# Infant Feeding Practices, Food-Borne Toxins and Immune Activation in HIV-Endemic South Africa

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**Abstract**

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Over 250,000 new mother to child transmission (MTCT) events occur each year, and at least 40% of these HIV infections are acquired through breastfeeding. Despite the risk of HIV acquisition via breastfeeding, exclusive breastfeeding of infants born to HIV-infected mothers is recommended by the WHO, since, even in the presence of HIV, breastfeeding results in reduced infant mortality, partly due to immune benefits from passive transfer of antibodies and other bioactive products. Therefore, HIV transmission via breast milk continues to be a major route of infant HIV acquisition.

This thesis explores four different exposures in early infancy that can alter immune activation and HIV target cell availability in South African infants born to HIV-infected mothers: 1. introduction of non-breast milk foods early in life, 2. maternal HIV disease progression, 3. oral candidiasis and 4. vaccination to infant immune activation. Findings from this work suggest that all 4 exposures may increase HIV susceptibility by inducing immune activation in HIV target cells.

Introduction of non-breast milk foods is associated with an increase in the level and activation state of HIV target cells with a T regulatory phenotype. These cells also may be recruited to the oral mucosa by increased levels of CCL5 and CCL22. Evaluation of two potential mechanisms for this increase in HIV target cell activation and recruitment, exposure to food borne toxins, specifically ochratoxin, and changes in the stool microbiome, demonstrate a potential role for altered gut microbiome, particularly an increase in *Prevotella copri*, in modulating HIV target cell activation and recruitment to the oral mucosa.

This thesis also demonstrates that: 1. As maternal CD4 count declines, a predictor of HIV disease progression, breastfed infants show an increase in T cell activation, suggesting that exposure to maternal HIV or HIV-induced immune activation via breast milk increases immune activation in infants; 2. Exposure to Candida, as occurs in oral candidiasis, a common infection of infants, increases CD4 T cell activation in adults; and 3. Vaccination with BCG, which is administered at birth to most infants around the globe, also can increase HIV target cells in the blood of infant macaques.

These studies add substantially to our understanding of environmental exposures that increase HIV susceptibility in South African infants, and suggest possible interventions, including probiotic administration, that may decrease HIV susceptibility in breastfed infants.

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## Chapter 1: Introduction

### Human Immunodeficiency Virus

The human immunodeficiency virus (HIV) and the disease it causes, acquired immunodeficiency syndrome (AIDS), is the sixth leading cause of death in all age groups, worldwide.[4] HIV is estimated to have infected a total of 78 million people worldwide, and currently 34 million people are living with this virus.[5]

HIV is a retrovirus of the lentivirus family that is closely related to the simian immunodeficiency viruses (SIVs) that are common in African non-human primate species. SIV viruses have been transmitted from at least three non-human primate species into humans.[6] These transmissions have resulted in two HIV species, HIV-1 and HIV-2.

HIV-2 was transmitted from sooty mangabeys to humans in West Africa, and this viral species has remained almost exclusively in West Africa. HIV-2 transmission is infrequent, with only a 4% risk of mother to child transmission (MTCT) through breastfeeding, and low level virus shedding in body fluids.[7] In addition, HIV-2 has nearly half of the mortality rate of HIV-1.[8] Therefore, HIV-2 has had a relatively limited impact, globally. In fact, HIV-2's prevalence is decreasing,[9] suggesting that this virus will not become an epidemic in humans.

HIV-1, although genetically quite similar to HIV-2, is a very different virus. HIV-1 has been transmitted from chimpanzees to humans multiple times between the 1850s and the 1960s, resulting in multiple subtypes, referred to as groups.[6] Although there are four known HIV groups, only one of these groups, group M, is responsible for nearly all HIV infections worldwide. Molecular clock estimates place the cross-species transmission of Group M HIV-1 between 1850 and 1930, and phylogenetic trees show that this virus is most closely related to SIV circulating in chimpanzees in Southern Cameroon, although it is believed that it initiated its epidemic spread after being transported south to what is now Kinshasa (formerly Leopoldville) in the Democratic Republic of Congo.[6] Group M HIV-1, itself, is also divided into 9, genetically divergent clades. In most of western and central Africa, multiple clades and recombinant viruses are present at relatively high levels in the HIV-infected population (Figure 1.1). However, as

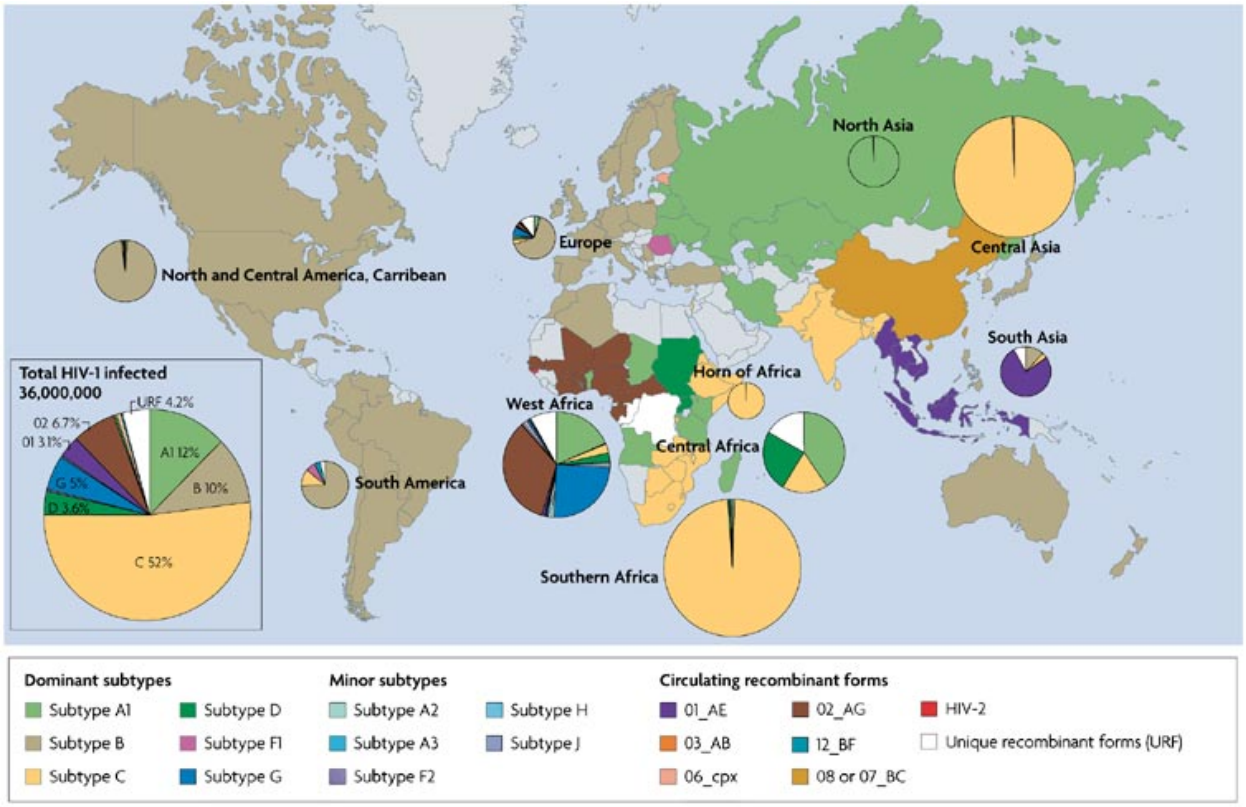



**Figure 1.1: HIV-1 diversity is greatest in West Africa.** Different subtypes dominate in Southern Africa, China and India, South Asia, the Americas and Europe, and North Asia.[1]

we move geographically further from the original cross-species transmission of group M HIV-1, the diversity in circulating virus clades rapidly decreases. In east Africa, HIV-1 infections are confined to clades A1, C, and D. In the Americas and Europe, clade B predominates, in Russia, clade A1 dominates, and in China and Southeast Asia, specific recombinant viruses, a clade B/C recombinant virus and a clade A/E recombinant virus, respectively, are responsible for most infections. However, the countries with the highest number of HIV-infected individuals, those in Southern Africa and the Indian subcontinent, have nearly exclusively clade C virus.[1] Therefore, just over 50% of HIV-infected individuals, globally, are infected with clade C virus.

Similar to other retroviruses, HIV requires a series of specific events in order to produce new, infectious virions (Figure 1.2), which has provided multiple targets for HIV treatment. An HIV virion first establishes infection of a target cell by binding its externally displayed envelope protein to its primary receptor, CD4, and one of its many coreceptors, most commonly CCR5, on the host cell. The viral membrane then fuses with the host cell's membrane, releasing the capsid, which contains the viral RNA genome and viral proteins, into the host cell cytosol. The capsid then uncoats, revealing its contents and permitting the initiation of reverse transcription, which copies the viral genomic RNA to DNA, using a viral protein, reverse transcriptase. This new viral DNA then enters the host nucleus and integrates into the host cell's genome. At this point, the cell may become latently infected with HIV or it can initiate the production of new HIV virions. Although we do not know all of the mechanisms that underlie the switch from latent infection to active virus production within a cell, immune activation in a latently infected target cell can initiate the production of new virions.[10] HIV then uses host cell machinery to transcribe new viral RNA genomes and translate viral proteins. Viral RNA and proteins then assemble to

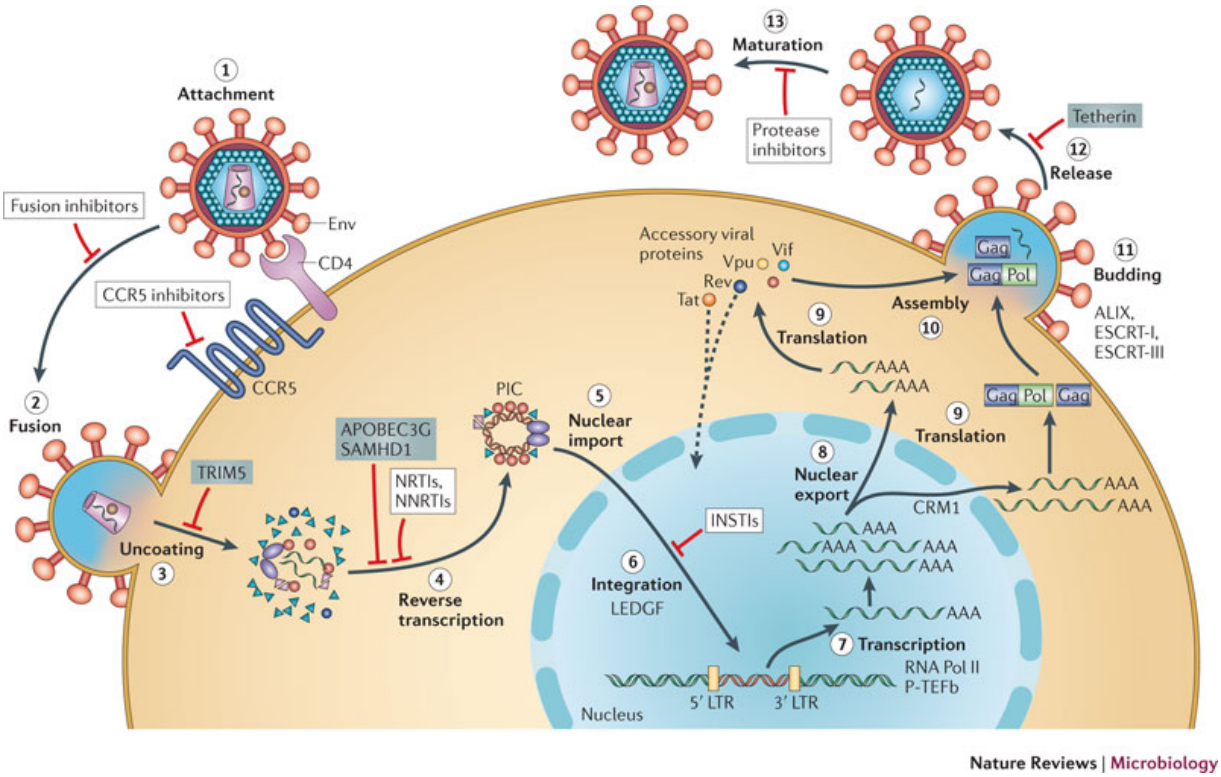



**Figure 1.2: HIV-1 lifecycle.** An HIV virion establishes by (1) binding its envelope protein to its primary receptor, CD4, and one of its coreceptors, most commonly CCR5, on the host cell. The viral membrane then (2) fuses with the host cell's membrane, releasing the capsid, into the host cell cytosol. The capsid then (3) uncoats, revealing its contents and permitting the initiation of (4) reverse transcription. The new viral DNA then (5) enters the host nucleus and (6) integrates into the host cell's genome. HIV can then use host cell machinery to (7) transcribe new viral RNA genomes and (9) translate viral proteins. Viral RNA and proteins then (10) assemble to form new virions, which in turn (11) bud off of the host cell plasma membrane. The viral proteins contained in each virion then must undergo (13) viral protease-mediated cleavage to form a mature virion that can efficiently infect additional cells.[3]

form new virions, which in turn bud off of the host cell plasma membrane. This process can result in the death of the host cell. The viral proteins contained in each virion then must undergo viral protease-mediated cleavage to form a mature virion that can efficiently infect additional cells. Anti-retroviral therapies that block CCR5 binding, viral fusion, reverse transcription, DNA integration and protease cleavage are all currently available in the clinic.

Although each of these drugs are highly effective in blocking replication for the vast majority of viruses, HIV has a very high mutation rate, due to the error-prone nature of reverse

transcriptase,[11] and, therefore, has large viral diversity even within a single infected patient. This results in rapid viral escape from a single drug.[12] Therefore, antiviral agents are only effective in sustaining viral suppression when administered as a cocktail of at least three drugs with at least two different mechanisms of action. Recently, cocktails of antiretroviral drugs have been co-formulated into a single pill, which significantly improves the ease of use for HIV-infected individuals.

Antiretroviral agents, in addition to slowing disease progression in HIV-infected individuals, can also be used to prevent HIV transmission. There are three ways that these drugs can be used to prevent new HIV infections: 1. Treatment as prevention, 2. Pre-exposure prophylaxis and 3. Post-exposure prophylaxis. Treatment as prevention, first employed for the prevention of mother-to-child transmission of HIV, is the treatment of HIV-infected individuals to suppress their viral load. HIV viral load positively correlates with the risk of transmitting HIV to another individual.[13,14] Therefore, suppression of viral load in HIV-infected individuals through antiretroviral treatment decreases the risk of virus transmission to other individuals.[15,16] Pre-exposure prophylaxis, the use of antiretrovirals by HIV-uninfected individuals at risk of HIV infection, particularly the use of oral antiretrovirals, has also been shown to dramatically decrease HIV infection.[17,18] Finally, post-exposure prophylaxis, the use of oral antiretroviral treatment to protect people recently exposed to HIV, can also effectively reduce transmission, and is commonly employed for exposed healthcare workers.[19] Together, these strategies have been used to dramatically reduced HIV transmission, including **mother-to-child transmission (MTCT).**

**Breast Milk Transmission of HIV**

Over 250,000 new MTCT events occur each year,[20] and at least 40% of these HIV infections are acquired through breastfeeding.[21] Despite the risk of HIV acquisition via breastfeeding, exclusive breastfeeding of infants born to HIV-infected mothers is recommended by the WHO, since, even in the presence of HIV, breastfeeding results in reduced infant mortality, partly due to immune benefits from passive transfer of antibodies and other bioactive products.[22] Therefore, HIV transmission via breast milk continues to be a major route of infant HIV acquisition.

Infants born to HIV-infected mothers, who do not acquire HIV *in utero* or perinatally, continue to be at risk of acquiring HIV through breastfeeding. This risk was first observed by Ziegler et al. in the mid-1980s[23] and subsequently quantified in a Kenyan trial that randomized women to breast or formula feeding.[24] In this large, randomized study, the risk of HIV transmission attributable to breastfeeding was 44.1%.[24] Since then, other studies have confirmed that, without intervention, between 20 and 40% of MTCTs occur during breastfeeding.[21,25] There are also a handful of case reports where postnatal MTCT of HIV occurred in the absence of breastfeeding where premastication of food by the mother may have been the route of transmission.[26] However, this route of transmission appears to be far less common than breast milk transmission of HIV.[27]

Because of the risk of HIV transmission through breastfeeding, formula feeding is universally recommended to HIV-infected mothers in developed countries.[28] However, in developing countries, breastfeeding is recommended even for HIV-infected women, due to increased morbidity and mortality from malnutrition, respiratory infections and gastroenteritis in formula fed infants.[22,29-33] Therefore, in developing countries, postnatal transmission continues to account for at least 39% of MTCTs.[21,25,34]

Multiple trials have demonstrated that mother and infant antiretroviral therapy during breastfeeding can dramatically reduce HIV breast milk transmission.[25,35-38] Implementation of these interventions has made breastfeeding remarkably safer for HIV-exposed infants around the world. In communities that have made these interventions widely available, rates of transmission during breastfeeding have decreased to 1-4%.[36] However, of the nearly 1 million HIV-infected pregnant women in 2012, only 62% received effective prophylaxis.[20] Therefore, over 250,000 infants are still infected with HIV each year.[20] In addition, the prophylactic use of antiretroviral drugs can result in drug resistance in infants that become infected despite prophylaxis.[39,40] Although much progress has been made in reducing breast milk transmission of HIV, additional interventions are still necessary to eliminate infant HIV acquisition.

Factors associated with increased risk of breast milk HIV transmission include high maternal viral load, particularly breast milk viral load,[41,42] and duration of breastfeeding.[21] Interestingly,

low maternal CD4 count is also a major determinant of transmission independent of viral load.[43] Clinical and subclinical mastitis (which are associated with increased milk viral load in the involved breast), breast abscesses or other lesions, and infant oral thrush have also been shown to increase the risk of transmission.[41,42,44-48] It is unclear if any particular period of time during lactation presents a greater risk for HIV-1 transmission. Some studies suggest that colostrum, the breast milk produced shortly after delivery, has a higher viral load than non-colostrum breast milk, and therefore, transmission may be higher early in life,[49] whilst others studies have not observed this phenomenon.[43,50] Regardless, the risk of MTCT persists until the infant is fully weaned.[21]

**Immune Activation and HIV Transmission**

Breast milk contains leukocytes, particularly macrophages,[51-53] that can harbor infectious virus, and cell-free virus, although the viral load in these fluids is generally lower than that observed in the blood.[49,52,54-58] However, the relative contribution of cell-free and cell-associated virus to HIV transmission is still unclear.

Multiple lines of evidence have suggested that cell-associated virus may be important in oral transmission of HIV. Cell-associated virus can withstand low pH environments, such as the stomach, better than cell-free virus;[53,59] HIV-infected macrophages can penetrate infant oral epithelium, allowing direct viral access to the HIV target cells of the lamina propria;[60] and epithelial transcytosis of virus (see below) is most efficient with cell-associated virus.[61-64]

However, treatment of HIV-infected mothers with antiretrovirals, which dramatically reduces the risk of HIV acquisition in breastfed infants, decreases cell-free viral load in breast milk without reducing either the DNA or RNA load in HIV-infected cells.[65-68] This would suggest that cell-free virus is more important than cell-associated virus in infant oral transmission of HIV. However, it is possible that ART may also reduce the infectivity of cell-associated virus, possibly due to transfer of antiretrovirals from mother to infant in breast milk.[69] Phylogenetic analysis suggests that both cell-free and cell-associated virus transmission can occur, although cell-free virus transmission occurs more frequently.[45,70-72] Interestingly, epidemiologic studies have shown that levels of cell-associated virus predict breast milk HIV transmission during the first 9 months of life, where cell-free viral titers better predict HIV transmission in older infants.[45,71,73] This suggests that both cell-associated and cell-free virus can mediate oral transmission of HIV.

Once HIV enters the oral cavity there are a number of distinct tissue sites along the GI tract that may permit viral entry in to the host's tissue. These histologically distinct tissues include areas of stratified squamous epithelium with (e.g. gingiva) or without (e.g. esophagus and buccal mucosa) keratinization, mucosa-associated lymphoid tissue (MALT) (e.g. tonsils), and columnar epithelium (e.g. salivary glands, stomach and intestinal mucosa). Macaque models indicate that virus, following high-dose oral exposure, most likely initiates infection across the mucosa of the upper gastrointestinal tract, namely the mucosa of the oral cavity, the tonsils and the esophagus (Table 1.1).[74,75] The tonsils have a relatively high proportion of HIV target cells,[76] and *ex vivo* analysis as well as macaque studies have suggested that the tonsils may play a particularly

important role in oral HIV transmission.[77-79] However, *ex vivo* studies of HIV replication in tonsil tissue have consistently used tissue obtained from therapeutic tonsillectomies, which are most commonly performed to remove tonsils that are enlarged due to infection or excessive

**Table 1.1: Macaque studies of oral HIV transmission.**[2]

| Finding | Virus strain | Route of virus exposure | References |
|---|---|---|---|
| Adult macaques can be orally infected by cell-free virus alone | SIVΔB67 | Drip with swallowing; variable dose | Baba, et al. 1996. AIDS RHR |
| Infants susceptible to oral SIV, and protected by prophylactic ART | SIVmac251 | Drip with swallowing; high dose | Van Rompay, et al. 1998. JID |
| SIV can initiate infection via tonsils | SIVmac251 | Direct application to tonsils; variable dose | Stahl Hennig, et al. 1999. Science |
| Attenuated SIV prevents SIV infection, implicating $\gamma\delta$ T cells in protection | SIVmac251 | Direct application to tonsils; high dose | Tenner-Racz, et al. 2004. PNAS |
| Oral SIV establishes infection in tissues proximal to the stomach | SIVmac251 | Drip with swallowing; high dose | Milush, et al. 2004. AIDS |
| Rapid induction of early innate genes in *infants* in tissues proximal to stomach | SIVmac251 | Drip with swallowing; low dose | Abel, et al. 2006. JVI |
| Rapid induction of early innate genes in *adults* in tissues proximal to stomach | SIVmac251 | Drip with swallowing; high dose | Milush, et al. 2007. JVI |
| Low dose challenge delays early innate gene activation | SIVmac251 | Drip with swallowing; low dose | Durudas, et al. 2011. Curr HIV Res |
| Mucosal disruption (acetic acid) increases SHIV oral transmission | SHIV-R5 clade | Drip on site of acetic acid treatment; low dose | Chenine, et al. 2010. JID |
| Mucosal inflammation (gingivitis) does not increase oral SIV transmission, but does impact immune activation following SIV infection | SIVmac251 | Drip on site of gingivitis; low dose | Giavedoni, et al. 2013. JVI |

immune response. Therefore, unlike most other mucosal tissues, tonsil tissues studied *ex vivo* are collected under inflammatory conditions,[76] which is known to increase HIV susceptibility (see Inflammation and HIV/SIV Susceptibility section below). Evaluating acute HIV infection with physiologically relevant, low-dose viral exposure (better representing natural exposure), is challenging, even in the macaque model, making it difficult to determine the exact site of HIV/SIV acquisition within the upper gastrointestinal tract.

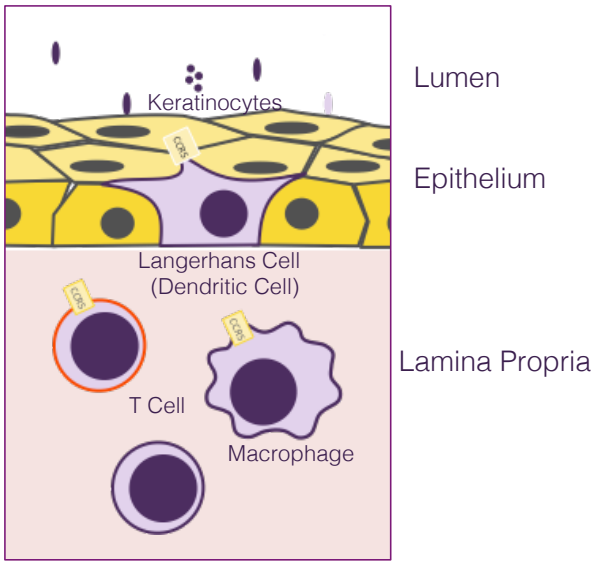
The predominant cell type in all mucosal tissues of the upper GI tract is epithelial cells, which do not express the classic HIV receptor CD4 and are not productively infected by HIV. However, cell-free and cell-associated HIV can be efficiently transcytosed across a wide range of epithelial tissues, allowing the virus to gain access to the HIV target cells of the lamina propria.[60,61,64,80-83] Although the transcytosis of HIV across mucosal epithelium has been well documented in *ex*

*vivo* studies, its role in natural transmission is still unknown. Virus transcytosed across the infant oral mucosa remains infectious.[60] However, in adult oral mucosa, transcytosed HIV is not infectious, and successful penetration by cell-free virus only occurs when tight junctions are disrupted.[64] Since epithelial cells are CD4 negative,[76,84] alternate receptors, namely galactosyl ceramide (GalCer) and heparin sulfate proteoglycans, are required for epithelial transcytosis.[60,61,80-83,85] The role of HIV coreceptors in transcytosis is still unclear. CXCR4 and CCR5 expression by mucosal epithelial cells varies, depending on the location of the mucosal tissue.[76] For example, gingival epithelium expresses CXCR4 but little CCR5[82,86] whereas jejunal epithelium expresses substantial levels of CCR5 but no detectable CXCR4.[83] In tissues that express HIV co-receptors, blockade of the receptors by mAbs or inhibitors reduces transcytosis of the virus,[82,83] indicating that when present, HIV co-receptors can facilitate HIV transcytosis. However, both CXCR4- and CCR5-tropic viruses can be successfully transcytosed, regardless of the co-receptor expression of the mucosal tissue.[60,82] Interestingly, only CCR5-tropic virus has been observed to establish infection (see discussion below), suggesting that CCR5-dependent transcytosis of HIV is particularly important in HIV transmission or that selection of co-receptor tropism in transmitted HIV variants is independent of transcytosis.

HIV target cells, including CD4+ T cells, macrophages and Langerhans cells, are present in adult and infant upper GI tissues (Figure 1.3).[60,64,87-89] However, in healthy upper GI tissue, Langerhans cells are the predominant immune cell type in the epithelium with CD4+ T cells and macrophages primarily observed in the lamina propria.[60,88,90] Langerhans cells are tissue dendritic cells that express the HIV receptor CD4[88] as well as the HIV co-receptor CCR5.[91]

Although the cells are capable of internalizing HIV, they do not appear to produce many infectious virions, following viral integration.[92,93] However, like other dendritic cells, they can efficiently transfer virus to other HIV target cells of the lamina propria, including CD4+ T cells and macrophages, via a mechanism known as trans-infection,[94,95] a process that occurs efficiently with HIV variants transmitted through breast milk.[96] Interestingly, the density of Langerhans cells in oral mucosa is significantly lower than that observed in the vaginal, cervical and foreskin mucosa, which may result in a lower risk of HIV acquisition across the oral mucosa.[88] If HIV can survive the inhospitable environment of the stomach, the intestinal mucosa also has numerous CD4+ T cells present in the epithelium, making it possible for virus at this site to directly infect cells that have the capacity to spread HIV without the assistance of additional cell types.

A.




B.

| Cell Type | Function in HIV Transmission |
|---|---|
| Keratinocyte | Transcytosis |
| Langerhans Cell | Trans-infection of HIV target cells |
| T Cell | Productive Infection |
| Macrophage | Productive Infection |

**Figure 1.3: Mucosal cells involved in HIV transmission. A.** Diagram showing the location of cells in the oral mucosa. **B.** Function of each cell type in HIV transmission.

Similar to HIV transmission through other mucosal routes, a transmission bottleneck occurs during breast milk HIV transmission. One or two viral variants are responsible for establishing

infection in infant oral transmission,[70,97,98] despite the diversity of HIV in breast milk. Higher SIV doses substantially increase the number of founder variants in macaque studies,[99-102] and a similar phenomenon has been observed in infants whose mothers seroconvert during breastfeeding,[98,103] likely due to high breast milk viral loads during acute infection. Also, similar to other routes of infection, only CCR5-tropic HIV viral variants establish infection *in vivo*.[97,103] Consistent with this observation, only macrophages infected with CCR5-tropic virus can migrate through infant oral mucosa to gain access to the HIV target cells of the lamina propria.[60,96] Interestingly, HIV variants transmitted during breastfeeding have fewer glycosylation sites and shorter Env sequences compared to maternal viral variants,[97] although this phenomenon was not recapitulated in the SIV/infant macaque model.[98] There is some evidence that virus populations in breast milk can harbor unique viral variants relative to those observed in the blood.[104] However, this compartmentalization is only observed in a subset of individuals, and the virus in breast milk remains diverse and does not differ from blood viral variants in glycosylation sites or Env sequence length.[44,54,57,105-108] Inflammatory lesions at the mucosa can result in an increase in the number of variants that establish infection, suggesting that the viral bottleneck is greatest at the mucosa itself.[109] However, it is also possible that an additional bottleneck may be present during dissemination of virus from mucosa to the systemic circulation. One potential mechanism for the observed bottleneck in mucosal transmission of HIV, despite the presence of cells that readily transcytose and replicate the virus, is that anti-viral responses in the upper GI tract may reduce, but not abolish, HIV transmission following oral exposure.

**Soluble, Innate and Adaptive Immunity in Breast Milk HIV Transmission**

The ability of salivary immune components to inhibit HIV has been well studied. This has led to the identification of a wide array of oral mucosa defenses against HIV, including both innate and adaptive immune responses.

Saliva can rapidly kill HIV-infected leukocytes; preincubation of HIV with saliva reduces HIV infectivity; and saliva inhibits HIV replication in infected cells.[110-119] Filtration of HIV-incubated saliva prior to application to target cells dramatically reduced the infectivity of the inoculum, a phenomenon not seen with filtered HIV alone.[114,116,120] This phenomenon may be due to the action of salivary components that can aggregate HIV, including thrombospondin-1 (TSP-1)[121,122] and mucins, particularly 5B, 7A and 7B,[123,124] some of which are also present in breast milk.

