PRENATAL PRIMING TO MALARIA ANTIGENS INCREASES SUSCEPTIBILITY TO HIV INFECTION

by

KEVIN LEE STEINER

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

Thesis advisor: Christopher L. King, M.D., Ph.D.

Department of Pathology

CASE WESTERN RESERVE UNIVERSITY

January 2012
We hereby approve the thesis/dissertation of

Kevin Lee Steiner

candidate for the Doctor of Philosophy degree *

(signed) Alan D. Levine, Ph.D.
(Chair of the committee)

Christopher L. King, M.D., Ph.D.

Eric J. Arts, Ph.D.

James W. Kazura, M.D.

Zahra Toossi, M.D.

(date) 9/16/2011

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

For my wife, in gratitude for her unwavering support and encouragement.
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 Figure 1-2B was originally published in “Global report: UNAIDS report on the global AIDS epidemic | 2010.” UNAIDS/ONUSIDA 2010. Used with permission.

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SPECIFIC CONTRIBUTIONS

Figures 2-3, 2-5, and 2-6: Latoya Myrie and Leben Tefera assisted with RT assays.

Table I: Elton Mzungu, Kefar Wambua, Charles NgaNga, Alex Osore, and Benjamin Kalayjian assisted with data collection and parasitic infection diagnosis.

Figure 2-4: Elton Mzungu, Kefar Wambua, Charles NgaNga, and Alex Osore performed CBMC malaria recall response cultures. Latoya Myrie assisted with multiplex cytokine assays.

Figures 3-1 and 3-6: Latoya Myrie and Jugnu Shrestha assisted with qPCR assays.
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<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>CBMC</td>
<td>cord blood mononuclear cells</td>
</tr>
<tr>
<td>CCL3</td>
<td>chemokine (C-C motif) ligand 3</td>
</tr>
<tr>
<td>CCL4</td>
<td>chemokine (C-C motif) ligand 4</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>CSA</td>
<td>chondroitin sulfate A</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
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<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ELISPOT</td>
<td>enzyme-linked immunosorbent spot assay</td>
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<tr>
<td>FMO</td>
<td>fluorescence minus one</td>
</tr>
<tr>
<td>geoMFI</td>
<td>geometric mean fluorescence intensity</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iMFI</td>
<td>integrated mean fluorescence intensity</td>
</tr>
<tr>
<td>IPTp</td>
<td>intermittent presumptive treatment protocol</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
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<tr>
<td>LTR</td>
<td>long terminal repeat</td>
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<tr>
<td>MIP-1α</td>
<td>macrophage-inflammatory protein-1α; also CCL3</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>macrophage-inflammatory protein-1β; also CCL4</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
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<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MSP1</td>
<td>merozoite surface protein-1</td>
</tr>
<tr>
<td>MTCT</td>
<td>mother-to-child transmission</td>
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<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>N</td>
<td>sample size</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NVP</td>
<td>nevirapine</td>
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<tr>
<td>OR</td>
<td>odds ratio</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>pg</td>
<td>picograms</td>
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<td>PLAP</td>
<td>placental alkaline phosphatase</td>
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<td>P. falciparum</td>
<td><em>Plasmodium falciparum</em></td>
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<tr>
<td>PfEMP1</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein 1</td>
</tr>
<tr>
<td>PfP0</td>
<td>plasmodium falciparum P0 ribosomal phosphoprotein</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
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<tr>
<td>pRBC</td>
<td>parasitized red blood cells</td>
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<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
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<td>RR</td>
<td>relative risk</td>
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<tr>
<td>RT-qPCR</td>
<td>reverse transcription quantitative polymerase chain reaction</td>
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<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>S. hematobium</td>
<td><em>Schistosoma hematobium</em></td>
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<tr>
<td>-ssDNA</td>
<td>minus strand strong-stop DNA</td>
</tr>
<tr>
<td>STD</td>
<td>sexually transmitted disease</td>
</tr>
<tr>
<td>TB</td>
<td>tuberculosis</td>
</tr>
<tr>
<td>T&lt;sub&gt;CM&lt;/sub&gt;</td>
<td>central memory T cell</td>
</tr>
<tr>
<td>T&lt;sub&gt;EM&lt;/sub&gt;</td>
<td>effector memory T cell</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
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Prenatal Priming to Malaria Antigens Increases Susceptibility to HIV Infection

Abstract

by

KEVIN LEE STEINER

Mother-to-child-transmission of HIV (MTCT) remains a significant cause of new HIV infections in many countries. Maternal risk factors such as high viral load, maternal immune status, vaginal delivery and presence of cervicovaginal infections increase the rate of HIV transmission to the newborn. However, all risk factors for MTCT during the in utero and perinatal periods, especially in developing countries, are not fully understood. To examine whether fetal immune activation as a consequence of prenatal exposure to parasitic antigens increases risk of MTCT, cord blood mononuclear cells (CBMC) from Kenyan versus North American newborns were examined for relative susceptibility to HIV infection in vitro. Kenyan CBMC were 3-fold more likely to be infected with HIV compared to North American CBMC (p=0.03). Fetal sensitization to malaria antigens further enhanced susceptibility to HIV when compared to Kenyan CBMC not sensitized to malaria (p=0.03). CD4+ T cells from malaria sensitized newborns expressed higher levels of CD25 and HLA-DR ex vivo, consistent with increased immune activation. CD4+ T cells were the primary reservoir of infection at day four following virus exposure.
To explore mechanisms for this selective susceptibility, we examined subpopulations of CBMC infected and molecular pathways involved. Effector memory CD3^+CD4^+ T cells (TEM) were the exclusive initial targets of HIV infection with rapid spread to central memory cells (TCM). HIV susceptibility correlated with increased expression of CD25 and HLA-DR on TEM. Virus entered all samples equally, however *gag/pol* RNA was only detected in HIV susceptible samples, suggesting regulation of proviral gene transcription. Targeted analysis of human genes in memory T cells showed greater expression of *IFNG*, *NFATc1*, *IRF1*, *FOS*, and *PPIA* and decreased expression of *YY1* and *TFCP2* in HIV susceptible samples.

Thus prenatal exposure and in utero priming to malaria increases the susceptibility of fetal cells to HIV infection by activating cellular pathways in effector memory T cells that enhance HIV proviral gene transcription. Prevention of parasitic coinfections in HIV positive women during pregnancy to prevent prenatal sensitization may reduce the risk of MTCT.
CHAPTER 1

Introduction
The global HIV/AIDS pandemic remains at the forefront of international health efforts and interventions. Of particular concern is the alarming burden of mother-to-child-transmission (MTCT) of HIV. Although a number of interventions, such as elective caesarean section, peri- and postpartum antiretroviral therapy, and elimination of breastfeeding have been shown to dramatically reduce the risk of MTCT, implementation of these interventions has been difficult in many developing countries (1–3). Indeed, recent estimates suggest that nearly 1800 new HIV infections are transmitted vertically from mother to child daily, translating into more than 500,000 new pediatric infections annually (Figure 1-1) (4). The vast majority of these new pediatric infections occur in sub-Saharan Africa (5).

While many parameters relevant to MTCT have been studied, many questions remain unanswered. Three major areas of focus have been the timing of viral transmission, mechanisms of fetal exposure to HIV, and factors that affect the rate of MTCT.

**Timing of mother-to-child-transmission (MTCT) of HIV**

MTCT can occur during pregnancy (in utero), during labor and delivery (peripartum), and through breastfeeding (postpartum) with up to 45% of HIV positive women transmitting the virus to their offspring in the absence of any intervention (1, 5–8). Much of our understanding of the timing of vertical transmission of HIV comes from the meta-analysis of clinical trials with a variety
of interventions (5–7). A randomized study performed in Nairobi, Kenya demonstrated substantial reduction in MTCT rates when exclusive formula feeding was substituted for breastfeeding, suggesting that nearly 40% of all transmission events occur during the postpartum period (9). Of the remaining 60% of transmission events, the majority occur late in gestation or during the delivery itself (5–7). The first major study to demonstrate this was the Pediatric AIDS Clinical Trials Group 076 (PACTG 076) clinical trial of the anti-retroviral drug zidovudine given intravenously to pregnant, HIV positive women beginning between 14 and 34 weeks of gestation and continuing through delivery with subsequent treatment of the newborn for six weeks (10). This drug therapy intervention reduced the transmission rate by two-thirds, suggesting that approximately 67% of MTCT events occur late in gestation (10). In a further study combining the PACTG 076 zidovudine regimen with elective caesarean section, the rate of MTCT was reduced to 1-2% (11). Similarly the PETRA study performed in Tanzania, South Africa, and Uganda demonstrated that maternal treatment with a combination of zidovudine and lamivudine from 36 weeks of gestation to delivery with subsequent treatment of the newborn for one week reduced MTCT by 52% (12). The HIVNET 012 study examined the impact on MTCT risk of the non-nucleoside reverse-transcriptase inhibitor nevirapine provided to mothers in a single, intrapartum dose with a single dose given to the offspring within seventy-two hours of delivery (13). Though concern has been raised regarding the emergence of nevirapine resistance in women and vertically infected newborns receiving this single-dose protocol, this intervention reduced
the percentage of HIV-infected infants to 8.2% at birth (13–19). The DITRAME Plus and Mashi studies supplemented single-dose, intrapartum nevirapine to zidovudine initiated at week 34-36 of gestation with resultant rates of MTCT of 6.5 and 5.3%, respectively (20, 21). Overall, these data suggest that HIV is transmitted in utero and prior to week 36 of gestation to approximately 5% of newborns of HIV infected women in the absence of any intervention. Further, HIV is transmitted to approximately 20% of offspring of HIV infected women from gestational week 36 through delivery without intervention.

