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Characterizing the Phenotype of Memory T cells in Cord Blood in Response to HIV-1

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B.Sc, Emory University 2013

Advisor: Rana Chakraborty, M.D. PhD

An abstract of

A thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Master of Science in Biology in 2014

Abstract

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Even in the absence of combination antiretroviral therapy (cART), a relatively low risk of mother-to-child transmission (MTCT) of HIV-1 *in utero* is documented (7%), suggesting natural correlates of protection exist at the feto-maternal interface, either in the placenta or in the cord blood. Little has been done to characterize memory T cell populations in cord blood, in the absence and presence of activation, as a correlate of protection or surrogate of transmission, respectively, during on-going HIV-1 exposure. In primate studies, natural hosts have been shown to down regulate HIV-1 co-receptor (CCR5) expression on central memory T cells (T_{cm}) and maintain low levels of T cell activation during the chronic phase of SIV infection. Studies in humans have shown that CCR5 expression is largely restricted to memory cells. Interestingly, low levels of memory cells are found in infants compared to adults. In order to investigate possible mechanisms of natural protection against HIV-1 transmission within fetal cord blood, we examined HIV-1 co-receptor expression, immune activation and apoptosis on stimulated and unstimulated cord blood mononuclear cells (CBMCs). We hypothesize that the unstimulated (immune quiescent) environment in cord blood may protect T_{cm} cells from HIV-1 infection by regulating CCR5 expression.

Here we show lower fractions of memory T cell populations and CCR5 expression in CBMCs in contrast to adult peripheral blood mononuclear cells (PBMCs). This is despite comparable proliferative capacities in response to stimulation in both cell types. Post-stimulation, the fraction of T_{cm} cells in both CD4+ and in CD8+ T cells increases five-fold, while the unstimulated T_{cm} subsets remain unchanged. In addition,

CCR5 expression is significantly upregulated under similar conditions. Intriguingly, CXCR4 (HIV-1 co-receptor) expression remained elevated in stimulated and unstimulated CD4+ T_{cm} cells post-infection. Lower levels of CCR5 expression in unstimulated CBMCs, independent of CXCR4 expression, may influence HIV entry and infection. In this regard, we show that HIV-1 replication is lower in unstimulated CBMCs compared to stimulated cells. High apoptotic levels are observed in stimulated T_{cm} cells post-infection. Taken together, these results suggest that the unstimulated (immune quiescent) environment in cord blood may protect central memory T cells from HIV-1 infection by regulating CCR5 expression.

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Introduction

Epidemiology

The human immunodeficiency virus, HIV, is a viral pathogen that affects millions of people worldwide and leads to acquired immunodeficiency syndrome (AIDS). Of the two types of HIV, HIV-1 is seen as a worldwide, global pandemic. An assessment of the HIV/AIDS epidemic at the end of 2010 saw more than 34 million people living with HIV-1, with 2.7 million new infections occurring globally [1]. Since the beginning of the epidemic, the virus has killed more than 20 million people, including 4.3 million children under 15 years of age [1]. In 2000, there were over 36 million people living with HIV/AIDS [1] (Figure 1). Women are disproportionately affected by the HIV/AIDS epidemic. Estimates suggest that more than half of the people living with HIV are women and adolescent girls. In sub-Saharan Africa, women ages 15-24 are eight times more likely than men to be HIV positive [2].

Vertical transmission of HIV-1 from infected mother to child can occur during pregnancy, at the time of delivery, or through breast-feeding. Impaired immunological conditions of pregnant women and fetus, increased viral shedding in breastmilk, and impaired epithelial barriers of protection in the placenta increase the likelihood of vertical transmission [72]. The fetal-maternal interface is particularly interesting, as the embryo inside the mother's womb creates an immunological dilemma. Peter Medawar initially proposed the idea that mother should reject their fetuses, because half of the fetal genes are derived from the father and thus the mother's immune system should in theory mount an immune response to this foreign, paternal allograft. However, most healthy mothers are able to carry their child to full term and ensure optimal nourishment and development

of the fetus. The placenta was traditionally thought to keep mother and child separate, however studies have shown that maternal cells have been found in fetal circulation. Using PCR genotyping, maternal cells have been detected in the umbilical cord blood of 16 out of 38 cases between mother and baby [74]. Furthermore, a substantial number of maternal cells cross the placenta to resideinfetal lymph nodes that induce the development of T regulatory cells that suppress antimaternal immunity [18]. In mouse models, fetal cell trafficking in mouse maternal circulation of immunocompetent mothers are eliminated and cleared from maternal circulation before maternal antigen-presenting cells are activated [75]. This bidirectional trafficking and microchimerism in mother and child highlights the close interaction between mother and child to ensure that maternal T cells specific for fetal antigens are not stimulated to attack the fetus during pregnancy. Fetomaternal microchimerism whereby fetal cells traffic to the placenta and maternal cells are found in the cord blood of the fetus suggests high vertical transmission of HIV-1 between mother and child if maternal cells were infected. However, despite intimate contact between mother and child, the risk of HIV-1 transmission *in utero* is only 7%, suggesting mechanisms of protection at the fetomaternal interface [19].

Most documented cases of HIV-1 from mother-to-child transmission (MTCT) occur intra-partum and post-partum [3]. Transplacental HIV transmission is the least efficient form of mother-to-child transmission [4]. These statistics underline the importance of understanding the pathogen and investigating natural mechanisms of protection against viral transmission. Understanding reservoirs of HIV-1 replication is particularly important in elucidating the potential mechanisms of protection at the fetomaternal interface.

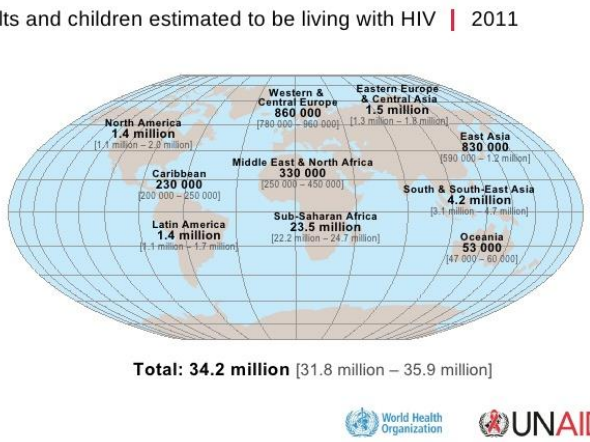


Figure 1. Estimated number of adults and children worldwide living with HIV in 2011.

Viral Structure

The virus comes from the family of *Retroviridae* known as lentiviruses and has shown a high potential for genetic diversity. The virus contains a single stranded RNA which can be transcribed to double stranded DNA before it is integrated into the host genome. The HIV-1 virion is comprised of an outer spiked envelope membrane with proteins gp120 and gp41. The virus enters cells by binding its gp120 envelope protein to a CD4 glycoprotein found on the surfaces of T-cells macrophages and microglial cells (Figure 2). However, CD4 attachment is not sufficient for viral entry. The V3 loop of gp120 must bind to a co-receptor to facilitate fusion of the viral envelope and the plasma membrane of the cell for eventual viral entry (Figure 2).

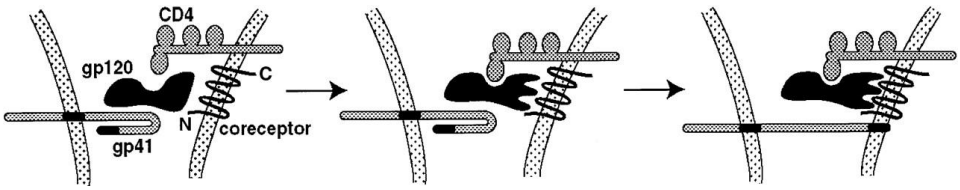


Figure 2. Upon binding to CD4, gp120 undergoes a conformational change that allows the gp41fusion peptide to insert into the target cell.

Co-receptors for HIV-1 Entry

The ability of HIV to enter specific cell types, known as cellular tropism, is determined by co-receptor expression. Before fusion and entry, gp120 on the virus must also bind to a co-receptor on the host cell membrane. While several co-receptors can serve to facilitate viral entry, chemokine receptors CCR5 and CXCR4 have been shown to be the main co-receptors involved in viral entry. CCR5 is a co-receptor used by macrophage-tropic HIV variants and is expressed mainly in dendritic cells, macrophages, and CD4 T cells. In contrast, CXCR4 is used by lymphocyte-tropic HIV variants and is preferentially expressed in activated T cells, T cells that have encountered the peptide-MHC complex and proliferate in response [5]. Binding of gp120 to the receptor and the co-receptor triggers fusion of the viral envelope and the target cell's membrane (Figure 3). It appears that that the macrophage-tropic strains of HIV are preferentially transmitted through sexual contact and account for the major production of HIV by CD4 T cells throughout life [5]. Infection of CD4 T cells occurs early in infection, and the virus continues to replicate for many years after initial infection before causing overt signs of disease. CD4+ T cell depletion is the hallmark of HIV-1 infection. HIV-1 infected CD4+ T cells die from direct virus-associated apoptosis, by acculmation of incomplete reverse transcripts, and indirect CD8+ cytotoxic T cell elimination through the release of perforin and granzymes [14]. CD4+ lymphocytes depletion in turn leads to increased immune activation and disruption of overall immune homeostasis.

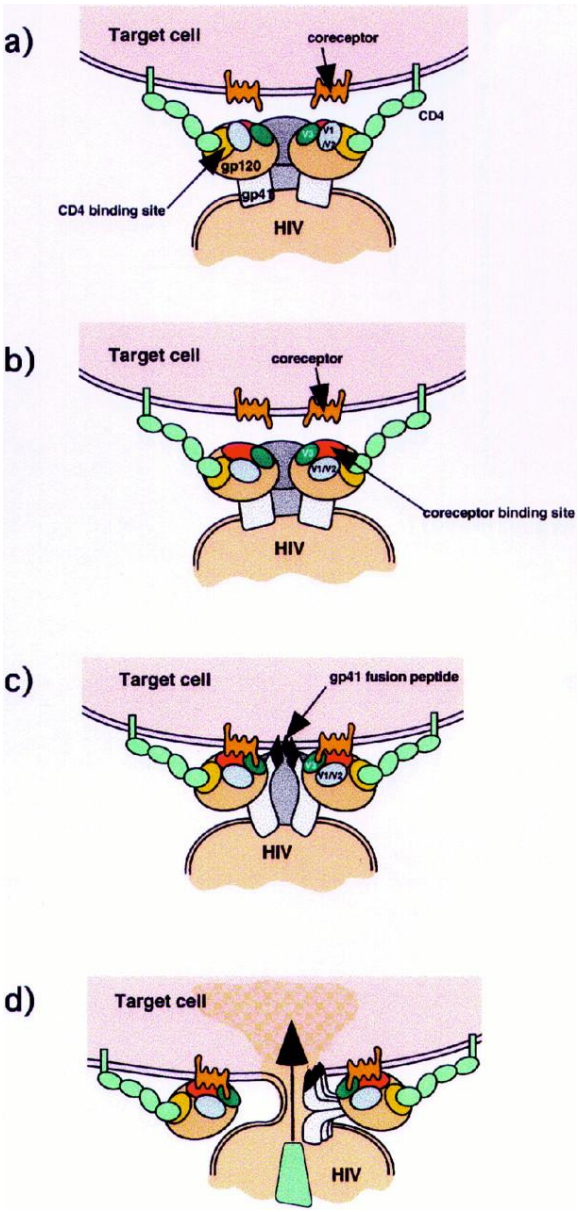


Figure 3. gp120 binding to CD4+ T cells exposes co-receptor binding sites CCR5 and CXCR4 necessary for HIV-1 entry. ©2001 Poignard et al

HIV-1 Genetic Diversity

HIV-1 rapidly evolves via changes in single nucleosides of the RNA (mutation) and via combinations of RNA sequences from two distinct HIV strains (recombination). Not only does the virus proliferate extremely rapidly during the acute clinical phase of infection, but its evolutionarily active state poses significant challenges to protection by vaccines and control by the immune system. The error-prone reverse transcriptase makes approximately five to ten errors per HIV-1 genome per round of replication in vivo [6]. Mutant virions encode peptide sequences that are able to escape recognition by antibodies that normally recognize the virions and thus have a selective proliferative advantage [5]. These mutant strains avoid recognition by antibodies and cytotoxic T cells, replicate rapidly, and contribute to the overall failure of the immune system to control or eradicate virus.

Vaccines for HIV-1

More than two dozen antiretroviral therapies have been designed to combat HIV-1 infection [7]. Despite ongoing developments in HIV prevention, however, HIV-1 continues to spread in many parts of the world especially in Sub-Saharan Africa. The genetic variability of HIV-1 has made developing an efficacious vaccine for the virus difficult. Initial immune control of the virus by CD8+ T cells is offset by rapid changes to the genetic material of the viral envelope protein (viral escape mutations) following a brief period of viremic control [7]. Changes to the envelope allow the virus to escape neutralizing antibodies as well further contributing to immune evasion.