In addition to HIV aggregation, there are also additional mechanisms by which salivary proteins protect against HIV. Mucin 1 and 4 from breast milk reduces DC-SIGN-dependent HIV infection by binding to specific carbohydrates on DC-SIGN.[125,126] Mucin-containing components of human saliva can also strip gp120 from HIV viral particles, rendering them uninfectious.[127] Salivary agglutinin, also known as gp340, inhibits CCR5- and CXCR4-tropic HIV infectivity by binding to gp120.[128,129] Additionally, TSP-1 binds CD4 to block HIV entry into target cells.[121] Lactoferrin, a protein present in high levels in both breast milk and saliva,

can also block HIV entry into target cells.[130-134] Cystatin also shows anti-HIV activity, including the ability to block infection of host cells, at concentrations observed in saliva.[115,123]

Interestingly, breast milk has the ability to both inhibit and enhance HIV infection in vitro, in a breast milk donor-specific manner.[135] Many saliva proteins associated with reduced HIV infectivity by saliva are also found in breast milk, most notably lactoferrin. Another, newly discovered breast milk protein, tenascin-C can also inhibit HIV-1 infection by binding to HIV-1 Env.[136] In addition, studies have also found a decreased risk of HIV transmission in mothers with elevated breast milk levels of sTLR2,[137,138] CCL4,[139] erythropoietin,[140] IL15,[141] and specific long-chain fatty acids[142] or polysaccharides,[143] indicating that many host factors could reduce HIV acquisition from breast milk. On the other hand, breast milk with high levels of pro-inflammatory cytokines and chemokines can enhance in vitro HIV infections, independent of the HIV infection status of the donor.[135]

Secretory Leukocyte Protease Inhibitor (SLPI) has strong anti-HIV activity *in vitro* at concentrations found in human saliva and breast milk[115,144] and is particularly effective at blocking HIV infection of monocytes.[145,146] Depletion of SLPI from whole saliva results in a substantial loss of salivary anti-HIV activity.[115,131,147] *In vivo*, higher SLPI salivary levels in HIV-exposed infants reduces their susceptibility to HIV.[148] However, breast milk levels of SLPI does not appear to correlate with reduced breastfeeding transmission of HIV.[149] SLPI does not bind to HIV proteins or CD4,[115] but does bind Annexin II on macrophages.[150] Blockade of

Annexin II by SLPI or siRNA knockout of Annexin II reduces macrophage infection *in vitro*, suggesting SLPI may inhibit HIV by acting on this cellular protein, although though the details of this interaction are still poorly understood.[150] In addition to expression in salivary glands, SLPI is expressed both intracellularly and extracellularly in infant tonsils, and inhibition of SLPI expression in adult human tonsil tissue results in decreased inactivation of transcytosed HIV.[64]

Another class of potent anti-microbial proteins that are abundant throughout the gastrointestinal tract and in breast milk,[151-159] human beta-defensins (hBD) also inactivate transcytosed HIV.[64] HBD-2 and 3, and possibly hBD1, reduce HIV replication in infected cells, and can reduce the expression of CXCR4, but not CCR5.[160,161] Alpha-defensins 1, 2 and 3, similar small proteins with anti-HIV activity,[162,163] are also expressed throughout the GI tract,[152,156] and breast milk levels of alpha-defensins correlate with reduced HIV acquisition in breastfed infants.[164] Theta-defensins, also known as retrocyclins, a new class of circular defensins first identified in non-human primates, have anti-HIV activity in humans by binding CD4, GalCer and glycosylated HIV Env.[165-167]

Interestingly, the gastrointestinal microbiome may also play an important role in protecting against HIV acquisition. Bacteria isolated from breast milk, particularly lactobacillus and pediococcal bacteria, common gastrointestinal microbiota, can inhibit HIV infection of target cells.[168] One potential mechanism for how specific microbes may modulate HIV susceptibility is the ability of bacteria, like lactobacilli, to produce hydrogen peroxide.[169] Saliva contains high

levels of peroxidases, which utilize hydrogen peroxide to produce reactive oxygen species that can rapidly inhibit HIV.[170]

It is important to note that most of these innate, anti-viral factors have been documented at other mucosal sites, including the vaginal and rectal mucosa. Although elevated mucosal expression of many of these same factors is correlated with reduced vaginal transmission of HIV,[171,172] HIV transmission still occurs even in the presence of innate anti-HIV factors. Although these factors may certainly be responsible for the low rate of HIV transmission per exposure, they are unable to entirely prevent HIV acquisition.

Anti-HIV adaptive immune responses can also provide protection against HIV acquisition, following oral exposure to HIV. Genetic variations in HLA genes modify the antigens targeted by adaptive immune responses, and infants that share HLA genotypes with their mother have increased susceptibility to breast milk transmission of HIV.[148] In addition, particular infant HLA genotypes are associated with a much higher risk of breast milk HIV acquisition,[173] highlighting the importance of adaptive immunity in reducing oral HIV transmission.

Antibody responses, both mucosal and systemic, appear to be particularly important in protecting against oral transmission of HIV. Strikingly, high dose intravenous administration of cocktails of neutralizing, anti-HIV antibodies to infant macaques protects against SHIV challenge.[174,175] However, it is important to note that only a subset of neutralizing antibody cocktails show full

protection in neonatal macaques.[174,175] Studies of anti-HIV responses in breast milk from HIV-infected mothers have shown that increased breast milk IgM responses[176] and antibody dependent cellular cytotoxicity (ADCC) activity[177] is associated with decreased risk of HIV infection in breastfed infants. Chronic exposure to HIV can induce anti-HIV antibody responses in the oral mucosa, namely IgA responses, as seen in HIV-uninfected partners of HIV-infected individuals.[178] However, no studies have demonstrated protection by salivary anti-HIV antibodies *in vitro*, although these studies particularly focused on IgA antibodies,[179] which are associated with increased risk of HIV transmission in breast milk.[180] The ability of anti-HIV antibodies to block HIV transcytosis is somewhat unclear. Although some studies have observed effective inhibition of HIV transcytosis with breast milk-derived anti-HIV IgA and IgG,[181,182] other researchers have failed to observe this phenomenon.[183] Interestingly, anti-HIV IgM and IgA applied to the basal side of epithelial cells can act within epithelial cells to block HIV transcytosis.[184]

The importance of T cell responses in protecting against oral HIV transmission is still unclear. Nearly 50% of HIV-exposed, uninfected infants in the absence of maternal ART have detectable T cell responses[185,186] and those responses are maintained for at least 6 months after birth.[187] A recent analysis found that the breadth and magnitude of breast milk HIV-gag T cell responses correlate with reduced risk of breast milk MTCT.[139] However, it is likely that both antibody and T cell responses are important for full protection against HIV. Therefore, an HIV vaccine that can effectively block oral transmission of HIV will most likely elicit robust antibody and T cell responses at the site of virus exposure, the upper digestive tract.

**Inflammation and Breast Milk Transmission of HIV**

Although infants are exposed to HIV-containing breast milk for many months, even in the absence of antiretroviral prophylaxis, only a relatively small number of infants born to HIV+ mothers will become infected.[188] This suggests that certain infants may be particularly susceptible to HIV. Understanding the factors that enhance oral HIV transmission will be critical for developing effective interventions to protect infants from postpartum transmission of HIV.

It is well documented that different types of mucosal inflammation at multiple sites impact sexual and vertical transmission of HIV.[189-197] Oral mucosa inflammation is also a risk factor for oral HIV transmission. For example, infant oral candidiasis increases the risk of MTCT of HIV through breastfeeding,[198] and CMV viral loads in breast milk, which can induce an immune response in breastfed infants,[199] is also an independent risk factor for HIV transmission.[200] In contrast, studies in HIV-uninfected, highly exposed individuals indicate that low levels of CD4+ T cell activation and quiescent CD4+ T cell phenotypes are associated with reduced HIV susceptibility.[201-204] Similarly, a low level of mucosal HIV target cells is associated with the low level of breast milk MTCT in sooty mangabeys, a natural host of SIV.[205] These studies suggest that inflammation at the oral mucosa increases susceptibility to HIV.

Two hypotheses offer a mechanism for the observed increase in HIV transmission, following

inflammatory events at the oral mucosa. The first is that inflammation leads to a break in the mucosal barrier, permitting viral access to target cells that reside beneath the difficult-to-penetrate stratified squamous epithelium of the oral cavity. Second, inflammatory events can recruit activated HIV target cells to mucosal tissues, while the mucosal barrier remains intact. Indeed, inflammatory cytokines can directly increase HIV replication,[206-209] indicating that an inflammatory mucosal environment alone may promote the spread of HIV virus to new cells and additional tissues.

Due to its unique ability to provide detailed mucosal information both before and during early infection, our understanding of inflammatory events that influence oral transmission relies on the SIV-macaque model. In one study, Chenine et al. utilized 10% acetic acid to induce a inflammatory sore on the inside cheek pouch with subsequent exposure to low dose oral SHIV challenge.[210] These investigators identified an increased risk in SHIV infection in treated macaques, which likely resulted in both increased access to underlying target cells, recruitment of additional target cells to the site of inflammation as well as an activation of the target cells themselves.[210] In contrast, a study from our laboratory experimentally induced gingival inflammation in adult rhesus macaques that mimicked the mild to moderate gingivitis common in humans.[211] This was accomplished by tying silk ligatures around the base of the teeth and softening the macaque's food with water. This treatment induced strong upregulation of multiple inflammatory markers in the oral tissue, including IL6, IL8, and IL18. Macaques were then orally exposed to repeated low dose challenge of SIVmac251 and, surprisingly, the rate of SIV transmission was similar in the gingivitis and control macaque groups, although an increase in

the number of viral variants that establish the SIV infection was observed.[211] One interesting distinction that may explain the different findings in these two studies is that while gingivitis does result in inflamed mucosa it does not necessarily compromised the mucosal barrier, which likely occurred with acetic acid treatment, potentially explaining the increased susceptibility in the acetic acid treated animals but not in the gingivitis animals.

In addition to inflammatory changes that are induced directly at the mucosa, activation of the systemic immune system may have an indirect impact on target cells at mucosal sites. Indeed, any infection, or even a vaccination, could potentially activate HIV target cells at both systemic and mucosal sites, potentially resulting in increased HIV transmission. This may have been the case in the STEP trial, where individuals who were previously exposed to the vaccine vector or were uncircumcised had an increased risk of acquiring HIV after vaccination.[212] This suggests a complex interplay between vaccine-induced immune activation and mucosal tissues, which may have resulted in increased HIV susceptibility.

The studies described here provide evidence that inflammation and activation of HIV target cells at the oral mucosa has the potential to increase the oral transmission of HIV.

**HIV Target Cells in the Infant**

The infant immune system has distinct features compared to that of adults. A combination of antigen inexperience and the need to not react to novel antigens, including maternal antigens, results in distinctive immune responses that are still being characterized. Because HIV specifically infects immune cells, the altered immune environment in infants may significantly alter the susceptibility of infants to HIV.

There is substantial variation in the published literature on the relative proportion of HIV target cells in infants. Nearly all studies have focused on blood levels, with a particular focus on umbilical cord blood. White blood cell and lymphocyte counts are highly dynamic within the first several years of life, increasing in the first year of life and then decreasing from infancy to adulthood.[213,214] Infants appear to have more T cells than adults.[215] However, the relative proportion of CD4 T cells within that pool is less clear. There are studies that report a decreased,[216,217] increased[213] or constant[214,218] CD4 T cell population in infants compared to adults.

In addition to modification in the HIV target cell pool in infancy, infant CD4 T Cells, monocyte/macrophages and dendritic cells also express distinctive surface markers. Infant CD4 T cells are nearly exclusively naïve, i.e. antigen inexperienced.[216,219,220] In fact, the proportion of CD4 T cells with a naïve phenotype increase within the first several years of life,[219] suggesting an active inhibition of memory phenotype induction in infants, at least in the blood. Memory cells represent less than 10% of CD4 T cells in infants, substantially lower than in adults.[217,221] Recently memory T cells have been divided into two populations, an effector

memory pool, which responds at sites of infection, and a central memory pool that remains resident in the lymphnodes and blood without entering tissue.[222] The effector memory pool is substantially smaller in infants, compared to adults.[216,220] In addition, the central memory compartment is either stable between adults and infants,[216,219] or lower in infancy.[220] A naïve phenotype on a CD4 T cell decreases the magnitude of its response to stimuli,[223] potentially resulting in lower activation induced by HIV (or non-HIV) exposures in infants. The effector memory population is responsible for the majority of HIV production in HIV-infected adults, therefore, the smaller population of effector memory CD4 T cells in infants may limit the ability of HIV to propagate in infants.

Infant CD4 T cells also have different responses to stimuli than what is seen in adults. In the first months of life, infant T cells are less likely to proliferate, compared to their adult counterparts, in response to a diverse set of stimuli.[220,224] Classically, infant CD4 T cells have been considered Th2-skewed, producing a more robust IL-4, IL-5 and IL-13 response to stimuli, with a paucity of Th1 responses, IFNg, TNFa and IL-6 production. Although this is the case in many settings,[213,217,220,225-234] there is more nuance to infant CD4 T cell responses. Some stimuli can induce the production of robust Th1 responses in infants that can either equal[235-237] or even surpass[227,230,233,238-243] the response seen in older children or adults. Furthermore, some investigators have found lower IL-4 production in either cord blood[226] or purified CD4 T cells,[227] compared to adults. In addition, infant T cells also generally have a reduced capacity to produce Th17 cytokines in response to stimuli.[225,238,244] However, infant cells produce a robust Th17 response to H pylori[225] and BCG.[240]

One potential mechanism for the reduced proliferative, Th1 and Th17 responses observed in infants is an increase in suppressive, T regulatory cells observed in infants.[224,238,244,245] Interestingly, a study of isolated hematopoietic stem cells from fetuses and adults showed that T cell precursors from fetal tissue were more than 3 times more likely to differentiate into T regulatory cells than their adult counterparts under the same conditions,[246] suggesting that infants cells at the very early stage of the hematopoietic progenitor are already primed to result in a regulatory T cell response. However, infant T regulatory cells have impaired functionality, similar to their Th1 and Th17 counterparts, expressing lower levels of FoxP3,[224,247-249] secreting lower levels of regulatory cytokines (IL10 and TGFb)[217,226,228,230,240,250,251] and less suppressive activity.[224,248,250,251] Therefore, it remains unclear if there is overall enhanced T regulatory activity in infants. This suggests that there are likely additional mechanisms for the altered TH1, TH17 and T regulatory responses observed in infants.

A number of potential mechanisms for the differences observed in T cell activation between infants and adults have been proposed with convincing evidence to support each model. This includes the observation that infants have lower circulating levels of arginine in their plasma, a key factor required for robust proliferative T cell responses.[252] In fact supplementation of infant PBMC cultures with arginine increases proliferation substantially, in an IL2R-independent manner.[252] There also appear to be significant differences in the kinetics and magnitude of calcium flux initiated upon T cell stimulation.[253,254] However, different studies have observed different patterns of calcium flux with different stimuli. These differences in calcium flux have

been attributed to expression of a specific microRNA (miR-181a)[254] and higher voltage-gated potassium channel expression in neonatal T cells.[253] In addition, lower levels of NFAT1, a key driver of Th1 differentiation, is induced at lower levels in stimulated infant T cells, also mediated by a microRNA (miR-184),[255] and a lower magnitude of MAPK3 induction, an intracellular signaling molecule involved in multiple steps in T cell activation and replication, after stimulation.[229] The diversity of potential mechanisms for reduced T cell responsiveness in infants suggests that there is a coordinated program that prevents excessive immune activation in infants.

These altered immune responses to stimulation result in altered HIV immune responses and disease progression. Infants that are exposed to HIV during infancy, through the consumption of breast milk, have, roughly, a 40% risk of acquiring HIV.[21] It is very difficult to compare the relative susceptibility of infants and adults to HIV, due to the very different mechanisms of HIV transmission to infant transmission (childbirth and breast feeding) and adult (sexual transmission). However, once infants acquire HIV, many are unable to control viral replication, and disease progression is far more rapid than that seen in adults.[256] In fact, nearly 50% of infants infected with HIV in Africa will not survive past 2 years of age.[256] This may be, at least partly, due to less effective immune response to HIV infection.[256] Therefore, the altered immune phenotype in infants makes pediatric HIV, in developing countries, a more rapid and lethal disease than adult HIV infection.

This thesis explores four different exposures in early infancy that can alter immune activation and HIV target cell availability in South African infants born to HIV-infected mothers. Chapter 2 will evaluate the role of immune activation in increased HIV susceptibility observed in infants introduced to non-breast milk foods early in life. Chapter 3 will then describe a series of smaller studies that evaluate the contribution of maternal HIV disease progression, oral candidiasis and vaccination to infant immune activation, to provide insights into the relative contribution of mixed feeding to HIV target cell levels and activation in infants.

## Chapter 2: HIV Target Cells and Infant Feeding Practices in Khayelitsha, South Africa

One perplexing observation in MTCT of HIV is the finding that mixed feeding, where infants receive breast milk and other foods, results in an increase in the rate of HIV breast milk transmission compared to exclusive breastfeeding. Indeed, in one study from KwaZulu-Natal, South Africa, infants who were mixed fed at any point after birth were almost 11 times more likely to acquire HIV than those who received breast milk alone.[43] In a second study, Kuhn and colleagues showed that the risk of postnatal HIV infection in the first four months of life was significantly lower among exclusively breastfed infants compared to mixed fed infants.[29] Multiple analyses have confirmed these findings.[29,30,257-259]

Many possible mechanisms for the increase in HIV susceptibility of mixed fed infants have been postulated. One study observed a higher level of cell free but not cell associated HIV in the breast milk of mothers that mixed fed relative to exclusively breastfed infants.[260] However, this increase in breast milk viral load was not observed in other studies.[41,261] Despite mucosal permeability being a clear risk factor for MTCT during breastfeeding,[262] mixed fed infants do not have an increase in GI permeability.[41,261,263] However, mixed feeding is associated with an increased risk of a number of inflammatory conditions, including asthma, eczema, atopic dermatitis, food allergies, ear infections and diarrhea.[264-266] In addition, formula feeding increases CD4 T cell activation in infants,[267] likely due to exposure to new food antigens and suggesting a possible mechanism for the increased HIV susceptibility of mixed fed infants.

I, therefore, hypothesized that immune activation, induced in mixed fed infants by exposure to food contaminants or changes in the microbiome is responsible for the observed increase in HIV transmission in mixed fed infants.

**Methods**

This hypothesis was evaluated in an observational, longitudinal study in HIV-unexposed infants in Khayelitsha, South Africa, which was designed to evaluate the mechanism underlying increased HIV transmission in mixed fed infants. HIV-unexposed infants were selected to control for the potentially confounding variables of maternal HIV viral load and HIV disease status, both of which are reflected in breast milk and can alter immune activation in the infant (Chapter 3). Blood, oral mucosa and stool samples were collected from the infants at birth, 6 and 14 weeks of age. Although all infants were Exclusively Breastfed at enrollment, per enrollment criteria, mothers were free to introduce non-breast milk foods whenever they wished. This study design was chosen since a significant body of data shows that for infants in communities with limited access to clean drinking water and high risk of infectious disease, there is substantially increased morbidity and mortality for infants that are not exclusively breast fed.[268-270] Therefore, even though the early introduction of non-breast milk foods is a frequent practice in Khayelitsha, any randomization of infants to non-breast milk foods would be unethical.

This study will be referred to as the **Feeding Study** throughout this thesis. This study was led by the Jaspan lab, in close collaboration with the Sodora laboratory.

*Community*

Khayelitsha is an informal settlement outside of Cape Town, South Africa. The community was founded in the 1980s by the South African government under the apartheid Group Areas Act, but has since become home to many South Africans, predominantly Xhosa from the Eastern Cape,[271] in search of work in affluent Cape Town. In 2011, Khayelisha's population was estimated at 390,000 by the City of Cape Town. Around 55% of households in the community live in informal housing, temporary housing structures often built of tin and cardboard. Nearly 20% of all households in the community have no income, and an additional 30% of households subsist on less than $300 (R1600) a month.[271] In contrast, the median income in Cape Town as a whole is approximately $600 (R3200) per month, making Khayelitsha one of the poorest communities in Cape Town.[272] This is, at least in part, a reflection of the intense economic disadvantage of the Black African community in Cape Town. Eighty seven percent of Black households in Cape Town make below the median income for the City of Cape Town, in contrast to 41% of Coloured (i.e. mixed race) households, 24% of Asian households and only 15% of White households.[272] This extreme divide in earning potential is present across South Africa. In fact, South Africa's Gini Index, 65.0, a metric of income inequality, is second only to that of the Seychelles, 65.8, measured in 2007. For comparison, the most recent Gini Index was 41.1 for the United States and 47.7 for Kenya.[273]

Income inequality and poverty are known risk factors for elevated HIV infection rates on a community level,[274-276] so it is no surprise that Khayelitsha has a very high HIV prevalence. Although it is difficult to estimate the population-level HIV infection rate for the community, 33% of pregnant women that attended an antenatal clinic in Khayletisha tested positive for HIV in 2010.[277] Due to the high rates of maternal HIV infection, governmental and non-profit

organizations have initiated aggressive programs to protect infants from acquiring HIV from their mothers. These programs were rolled out on a large scale in 2008, and since their implementation, infant HIV infection has fallen from 12% to less than 5%.[277] Despite this great success, it is estimated that half of all under-5 mortality in South Africa in 2011 was due to HIV infection.[278] Therefore, it is critical to identify additional interventions that can further decrease, and, ideally, eliminate MTCT of HIV in this setting.

Although infants born to HIV-infected mothers have lower rates of mixed feeding than HIV-uninfected mothers, roughly 30% of HIV-exposed infants are exposed to both breast milk and non-breast milk foods.[279] Therefore, Khayelitsha is an ideal community to recruit for and benefit from this study.

*Sample Collection*

A total of 156 infants were recruited at birth from the maternity ward at the Site B Clinic in Khayelitsha, Western Cape, South Africa, into a prospective cohort. At the time of enrollment, the mothers of all enrolled infants indicated that they planned to exclusively breastfeed their infant. Infants who were born to HIV-infected mothers, as self-reported by the mother, and to mothers under 18 years of age were excluded from the study. All mothers of recruited infants were orally informed of potential risks and benefits of the study, and signed an IRB-approved consent form in either English or Xhosa. Mothers of enrolled infants were also provided with a consent form in their preferred language to take home. At 6 and 14 weeks of age, mothers orally completed a survey that included detail information about the foods consumed by each infant and the health status of each infant since the last study visit, including receipt of vaccinations. In

addition, infants received a full physical exam, including anthropometrics and evaluation for any oral or skin conditions. Finally, blood (EDTA by venipuncture; vendor), stool, oral cytobrush samples (OralCDx Brush; OralCDx Diagnostics, New York, USA) and saliva samples (Salivette; Sarstedt, Germany) were collected at each study visit.

*Blood Processing*

Blood was collected to identify immune activation in the systemic circulation. Blood samples were stored at room temperature and transported to the lab within 6 hours of collection. PBMCs were isolated from each sample, using Ficoll (Sigma, Missouri, USA) density gradient separation. PBMCs were slowly cooled to -80°C in DMSO (Sigma, Missouri, USA) + 10% FBS (Biochrom, UK), and transferred to liquid nitrogen for storage. Blood processing was performed by the Jaspan Lab in Cape Town, South Africa.

*Blood Flow Cytometry*

PBMCs were evaluated by flow cytometry to identify HIV target cells in the systemic circulation, with a particular focus on identifying **regulatory T cells (Tregs)** and HIV target cell activation. Cryopreserved PBMCs were rapidly thawed at 37°C, washed and resuspended in PBS (Sigma, Missouri, USA) + 1% FBS (Biochrom, UK). Each sample was then stained with Live/Dead-Violet (Life Technologies, New York, USA) for 20 min at room temperature, stained with anti-CCR5-FITC (2D7, BD Bioscience, California, USA) at 37C for 15 minutes, and then stained with anti-CD4-QDot 655 (S3.5, Life Technologies, New York, USA), CD39-PE (eBioA1, eBioscience, California, USA), CD14-PE-TexasRed (RMO52, Beckman Coulter), HLA-DR-PE-Cy5 (LN3, eBioscience, California, USA), a4b7-APC (A4B7, NHP Reagent

Resource, Massachusetts, USA), CD3-Alexa680 (UCHT1, BD Bioscience, California, USA) and CD25-APC-Cy7 (M-A251, BD Bioscience, California, USA) for 20 minutes at room temperature. Samples were then washed, permeabilized (Cytofix Cytoperm, BD Bioscience, California, USA), and stained with anti-TNF-PE-Cy7 (Mab11, BD Bioscience, California, USA) for 20 minutes at room temperature. A final wash was performed and then cells were resuspended in 2% paraformaldehyde and collected on an LSR II within 24 hours of staining. Blood processing and flow cytometry staining was performed in the Jaspan Lab in Cape Town, South Africa.

*Stool Processing*

Stool samples were collected to evaluate the stool microbiome of mixed and exclusively breastfed infants. If an infant had stool in their diaper at their study visit, a stool sample was collected, with caution to avoid stool that was in direct contact with the diaper. If stool was not present at the study visit, mothers were provided with a sample cup, and instructed to collect stool and return the sample cup to the clinic within one day of collection. Samples were returned to the clinic no more than 2 days after collection. After receipt at the clinic, stool was stored at 4°C and transported to the laboratory within 6 hours, where the stool was immediately placed at -20°C. Stools were thawed, and DNA was then extracted, using the MioBio Powerfecal DNA kit. The quantity of extracted DNA was then assessed by Quant-iT™ PicoGreen® reagent (Invitrogen, California, USA). Isolated DNA, suspended in water, was then frozen at -80C for shipment to the Brodie laboratory in Berkeley, California, USA for library preparation and sequencing. Stool processing was performed by the Jaspan Lab in Cape Town, South Africa.

*Stool Microbiome DNA Amplification and Sequencing*

To identify bacterial species in the stool samples, the 16S rRNA gene, which is specific to bacteria, was amplified and sequenced. DNA was thawed, and the V4 region of the 16SrRNA gene was amplified using 515F and 806R primers.[280] 10 ng of stool DNA was amplified in triplicate by PCR with 0.025U Hot Start ExTaq (Clontech, California, USA), 0.56mg/mL BSA (Roche, Switzerland), 1x PCR buffer (Clontech, California, USA), 200uM dNTP mixtures (Clontech, California, USA) and 400mM each of forward reverse primers. The primers used for amplification are shown in Table 2.1. Total reaction volume was brought to 25uL with sterile water, and the samples were amplified under the conditions detailed in Table 2.2. After amplification, replicates were combined. Amplified DNA was purified with SPRI magnetic beads (Beckman Coulter, California, USA). 90uL of SPRI beads were added to 75uL of amplified DNA and incubated at room temperature for 10 minutes with gentle tapping. Samples were then placed on the magnetic plate for 2 minutes, the supernatant removed and discarded, and then washed twice with 120uL of 80% ethanol. Excess ethanol was allowed to evaporate before tube was removed from the magnetic plate. 50uL of 1X TE buffer was added to the beads and pipetted to release the purified PCR product. Tubes were then returned to the magnetic plate for 1 minute and the supernatant, which contains the purified PCR product, removed and stored at -80C until sequencing. Purity of pooled amplicons diluted to 10nM were evaluated on a bioanalyzer (Agilent, California, USA). Purified amplicons were then sequenced, using an Illumina Miseq platform (Illumina, California, USA).

**Table 2.1: Primer sequences for amplification of 16S rRNA gene.** Illumina adapter sequences are in bold and 16S rRNA gene-specific sequences are italicized.

| | |
|---|---|
| Forward Primer | **AATGATACGGCGACCACCGAGATCTACAC**TATGGTAATTGT*GTGCCAGCMGCCGCGGTAA* |
| Reverse Primer | **CAAGCAGAAGACGGCATACGAGAT**AGTCAGTCAG*GGACTACHVGGGTWTCTAAT* |

**Table 2.2: PCR amplification cycles.**

| | | |
|---|---|---|
| 1 cycle | 98C | 2 min |
| 30 cycles | 98C | 20s |
| | 50C | 30s |
| | 72C | 45s |
| 1 cycle | 72C | 10 min |
| | 4C | Hold |

All stool 18S rRNA DNA amplification and sequencing was performed by the Brodie Lab in Berkeley, California.

*Stool Microbiome Data Analysis*

Computational analysis of the 16S rRNA gene sequences was used to identify bacterial species in stool samples and to identify variation in the bacterial populations between feeding groups. Of the 140 samples for which sequencing data was obtained, 9 were exclude based on the fact that they had < 500 reads (arbitrary cutoff), leaving 131 samples. Four samples did not have feeding annotation available. Across the 131 samples, 452 operational taxonomic units (OTUs defined as having ≥97% similarity within an OTU) were identified.

Alpha diversity was calculated, using the Shannon Diversity Index.

Differential abundance testing was performed using the R package metagenomeSeq. The following filters were applied to the results:

- Keep features where column 'adjPvalues' OR column fisherAdjP' ≤ 0.05 (previously only based on adjPvalues)
- Keep features where absolute coefficient ≥ 1.5 (for binary variables the coeff. can be interpreted as a fold change)
- Discards OTUs where neither mixed or exclusively breastfed infants has ≥ 30% positive groups UNLESS the feature has a fisherAdjP ≤ 0.05 (then keep)

For unsupervised hierarchical analysis, OTUs were filtered so that only OTUs that were present in at least 10% of samples were included. Clustering was performed using Euclidian distance with complete linkage. All microbiome data analysis was performed by the Jaspan Lab in Cape Town, South Africa.

*Oral Cytobrush RNA Processing*

Samples of the oral epithelium were collected to evaluate if immune activation was evident at the site of HIV exposure in breastfed infants. Samples were collected with the Oral CDx brush, which was immediately placed into RLT lysis buffer (Qiagen, Netherlands) and stored at 4C until transport to the Jaspan laboratory. Samples were then placed at -80C and shipped on dry ice to the Sodora laboratory in Seattle, Washington, USA for processing. RNA was isolated from each sample, using an RNeasy kit (Qiagen, Netherlands). RNA was eluted in water, and then quantified on a Nanodrop (NanoDrop, Delaware, USA). All oral sample mRNA processing and analysis was performed in the Sodora Lab in Seattle, WA.

*Oral Cytobrush Nanostring Sample Processing and Analysis*

The Nanostring platform was used to evaluate expression of inflammatory mediators in the oral mucosa. The 24 samples collected at 14 weeks of age with the highest oral cytobrush RNA levels were selected for Nanostring (Nanostring, Washington, USA) analysis. 150ng of each sample was diluted to 30ng/uL and run on a custom Nanostring chip with probes against a total of 248 human genes associated with HIV infection and inhibition as well as cytokines and chemokines that may mediate inflammatory responses (Figure 2.1)(supplemental table 1). Raw data for each gene was normalized by GAPDH or MAPK3 copy number. The water used to

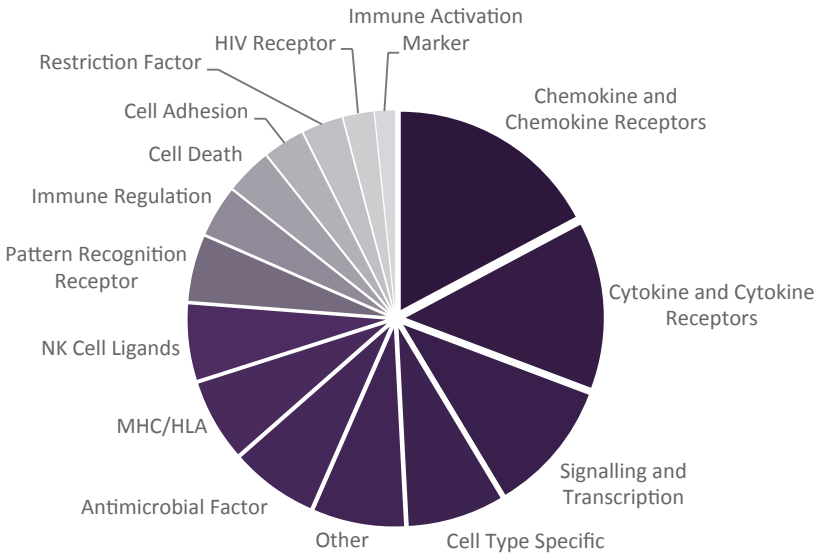



**Figure 2.1: Genes analyzed by Nanostring by function.** The thickness of each slice represents the proportion of genes that are in each category. Genes that match more than one category were placed in the category with the greatest role in defining HIV susceptibility.

dilute each sample was run in triplicate on the same chip. The lower limit of detection for each gene was defined as the average+2*(standard error) for the three water controls or the average+2*(standard error) for negative control probes (probes directed against sequences not found in nature), whichever was greater (supplemental table 1). Only the 161 genes detectable in at least 25% of samples when normalized by either GAPDH or MAPK3 (supplemental table 1) were used for subsequent statistical analysis. All oral sample mRNA processing and analysis was performed in the Sodora Lab in Seattle, WA.

