should this chemokine function as an antimicrobial peptide, we are curious about the factors that regulate its expression and release from Paneth cells? Additionally, antimicrobial studies are necessary to determine pathogens targeted by CXCL13. An *ex vivo* study utilizing intestinal tissue explants could be optimized to stimulate Paneth cells. A measurement of the amount released AMPs and detection of CXCL13 can be carried out.

6.2 PUBLIC HEALTH IMPACT AND SIGNIFICANCE

The 30th anniversary since the emergence of the AIDS epidemic recently passed and there has yet to be a vaccine developed to prevent the further spread of the HIV-1 virus. A hallmark of infection is B cell dysfunction and an inability for the host to develop high-affinity antibodies. The production of these antibodies occurs in GCs of lymphoid tissues. CXCL13 is a

constitutively expressed chemokine whose function is to drive the migration of lymphocytes into GCs. Modifications to the expression levels of this chemokines during SIV infection provide evidence that warrant further investigation of this homeostatic chemokines and its role in the interruption of B cell maturation and antibody production.

A unique finding in this study was the expression of CXCL13 by macaque Paneth cells. In conjunction, we also show other AMPs expressed by macaque Paneth cells that had not previously been described. These results provide the basis for further inquiry into their role in maintaining the microbiome of the gastrointestinal tract.

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