A successful vaccine against HIV-1 should be safe, affordable and offer long-lasting protection against infection in the majority of recipients. The external

glycoprotein (gp120) and its uncleaved precursor protein (gp160) have been manipulated in dozens of clinical trials. gp120 that had been produced in mammalian cell lines induced the highest level of neutralizing antibodies against HIV-1 *in vitro* [7]. However, later experiments demonstrated that antiserum failed to neutralize isolates of HIV-1 in peripheral blood cells. Both the AIDSVAX B/B HIV vaccine and the MRKAd5 vaccine failed to prevent infection. In fact, the MRKAD5 vaccine, a trivalent vaccine that was a 1:1:1 mixture of a replication-defective adenovirus expressing HIV gag, pol, and nef genes, increased susceptibility to HIV-1 [8-9]. Of all vaccines, the Thai Trial vaccine, a CD8+ T cell based vaccine against the virus,, has been the only one to show modest success. We have learned from these trials that a good preventive candidate vaccine against HIV-1 would, in theory, prevent the establishment of the virus by clearing the virus from the immune system before latent viral reservoirs are established.

CD8+ T cells in response to HIV-1 Infection

Once the viral core enters the cell, the host cell mounts HIV-specific immune responses in the forms of cellular (cell-mediated) response and humoral (antibody-mediated response). In the cell-mediated response, viral peptides, termed antigens, are loaded onto MHC class I proteins after phagocytosis by dendritic cells, which present the antigens on the surface of infected cells. At the site of infection, primed CD8+ cytotoxic T cells can recognize infected cells via the expression of pMHC-I presenting peptides derived from the intracellular pathogen [10]. Naïve CD8+ T cells must be primed by dendritic cells in the secondary lymphoid organs (spleen, lymph nodes) before they can function efficiently [10]. Once binding occurs, CD8+ cytotoxic T cells destroy the infected cell (Figures 4 and 5) by the release of granzymes and perforin.

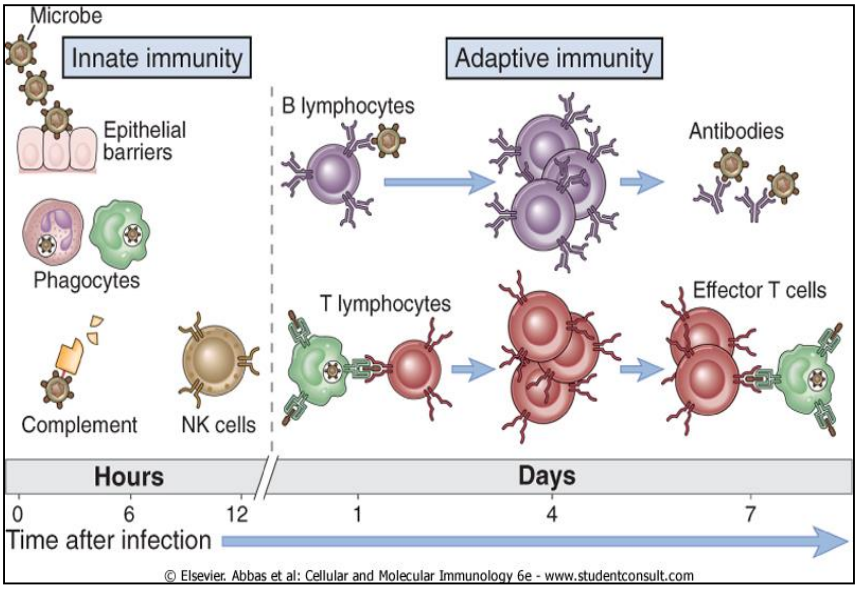
CD4+ T cell Response to HIV-1 Infection

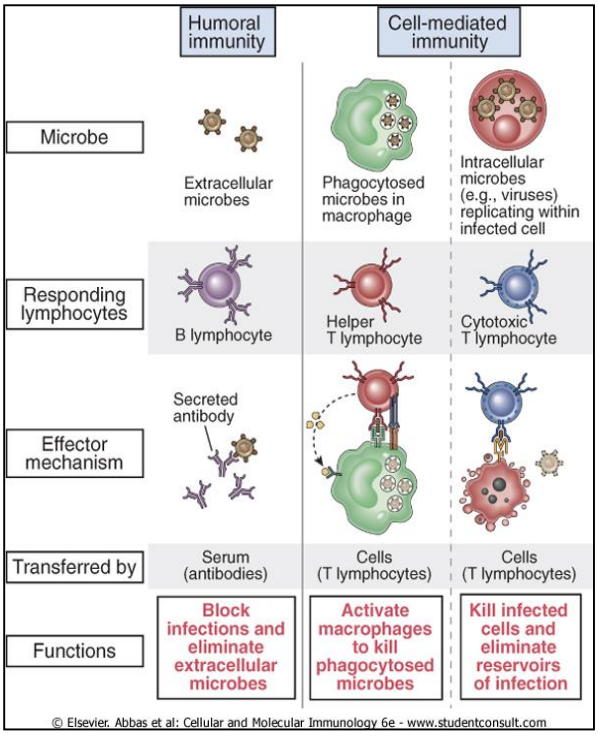
CD4+ helper T cells also play an important role in the cell-mediated response by assisting the activation of other lymphocytes.CD4+ T cells mount an immunogenic reponse to HIV-1, but they are also targets of the virus. CD4+ T cells respond to and are infected by HIV-1. To mount a response against HIV-1, binding between T cells and antigen-presenting cells such as dendritic cells activates CD4+ T cells. Three main signals from the antigen-presenting cell are necessary for full activation of a CD4+ helper T cell: ligation of the T cell receptor on the surface of the T cell with pMHC-II (MHC Class II), ligation of CD28 with CD80 or CD86 expressed on antigen-presenting cells, and signals received by cytokine receptors to allow for further expansion and differentiation of CD4+ T cells [10].

Once CD4+ T cells have been activated, they proliferate into antigen-specific T cells to effectuate an immune response. During priming of CD4+ T cells, secreted chemical molecules that act on nearby cells called cytokines, polarize the CD4+ T cells into several different phenotypes. CD4+ T cells that differentiate into Th_1 can activate CD8+ lymphocytes while CD4+ T cells that differentiate into Th_2 activate B lymphocytes to promote an antibody-mediated immunity found in the humoral response. If a cell differentiates into a Th_1 cell, it can activate macrophages, a phagocytic cell that engulfs pathogens, and cytotoxic cells to kill infected cells. Th_1 cells activate cytotoxic T cells in the peripheral lymphoid organs by stimulating dendritic cells to produce more costimulatory cells on the dendritic cell [11]. Costimulatory molecules along with the antigen provide signals to stimulate the T lymphocyte to proliferate. In the humoral

response, the recruitment of CD4+ T cells is essential for B cell proliferation and increased affinity of antibodies of the B cell for the antigen [11]. The ligation of CD40 on the B cell surface with the CD40 ligand on the T cell is essential to induce B cell proliferation. Antibodies bind to the surface of the virus and the Fc portion of the antibody binds to a natural killer cell to destroy the infected cell.

CD4+ T cells are also readily infected by the HIV-1 virus, as the viral envelope glycoprotein (Env) has a high affinity to CD4, the primary receptor expressed on target cells. During infection, HIV-1 enters target CD4+ T cells by using the CD4 receptor and an additional co-receptor as main routes of entry. HIV-1 binding to CD4 receptors via binding sites on gp120 leads to conformational changes that expose binding sites that allow binding to the co-receptors CCR5 and CXCR4. Once bound to CD4 and the co-receptor, the HIV envelope glycoprotein undergoes additional conformational changes that facilitate fusion of the viral membrane with target host cell membrane. CD4 receptors are thus integral in facilitating the initial stages of HIV-1 entry into target cells.





Figures 4 and 5. Both the innate and adaptive immune system play crucial roles in response to a pathogen. The innate immune system acts quickly and recruits phagocytes (macrophages, neutrophils and other granulocytes) to the site of infection (Figure 3). The adaptive immune plays a key role in immune response against pathogens. Within the adaptive immune response are cell-mediated (T lymphocyte) and humoral (antibody-mediated) response types (Figure 4). In general, the location of the infection determines the type of adaptive immune response.

Naïve T Cells as Precursors of Memory Cells

Naïve cells, or differentiated B and T cells that have not encountered antigens in the periphery, are precursors to activated and memory cells. During the course of an infection, naïve cells driven by antigen and costimulation become activated and proliferating B and T cells. B cells can become short-lived plasmablasts that produce antibodies to the pathogen or can clonally expandand differentiate in the germinal center to become memory cells. Similarly naïve T cells are the precursors to memory T cells and activated T cells. Once a naïve T cell encounters an antigen by an antigen-presenting cell, or an APC such as a dendritic cell, the T cell is activated and become effector cells. Some of these activated/effector cells become long-lived memory cells that can mediate better protection than naïve cells. Memory cells can mediate better protection because they are relatively less dependent upon their requirement for co-stimulatory molecules and antigen presenting cells for acquiring effector functions to response to the antigen if encountered again.

Development of Memory Cells from Naïve Cells

During the course of the immune response, some of the B and T cells activated by antigen differentiate into memory cells, lymphocytes that are responsible for long-lasting immunity that can follow re-exposure to disease or vaccination [12] (Figure 6). These memory cells provide a rapid response should an infection of the same antigen occur again. Naïve T lymphocytes can differentiate into effector or memory cells. Memory T cells are long-lived cells with a particular set of cell-surface proteins, responses to stimuli, and expression of genes that control cell survival [12]. Memory lymphocytes confer immediate protection in peripheral tissues and mount recall responses in

secondary lymphoid organs should the pathogen be encountered again [13]. Within the memory T cells is a subset of central memory, T_{CM}, and effector memory, T_{EM}, T cells. Effector memory T cells rapidly mature into an effector T cell and secrete large amounts of IFNγ, IL-4, and IL-5 early after restimulation [13]. These cells have lost the constitutive expression of CCR7, are heterogeneous for CD62L expression, and display chemokine receptors and adhesion molecules necessary for homing to inflamed tissue [13] (Figure 7). These cells offer protective memory and migrate to inflamed peripheral tissues to display immediate effector function [13]. Central memory T cells are very sensitive to cross-linking of their T cell receptors and rapidly express CD40 ligand in response. Human T_{CM} are CD45R0+ memory cells that constitutively express CCR7 and CD62L [13]. They take longer than effector memory cells to differentiate into effector cells and thus do not secrete large amounts of cytokines after restimulation [12]. Central memory T cells home to T cell areas of secondary lymphoid organs, but have little or no effector function. They proliferate and differentiate into effector cells in response to antigenic stimulation [13]. Ultimately, memory cells can be maintained for long periods of time in a resting state. Immunological memory produces strong responses with a shorter lag time and is essential to immediate defense against foreign antigens.

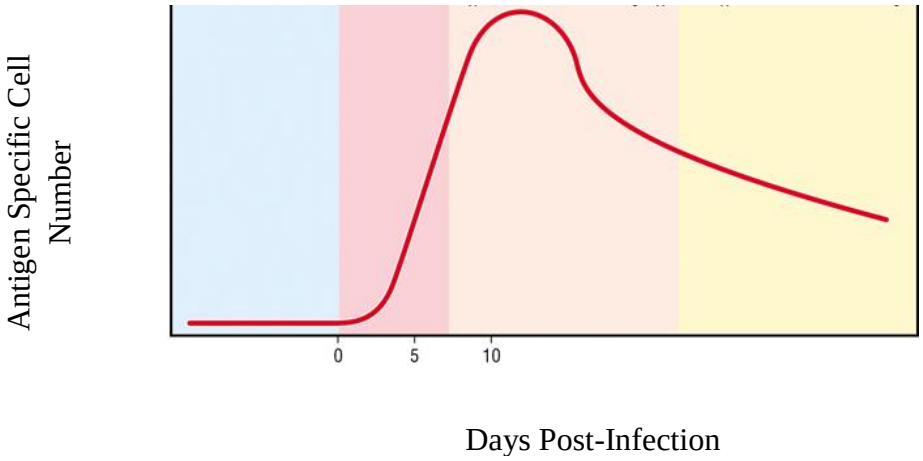


Figure 6. At later stages of infection following initial T cell response, many of the effector cells die whereas some live to differentiate into long lasting protective immune memory cells. ©Murali Kaja

CD3+ T cell subsets	Properties and Receptors	Function	Homing
CD4+ T naïve	non-antigen experienced CD62L+ CD127+ CD40L+	1. response to novel pathogens the immune system has not encountered 2. quiescent and non-dividing	secondary lymphoid organs
CD4+ T central memory	antigen-experienced CCR5+ CXCR3+ CD62L+ CD40L+	1. absence of immediate effector function 2. secrete IL2 3. capacity for self-renewal higher antigenic stimulation compared to naïve T	secondary lymphoid organs TCM predominant in blood, lymph nodes, tonsils
CD4+ T effector memory	antigen-experienced CCR5+ CXCR3+ HLADR+	1. immediate effector function 2. support cytotoxic function of CD8+ T cells 3. production of effector cytokine IFNy and IL4	peripheral site of inflammation TEM proportion predominant in lung, liver, gut

Figure 7. Immunological memory is necessary to mount a strong response against recurring pathogens. Memory lymphocytes can be subdivided into central memory (T_{CM}) and effector memory (T_{EM}) T cells. Central memory T cells express the receptors CD45RO+

CCR7+ and reside in secondary lymphoid organs. Effector memory Tcells express CD45RO+ CCR7- and is predominant in the lung, liver, and gut.