Combined, these and other clinical trials with varying drug and timing regimens present strong evidence that the majority of non-postpartum transmission events occur late in pregnancy or peripartum with very little transmission occurring prior to 14 weeks gestation. Using these data, an estimation of MTCT timing has been developed in which approximately 25% of infants born to HIV positive women will become infected in the absence of any anti-retroviral intervention and without breastfeeding (5, 7). Of these transmission events, 80% occur from 36 weeks through delivery, while 20% occur earlier in gestation (5, 7).

Mechanisms of fetal HIV exposure

Routes of exposure to HIV, particularly during the in utero and peripartum periods remain controversial and the subject of ongoing study. One hypothesis put forth by Van de Perre is the so-called “all mucosal” route of exposure (22). Under this hypothesis, most vertically transmitted infections occur as the result of
fetal mucosal exposure to free-virus containing amniotic fluid in utero, cervicovaginal secretions intrapartum, or breast milk postpartum (22). This hypothesis is bolstered by in vitro evidence that human intestine is capable of being infected through a non-CD4/chemokine coreceptor pathway (23). Further, the beneficial effect of elective caesarean section on reduction of MTCT and the converse observation that elevated levels of free HIV virions in birth canal secretions increases risk of MTCT provide support for a role of mucosal exposure in intrapartum vertical transmission (24, 25). However, neither birth canal disinfection with chlorhexidine prior to vaginal delivery nor emergent caesarean section, both of which reduce mucosal viral exposure in the birth canal, have been demonstrated to significantly reduce the rate of MTCT, suggesting that alternative mechanisms of fetal viral exposure may also be important (25, 26).

One such alternative mechanism is disruption of the maternal-fetal barrier with placental microtransfusions of maternal blood into the fetal circulation (27, 28). In addition to HIV, placental microtransfusions have been implicated in the vertical transmission of a variety of viral agents including multiple hepatitis subtypes (29–31). Utilizing an assay to measure placental alkaline phosphatase (PLAP), a glycoprotein synthesized by maternal placental components and size-excluded from diffusion into the fetal circulation in the absence of placental disruption, maternal-fetal microtransfusion was shown to be an independent risk factor for MTCT of HIV (27). This observation suggests that direct maternal
blood-fetal blood contact may mediate viral transmission and establishes circulating fetal leukocytes as potential targets of infection.

**Maternal factors impacting MTCT**

A variety of maternal factors have been studied and demonstrated to increase the risk of MTCT. The most consistently reported risk factor is elevated maternal plasma viral load (2, 5–7, 32–34). There is no threshold below which MTCT cannot occur, but a clear correlation between maternal viral load and vertical transmission has been described with the reported risk increasing 65–800% for each log₁₀ increase in viral load (27, 32, 35). Thus maternal viral load represents an important risk factor for MTCT; however the wide variation in reported risk implies that other factors may also play determinant roles. Indeed, vaginal delivery, prolonged rupture of membranes, premature delivery, chorioamnionitis, and maternal infection with other sexually transmitted diseases (STD) have all been associated with increased MTCT (2, 36–38). Though some of these factors such as maternal syphilis infection can increase maternal HIV viral load, all remain independent risk factors for MTCT after statistical adjustment for maternal viral load (38, 39). The mechanism by which these conditions increase the risk of MTCT has not been established. It has been hypothesized that, particularly in the cases of maternal STD, chorioamnionitis, and ascending bacterial infections of the female reproductive tract, fetal exposure to pathogens or pathogen antigens may increase the susceptibility of the fetus to
HIV transmission (40). However, few in vitro or mechanistic studies have been undertaken to address this hypothesis.

The impact of maternal malaria infection during pregnancy on MTCT is of particular interest in the context of sub-Saharan Africa where significant geographic overlap of malaria and HIV prevalence exists and where coinfections are common (Figure 1-2). There is increasing evidence for a bi-directional interaction between malaria and HIV in pregnancy leading to changes in maternal immune responses and disease progression (41, 42). Coinfection with HIV increases peripheral, placental, and fetal cord blood malaria parasite densities, and peripheral malaria parasitemia increases peripheral HIV viral load (43–45). Further, elevations in both peripheral and placental HIV viral load have been associated with the presence of the clinical condition termed placental malaria (46). These observations, coupled with the correlation between maternal plasma viral load and increased risk of MTCT, have led some to hypothesize that placental malaria may increase the risk of vertical transmission of HIV.

**Placental malaria and MTCT**

Infection with *Plasmodium falciparum*, the predominant malaria species in sub-Saharan Africa, during pregnancy may result in accumulation of parasitized red blood cells (pRBC) in the intervillous space of the placenta, a condition termed placental malaria (47) (Figure 1-3). Clinically, placental malaria may
Figure 1-2: Global prevalence of malaria due to *P. falciparum* and HIV. A) Global limits and endemicity of *P. falciparum* in 2007. The land area was defined as no risk (light grey), unstable risk (medium grey areas, where *PfAPI* <0.1‰ PA), and stable risk (where *PfAPI* >0.1‰ PA) with endemicity (*PfPR* in the 2- up to 10-year age group, *PfPR*2–10) displayed as a continuum of yellow to red between 0% and 100%. The dashed lines separate the Americas, Africa+, and the CSE Asia region, respectively, from left to right. The seven countries with thick blue borders have very low *P. falciparum* burden and reliable national health information systems. Credit: originally published in Hay SI, Okiro EA, Gething PW, Patil AP, Tatem AJ, et al. (2010) Estimating the Global Clinical Burden of *Plasmodium falciparum* Malaria in 2007. PLoS Med 7(6): e1000290. doi:10.1371/journal.pmed.1000290. Used with permission. B) Global prevalence of HIV, 2009. Credit: originally published in “Global report: UNAIDS report on the global AIDS epidemic | 2010.” UNAIDS/ONUSIDA 2010. Used with permission.
Figure 1-3: The life cycle of *Plasmodium falciparum* malaria in a pregnant woman. Malaria infection occurs following (a) the injection of sporozoite-stage parasites during the blood-meal of an infected, female mosquito of the genus *Anopheles*. Within minutes, these sporozoites rapidly invade (b) hepatocytes in the liver; (c) extensive parasite multiplication occurs in these cells over the following 5–8 days. (d) Eventually, the infected hepatocytes burst and each one releases as many as tens of thousands of merozoite-stage parasites into the blood circulation. (e) The merozoites invade red blood cells (RBCs), and (f) parasites continue to multiply in the RBCs. Each RBC-multiplication cycle takes 48 h and results in the production of 20–32 new merozoites, each capable of invading a new RBC. During their development in the RBCs, the parasites insert proteins into the RBC membrane, including the very variable molecule *P. falciparum*-infected erythrocyte membrane protein 1 (PfEMP1). The PfEMP1 molecules mediate adhesion of the parasitised RBCs to host tissues (and therefore sequestration in some tissues), thus allowing them to avoid destruction during the otherwise inevitable passage through the spleen. PfEMP1 variants exist that have specificity for many different host ligands, including variants that are specific for adhesion to chondroitin sulphate A (CSA). (g) CSA is a host-derived molecule, prevalent in the syncytiotrophoblast of (the placenta of) pregnant women. (h) Some merozoites differentiate into non-dividing male or female gametocytes, which can be transmitted to female mosquitoes as they feed. (i) Once inside the mosquito, gametocytes transform into male and female gametes, respectively, and unite to form a zygote in the mosquito midgut. Eventually, zygotes mature to form oocysts, which divide within oocysts on the external gut wall to form thousands of sporozoites. The sporozoites migrate through the mosquito haemocoele to the salivary glands, ready for injection into a human host, thereby completing the life cycle. Credit: originally published in Lars Hviid (1998) Clinical disease, immunity and protection against *Plasmodium falciparum* malaria in populations living in endemic areas. Exp. Rev. Mol. Med. 24 June, http://www-ermm.cbcu.cam.ac.uk/lhc/txt001lhc.htm. Used with permission from Cambridge University Press.
result in severe maternal anemia, intrauterine growth retardation, preterm delivery, and an increased risk of fetal and maternal death (48–55). This placental accumulation is thought to be mediated in part by the interaction of parasite derived *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) expressed on the surface of pRBCs with chondroitin sulfate A (CSA) which is highly expressed in the syncytiotrophoblast of the human placenta (56, 57). Placental malaria is more common in primigravid and secundigravid women suggesting that women may eventually develop an immune response capable of disrupting the PfEMP1-CSA interaction (57). However, in the setting of maternal coinfection with HIV and malaria, the protective effect of multiparity on the occurrence of placental malaria is diminished (42, 43, 48, 58).