*Oral Cytobrush qPCR Sample Processing and Analysis*

**Quantitative PCR (qPCR)** was employed to verify expression differences by feeding pattern and age in genes identified in the Nanostring analysis. Based on the data from the Nanostring analysis, 10 genes differentially expressed by feeding pattern were selected for validation by qPCR. A total of 26 samples from 6 weeks of age and 33 samples from 14 weeks of age had sufficient RNA for qPCR analysis. RNA was converted to cDNA with SuperScript III (Invitrogen, California, USA). Taqman Universal Master Mix (Applied Biosystems, California,

USA) and Single Tube Taqman Assays (Applied Biosystems, California, USA) were used to set up qPCR amplification reactions, which were run on a 7500 Fast Real Time PCR System (Applied Biosystems, California, USA) for 40 cycles. Thresholds were set for each gene in the linear range of the curve. Fold changes were then calculated, using the DDCt method, using RPLP0 or MAPK3 as endogenous control gene and the median exclusively breastfed value for each gene as the reference sample. All oral sample mRNA processing and analysis was performed in the Sodora Lab in Seattle, WA.

*Oral Cytobrush Flow Cytometry*

Flow cytometry was employed to identify the cell populations, specifically HIV target cells, present in oral mucosa. Immediately after sample collection, one oral cytobrush was placed in RPMI, and stored at 4C for less than 6 hours. Samples were then transported to the Jaspan laboratory at Groote Schuur Hospital, Cape Town, Western Cape, South Africa. Any cells remaining on the brush were washed off by repeated flushing of the brush with a Pasteur pipette. Cells were pelleted, resuspended in PBS (Sigma, Missouri, USA) + 1% FBS (Biochrom, UK) and transferred to a 96 well plate for staining. Cells were washed and 1uL undiluted Live/Dead Aqua was added to the decanted wells and allowed to sit for 20 minutes at room temperature in the dark. The plate was then washed twice, and incubated with 50uL of the extracellular master mix. The extracellular master mix included: 5uL/well CD45 APC (HI30, Biolegend, California, USA) and 1uL/well CD4 PerCP-Cy5 (L200, BD, California, USA). Antibodies were incubated with the sample for 20 minutes at room temperature in the dark. Cells were then fixed with cytofix/cytoperm and acquired on an LSR II. Flow cytometry sample processing was performed by myself and the Jaspan Lab in Cape Town, South Africa.

*Flow Cytometry Data Analysis*

All flow data was compensated and analyzed, using Flow Jo v10.0.7r2 (Treestar, Oregon, USA). Any sample where less than 5% of singlet events were live were excluded from analysis. If more than 5% of events for any population was above the maximum signal detectable by the flow cytometer or if fewer than 50 cells were detected in the denominator of the population percentage calculation, that population was excluded from analysis. Finally, if no events were detectable, the population was calculated for 1 event to allow for log transformation. All flow cytometry analysis was performed by me in Seattle, WA.

*Plasma Protein Quantification*

Plasma levels of cytokines and chemokines whose expression varied in the oral mucosa were measured by ELISA to determine whether the variation observed in the oral mucsoa was also present in the systemic circulation. Plasma protein levels of CCL22, IL7, IL10 and IL2 (R&D Systems, Minnesota, USA), plasma diluted 1:2, and CCL5 (Affymetrix, California, USA), plasma diluted 1:50, were measured in all available samples. Plasma protein quanitification was performed in the Sodora Lab in Seattle, WA.

*Ochratoxin Exposure Quantification*

Ochratoxin A was measured in a subset of stored plasma from 2 and 6 week old infants. Ochratoxin was quantified by competitive ELISA in accordance with the manufacturer's instructions (Helica, California, USA). Finished plates were quantified on a plate reader (Helica, California, USA). Ochratoxin levels in each sample were calculated based on a 5-parameter

regression of the standard curve values. Values calculated below the lowest standard were assigned the value of the lowest standard. Ochratoxin quantification was measured by the Sodora lab in Seattle, WA.

## Results

### *Study Enrollment*

A total of 156 infants were recruited to the study. Study retention was sub-optimal, with less than 2/3 of the mothers returning for the 6 week visit and just over half returning at 14 weeks (Figure 2.2). Although most mothers that did not return for study visits could not be reached (60%), the most common reason (21%) provided for not returning for later visits was that the mother and/or infant had relocated to outside of the community. Khayelitsha's population is relatively transient. Fewer than 38% of Khayelitsha residents are employed and 50% of the population is under the age of 25.[271] In addition, most residents of Khayelitsha were born in the Eastern Cape,[281] and several study participants reported returning to live near family in the Eastern Cape as the reason for their relocation. Only 8% of all infants enrolled in the study were formally withdrawn from the study by their mothers, suggesting that mothers were not overly concerned that the study would compromise the welfare of their child. In addition, 1 mother that was initially enrolled in the study tested positive for HIV, and therefore was removed from the study. The retention observed in this study is lower

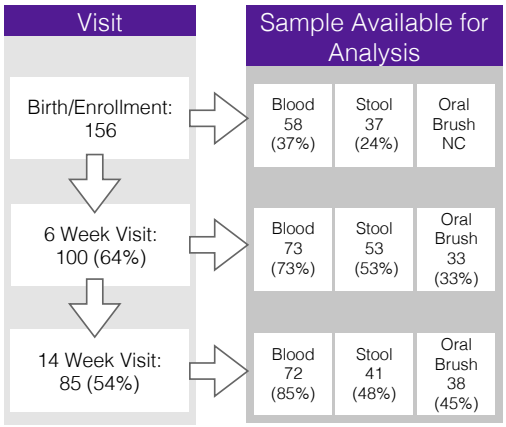



**Figure 2.2: Feeding Study enrollment.** On the left, the number of infants that attended each visit are listed along with the percentage of all enrollees that that attended that visit. On the right are the number and percentage of infants that attended each visit that had blood, stool or oral brush samples available for analysis. NC = not collected

than that observed in cohorts in this community,[282-284] but similar to retention in cohorts of HIV-uninfected mothers in other studies.[285,286]

*Feeding Patterns in Khayelitsha, South Africa*

By 6 weeks of age, about half of the infants in the cohort had been introduced to non-breast milk foods, which rose to 80% of infants by 14 weeks of age (Figure 2.3A). For this and all subsequent analyses, **Exclusively Breastfed** infants were defined as infants whose mothers only reported feeding their infants breast milk at that and any previous time point. **Mixed Fed** infants were defined as any infants whose mothers reported feeding their child breast milk and cereal, formula and/or fruits and vegetables at that time point. Infants that consume cereal, formula

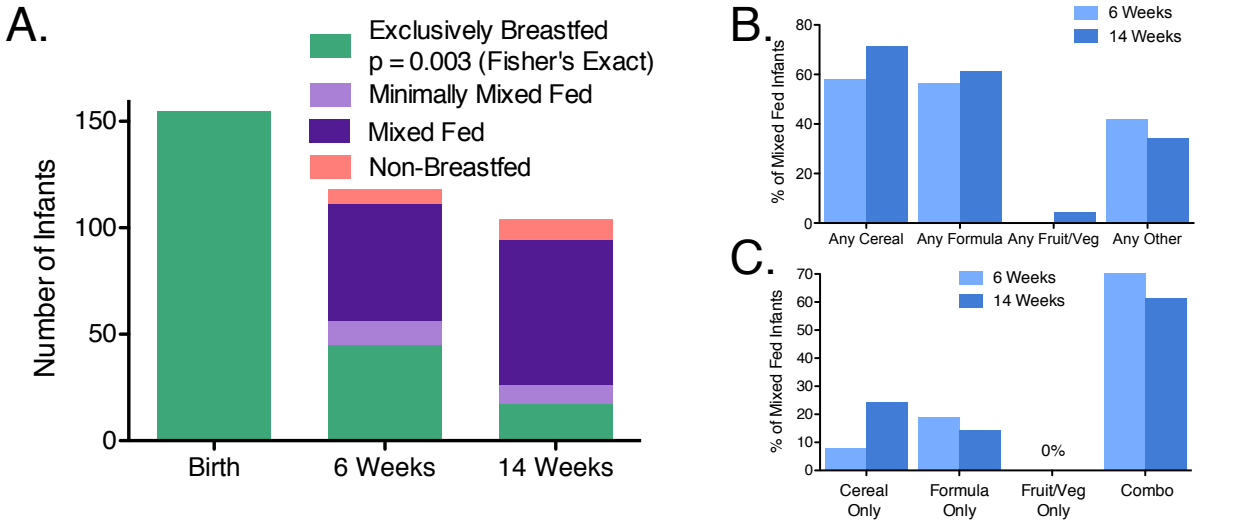



**Figure 2.3: Mixed feeding is common in infants in Khayelitsha, South Africa. A.** Number of infants consuming breast milk only (Exclusively Breastfed), infants that consume cereal, formula and/or fruits/vegetables (Mixed Fed), infants that consume breast milk and other non-breastmilk foods (Minimally Mixed Fed) and infants that no longer consume breast milk (Non-Breastfed) at each study timepoint. **B.** Percentage of Mixed Fed infants that consume cereal, formula, water or other food types. **C.** Percentage of Mixed Fed infants that consumed only one type of food or consumed a combination of different foods (Combo).

and/or fruits and vegetables, while still breastfeeding, were chosen as the most extreme population of mixed fed infants because these foods can be fed as a replacement for breast milk.

In addition, these particular foods contain high levels of diverse antigens, either originating from plants or microorganisms, that could trigger immune activation. These conservative definitions of exclusive breastfeeding and mixed feeding were chosen to maximize our ability to observe differences between Exclusively Breastfed and Mixed Fed infants. Breastfed infants that had been introduced to non-breast milk foods that did not include cereal, formula or fruits and vegetables were classified as **Minimally Mixed Fed**. The foods introduced to Minimally Mixed Fed infants include water and traditional medicine. In addition, infants that were no longer being breastfed were classified as **Non-Breastfed**. Due to very small numbers at either time point, both Minimially Mixed Fed and Non-Breastfed infants were excluded from most subsequent analyses.

Within Mixed Fed infants, there was a high rate of consumption of a wide variety of non-breast milk foods at both 6 and 14 weeks (Figure 2.3B). Over half the Mixed Fed infants consumed some amount of infant cereal, and over half consumed some amount of infant formula. Few to no infants were introduced to fruit or vegetables at either timepoint. Finally, around 40% of infants consume other foods, which include water, traditional medicine and gripe water.

Between 6 and 14 weeks of age, there were some changes observed in infant feeding practices. At both timepoints, most Mixed Fed infants consumed a mixture of different food types, but at 6 weeks, few infants only consumed cereal, where at 14 weeks of age, nearly 20% of all Mixed Fed infants had been only introduced to cereal (in addition to breast milk) (Figure 2.3C).

Demographic characteristics, including gestational age, age at the time of sample collection, weight at any study visit or time of enrollment into the study, did not differ significantly between Mixed and Exclusively Breastfed infants at either time point (Table 2.3).

**Table 2.3: Demographic characteristics of Mixed Fed and Exclusively Breastfed infants in the Feeding Study.**

| | | 6 Weeks | | 14 Weeks | |
|---|---|---|---|---|---|
| | | **Exclusively Breastfed (n=45)** | **Mixed Fed (n=37)** | **Exclusively Breastfed (n=17)** | **Mixed Fed (n=68)** |
| Gestational Age | Median Gestational Age at Birth in Weeks (25-75th Percentile) | 39 (38-40) | 39 (39-40) | 38 (38-39) | 39 (38-40) |
| Age at Sample Collection | Median Age in Days (25-75th Percentile) | 43 (42-46) | 43 (42-51) | 99 (98-102) | 99 (98-105) |
| Infant Weight | Median Birth Weight in kg (25-75th Percentile) | 3.2 (2.9-3.4) | 3.2 (2.9-3.6) | 3.1 (2.9-3.2) | 3.2 (3.0-3.6) |
| | Median Weight at 6 weeks in kg (25-75th Percentile) | 4.8 (4.5-5.3) | 4.9 (4.5-5.5) | 4.7 (4.5-5.4) | 4.9 (4.6-5.3) |
| | Median Weight at 14 weeks in kg (25-75th Percentile) | 6.7 (6.1-6.9) | 6.6 (6.1-7.4) | 6.7 (6.1-7.1) | 6.6 (6.2-7.2) |
| Enrollment Date | Median Days Since First Study Subject Enrolled (25-75th Percentile) | 181 (123-378) | 197 (84-402) | 276 (137-526) | 217 (137-384) |
| Illness | % Reporting Any Illness at Study Visit (#) | 9% (5) | 8% (3) | 35% (6) | 18% (9) |

*HIV Target Cells in Mixed Fed Infants*

HIV Target Cells in the Blood

To evaluate the contribution of mixed feeding to immune activation in the systemic circulation, blood samples were collected at each study time point, and PBMCs were stained for flow cytometric analysis. The gating strategy used in this analysis is shown in Figure 2.4. This

analysis allowed for the assessment of activation in two HIV target cell populations, CD4 T cells (Figure 2.4A) and monocytes (Figure 2.4B).

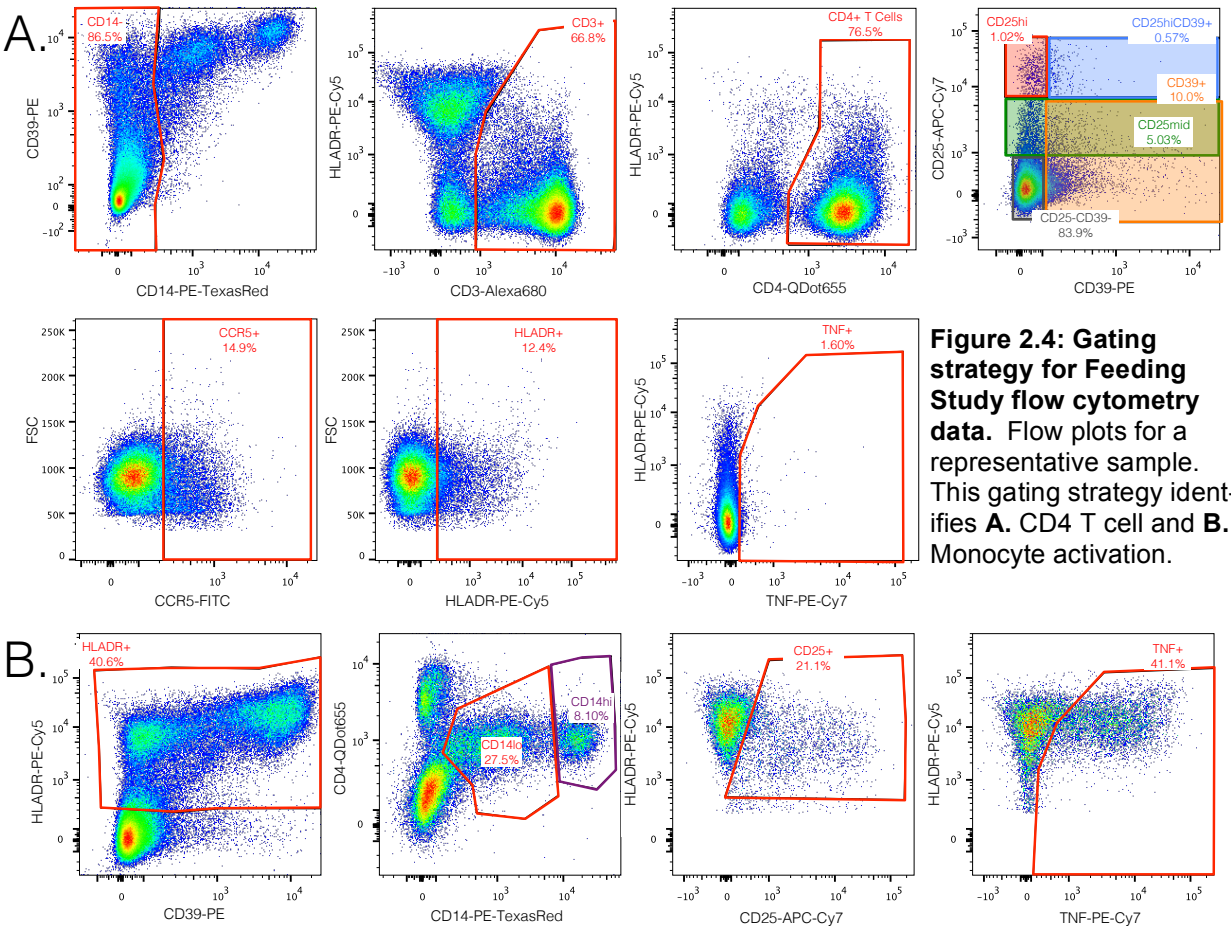



**Figure 2.4: Gating strategy for Feeding Study flow cytometry data.** Flow plots for a representative sample. This gating strategy identifies **A.** CD4 T cell and **B.** Monocyte activation.

Activation of T cells was evaluated, using markers that facilitate HIV infection of cells, specifically the HIV coreceptor CCR5,[287,288] and drive HIV production in HIV-infected cells, including HLA-DR,[289,290] TNF[291-294] and the receptor for IL2 (CD25).[290,293,295,296] CCR5 expression in CD4 T cells increased in Mixed Fed infants at 14 weeks of age, but not at 6 weeks of age (Figure 2.5A). However, both HLA-DR and TNF expression on CD4 T cells did not vary between Mixed and Exclusively Breastfed infants at either time point.

The percentage of activated monocytes was also calculated, based on the percentage of monocytes that: 1. downregulated CD14 expression,[297] 2. expressed CD25 (IL-2R) [298,299] and 3. produced TNF. A decrease in the percent of CD14lo monoyctes and an increase in CD25+ monocytes in 14 week old infants compared to 6 week old infants (Figure 2.5B). However, there was no clear difference in monocyte expression of either marker between Mixed Fed and

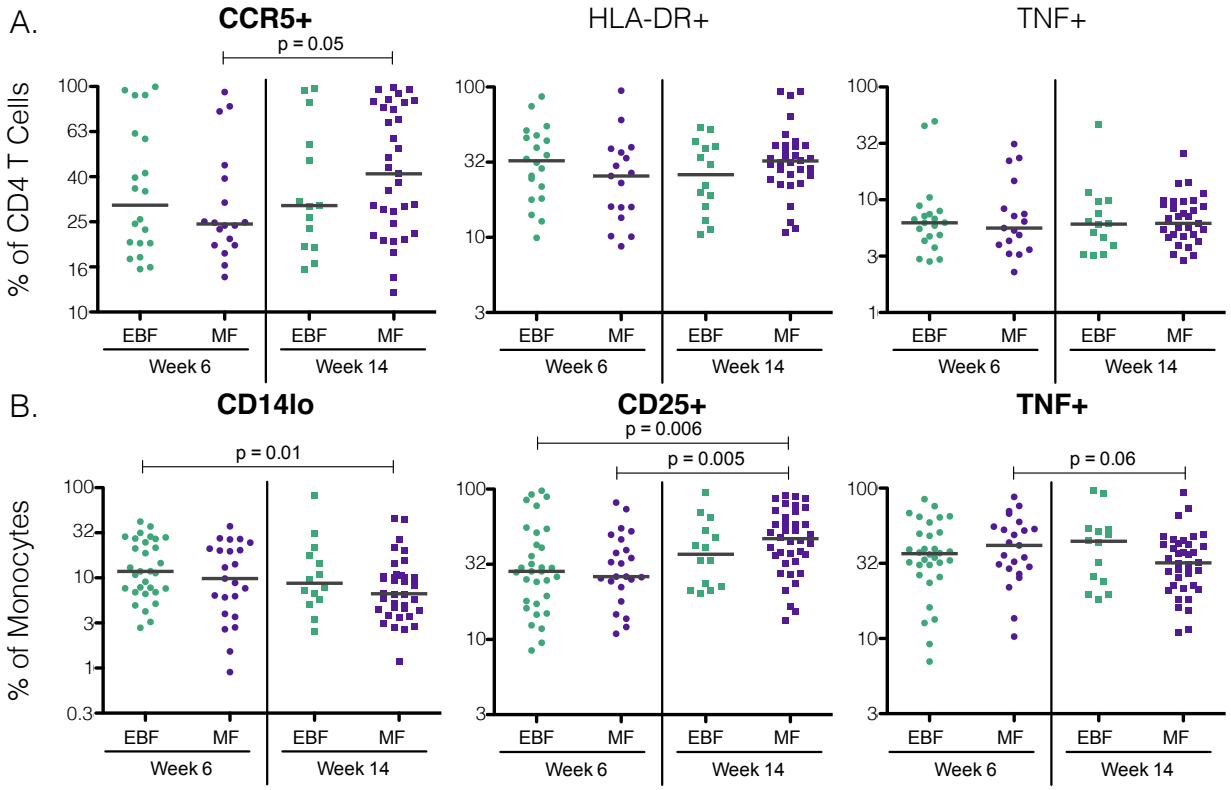



**Figure 2.5: Increased CCR5 expression on CD4 T cells but no increase in activation on monocytes during mixed feeding.** Flow plots for a representative sample is shown. This gating strategy identifies **A.** CD4 T cell and **B.** Monocyte activation. EBF = Exclusively Breast Fed, MF = Mixed Fed

Exclusively Breastfed infants at either time point. In addition, there is a trend towards decreased TNF expression in monocytes of 14 week old Mixed Fed infants. However, this difference did not reach significance and no significant alterations in TNF expression within any subset of monocytes was present between feeding groups.

In addition, this panel was also designed to identify Tregs, a key HIV target cell population.[300-302] These cells are particularly intriguing as HIV target cells since they can express high levels of activation markers that facilitate HIV infection and drive virus production while actively suppressing anti-HIV immune responses that could facilitate the control of virus.[303-306] There are many different ways to identify Tregs, each with their own benefits and disadvantages.[307] Here, we used the markers CD25, the receptor for IL-2, which although also expressed on non-Treg, activated T cell populations, is expressed at higher levels in Tregs,[307] and CD39, an extonucleotidase that depletes extracellular pools of ATP and ADP that are important for immune activation and plays an important role in the suppressive activity of Tregs,[308,309] including their ability to suppress HIV-specific responses.[310,311] Interestingly, CD39, which is also expressed at high levels on monocytes and macrophages (Figure 2.4B), has been shown to potently enhance HIV

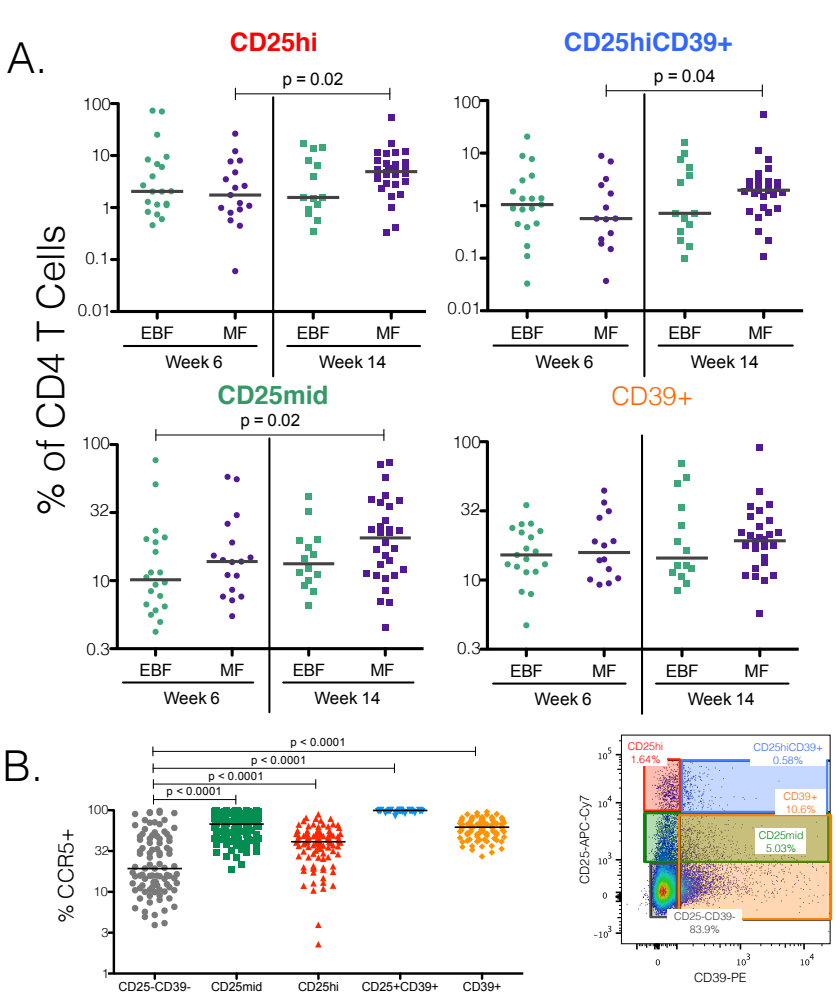



**Figure 2.6: Mixed fed infants show increase in populations expressing high levels of CCR5.** **A.** Percentage of CD4 T cells within CD25hi, CD25hiCD39+, CD25mid and CD39+ gates by age and feeding practice. **B.** Percent of each population expressing CCR5.

infection of macrophages.[312] This was true whether CD39 was present on the outside of virions or on the outside of macrophages,[312] suggesting that any cell type, including Tregs, that express high levels of CD39 are at increased risk of acquiring HIV. In fact, CD39+ cells increase with HIV disease progression,[310,313,314] and a human genetic phenotype that decreases CD39 expression results in reduced rates of HIV disease progression.[310]

Two distinct populations of CD25-expressing cells, one CD25mid and one CD25hi population can be identified (Figure 2.4). We also observed a single CD39+ population, some of which also expressed CD25 at high levels. We, therefore, looked separately at: 1. all CD25hiCD39- cells, 2. all CD25mid/-CD39+ cells, and 3. cells that expressed both CD25 at a high level and CD39, simultaneously. Mixed Fed infants at 14 weeks of age showed an increased percentage of CD25hi and CD25hiCD39+ Treg populations (Figure 2.6A). In addition, this same group of infants had increased levels of activated, CD4+CD25mid cells (Figure 2.6A). CCR5 expression was very high on CD25hi and CD25mid T cells and nearly ubiquitous on CD25hiCD39+ Treg (Figure 2.6B). Therefore, mixed fed infants at 14 weeks of age have an increase in CD4 T cell subsets that express both CD4 and CCR5, specifically CD25hi, CD25mid and CD25hiCD39+ CD4 T cells.

To determine if CCR5 expression in CD4 T cell subsets was altered by mixed feeding, we looked at changes in CCR5 expression on each of the CD4 T cell subsets described above. Only CD25hiCD39+ CD4 T cells showed a subtle increase in CCR5 expression in mixed fed infants, again, specifically at 14 weeks of age (Figure 2.7). Although there is some increase in CCR5

expression in week 14 mixed fed infants, the increase is small. Neither CD25mid nor CD39+ T cells show alterations in CCR5 expression by age or feeding pattern (data not shown).

CCL5, also known as RANTES, is an important ligand for CCR5 that induces chemotaxis of CCR5-expressing cells.[315] CCL5 plasma levels were measured in week 14 infants by Luminex. Plasma CCL5 levels did not correlate with overall CCR5+ CD4+ T cells, but there was a significant correlation with the percent of CD25hi and CD25hiCD39+ cells (Figure 2.8), the two cell types with the highest expression of CCR5 (Figure 2.6). These data suggests that CD25hi and CD25hiCD39+ cells, but not all CCR5+ T cells, migrate in response to CCL5 in infants. The lack of response to CCR5 by non-Treg, CCR5-

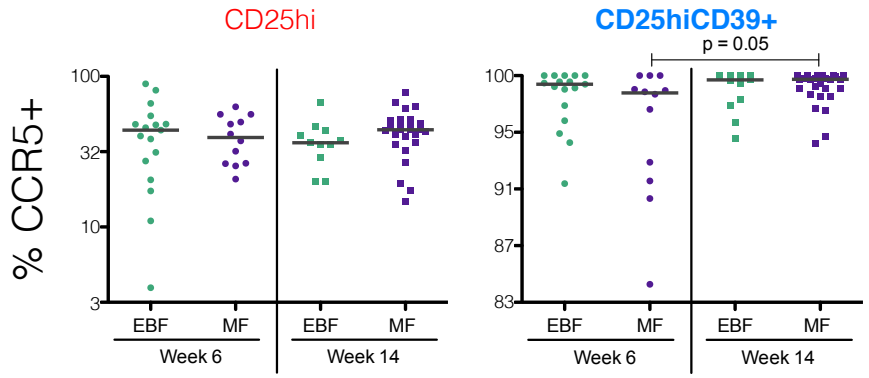



**Figure 2.7: CCR5 expression increases on regulatory T cell subsets in mixed fed infants.** Percent of CD25hi and CD25hiCD39+ T cells that express CCR5 by age and feeding pattern.

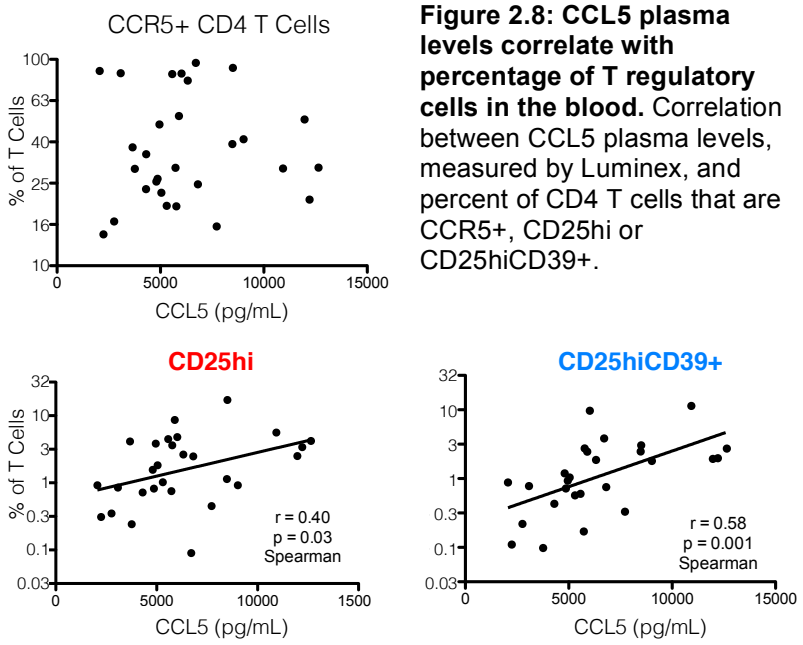



**Figure 2.8: CCL5 plasma levels correlate with percentage of T regulatory cells in the blood.** Correlation between CCL5 plasma levels, measured by Luminex, and percent of CD4 T cells that are CCR5+, CD25hi or CD25hiCD39+.

expressing T cells may be due to expression of other chemokine receptors at higher levels, which are more important for chemotaxis of non-Treg, CCR5-expressing T cells.