CD4+ T cell depletion is the hallmark of HIV-1 Infection

The direct effects on CD4 T cell count include lysis of infected cells and toxic effects of viral gene products. During the clinical latency period, the period following an initial burst of viraemia, CD4+ T cells start to decline slowly. During this period, HIV-1 replicates at low levels, and the infected indvidual does not appear to exhibit symptoms of HIV-1 infection [73]. However, the latency period is a highly dynamic state with high levels of virus turnover. This high rate of turnover allows for generation of viral diversity and subsequent opportunity for viral escape [14]. It has been previously proposed that high lymphocyte turnover leads to a new balance between production and death of lymphocytes, with increasing CD4+ T-cell renewal capacity and eventual clonal exhaustion of CD4+ T cells [15]. In addition to high rates of viral turnover, Finkel et al showed the number of apoptotic cells is greater than the number of infected cells. Most apoptosis occurs in the CD8+ T cell populations which are not thought to become HIV-infected, and only few of the infected cells actually undergo apoptosis. Apoptosis of bystander cell CD4+ and CD8+ T cells, as a result of the host response to HIV, affects T cell homeostatis and impaired T cell regeneration. Though many potential mechanisms of CD4+ T cell depletion have been investigated, chronic immune activation has been the most important correlate of HIV progression. Increased division of T cells in HIV-1 infection reflects persistent activation of the immune system [15].

Natural hosts for SIVs (Simian Immunodeficiency Virus) remian healthy despite high viremia. Silvestri et al proposed that mild and transient immune activation, coupled with minimal apoptosis and central memory T cell preservation are key to maintaining stable levels of CD4+ T cells over time. Natural hosts have low levels of CCR5

expression on their CD4+ central memory T cells. Low levels of CCR5 expression reduces infection by SIV and favors preservation of CD4+ T cell homeostasis and promotes an AIDS-free status.

The Importance of the Placenta in HIV-1 Prevention

The placenta is a vital organ for the developing fetus *in utero*. It supplies oxygen and nutrients to the baby and removes waste products from the baby via the umbilical cord. The mother's blood and the baby's blood come very close in contact to each other in the placenta, but the trophoblast cells of the placenta provides a barrier to prevent the two from mixing. Gases get across the placenta by differences in partial pressure between fetal blood and maternal blood. Oxygen in the maternal blood is higher than in fetal blood and thus oxygen diffuses to fetal blood. Along those same lines, the partial pressure of carbon dioxide is fetal blood is higher than maternal blood and thus carbon dioxide diffuses from fetal blood to maternal blood. Glucose cannot passively diffuse and is transported across the membrane by hexose transporters GLUT1 and GLUT3. In addition to offering protection, a mechanism of gas exchange and waste deposition, the placenta also allows maternal antibodies to enter the developing baby via immunoglobulin-binding proteins.

There are six layers of tissue separating maternal blood from fetal blood. On the maternal side, there are endometrial epithelial cells, maternal connective tissue and maternal endothelial cells. On the fetal side, there are chorionic epithelial cells (closest to the endometrial epithelial cells), fetal connective tissue, and fetal endothelial cells. Placental villi, grapelike structures in the placenta near the fetal side that are bathed in

maternal blood supplied by the maternal decidual branches, are chorionic epithelium that cuts into the maternal endothelial layer.

Retroviruses and the Placenta

Endogenous retroviruses (ERV) are present within the placenta of humans and other mammals [16]. It appears that these retroviruses have a particular affinity for placentas. Human endogenous retroviruses have been proposed to be footprints of ancient germ-cell infections, exogenous progenitors of ERV that have inserted into the germ line and replicated with the hosts' cellular genes [17]. BaEV, the baboon endogenous retrovirus, is expressed in embryonic tissue such as the placenta. While endogenous retroviruses exist, no related exogenous strains have been identified and thus it is believed that ERV confer protection to the host to infection with a closely related exogenous retrovirus [17]. From an evolutionary point of view, resistance to superinfection by exogenous retroviruses may have imparted a survival advantage to the offspring of individuals in which integration of the virus to the cell line occurred. The endogenous form of the virus is no longer subject to the selective pressure exerted on the exogenous form. ERV have been detected within the fetal interstitial compartment of the human placenta [16]. Previous research has shown that these human endogenous retroviruses can elicit an immunosuppressive response and control immune activation [16]. In addition, the 17-amino acid peptide of HIV-1 gp41 shows immunosuppression and sequence homology with the evolutionarily conserved, human ERV-R [16]. These findings suggest that retroviruses have evolutionarily evolved within the human placenta to confer a survival advantage to the host by eliciting immune suppression and perhaps providing a barrier against exogenous retroviruses such as HIV-1.

Mother to Child Transmission of HIV-1

It has been previously estimated that 430,000 new HIV-1 infections occurred in children under 15 years of age in 2008 alone, with most occurring via mother-to-child transmission (MTCT) intra-partum and post-partum [3]. The maternal-fetal interface is characterized by intimate contact between the maternal tissue and extravillious trophoblastic cells that invade the decidua (Figure 8). The barrier between maternal and fetal circulations is only several layers thick and consists of trophoblast, Hofbauer cells and intervening stroma. During pregnancy, a large number of maternal cells cross the placenta to reside in fetal lymph nodes [18]. These maternal cells induce the development of specific T regulatory cells that suppress fetal anti-maternal immunity and exhibit maternal tolerance [18]. Despite close contact between infected maternal cells and the placenta during pregnancy, however, the risk of *in utero* transmission is less than 7%, making transplacental HIV transmission the least efficient form of MTCT [3]. In the absence of antiretroviral therapy, over 90% of newborns of infected mothers are naturally protected [19]. These findings suggest that there exists a natural mechanism restricting the vertical transmission of HIV-1 *in utero* (Figure 9).

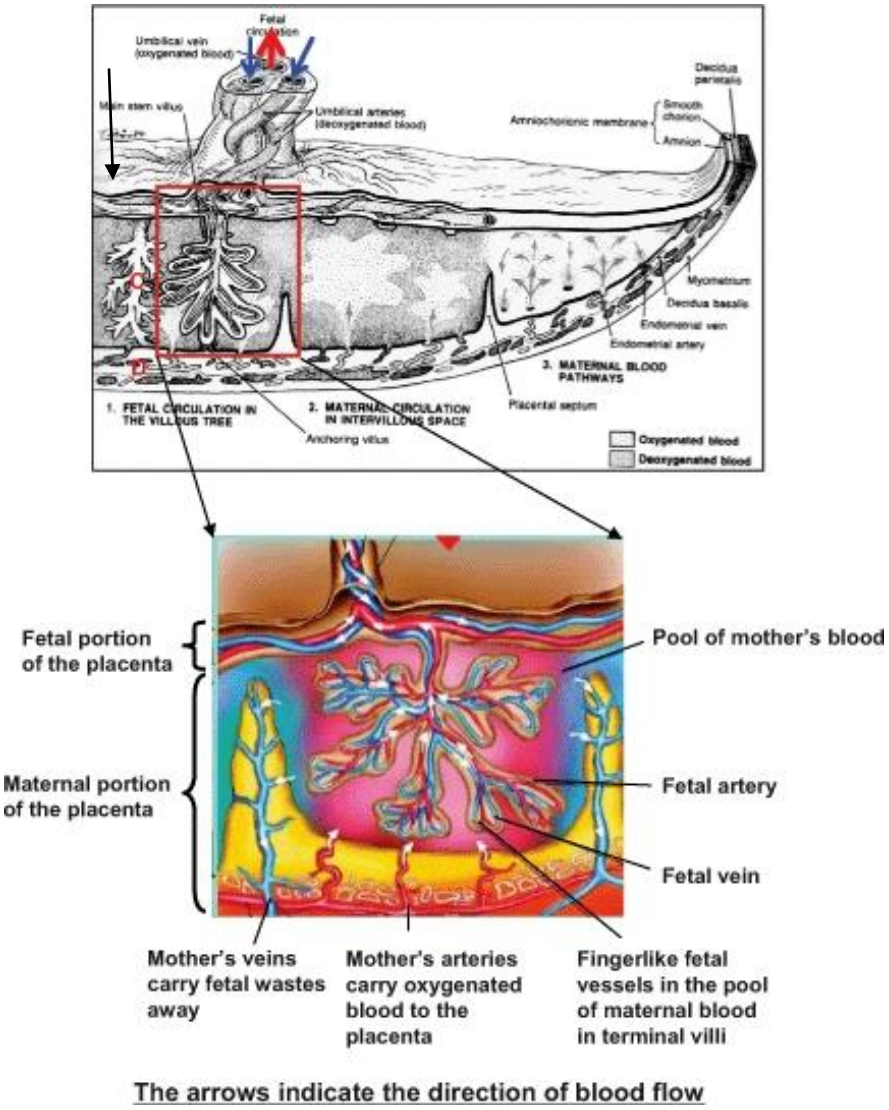


Figure 8. The barrier between maternal and fetal circulation is only a few layers thick. Many studies have demonstrated bidirectional trafficking of immune cells between the mother and fetus. A pool of maternal blood surrounds trophoblasts of the villi which contain both fetal vein and artery. It is plausible that the fetus is exposed to millions of virions and other pathogens during gestation. ©Wang Y, Zhao S. Vascular Biology of the Placenta

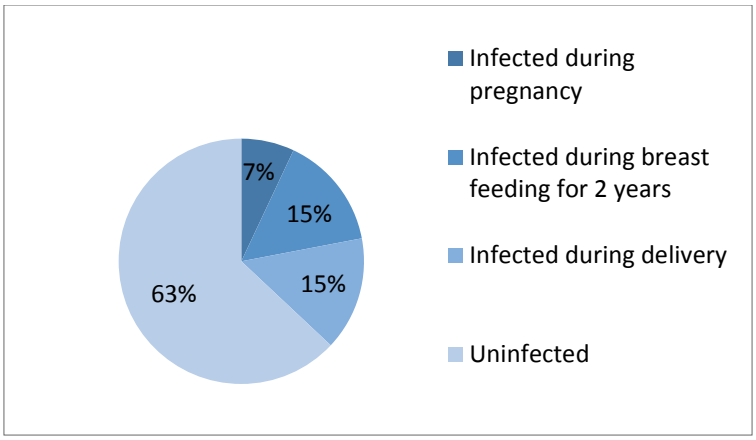


Figure 9. In a scenario of 100 seropositive HIV mothers, one would expect 7 of their infants to be infected with HIV in-utero (during pregnancy), another 15 would become infected during labor and delivery, and another 15 would become infected with HIV over the course of about 2 years of breastfeeding. 63 infants would not become infected with HIV, even if breastfed.

HIV-1 Infection in Cord Blood and in Adult Blood

Compared to HIV-1 infection in adults, infected neonates and infants exhibit a higher level of viremia, develop more rapid progression to AIDS, and have a higher susceptibility to opportunistic infections [21]. In comparison to adult peripheral blood mononuclear cells (PBMCs), CBMCs exhibit an increased susceptibility to HIV-1_{BAL} infection *in vitro* as well as a higher level of HIV-1_{Bal} viral replication *in vitro*. There exists a higher number of circulating T cells, and therefore more targets for infection, in CBMCs [22]. Despite the difference in circulating T cells, the risk of *in utero* transmission is particularly low, suggesting that a correlate of protection exists within cord blood.

Central memory T cell preservation protective in HIV-1 infection

Previous studies have found that Sooty Mangabeys and African Green Monkeys, are natural hosts for SIV since they remain healthy despite high levels of viremia [23]. Silvestri et al showed that these natural hosts exhibit mild and transient immune

activation upon infection, minimal cell death, and a minor decrease in CD4 cells but no progression to AIDS [23]. The preservation of central memory T cells is key to a mild immune response from these natural hosts. Stimulating T cells usually increases CCR5 expression, facilitating infection of the cell. In Sooty Mangabeys, however, central memory T cells maintain low levels of CCR5 relative to effector memory T cells upon activation. Preservation of central memory T cells is particularly important because these cells are long-lived as opposed to the shorter life span of effector memory T cells. Central memory T cell depletion in pathogenic HIV and SIV infection is thought to trigger CD4+ T cell proliferation associated with a robust proinflammatory response. This inflammatory response from central memory T cell depletion is both chronic and pro-apoptotic [23]. Central memory T cell depletion triggers chronic immune responses and pro-apoptotic cytokines against the pathogen that ultimately lead to T cell exhaustion [23]. By protecting central memory T cells, Sooty Managabeys avoid significant loss of T cells and chronic immune activation. In addition, it has been proposed in rhesus macaques and non-natural hosts that CD4+ effector memory T cells are dependent on the proliferation of CD4+ central memory T cells for their homeostasis following persistent immune activation [24]. Because these non-natural hosts are not able to control the chronic immune response that follows, their CD4+ central memory T cells decline and further decrease CD4+ effector memory T cell counts. Altogether, this suggests that an AIDS-free status is promoted in sooty mangabeys due to the preservation of CD4+ central memory T cells and the maintenance of CD4+ T cell homeostasis (Figure 10).