Placental malaria alters the local placental environment in a variety of ways (51). Histologically, in addition to the accumulation of trophozoite and schizont stage pRBCs, placental malaria is characterized by increased numbers of infiltrating maternal phagocytic cells and deposition of haemozoin, a pigment created by degradation of hemoglobin by malaria parasites (51, 59). Structurally, the syncytiotrophoblast becomes thickened resulting in abnormal blood flow and often placental insufficiency (47). Alterations in the immunologic milieu of the placenta have been noted. Parasite sequestration stimulates production of the β-chemokines macrophage-inflammatory protein-1α and β (MIP-1α or CCL3 and MIP-1β or CCL4) by maternal monocytes (60). Further, surface expression of the HIV β-chemokine coreceptor CCR5 is upregulated on placental macrophages by malaria infection (61). Both pro- and anti-inflammatory cytokines are
enhanced in maternal placental samples as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-10 (IL-10) may be upregulated in the context of placental malaria (60, 62).

Yet epidemiological studies of the impact of placental malaria on MTCT of HIV have yielded conflicting results (Table 1). Several studies have described increased risk of MTCT in the setting of placental malaria. Within a Ugandan-based, prospective study of the impact of STD treatment during pregnancy, Brahmbhatt et al. found a marked impact of placental malaria on MTCT of HIV (63). Though the sample sizes were limited, histopathological diagnosis of placental malaria was associated with a statistically significant relative risk (RR) of 2.60 for HIV-1 vertical transmission, an association that became stronger (RR = 2.89) after adjustment for maternal HIV viral load (63). A subsequent paper from the same group based on a larger cohort and more sensitive methods of detecting placental malaria revealed a more striking impact with an overall relative risk for MTCT of 7.9, adjusted for maternal viral load, in the presence of placental malaria compared to its absence (64). Considering solely mother-infant pairs where the maternal viral load was low, presence of placental malaria resulted in a MTCT rate of 28.6% compared to a reported rate of 0% in its absence (64). A similar impact of placental malaria was described in a recently published retrospective, nested case-control study from a cohort recruited in Rwanda during the early 1990s (65). Univariate analysis showed a significantly increased risk of MTCT with placental malaria at an odds ratio (OR) of 5.6 (65).
**Table I: Epidemiologic studies of impact of placental malaria on risk of MTCT of HIV.**

<table>
<thead>
<tr>
<th>Study site</th>
<th>Study purpose</th>
<th>PM diagnosis</th>
<th>Freq. PM in HIV+ women</th>
<th>ARV</th>
<th>Freq. MTCT</th>
<th>Stratified</th>
<th>Unadjusted risk of PM</th>
<th>Adjusted risk of PM</th>
<th>MTCT by PM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brahmbhatt et al.</td>
<td>Uganda: STD prevention during pregnancy on risk of MTCT</td>
<td>histopathology</td>
<td>13.6%</td>
<td>none</td>
<td>18/93</td>
<td></td>
<td>RR = 2.60 (1.16-5.84)</td>
<td>aRR = 2.89 (1.12-7.52)</td>
<td>0.03</td>
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<tr>
<td>Brahmbhatt et al.</td>
<td>Uganda: STD prevention during pregnancy on risk of MTCT</td>
<td>histopathology, immunohistochemistry</td>
<td>37.1%</td>
<td>none</td>
<td>19/109</td>
<td></td>
<td>RR = 3.6 (1.3-10.1)</td>
<td>&lt;0.05 aRR = 7.9 (1.4-58.5)</td>
<td>0.03</td>
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<tr>
<td>Bulterys et al.</td>
<td>Rwanda: Prospective cohort study of impact of HIV on pregnancy and outcomes</td>
<td>histopathology, immunohistochemistry</td>
<td>55.0%</td>
<td>none</td>
<td></td>
<td></td>
<td>OR = 5.6 (1.4-21.9)</td>
<td>aOR = 6.3 (1.4-29.1)</td>
<td>0.51</td>
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<td>Inion et al.</td>
<td>Kenya: Chlorhexidine vaginal lavage on risk of MTCT</td>
<td>histopathology</td>
<td>9.1%</td>
<td>none</td>
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<tr>
<td>Mwapasa et al.</td>
<td>Malawi: Maternal syphilis infection on risk of MTCT</td>
<td>histopathology; active vs. past</td>
<td>Overall 43%</td>
<td>NVP</td>
<td>in utero: 65/751 Past: 89/507</td>
<td>Past PM MTCT in utero:</td>
<td>RR=1.28 (0.73-2.24)</td>
<td>0.39</td>
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<td>Past: 23.7%</td>
<td></td>
<td>Intrapartum/ postnatal: 46/324</td>
<td>Past PM MTCT intrapartum/postnatal</td>
<td>RR = 1.13 (0.69-1.83)</td>
<td>0.63</td>
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<td>Active: 19.2%</td>
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<td>Active PM MTCT in utero:</td>
<td>RR = 1.02 (0.53-1.97)</td>
<td>0.94</td>
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<td></td>
<td>Active PM MTCT intrapartum/postnatal</td>
<td>RR = 0.7 (0.55-1.71)</td>
<td>0.92</td>
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<tr>
<td>Study site</td>
<td>Study purpose</td>
<td>PM diagnosis</td>
<td>Freq. PM in HIV+ women</td>
<td>ARV</td>
<td>Freq. MTCT</td>
<td>Stratified</td>
<td>Unadjusted risk of PM</td>
<td>P</td>
<td>Adjusted risk of PM</td>
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<tr>
<td>Msamanga et al.</td>
<td>Malawi, Zambia, Tanzania</td>
<td>Antibiotics to reduce chorioamnionitis on risk of MTCT</td>
<td>6.3%</td>
<td>NVP</td>
<td>126/1662</td>
<td></td>
<td>OR = 1.17 (0.57-2.37)</td>
<td>0.67</td>
<td>aOR = 1.06 (0.51-2.20)</td>
<td>0.87</td>
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<td>PM with maternal viral load &lt;55,000 copies/ml</td>
<td>0.11</td>
<td>aOR = 1.99 (0.81-4.90)</td>
<td>0.14</td>
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<td>PM with maternal viral load &gt;55,000 copies/ml</td>
<td>0.21</td>
<td>aOR = 0.51 (0.15-1.74)</td>
<td>0.28</td>
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<td>PM with maternal viral load &lt;55,000 copies/ml</td>
<td>0.11</td>
<td>aOR = 1.99 (0.81-4.90)</td>
<td>0.14</td>
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<td></td>
<td>PM with maternal viral load &gt;55,000 copies/ml</td>
<td>0.21</td>
<td>aOR = 0.51 (0.15-1.74)</td>
<td>0.28</td>
</tr>
<tr>
<td>Naniche et al.</td>
<td>Mozambique</td>
<td>IPTp on risk of MTCT</td>
<td>NVP</td>
<td>19/153</td>
<td></td>
<td></td>
<td>OR = 0.41 (0.13-1.25)</td>
<td>0.01</td>
<td>aOR = 0.23 (0.06-0.89)</td>
<td>0.03</td>
</tr>
<tr>
<td>Ayisi et al.</td>
<td>Kenya</td>
<td>Placental malaria on risk of perinatal MTCT</td>
<td>25.0%</td>
<td>none</td>
<td>102/512</td>
<td></td>
<td>RR = 0.6 (0.4-1.0)</td>
<td>0.05</td>
<td>aRR = 0.4 (0.3-0.7)</td>
<td>0.001</td>
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<td></td>
<td>PM low density PM&lt;sub&gt;h&lt;/sub&gt; vs high density PM&lt;sub&gt;1&lt;/sub&gt;</td>
<td>aRR = 0.4 (0.2-0.6)</td>
<td>0.001</td>
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<td></td>
<td>low density PM&lt;sub&gt;h&lt;/sub&gt; vs high density PM&lt;sub&gt;1&lt;/sub&gt;</td>
<td>aRR = 2.0 (1.1-3.9)</td>
<td>0.04</td>
<td></td>
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</tbody>
</table>

Abbreviations: ARV - anti-retroviral treatment, PM - placental malaria, NVP - nevirapine, OR - odds ratio, aOR - adjusted odds ratio, RR - relative risk, aRR - adjusted RR

<sup>a</sup> Adjusted for maternal viral load.
<sup>b</sup> Adjusted for maternal CD4% and ultrasensitive p24 antigen.
<sup>c</sup> Adjusted for maternal plasma viral load, CD4<sup>+</sup> cell counts, trichomoniasis, bacterial vaginosis, preterm birth, low birth weight, and randomization arm.
<sup>d</sup> Adjusted for CD4<sup>+</sup> cell counts, trichomoniasis, bacterial vaginosis, preterm birth, low birth weight, and randomization arm.
<sup>e</sup> Includes active and past placental malaria.
<sup>f</sup> Adjusted for age, gravidity, and IPTp treatment group.
<sup>g</sup> Adjusted for plasma viral load and episotomy or perineal tear.
<sup>h</sup> <10,000 parasites/μl
<sup>i</sup> ≥10,000 parasites/μl
Adjustment for the maternal CD4⁺ T cell percentage and ultrasensitive p24 antigen level (a surrogate for the unavailable maternal viral load) resulted in a calculated OR of 6.3 for MTCT with placental malaria (65). Together these studies present evidence that placental malaria may be an important risk factor for MTCT, particularly when maternal viral loads are low.