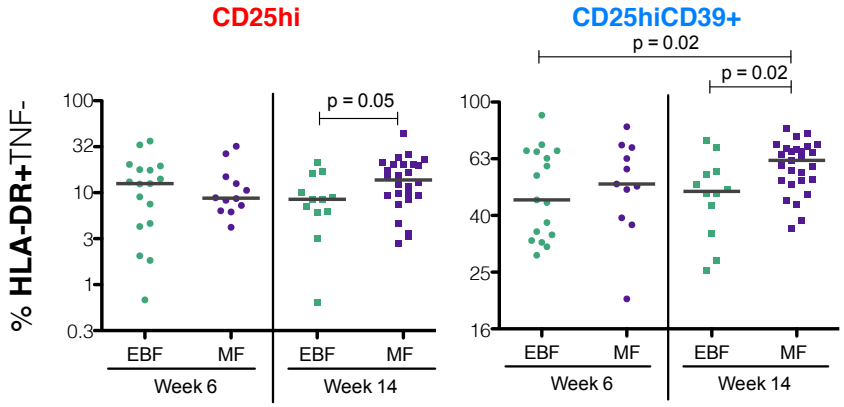



**Figure 2.9: Mixed feeding increases HLA-DR expression on T regulatory cells.** Percent of CD25hi or CD25hiCD39+ CD4 T cells that express HLA-DR but not TNF.

We then evaluated the expression of activation markers HLA-DR and TNF on each Treg population. Although neither of these activation markers showed increased expression in bulk CD4 T cells, the percentage of HLA-DR+TNF- cells was significantly increased for both CD25hi and CD25hiCD39+ Tregs in 14 week old Mixed Fed infants (Figure 2.9). HLA-DR-TNF+ cells were not altered in either of these populations (data not shown). However, HLA-DR+TNF+ double positive cells were increased in CD25hi CD4 T cells, but not in

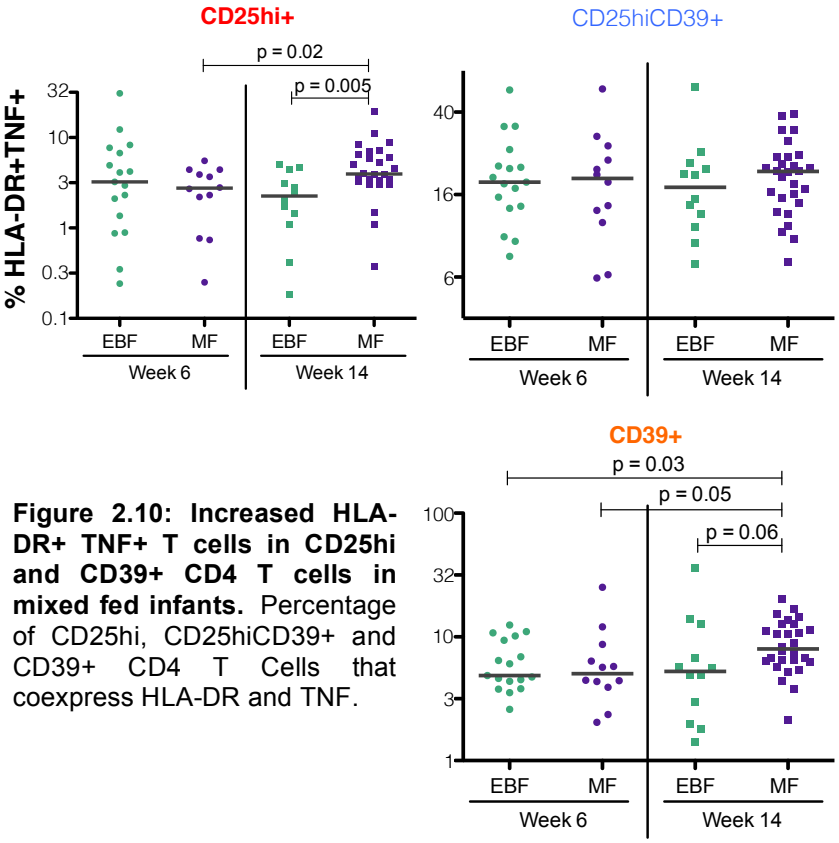



**Figure 2.10: Increased HLA-DR+ TNF+ T cells in CD25hi and CD39+ CD4 T cells in mixed fed infants.** Percentage of CD25hi, CD25hiCD39+ and CD39+ CD4 T Cells that coexpress HLA-DR and TNF.

CD25hiCD39+ T cells (Figure 2.10). CD39+ T cells also showed an increase in HLA-DR+TNF+ cells (Figure 2.10). No alteration in HLA-DR+TNF-, HLA-DR+TNF+ or HLA-DR+TNF+ cells was observed for CD25mid or CD25-CD39- CD4 T cells (data not shown).

HIV Target Cells at the Oral Mucosa

In addition, we wanted to evaluate immune activation at the first site of HIV exposure in breastfed infants, the oral mucosa. Animal studies suggest that HIV most likely crosses the mucosa in the upper gastrointestinal tract (i.e. oral mucosa, tonsils and/or esophagus).[74,75] Therefore, oral mucosa samples were collected from infants at each time point and were analyzed by flow cytometery and mRNA analysis. Even if the oral mucosa is not the primary site of virus entry, sampling at the oral mucosa may provide insight into the immune activation at lower sites of the gastrointestinal tract.

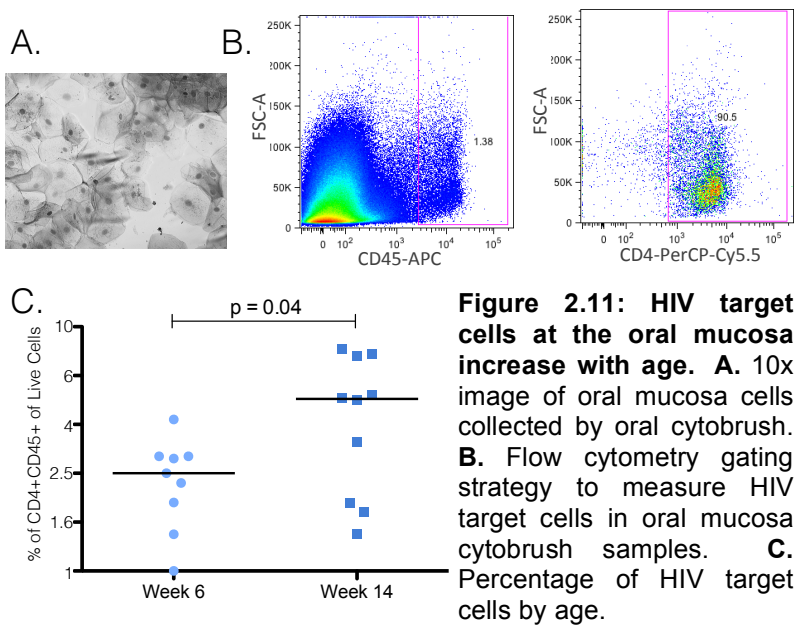



**Figure 2.11: HIV target cells at the oral mucosa increase with age. A.** 10x image of oral mucosa cells collected by oral cytobrush. **B.** Flow cytometry gating strategy to measure HIV target cells in oral mucosa cytobrush samples. **C.** Percentage of HIV target cells by age.

Oral mucosa samples were collected with an oral cytobrush that collects cells from the epithelium of the oral mucosa, which are predominantly epithelial cells (Figure 2.11A&B). A limited flow cytometry panel on a subset of samples was used to evaluate the cell types present in the oral mucosa samples. CD45 was used to identify cells of lymphocytic or monocytic origin, and then cells were subsequently gated on CD4 to identify cells that may be

susceptible to HIV infection (Figure 2.11B). In all samples, between 1% and 9% of all live cells in the oral mucosa are HIV target cells (Figure 2.11C). When we divided the samples by age, we observed a substantial (roughly 2 fold) increase in HIV target cells in the oral mucosa of 14 week old infants compared to 6 week old infants. Unfortunately, the small number of samples collected for this analysis prevent meaningful analysis of the difference in oral HIV target cell levels between Mixed Fed and Exclusively Breastfed infants at either time point.

To determine the type of HIV target cells present in these oral mucosa samples, we used mRNA data from 14 week old infants, collected on the Nanostring platform, to identify genes with

A.

| | | spearman correlation | |
|---|---|---|---|
| **Gene** | **Function** | **r value** | **p value** |
| CSF1R | specific for myeloid cells | 0.78 | <0.0001 |
| IFNGR1 | cytokine receptor | 0.76 | <0.0001 |
| HLA-DMA | MHC Class II loading | 0.75 | <0.0001 |
| ATF7 | TNF signaling transcription factor | 0.74 | 0.0001 |
| HLA-DPA1 | MHC Class II | 0.74 | 0.0001 |
| HLA-DPB1 | MHC Class II | 0.72 | 0.0002 |
| TYK2 | Type 1 IFN signaling | 0.72 | 0.0002 |
| HLA-DRB3 | MHC Class II | 0.70 | 0.0004 |
| APOL6 | lipid transport | 0.69 | 0.0005 |
| HLA-DRA | MHC Class II | 0.69 | 0.0006 |
| CD207 | specific for Langerhans' cells | 0.68 | 0.0007 |

B.

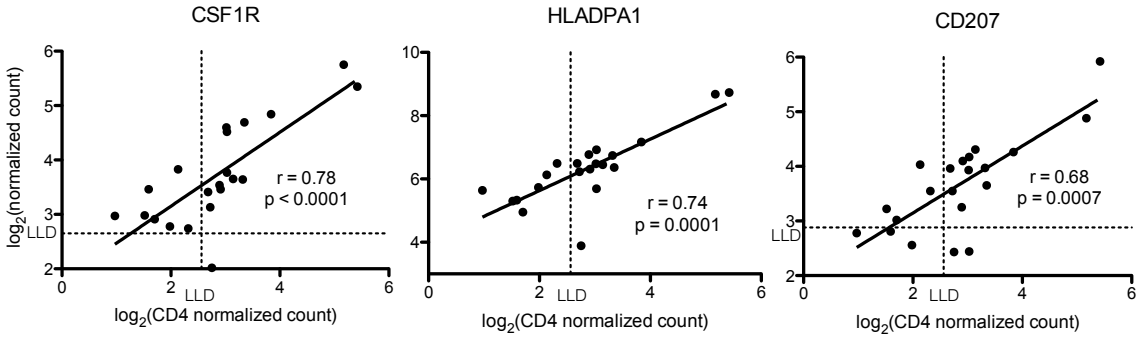



**Figure 2.12: Oral mucosa CD4 expression correlates with Langerhan cell markers. A.** Table of spearman correlation parameters for all genes, measured by Nanostring, whose mRNA correlate with CD4 mRNA expression, and **B.** a selection of the correlation graphs.

mRNA levels that correlate with the expression of CD4 mRNA. There were a total of 11 genes that significantly correlated with CD4 mRNA expression (Figure 2.12). Among these genes, 4 MHC Class II molecules and 1 MHC Class II loading protein correlated with CD4 expression, suggesting that HIV target cells in the oral mucosa are professional antigen presenting cells, which include dendritic cells, macrophages and B cells. The gene whose expression has the strongest correlation with CD4 mRNA expression is CSF1R, the receptor for M-CSF, which is uniquely expressed on cells of myeloid lineage, narrowing oral mucosa CD4 T cells as either dendritic cells or macrophages.[316] Finally, CD207, also known as Langerin, strongly correlates with CD4 gene expression. CD207 is specifically expressed on dermal dendritic cells, predominantly Langerhans cells, a dendritic cell subtype also known to be present in the epithelium of the oral mucosa,[317,318] suggesting that Langerhans cells are the primary population of HIV target cells present in the epithelium of the oral mucosa.

To determine if the oral mucosa was actively producing factors that could recruit HIV target cells to this site of HIV exposure, we analyzed the mRNA expression of a wide range of chemotactic molecules, again, using the Nanostring platform. Although there were few Exclusively Breastfed infants with sufficient oral brush mRNA for this analysis, two factors were particularly intriguing in this analysis, CCL22 and CCL5. Both of the chemotactic factors showed much higher mRNA expression in Mixed Fed infants than in the Exclusively Breastfed infants (data not shown). Therefore, a more sensitive qPCR analysis was performed on these two chemokines. Consistent with the observation in the nanostring mRNA expression data, both CCL5 and CCL22 were elevated in week 14 Mixed Fed infants by qPCR (Figure 2.13A). To determine if this increase in CCL22 and CCL5 expression was unique to the oral mucosa, we

also measured CCL22 and CCL5 plasma levels in 14 week old infants and saw no difference in plasma levels of either protein (Figure 2.13B). Therefore, CCL22 and CCL5 are upregulated in the oral mucosa but not the systemic circulation, potentially creating a gradient that could induce the migration of HIV target cells from the blood into the oral mucosa.

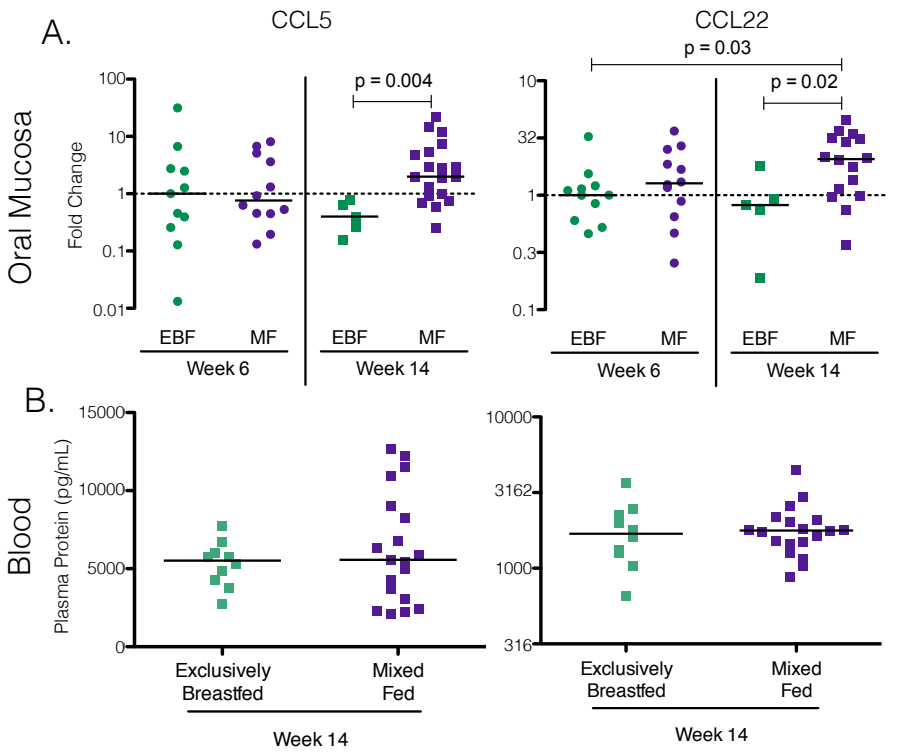



**Figure 2.13: Mixed feeding increase mucosal but not systemic CCL5 and CCL22 expression. A.** Fold change in CCL5 and CCL22 mRNA expression, measured by qPCR, relative to median of week 6 exclusively breastfed infants. **B.** Plasma levels of CCL5 and CCL22 measured by Luminex .

*Source of Immune Modulation in Mixed Fed Infants*

The introduction of non-breast milk foods exposes infants to a wide variety of new, potentially immunomodulatory stimuli. Non-breast milk food itself is a rich source of novel antigens for the exclusively breast fed infant. Each new food contains potentially immunogenic B cell and T cell epitopes that can stimulate the immune system.[319,320] In addition, non-breast milk foods can also include whole pathogens and/or PAMPs, particularly in communities with limited access to clean water and sanitation services.[321] Food, particularly in developing countries with limited regulatory oversight, may also be contaminated with immunomodulatory food contaminants,

which may also drive the changes in immune responses observed in Mixed Fed infants. Since breast milk, itself, is immunomodulatory,[322] decreasing the consumption of breast milk may also result in changes in an infant's immune system. Finally, food, both breast milk or non-breast milk foods, can significantly alter the composition of the microbiota in the gut,[323-339] which has been shown to have substantial effects on the immune system both at mucosal sites and in the systemic circulation.[340,341]

In this study, we selected two exposures, ochratoxin exposure and microbiome alterations, that may be counteracted by particular interventions, namely antioxidant treatment and probiotics, respectively, for further analysis. First, we evaluated the contribution of a specific, immunomodulatory, food-borne toxin, called ochratoxin, to the HIV target cell activation observed in Mixed Fed infants in Khayelitsha. Second, we investigated the contribution of altered stool microbiome on the immune system of Mixed Fed infants.

Ochratoxin Exposure

Mycotoxins are a class of cytotoxic small molecules produced by a range of fungal species, and are most commonly associated with cereals such as wheat, rice, corn, rye, barley and oats.[342] The risk of cancer and acute toxicity has led to regulation of mycotoxin levels in food products in developed countries, including the European Union.[343] More stringent regulations have been implemented for foods intended for infant consumption due to their high food intake relative to their body size and developing organ systems. However, lack of food safety legislation or enforcement in developing countries results in mycotoxin exposure of more than 90% of young children.[344,345] Ochratoxin A (OTA) is one of the most common mycotoxins worldwide and

remains stable during both heating and food processing.[346,347] OTA was first isolated in 1965 from *Aspergillus ochraceus* in South Africa,[348] and has since been isolated from a number of species of both *Aspergillus* and *Penicillium* fungi.[349,350] There is an established relationship between mycotoxin exposure and increased pathogenicity of a number of parasitic, bacterial and viral infections in both laboratory and farm animals,[351-353] likely due to the immunomodulatory activity of mycotoxins. At high doses, OTA is cytotoxic, resulting in immunosupression. However, with lower level exposure, similar to chronic environmental exposure, OTA induces robust inflammatory responses, including an increase in TNF-a, IL-6 and IL-1 release by multiple cell types.[354] In fact, OTA induces more TNF-alpha production than other mycotoxins in rats.[354]

Due to its immunomodulatory activity,[354] we performed a screen of plasma from infants enrolled in a vaccination study in South Africa. The infant samples were collected in Khayelitsha, as part of the BCG Infant Study (see Chapter 3 for methods), and were divided by feeding type: Breastfed (infants that received any breast milk) or Non-Breastfed (infants that received no breast milk). Infants that were breastfed had low to undetectable levels of serum OTA at both 2 and 6 weeks of age, whereas infants that were cereal or formula fed had a significant increase in serum OTA over the same time period (Figure 2.14A). These findings are in agreement with a study from Benin and Togo, which demonstrated that fully weaned infants had higher mycotoxin exposure than those who continued to breastfeed.[344] Therefore, we conclude that African infants are routinely exposed to significant levels of OTA through consumption of non-breast milk foods.

We also found that plasma levels of ochratoxin correlate with CD4 T cell activation, including CCR5 and HLA-DR expression (Figure 2.14B). Since these markers also increased in CD39+ and CD25hiCD39+ CD4 T cells in Mixed Fed infants in the Feeding Study, we evaluated whether ochratoxin exposure may be responsible for the immune activation observed in these cell subsets.

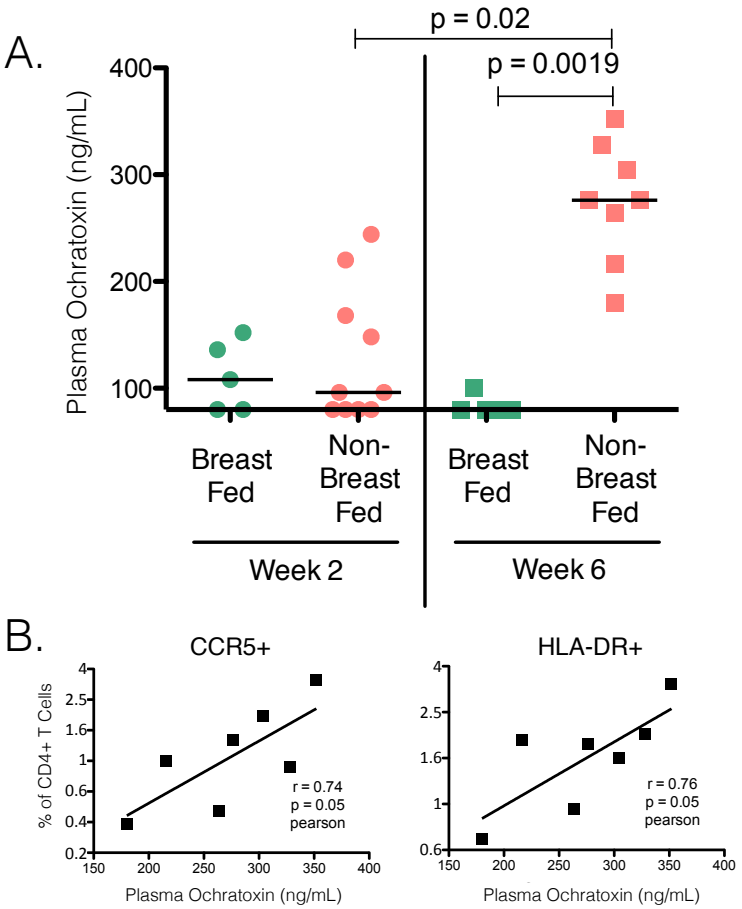



**Figure 2.14: Ochratoxin exposure correlates with CD4+ T Cell activation in non-breastfed infants. A.** Plasma levels ofochratoxin, measured by competitive ELISA in HIV-exposed infants from the BCG Study. **B.** Correlations between percent of activated CD4+ T cells (flow cytometry) and plasma ochratoxin levels (ELISA) in non-breastfed 6 week old, HIV-exposed infants.

We measured ochratoxin plasma levels in Mixed Fed and Exclusively Breastfed infants in the Feeding Study. Similar to the finding in the BCG Infant Study (Figure 2.14), Exclusively Breastfed infants had low to undetectable plasma levels of OTA, and infants that consumed no breast milk had similarly elevated plasma OTA as those seen in the non-breastfed infants in the BCG Infant Study (Figure 2.15). However, plasma levels of OTA in Mixed Fed infants were similar to those observed in Exclusively Breastfed infants (Figure 2.15). Plasma OTA levels in this cohort also did not correlate with any of the HIV target cell populations found to increase in Mixed Fed infants (data no shown).

Stool Microbiome of Mixed Fed Infants

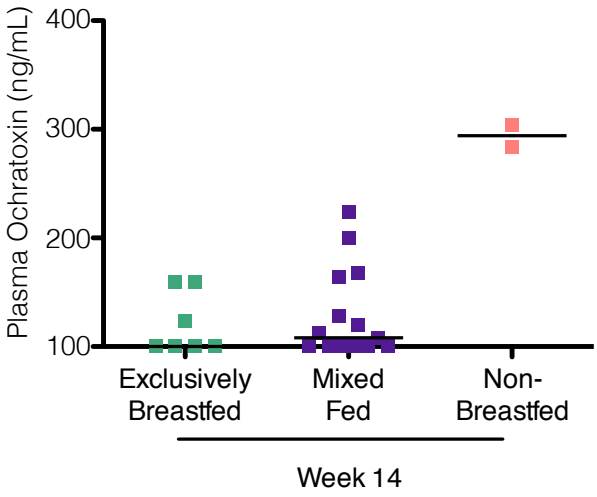



**Figure 2.15: Plasma ochratoxin levels are not increased in most mixed fed infants.** Ochratoxin plasma levels, measured by ELISA in 14 week old, HIV-unexposed infants from the Feeding Study.

The gut microbiome makes multiple, substantial transitions during the first year of life. The introduction of non-breast milk foods contributes new nutrients to microbes in the gut while also seeding the gut with new microrganisms. Therefore, it is not surprising that one of the largest transitions in the infant gut microbiome occurs at the time of weaning.[355] In addition, feeding practices in infants can influence the microbiome into adulthood.[356] Furthermore, the gut microbiome can substantially alter an individual's immune system, with particular bacteria associated with a number of atopic diseases,[357] altered immune mRNA profiles,[337] and the development of specific T cell responses.[358] However, few studies have evaluated the effects of early introduction of non-breast milk foods while the infant continues to breastfeed. Additionally, there are substantial differences across the globe in microbiome composition,[326,327,330,359] and most studies performed to date on the infant microbiome have been based in the United States or Europe.[324,326,328,335,339] In fact, only one study of the microbiome of healthy, African infants has been published to date,[329] making it important to define how the gut microbiota is altered in infants that live in HIV endemic communities

Therefore, in collaboration with the Brodie laboratory and the Jaspan laboratory, DNA was isolated from stool samples from Mixed and Exclusively Breastfed infants and the DNA for 16S rRNA was sequenced to determine the bacteria present in the stool. Sequences were mapped to OTUs for further analysis.

To determine if alterations in the overall bacterial microbiome structure exist between the Mixed Fed and Exclusively Breastfed infants, unsupervised hierarchical clustering was performed on the log2 normalized proportion of reads from each OTU present in at least 10% of samples, including samples from infants both 6 and 14 week of age. A cluster of Exclusively Breastfed infants was apparent on the left of the heat map where a Mixed Fed cluster was evident on the

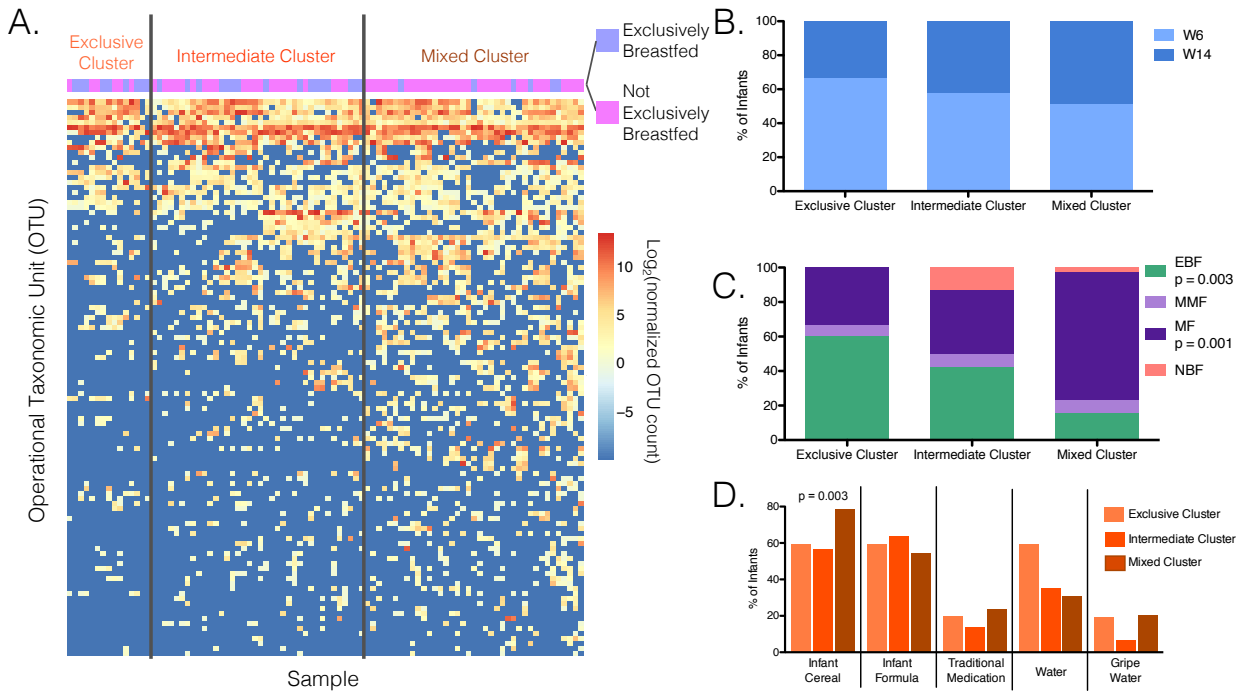



**Figure 2.16: Microbiome data clusters by feeding pattern.** **A.** Unsupervised hierarchical clustering was performed on week 6 and week 14 samples for all taxonomic units present in at least 10% of samples, based on 16S rRNA DNA sequencing. Euclidian distance with complete linkage was used for this analysis. Clusters were defined by visual grouping Exclusively Breastfed and Not Exclusively Breastfed infants. Percentage of each cluster that are **B.** 6 or 14 weeks old, **C.** and Exclusively Breastfed (EBF), Minimally Mixed Fed (MMF), Mixed Fed (MF) and Not Breastfed (NBF). **D.** Percentage infants in each cluster that consume and amount of infant cereal, formula, traditional medicine, water and gripe water.

right side of the heat map (Figure 2.16A). Between these two cluster was an intermediate cluster that included a combination of both Mixed Fed and Exclusively Breastfed infants, which we have labeled Intermediate Cluster. Each of these three clusters had a similar proportion of samples from 6 and 14 week old infants (Figure 2.16B). However, there was significant divergence between the feeding patterns of the infants in each group, with a higher proportion of Exclusively Breastfed infants whose microbiomes mapped to the Exclusive Cluster and a higher proportion of Mixed Fed infants whose microbiomes mapped to the Mixed Cluster (Figure 2.16C). Finally, Mixed Fed infants in the Mixed Cluster were more likely to consume infant cereal than infants in either of the other clusters. This suggests that the introduction of infant cereal may be resposible for the greatest divergence in the microbiomes between these three clusters.

**Table 2.4: Alterations in specific bacterial populations in mixed fed infants.** Differential abundance testing on week 6 and 14 stool 16S rRNA DNA sequence counts by OTU, using age as a covariant. Family, genus and specie for each OTU are provided. Reported p value was adjusted for multiple comparisons.

| Genus | Species | Coefficient (Mixed/Exclusive) | P value |
|---|---|---|---|
| Prevotella | copri (1) | 3.4 | 9E-6 |
| Eubacterium | biforme | 3.2 | 1E-9 |
| Megasphaera | | 2.4 | 1E-4 |
| Faecalibacterium | prausnitzii | 2.3 | 0.003 |
| Veillonella | | 2.2 | 0.03 |
| Lactobacillus | reuteri | 1.9 | 0.03 |
| Prevotella | copri (2) | 1.8 | 0.05 |

Next, we looked at specific bacterial members of the microbiome to determine how individual bacteria varied by feeding pattern. Differential abundance testing on OTUs from stool microbiome samples from both 6 and 14 week old infants was performed (Table 2.4). Seven OTUs were increased in the microbiomes of Mixed Fed infants with no OTUs decreased in the same group of infants. Of these 7 OTUs, 2 mapped to *Prevotella*

*copri*, 1 to *Eubacterium biforme*, 1 to the genus Megasphaera, 1 to *Faecalibacterium parusnitzii*, 1 to the genus Veillonella and 1 to *Lactobacillus reuteri*. Of these 6 bacterial species/genera, *Prevotella copri* showed the greatest increase in Mixed Fed infants and was the only bacterial species that showed increase in two separate OTUs.

To further evaluate whether the alterations oberved in Mixed Fed infants may be responsible for the alterations observed in HIV target cells in Mixed Fed infants, we compared the levels of the bacterial OTUs that were increased in Mixed Fed infants to HIV target cells in the blood and immune activation in the oral mucosa. P copri levels in the stool positively correlate with the percentage of CCR5-expressing CD4 T cells in the blood as well as CCL5 expression in the oral mucosa (Figure 2.17).