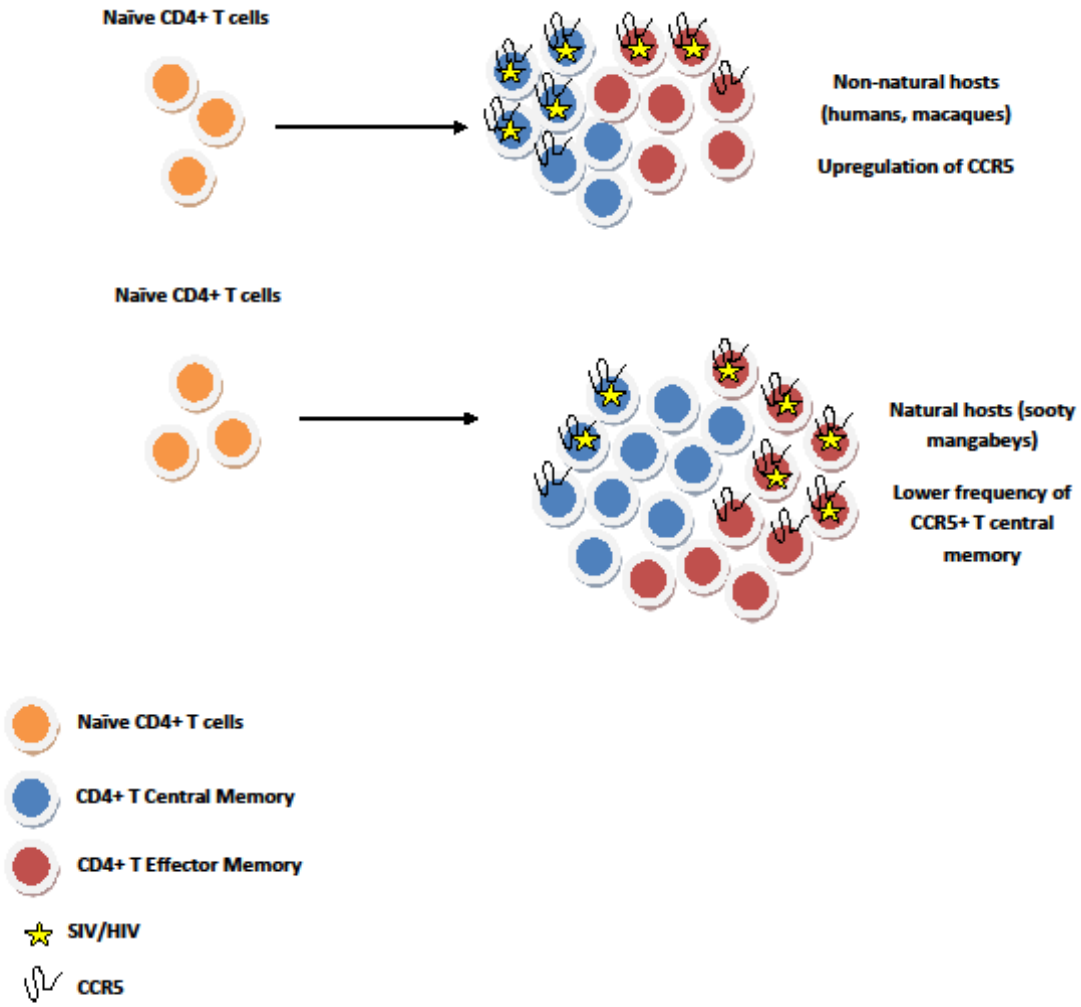


Figure 10. Proposed mechanisms of target cell restriction in natural host species. In pathogenic hosts such as Rhesus Macaques, activation of CD4+ T cells typically leads to robust surface expression of CCR5, providing targets for infection and viral replication. Infection of T_{CM} has proved to disrupt CD4+ T cell homeostasis *in vivo*. In Sooty Mangabeys, CD4+ T_{CM} maintain low levels of CCR5 relative to CD4+ T_{EM} upon activation.

Activation and Transmission

Immune activation has been shown to be associated with faster disease progression and increased HIV replication. Multiple studies have examined HIV-1 transmission with respect to immune activation. gp120, the glycoprotein on the virus, has been shown to enhance responsiveness to the virus, and binding of gp120 can activate cells to trigger release of cytokines and chemokines. Proinflammatory cytokines such as TNFα, IL-6, and IL-1B in return create an inflammatory environment. Immune activation may drive naïve T cell depletion and eventual T cell exhaustion [25]. During infection, T cells that are primed to respond to the virus are activated and infected. These cells eventually undergo apoptosis. The T cell pool is replenished by naïve T cells that undergo the same fate as primed T cells. Eventually, the immune system depletes the T cell pool as the body fails to replenish these cells efficiently. Chronic immune activation has been shown to result in decrease IL-2 production as the T cell repertoire undergoes exhaustion and lose the ability to differentiate and proliferate. CD4+ T cell depletion has been shown to disrupt the mucosal barrier to cause microbial translocation. Higher levels of LPS, lipopolysaccharides of bacterium, are found in plasma of patients with chronic immune activation from HIV-1 [26]. Furthermore, T cell loss generates fibrosis within the thymus and damages the architecture of the thymus. Damage to the thymus further promotes T cell dysfunction and disruption of T cell homeostasis [27]. Taken as a whole, chronic immune activation has been demonstrated to increase immune dysfunction associated with chronic HIV -1 infection [28-29].

Rationale for Study and Hypothesis

CCR5 expression is largely restricted to the memory cell subset with low levels of memory T cells in CBMCs [20]. The scarcity of memory T cells in CBMCs, and therefore a lower expression of CCR5, may serve as one mechanism behind low rates of mother-to-child transmission of HIV-1. Secondly, immune quiescence *in utero* as a background for central memory T cell preservation may be a mechanism to limit transmission between mother and child.

Placentas from transmitting mothers show a pro-inflammatory cytokine release when compared with mothers who do not transmit [19]. Fetomaternal cellular exchanges promote the development of fetal T-regulatory cells that actively suppress the immune system [4]. Immune unresponsiveness has also been shown in pregnant mice, where the uterus is particularly unresponsive to immune challenge around the time of implantation and through gestation [30]. Maternal decidual reaction in these mouse models block the afferent lymphatic vessels that traffic into the lymph nodes [30]. This mechanism may provide a model for a human understanding of immune quiescence during pregnancy.

Most studies examining cord blood *in vitro* stimulate CBMCs with PHA and IL-2 to promote viral replication that may not be susceptible *in vivo* [31]. Cellular activation enhances viral entry, reverse transcription, and proviral transcription that result in increased viral replication from activated CD4+ T cells. Many experiments look at viral replication under cellular activation, because it is believed that cellular activation is required for efficient viral spread [32]. While cellular activation may promote viral spread, stimulating CBMCs *in vitro* may not accurately serve as a physiologic surrogate of the cellular milieu in which HIV replicates *in utero*. Therefore to more accurately

characterize the cellular milieu *in utero,* we investigate the CCR5 expression in both the stimulated and unstimulated environment.

We hypothesize that CCR5 expression is lower in central memory T cells of unstimulated CBMCs as the environment of unstimulated CBMCs may be more characteristic of the quiescent, placental-fetal environment *in utero*. Infection would occur at a lower rate in these memory cell subsets and central memory T cell preservation could account for low levels of transmission at the fetomaternal interface. Our goal was to characterize memory cell and co-receptor expression in CBMCs to understand the ability of CBMCs to proliferate and undergo apoptosis in response to HIV-1_{Bal}. Our ultimate goal is to propose a mechanism by which fetomaternal immune regulation occurs in humans.

Methods

Ethics statement

All participants provided written informed consent, and the study protocol was reviewed and approved by Emory University Institutional Review Board and Grady Research Oversight Committee.

Study Subjects

With written informed consent, umbilical cord blood was collected from 10 HIV-1 seronegative women immediately following delivery at Grady Memorial Hospital and Emory Midtown Hospitals in Atlanta, GA. Cord blood was collected in Vacutainer tubes containing Heparin (Becton Dickinson, USA).

Isolation and Stimulation of CBMCs

Following collection, fresh cord blood mononuclear cells, CBMCs, and peripheral blood mononuclear cells, PBMCs, were isolated by density centrifugation over a Ficoll gradient, and re-suspended in complete medium (RPMI supplemented with 10% FBS, 1mM L-glutamine, and 1% pen/strep) [Mediatech, VA] following two washes with DPBS. Frozen samples were also defrosted from cryovials and placed in complete medium. After cell count, half of the CBMCs and half of the PBMCs were cultured at 37°C in the presence of PHA (5ug/mL) and 5% IL-2 while the rest were cultured at 37°C in the presence of only 5% IL-2 at the concentration of $1x10^6$cells/ml. The expression of CCR5 on CD4+ T-cells was assessed every 48 hours over the course of 4 days by flow cytometry.

Flow Cytometry

The staining for flow cytometry studies was performed using monoclonal antibodies that are cross-reactive with PBMCs and CBMCs. Cells were labeled with the following antibodies: anti-CD3 (A700), -CD4 (PerCp-Cy5.5), -CD8 (PE-Cy7), -CD45RO (APC), -

CD27 (PE), -Ki67 (V450), -CD38 (PE-Cy5), -HLADR (APC-H7), -KC57 (FITC), and -CCR5 (PE-CF594) [BD Biosciences, CA]. For staining, cells were incubated with 2uL of the respective antibody for 30 minutes at 4 degrees in the dark. They were thereafter washed twice before analysis. Ten-parameter flow cytometric analysis was performed on a LSR II flow cytometer driven by the DiVA software package (Becton Dickinson). Analysis of the acquired data was performed using FlowJo software (Tree Star).

Cell Gating

Gating CD4+ T_{EM} and T_{CM} from PBMCs and CBMCs, sub-categorized infected and uninfected, stimulated and unstimulated was performed on an LSR II flow cytometer (Becton Dickinson). Cells were initially gated on the basis of light scatter, followed by positive staining for CD3 and CD4. CD4+ T effector memory and T central memory were gated on the basis of characteristic expression patterns of CD45RO and CD27 (Figure 11). Central memory T cells were CD45RO+ CD27+ while effector memory t cells were CD45RO+ CD27-.

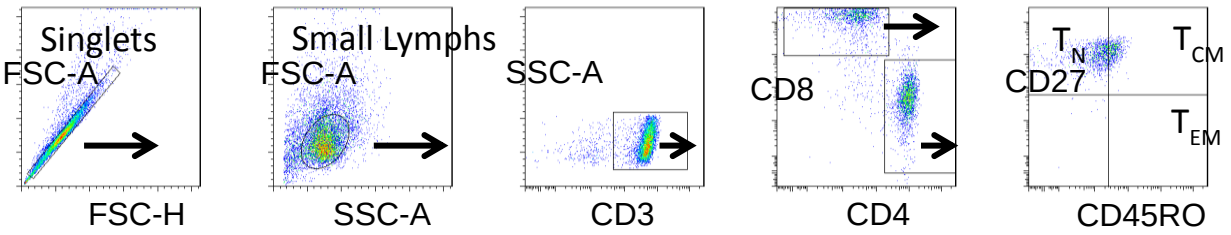


Figure 11. Flow cytometry analysis allows selective visualization of the cells of interest while eliminating results from unwanted particles. Use of fluorescently labeled antibodies allows individual cells to be identified by their cell-surface antigens and to characterize populations. Spectral overlap of fluorescent emission is overcome by compensation.

Infection

HIV-1_{Bal} was a gift from Jason Hammonds PhD of Emory University School of Medicine. PBMCs and CBMCs were infected at 0.2 TCID50/cell for 4 hours at 37°C. Cells were washed and fresh media added to the cultures. To monitor viral production and particle

release, cell supernatants and lysates were collected at days 0, 2, 3, 4, 6, 8, 9 10, 12, and 15 post-infection. Viral replication and particle release was detected by p24 released into the supernatant and cell-associated p24 by ELISA [Advanced BioScience Laboratories, Inc., MD].

Statistical Analysis

The performed analyses for comparison of means between groups include the independent samples T test. Significance was assessed at $P < 0.05$ levels. All analyses were performed using the IBM SPSS Statistics 21.0 software from Emory University.

Results

Lower fraction of memory T cells in cord blood when compared to adult blood

To investigate the fraction of memory T cells in CBMCs in comparison to the fraction of memory T cells in PBMCs, we isolated lymphocytes from CBMCs and PBMCs of ten donors by density centrifugation over a Ficoll gradient, stained, and analyzed fluorescently labeled T cells in flow cytometry and FlowJo respectively. Cells were cultured in 5% IL-2 with no PHA stimulation. We compared the means of CD4+ T cells from CBMCs (M=60.40, SD=18.12) and PBMCs (M=77.63, SD=4.69) and CD8+ T cells from CBMCs (M=16.35, SD=11.85) and PBMCs (M=13.00, SD=3.10) using an independent samples T test (Figure 12). We found no significant difference in CD4+ T cells levels between PBMCs and CBMCs ($t=1.84$, $p=0.115$) and CD8+ T cell levels ($t=0.56$, $p=0.60$) between PBMCs and CBMCs, results which could be attributed to donor variability.