In contrast to these results, several studies have found no evidence of increased risk of MTCT with placental malaria. A retrospective analysis of the nontreatment arm of a chlorhexidine vaginal lavage study on the coast of Kenya found that placental malaria was not associated with an increased risk of MTCT (66). Interestingly, no relationship between maternal malaria and viral load was found in contrast to other published reports (44–46, 66). While aimed primarily at determining the impact of syphilis on MTCT, Mwapasa et al. also found no association between placental malaria and in utero or peripartum MTCT (67). Similarly, a large, multi-country clinical trial of antibiotics to reduce chorioamnionitis-associated MTCT found no overall association between placental malaria and MTCT, though there was a trend toward increased MTCT with placental malaria at low baseline maternal viral loads (68). In a study based in Mozambique and designed to assess the efficacy of an intermittent presumptive therapy protocol (IPTp) for the prevention of malaria during pregnancy, univariate analysis revealed no association between active or past placental malaria and MTCT (69). After adjusting for maternal viral load, anemia and malaria prophylaxis, placental malaria was significantly associated with lower MTCT with an adjusted OR of 0.23 suggesting a possible protective effect (69).
Finally, a study performed in Western Kenya described a complex relationship between parasite density of placental malaria and vertical transmission of HIV (44). When stratifying solely on the presence or absence of histopathologically diagnosed placental malaria, a striking 40% reduction in risk for MTCT was found (44). Further analysis demonstrated that the protective effect of placental malaria remained significant in the context of low density parasitemia (<10,000 parasites/μl), but high density parasitemia (>10,000 parasites/μl) was associated with a two-fold increased risk of MTCT (44). Mechanisms for this discordant effect of low versus high density parasitemia were not explored, though postulated roles for the timing of maternal malaria infection and changes in local immunologic placental environment due to parasitemia were put forth (44).

These epidemiological studies and their conflicting results suggest the relationship between placental malaria and MTCT of HIV is complex. Some have sought to attribute the variability in the results to differences in technique of placental malaria diagnosis, statistical methods and power, epidemiology of malaria transmission, or the ecology of study locations whether urban or rural (6, 42, 48, 63–65). However, none of these studies took into account the potential impact of fetal factors which may influence susceptibility of fetal cells to HIV infection and thus MTCT. One such potential factor is activation of the fetal immune system in response to malaria antigens, a situation termed prenatal sensitization.
Prenatal sensitization to malaria antigens

Prenatal sensitization to malaria has been demonstrated in various countries across Africa and in response to in vitro stimulation of CBMC with a variety of *P. falciparum* antigens (70–75). In these studies, sensitization has been defined as lymphoproliferation and/or production of cytokines as measured by enzyme-linked immunosorbent assay (ELISA) or enzyme-linked immunosorbent spot assay (ELISPOT) in response to in vitro culture with primarily malaria asexual stage antigens. The reported frequency of prenatal sensitization has ranged from being a rare event to occurring in a majority of newborns depending on the type and number of antigens used (70, 72, 74, 76, 77). Importantly, prenatal sensitization may be observed despite a lack of documented maternal peripheral or placental malaria parasitemia during gestation or at delivery and conversely, not all malaria-infected women expose their fetus to antigens resulting in sensitization (70–73). While factors influencing prenatal sensitization are not clear, it is likely impacted by the duration, timing, and intensity of parasite infection during pregnancy. Additional factors may include the integrity of the placenta and the ability of the pregnant woman to generate appropriate antibody responses to parasite antigens as immune complexes are known to be an important mechanism for transplacental transfer of malaria antigens (78).

On the coast of Kenya, several studies have demonstrated prenatal sensitization to malaria (70, 73, 79–81). When a panel of three *P. falciparum* blood stage antigens were used (MSP-119, PfP0, EBA-175), prenatal
sensitization was reported for 66% of all newborns (70). Of note, CBMC cytokine responses differed from maternal peripheral blood mononuclear cell responses. Maternal responses typically showed prominent IFN-γ and IL-2 production while cord blood responses had greater variability with 38% demonstrating elevated IFN-γ and IL-2, 38% elevated IL-5 and IL-13, and 24% a mixture of Th1 and Th2 cytokines (70). Several studies have also revealed prominent production of IL-10 by malaria sensitized CBMC, suggesting a potential immunoregulatory response (73, 75, 81). Indeed, the existence of a malaria-specific CD4⁺CD25⁺ population of CBMC has been described that is capable of broad immunosuppressive effects on antigen presenting cells and both CD4⁺ and CD8⁺ T cells (75, 82).

Thus, prenatal sensitization to malaria antigens is a consistently reported phenomenon occurring with variable frequency and the presence of these recall responses to malaria antigens provides de facto evidence of a prior, in utero immune activation event (70, 73–77, 79). As immune activation is crucial to both susceptibility of target cells to infection and HIV disease progression, the major hypothesis explored here is that prenatal activation of the fetal immune system renders cord blood mononuclear cells (CBMC) more susceptible to infection by HIV.

*Immune activation and the HIV life cycle*

The life cycle of HIV may be broadly separated into two distinct phases (83–85). Early events include binding of free virus to CD4 and a chemokine coreceptor on the target cell, fusion of the viral and cell membranes with insertion
of the viral genome, reverse transcription from single-stranded RNA to double-stranded DNA, translocation to the nucleus, and integration of the double-stranded DNA into the host genome (84, 85). Subsequent late events occur throughout the lifetime of the infected cell and are characterized by transcription and translation of virally encoded genes, assembly into viral particles, and the release of infectious virions (84, 85). While regulation of the progression through these stages is complex, immune activation has consistently been shown to facilitate successful HIV replication (84, 85). Three stages of the HIV life cycle have been particularly linked with cellular activation state: expression of CCR5 on the target cell surface and viral binding, progression through reverse transcription, and production of virally encoded genes (40).

Binding of free HIV virus to a target cell is mediated by the interaction of the viral envelope protein gp120 with CD4 and a chemokine receptor on the surface of the host cell (84–86). Slight differences in sequence of the V3 loop of gp120 determine usage of CXCR4 or CCR5 with most transmitted strains utilizing CCR5 (85, 87, 88). Though expression of CD4 alone is sufficient for viral attachment to the cell surface, viral entry and subsequent establishment of infection do not occur in the absence of an appropriate chemokine coreceptor (86). Individuals with a genetic deficiency of CCR5 are largely resistant to HIV infection (89). Therefore surface expression of functional CCR5 is an important factor in the context of newly acquired infection. Adult lymphocytes tend to upregulate this chemokine receptor upon activation with effector T cells and monocytes expressing high levels of CCR5 while naïve T cells do not (90–92).
Similarly, though ex vivo expression of CCR5 on CBMC from healthy newborns is low, in vitro activation results in dramatic upregulation with a corresponding increase in susceptibility to HIV infection (93, 94).

Upon successful virus binding to a host cell, fusion of the viral and cellular membranes occurs and the viral genome is inserted in the cytoplasm where the highly ordered process of reverse transcription is initiated (84, 85). Incompletely reverse-transcribed and highly labile HIV genome fragments have been found in resting lymphocytes from both adult and cord blood following in vitro infection (95–97). When these resting lymphocytes containing partial reverse transcripts were subsequently activated in vitro, patent infection with viral replication was achieved (97). It has been hypothesized that low availability of cytoplasmic deoxynucleotides in resting lymphocytes may account for the observed block in reverse transcription (86). These observations suggest that, while binding to and insertion of the HIV genome may occur in quiescent lymphocytes, completion of reverse transcription may be a bottleneck for completion of the HIV life cycle.

Upon successful completion of reverse transcription, the double-stranded DNA proviral genome is transported into the nucleus and integration into the host genome occurs (84–86). Though some retroviruses depend upon host cell progression through mitosis for successful nuclear entry and integration, HIV does not (86). However, while nuclear import and integration of HIV may be less dependent on activation state, transcription of virally encoded genes is greatly influenced by cellular activation signals acting on the proviral 5’ long terminal repeat (LTR) (40, 85, 86). Within the 5’ LTR is a modulatory enhancer region
containing binding sites for a variety of host cellular transcription factors, most notably nuclear factor-κB (NF-κB), activator protein 1 (AP-1), and nuclear factor of activated T cells (NFAT) (40, 85). Cell signal pathways due to exposure of the cell to stimuli – whether cytokines such as TNF-α, IL-1, and IL-6; toll-like receptor ligands; or TCR engagement – enhance the activity of these transcription factors on both host and proviral gene response elements leading to increased transcription (40, 85, 98–100). The 5’ LTR also contains binding sites for transcription factors that suppress gene expression such as YY1 and TFCP2 which cooperate in the recruitment of histone deacetylase resulting in alteration of the chromatin structure to a transcriptionally inactive form (101–104). Thus host cell activation status has a significant and direct effect on proviral gene transcription.