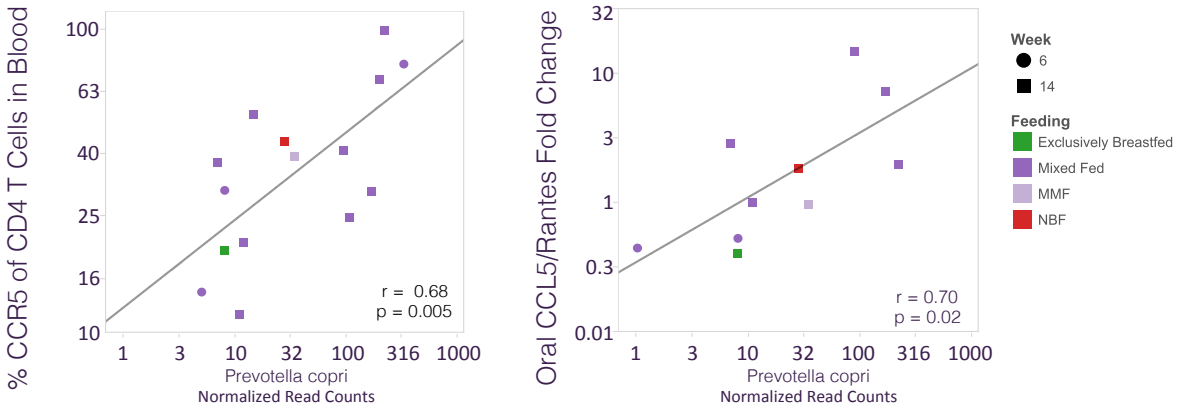



**Figure 2.17: Stool *P copri* population correlates with % CCR5+ T cells and mucosal CCL5 mRNA.** Stool microbial population of *P copri* were measured by 16S rRNA DNA sequencing, CCR5 positive CD4 T cells were identified by flow cytometry and oral mucosa CCL5 levels were measured by qPCR. Data from week 6 and 14 infants with detectable *P copri* are included in this analysis.

## Discussion

This non-randomized, observational study successfully captured an illuminating picture of immune activation in HIV target cells and activation at the site of HIV virus exposure in breastfed infants, the oral mucosa.

The feeding patterns we observed in this cohort are consistent with previously reported data from the Western Cape in South Africa, with a large proportion of HIV-unexposed infants consuming non-breast milk foods within the first few weeks of life.[279] There was a higher rate of reported illness in Exclusively Breastfed infants than Mixed Fed infants at 14 weeks of age. This may be due to mothers extending exclusive breastfeeding in infants that are ill, or mothers being more reluctant to report mixed feeding in infants that were actively ill at the time of the clinic visit. However, the difference observed in reported illness between Mixed Fed and Exclusively Breastfed infants did not reach statistical significance, making it also likely that the observed difference was entirely due to random chance. Despite the observational nature of this study, the mixed and Exclusively Breastfed infants in this cohort are comparable by all demographics metrics reported, with minimal variation between the groups. Therefore, this study is well positioned to evaluate any immune activation elicited by mixed feeding in infants in Khayelitsha, South Africa.

Our study suffered poor retention, which may have been due to our requirement that mothers find their own transportation to the clinic site, although study participants were compensated after attending visits. The compensation amount was selected to reflect the cost of taxi fare to the clinic. Studies with higher retention often performed study visits at the homes of study participants or collected study participants from their homes to bring them to the clinic.[282-284]

Therefore, it would be likely that retention could be improved in this study by providing transportation for study participants to reach clinic.

In the blood, we observed an increase in activation markers on Treg populations. These same cell population demonstrated high rates of CCR5 expression, indicating that these cells would not only be permissive to HIV infection but could also produce high levels of HIV. HLA-DR expression on Tregs is a marker of cells with a particularly suppressive phenotype,[360,361] so in addition to expressing markers of highly HIV susceptible cells, these would actively suppress other cells from mounting a productive anti-HIV response. The Treg populations increased in Mixed Fed infants also express CD39, which has been shown to facilitate the infection of cells with HIV. Therefore, mixed fed infants have increased HIV target cells with a phenotype that suggests high levels of HIV susceptibility both in the blood and, possibly, in the oral mucosa. Therefore, the increase in activated Treg cells observed in Mixed Fed infants may be responsible for the dramatic increase in HIV susceptibility observed in mixed fed infants in previous studies.

Observing an increase in T cells with a regulatory phenotype is consistent with the need of infants, and adults, to not mount a robust pro-inflammatory response to food, as well as the observation that oral administration of antigens, specifically allergens, can result in the development of a toleragenic response.[362]

Two chemokines, CCL5 and CCL22, were increased in the oral mucosa of Mixed Fed infants. Both of these proteins recruit specific cell populations to sites of immune activation by binding to their respective chemokine receptors. CCL5 binds to CCR5 and CCL22 binds to CCR4.

CCR5 was shown to be highly expressed on Treg cells, which were increased in 14 week old mixed fed infants, suggesting that these cells could be recruited to the oral mucosa by CCL5. Although not directly measured in our peripheral blood analyses, CCR4 has been shown to be expressed at high levels on effector Treg,[363] suggesting that CCL22 could also recruit Treg HIV target cells to the oral mucosa.

All of the immune activation observed either in HIV target cells or at the oral mucosa were specifically increased in 14 week old mixed fed infants. Fourteen week old infants may be uniquely susceptible to the stimuli that induce Treg and oral mucosa activation. Alternatively, 14 week old infants may have a more prolonged exposure to non-breast milk foods, which may be required to induce the observed immune activation. Therefore, identifying the exposure associated with mixed feeding that causes this immune activation would greatly enhance our ability to reduce HIV susceptibility in mixed fed infants.

We evaluated the role of exposure to the food contaminant, ochratoxin, in increasing HIV target cells in mixed fed infants. However, the level of ochratoxin exposure in mixed fed infants in this community was largely below the lower limit of detection for our assay. It is possible that a more sensitive test of OTA exposure may be able to detect differences in OTA exposure between Exclusively Breastfed and Mixed Fed infants. Although ochratoxin may alter immune responses in infants consuming non-breast milk foods, we were not able to link ochratoxin exposure to increased HIV susceptibility of mixed fed infants.

This study also evaluated the possible contribution of the gut microbiome to altered HIV target cell activation and oral mucosa activation.

Mixed fed and exclusively breastfed infants showed divergent microbiomes, with an increase in inflammation-associated bacteria in mixed fed infants. Lactobacillus, which is typically associated with reduced inflammation,[325] increased as a genus in Mixed Fed infants but *Lactobacillus ruminis* decreased. This is particularly interesting since *Lactobacillus ruminis* is the only motile Lactobacillus isolated from mammals to date.[364] The role this would play in altering the immune system of an infant is unclear, however. Veillonella, which is elevated in Mixed Fed infants, also increases in the stool of individuals with Inflammatory Bowel Syndrome.[365] *Prevotella copri*, which increased in Mixed Fed infants, is associated with activation of mDCs in the gut, specifically in HIV-infected individuals,[366] and correlates with an increased risk of arthritis in mice.[367] Megasphaera, which also increases in Mixed Fed infants, is more commonly associated with the oral or vaginal microbiome, but megasphaera in the oral cavity is found to increase when dental caries are present, suggesting that this genus may also be associated with an increased inflammatory state.[332] Together these data suggests that Mixed Fed infants have a more inflammatory microbiome profile in their gut.

Importantly, levels of *P copri* correlated with increased HIV target cell levels in the blood and levels of chemokines that recruit HIV target cells in the oral mucosa of mixed fed infants, suggesting a close link between alterations in the gut microbiome and increased HIV target cells in mixed fed infants.

Although the microbiomes of the stool and oral mucosa are divergent, the microbiota are more similar than any other two microbial populations in the body.[356] Therefore, the alteration in *P copri* in the stool may reflect alterations in Prevotella in the oral mucosa, which may, in turn, induce the oral mucosa to produce CCL5.

Whether the alterations we observe in stool bacteria drive the increase in HIV target cells we observe in Mixed Fed infants, or are, perhaps, an independent response to mucosal inflammation is not clear from these data. However, altering the gut microbiome can alter immune responses

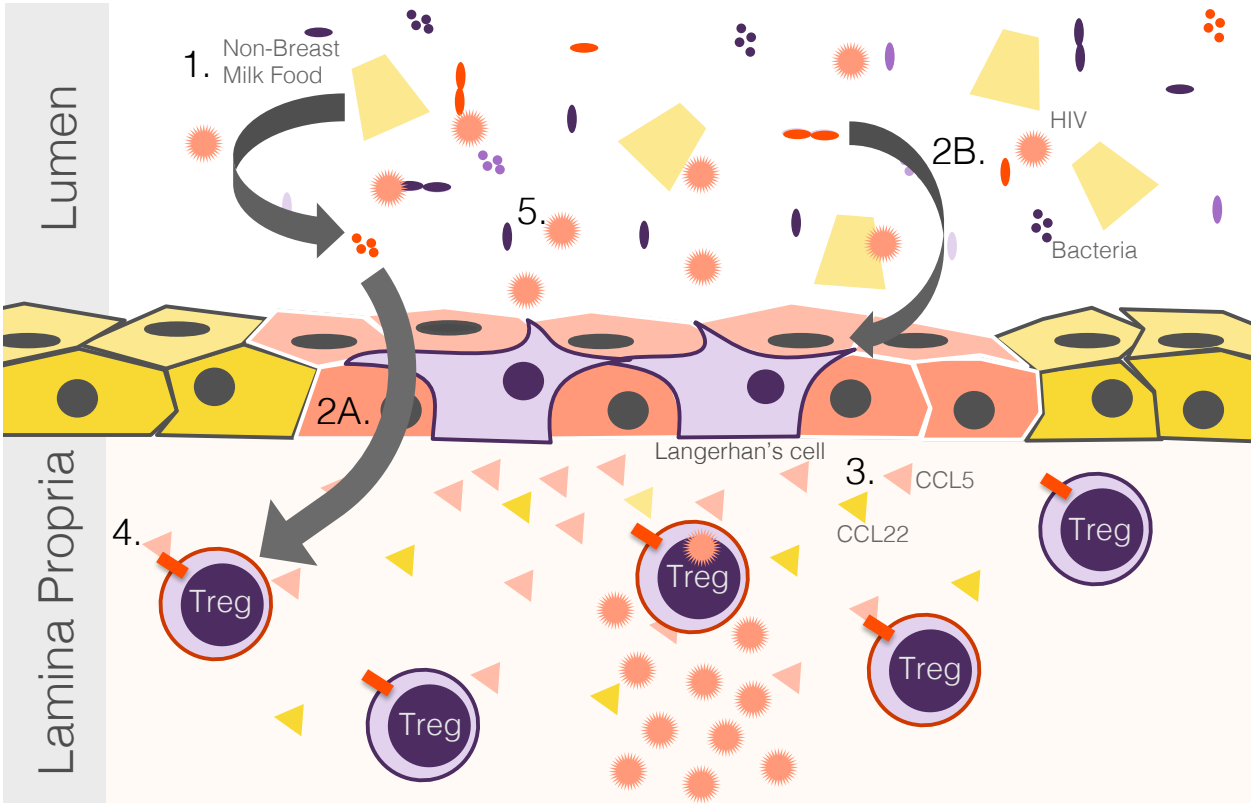



**Figure 2.18: Model of increased HIV susceptibility in mixed fed infants.** **1.** The introduction of non-breast milk foods alters the microbiome throughout the gastrointestinal tract, increasing bacterial species associated with increased inflammation. These bacterial species **2A.** increase Treg activation, including CCR5 and HLADR expression and **2B.** alter activation of the gut mucosa, resulting in **3.** an increase in CCL5 and CCL22 production by the gut mucosa. This, in turn, results in **4.** the recruitment of CCR5-expressing Tregs to the lamina propria of the gut mucosa. Therefore, **5.** when an infant is exposed to HIV via breast milk, it can readily transverse the oral mucosa through Langerhan's cells to reach the activated, HIV susceptible Tregs that are increased in the lamina propria, where the virus can establish infection and rapidly produce additional virions.

in mice,[368] suggesting that perturbing the microbiome of Mixed Fed infants may be able to counteract the increase in HIV target cells observed in these infants with the hope of preventing the increase in HIV susceptibility observed in Mixed Fed infants.

Together, these data suggest a potential model for increased HIV susceptibility in mixed fed infants (Figure 2.18). The introduction of non-breast milk foods alters the microbiome throughout the gastrointestinal tract, increasing the proportion of bacterial species associated with increased inflammation. *P copri*, which is increased in response to mixed feeding, increases Treg activation, including CCR5 and HLA-DR expression. This may be through direct recognition of bacterial proteins by regulatory T cells or may be mediated by additional cell types, including monocytes and dendritic cells. In addition, this increase in *P copri* alters activation of the gut mucosa, resulting in an increase in CCL5 and CCL22 production. This, in turn, recruits CCR5-expressing Tregs to the lamina propria of the gut mucosa. Therefore, when an infant is exposed to HIV via breast milk, the virus can readily transverse the oral mucosa through Langerhans cells to reach the activated, HIV susceptible Tregs that are increased in the lamina propria, where the virus can establish infection and rapidly produce additional virions.

## Chapter 3: Relative Contribution of Mixed Feeding to Immune Activation in South African Infants

The infant immune system can be modulated by many factors, including the type of feeding and environmental exposure to fungally-derived toxins. It is difficult to discern which of these immune modulatory factors will have the largest impact on an infant's susceptibility to HIV. In this chapter, we will explore three additional early childhood exposures that may induce immune activation in infants in developing countries: 1. immunomodulatory factors in maternal breast milk associated with maternal HIV disease progression, 2. oral candidiasis and 3. early childhood vaccinations (Figure 3.1) . Figure 3.1A shows when, during the first year of life, infants are at risk of exposure to each of these factors.

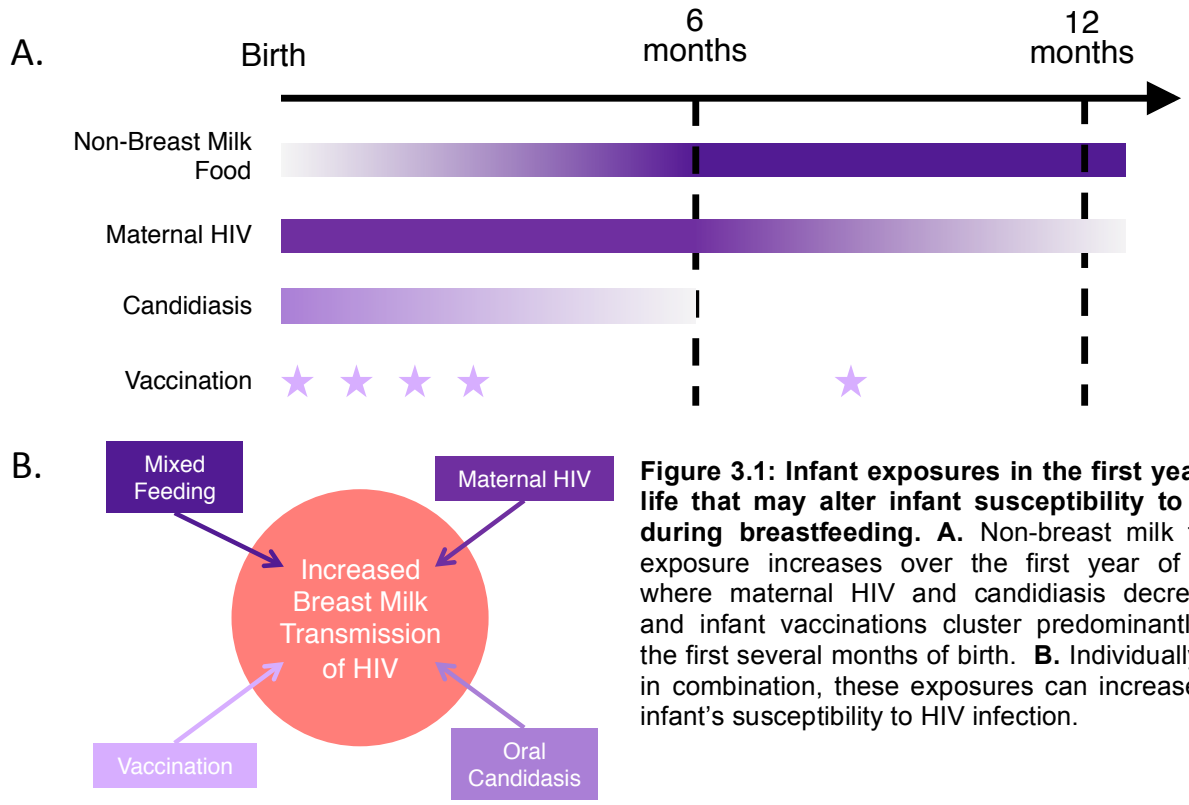



**Figure 3.1: Infant exposures in the first year of life that may alter infant susceptibility to HIV during breastfeeding. A.** Non-breast milk food exposure increases over the first year of life, where maternal HIV and candidiasis decrease, and infant vaccinations cluster predominantly in the first several months of birth. **B.** Individually, or in combination, these exposures can increase an infant's susceptibility to HIV infection.

Breast milk factors that are altered during maternal HIV infection have the greatest ability to impact immune activation in infants while the child is actively breastfeeding. The volume of breast milk consumed by an infant generally remains constant during exclusive breastfeeding,[369-373] which, according to WHO recommendations for HIV-infected mothers, is roughly 6 months.[22] Following the introduction of non-breast milk foods, breast milk consumption in turn declines as more calories are gained from non-breast milk sources. Exposure to HIV or other immune factors associated with HIV disease status in breast milk would, therefore, decline as the infants progressively decrease breast milk consumption (Figure 3.1A).

An infant's risk of candidiasis is highest within the first several weeks of life, often associated with exposure to vaginal candida at the time of delivery. This risk then progressively declines with little risk of candidiasis after the first 6 months of life (Figure 3.1A).[374]

Finally, infants receive a series of vaccines throughout the first year of life. Although the timing of vaccine administration can vary from country to country, our international collaborations are based in South Africa, and therefore the South African vaccination schedule is depicted (Figure 3.1A).

I hypothesized that each of these factors, mixed feeding, maternal HIV disease status, oral candidiasis and vaccinations, each has the potential to increase infant immune activation. However, in combination these four exposures may result in a highly activated immune environment, a significant increase in an infant's risk of MTCT of HIV via breast milk (Figure 3.1B).

**Maternal HIV Disease Progression**

The breast milk of HIV infected mothers is altered by their infection. This includes cell-free HIV virus and HIV-infected cells in breast milk, both of which correlate with maternal plasma viral load.[49,52,54-58] Breast milk also contains substantial levels of maternal immune cells, primarily macrophages,[51-53] whose activation status is likely to be modified by maternal HIV disease progression, similar to macrophages at other tissue sites.[375-379] In addition, breast milk contains multiple cytokines and chemokines that can impact an infant's immune responses. Levels of cytokines and chemokines in breast milk are also altered by HIV disease progression and the resulting maternal immune activation,[141,143,380-383] and alterations in these factors can, in turn, alter an infant's immune responses.[384] Together, exposure to maternal HIV virus and immune activation through breast milk may alter the activation profile of an infant's immune system and, ultimately, their susceptibility to HIV during breastfeeding.

*Methods*

To investigate this question, plasma samples from a study of infants born to HIV-infected mothers were evaluated to determine the contribution of maternal HIV disease status on infant immune activation. This study, a randomized study of HIV-exposed infants, delayed BCG vaccination from birth to 8 weeks of age to determine the contribution of BCG vaccine administration to HIV susceptibility. In addition to monitoring immune activation in this cohort, maternal CD4 count and detailed information on feeding patterns were also recorded, allowing for additional analysis of these data. This study will be referred to as the **BCG Infant Study**.

Community

Study participants were recruited in Khayelitsha, South Africa. This community is described in detail in Chapter 2.

Sample Collection

A total of 151 infants were recruited at birth from the maternity ward at the Site B Clinic in Khayelitsha, Western Cape, South Africa, into a randomized control study, designed to evaluate the contribution of BCG vaccination to HIV susceptibility. Infants were born to HIV-infected women and were randomized to receive BCG vaccination either at birth or 8 weeks of age. Infants who were born prematurely (<36 weeks gestational age), weighed less than 2.5kg at birth, were born to mothers under 18 years of age or had household tuberculosis contact were excluded from the study. Infants were tested for HIV infection by PCR at time of study enrollment (within 24 hours of birth) and again at 6 weeks of age. If an infant tested positive, for HIV, they were not vaccinated with BCG and were referred immediately for antiretroviral therapy. If the HIV PCR at birth was negative, infants were randomized to receive BCG vaccination immediately or at 8 weeks of age. All mothers of recruited infants were orally informed of potential risks and benefits of the study, and signed an IRB-approved consent form in either English or Xhosa. Mothers of enrolled infants were also provided with a consent form in their preferred language to take home. At birth, 2, 6, 8 and 14 weeks of age, mothers orally completed a survey that included detail information about the foods consumed by each infant, the health status of each infant since the last study visit, including receipt of vaccinations, and maternal health status, including their most recent CD4 count. In addition, infants received a full

physical exam, including anthropometrics and evaluation for any oral or skin conditions. Finally, blood (EDTA by venipuncture; vendor) and saliva samples (Salivette, Sarstedt, Germany) were collected at each study visit.

Blood Processing

Blood was collected to evaluate systemic immune activation in study participants. Blood samples were stored at 4C for no more than 6 hours before transport from Khayelitsha to the Jaspan laboratory at Goote Schuur Hospital, Cape Town, Western Cape, South Africa. PBMCs were isolated from each sample, using Ficoll (Sigma, Missouri, USA) density gradient separation. PBMCs were slowly cooled to -80C in DMSO (Sigma, Missouri, USA) + 10% FBS (Biochrom, UK), and transferred to liquid nitrogen for storage. PBMCs were stored in liquid nitrogen. All blood processing was performed by the Jaspan Lab in Cape Town, South Africa.

Blood Flow Cytometry

Flow cytometry was performed on PBMCs to quantify immune activation in T cells. Frozen PBMCs were rapidly thawed at 37C, washed and resuspended in PBS (Sigma, Missouri, USA) + 1% FBS (Biochrom, UK), and cell counts quantified with a Guava automated cell counter (EMD Millipore, Darmstadt, Germany).

Each sample was then stained with a flow panel to evaluate T cell activation. One million cells were stained in a 96 well plate with Live/Dead-Violet (Invitrogen, California, USA) for 20 minutes at room temperature, stained with anti-CCR5-APC (BD, California, USA) at 37C for 15 minutes, and then stained with anti- CD4-PerCP-Cy5.5 (BD, California, USA), CD38-PE-Cy7(e-

bioscience, California, USA), HLA-DR-PE (BD, California, USA), and CD8-QDot605(Invitrogen, California, USA) and for 20 minutes at room temperature. Samples were then washed with PBS, permeabilized with CytoFix/CytoPerm (BD, California, USA), washed twice with Permawash (BD, California, USA), and then stained intracellularly with anti-Ki-67-FITC and CD3-AlexaFlour700 (BD, California, USA) for 20 minutes at room temperature. A final wash was performed and then cells were resuspended in 150uL Cell Fix (BD, California, USA), transferred to a polysterene FACS tube and collected on an LSR II within 24 hours of staining. All flow cytometry data collection was performed by the Jaspan Lab in Cape Town, South Africa.

Flow Cytometry Data Analysis

All flow data was compensated and analyzed, using Flow Jo v10.0.7r2 (Treestar, Oregon, USA). Flow cytometry analysis for this study was performed by the Jaspan Lab in Cape Town, South Africa with the gating strategy shown in Figure 3.2.

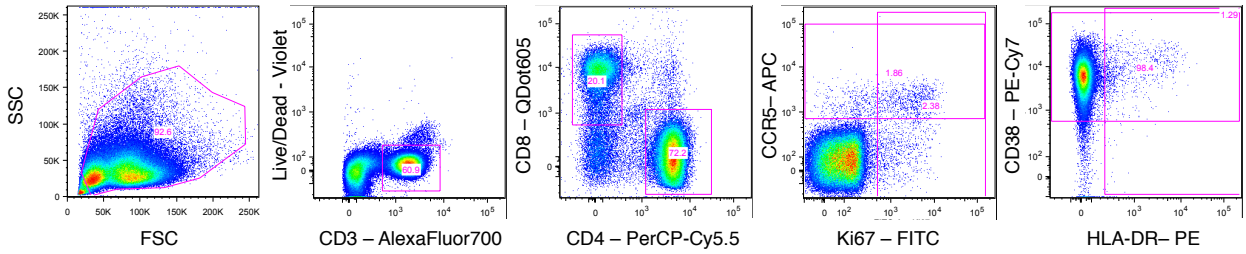



**Figure 3.2: Gating strategy for BCG Infant Study flow data.**

Ochratoxin Exposure Quantification

To determine if Ochratoxin contributed to immune activation in South African infants, Ochratoxin A was measured in a subset of stored plasma from 2 and 6 week old infants (findings described in Chapter 2). Ochratoxin was quantified by competitive ELISA in accordance with

the manufacturer's instructions (Helica, California, USA). Finished plates were quantified on a plate reader (Helica, California, USA). Ochratoxin levels in each sample were calculated based on a 5-parameter regression of the standard curve values. Values calculated below the lowest standard were assigned the value of the lowest standard, to reflect the highest possible value. Ochratoxin quantification was measured by the Sodora lab in Seattle, WA.

*Results*

The evaluated T cell activation markers included: 1. CCR5, the primary coreceptor used by HIV when establishing a new infection and a chemokine receptor that recruits T cells to sites of active inflammation, 2. HLA-DR, an MHC Class II receptor upregulated on T cells within 3-5 days of stimulation, i.e. a late activation marker,[385] 3. CD38, a receptor that mediates activated T cell binding to endothelium,[386] but is also expressed at high levels in nearly all infant T cells,[387] and 4. Ki-67, a nuclear protein whose expression is tightly connected to cell proliferation.[388]

Six week old infants that were breastfed showed a clear negative correlation between the number of activated CD4 (Figure 3.3) and CD8 (Figure 3.4) T cells and maternal CD4 count. Specifically, proliferating, CCR5+ T cells that lack HLA-DR or CD38 expression (CCR5+HLA-DR-CD38-Ki67+) and non-proliferating CCR5+ T cells that express both HLA-DR and CD38 (CCR5+ HLA-DR+CD38+Ki67-) increase in infants born to mothers with lower CD4 T cell count. Additionally, highly activated CD4 T cells that express all four activation markers (CCR5+HLA-DR+CD38+Ki67+) showed the same increase with decreasing maternal CD4 count. In addition, CCR5-expressing CD8 T cells, a key activated population associated with progressive disease in HIV and SIV infection,[389,390] are also inversely correlated with maternal

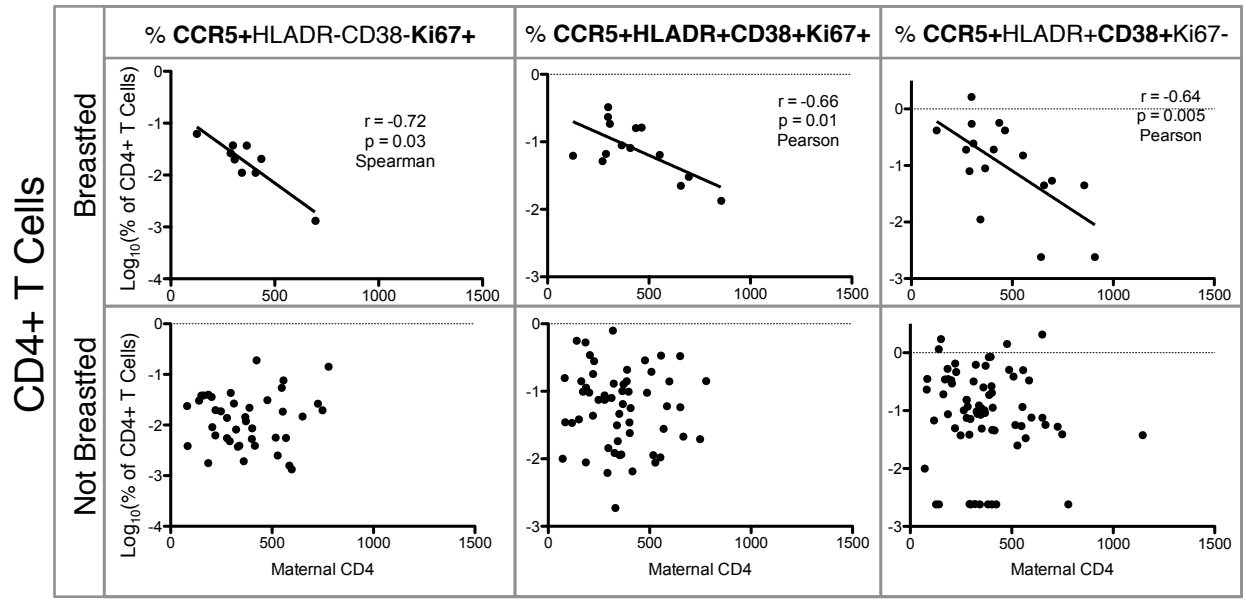



**Figure 3.3: Breast Milk HIV-Exposure Correlates with CD4 T Cell Immune Activation in 6 week old South African Infants.** CD4+ T cell immune activation marker expression measured by flow cytometry in HIV-uninfected infants born to HIV-infected mothers. T cells expressing multiple activation markers increase in infants born to HIV-infected mothers and are breastfed but not in infants that are not breastfed.

CD4 count (Figure 3.4). This correlation between maternal CD4 count and the activation status of the infant's T cells was not observed in infants that were not breastfed (Figures 3.3 and 3.4). Additionally, younger infants (6 weeks of age), whether breastfed or not breastfed do not show any correlation between maternal CD4 count and T cell activation (data not shown), suggesting that the activation observed at 6 weeks was not induced at birth but rather developed during the first several weeks of life.

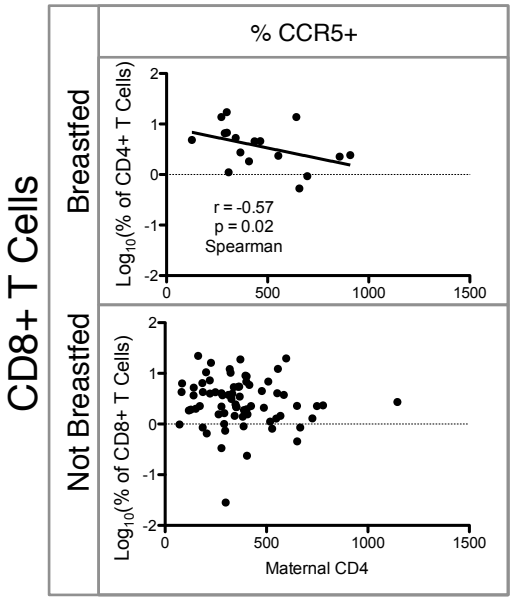



**Figure 3.4: Breast milk HIV-exposure correlates with CD8 T cell immune activation in South African infants.** CD8+ T cell immune activation marker expression measured by flow cytometry in HIV-uninfected infants born to HIV-infected mothers. CCR5 expression increases as maternal CD4 count decreases, but only in infants that were breastfed.