To further investigate memory T cell subsets in unstimulated CBMCs and PBMCs, we assessed the fraction of T_{CM} (CD45RO+ CD27+) and T_{EM} (CD45RO+ CD27-) in CD4+ T cells. We found a lower fraction (32.8% lower) of memory T cells in CD4+ T cells of CBMCs in comparison to CD4+ T cells of PBMCs (Figure 13). Central memory T cells were found in much higher levels in CD4+ T cells of PBMCs (M=5.48, SD=2.79) than CBMCs (M=1.36, SD=1.66) and the overall fraction of memory T cells was much higher in CD4+ T cells of PBMCs. We found negligible levels of effector memory T cells in CBMCs, where most memory T cells were found to express central memory phenotype. On the other hand, we found that most of the memory T cells in PBMCs were of the effector memory phenotype (M=28.68, SD=14.58). These data indicate that though CD4+ T cell levels are similar in PBMCs and CBMCs, a smaller

fraction of the CD4+ T cells in CBMCs are actually memory T cells. One would expect a smaller fraction of memory T cells in CD4+ T cells of infants, as fetal cells have a only a brief life-time exposure to environmental antigens. Following maturation in the thymus, naïve T cells circulate the body in a naïve state must be primed by antigens to express the memory phenotype [33]. When a naïve T cell reacts to antigen during an immune response, a small proportion of the responding cells survive and become antigen-specific memory cells capable of expressing effector functions upon re-infection with the same antigen [34]. In the presence of antigen, naïve cells become memory cells. However, a lack of antigen present *in utero* renders naïve fetal cells unable to be primed into the memory phenotype. Collectively, this data confirms previous studies showing low levels of CD4+ T cells with the memory phenotype in both infants and children from HIV infected mothers [20, 35].

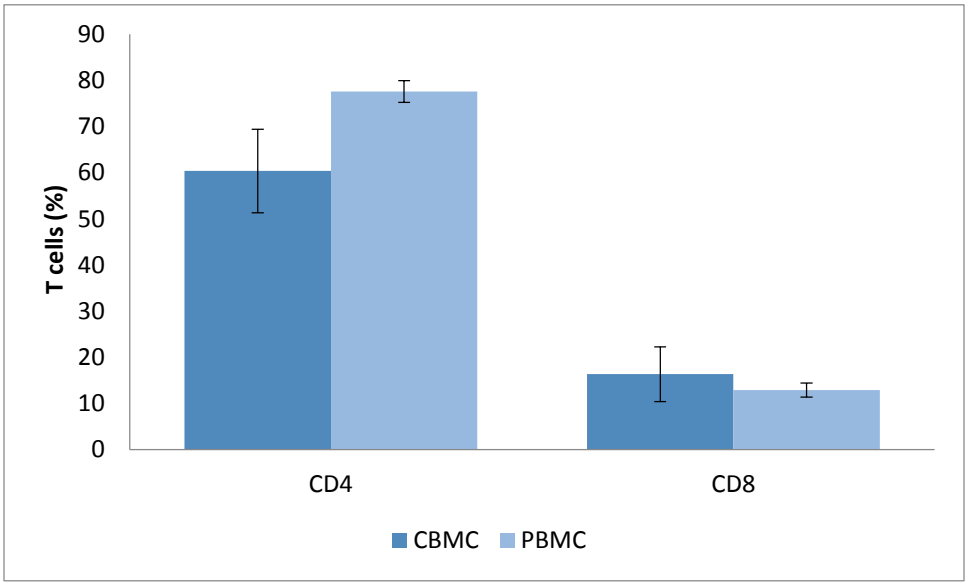


Figure 12. A comparison of the means between CBMC and PBMC CD4+ T cell levels and CD8+ T cell levels show no significance difference which is likely attributed to donor variability. Numbers indicate the mean across 10 donors. The error bars represent standard error.

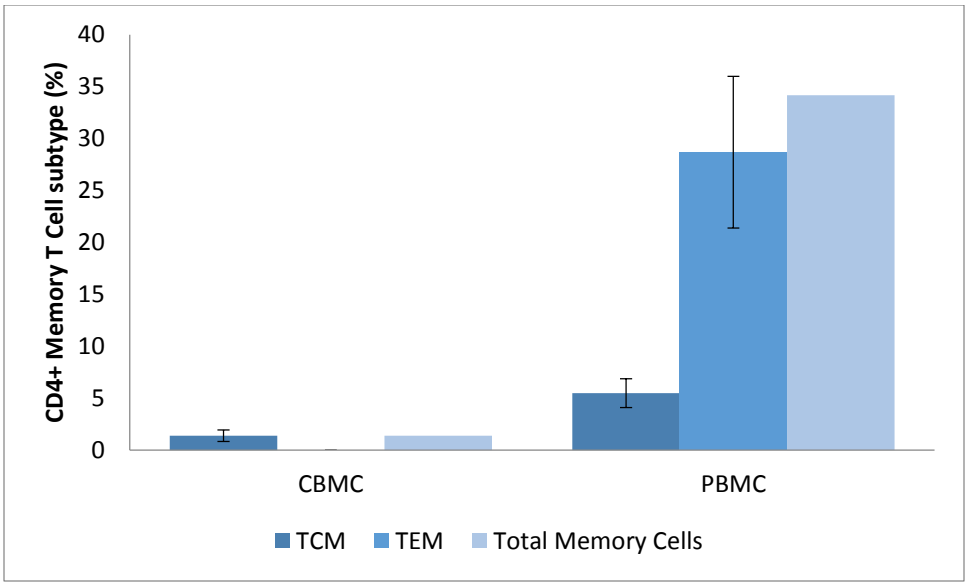


Figure 13. Comparable fractions of CD4+ T cells are apparent in both PBMCs and CBMCs, but only a small fraction of CD4+ T cells in CBMCs are memory T cells and indicate that cord blood is enriched with cells of the naïve phenotype. Numbers indicate the mean among 10 donors. The error bars represent standard error. Central memory T cells t=3.37, 0.006. Effector memory T cells t=6.27, 0.00.

Low levels of T_{CM} maintained in unstimulated CD4+ T cells of CBMCs over time compared to a five-fold increase in stimulated CD4+ T_{CM} levels over time

In order to investigate the effects of *in vitro* activation on the fraction of CD4+ memory T cells in infected CBMCs, we separated the CBMCs into stimulated CBMCs and unstimulated CBMCs. Both groups were infected with HIV-1_{Bal} on Day 3. We stimulated the cells with 5ug/mL of PHA and 5% IL-2 over a period 6 days, infecting the cells with HIV-1_{Bal} on Day 3. CD4+ T_{CM} remained low in unstimulated CBMCs post-infection (M=0.66, SD=0.54 for Day 2 post-infection) while stimulated CD4+ T_{CM} increased five-fold post-infection (M=4.82 on Day 0, M=22.46 on Day 2 post-infection) (Figure 14). In addition, we found that unstimulated CBMCs exhibited a significant increase in T_{EM} post-infection.

Low levels of T_{CM} maintained in unstimulated CD8+ T cells of CBMCs over time compared to a six-fold increase in stimulated CD8+ T_{CM} over time

In order to determine the effects of *in vitro* activation in CD8+ central memory T cells, we separated CBMCs into stimulated and unstimulated CBMCs. CBMCs in the stimulated subtype were activated with 5ug/mL of PHA and 5% IL-2 over a period of 6 days. Both groups were infected with HIV-1_{BAL} on Day 3. CD8+ central memory T cells remained low in unstimulated CBMCs post-infection (M=0.58, 3.32, 2.15, SD=0.93, 4.29, 3.54) while the fraction of central memory T cells in stimulated CBMCs increased from M=2.03 (SD=1.77, Day of infection) to M=11.74 (SD= 9.33, Day 2 post-infection) (Figure 15). Maintenance of low levels of CD8+ central memory T cells in unstimulated CBMCs correlate with low levels of TCM in unstimulated CD4+ T cells of CBMCs (M=0.81, 0.66, 1.12, SD=0.75, 0.31, 0.57) while CD4+ T_{CM} increase five-fold in stimulated CBMCs, and CD8+ T_{CM} increase six-fold in stimulated CBMCs.

Low levels of HIV-1 replication, measured by p24 levels, in unstimulated CBMCs compared to higher levels of HIV-1 replication in stimulated CBMCs

In order to investigate the effects of stimulation on HIV-1 susceptibility of CBMCs, we measured p24 levels in the supernatant of infected, stimulated CBMCs versus infected, unstimulated CBMCs over a period of 12 days. p24 is a component of the HIV particle capsid and can be detected in the supernatant of infected cells via a p24 HIV Antigen ELISA [Perkin Elmer]. We found higher levels of p24 in stimulated CBMCs, suggesting higher levels of HIV-1 replication in stimulated CBMCs. These findings suggest a strong association between low levels of T_{CM} in unstimulated CBMCs and a lower level of HIV-1 replication rate in unstimulated CBMCs (Figure 16). We interpret these findings to be reflective of immune quiescence that is mirrored in infected

sooty mangabeys, where central memory T cells are found in much smaller fractions that effector memory T cells [6].

The temporal maintenance of CD4+ central memory T cells in unstimulated CBMCs and CD8+ central memory T cells in unstimulated CBMCs post-infection may serve as an important mechanism to avoid chronic immune activation and effector memory T cell depletion as as found by Paiardini et al. [23-24]. Persistent HIV infection and disruption of central memory T cell triggers further chronic immune responses and pro-apoptotic cytokines that ultimately lead to exhaustion of the T cell renewal capacity [24, 36]. Recent studies have shown that apoptosis detected in T cell subsets is related and caused by continuous stimulation of the immune system [37-38]. In short term culture, continuous stimulation from activation induces apoptosis of both CD4 and CD8 populations [39]. Cells that eventually undergo apoptosis display the phenotype of activated lymphocytes [39]. The ability to maintain levels of CD4+ and CD8+ central memory T cells in unstimulated CBMCs in key in controlling immune response to the antigen, as a chronic immune response correlates with disease progression.

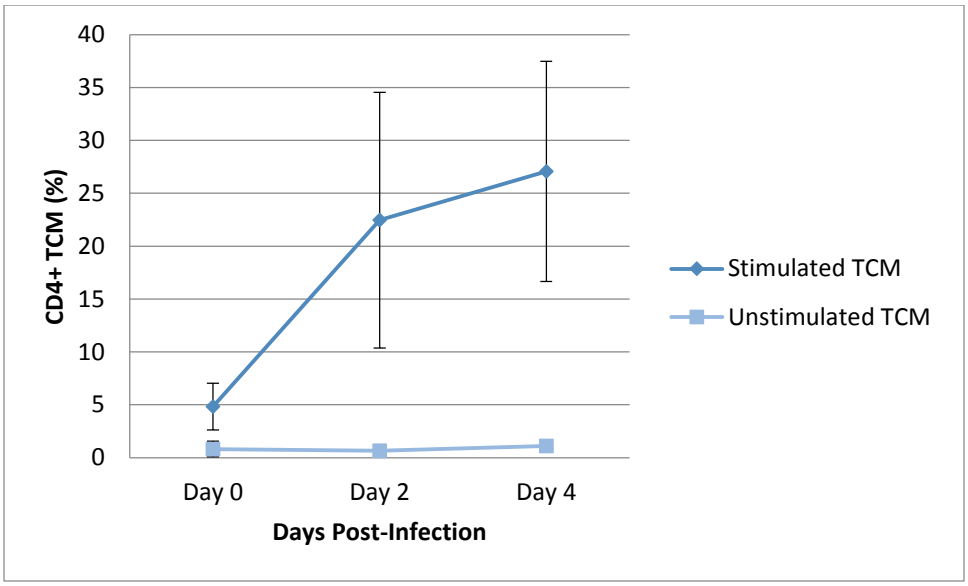


Figure 14. Unstimulated CBMCs show lower fractions of central memory T cells post-infection and a maintenance of CD4+ central memory T cell levels. Values indicate the mean of 10 donors. The error bars represent standard error.

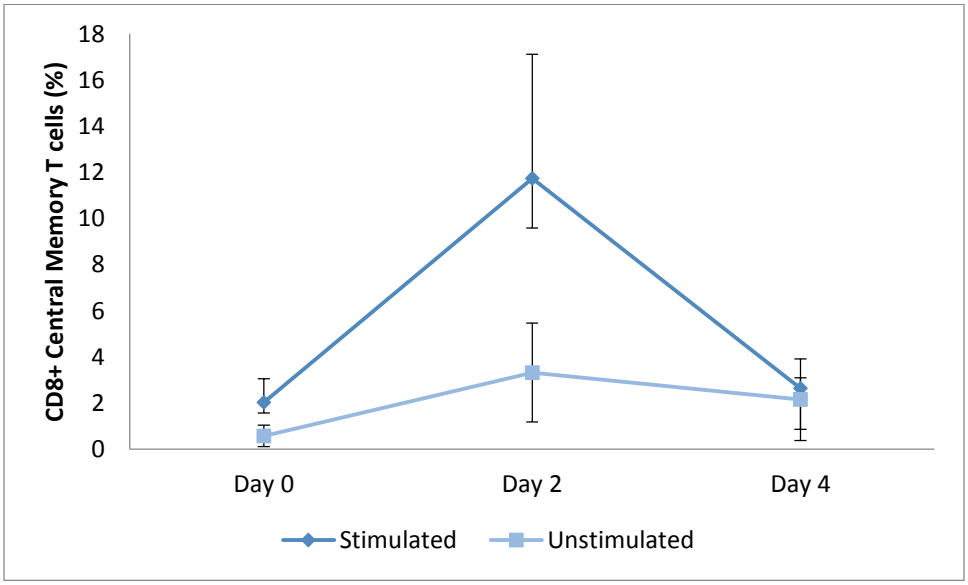


Figure 15. Unstimulated CBMCs show a lower fraction central memory T cells post-infection and maintenance of CD8+ central memory T cell levels. Values indicate the mean of 10 donors. The error bars represent standard error.