Central hypothesis

Therefore, as prenatal sensitization by definition implies fetal immune activation, this thesis sought to test the hypothesis that CBMC from malaria sensitized newborns are more susceptible to in vitro HIV infection compared to malaria not sensitized CBMC, to identify the initial target cells of HIV in this model system, and to describe the mechanisms by which prenatal immune activation mediates enhanced susceptibility to HIV infection.
CHAPTER 2

Fetal immune activation to malaria antigens enhances susceptibility to in vitro HIV infection in cord blood mononuclear cells.
Abstract

Mother-to-child-transmission of HIV (MTCT) remains a significant cause of new HIV infections in many countries. To examine whether fetal immune activation as a consequence of prenatal exposure to parasitic antigens increases risk of MTCT, cord blood mononuclear cells (CBMC) from Kenyan versus North American newborns were examined for relative susceptibility to HIV infection in vitro. Kenyan CBMC were 3-fold more likely to be infected with HIV compared to North American CBMC (p=0.03). Kenyan CBMC with recall responses to malaria antigens demonstrated enhanced susceptibility to HIV when compared to Kenyan CBMC lacking recall responses to malaria (p=0.03). CD4+ T cells from malaria sensitized newborns expressed higher levels of CD25 and HLA-DR ex vivo, consistent with increased immune activation. CD4+ T cells were the primary reservoir of infection at day four following virus exposure. Thus prenatal exposure and in utero priming to malaria may increase the risk of MTCT.
Introduction

Malaria and HIV infections are leading causes of death in children and adults respectively in Africa (4, 105). Morbidity attributable to these pathogens is particularly high during pregnancy and the neonatal time period. Pregnant women infected with malaria or HIV have increased risk of adverse events and outcomes including anemia, spontaneous abortion, premature delivery, low neonatal birth weight and increased infant mortality (54, 55, 106–109). Maternal co-infection with both malaria and HIV may result in worse birth outcomes than either infection alone. Malaria infected, HIV positive pregnant women experience increased frequency of placental malaria, higher parasitemia, and clinical disease compared to HIV negative women (43, 48, 64, 66, 108, 110, 111). Conversely, malaria infection has been shown to increase HIV viral load and damage the placenta causing microtransfusions from maternal to fetal circulation, both of which are important risk factors in the vertical transmission of HIV from mother to child (MTCT) (46, 112–115).

It is unclear if malaria coinfection of HIV positive women increases the risk for MTCT. Epidemiologic studies addressing this question have produced conflicting results (41). A study in Uganda found placental malaria was associated with an approximately 3-fold greater risk for HIV vertical transmission (63). Similarly, a study in Cameroon found a positive association between MTCT and the peak rainy season when malaria transmission was high, suggesting a possible correlation between MTCT and maternal malaria infection, though malaria burden was not directly assessed (116). By contrast, no association
between placental malaria and increased risk of MTCT was found on the Kenyan coast, which had lower infection levels compared to the Uganda and Cameroon studies (66). Interestingly, a large study from western Kenya showed that high density placental parasitemia was associated with increased risk of MTCT while low density placental parasitemia was not (44), suggesting intensity or frequency of placental malaria may explain differences among studies.

These studies suggest the relationship between placental malaria and MTCT is complex. No study, however, has evaluated the potential impact of fetal factors on the risk of MTCT. For example, immune activation is vital for HIV infection, thus in utero exposure to exogenous antigens and consequent fetal lymphocyte activation may increase susceptibility to HIV infection (96, 97). Newborns of mothers infected with malaria and other intravascular parasites such as schistosomiasis and lymphatic filariasis are often primed to parasite antigens (70, 76, 77, 117, 118). This activation of cord blood mononuclear cells (CBMC) could increase risk for HIV infection if exposed to virus in utero or perinatally. In support of this hypothesis, we previously demonstrated that helminth coinfections in HIV positive women during pregnancy increase the risk for MTCT, especially among newborns demonstrating in utero sensitization to helminth antigens (80). Here we tested the hypothesis that CBMC from Kenyan newborns of women infected with malaria exhibit greater susceptibility to HIV infection in vitro compared to CBMC collected from newborns of parasite non-infected Kenyan or North American women. We further show that Kenyan
CBMC demonstrating in utero priming to malaria blood stage antigens exhibit the greatest susceptibility to HIV infection in vitro.
Materials and Methods

Study population

Umbilical cord blood was obtained from newborns delivered at the Msambweni District Hospital, Coast Province, Kenya, an area endemic for malaria, schistosomiasis, lymphatic filariasis and intestinal helminthes (81, 117). The mothers were assessed for helminth and malaria infection during repeated assays collected during antenatal clinical visits. All women were tested for HIV with the offspring of HIV positive women excluded from the current study. Negative control cord blood was collected from healthy North American newborns delivered at University Hospitals of Cleveland (Cleveland, OH). Ethical approval was obtained from the Institutional Review Boards of University Hospitals of Cleveland and the Kenya Medical Research Institute in Nairobi, and informed consent for the study was obtained from study subjects.

Determination of maternal parasite infection

Malaria infection was determined by blood smear and/or PCR of maternal peripheral and intervillous blood; urinary schistosomiasis was assessed by presence of ova in the urine; lymphatic filariasis by antigen detection of peripheral blood; and intestinal helminth infections by stool examination as previously described (81).
CBMC sample collection and sensitization determination

CBMC were isolated by Ficoll-Hypaque density gradient separation. A subset of freshly collected CBMC was immediately cultured with media alone, malaria antigens: recombinant malaria surface protein 1-42 (MSP1-42) 3D7 and MSP1-42 FVO (5 µg/ml each, kindly provided by Sanjay Singh, Carole Long and David Narum of Malaria Vaccine Development Unit, National Institutes of Health), pooled MSP1-42 peptides previously determined to be dominant CBMC epitopes in this population (79) (10 µg/ml for each peptide), *Plasmodium falciparum* ribosomal phosphoprotein peptides (PfP0, 10 µg/ml), and phytohemagglutinin (PHA, 1 µg/ml). Remaining CBMC were cryopreserved.

Quantification of IL-2, IL-5, IFN-γ, IL-10, and IL-13 was performed on culture supernatants collected at 96 hours using multiplex immunoassay (Millipore). Results were expressed in pg/ml by interpolation from standard curves. A positive response was scored when the following two criteria were fulfilled: 1) a net value for antigen-stimulated cells that was at least 2-fold greater than that of parallel cultures containing medium alone and 2) responses to two or more malaria antigens. If cytokine production was not detectable in the negative control cultures, then >40 pg/ml cytokine was considered to be a positive response. Figure 2-4 depicting recall responses was created using Mayday software (119).
In vitro HIV infection

Cryopreserved CBMC were thawed (all samples showed >85% viable cell recovery with preservation of immune function as indicated by similar recall responses to malaria antigens between fresh and cryopreserved aliquots from the same cord blood sample) and rested overnight in RPMI-1640 + L-glutamine supplemented with penicillin/streptomycin (100 units/100mg/ml), HEPES (10 mM), and 10% pooled human AB serum (cRPMI+10%). From this point forward, culture media consisted of cRPMI+10% supplemented with 1.0 ng/ml recombinant human IL-2 (rhIL-2, BD Biosciences). Infection with the R5 tropic virus HIVBal (kindly provided by Dr. Eric Arts, Case/UHC Center for AIDS Research) was performed as follows: triplicate wells of 200,000 cells/well (1 x 10^6 cell/ml) in a 96-well tissue culture plate were cultured in the following conditions: 1) media alone, 2) immediate HIVBal exposure with subsequent culture in media alone (virus + media), 3) immediate HIVBal exposure with subsequent culture in media containing 5.0 µg/ml PHA (Sigma-Aldrich, virus + PHA), 4) three days of culture in media followed by HIVBal exposure (media + virus), 5) three days of culture in media containing pooled MSP1-42 peptides at 10 µg/ml each followed by HIVBal exposure (malaria + virus), and 6) three days of culture in media containing 5.0 µg/ml PHA followed by HIVBal exposure (PHA + virus). Initially, a dose response curve was created to determine the optimal multiplicity of infection (MOI) (Figure 2-1). Subsequently, cells were exposed to virus at an MOI of 0.001 for four hours at 37°C after which cells were centrifuged, washed with fresh media and resuspended according to the conditions outlined above. Every three
days, half the culture volume was removed, frozen and replaced with fresh media containing appropriate supplements.

**RT Assay**

The removed culture media was stored at -80°C prior to the RT assay which was performed according to standard protocols (120). Briefly, 10 μl culture supernatant was incubated with 25 μl RT mix composed of Tris-HCl, KCl, dithiothreitol (DTT), MgCl2, Poly r(A) template, poly d(T) primer, NP-40, and α-32P dTTP for two hours at 37°C. 10 μl of this reaction mixture was spotted onto Whatman filter paper which was washed successively with 1X SSC and 85% ethanol and allowed to dry. A known quantity of virus stock was included in all RT assays to verify consistency. Counts per minute per microliter culture supernatant (cpm/μl) were quantified using a beta counter.