*Discussion*

Infants exposed to HIV through breast milk demonstrate clear correlations between maternal HIV disease status, measured by CD4 count, and immune activation in T cells. Most importantly, activation and proliferation of T cells that express the HIV receptors CD4 and CCR5 increased with more advanced maternal HIV disease (i.e. lower maternal CD4 counts). This increase in immune activation may be a direct effect of infant exposure to HIV, since HIV viral load in breast milk increases as maternal CD4 count decreases.[49,56,391] However, this may also be due to changes in the inflammatory cytokine profile of maternal breast milk as HIV progresses,[141,381] driven by the increase in immune activation observed in advanced HIV.[392] Alternatively, other milk proteins, particularly defensins, chemokines, specific fatty acids and sTLR2,[138,142,382,383] are found at altered levels in the breast milk of HIV-infected mothers, and these proteins can also play a role in altering an infant's immune responses.[384] Although this study was unable to differentiate between these mechanisms, it is clear that maternal HIV disease progression increases the population of HIV-susceptible cells in infants. This suggests that infants born to mothers with advanced HIV are at a higher risk for HIV infection not just because of higher maternal viral load in breast milk, but also due to an increase in the infant's population of HIV-susceptible cells.

**Candida Exposure**

Infants, have an increased risk of oral candidiasis,[374] also known as oral thrush, an invasive colonization of the oral mucosa with Candida, most commonly *Candida albicans*. This mucosal infection has the potential to induce immune activation that may in turn result in an increase in HIV susceptibility. Indeed, one report determined that oral candidiasis in HIV-exposed infants

can predict an increased risk an infant testing positive for HIV.[198] However, it is difficult to determine causation in this setting, since HIV infection also increases an individual's risk of oral candidiasis,[393] and infants often do not test positive for HIV until up to a week after virus infection.[394] Candida contains a host of pathogen-associated molecular patterns (PAMPs) that can be recognized by a diverse array of immune cells. PAMPs produced by Candida and recognized by human cells include: 1. beta-linked glucans, which are recognized by the host cell receptor Dectin-1, 2. beta-linked mannans, recognized by TLR2, and Galectin-3, 3. alpha-linked mannans, recognized by TLR4, DC-SIGN, Mannose Receptor, Mincle and Dectin-2, and 4. Zymosan, a more complex molecule composed of both alpha-linked mannans and beta-linked glucans, recognized by Dectin-2 and the TLR2/6 complex. Most of these PAMPs induce a pro-inflammatory signal in the recognizing cell.[395] Receptors for these PAMPs are expressed on classic innate cells, including macrophages, neutrophils, NK cells, mast cells and dendritic cells,[396-402] but also cell types associated with adaptive responses to pathogens, including B cells and activated T cells,[396,398,401-403] and cells not typically associated with pathogen immune responses, like epithelial cells, endothelial cells and glomerular cells.[398-400,404] Here we evaluate human whole blood responses to Candida exposure to determine if Candida can influence the expression of cytokines that can enhance HIV susceptibility.

*Methods*

Samples

Whole blood samples were collected in heparin (BD, California, USA) from healthy, adult donors through the Seattle BioMed (Washington, USA) blood draw program.

Stimulation

Whole blood was stimulated with *Candida glabrata* grown to log phase with CSM complete media with 20% dextrose at a ratio of 1 yeast to 5 monocytes. Monocytes in each blood sample were estimated as 500 monocytes/mL of blood. *C glabrata* levels were calculated by estimating $2 \times 10^7$ cells/mL per OD unit. Blood samples were stimulated with yeast as indicated above or with 20ug/mL of LPS (Sigma, Missouri, USA) for 4, 6 or 12 hours at 37C, 5% $CO_2$. Cells that were used for ICS had 20ug/mL of Brefeldin A (Sigma, Missouri, USA) added 1 hour after the beginning of the stimulation. Samples were then either placed at 4C overnight before intracellular cytokine staining or were lysed with Buffer EL (Qiagen, Netherlands) per the manufacturer instructions for RNA isolation.

Quantitative PCR

To determine the expression of multiple cytokines by stimulated blood cells, mRNA transcripts of pro-inflammatory cytokines were measured in stimulated whole blood. RNA was isolated from lysed sample, using the QIAamp RNA Blood Mini Kit (Qiagen, Netherlands) per the manufacturer's protocol. RNA was converted to cDNA with SuperScript III (Invitrogen, California, USA). Taqman Universal Master Mix (Applied Biosystems, California, USA) and Single Tube Taqman Assays (Applied Biosystems, California, USA) were used to set up qPCR amplification reactions, which were run on a 7500 Fast Real Time PCR System (Applied Biosystems, California, USA) for 40 cycles. Thresholds were set for each gene in the linear range of the curve. Fold changes were then calculated, using the ΔΔCt method, using GAPDH

as endogenous the control gene and the median unstimulated sample value for each gene as the reference sample.

Intracellular Cytokine Staining

To identify cytokine responses in specific cell populations after stimulation, intracellular cytokine staining was performed on stimulated whole blood. After overnight storage at 4C, stimulated blood was stained with 1uL Live/Dead-Aqua (Invitrogen, California, USA), 5uL CD3-APC-Cy7, 0.5uL CD14 PE-Cy7 at room temperature for 30 minutes at 4C. Samples were then washed PBS (Sigma, Missouri, USA) + 2% FBS (Biochrom, UK), and cells were then permeabilized with 750uL 20% BD FACS Lysing Solution (BD, California, USA) + 0.05% Tween 20 (Fisher Scientific, Massachusetts, USA) for 10 minutes at 4C with vortexing. Samples were then washed twice and then stained intracellularly with 5uL TNF-APC for 30 minutes at 4C. Cells were then washed a resusupended in 2% paraformaldehyde (VWR, Pennsylvania, USA). Samples were then run on an LSR II (BD, California, USA). All flow data was compensated and analyzed, using Flow Jo v10.0.7r2 (Treestar, Oregon, USA).

*Results*

Cytokines associated with Th1 responses were upregulated in response to Candida stimulation, including TNFa, IFNg and IL6 (Figure 3.5A). However, regulatory responses (Figure 3.5B), measured by IL-10, and antiviral responses (Figure 3.5C), measured by the type I IFN-induced gene OAS, did not show a similar level of induction by Candida. In general, these responses were very similar to that seen following LPS stimulation. The one exception was a higher

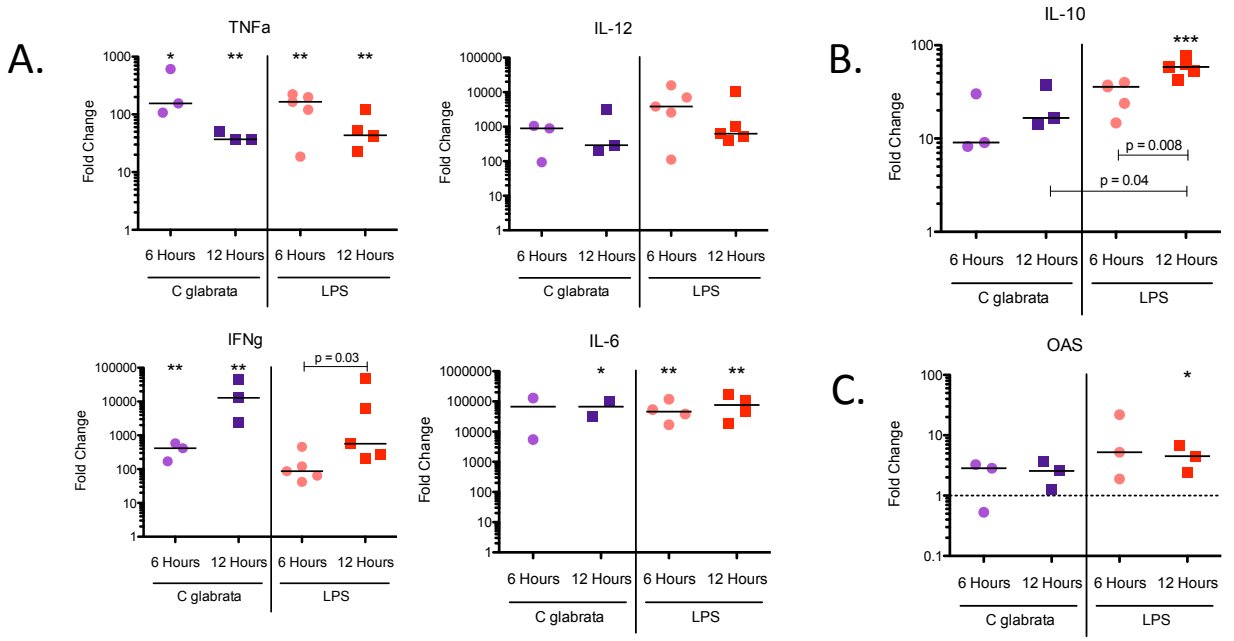



**Figure 3.5: Candida stimulation of whole blood induces robust Th1 responses but minimal regulatory and anti-viral responses.** Cytokine responses in healthy, human whole blood to Candida glabrata stimulation (1:5 yeast:monocytes). **A.** Th1 cytokine **B.** Regulatory cytokine and **C.** anti-viral mRNA quantification by qPCR. Fold changes calculated by the ddCt method relative to GAPDH mRNA and PBS stimulated controls. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.0001$ compared to no change in mRNA expression.

induction of IL-10 in response to LPS stimulation, suggesting that LPS induces a higher regulatory response than Candida.

Using flow cytometry, it was possible to identify the whole blood population responsible for TNFa production as primarily T cells (40-70% of TNF-producing cells), although there was a substantial contribution by CD3-CD14- cells (5-50% of TNF-producing cells) (Fig. 3.6). Although some of the TNFa+CD3-CD14- cells may be monocytes that have down-regulated CD14 expression in response to stimulation, side and forward scatter properties of the majority of this population are more consistent with granulocytes, most likely neutrophils, which also produce TNFa in response to Candida.[405-409]

*Discussion*

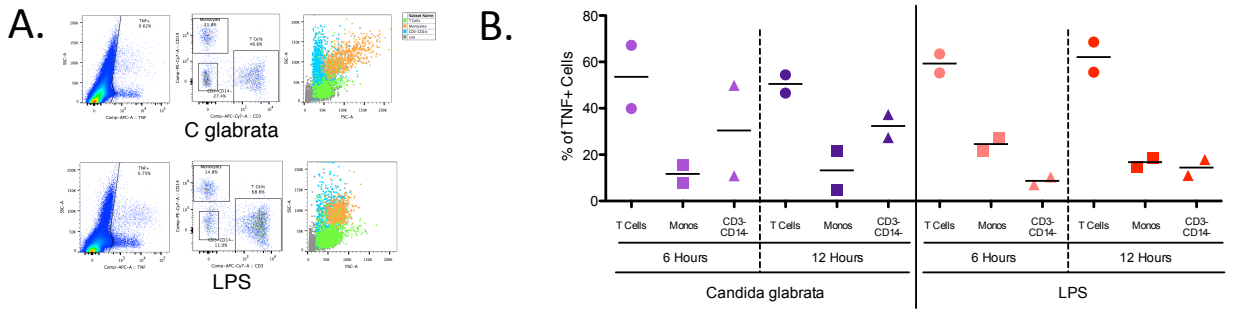



**Figure 3.6: Candida induces Th1 cytokine production primarily in T cells.** Cytokine responses in healthy, human whole blood to Candida glabrata stimulation (1:5 yeast:monocytes). Flow cytometry revealed that Th1 cytokine TNFa was predominantly produced by T cells following C glabrata or LPS stimulation for **A.** 4 hours or **B.** 6 and 12 hours in adult human whole blood. Gating shown in **A**. was used for data shown in **B.**

Studies have shown that Th1 cells express high levels of CCR5, and are more susceptible to HIV infection than other T cell populations.[410,411] However, Th1 cells appear to be somewhat resistant to HIV replication.[410-412] Interestingly, dendritic cells that elicit a Th1 response are particularly effective at transferring HIV to T cells.[413] The lower antiviral response to Candida may result in Candida-exposed infants having a particularly high risk of acquiring HIV compared to infants with immune activation with a more robust induction of anti-viral responses. Similarly, the minimal induction of regulatory cytokines by Candida suggests that the immune activation induced by Candida is not rapidly controlled, resulting in a more sustained period of enhanced HIV susceptibility, compared to other immune stimuli. Finally, neutrophils also play an under-studied, but important role in the establishment of HIV infection.[414-416] Therefore, alterations in the phenotype of neutrophils during oral candidiasis may alter control of HIV after exposure to virus-containing breast milk.

However, these studies were performed in adult blood, which may have much more robust Th1 responses to Candida than infant blood.[213,217,220,225-234] Although Candida infection is far more common in infants than in adults, with estimates from ranging from 1-37% of infants.[374] there

are no recent studies of infant candidiasis rates in full term infants, particularly in developing countries. Therefore, it is difficult to determine the number of HIV-exposed infants that may have increased risk of acquiring HIV due to oral candidiasis.

**Early Childhood Vaccinations**

Another potential source of immune activation that may alter HIV susceptibility in infancy are vaccinations. Although the protective benefits of early childhood vaccinations far outweigh any risk in healthy infants, the very characteristics of vaccines that result in their success, the robust induction of immune activation, which drive development of adaptive immune responses, may in fact enhance HIV susceptibility. This is particularly true of BCG, a vaccine against tuberculosis administered to 89% of infants born in 2012, globally.[417] Infants notoriously mount very weak T cell responses to most vaccines (as described in detail in the introduction). However, BCG is a special exception. Even when administered at birth, BCG elicits robust Th1 T cell responses. In fact, infants appear to have a better T cell response to BCG than older children or even adults.[239,240] This may be the reason why BCG vaccination appears to be particularly effective at protecting infants from tuberculosis, especially disseminated forms of the disease, but has shown inconsistent benefit in reducing

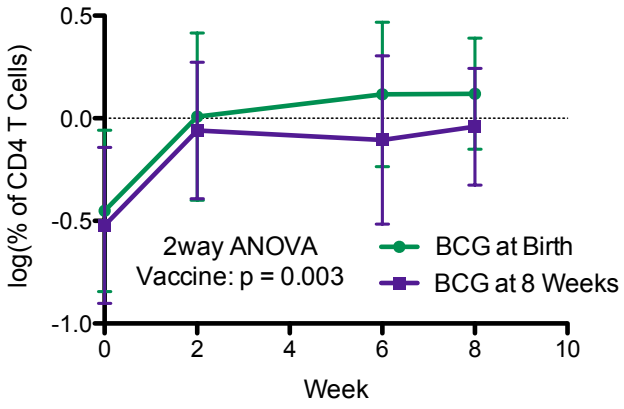



**Figure 3.7: BCG vaccination increases CCR5 expression on South African infant CD4+ T cells.** Longitudinal changes in CCR5 expression in South African infants vaccinated with BCG at birth or 8 weeks of life. Flow data was gated as shown in Figure 2.1.

adult tuberculosis burden.[418,419] Preliminary data collected by Heather Jaspan in Khayelitsha, South Africa to evaluate the impact of BCG (BCG Infant Study) has demonstrated that infants receiving BCG vaccination at birth exhibit a significantly higher percentage of CCR5+ CD4 T cells for up to 8 weeks post-vaccination compared to infants that remained unvaccinated until 8 weeks of age (Figure 3.7). Therefore, we designed a study to evaluate the impact of BCG vaccination on HIV susceptibility, using the infant rhesus macaque/SIV model.

*Methods*

I played a role in the design and implementation of these studies, which are ongoing in the Sodora laboratory. To date, data from two SIV-uninfected infant macaques, vaccinated with BCG at 7 months of age, have been analyzed.

Animals

Two infant macaques were purchased from the Oregon National Primate Center (Oregon, USA) and transported to the Washington National Primate Center (Washington, USA) at 1 month of age.

Vaccination

Macaques were vaccinated subcutaneously with BCG Danish 1331 at 7 months of age.

Blood Collection and Processing

Blood was collected in EDTA 3 weeks before vaccination, 3 days, 1 week, 4 weeks, 6 weeks, and 9 weeks after vaccination. PBMCs were isolated from each sample, using Ficoll (VWR, Pennsylvania, USA) density gradient separation. Flow staining was performed immediately after PBMC isolation.

Flow Cytometry

Flow cytometry was used to quantify HIV target cell activation in PBMCs. One million PBMCs were washed and 20uL of CCR5-APC (3A9, BD, California, USA) was added to each sample in a polysterene FACS tube. Cells were incubated for 15 minutes at 37C, and then 86uL of extracellular master mix (Table 2.3) was added without washing. Cells were then incubated at room temperature in the dark for 20 minutes and then washed with PBS (Sigma, Missouri, USA) + 2% FBS (Biochrom, UK). Cells were permeabilized with 750uL, 20% BD FACS Lysing Solution (BD, California, USA) + 0.05% Tween 20 (Fisher Scientific, Massachusetts, USA) for 10 minutes on ice with vortexing, and then washed twice as described above. 65uL of intracellular master mix (Table 3.1) was then added to the cells and the mixture was incubated for 20 minutes at room temperature in the dark. A final wash was then performed before fixing the cells with 250uL of 1% paraformaldehyde (VWR, Pennsylvania, USA). All flow data was compensated and analyzed, using Flow Jo v10.0.7r2 (Treestar, Oregon, USA).

**Table 3.1: BCG macaque study intracellular master mix**

| **Antibody** | **CD4** BV650 | **Ki-67** PE | **BV Buffer** |
|---|---|---|---|
| **Manufacturer** Clone | BioLegend OKT4 | BD B56 | BD |
| Volume | 5 | 10 | 50 |

*Results*

To date, data from two SIV-uninfected infant macaques, vaccinated with BCG at 7 months of age, have been analyzed and are described below (Figure 3.8). These macaques were followed

longitudinally until necropsy at 9 weeks post-vaccination to determine the kinetics of BCG-induced immune activation. These data show a robust increase in CCR5 expression within CD4+ T cells between 2 and 6 weeks post-vaccination (Figure 3.8). However, in these macaques, CCR5 expression returned to baseline between 6 and 8 weeks post-vaccination.

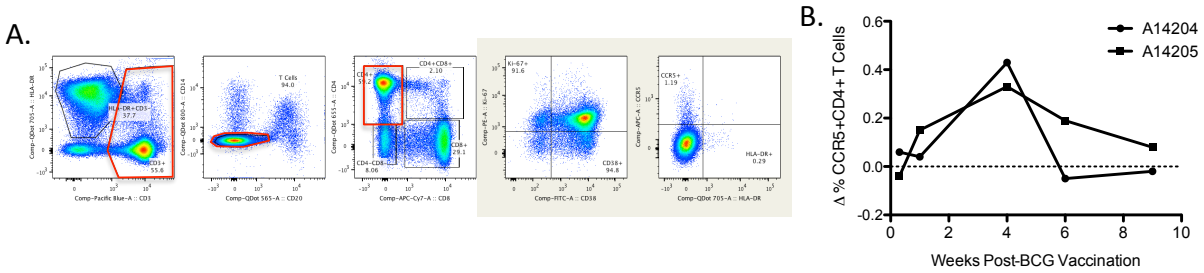



**Figure 3.8: BCG vaccination increases CCR5 expression on macaque infant CD4+ T cells. B.** Change in percent of CCR5 expressing CD4+ T Cells in PBMCs from two infant macaques vaccinated at 7 months of age. Macaque BCG response flow data was gated as shown in **A.** BCG vaccination induces a robust but transient induction of CCR5 on CD4 T cells in infant rhesus macaques.

*Discussion*

Infant vaccinations are nearly ubiquitous in most communities with high HIV prevalence. The data presented above clearly demonstrates that HIV target cells, particularly T cells that express both CD4 and CCR5, increase in response to BCG vaccination. Although the most likely source of this immune activation is the BCG vaccine, without unvaccinated control animals, we can not rule out the possibility that an environmental exposure that was not part of our study design elicited the observed T cell activation.

There is some variation in the kinetics of the CD4 T cell responses induced in the human and this macaque study. It is possible that the differences observed between these two study groups are due to a difference in immune responses in macaques and humans. However, Jensen et al. recently reported that in their study of infants administered BCG at birth, they also saw sustained, CCR5 activation that resulted in an increase in SIV susceptibility in BCG-vaccinated

infant macaques.[420] This suggests that the age of the infant at the time of vaccination likely alters the kinetics of BCG vaccine T cell responses with younger infants sustaining T cell activation for a more extended period of time than older infants. Therefore, infants vaccinated with BCG at birth likely sustain a greater level of immune activation for weeks to months after vaccination.

BCG is a very unique vaccine in infants, and although it is possible that other infant vaccines also result in HIV target cell activation, *ex vivo* stimulation assays suggest that the magnitude of the response to other vaccines is much lower.

**Relative Contribution of Environmental Exposures to Infant Immune Activation**

In this chapter I evaluated three additional factors that may alter an infant's susceptibility to HIV while breastfeeding. All three show potential to increase immune activation and, therefore, HIV susceptibility.

The relative contribution of each environmental exposure to an individual infant's risk of acquiring HIV certainly vary from infant to infant. Nevertheless, it is possible to use the data presented above to estimate which environmental exposures have the greatest potential to impact MTCT of HIV at a population level.

First, the magnitude and nature of the immune activation elicited by each exposure is important in determining the relative contribution of these exposures to increased risk of MTCT. Despite

the differential assays utilized to assess these different factors, there is evidence that maternal HIV disease progression, candidiasis and BCG vaccination all likely contribute to HIV infection of infants.

In addition, the percentage of infants that are exposed to any particular risk factor is a key determinant of the relative contribution of each environmental exposure to the population-level risk of MTCT. Only BCG vaccination is a risk factor for all HIV-exposed infants. Only a subset of infants will acquire candidiasis and, similarly, only a fraction of infants will be born to mothers with advanced HIV and low CD4 counts who choose to breastfeed. In fact, as HIV treatment becomes increasingly more universal and robust, the likelihood of any mother having a low CD4 count while breastfeeding should continue to decline.

Finally, the duration of exposure to each potential risk factor should alter their impact on MTCT rates. There is a relatively narrow window of time when infants are at increased risk of candidiasis, where maternal HIV disease progression will continue to alter immune activation in an infant for the duration of breastfeeding, which can often continue for a year or more (Fig. 4.1). It is a bit harder to determine the duration of the immune activation induced by BCG vaccination, and as discussed, above, it is likely that the age of the infant at the time of vaccination likely impacts the length of time that an infant sustains an increase in HIV target cells. However, since infants in South Africa currently receive BCG vaccination at birth regardless of maternal HIV infection status, it is likely that the immune activation induced by BCG vaccination in South African infants is sustained for many weeks post-vaccination.

Therefore, I predict that, although all three factors assessed here may increase HIV infection rates, the induction of immune activation by BCG vaccination likely has the greatest impact on MTCT of HIV in South Africa.

## Chapter 4: Discussion

This thesis presented data from several cohorts that together define a wide range of immunomodulatory environmental exposures that may increase HIV suscpetible cells in South African infants.

First, data from a prospective, observational study of mixed feeding in Khayelitsha, South Africa suggests that not only is mixed feeding common, but it results in an increase in HIV target cells with a Treg phenotype that is consistent with high HIV susceptibility as well as high virus production following infection. In addition, these Treg, HIV target cells may be recruited to the site of HIV exposure, the oral mucosa, by CCL5 and possibly CCL22. Although there are many possible triggers that could result in an altered immune response in mixed fed infants, we present evidence that alterations in the microbiome of mixed fed infants, particularly an increase in P copri, is responsible for the observed alterations in Treg activation and recruitment.

Additionally, this work indicates that evaluating both the systemic circulation and the site of HIV exposure, which in the case of MTCT of HIV is the gastrointestinal tract, is practical and can lend substantial insight into the mechanisms underlying increased HIV susceptibility.

By identifying alterations in HIV target cells associated with mixed feeding, it is now possible to consider evaluating potential interventions to reduce HIV transmission during mixed feeding. These interventions can include targeted counseling, including the use of support groups, for mothers that feed particular food products their infant. The microbiome data suggests that the

greatest alteration in the gut microbiota occurs with the introduction of infant cereal, suggesting that delaying the introduction of cereal may reduce HIV transmission. Intensification of HIV treatment to further suppress maternal viral load in mothers whose infants have elevated HIV target cells in their blood may also be a viable intervention to reduced HIV transmission during mixed feeding. Finally, the administration of probiotics that can prevent the accumulation of HIV target cell-inducing bacteria in the gastrointestinal tract of infants that mixed feed may also be an effective intervention to reduce HIV transmission. A particularly attractive delivery method for probiotics would be to add specifically selected probiotics to infant food commercially available in South Africa.

Although we were unable to demonstrate a role for ochratoxin in activation of HIV target cells in mixed fed infants, it is possible that ochratoxin exposure below our assay's lower limit of detection may still contribute to the activation observed in mixed fed infants. If this is the case, direct inhibitors of ochratoxin, specifically phenylalanine or aspartame, or inhibitors of reactive oxygen species may be effective therapies in reducing HIV susceptibility in mixed fed infants.

Candida and maternal HIV disease progression seem to induce a much less regulatory and a more proinflammatory immune response than mixed feeding. These exposures may also increase HIV susceptibility, but it is likely that different interventions will be necessary to counteract any increase in HIV susceptibility observed with these childhood exposures.

The immune response induced in HIV target cells in response to BCG vaccination has not yet been fully characterized, so it is, as of yet, difficult to determine what intervention would be most appropriate to reduce HIV susceptibility in BCG vaccinated infants.

Although many questions remain, these studies add substantially to our understanding of environmental exposures that increase HIV susceptibility in South African infants.

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## Appendix:

Nanostring Gene Panel

| Gene | Accession | Targ Region | Target Sequence |
|---|---|---|---|
| AGRN | NM_198576.3 | 3581-3681 | CGGAATTCAGACGTCAAGAAGGATTTTCGGAGTGTCCGCTTGCGGGACCTGGGGCCCGGCAAATCCGTCCGCGCCATTGTGGATGTGCACTTTGACCCCA |
| ALAS1 | NM_000688.4 | 395-495 | AGAAAGCAGGCAAATCTCTGTTGTTCTATGCCCAAAACTGCCCCAAGATGATGGAAGTTGGGGCCAAGCCAGCCCCTCGGGCATTGTCCACTGCAGCAGT |
| ANXA3 | NM_005139.2 | 599-699 | AGCAGCTAAAGAAATCCATGAAGGGCGCGGGAACAAACGAAGATGCCTTGATTGAAATCTTAACTACCAGGACAAGCAGGCAAATGAAGGATATCTCTCA |
| APOBEC3F | NM_145298.5 | 3315-3415 | GCTGGTGTGAGCCACAGCGCCTAGCCCATTTCTCCTTTTAATAGGACCTGTTGCTGTCTCTGTTCTCCCAACATGGTGAACACCACCCGGACTGCGTGTA |
| APOBEC3G | NM_021822.2 | 761-861 | GAAAAGAGACGGTCCGCGTGCCACCATGAAGATCATGAATTATGACGAATTTCAGCACTGTTGGAGCAAGTTCGTGTACAGCCAAAGAGAGCTATTTGAG |
| APOBEC3H | NM_181773.2 | 190-290 | CCACGAGAGGCTACTTTGAAAACAAGAAAAAGTGCCATGCAGAAATTTGCTTTATTAACGAGATCAAGTCCATGGGACTGGACGAAACGCAGTGCTACCA |
| APOL6 | NM_030641.3 | 9055-9155 | GGAACAAAGTTAATTGGTTCCAGAGATTCAAAGCCAGAGTTGCTGTCAGTTCATTGGTAGAGATGCCATCACTGGGCAAGTGTTCTGAAAACATCTTATC |
| ARG1 | NM_000045.2 | 505-605 | AAGGAACTAAAAGGAAAGATTCCCGATGTGCCAGGATTCTCCTGGGTGACTCCCTGTATATCTGCCAAGGATATTGTGTATATTGGCTTGAGAGACGTGG |
| ATF1 | NM_005171.2 | 710-810 | CAGATACTTGTGCCCAGCAATCAGGTGGTCGTACAAACTGCATCAGGAGATATGCAAACATATCAGATCCGAACTACACCTTCAGCTACTTCTCTGCCAC |
| ATF7 | NM_001130060.1 | 203-303 | CAGAGATTTACAAACGAGGACCACCTGGCAGTTCATAAACACAAGCATGAGATGACATTGAAATTTGGCCCAGCCCGAACTGACTCAGTCATCATTGCAG |
| BATF | NM_006399.3 | 825-925 | CACTGTGGGTTGCAGGCCCAATGCAGAAGAGTATTAAGAAAGATGCTCAAGTCCCATGGCACAGAGCAAGGCGGGCAGGGAACGGTTATTTTTCTAAATA |
| BCL2 | NM_000657.2 | 947-1047 | AGTTCGGTGGGGTCATGTGTGTGGAGAGCGTCAACCGGGAGATGTCGCCCCTGGTGGACAACATCGCCCTGTGGATGACTGAGTACCTGAACCGGCACCT |
| BCL2L1 | NM_138578.1 | 1560-1660 | CTAAGAGCCATTTAGGGGCCACTTTTGACTAGGGATTCAGGCTGCTTGGGATAAAGATGCAAGGACCAGGACTCCCTCCTCACCTCTGGACTGGCTAGAG |
| CAMP | NM_004345.3 | 220-320 | CATCATTGCCCAGGTCCTCAGCTACAAGGAAGCTGTGCTTCGTGCTATAGATGGCATCAACCAGCGGTCCTCGGATGCTAACCTCTACCGCCTCCTGGAC |
| CASP1 | NM_001223.3 | 971-1071 | TGGAGACATCCCACAATGGGCTCTGTTTTTATTGGAAGACTCATTGAACATATGCAAGAATATGCCTGTTCCTGTGATGTGGAGGAAATTTTCCGCAAGG |
| CASP10 | NM_001206524.1 | 1522-1622 | TTCCTACTTGGTCTGGCCACTGTCCCAGGCTATGTATCCTTTCGGCATGTGGAGGAAGGCAGCTGGTATATTCAGTCTCTGTGTAATCATCTGAAGAAAT |
| CASP3 | NM_032991.2 | 685-785 | ACTCCACAGCACCTGGTTATTATTCTTGGCGAAATTCAAAGGATGGCTCCTGGTTCATCCAGTCGCTTTGTGCCATGCTGAAACAGTATGCCGACAAGCT |
| CASP8 | NM_033355.3 | 325-425 | GGATGCCTTGATGTTATTCCAGAGACTCCAGGAAAAGAGAATGTTGGAGGAAAGCAATCTGTCCTTCCTGAAGGAGCTGCTCTTCCGAATTAATAGACTG |
| CASP9 | NM_001229.2 | 1805-1905 | CCTGGGGTATAAAACTTTCCTCGCCTGACAACCACTGGTCTGTAGGGATTTTTGGCTACACACAAACCAGTATCGCTCATAGATCAGCAAACCGGGGCCT |
| CCL1 | NM_002981.1 | 157-257 | CCTTCTCCAGATGTTGCTTCTCATTTGCGGAGCAAGAGATTCCCCTGAGGGCAATCCTGTGTTACAGAAATACCAGCTCCATCTGCTCCAATGAGGGCTT |
| CCL11 | NM_002986.2 | 334-434 | AGACCAAACTGGCCAAGGATATCTGTGCCGACCCCAAGAAGAAGTGGGTGCAGGATTCCATGAAGTATCTGGACCAAAAATCTCCAACTCCAAAGCCATA |
| CCL13 | NM_005408.2 | 320-420 | CCAGAATTATATGAAACACCTGGGCCGGAAAGCTCACACCCTGAAGACTTGAACTCTGCTACCCCTACTGAAATCAAGCTGGAGTACGTGAAATGACTTT |
| CCL14 | NM_032963.3 | 274-374 | TTCATCACCAAAAGGGGCCATTCCGTCTGTACCAACCCCAGTGACAAGTGGGTCCAGGACTATATCAAGGACATGAAGGAGAACTGAGTGACCCAGAAGG |
| CCL16 | NM_004590.2 | 367-467 | TTGTCCACGGTTAAAATTATTACAGCAAAGAATGGTCAACCCCAGCTCCTCAACTCCCAGTGATGACCAGGCTTTAGTGGAAGCCCTTGTTTACAGAAGA |
| CCL17 | NM_002987.2 | 229-329 | GCCTGGAGTACTTCAAGGGAGCCATTCCCCTTAGAAAGCTGAAGACGTGGTACCAGACATCTGAGGACTGCTCCAGGGATGCCATCGTTTTTGTAACTGT |
| CCL18 | NM_002988.2 | 585-685 | CCCCTTTCCCTTCAACTCTTCGTACATTCAATGCATGGATCAATCAGTGTGATTAGCTTTCTCAGCAGACATTGTGCCATATGTATCAAATGACAAATCT |
| CCL19 | NM_006274.2 | 149-249 | ACTGGCCCTCAGCCTGCTGGTTCTCTGGACTTCCCCAGCCCCAACTCTGAGTGGCACCAATGATGCTGAAGACTGCTGCCTGTCTGTGACCCAGAAACCC |
| CCL2 | NM_002982.3 | 250-350 | CCCAAAGAAGCTGTGATCTTCAAGACCATTGTGGCCAAGGAGATCTGTGCTGACCCCAAGCAGAAGTGGGTTCAGGATTCCATGGACCACCTGGACAAGC |
| CCL20 | NM_004591.1 | 35-135 | ATCTGTTCTTTGAGCTAAAAACCATGTGCTGTACCAAGAGTTTGCTCCTGGCTGCTTTGATGTCAGTGCTGCTACTCCACCTCTGCGGCGAATCAGAAGC |
| CCL21 | NM_002989.2 | 180-280 | CTCAAGTACAGCCAAAGGAAGATTCCCGCCAAGGTTGTCCGCAGCTACCGGAAGCAGGAACCAAGCTTAGGCTGCTCCATCCCAGCTATCCTGTTCTTGC |
| CCL22 | NM_002990.3 | 797-897 | CTCGCCCAAGCAGCTGGTAATTCCATTTCATGTATTAGATGTCCCCTGGCCCTCTGTCCCCTCTTAATAACCCTAGTCACAGTCTCCGCAGATTCTTGGG |
| CCL23 | NM_145898.1 | 336-436 | AGGGGCGACGTTTCTGTGCCAACCCCAGTGATAAGCAAGTTCAGGTTTGCGTGAGAATGCTGAAGCTGGACACACGGATCAAGACCAGGAAGAATTGAAC |
| CCL24 | NM_002991.2 | 18-118 | ATAGTAACCAGCCTTCTGTTCCTTGGTGTCTGTGCCCACCACATCATCCCTACGGGCTCTGTGGTCATCCCCTCTCCCTGCTGCATGTTCTTTGTTTCCA |
| CCL28 | NM_148672.2 | 236-336 | GGGATTGTGACTTGGCTGCTGTCATCCTTCATGTCAAGCGCAGAAGAATCTGTGTCAGCCCGCACAACCATACTGTTAAGCAGTGGATGAAAGTGCAAGC |
| CCL3 | NM_002983.2 | 681-781 | CTGTGTAGGCAGTCATGGCACCAAAGCCACCAGACTGACAAATGTGTATCGGATGCTTTTGTTCAGGGCTGTGATCGGCCTGGGGAAATAATAAAGATGC |
| CCL4 | NM_002984.2 | 201-301 | GAAGCTTCCTCGCAACTTTGTGGTAGATTACTATGAGACCAGCAGCCTCTGCTCCCAGCCAGCTGTGGTATTCCAAACCAAAAGAAGCAAGCAAGTCTGT |
| CCL5 | NM_002985.2 | 148-248 | CTCGGACACCACACCCTGCTGCTTTGCCTACATTGCCCGCCCACTGCCCCGTGCCCACATCAAGGAGTATTTCTACACCAGTGGCAAGTGCTCCAACCCA |
| CCL7 | NM_006273.2 | 120-220 | CAGCCCCCAGGGGCTTGCTCAGCCAGTTGGGATTAATACTTCAACTACCTGCTGCTACAGATTTATCAATAAGAAAATCCCTAAGCAGAGGCTGGAGAGC |