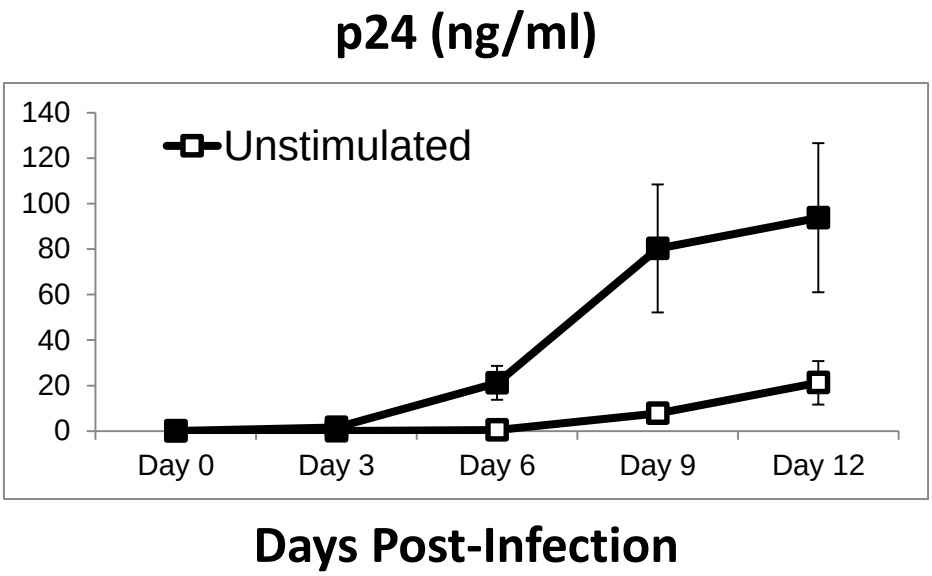


Figure 16. HIV-1 replication rates are lower in unstimulated CBMCs post-infection. Values indicate the mean of 10 donors. The error bars represent standard error.

Rapid CD4+ T cell proliferation in CBMCS upon stimulation, but low CCR5 levels in unstimulated CBMCs compared to stimulated PBMCs

We next investigated CCR5 expression in relation to T cell proliferation in both cord and adult blood. We used Ki67, a widely used cellular marker for proliferation, to measure fractions of proliferating CD4+ T cells. PBMCs and CBMCs were stimulated but not infected. Our results showed that CD4+ T cells in PBMCs took several days to generate a robust proliferative response to stimulation while CD4+ T cells in CBMCs showed robust proliferation by Day 2 post-stimulation (Figure 17). The quick capacity for CD4+ T cells in CBMCs to proliferate may explain increased susceptibility of CBMCs to HIV-1_{BAL} infection *in vitro* as well as a higher level of HIV-1_{BAL} viral replication *in vitro* found in previous studies [21]. However, when we investigated CCR5 expression post-stimulation in cord and adult blood, cord blood CD3+ CD4+ T cells exhibited lower levels of CCR5 (M=1.99, SD=1.19) in comparison to CD3+ CD4+ T cells in peripheral blood (M=2.723, SD=2.52). CCR5 expression is reduced in T cells of

cord blood (Figure 18, 19). The kinetics of CCR5 expression *in vivo* post-stimulation may be important in explaining low levels of MTCT of HIV-1 as CCR5 serves as a co-receptor for HIV. In addition, lower levels of CCR5 expression in CBMCs correlate with previous experiments that show CCR5 expression mostly on memory T cells. We have previously shown lower levels of memory T cells in CBMCs in comparison to PBMCs and thus it is fitting to note lower levels of CCR5 expression in CBMCs in comparison to PBMCs [20].

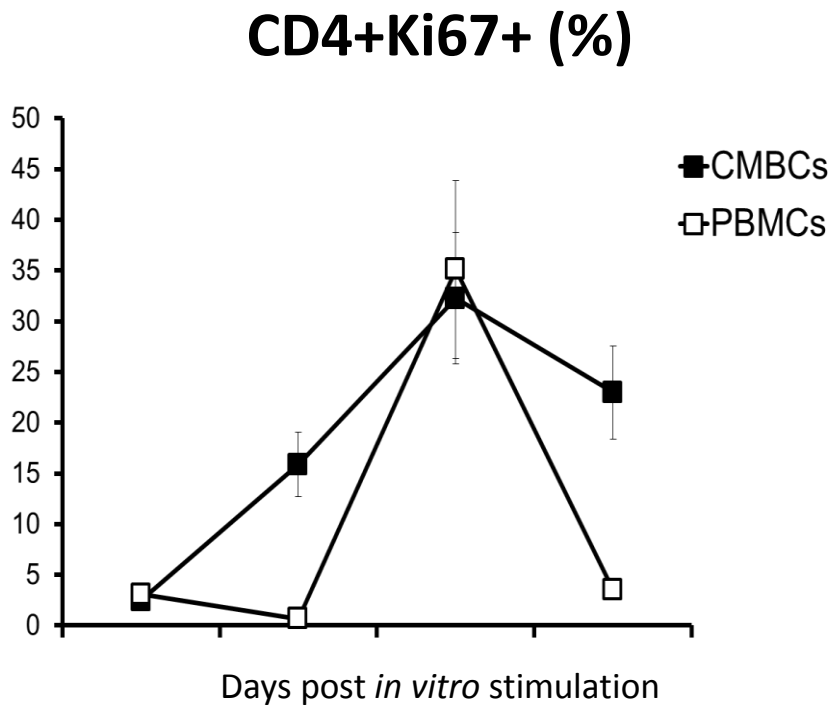


Figure 17. CD4+ T cells in both CBMCs and PBMCs show proliferative capacity. Ki67, a cellular marker for proliferation, was used to measure proliferation levels over time. CD4+ T cells in CBMCs showed a robust proliferative response early on, while PBMCs did not proliferate until Day 2 post-stimulation. Values indicate the mean of 10 donors. The error bars represent standard error.

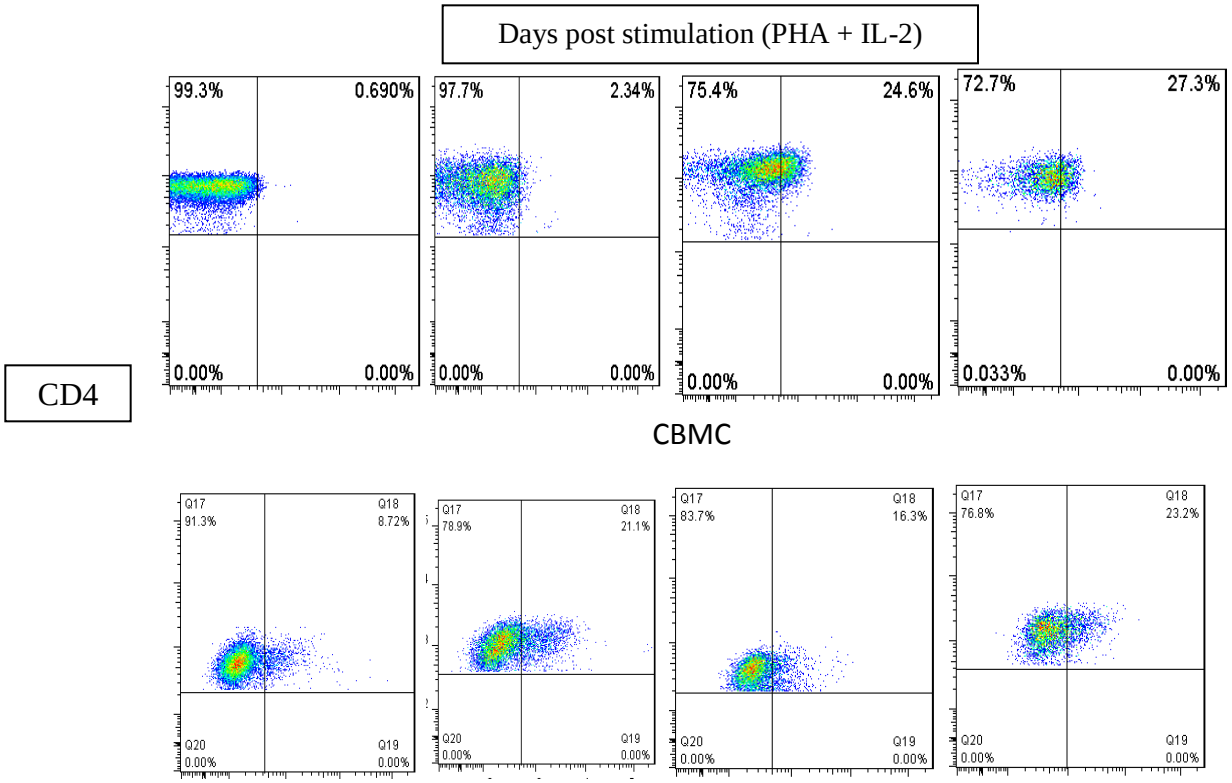


Figure 18. CCR5 expression is found at a much lower level in cord blood CD3+ CD4+ T cells in comparison with adult blood. The flow plot above shows CCR5 expression differences in one sample of CBMC and one sample of PBMC

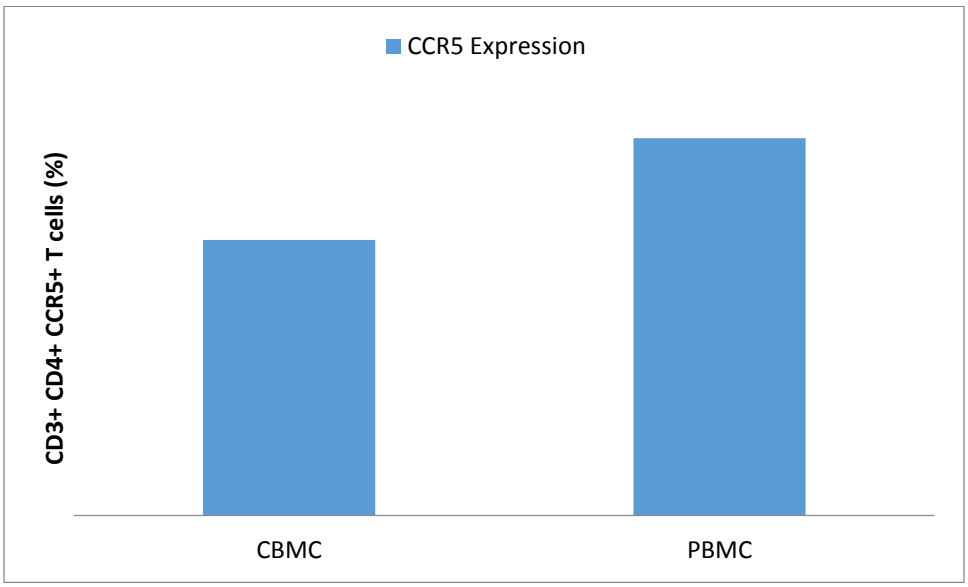


Figure 19. CCR5 expression is found at a much lower level in cord blood CD3+ CD4+ T cells in comparison with adult blood. The bar graph shows means of CD3+ CD4+ CCR5+ T cells across 10 different donors in CBMCs and PBMCs (M=2.72, SD=2.52 for PBMCs, M=1.99, SD=1.19 for CBMCs)

Expression of activation marker on CD4+ cells may correlate with HIV-1 susceptibility

We further explored the idea of central memory T cell preservation by looking at activation levels of central memory T cells in unstimulated CBMCs versus stimulated CBMCs. CD38 is a marker of activation and immaturity which is thought to represent immature T cells released in late HIV disease as a consequence of lymphopoietic exhaustion [40]. CD38+ cells are a strong marker of poor prognosis in HIV-infected patients [41-43]. We found that that unstimulated central memory T cells show a decrease in CD38+ expression over time while CD38 levels increased in stimulated central memory T cells. Unstimulated CD4+ central memory T cells are significantly lower ($t=7.68$, $p=0.002$) by day 4 post-infection ($M=8.07$, $SD=13.03$ for unstimulated CD4+ central memory T cell, $M=77.2$, $SD=8.46$ for stimulated CD4+ central memory T cells) (Figure 20). One of the key mechanisms of central memory T cell preservation in sooty mangabeys is an ability to avoid chronic immune activation [23]. Here we demonstrate that unstimulated central memory T cells exhibit a decline in activation post-infection while stimulated CBMCs show an increase in the fraction of activated central memory T cells. In addition, CD38+ cells have been shown to increase with disease progression [44]. As such, expression of CD38, the activation marker on CD4+ T cells, may correlate with HIV-1 susceptibility. CD4+ central memory T cells express lower levels of CD38 and which may be correlated with immune quiescence. In contrast, higher levels of CD38 in stimulated central memory T cells may correlate with HIV progression and a chronic inflammatory response.