**Flow Analysis**

One million cells were washed and stained with fluorochrome labeled antibodies for 30 minutes in the dark at 4°C. For analysis of HIV infection the following antibodies were used: CD3-PerCP (BD Biosciences); CD4-APC-Cy7, CD14-PE (eBioscience); and p24-FITC (Beckman Coulter). For analysis of CD4+ T cell activation markers the following antibodies were used: CD3-PerCP, Ki-67-PE (BD Biosciences); CD4-APC-Cy7, CD25-PE-Cy7, HLA-DR-FITC, CCR5-APC (eBioscience). Samples were processed (50,000 events) on a BD LSRII flow cytometer and data analyzed with FlowJo software (Tree Star, Inc). Gating was
determined by fluorescence minus one staining and compensation calculated using Comp Beads (BD Biosciences) and compensation platform in FlowJo. Integrated mean fluorescent indices were calculated as the product of percent positive cells and the geometric mean fluorescence intensity of the positive cells.

**Statistical Analysis**

Fisher’s exact test was used to make comparisons between groups for frequency of infection. Differences in peak viral replication between groups were determined by either $t$ test or ANOVA. For expression of activation markers by flow cytometry, integrated mean fluorescent indices were log transformed and compared by ANOVA.
Results

Determination of Optimal MOI

To determine the optimal MOI for distinguishing potential differences in susceptibility to HIV in vitro, CBMC from Kenyan (N=6) and North American (N=3) subjects were exposed to varying viral quantities (Figure 2-1). Maximal viral replication was observed at day 15 for all samples productively infected, as indicated by incorporation of $\alpha^{-32}$P dTTP at multiple MOI (Figure 2-2). At day 15, an MOI of 0.005 infected all samples tested, whereas an MOI of 0.001 infected 4 of 6 Kenyan CBMC and none of the North American CBMC. This difference in infection at lower MOI suggested heightened susceptibility to HIV of Kenyan compared to North American CBMC.

Kenyan CBMC are more susceptible to in vitro HIV infection than North American CBMC

To further examine this increased susceptibility of CBMC from Kenyan compared to North American newborns to HIV in vitro, additional cryopreserved CBMC from Kenyan (N=60) and North American newborns (N=25) were examined. Samples were considered infected for a given culture if the measured cpm/µl was at least 5-fold greater than the no virus condition at two or more time points. In the absence of any exogenous stimulation (virus + media), CBMC from Kenyan newborns were significantly more likely to be infected with HIV compared to North American CBMC (21/60 versus 3/25, p=0.03, Fig. 2-3, A). This difference was further enhanced when viral exposure was followed by exogenous
Figure 2-1: Multiplicity of infection (MOI) dose response curve of HIVBaL.

$^{32}$P-dTTP incorporation for individual CBMC samples at varying MOI fifteen days after virus exposure. N=6 Kenyan, N=3 North American. Mean $^{32}$P-dTTP incorporation was significantly different between Kenyans and North Americans at MOI=0.001 (p=0.02, t test).
Figure 2-2: Kinetics of HIV\textsubscript{BaL} replication in CBMC productively infected. \textsuperscript{32}P-dTTP incorporation was determined at varying time points after virus exposure.  \textbf{A)} HIV\textsubscript{BaL} MOI=0.005. N=6 Kenyan, N=3 North American. \textbf{B)} HIV\textsubscript{BaL} MOI=0.001. N=4 Kenyan.
Figure 2-3: CBMC from Kenyan newborns demonstrate increased susceptibility to in vitro HIV infection compared to North Americans. The frequency of infection (A) and levels of viral replication (B) of in vitro CBMC in media alone (virus+media), HIV exposure prior to addition of PHA (virus+PHA), and preactivation with PHA (PHA+virus). B) Lines indicate mean cpm/µl of samples meeting definition of productive HIV infection. p-values were determined by Fisher’s exact test. N= 60 Kenyan CBMC, N=25 North American.
stimulation (virus + PHA) (36/60 versus 6/25, p=0.004). PHA stimulation proceeding viral exposure (PHA + virus) resulted in HIV infection of 59/60 (98%) of Kenyan and 25/25 (100%) of North American CBMC.

Peak virus replication, as indicated by incorporation of $\alpha^{-32}$P dTTP, was observed at day 15 for all samples and all culture conditions except when PHA stimulation preceded virus exposure (PHA + virus) when the peak was consistently observed at day 12. Among samples that were infected, peak viral production tended to be higher in Kenyan CBMC compared to North American controls at day 15 for virus+media and virus+PHA, although differences were not significant (Fig. 2-3, B).

To confirm reproducibility of results, a second aliquot of Kenyan (N=10) and North American (N=10) CBMC was thawed and infected as before. These replicates exactly matched the results from earlier experiments in terms of classification of productive HIV infection while peak levels of viral replication as determined by RT assay were also consistent (<20% difference in cpm/µl between replicate experiments).

*CBMC of newborns sensitized to malaria antigens have increased HIV susceptibility*

There was no clear association of increased CBMC susceptibility to HIV infection with either various maternal parasitic infections during pregnancy or parity (Table II). To examine the possibility that in utero immune priming to parasite antigens may increase susceptibility of Kenyan CBMC to HIV infection in
vitro, individual samples were stratified based on prenatal immune priming to malaria blood stage antigens. Half of Kenyan CBMC 30/60 (50%) demonstrated recall responses to two or more malaria antigens, and were defined as “malaria sensitized” (Figure 2-4). Samples classified as “malaria not sensitized” generally did not produce any malaria antigen-driven cytokine. A few samples, however demonstrated an isolated recall response to a single antigen, thereby failing to meet our criteria for immune priming.

Malaria sensitized CBMC were significantly more likely to be infected with HIV in vitro in the absence of exogenous stimulation (virus + media) when compared to either malaria not sensitized (15/30 vs 6/30, p=0.03) or to North American CBMC (15/30 vs 3/25, p=0.004, Fig. 2-5, A). While malaria sensitized and malaria not sensitized CBMC were similarly susceptible when virus exposure was followed by PHA stimulation (20/30 vs 16/30, p=0.43), malaria sensitized CBMC remained more likely to be infected compared to those of North Americans (20/30 vs 6/25, p=0.003). Among infected CBMC, peak viral replication was similar for malaria sensitized versus malaria not sensitized CBMC (Fig. 2-5, B).

*Stimulation with malaria antigens increases susceptibility of malaria sensitized CBMC to HIV infection*

We further examined whether recall responses to malaria blood stage antigens increased susceptibility to HIV infection in vitro. Prior to virus exposure, a subset of CBMC samples from newborns described in Figures 2-3 and 2-5
Table II. Association of maternal parasite infections and parity with susceptibility of CBMC from their offspring to HIV infection in vitro

<table>
<thead>
<tr>
<th>Maternal Infection:</th>
<th># samples susceptible to HIV in vitro / total # tested</th>
<th>p-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>2/5</td>
<td>0.6</td>
</tr>
<tr>
<td>-</td>
<td>11/44</td>
<td></td>
</tr>
<tr>
<td>S. hematobium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>3/9</td>
<td>1.0</td>
</tr>
<tr>
<td>-</td>
<td>16/51</td>
<td></td>
</tr>
<tr>
<td>Geohelminthes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>5/18</td>
<td>0.38</td>
</tr>
<tr>
<td>-</td>
<td>14/34</td>
<td></td>
</tr>
<tr>
<td>Any parasitic infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>8/27</td>
<td>0.79</td>
</tr>
<tr>
<td>-</td>
<td>11/33</td>
<td></td>
</tr>
<tr>
<td>Parity:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primi/secundi</td>
<td>10/25</td>
<td>0.27</td>
</tr>
<tr>
<td>Multi</td>
<td>9/35</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Fisher’s exact test.
Figure 2-4: Cytokine production profiles by Kenyan CBMC in response to recall stimulation with malaria antigens. Each row represents an individual CBMC sample; each column represents a cytokine:antigen combination. The grayscale corresponds to varying levels of cytokine production with darkest color indicating highest production. Malaria antigens used for the columns as follows: 1-recombinant MSP1_{42-3D7}, 2-recombinant MSP1_{42-FVO}, 3-pooled MSP1 peptides 2+3, 4-PfP0, 5-MSP1_{42} peptide K49, 6-MSP1_{42} peptide K30, 7-MSP1_{42} peptide M42.
Figure 2-5: CBMC from malaria sensitized newborns demonstrate increased susceptibility to in vitro HIV infection compared to malaria not sensitized and North Americans. The frequency of infection (A) and levels of viral replication (B) of in vitro Kenyan CBMC stratified by prenatal immune sensitization status as described in the materials and methods in media alone (virus+media), HIV exposure prior to addition of PHA (virus+PHA), and preactivation with PHA (PHA+virus). B) Lines indicate mean cpm/µl of samples meeting definition of productive HIV infection. p-values were determined by Fisher’s exact test. N=30 malaria sensitized, N=30 malaria not sensitized, N=25 North American.
(samples with sufficient numbers of cryopreserved CBMC) were exposed to pooled MSP1-42 peptides previously demonstrated to be dominant T cell epitopes in CBMC from newborns on the coast of Kenya (79). In contrast to exposure to HIVBaL at the beginning of culture, preculture of CBMC with media alone for 3 days prior to virus exposure failed to establish a productive infection in most samples for both Kenyan and North American CBMC (4/20 vs 2/20, open bars, Fig. 2-6, A). As expected, addition of malaria peptides to North American CBMC failed to enhance CBMC susceptibility to HIV infection (1/20, black bars). In contrast, addition of malaria peptides to CBMC classified as malaria sensitized showed a trend toward augmented HIV susceptibility compared to parallel cultures of malaria not sensitized CBMC (11/20 vs 6/20, p=0.2, Fig. 2-6, A). Malaria antigen- and PHA- driven Kenyan CBMC cultures infected with HIV produced similar levels of virus production, regardless of malaria sensitization status (Fig. 2-6, B).