| | | | |
|---|---|---|---|
| CCL8 | NM_005623.2 | 497-597 | GCCACTTTCAGCCCTCAGGGACTTGCTCAGCCAGATTCAGTTTCCATTCCAATCACCTGCTGCTTTAACGTGATCAATAGGAAAATTCCTATCCAGAGGC |
| CCR2 | NM_001123041.2 | 743-843 | TCTGATCTGCTTTTTCTTATTACTCTCCCATTGTGGGCTCACTCTGCTGCAAATGAGTGGGTCTTTGGGAATGCAATGTGCAAATTATTCACAGGGCTGT |
| CCR3 | NM_001837.2 | 980-1080 | CAGTGCTCTTTACCCAGAGGATACAGTATATAGCTGGAGGCATTTCCACACTCTGAGAATGACCATCTTCTGTCTCGTTCTCCCTCTGCTCGTTATGGCC |
| CCR5 | NM_000579.3 | 366-466 | CAAGTGTCAAGTCCAATCTATGACATCAATTATTATACATCGGAGCCCTGCCAAAAAATCAATGTGAAGCAAATCGCAGCCCGCCTCCTGCCTCCGCTCT |
| CCR7 | NM_001838.3 | 86-186 | GAAACCAATGAAAAGCGTGCTGGTGGTGGCTCTCCTTGTCATTTTCCAGGTATGCCTGTGTCAAGATGAGGTCACGGACGATTACATCGGAGACAACACC |
| CCR8 | NM_005201.2 | 245-345 | TTGCCTCCTGTTTGTATTCAGTCTTCTGGGAAACAGCCTGGTCATCCTGGTCCTTGTGGTCTGCAAGAAGCTGAGGAGCATCACAGATGTATACCTCTTG |
| CCR9 | NM_031200.1 | 1095-1195 | CCCTGTTCTCTATGTTTTTGTGGGTGAGAGATTCCGCCGGGATCTCGTGAAAACCCTGAAGAACTTGGGTTGCATCAGCCAGGCCCAGTGGGTTTCATTT |
| CD14 | NM_000591.2 | 885-985 | GCCCAAGCACACTCGCCTGCCTTTTCCTGCGAACAGGTTCGCGCCTTCCCGGCCCTTACCAGCCTAGACCTGTCTGACAATCCTGGACTGGGCGAACGCG |
| CD163 | NM_203416.2 | 1784-1884 | CATCTTTCACTCTGCCCAGTAGCACCCCGCCCAGAAGGAACTTGTAGCCACAGCAGGGATGTTGGAGTAGTCTGCTCAAGATACACAGAAATTCGCTTGG |
| CD19 | NM_001178098.1 | 938-1038 | TCAGCTGTGACTTTGGCTTATCTGATCTTCTGCCTGTGTTCCCTTGTGGGCATTCTTCATCTTCAAAGAGCCCTGGTCCTGAGGAGGAAAAGAAAGCGAA |
| CD2 | NM_001767.3 | 687-787 | GCAGCCTCTTGATGGTCTTTGTGGCACTGCTCGTTTTCTATATCACCAAAAGGAAAAAACAGAGGAGTCGGAGAAATGATGAGGAGCTGGAGACAAGAGC |
| CD207 | NM_015717.2 | 210-310 | CCTCCGTCCTGCTGCAGGCCGTCCTTTATCCCCGGTTTATGGGCACCATATCAGATGTAAAGACCAATGTCCAGTTGCTGAAAGGTCGTGTGGACAACAT |
| CD209 | NM_021155.3 | 802-902 | AAGGCTGCAGTGGAACGCCTGTGCCACCCCTGTCCCTGGGAATGGACATTCTTCCAAGGAAACTGTTACTTCATGTCTAACTCCCAGCGGAACTGGCACG |
| PDCD1 | NM_005018.2 | 679-779 | CACCGGCCAGCCCCTGAAGGAGGACCCCTCAGCCGTGCCTGTGTTCTCTGTGGACTATGGGGAGCTGGATTTCCAGTGGCGAGAGAAGACCCCGGAGCCC |
| FCGR2A | NM_021642.3 | 60-160 | TGGAGACCCAAATGTCTCAGAATGTATGTCCCAGAAACCTGTGGCTGCTTCAACCATTGACAGTTTTGCTGCTGCTGGCTTCTGCAGACAGTCAAGCTGC |
| CD3E | NM_000733.2 | 75-175 | AAGTAACAGTCCCATGAAACAAAGATGCAGTCGGGCACTCACTGGAGAGTTCTGGGCCTCTGCCTCTTATCAGTTGGCGTTTGGGGGCAAGATGGTAATG |
| CD4 | NM_000616.4 | 975-1075 | TGGCAGGCGGAGAGGGCTTCCTCCTCCAAGTCTTGGATCACCTTTGACCTGAAGAACAAGGAAGTGTCTGTAAAACGGGTTACCCAGGACCCTAAGCTCC |
| CD40LG | NM_000074.2 | 533-633 | CCTGGAAAATGGGAAACAGCTGACCGTTAAAAGACAAGGACTCTATTATATCTATGCCCAAGTCACCTTCTGTTCCAATCGGGAAGCTTCGAGTCAAGCT |
| NCAM1 | NM_000615.6 | 415-515 | CAGTTTCTCTGCAGGTGGATATTGTTCCCAGCCAGGGGGAGATCAGCGTTGGAGAGTCCAAATTCTTCTTATGCCAAGTGGCAGGAGATGCCAAAGATAA |
| FCGR1A | NM_000566.3 | 1545-1645 | CCAGGAGTGGGTAGATTTCAGGGAGACAAGAGGGAATAGTATAGACAATAAGGAAGGAAATAGTACTTACAAATGACTCCTAAGGGACTGTGAGACTGAG |
| CD68 | NM_001251.2 | 1140-1240 | ACCGGTCCATCTTGCTGCCTCTCATCATCGGCCTGATCCTTCTTGGCCTCCTCGCCCTGGTGCTTATTGCTTTCTGCATCATCCGGAGACGCCCATCCGC |
| CD69 | NM_001781.1 | 460-560 | AGGACATGAACTTTCTAAAACGATACGCAGGTAGAGAGGAACACTGGGTTGGACTGAAAAAGGAACCTGGTCACCCATGGAAGTGGTCAAATGGCAAAGA |
| CD8A | NM_001768.5 | 1320-1420 | GCTCAGGGCTCTTTCCTCCACACCATTCAGGTCTTTCTTTCCGAGGCCCCTGTCTCAGGGTGAGGTGCTTGAGTCTCCAACGGCAAGGGAACAAGTACTT |
| KLRD1 | NM_002262.3 | 393-493 | CTCCAGGACCCAACATAGAACTCCAGAAAGACTCTGACTGCTGTTCTTGCCAAGAAAAATGGGTTGGGTACCGGTGCAACTGTTACTTCATTTCCAGTGA |
| CIITA | NM_000246.3 | 415-515 | TATGCCAATATCGCGGAACTGGACCAGTATGTCTTCCAGGACTCCCAGCTGGAGGGCCTGAGCAAGGACATTTTCAAGCACATAGGACCAGATGAAGTGA |
| COMMD1 | NM_152516.2 | 232-332 | ATTCTTGACTGCTCAAACCAAAAAGCAAGGTGGGATCACATCTGACCAAGCTGCTGTCATTTCCAAATTCTGGAAGAGCCACAAGACAAAAATCCGTGAG |
| CSF1R | NM_005211.3 | 1230-1330 | GACCGTGGGGGAGGGGCTCAACCTCAAAGTCATGGTGGAGGCCTACCCAGGCCTGCAAGGTTTTAACTGGACCTACCTGGGACCCTTTTCTGACCACCAG |
| CTLA4 | NM_005214.3 | 405-505 | AGTCTGTGCGGCAACCTACATGATGGGGAATGAGTTGACCTTCCTAGATGATTCCATCTGCACGGGCACCTCCAGTGGAAATCAAGTGAACCTCACTATC |
| CX3CL1 | NM_002996.3 | 140-240 | AGCACCACGGTGTGACGAAATGCAACATCACGTGCAGCAAGATGACATCAAAGATACCTGTAGCTTTGCTCATCCACTATCAACAGAACCAGGCATCATG |
| CX3CR1 | NM_001337.3 | 1040-1140 | GGGCGCTCAGTCCACGTTGATTTCTCCTCATCTGAATCACAAAGGAGCAGGCATGGAAGTGTTCTGAGCAGCAATTTTACTTACCACACGAGTGATGGAG |
| CXCL1 | NM_001511.1 | 742-842 | TATGTTAATATTTCTGAGGAGCCTGCAACATGCCAGCCACTGTGATAGAGGCTGGCGGATCCAAGCAAATGGCCAATGAGATCATTGTGAAGGCAGGGGA |
| CXCL10 | NM_001565.3 | 168-268 | GTACCTCTCTCTAGAACTGTACGCTGTACCTGCATCAGCATTAGTAATCAACCTGTTAATCCAAGGTCTTTAGAAAAACTTGAAATTATTCCTGCAAGCC |
| CXCL11 | NM_005409.4 | 282-382 | TTCAAAAGAGGACGCTGTCTTTGCATAGGCCCTGGGGTAAAAGCAGTGAAAGTGGCAGATATTGAGAAAGCCTCCATAATGTACCCAAGTAACAACTGTG |
| CXCL12 | NM_000609.5 | 210-310 | CCAGAGCCAACGTCAAGCATCTCAAAATTCTCAACACTCCAAACTGTGCCCTTCAGATTGTAGCCCGGCTGAAGAACAACAACAGACAAGTGTGCATTGA |
| CXCL13 | NM_006419.2 | 0-100 | GAGAAGATGTTTGAAAAAACTGACTCTGCTAATGAGCCTGGACTCAGAGCTCAAGTCTGAACTCTACCTCCAGACAGAATGAAGTTCATCTCGACATCTC |
| CXCL16 | NM_001100812.1 | 850-950 | CCATGGGTTCAGGAATTGATGAGCTGTCTTGATCTCAAAGAATGTGGACATGCTTACTCGGGGATTGTGGCCCACCAGAAGCATTTACTTCCTACCAGCC |
| CXCL2 | NM_002089.3 | 854-954 | ATCACATGTCAGCCACTGTGATAGAGGCTGAGGAATCCAAGAAAATGGCCAGTGAGATCAATGTGACGGCAGGGAAATGTATGTGTGTCTATTTTGTAAC |
| CXCL3 | NM_002090.2 | 540-640 | TCCCTGCCCTTACCAGAGCTGAAAATGAAAAAGAGAACAGCAGCTTTCTAGGGACAGCTGGAAAGGACTTAATGTGTTTGACTATTTCTTACGAGGGTTC |
| CXCL5 | NM_002994.3 | 250-350 | AGAGAGCTGCGTTGCGTTTGTTTACAGACCACGCAAGGAGTTCATCCCAAAATGATCAGTAATCTGCAAGTGTTCGCCATAGGCCCACAGTGCTCCAAGG |
| CXCL6 | NM_002993.3 | 539-639 | AGTAACAAAAAAGACCATGCATCATAAAATTGCCCAGTCTTCAGCGGAGCAGTTTTCTGGAGATCCCTGGACCCAGTAAGAATAAGAAGGAAGGGTTGGT |
| CXCL9 | NM_002416.1 | 87-187 | CTGATTGGAGTGCAAGGAACCCCAGTAGTGAGAAAGGGTCGCTGTTCCTGCATCAGCACCAACCAAGGGACTATCCACCTACAATCCTTGAAAGACCTTA |
| CXCR1 | NM_000634.2 | 653-753 | CATCCAAACAATTCCAGTCCAGTTTGCTATGAGGTCCTGGGAAATGACACAGCAAAATGGCGGATGGTGTTGCGGATCCTGCCTCACACCTTTGGCTTCA |
| CXCR3 | NM_001504.1 | 80-180 | GTGAGTGACCACCAAGTGCTAAATGACGCCGAGGTTGCCGCCCTCCTGGAGAACTTCAGCTCTTCCTATGACTATGGAGAAAACGAGAGTGACTCGTGCT |
| CXCR4 | NM_001008540.1 | 616-716 | AACTGGTACTTTGGGAACTTCCTATGCAAGGCAGTCCATGTCATCTACACAGTCAACCTCTACAGCAGTGTCCTCATCCTGGCCTTCATCAGTCTGGACC |

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|---|---|---|---|
| CXCR6 | NM_006564.1 | 983-1083 | ACTTGTGAAGGACATTGGTTGCCTCCCTTACCTTGGGGTCTCACATCAATGGAAATCTTCTGAGGACAATTCCAAGACTTTTTCTGCCTCCCACAATGTG |
| CXCR7 | NM_020311.2 | 1704-1804 | TGTTTGGGAGGCATAGTGCTGACATATATTCAGAGTGTTGTAGTTTTAAGGTTAGCGTGACTTCAGTTTTGACTAAGGATGACACTAATTGTTAGCTGTT |
| DDX58 | NM_014314.3 | 1368-1468 | AGGTCATTGGGCTGACTGCCTCGGTTGGTGTTGGGGATGCCAAAAACACAGATGAAGCCTTGGATTATATCTGCAAGCTGTGTGCTTCTCTTGATGCGTC |
| DEFA3 | NM_005217.3 | 276-376 | GGACTGCTATTGCAGAATACCAGCGTGCATTGCAGGAGAACGTCGCTATGGAACCTGCATCTACCAGGGAAGACTCTGGGCATTCTGCTGCTGAGCTTGC |
| DEFA5 | NM_021010.1 | 235-335 | TATTGCCGAACCGGCCGTTGTGCTACCCGTGAGTCCCTCTCCGGGGTGTGTGAAATCAGTGGCCGCCTCTACAGACTCTGCTGTCGCTGAGCTTCCTAGA |
| DEFB1 | NM_005218.3 | 40-140 | CTTATAAATACAGTGACGCTCCAGCCTCTGGAAGCCTCTGTCAGCTCAGCCTCCAAAGGAGCCAGCGTCTCCCCAGTTCCTGAAATCCTGGGTGTTGCCT |
| DEFB103A | NM_001081551.2 | 4-104 | TCCATAGGGAGCTCTGCCTTACCATTGGGTTCCTAATTAACTGAGTGAGTGGGTGTGTTCTGCATGGTGAGAGGCATTGGAATGATGCATCAGAAAACAT |
| DEFB4A | NM_004942.2 | 97-197 | TTTTTGGTGGTATAGGCGATCCTGTTACCTGCCTTAAGAGTGGAGCCATATGTCATCCAGTCTTTTGCCCTAGAAGGTATAAACAAATTGGCACCTGTGG |
| EIF2A | NM_032025.3 | 220-320 | CTACTGCACTCCTTCGACCTCCTGAAGGCAGTTTGCCTTGAATTCTCACCCAAAAATACTGTCCTGGCAACGTGGCAGCCTTACACTACTTCTAAAGATG |
| EIF2B4 | NM_172195.3 | 1390-1490 | ATGTACCAGTGCTGGTTTGCTGTGAAACATACAAGTTCTGTGAGCGTGTGCAGACTGATGCCTTTGTCTCTAATGAGCTAGATGACCCTGATGATCTGCA |
| EMR1 | NM_001974.3 | 2043-2143 | CGGGCTTCCTGCACTACCTTTTCCTTGCCTGCTTCTTCTGGATGCTGGTGGAGGCTGTGATACTGTTCTTGATGGTCAGAAACCTGAAGGTGGTGAATTA |
| FCGR3A | NM_000569.6 | 873-973 | GGCAGTGTCAACCATCTCATCATTCTTTCCACCTGGGTACCAAGTCTCTTTCTGCTTGGTGATGGTACTCCTTTTTGCAGTGGACACAGGACTATATTTC |
| FOXP3 | NM_014009.3 | 1230-1330 | GGGCCATCCTGGAGGCTCCAGAGAAGCAGCGGACACTCAATGAGATCTACCACTGGTTCACACGCATGTTTGCCTTCTTCAGAAACCATCCTGCCACCTG |
| FYN | NM_153048.3 | 1455-1555 | CCCTGAAGAACGCCCCACTTTTGAGTACTTGCAGAGCTTCCTGGAAGACTACTTTACCGCGACAGAGCCCCAGTACCAACCTGGTGAAAACCTGTAAGGC |
| GAL3ST1 | NM_004861.1 | 1378-1478 | CATGCTCACGCCCGAGATCCAGTACCTGATGGACCTCGGCGCCAACCTGTGGGTCACCAAGCTCTGGAAGTTCATTCGCGATTTCCTGCGGTGGTGACGT |
| GAPDH | NM_002046.3 | 104-204 | GGGGAAGGTGAAGGTCGGAGTCAACGGATTTGGTCGTATTGGGCGCCTGGTCACCAGGGCTGCTTTTAACTCTGGTAAAGTGGATATTGTTGCCATCAAT |
| GATA3 | NM_001002295.1 | 1691-1791 | GTGCATGACTCACTGGAGGACTTCCCCAAGAACAGCTCGTTTAACCCGGCCGCCCTCTCCAGACACATGTCCTCCCTGAGCCACATCTCGCCCTTCAGCC |
| GZMB | NM_004131.4 | 112-212 | CAGATGCAGGGGAGATCATCGGGGGACATGAGGCCAAGCCCCACTCCCGCCCCTACATGGCTTATCTTATGATCTGGGATCAGAAGTCTCTGAAGAGGTG |
| HAVCR2 | NM_032782.3 | 955-1055 | TATATGAAGTGGAGGAGCCCAATGAGTATTATTGCTATGTCAGCAGCAGGCAGCAACCCTCACAACCTTTGGGTTGTCGCTTTGCAATGCCATAGATCCA |
| HLA-A | NM_002116.5 | 1000-1100 | GGAAGAGCTCAGATAGAAAAGGAGGGAGTTACACTCAGGCTGCAAGCAGTGACAGTGCCCAGGGCTCTGATGTGTCCCTCACAGCTTGTAAAGTGTGAGA |
| HLA-B | NM_005514.6 | 937-1037 | CCCTGAGATGGGAGCCGTCTTCCCAGTCCACCGTCCCCATCGTGGGCATTGTTGCTGGCCTGGCTGTCCTAGCAGTTGTGGTCATCGGAGCTGTGGTCGC |
| HLA-C | NM_002117.4 | 895-995 | AGCTGGGAGCCATCTTCCCAGCCCACCATCCCCATCATGGGCATCGTTGCTGGCCTGGCTGTCCTGGTTGTCCTAGCTGTCCTTGGAGCTGTGGTCACCG |
| HLA-DMA | NM_006120.3 | 255-355 | TGGGACTCTCTGAGGCCTACGACGAGGACCAGCTTTTCTTCTTCGACTTTTCCCAGAACACTCGGGTGCCTCGCCTGCCCGAATTTGCTGACTGGGCTCA |
| HLA-DMB | NM_002118.3 | 20-120 | CCCGTGAGCTGGAAGGAACAGATTTAATATCTAGGGGCTGGGTATCCCCACATCACTCATTTGGGGGGTCAAGGGACCCGGGCAATATAGTATTCTGCTC |
| HLA-DOA | NM_002119.3 | 3075-3175 | CAGTGAACATGTCCACCCGACAGCTCCTGAGTTTATATCATCTCAACCCTCACAACCCACAGAGGCTGTGTCTCCTAGTCACAGCTTTAAATTACTGGAA |
| HLA-DOB | NM_002120.3 | 230-330 | ACGGGACAGAAAAGGTGCAGTTTGTGGTCAGATTCATCTTTAACTTGGAGGAGTATGTACGTTTCGACAGTGATGTGGGGATGTTTGTGGCATTGACCAA |
| HLA-DPA1 | NM_033554.3 | 305-405 | CTGGACAAGAAGGAGACCGTCTGGCATCTGGAGGAGTTTGGCCAAGCCTTTTCCTTTGAGGCTCAGGGCGGGCTGGCTAACATTGCTATATTGAACAACA |
| HLA-DPB1 | NM_002121.4 | 931-1031 | TCCAAATTGGATACTGCTGCCAAGAAGTTGCTCTGAAGTCAGTTTCTATCATTCTGCTCTTTGATTCAAAGCACTGTTTCTCTCACTGGGCCTCCAACCA |
| HLA-DQA1 | NM_002122.3 | 261-361 | GGTGGCCTGAGTTCAGCAAATTTGGAGGTTTTGACCCGCAGGGTGCACTGAGAAACATGGCTGTGGCAAAACACAACTTGAACATCATGATTAAACGCTA |
| HLA-DQB1 | NM_002123.3 | 384-484 | GGGGACCCGGGCGGAGTTGGACACGGTGTGCAGACACAACTACGAGGTGGCGTTCCGCGGGATCTTGCAGAGGAGAGTGGAGCCCACAGTGACCATCTCC |
| HLA-DRA | NM_019111.3 | 335-435 | GGCCAACATAGCTGTGGACAAAGCCAACCTGGAAATCATGACAAAGCGCTCCAACTATACTCCGATCACCAATGTACCTCCAGAGGTAACTGTGCTCACG |
| HLA-DRB1 | NM_002124.2 | 104-204 | TGCTGAGCTCCCCACTGGCTTTGTCTGGGGACACCCGACCACGTTTCCTGTGGCAGCCTAAGAGGGAGTGTCATTTCTTCAATGGGACGGAGCGGGTGCG |
| HLA-DRB3 | NM_022555.3 | 698-798 | GGTCTGAATCTGCACAGAGCAAGATGCTGAGTGGAGTCGGGGGCTTTGTGCTGGGCCTGCTCTTCCTTGGGGCCGGGCTGTTCATCTACTTCAGGAATCA |
| HLA-DRB4 | NM_021983.4 | 194-294 | TGTGAGTGTCATTTCCTCAATGGGACGGAGCGAGTGTGGAACCTGATCAGATACATCTATAACCAAGAGGAGTACGCGCGCTACAACAGTGACCTGGGGG |
| HLA-DRB5 | NM_002125.3 | 131-231 | GAGTGTCATTTCTTCAACGGGACGGAGCGGGTGCGGTTCCTGCACAGAGACATCTATAACCAAGAGGAGGACTTGCGCTTCGACAGCGACGTGGGGGAGT |
| HPRT1 | NM_000194.2 | 545-645 | GGAAAGAATGTCTTGATTGTGGAAGATATAATTGACACTGGCAAAACAATGCAGACTTTGCTTTCCTTGGTCAGGCAGTATAATCCAAAGATGGTCAAGG |
| ICAM1 | NM_000201.2 | 1530-1630 | GGGAAACTGGACGTGGCCAGAAAATTCCCAGCAGACTCCAATGTGCCAGGCTTGGGGGAACCCATTGCCCGAGCTCAAGTGTCTAAAGGATGGCACTTTC |
| ICAM2 | NM_000873.3 | 415-515 | ACCTCTCTAGATAAGATTCTGCTGGACGAACAGGCTCAGTGGAAACATTACTTGGTCTCAAACATCTCCCATGACACGGTCCTCCAATGCCACTTCACCT |
| ICOS | NM_012092.3 | 549-649 | TTGTTGGCTTACAAAAAAGAAGTATTCATCCAGTGTGCACGACCCTAACGGTGAATACATGTTCATGAGAGCAGTGAACACAGCCAAAAAATCTAGACTC |
| IDO1 | NM_002164.5 | 219-319 | TATAATGACTGGATGTTCATTGCTAAACATCTGCCTGATCTCATAGAGTCTGGCCAGCTTCGAGAAAGAGTTGAGAAGTTAAACATGCTCAGCATTGATC |
| IFNA1 | NM_024013.2 | 556-656 | GAGCAGAAATCATGAGATCCCTCTCTTTATCAACAAACTTGCAAGAAAGATTAAGGAGGAAGGAATAACATCTGGTCCAACATGAAAACAATTCTTATTG |
| IFNAR1 | NM_000629.2 | 782-882 | TGGTGTCTATAGTCCAGTACATTGTATAAAGACCACAGTTGAAAATGAACTACCTCCACCAGAAAATATAGAAGTCAGTGTCCAAAATCAGAACTATGTT |
| IFNB1 | NM_002176.2 | 92-192 | CCTCCAAATTGCTCTCCTGTTGTGCTTCTCCACTACAGCTCTTTCCATGAGCTACAACTTGCTTGGATTCCTACAAAGAAGCAGCAATTTTCAGTGTCAG |
| IFNG | NM_000619.2 | 484-584 | ATTATTCGGTAACTGACTTGAATGTCCAACGCAAAGCAATACATGAACTCATCCAAGTGATGGCTGAACTGTCGCCAGCAGCTAAAACAGGGAAGCGAAA |