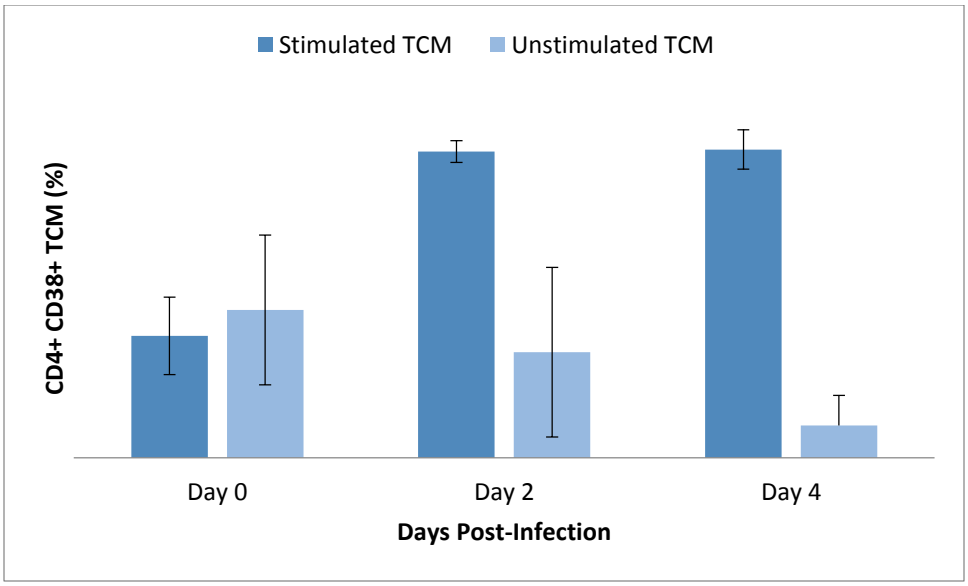


Figure 20. CD38, a marker for activation and immaturity, was used to measure activation levels in central memory T cells post-infection. Unstimulated central memory T cells showed a decline in levels of activation post-infection while stimulated central memory T cells increased in activation levels post-infection. Values indicate the mean of 10 donors while error bars represent standard error.

Similar levels of CXCR4 expression observed in both stimulated and unstimulated T cells of CBMCs over time

We next investigated CXCR4 expression in both stimulated and unstimulated CD4+ central memory T cells of CBMCs. CCR5 and CXCR4 both serve as entry cofactors for HIV-1 [45-46]. During the early stages of HIV-1 infection, viral isolates tend to use CCR5 for viral entry and shift to use CXCR4 during later courses [47]. Co-receptor expression is important in controlling T cell infection and regulation as chemokines and their receptors are thought to direct migration of leukocyte, white blood cell, subsets to the site of infection [45, 48] and thus play an important role in AIDS pathogenesis. Previous studies have shown that individuals deficient in CCR5 are resistant to infection by HIV-1[49-53].

CBMCs in the stimulated subtype were activated with 5ug/mL of PHA and 5% IL-2 over a period of 6 days. Both groups were infected with HIV-1_{BAL} on Day 3. We

found similar levels of CD4+ CXCR4+ central memory T cells in both stimulated (M=34.95, 95.23, 88.17, SD= 31.47, 4.22, 2.63) and unstimulated CBMCs (M=30.15, 92.5, 88.55, SD=4.17, 2.69, 6.72) over time (Figure 21). Stimulated and unstimulated CD4+ CXCR4+ central memory T cells increased three-fold from day of infection to two days post infection. We found no significant difference in CXCR4 expression between stimulated and unstimulated CBMCs post-infection (t=0.21, p=0.85 for stimulated CBMCs on day of infection, t=0.21, p=0.87 for unstimulated CBMCs on day of infection, t=0.79, p=0.49 for stimulated CBMCs two days post-infection, t=0.89, p=0.44 for unstimulated CBMCs two days post-infection). High levels of CXCR4 expression post-infection in both subtypes appear to be independent of phytohemagglutinin (PHA) stimulation and interleukin 2 (IL-2) stimulation and priming.

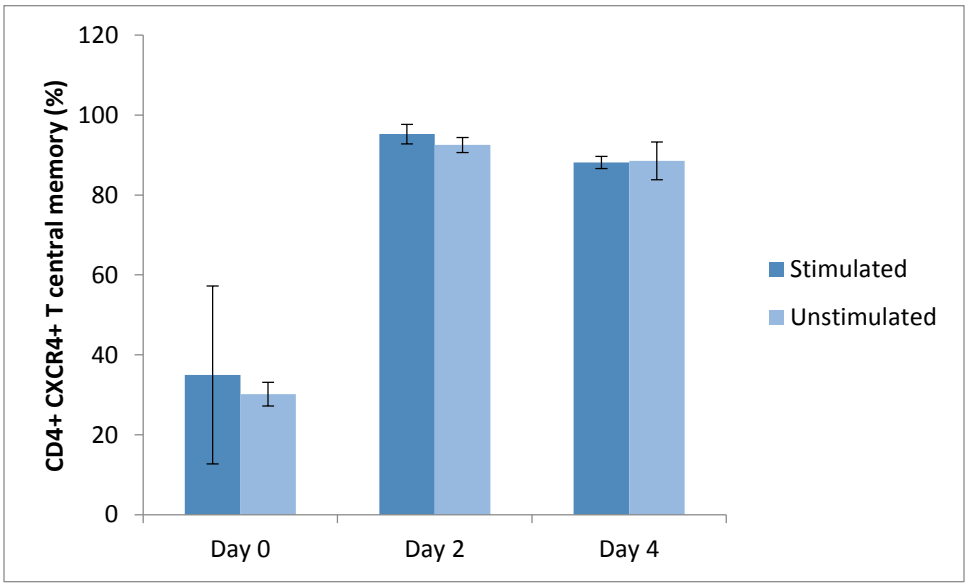


Figure 21. CXCR4, a coreceptor for HIV-1, was found to be expressed in comparable fractions on stimulated and unstimulated central memory T cells of CBMCs. Stimulated and unstimulated CD4+ CXCR4+ central memory T cells increased three-fold (M=34.95, 95.23, 88.17, M=30.15, 92.5, 88.55 respectively) from date of infection to Day 2 post-infection. Values indicate the mean of 10 donors while error bars represent standard error.

Reduced CCR5 expression in unstimulated T cells of CBMCs

We next investigated CCR5 expression in both naïve and central memory T cells of CBMCs. As mentioned before, both CXCR4 and CCR5 are coreceptors for HIV-1. Both central memory and naïve T cells showed much higher levels of CCR5 expression post-infection when stimulated (Figure 22). Central memory T cells were gated as CD3+ CD4+ CD45RO+ CD27+ T cells while naïve T cells were gated as CD3+ CD4+ CD45RO-. We conclude that stimulated CD4+ central memory cells may be more permissive to HIV-1 infection *in vitro* due to increased expression of CCR5 [47, 54]. Unstimulated cells may be less permissive to HIV-1 infection because they show a markedly lower level of CCR5 expression post-infection. Taken together, unstimulated cells may reflect the immune quiescent environment *in utero* that modulates HIV-1 susceptibility.

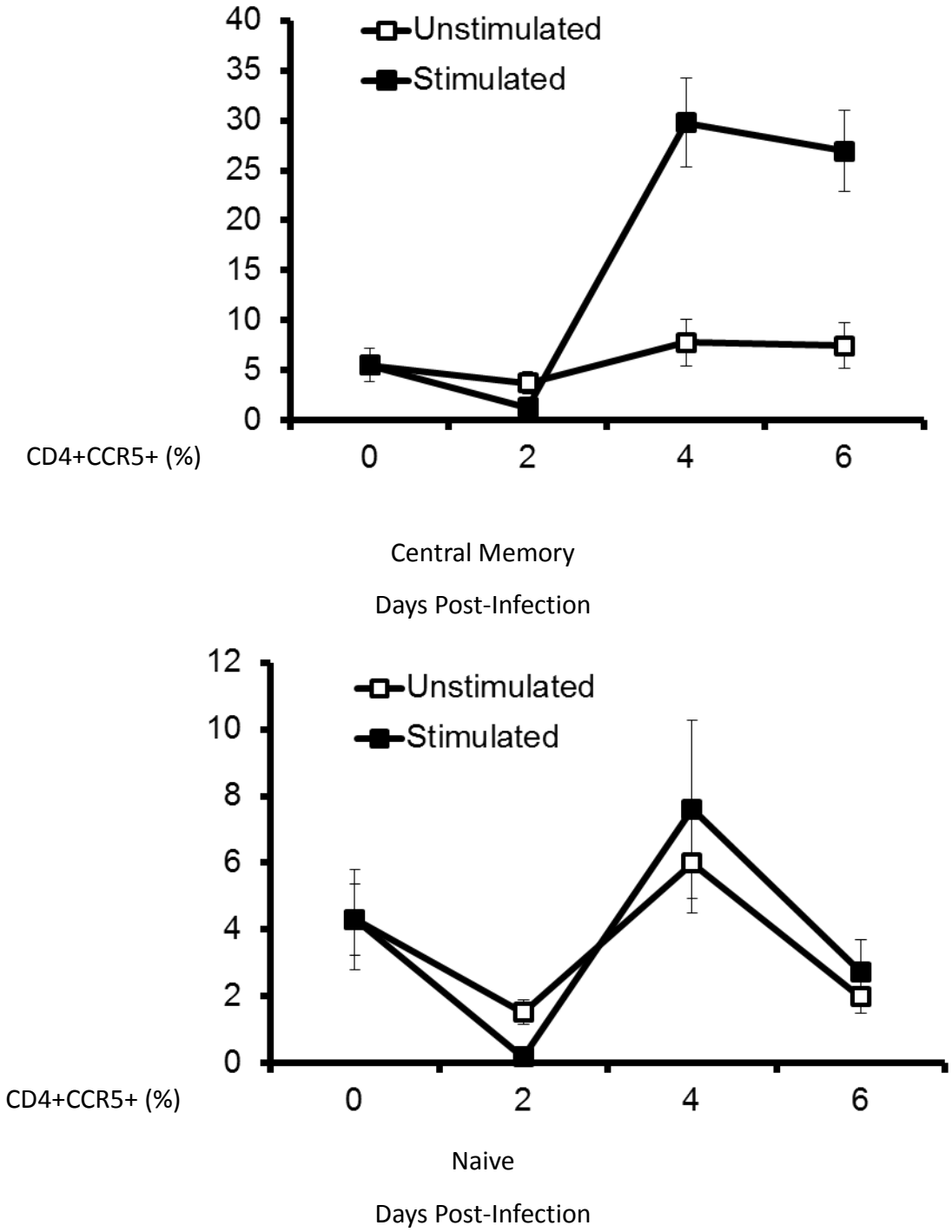


Figure 22. Analysis of central memory and naïve T cells show lower levels of CCR5 expression in the unstimulated subset. This may be key explaining lower levels of fetomaternal transmission of HIV-1. Central memory cells were gated as CD3+ CD4+ CD45RO+ CD27+ T cells while naïve cells were gated as CD3+ CD4+ CD45RO- T cells. Values indicate the mean of 10 donors. Error bars represent standard error.

CD4+ T cell death, CD4+ central memory T cell death in stimulated CBMCs

We assessed cell death, apoptosis, in stimulated CD4+ T cells as a whole and in stimulated CD4+ central memory T cells by using Annexin V. Changes at the cell surface occur in the early stages of apoptosis, including translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the external, outer layer. Annexin V, a calcium dependent phospholipid-binding protein, has a high affinity for PS and binds to it at the external surface [55]. HIV infection is one of the situations in which apoptosis is thought to occur at a high degree where the lost of circulating CD4+ T cells is the hallmark of *in vivo* HIV infection [55-58] (Figure 23).

We found a peak in the fraction of stimulated CD4+ Annexin V+ T cells post-infection, from M=44.1 (SD=4.63) on the day of infection to M=76.2 (SD=43.27) two days post-infection. We found similar apoptotic levels in stimulated CD4+ Annexin V+ central memory T cells (M=41.1, 89.05, SD=4.53, 14.35) showing that CD4+ memory T cells undergo apoptosis at high rates post-infection in stimulated CBMCs. A loss of CD4+ homeostasis and decline in CD4+ T cell count is followed by a comparable loss of CD4+ central memory T cell homeostasis, with CD4+ T cells and CD4+ central memory T cells each expressing almost a two-fold increase in Annexin V expression. Taken together, these results provide an explanation for chronic immune activation and illustrate the initial depletion of CD4+ T cells post-infection as found in previous studies. These results also demonstrate that stimulated central memory T cells also undergo high levels of apoptosis post-infection with the HIV-1 antigen.

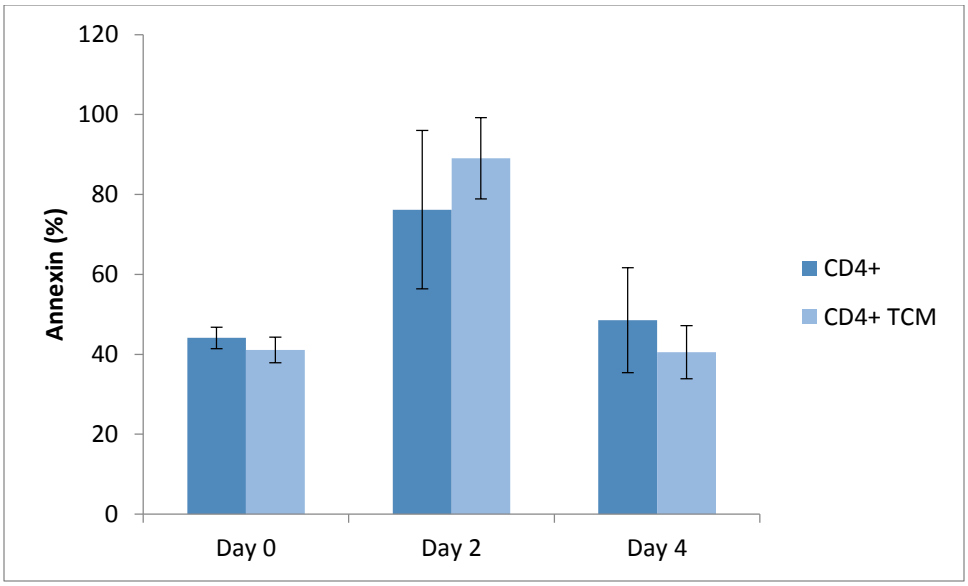


Figure 23. Annexin V, a marker for apoptosis, was used to measure cell death in stimulated CD4+ T cellls and CD4+ central memory T cells. There is a two-fold increase in Annexin V expression post-infection both stimulated CD4+ T cells and CD4+ central memory T cells. Values indicate the mean of 10 donors while error bars represent standard error.