**CD4⁺ T cells are the primary reservoir for replicating HIV at day 4 in vitro.**

Prenatal sensitization to malaria antigens has been shown to be predominantly attributable to CD4⁺ T cell responses (70). Therefore we hypothesized that CD4⁺ T cells were the early target of HIV infection in this in vitro model. Flow cytometric analysis of p24 expression was performed in CD3⁺CD4⁺ T cells and CD14⁺ cells from malaria sensitized samples known to be susceptible to HIV infection in the absence of exogenous stimulation. Expression
Figure 2-6: Pretreatment with malaria peptides increases susceptibility to in vitro HIV infection of malaria sensitized CBMC.  
A) Frequency of CBMC samples productively infected with HIV in vitro.  
B) $^{32}$P-dTTP incorporation for individual CBMC samples.  
Lines indicate mean cpm/µl of samples with productive HIV infection.  
N=20 for all conditions except N=10 for media+virus for both malaria sensitized and malaria not sensitized.
of p24 was undetectable in all samples 24 hours after virus exposure. However, at 96 hours, 1.3% of CD3+ lymphocytes stained positively for p24 (Fig. 2-7). Further analysis indicated all CD3+ cells expressing p24 also expressed CD4, but p24 was not detected in CD14+ cells. Thus, productive viral infection is primarily detectable in CD3+CD4+ cells at 96 hours after virus exposure.

Ex vivo flow staining of activation markers on CD4+ T cells

To determine whether malaria sensitized newborns had evidence of increased immune activation compared to malaria not sensitized or North American CBMC, ex vivo flow cytometric analysis of various CD4+ T cell activation markers was performed (N=10 for each group). Little ex vivo proliferation, measured by Ki-67 expression, was observed with no differences between groups and no differences were observed in the expression of CCR5 on CD4+ cells. Malaria sensitized CD3+CD4+ T cells demonstrated higher frequency and expression levels of CD25 (integrated mean fluorescent index, iMFI = 6224) compared to malaria not sensitized (iMFI = 2049, p=0.04) and North American CBMC (iMFI = 1688, p=0.04, Figure 2-8). Similarly, the frequency and expression of HLA-DR on CD4+ T cells was highest for malaria sensitized CBMC (iMFI = 995) compared to malaria not sensitized (iMFI = 304, p=0.05) and North American CBMC (iMFI = 328, p=0.03). Thus malaria sensitized CBMC contained CD4+ T cells demonstrating increased ex vivo activation that correlated with increased susceptibility to HIV infection in vitro.
Figure 2-7: Detection of p24 in CD3⁺ T cells of malaria sensitized CBMC by flow cytometry at 96 hours. Gated on viable lymphocytes. CD3 expression is plotted on y-axis and p24 expression on x-axis. Left panel: no virus exposure (negative control). Right panel: virus+media. Plots shown are representative of data from five samples.
Figure 2-8: Ex vivo expression of CD25 and HLA-DR is increased on CD3⁺CD4⁺ cells from malaria sensitized CBMC. Integrated mean fluorescent indices (iMFI) for CD25 and HLA-DR gated on viable CD3⁺CD4⁺ lymphocytes. N=10 for each group. Lines indicate geometric mean of iMFI. Statistics calculated by ANOVA.
Discussion

Here we show CBMC from Kenyan newborns in an area endemic for multiple chronic parasitic infections are more susceptible to infection with an R5-tropic strain of HIV in vitro compared to CBMC from offspring born in North America. This increased susceptibility of Kenyan CBMC to HIV infection was associated with in utero priming to malaria blood stage antigens and upregulation of CD25 and HLA-DR on CD4\(^+\) T cells. Stimulation of malaria sensitized CBMC further enhanced their susceptibility to HIV infection in vitro. HIV infected women are often coinfected with malaria and other chronic helminth infections which may lead to activation of fetal lymphocytes and therefore potentially increase the risk of MTCT. Thus prevention of malaria and chronic helminth infections such as schistosomiasis, lymphatic filariasis and geohelminthes in pregnant women may reduce MTCT, especially in conditions where anti-retroviral treatment may be difficult to reliably administer during pregnancy.

The failure to observe an association of maternal malaria or helminth infection with in vitro susceptibility to HIV is not surprising, since not all women may expose their fetus to parasite antigens necessary for in utero priming. The circumstances that might affect in utero sensitization are not clear, but it is likely affected by the duration, timing and intensity of the parasite infection during gestation. Additional factors may include the integrity of the placenta and the pregnant woman’s ability to generate appropriate antibodies to certain parasite antigens, since we have shown that immune complexes may be an important mechanism for transplacental transfer of malaria antigens (78).
Viral dose affected CBMC susceptibility to infection. At higher viral doses, all CBMC tested became infected in vitro, however at lower viral doses, more Kenyan samples remained susceptible compared to North American samples. This increased susceptibility of Kenyan CBMC occurred in the absence of exogenous stimulation, implicating in utero activation to exogenous antigens, particularly malaria and potentially other intravascular parasites such as schistosomiasis and lymphatic filariasis which are common in our population. In the event that the fetus or newborn should be exposed to HIV, particularly at lower viral doses, this prenatal priming may increase the likelihood of MTCT. Importantly, when strong polyclonal activation (PHA) preceded virus exposure in vitro, both Kenyan and North American samples were equally susceptible to infection indicating that intrinsic or genetic differences between Kenyan and North American CBMC were unlikely to contribute to the observed differences in HIV susceptibility.

One potential mechanism for fetal activation leading to increased viral susceptibility is by upregulation of CCR5 thereby facilitating viral invasion (121, 122). Interestingly such upregulation was not observed in the current study. This suggests viral entry may not be the limiting step, an observation supported by similar levels of HIV minus strand strong-stop DNA (–ssDNA) detected by qPCR 24 hours post virus exposure among Kenyan and North American CBMC (see chapter 3). Rather activated cells are likely to have greater pools of nucleotide precursors necessary for viral progression through reverse transcription, and/or proviral gene transcription and translation is enhanced by
host cell transcription factors such as NF-κB, AP-1, and/or NFAT (40). Although p24 was detected only in CD3⁺CD4⁺ T cells three days after virus exposure, the initial cell type infected in CBMC remains unclear and warrants further study.

There are several limitations to this study. It is generally accepted that initial HIV infection occurs at mucosal surfaces (22, 24, 123–125), with dissemination to lymph nodes (LN) where CD4⁺ cells act a reservoir (126–128). Circulating CBMC may not reflect those lymphocytes observed either at mucosal surfaces or in LN. Thus our in vitro model of HIV infection may not fully represent in vivo conditions. Further, though maternal infection with helminthes during gestation was documented and failed to account for differences in observed in vitro HIV susceptibility, fetal sensitization to helminth antigens was not fully assessed. As the frequency of maternal helminth infection was high in the present cohort and since we have previously shown that helminth-sensitized newborns of HIV positive mothers had an increased risk of MTCT in vivo, we cannot rule out the likelihood of fetal helminth sensitization contributing to the observed increased HIV susceptibility in vitro for Kenyan CBMC (80).