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|---|---|---|---|
| IFNGR1 | NM_000416.2 | 592-692 | GAAACTACCTGTTACATTAGGGTGTACAATGTGTATGTGAGAATGAACGGAAGTGAGATCCAGTATAAAATACTCACGCAGAAGGAAGATGATTGTGACG |
| NFKBIA | NM_020529.2 | 606-706 | CCTGCACCACCCCGCACCTCCACTCCATCCTGAAGGCTACCAACTACAATGGCCACACGTGTCTACACTTAGCCTCTATCCATGGCTACCTGGGCATCGT |
| IL10 | NM_000572.2 | 230-330 | AAGGATCAGCTGGACAACTTGTTGTTAAAGGAGTCCTTGCTGGAGGACTTTAAGGGTTACCTGGGTTGCCAAGCCTTGTCTGAGATGATCCAGTTTTACC |
| IL12A | NM_000882.2 | 775-875 | CTTTCTAGATCAAAACATGCTGGCAGTTATTGATGAGCTGATGCAGGCCCTGAATTTCAACAGTGAGACTGTGCCACAAAAATCCTCCCTTGAAGAACCG |
| IL12B | NM_002187.2 | 73-173 | TTTCCCTGGTTTTTCTGGCATCTCCCCTCGTGGCCATATGGGAACTGAAGAAAGATGTTTATGTCGTAGAATTGGATTGGTATCCGGATGCCCCTGGAGA |
| IL12RB1 | NM_005535.1 | 448-548 | GTGACCCTGCAGCTCTACAACTCAGTTAAATATGAGCCTCCTCTGGGAGACATCAAGGTGTCCAAGTTGGCCGGGCAGCTGCGTATGGAGTGGGAGACCC |
| IL13 | NM_002188.2 | 304-404 | GAGGATGCTGAGCGGATTCTGCCCGCACAAGGTCTCAGCTGGGCAGTTTTCCAGCTTGCATGTCCGAGACACCAAAATCGAGGTGGCCCAGTTTGTAAAG |
| IL15 | NM_172174.1 | 1685-1785 | AGGGTGATAGTCAAATTATGTATTGGTGGGGCTGGGTACCAATGCTGCAGGTCAACAGCTATGCTGGTAGGCTCCTGCCAGTGTGGAACCACTGACTACT |
| IL15RA | NM_002189.3 | 257-357 | AAGAGCTACAGCTTGTACTCCAGGGAGCGGTACATTTGTAACTCTGGTTTCAAGCGTAAAGCCGGCACGTCCAGCCTGACGGAGTGCGTGTTGAACAAGG |
| IL17A | NM_002190.2 | 240-340 | TACTACAACCGATCCACCTCACCTTGGAATCTCCACCGCAATGAGGACCCTGAGAGATATCCCTCTGTGATCTGGGAGGCAAAGTGCCGCCACTTGGGCT |
| IL17F | NM_052872.3 | 210-310 | GCCCGCCTGTGCCAGGAGGTAGTATGAAGCTTGACATTGGCATCATCAATGAAAACCAGCGCGTTTCCATGTCACGTAACATCGAGAGCCGCTCCACCTC |
| IL18 | NM_001562.3 | 364-464 | AGAAATTTGAATGACCAAGTTCTCTTCATTGACCAAGGAAATCGGCCTCTATTTGAAGATATGACTGATTCTGACTGTAGAGATAATGCACCCCGGACCA |
| IL18R1 | NM_003855.2 | 1302-1402 | GATGAAATCCACTCACTGATAGAGAAAAGCCGAAGACTAATCATTGTCCTAAGTAAAAGTTATATGTCTAATGAGGTCAGGTATGAACTTGAAAGTGGAC |
| IL1A | NM_000575.3 | 1085-1185 | ACTCCATGAAGGCTGCATGGATCAATCTGTGTCTCTGAGTATCTCTGAAACCTCTAAAACATCCAAGCTTACCTTCAAGGAGAGCATGGTGGTAGTAGCA |
| IL1B | NM_000576.2 | 560-660 | CTCCATGTCCTTTGTACAAGGAGAAGAAAGTAATGACAAAATACCTGTGGCCTTGGGCCTCAAGGAAAAGAATCTGTACCTGTCCTGCGTGTTGAAAGAT |
| IL1R1 | NM_000877.2 | 1205-1305 | TCCCAATAAAAGCTTCAGATGGAAAGACCTATGACGCATATATACTGTATCCAAAGACTGTTGGGGAAGGGTCTACCTCTGACTGTGATATTTTTGTGTT |
| IL2 | NM_000586.2 | 300-400 | AGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACTTGTCACAAACAGTGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACTGGAGC |
| IL21R | NM_021798.3 | 492-592 | AACGTGACTGTGACCTTCTCAGGACAGTATAATATCTCCTGGCGCTCAGATTACGAAGACCCTGCCTTCTACATGCTGAAGGGCAAGCTTCAGTATGAGC |
| IL22 | NM_020525.4 | 319-419 | CTATCTGATGAAGCAGGTGCTGAACTTCACCCTTGAAGAAGTGCTGTTCCCTCAATCTGATAGGTTCCAGCCTTATATGCAGGAGGTGGTGCCCTTCCTG |
| IL23A | NM_016584.2 | 411-511 | CAGGGACAACAGTCAGTTCTGCTTGCAAAGGATCCACCAGGGTCTGATTTTTTATGAGAAGCTGCTAGGATCGGATATTTTCACAGGGGAGCCTTCTCTG |
| IL2RA | NM_000417.2 | 359-459 | GAACTGTGAATGCAAGAGAGGTTTCCGCAGAATAAAAAGCGGGTCACTCTATATGCTCTGTACAGGAAACTCTAGCCACTCGTCCTGGGACAACCAATGT |
| IL3 | NM_000588.3 | 130-230 | GCCCTTGAAGACAAGCTGGGTTAACTGCTCTAACATGATCGATGAAATTATAACACACTTAAAGCAGCCACCTTTGCCTTTGCTGGACTTCAACAACCTC |
| IL31 | NM_001014336.1 | 349-449 | TAGAGCACCTCGACAAACTCATATTTCAAGATGCACCAGAAACAAACATTTCTGTGCCAACAGACACCCATGAATGTAAACGCTTCATCCTGACTATTTC |
| IL4 | NM_000589.2 | 625-725 | GACACTCGCTGCCTGGGTGCGACTGCACAGCAGTTCCACAGGCACAAGCAGCTGATCCGATTCCTGAAACGGCTCGACAGGAACCTCTGGGGCCTGGCGG |
| IL5 | NM_000879.2 | 105-205 | CCACAGAAATTCCCACAAGTGCATTGGTGAAAGAGACCTTGGCACTGCTTTCTACTCATCGAACTCTGCTGATAGCCAATGAGACTCTGAGGATTCCTGT |
| IL6 | NM_000600.1 | 220-320 | TGACAAACAAATTCGGTACATCCTCGACGGCATCTCAGCCCTGAGAAAGGAGACATGTAACAAGAGTAACATGTGTGAAAGCAGCAAAGAGGCACTGGCA |
| IL7R | NM_002185.2 | 1610-1710 | TTGCTTTGACCACTCTTCCTGAGTTCAGTGGCACTCAACATGAGTCAAGAGCATCCTGCTTCTACCATGTGGATTTGGTCACAAGGTTTAAGGTGACCCA |
| IL8 | NM_000584.3 | 221-321 | AGTTTTGCCAAGGAGTGCTAAAGAACTTAGATGTCAGTGCATAAAGACATACTCCAAACCTTTCCACCCCAAATTTATCAAAGAACTGAGAGTGATTGAG |
| IRF3 | NM_001571.5 | 1303-1403 | TCATGGCCCCAGGACCAGCCGTGGACCAAGAGGCTCGTGATGGTCAAGGTTGTGCCCACGTGCCTCAGGGCCTTGGTAGAAATGGCCCGGGTAGGGGGTG |
| IRF4 | NM_001195286.1 | 1365-1465 | TATTTTGCTCAACAAAACAGTGGACATTTCCTGAGGGGCTACGATTTACCAGAACACATCAGCAATCCAGAAGATTACCACAGATCTATCCGCCATTCCT |
| IRF7 | NM_001572.3 | 1583-1683 | TCGACTTCAGAGTCTTCTTCCAAGAGCTGGTGGAATTCCGGGCACGGCAGCGCCGTGGCTCCCCACGCTATACCATCTACCTGGGCTTCGGGCAGGACCT |
| ITGA4 | NM_000885.4 | 975-1075 | GCCCACTGCCAACTGGCTCGCCAACGCTTCAGTGATCAATCCCGGGGCGATTTACAGATGCAGGATCGGAAAGAATCCCGGCCAGACGTGCGAACAGCTC |
| ITGAL | NM_002209.2 | 1113-1213 | TTCTGGACACATTTGAGAAGCTGAAAGATCTATTCACTGAGCTGCAGAAGAAGATCTATGTCATTGAGGGCACAAGCAAACAGGACCTGACTTCCTTCAA |
| ITGAM | NM_000632.3 | 515-615 | GCCCTCCGAGGGTGTCCTCAAGAGGATAGTGACATTGCCTTCTTGATTGATGGCTCTGGTAGCATCATCCCACATGACTTTCGGCGGATGAAGGAGTTTG |
| ITGAX | NM_000887.3 | 700-800 | CCCCTCAGCCTGTTGGCTTCTGTTCACCAGCTGCAAGGGTTTACATACACGGCCACCGCCATCCAAAATGTCGTGCACCGATTGTTCCATGCCTCATATG |
| ITGB1 | NM_002211.3 | 355-455 | TGGGTGGTGCACAAATTCAACATTTTTACAGGAAGGAATGCCTACTTCTGCACGATGTGATGATTTAGAAGCCTTAAAAAAGAAGGGTTGCCCTCCAGAT |
| ITGB7 | NM_000889.1 | 1278-1378 | CAACGTGGTACAGCTCATCATGGATGCTTATAATAGCCTGTCTTCCACCGTGACCCTTGAACACTCTTCACTCCCTCCTGGGGTCCACATTTCTTACGAA |
| JAK1 | NM_002227.1 | 285-385 | GAGAACACCAAGCTCTGGTATGCTCCAAATCGCACCATCACCGTTGATGACAAGATGTCCCTCCGGCTCCACTACCGGATGAGGTTCTATTTCACCAATT |
| KIR3DS1 | NM_001083539.1 | 1146-1246 | GGGAACAGAAGTGAACAGCGAGGATTCTGATGAACAAGACCATCAGGAGGTGTCATACGCATAATTGGAACACTGTGTTTTCACACAGAGAAAAATCACT |
| KIR2DS1 | NM_014512.1 | 718-818 | TCCGAAACCGGTAACCCCAGACACCTACATGTTCTGATTGGGACCTCAGTGGTCAAAATCCCTTTCACCATCCTCCTCTTCTTTCTCCTTCATCGCTGGT |
| KIR2DL1 | NM_014218.2 | 872-972 | CAAGAGTCTGCAGGAAACAGAACAGCGAATAGCGAGGACTCTGATGAACAAGACCCTCAGGAGGTGACATACACACAGTTGAATCACTGCGTTTTCACAC |
| KIR2DL3 | NM_014511.3 | 592-692 | GGGAACAGAACAGTGAACAGGGAGGACTCTGATGAACAAGACCCTCAGGAGGTGACATATGCACAGTTGAATCACTGCGTTTTCACACAGAGAAAAATCA |
| KIR2DL4 | NM_002255.5 | 15-115 | GCGTCCTGGCAGCAGAAGCTGCACCATGTCCATGTCACCCACGGTCATCATCCTGGCATGTCTTGGGTTCTTCTTGGACCAGAGTGTGTGGGCACACGTG |
| KIR3DL1 | NM_013289.2 | 1691-1791 | GGCTGCAATCACACTGAGGAACTCACAATTCCAAACATACAAGAGGCTCCCTCTTGACGTGGCACTTACCCACGTGCTGTTCCACCTTCCCTCATGCTGT |

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| KIR3DL2 | NM_006737.2 | 884-984 | TGCCACCCACGGAGGGACCTACAGATGCTTCGGCTCTTTCCGTGCCCTGCCCTGCGTGTGGTCAAACTCAAGTGACCCACTGCTTGTTTCTGTCACAGGA |
| KIR3DL3 | NM_153443.3 | 539-639 | GGGTTCCCAGGTCAACTATTCCATGGGTCCCATGACACCTGCCCTTGCAGGGACCTACAGATGCTTTGGTTCTGTCACTCACTTACCCTATGAGTTGTCG |
| KLF4 | NM_004235.4 | 909-1009 | GACTTTATTCTCTCCAATTCGCTGACCCATCCTCCGGAGTCAGTGGCCGCCACCGTGTCCTCGTCAGCGTCAGCCTCCTCTTCGTCGTCGCCGTCGAGCA |
| KLRG1 | NM_005810.3 | 65-165 | CAGAATGACTATGGACCACAGCAAAAATCTTCCTCTTCCAGGCCTTCTTGTTCTTGCCTTGTGGCAATAGCTTTGGGGCTTCTGACTGCAGTTCTTCTGA |
| KRT1 | NM_006121.3 | 988-1088 | GGAAATTGATTTCCTTACAGCACTCTACCAAGCAGAGTTGTCTCAGATGCAGACTCAAATCAGTGAAACTAATGTCATCCTCTCTATGGACAACAACCGC |
| KRT10 | NM_000421.3 | 894-994 | AAATGAAAGACCTTCGAAATGTGTCCACTGGTGATGTGAATGTGGAAATGAATGCTGCCCCGGGTGTTGATCTGACTCAACTTCTGAATAACATGAGAAG |
| KRT15 | NM_002275.3 | 1243-1343 | CGAGACAGAGTGCCGCTATGCCACGCAGCTGCAGCAGATCCAGGGGCTCATTGGTGGCCTGGAGGCCCAGCTGAGTGAGCTCCGATGCGAGATGGAGGCT |
| KRT4 | NM_002272.3 | 1518-1618 | CTTTGGCTCCGGCTCTGGAAGTGGCTTTGGGTTTGGTGGCAGTGTCTCTGGCAGTTCCAGCAGCAAGATCATCTCTACCACCACCCTGAACAAGAGACGA |
| KRT5 | NM_000424.3 | 1950-2050 | TCACTGCCTTCCAAGTGCAGCAACCCAGCCCATGGAGATTGCCTCTTCTAGGCAGTTGCTCAAGCCATGTTTTATCCTTTTCTGGAGAGTAGTCTAGACC |
| LAT | NM_001014987.1 | 843-943 | CATGGAGTCCATTGATGATTACGTGAACGTTCCGGAGAGCGGGGAGAGCGCAGAAGCGTCTCTGGATGGCAGCCGGGAGTATGTGAATGTGTCCCAGGAA |
| LCK | NM_005356.3 | 510-610 | GAACCCTGGTTCTTCAAGAACCTGAGCCGCAAGGACGCGGAGCGGCAGCTCCTGGCGCCCGGGAACACTCACGGCTCCTTCCTCATCCGGGAGAGCGAGA |
| LCN2 | NM_005564.3 | 505-605 | AAAGTTTCTCAAAACAGGGAGTACTTCAAGATCACCCTCTACGGGAGAACCAAGGAGCTGACTTCGGAACTAAAGGAGAACTTCATCCGCTTCTCCAAAT |
| LTF | NM_002343.2 | 590-690 | TTCCCCAACCTGTGTCGCCTGTGTGCGGGGACAGGGGAAAACAAATGTGCCTTCTCCTCCCAGGAACCGTACTTCAGCTACTCTGGTGCCTTCAAGTGTC |
| LY9 | NM_001033667.1 | 260-360 | GTAGACACAGAGATTGAGAACGTCATCTGGATTGGTCCCAAAAATGCTCTTGCTTTCGCACGTCCCAAAGAAAATGTAACCATTATGGTCAAAAGCTACC |
| MRC1 | NM_002438.2 | 2693-2793 | TATTGATCAGCTTGGATAAAAAGTTTGCTTGGATGGATGGAAGCAAAGTGGATTACGTGTCTTGGGCCACAGGTGAACCCAATTTTGCAAATGAAGATGA |
| MAPK3 | NM_001040056.1 | 580-680 | AACGTGCTCCACCGAGATCTAAAGCCCTCCAACCTGCTCATCAACACCACCTGCGACCTTAAGATTTGTGATTTCGGCCTGGCCCGGATTGCCGATCCTG |
| MAPK8 | NM_002750.2 | 945-1045 | TCTCTGTAGATGAAGCTCTCCAACACCCGTACATCAATGTCTGGTATGATCCTTCTGAAGCAGAAGCTCCACCACCAAAGATCCCTGACAAGCAGTTAGA |
| MARCO | NM_006770.3 | 61-161 | CTCCAAGTGGTTCCTCTTGAGGGGAGCATTTCTGCTGGCTCCAGGACTTTGGCCATCTATAAAGCTTGGCAATGAGAAATAAGAAAATTCTCAAGGAGGA |
| MICA | NM_000247.1 | 550-650 | AAGATGCCATGAAGACCAAGACACACTATCACGCTATGCATGCAGACTGCCTGCAGGAACTACGGCGATATCTAAAATCCGGCGTAGTCCTGAGGAGAAC |
| MICB | NM_005931.3 | 2317-2417 | TGAACTTTATGAGTGAGTATCTTGGTGATGACTCCTCCTGACCAGCCCAGGACCAGCTTTCTTGTCACCTTGAGGTCCCCTCGCCCCGTCACACCGTTAT |
| MKI67 | NM_002417.4 | 2605-2705 | GTTTTGGAGGAAATGTGTTCTTCAGTGCACAGAATGCAGCAAAACAGCCATCTGATAAATGCTCTGCAAGCCCTCCCTTAAGACGGCAGTGTATTAGAGA |
| MPO | NM_000250.1 | 545-645 | AGACCTGCTGGAGAGGAAGCTGCGGTCCCTGTGGCGAAGGCCATTCAATGTCACTGATGTGCTGACGCCCGCCCAGCTGAATGTGTTGTCCAAGTCAAGC |
| MRC1 | NM_002438.2 | 2162-2262 | TAGCTAGCATCAATAACAAAGAGGAACAGCAAACAATATGGCGATTAATAACAGCTAGTGGAAGCTACCACAAACTGTTTTGGTTGGGATTGACATATGG |
| MSR1 | NM_002445.3 | 326-426 | AGAGAAACTGAAGTCCTTCAAAGCTGCACTGATTGCCCTTTACCTCCTCGTGTTTGCAGTTCTCATCCCTCTCATTGGAATAGTGGCAGCTCAACTCCTG |
| MUC1 | NM_001018017.1 | 725-825 | CCCTAGCAGTACCGATCGTAGCCCCTATGAGAAGGTTTCTGCAGGTAATGGTGGCAGCAGCCTCTCTTACACAAACCCAGCAGTGGCAGCCACTTCTGCC |
| MUC5B | NM_002458.1 | 16311-16411 | GGGACCCGATGGGTTTCCTAAATTTCCCGGGGAGCGGTGGGTCAGCAACTGCCAGTCCTGCGTGTGTGACGAGGGTTCAGTGTCGGTGCAGTGCAAGCCC |
| MUC6 | NM_005961.2 | 367-467 | TCACTGTGAGCGAAGCCATCATCTCAGTCAAGGACATCGGGGTCATCAGCCTGCCCTATACCAGCAATGGACTCCAGATCACACCCTTCGGCCAGAGCGT |
| MUC7 | NM_001145007.1 | 206-306 | CACCAGGAGACATCAGAAAGAATGAAAACTCTGCCGCTGTTTGTGTGCATCTGTGCACTGAGTGCTTGCTTCTCGTTCAGTGAAGGTCGAGAAAGGGATC |
| MX1 | NM_002462.3 | 2277-2377 | AGGAGGCCAGCAAGCGCATCTCCAGCCACATCCCTTTGATCATCCAGTTCTTCATGCTCCAGACGTACGGCCAGCAGCTTCAGAAGGCCATGCTGCAGCT |
| NFKB1 | NM_001165412.1 | 2305-2405 | CTTGGGTAACTCTGTTTTGCACCTAGCTGCCAAAGAAGGACATGATAAAGTTCTCAGTATCTTACTCAAGCACAAAAAGGCAGCACTACTTCTTGACCAC |
| KLRK1 | NM_007360.1 | 760-860 | GGACCAGGATTTACTTAAACTGGTGAAGTCATATCATTGGATGGGACTAGTACACATTCCAACAAATGGATCTTGGCAGTGGGAAGATGGCTCCATTCTC |
| NCR3 | NM_147130.2 | 655-755 | ATCCTCAGCTAGGGGCTGGTACAGTCCTCCTCCTTCGGGCTGGATTCTATGCTGTCAGCTTTCTCTCTGTGGCCGTGGGCAGCACCGTCTATTACCAGGG |
| NCR1 | NM_004829.5 | 602-702 | CGATGTTTTGGCTCCTATAACAACCATGCCTGGTCTTTCCCCAGTGAGCCAGTGAAGCTCCTGGTCACAGGCGACATTGAGAACACCAGCCTTGCACCTG |
| NLRP3 | NM_001079821.2 | 415-515 | AGTGGGGTTCAGATAATGCACGTGTTTCGAATCCCACTGTGATATGCCAGGAAGACAGCATTGAAGAGGAGTGGATGGGTTTACTGGAGTACCTTTCGAG |
| NOS2 | NM_000625.4 | 605-705 | TTGCCTGGGGTCCATTATGACTCCCAAAAGTTTGACCAGAGGACCCAGGGACAAGCCTACCCCTCCAGATGAGCTTCTACCTCAAGCTATCGAATTTGTC |
| OAS1 | NM_001032409.1 | 805-905 | CTCCTGACGGTCTATGCTTGGGAGCGAGGGAGCATGAAAACACATTTCAACACAGCCCAGGGATTTCGGACGGTCTTGGAATTAGTCATAAACTACCAGC |
| PAK6 | NM_020168.3 | 3480-3580 | CCCATTGTTCTCTTCAGAACCCTATCTGGGAGAGAAGGCGAACCACCTCCGGGTTTCCATCATGTCAAGGTCACAGGCATCCATGTGTGCAAACCATCTG |
| PDGFA | NM_002607.5 | 1623-1723 | GTCCGAGAACACTCGGGAGAACAAAGAGACAGTGCACATTTGTTTAATGTGACATCAAAGCAAGTATTGTAGCACTCGGTGAAGCAGTAAGAAGCTTCCT |
| PF4 | NM_002619.2 | 0-100 | CCATCGCACTGAGCACTGAGATCCTGCTGGAAGCTCTGCCGCAGCATGAGCTCCGCAGCCGGGTTCTGCGCCTCACGCCCCGGGCTGCTGTTCCTGGGGT |
| PRF1 | NM_001083116.1 | 2135-2235 | ATCAGACAGATGGAAAAGGGAGATTCAGACTGTTTTTCAGGGAGGTGGCTGGGTTTACACGCTAATCCCGATTCACCCTGTCCAAACTGCCTAAGCCCTC |
| PTGS2 | NM_000963.2 | 2026-2126 | AACTCCTTATGTTACTTAACATCTTCTGTAACAGAAGTCAGTACTCCTGTTGCGGAGAAAGGAGTCATACTTGTGAAGACTTTTATGTCACTACTCTAAA |
| RORC | NM_001001523.1 | 1350-1450 | CTCATCAATGCCCATCGGCCAGGGCTCCAAGAGAAAAGGAAAGTAGAACAGCTGCAGTACAATCTGGAGCTGGCCTTTCATCATCATCTCTGCAAGACTC |
| SAMHD1 | NM_015474.2 | 640-740 | GTGGGGTATCTAGCAGGATGTCTAGTTCACGCACTGGGTGAAAAACAACCAGAGCTGCAGATAAGTGAACGAGATGTTCTCTGTGTTCAGATTGCTGGAC |

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| SDC2 | NM_002998.3 | 815-915 | AGAGCTGACAACATCTCGACCACTTCCAAAGATACTGTTGACTAGTGCTGCTCCAAAAGTGGAAACCACGACGCTGAATATACAGAACAAGATACCTGCT |
| SIGLEC7 | NM_014385.2 | 1475-1575 | AAATGCAGAGGCTCGGGCTTGTTTGAGGGTTCACGACCCCTCCAGCAAAGGAGTCTGAGGCTGATTCCAGTAGAATTAGCAGCCCTCAATGCTGTGCAAC |
| SLC22A17 | NM_020372.2 | 685-785 | TAAGGATTGGCGATTCCTACAGCGAATGATCACCGCTCCCTGCATCCTCTTCCTGTTTTATGGCTGGCCTGGTTTGTTCCTGGAGTCCGCACGGTGGCTG |
| SLPI | NM_003064.2 | 330-430 | TTTCTGTGAGATGGATGGCCAGTGCAAGCGTGACTTGAAGTGTTGCATGGGCATGTGTGGGAAATCCTGCGTTTCCCCTGTGAAAGCTTGATTCCTGCCA |
| SOCS2 | NM_003877.3 | 720-820 | GTCAGACAGGATGGTACTGGGGAAGTATGACTGTTAATGAAGCCAAAGAGAAATTAAAAGAGGCACCAGAAGGAACTTTCTTGATTAGAGATAGCTCGCA |
| SOCS3 | NM_003955.3 | 1028-1128 | GAGAAAGTCACCCAGCTGCCGGGGCCCATTCGGGAGTTCCTGGACCAGTACGATGCCCCGCTTTAAGGGGTAAAGGGCGCAAAGGGCATGGGTCGGGAGA |
| SPRED1 | NM_152594.2 | 945-1045 | TTCAGAGCAGAAGTATGGAATACGTACAGCGGCAAATATCCAAGGAATGTGGAAGCCTAAAGTCCCAAAATAGGGTCCCTTTGAAATCAATCAGACATGT |
| STAT1 | NM_139266.1 | 455-555 | ACAGTGGTTAGAAAAGCAAGACTGGGAGCACGCTGCCAATGATGTTTCATTTGCCACCATCCGTTTTCATGACCTCCTGTCACAGCTGGATGATCAATAT |
| STAT3 | NM_003150.3 | 2915-3015 | TTCTGCCTGTTTCTGTAAGCAAATGCCACAGGCCACCTATAGCTACATACTCCTGGCATTGCACTTTTTAACCTTGCTGACATCCAAATAGAAGATAGGA |
| STAT5A | NM_003152.3 | 2523-2623 | ACCATCGCCTGGAAGTTTGACTCCCCGGAACGCAACCTGTGGAACCTGAAACCATTCACCACGCGGGATTTCTCCATCAGGTCCCTGGCTGACCGGCTGG |
| STAT5B | NM_012448.3 | 200-300 | AAGGAGAAGCCCTTCATCAGATGCAAGCGTTATATGGCCAGCATTTTCCCATTGAGGTGCGGCATTATTTATCCCAGTGGATTGAAAGCCAAGCATGGGA |
| TBX21 | NM_013351.1 | 890-990 | ACACAGGAGCGCACTGGATGCGCCAGGAAGTTTCATTTGGGAAACTAAAGCTCACAAACAACAAGGGGGCGTCCAACAATGTGACCCAGATGATTGTGCT |
| TGFB1 | NM_000660.3 | 1260-1360 | TATATGTTCTTCAACACATCAGAGCTCCGAGAAGCGGTACCTGAACCCGTGTTGCTCTCCCGGGCAGAGCTGCGTCTGCTGAGGCTCAAGTTAAAAGTGG |
| TLR1 | NM_003263.3 | 645-745 | TGCATTTGATGCCCTGCCTATATGCAAAGAGTTTGGCAATATGTCTCAACTAAAATTTCTGGGGTTGAGCACCACACACTTAGAAAAATCTAGTGTGCTG |
| TLR2 | NM_003264.3 | 668-768 | ATTGCAAATCCTGAGAGTGGGAAATATGGACACCTTCACTAAGATTCAAAGAAAAGATTTTGCTGGACTTACCTTCCTTGAGGAACTTGAGATTGATGCT |
| TLR3 | NM_003265.2 | 1688-1788 | GAGAAACTAGAAATTCTCGATTTGCAGCATAACAACTTAGCACGGCTCTGGAAACACGCAAACCCTGGTGGTCCCATTTATTTCCTAAAGGGTCTGTCTC |
| TLR4 | NR_024168.1 | 2575-2675 | TCATCCATGAAGGTTTCCATAAAAGCCGAAAGGTGATTGTTGTGGTGTCCCAGCACTTCATCCAGAGCCGCTGGTGTATCTTTGAATATGAGATTGCTCA |
| TLR6 | NM_006068.4 | 1280-1380 | ATGGATTAAAAGACCTTTTCAAAGTAGGTCTCATGACGAAGGATATGCCTTCTTTGGAAATACTGGATGTTAGCTGGAATTCTTTGGAATCTGGTAGACA |
| TLR7 | NM_016562.3 | 187-287 | AACATAATCCTAATTTCCAAACTCCTTGGGGCTAGATGGTTTCCTAAAACTCTGCCCTGTGATGTCACTCTGGATGTTCCAAAGAACCATGTGATCGTGG |
| TLR8 | NM_138636.3 | 2795-2895 | GACAAAAACGTTCTCCTTTGTCTAGAGGAGAGGGATTGGGATCCGGGATTGGCCATCATCGACAACCTCATGCAGAGCATCAACCAAAGCAAGAAAACAG |
| TLR9 | NM_017442.2 | 985-1085 | ACCTTCTTGGCTGTGCCCACCCTGGAAGAGCTAAACCTGAGCTACAACAACATCATGACTGTGCCTGCGCTGCCCAAATCCCTCATATCCCTGTCCCTCA |
| TNF | NM_000594.2 | 617-717 | TGCTCCTCACCCACACCATCAGCCGCATCGCCGTCTCCTACCAGACCAAGGTCAACCTCCTCTCTGCCATCAAGAGCCCCTGCCAGAGGGAGACCCCAGA |
| TNFAIP3 | NM_006290.2 | 45-145 | GGAGAGGTGTTGGAGAGCACAATGGCTGAACAAGTCCTTCCTCAGGCTTTGTATTTGAGCAATATGCGGAAAGCTGTGAAGATACGGGAGAGAACTCCAG |
| FAS | NM_000043.4 | 1089-1189 | TGTTCGAAAGAATGGTGTCAATGAAGCCAAAATAGATGAGATCAAGAATGACAATGTCCAAGACACAGCAGAACAGAAAGTTCAACTGCTTCGTAATTGG |
| TNFRSF8 | NM_152942.2 | 122-222 | GGAGGGCCTGCAGGAAGCGAATTCGGCAGAAGCTCCACCTGTGCTACCCGGTCCAGACCTCCCAGCCCAAGCTAGAGCTTGTGGATTCCAGACCCAGGAG |
| TNFRSF9 | NM_001561.4 | 255-355 | AGATTTGCAGTCCCTGTCCTCCAAATAGTTTCTCCAGCGCAGGTGGACAAAGGACCTGTGACATATGCAGGCAGTGTAAAGGTGTTTTCAGGACCAGGAA |
| TNFSF10 | NM_003810.3 | 966-1066 | TAACTGACCTGGAAAGAAAAAGCAATAACCTCAAAGTGACTATTCAGTTTTCAGGATGATACACTATGAAGATGTTTCAAAAAATCTGACCAAAACAAAC |
| FASLG | NM_000639.1 | 636-736 | GGAATGGGAAGACACCTATGGAATTGTCCTGCTTTCTGGAGTGAAGTATAAGAAGGGTGGCCTTGTGATCAATGAAACTGGGCTGTACTTTGTATATTCC |
| TNFSF9 | NM_003811.3 | 239-339 | CCGAGACTCCGCGAGGGTCCCGAGCTTTCGCCCGACGATCCCGCCGGCCTCTTGGACCTGCGGCAGGGCATGTTTGCGCAGCTGGTGGCCCAAAATGTTC |
| TRIM5 | NM_033093.1 | 117-217 | CTGGTGTCTGAAGGTGTATTGAGTGATTTTGTGGAGGGCAGAAGTAGGAAGTCTTTGGGACAAAACTGTATTTACCTTGGGATCTGTGAACAAGAGGAAC |
| TYK2 | NM_003331.3 | 485-585 | TCATCGCTGACAGCTGAGGAAGTCTGCATCCACATTGCACATAAAGTTGGTATCACTCCTCCTTGCTTCAATCTCTTTGCCCTCTTCGATGCTCAGGCCC |
| UGT8 | NM_003360.3 | 670-770 | GTGTTCCTCCTCTCTGAAGGCAGAGACATCGCCCCATCTAATCATTACAGCCTCCAGCGCTACCCAGGGATCTTTAACAGTACCACCTCAGATGCTTTCC |
| ULBP1 | NM_025218.2 | 1635-1735 | TCAACCTGCACACCTACCGCCATCTAATCTTCAATAGAAATGGGCAATGTGGGAAAGACTCCCTATTCGAAAATTAGTGCTGGAATATCTGGCCAACCAT |
| ULBP2 | NM_025217.2 | 905-1005 | TGCCCACGACCTACGGTGTATGTCCAGTGGCCTCCAGCAGATCATGATGACATCATGGACCCAATAGCTCATTCACTGCCTTGATTCCTTTTGCCAACAA |
| ULBP3 | NM_024518.1 | 30-130 | CCGCGCCTCGCGATTCTTCCGTACCTGCTATTCGACTGGTCCGGGACGGGGCGGGCCGACGCTCACTCTCTCTGGTATAACTTCACCATCATTCATTTGC |