Discussion

Despite high viral loads in the mother, the low risk of mother-to-child transmission of HIV-1 *in utero* suggests that a natural correlate of protection exists at the feto-maternal interface. One such correlate of protection could be explained by low levels of CCR5 expression in central memory T cells *in utero*. CCR5, the co-receptor for HIV-1, plays an integral role in HIV-1 entry into the target cell. By monitoring co-receptor expression for HIV-1 entry on memory cells, the immunoregulatory environment *in utero* protects the fetus from rapid viral entry.

The endometrium expresses certain immunosuppressive factors (TGF-β) to control immune responses and both mother and fetal cells work together to ensure attacks by pathogens are regulated and cleared [59]. Strict immunoregulatory patterns work to maintain control of immune responses and prevent chronic activation. In contrast, hyperactive cell-mediated immunological reactivity leading to measurable levels of cytotoxic cells was found at miscarriage in mothers [60]. Fetomaternal immune regulation is thus an important mechanism to maintain fetal growth and pathogen control. Our experiments served to characterize and investigate fetoimmunoregulatory patterns by comparing stimulated CBMCs with unstimulated CBMCs. While most studies examining cord blood in vitro stimulate CBMCs to promote viral replication, cellular activation may promote viral spread that may not be an accurate physiologic surrogate of HIV replication in utero [31-32].

Low levels of CD4+ and CD8+ central memory T cells in an unstimulated, quiescent immune environment

The hallmark of HIV infection is the progressive loss of CD4+ T cells through destruction and decreased production. CD4+ T lymphocytes play key roles in regulating

immunological response during pregnancy and physiologic regulation of the innate immune response must be implemented to prevent fetal allograft rejection [62]. In addition, CD8+ T cells are believed to be important in the control of HIV infection, because the emerging HIV-specific cytotoxic T lymphocyte response follows a close association with acute viral load reduction [61].

When we looked at central memory T cells in CD4+ T cells and CD8+ T cells, we found that unstimulated CD4+ T cells and CD8+ T cells were able to maintain low levels of central memory T cells post-infection, a mechanism that may serve an important correlate of protection at the feto-maternal interface. Temporal maintenance of CD4+ central memory T cells and CD8+ central memory T cells is important in T cell homeostasis and in avoiding chronic immune activation and T cell exhaustion. T cell homeostasis is critical in both regulating a immune response and in reducing viral load. Disrupting T cell homeostasis leads to pro-inflmmatory cytokines and a cycle of T cell destruction as lymphocytes are activated to mount a response against the pathogen. Individuals who are able to maintain CD4+ T cell counts and low viral loads appear to have no evidence of HIV-1 related disease manifestations [20]. Here note that steady levels of central memory T cells are maintained in an immune quiescent, unstimulated milieu which may be reflective of the immune environment *in utero*.

Downregulation of memory T cells post-infection serves as a possible correlate of protection against HIV-1

Fetal cells appear to downregulate memory T cell levels post-activation and infection. The ability to regulate memory T cell levels post-activation and infection may be key to memory T cell preservation during infection. HIV-1 induces a strong HIV-1-specific proliferative response in persons who are controlling viremia in the absence of

antiretroviral therapy [63]. Here we see that CD4+ central memory T cells showed a decline in levels of activation post-infection. Maintenance of CD4+ central memory T cells in CBMCs post-infection may serve as an important mechanism to avoid chronic immune activation as persistent HIV infection and disruption of central memory T cells trigger further immune responses and pro-apoptotic cytokines that lead to T cell exhaustion and inhibited capacity for renewal [23-24, 36]. Non-human primates that are natural hosts for SIV are able to preserve their CD4+ central memory T cells and thus maintain T cell homeostasis and avoid chronic immune activation [23]. In addition, initiating antiviral therapy in primates leads to improved CD4+ central memory T cell restoration and regeneration, linked to viral suppression and a reduced inflammatory profile [64]. Maintaining stable levels of central memory T cells is important in controlling the body's immune response to an antigen and in avoiding chronic immune activation and disease progression [25-28].

Lower levels of memory cells in cord blood compared to adult blood and similar levels of CXCR4 expression both unstimulated CBMCs and stimulated CBMCs

HIV entry into CD4+ T cells is mediated by interactions between the virus and its receptors on the cell surface [20]. The CC-chemokine receptors CCR5 and CXCR4 are used by the virus as coreceptors necessary for entry into the target cell. CCR5 is the coreceptor most commonly associated with macrophage-tropic viruses while CXCR4 is the dominant coreceptor for viruses that infect the T cell line [20]. It has previously been shown that viruses use CCR5 early during infection and switch to use CXCR4 during later course of infection [20]. In pediatric HIV-1 infection, the immune system of infants is enriched in the naïve phenotype and CCR5 expression is largely restricted to memory cells in cord blood. From our experiments, we noted a paucity of memory cells in cord

blood in comparison with adult blood. We examined CXCR4 and CCR5 expression in stimulated and unstimulated CD4+ central memory T cells post-infection with HIV-1_{BAL}. CXCR4 expression increased three-fold post-infection in both stimulated and unstimulated CD4+ central memory T cells. A two-fold increase in CXCR4 expression in both stimulated and unstimulated central memory T cell post-infection does not appear to correlate with stimulation with PHA and IL-2. Donor dependent variation was also observed in CXCR4 expression levels in CBMCs. We assessed previous studies showing lower levels of CCR5 expression in cord blood and show that low fractions of memory cells in cord blood is directly correlated with lower levels of CCR5 expression, as CCR5 expression is largely restricted to memory T cells [20, 34]. Low levels of memory-type cells in cord blood are thought to be a result of scarce antigen stimulation [65]. Naïve fetal cells are not primed into the memory phenotype due to a lack of antigen presence outside maternally derived antigens. CD4+ T cells of infants are enriched in the naïve phenotype, reflecting much briefer life-time exposure to environmental antigens compared with more fractions of memory T cells in adults.

Low levels of CCR5 expression in cord blood correlates with lower HIV-1 infection

We found that levels of CCR5 were much lower in both unstimulated naïve and memory T cells in comparison with stimulated naïve and memory T cells. Activation with IL-2 and PHA substantially increased CCR5 expression in cord blood. CCR5 upregulation by IL-2 and PHA is consistent with previous reports and with an increase in CCR1 and CCR2b mRNA expression in T cells post-stimulation [66]. The fraction of CCR5+ T cells also increased post-stimulation, perhaps reflecting activation of naïve cells. We measure p24, the internal structural viral protein Gag, by ELISA in the supernatant of unstimulated and stimulated CD4+ central memory T cells of CBMCs

[67].We found that p24 levels were much lower in unstimulated CD4+ central memory T cells of CBMCs. From these experiments, lower levels of p24 in unstimulated CD4+ central memory T cells are presumably correlated with lower levels of CCR5 expression in these cells. CXCR4 are expressed at high levels in both stimulated and unstimulated central memory T cells, whereas CCR5 is expressed at a much lower level in unstimulated central memory T cells compared to stimulated central memory T cells. Lower levels of p24 in unstimulated central memory T cells, thus, seem to correlate with lower levels of CCR5 and less with CXCR4 expression. The expression pattern of CCR5 as a coreceptor for HIV-1 entry presumably accounts for observations that stimulated cord blood T cells harbor a majority of proviral DNA.

High levels of apoptosis in stimulated CD4+ T cells lead to loss of T cell homeostasis

HIV infection has been shown to deplete CD4+ T cells and cause apoptosis of other lymphocytes. In HIV-1-infected patients, the loss of CD4+ cells is associated with lymphocyte activation. Cells undergoing apoptosis lose their structural integrity and exhibit nuclear collapse [68]. We saw a two-fold increase in apoptosis of CD4+ T cells post-infection after stimulation with IL-2 and PHA. *In vivo* priming of CD4 T cells by gp120 and subsequent deletion following T cell triggering may the cause of CD4 destruction and depletion and eventual exhaustion of the T cell repertoire. The envelope protein expressed on the surface of HIV-1-infected cells may prime neighboring uninfected cells for apoptosis by cross-linking the CD4 molecule and therefore induce these uninfected cells for cell death. Apoptosis of CD4+ T cells during chronic immune activation is seen in stimulated CD4+ T cells of CBMCs. The extent of activation-induced apoptosis in CD4+ T cells seems to correlate with disease progression

Taken together, unstimulated CBMCs show a low fraction of central memory T cells, have an ability to modulate central memory T cell activation levels and express much lower levels of CCR5 in contrast to the inflammatory response of stimulated CBMCs. If our postulate that unstimulated CBMCs aptly reflect the quiescent, immunoregulatory environment in utero, then low levels of CCR5 expression in unstimulated CBMCs coupled with an ability to regulate activation levels of central memory T cells may provide important explanations to low levels of MTCT of HIV-1. A comparison between SIV-vaccinated monkeys and untreated monkeys showed a vast difference between the central memory CD4+ T lymphocyte population of each treatment, with the vaccinated group expressing a higher count of CD4+ central memory T cells [64, 69]. A clear survival advantage was seen in rhesus monkeys with the highest counts of central memory T cells. Monkeys with the highest CD4+ central memory T cell counts were able to illicit the strongest immune responses to SIV (IFN-γ, TNF-α, IL-2) [69]. In humans, T cell regenerative capacity is progressively lost in later stages of HIV infection. The loss of T cell regeneration capacity may reflect insufficient regeneration of central memory T cells as a result of excessive differentiation from chronic inflammation [70]. Central memory T cell preservation may be a crucial mechanism to prevent immune dysfunction and loss of T cell homeostasis in humans. Understanding immunoregulatory mechanisms in fetal cells and mapping out the method of central memory T cell preservation may prove necessary to overcoming current challenges in HIV vaccine development as current vaccines fail to induce large fractions of neutralizing antibodies and do not account for the genetic variability of the viral envelope proteins [71]. Further investigation on the mechanisms of immune quiescence and central memory T cell

preservation may aid in moving the field toward the development of vaccines that are designed to elicit lasting levels of HIV protection and cellular immunity.

Immune quiescence in the fetomaternal environment

While immune quiescence in fetal cells is an attractive hypothesis, additional amniotic and placental factors may come into play. Previous observations have suggested that placental Hofbauer cells potentially offset vertical transmission of HIV-1 through induction of immunoregulatory cytokines, providing immunomodulatory and/or antiviral properties [19]. In addition, it is important to study coreceptor expression on T cells of infants who are already infected with HIV-1, as receptor expression in these individuals may serve as a better model for understanding receptor expression and receptor change post-infection. There is also a considerable amount of donor variability in CCR5 and CXCR4 expression in cord blood. Genetic factors play a large role in the amount of CCR5 expression and CXCR4 expression on a cell, and these factors may change over time in cord and adult blood. Elucidating the affinity of viruses for different receptors is crucial in understanding the efficiency of viral replication. New receptors have been found to support HIV-1 replication and should be characterized to see if there is differential expression on various cell types.

This experiment investigated central memory T cell populations in relation to an immune quiescent environment in 10 donors. A greater number of replicates and donors are needed for more conclusive data, as means of CD4+ and CD8+ T cell populations in both cord blood and adult blood could be attributed to donor variability in the subsets. In addition, very low fractions of effector memory T cells were detected in the adult and cord blood samples provided. A larger number of donors would allow for better insight into characterizing this population as response to HIV-1 infection. Futhermore, while a

large fraction of the naïve cell population can be found in cord blood, more analysis is necessary to characterize and understand this population post-infection. While this study examined central memory T cell populations over the course of three days post-infection, further experimentation over a longer period of time would allow for better insight into stimulated and unstimulated memory cell subsets.

Alternate Hypotheses

While CCR5 regulation on central memory T cells in an immune quiescent milieu is an attractive hypothesis to explain the low rate of HIV-1 transmission *in utero*, the paucity of memory cells in cord blood may serve as alternate mechanism of protection against HIV-1 transmission. Past studies have also shown that CCR5 expression is largely restricted to the memory cell subset, with a paucity of memory T cells in cord blood mononuclear cells, or CBMCs [20]. The scarcity of memory T cells in CBMCs, and therefore a lower expression of CCR5, may therefore serve as a mechanism behind low rates of *in utero* transmission of HIV-1. A third explanation for low rates of vertical transmission *in utero* bridges the two aforementioned hypotheses, where CCR5 is not only scarce in cord blood as a result of the low memory population in cord blood but is downregulated on central memory cells in the immune quiescent environment *in utero*.

Conclusion

In conclusion, this study demonstrates how central memory T cell preservation in an immune quiescent environment may serve as a mechanism to limit HIV-1 infection in fetal cells. Understanding correlates of protection at the feto-maternal interface and mechanisms whereby CD4+ cells contribute to the maintenance of immunity may provide pathways to effective antiviral therapies. While this study demonstrate one mechanism of protection against HIV-1infection by central memory T cells, how memory

T cells interplay with other immunomodulatory factors remains evasive and there is still much to be understood in both targeting and defining reservoirs of HIV replication.

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