In conclusion this study demonstrates that CBMC from Kenyan newborns primed in utero to parasite antigens are more likely to become infected with HIV. Prevention of malaria and other chronic parasitic infections in HIV positive pregnant women to block prenatal sensitization could reduce the risk of MTCT.
CHAPTER 3

In utero activation of fetal memory T cells alters host regulatory gene expression and affects HIV susceptibility
Abstract

We previously showed that fifty percent of unstimulated cord blood mononuclear cell (CBMC) samples from offspring primed to malaria antigens were susceptible to productive HIV infection in vitro which was a significantly higher percentage compared to CBMC from offspring lacking recall responses to malaria. To explore mechanisms for this selective susceptibility we examined the subpopulations of CBMC infected and molecular pathways involved. Effector memory CD3⁺CD4⁺ T cells (TEM) were the exclusive initial targets of HIV infection with rapid spread to central memory cells (TCM). HIV susceptibility correlated with increased expression of CD25 and HLA-DR on TEM and TCM. Virus entered all samples equally; however gag/pol RNA was only detected in HIV susceptible samples, suggesting regulation of proviral gene transcription. Targeted analysis of human genes in memory T cells showed greater expression of IFNG, NFATc1, IRF1, FOS, and PPIA and decreased expression of YY1 and TFCP2 in HIV susceptible samples. Thus fetal priming to exogenous antigens activates cellular pathways in effector memory cells that enhance proviral gene transcription.
Introduction

Mother-to-child transmission (MTCT) of HIV remains an important global public health concern despite advances in targeted antiretroviral therapy among HIV infected pregnant women and newborns (3). Maternal coinfections with pathogens such as malaria, tuberculosis and helminthes may influence the risk of transmitting the virus to offspring and contribute to high rates of MTCT in many parts of the world (43, 44, 63, 66, 80, 129, 130). These coinfections may affect the risk of MTCT of HIV by several mechanisms including increased viral loads in pregnancy, formation of genital lesions and/or by activation of fetal lymphocytes.

HIV infection in neonates and infants differs from adults. Children infected early in life often exhibit higher levels of viremia, more rapid progression to AIDS, and increased frequency of opportunistic infections (131–133). The mechanisms underlying this differential disease course remain unclear. A contributing factor may be a higher total number of circulating T lymphocytes, predominantly naïve CD3⁺CD4⁺ cells, undergoing homeostatic proliferation to fill different lymphocyte compartments (134). Several studies have demonstrated increased susceptibility to HIV in CBMC compared to adult peripheral blood mononuclear cells (PBMC) (93, 135). Both naïve (CD45RA⁺) and memory (CD45RO⁺) CD3⁺CD4⁺ cells isolated from CBMC showed increased capacity to sustain viral gene transcription and replication compared to adult PBMC (135). This increased susceptibility correlated with differential mRNA expression of a variety of host factors associated with the HIV viral lifecycle including upregulation of several STAT family signal transducers, transcription factors such
as NF-κB, E2F, and HAT, and several matrix metalloproteinases while the repressive transcription factor YY1 was downregulated (136). A significant limitation to these in vitro studies is the necessity of mitogenic activation of CBMC prior to virus exposure to allow HIV infection. This stimulation likely alters target cell phenotype, gene expression profiles, and HIV susceptibility of CBMC.

We have previously shown that cord blood mononuclear cells (CBMC) collected from newborns having evidence of in utero exposure and immune activation to malaria demonstrated increased in vitro susceptibility to HIV\textsubscript{BaL} infection (129). In this in vitro HIV infection system, 50% of malaria sensitized CBMC were productively infected with HIV\textsubscript{BaL} without exogenous activation. This differential susceptibility to HIV infection in the absence of mitogenic stimulation enables us to characterize the targets and mechanisms that may affect lymphocyte susceptibility to HIV infection in vivo. We demonstrate that CD3\textsuperscript{+}CD4\textsuperscript{+} cells are the sole targets of HIV in our system and show that only CD45RO\textsuperscript{+} memory cells are productively infected. Further, by utilizing the protease inhibitor saquinavir, we demonstrate that CD45RO\textsuperscript{+}CD27\textsuperscript{-} effector memory cells are the primary initial targets of HIV\textsubscript{BaL} with rapid spread to other memory cells including those of the CD45RO\textsuperscript{+}CD27\textsuperscript{+} central memory phenotype. We show that differences in activation marker expression and gene expression profiles within the memory cell compartment correlate with susceptibility to HIV\textsubscript{BaL} infection and provide evidence that viral entry is not the limiting step in establishing productive HIV infection in CBMC.
Materials and Methods

Study population

Characteristics of the study population and procedures for CBMC sample collection and handling have been described previously (129). Briefly, samples were obtained from newborns born at the Msambweni District Hospital, Coast Province, Kenya an area endemic for malaria, schistosomiasis, lymphatic filariasis and intestinal helminthes (81, 117). All women were tested for HIV with the offspring of HIV positive women excluded from the current study. Additional CBMC were prepared from healthy North American newborns delivered at University Hospitals of Cleveland (Cleveland, OH) following the same procedures. Ethical approval was obtained from the Institutional Review Boards of University Hospitals of Cleveland and the Kenya Medical Research Institute in Nairobi and informed consent for the study was obtained from study subjects.

In vitro HIV infection

Ficoll-Hypaque density gradient separation was used to isolate CBMC which were promptly cyropreserved. Cryopreserved CBMC were thawed (all samples showed >85% viable cell recovery with preservation of immune function as indicated by similar recall responses to malaria antigens between fresh and cryopreserved aliquots from the same cord blood sample) and rested overnight in RPMI-1640 + L-glutamine supplemented with penicillin/streptomycin (100 units/100mg/ml), HEPES (10 mM), and 10% pooled human AB serum (cRPMI+10%). From this point forward, culture media consisted of cRPMI+10%
supplemented with 1.0 ng/ml recombinant human IL-2 (rhIL-2, BD Biosciences). Infection with the R5 tropic virus HIV_{BaL} (kindly provided by Dr. Eric Arts, Case/UHC Center for AIDS Research) was performed at an experimentally determined optimal multiplicity of infection (MOI) of 0.001 (129). Cells were exposed to HIV_{BaL} for four hours at 37°C after which cells were centrifuged, washed with fresh media and resuspended according to appropriate experimental culture conditions. The protease inhibitor saquinavir (5 ng/ml) was added to cultures following resuspension as appropriate.

*Isolation of CD3⁺CD4⁺ cell subset*

Twenty-four hours after virus exposure, the CD3⁺CD4⁺ cell subset was separated from the total cultured CBMC using the CD4⁺ T Cell Isolation Kit II (Miltenyi Biotech) following manufacturer’s instructions. Both the flow through (CD3⁺CD4⁺) and column-bound (non CD3⁺CD4⁺) cells were collected. Flow cytometric analysis consistently demonstrated greater than 95% purity of the CD3⁺CD4⁺ fraction.

*Nucleic acid extraction*

Twenty-four hours after virus exposure, cells were centrifuged and culture supernatant removed. Cells were harvested using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) to allow efficient extraction of both RNA and DNA which was performed according to the manufacturer’s recommended protocol. RNA and DNA concentrations were determined spectrophotometrically.
with dilution in Phosphate Buffer (Molecular Research Center, Inc., Cincinnati, OH).

**Minus strand strong stop DNA and gag/pol RNA detection**

Detection of HIV minus-strand strong stop DNA (-ssDNA) was performed using real time quantitative PCR (qPCR) methods. Primers and probes were as follows: for -ssDNA [probe (6FAM-AACAGACGGGCACACACTACTTTGAAGCA-TAMRA) and primers (5′-GCCTCAATAAAGCTTGCTGA-3′, and 5′-CTGAGGGATCTCTAGTTACCAGAGTCA-3′)], and for β-globin DNA [probe (6FAM-CACCTTTGCTACACTGAGTGACCTGCACTG-TAMRA), and primers (5′-GTTATGCTACGGATGACCTCAA-3′ and 5′-GGTCCACGTGCAGCTTGTGT-3′)]. Quantities of DNA were determined by serial dilution of cloned target cDNA in each assay and normalized to the copy number of β-globin. Detection of HIV gag/pol RNA was performed using reverse transcription quantitative PCR (RT-qPCR) methods. Primers were as follows: for HIV gag/pol [(5′-CATGTGTTTGCAGCATTATCAGAAGGA-3′) and (5′-CCACTGTGTTTAGCATGTTTGA-3′)] and for R18 [(5′-CGCCGTAGAGGTGAAATTC-3′) and (5′-CATTCTTGCAATGCTTTCG-3′)]. SYBERGreen (Applied Biosystems) was used for detection of PCR product. Results were normalized to the copy number of R18. The real-time PCR quantification was performed using Applied Biosystems 7300 Real Time PCR System.
Detection of virally encoded EGFP at early time points

CBMC from HIV susceptible and North American samples were thawed and plated in 48-well plates at a concentration of 1x10^4 cells/well. Cells were exposed to the mutant virus HIV-1 p83-10-EGFP modified with a Yu envelope (Yu-GFP, kindly provided by Dr. Miguel Quinones-Mateu, Cleveland Clinic Foundation, and Dr. David McDonald, Case/UHC Center for AIDS Research) at MOI=0.001 (137). EGFP production served as a marker of proviral gene transcription and translation. At subsequent twenty-four hour intervals, the entire well was thoroughly scanned at 20X power using a Deltavision RT epifluorescent deconvoluting microscope system and all EGFP-expressing cells were counted. Additionally, wells were imaged at 4X power with the resulting images stitched together using the Softworks deconvolution software to form a full well view.

Flow Analysis

One million cells were washed and stained with fluorochrome labeled antibodies for 30 minutes in the dark at 4°C. For analysis of HIV infection the following antibodies were used: CD3-APC-eFluor780, CD4-PE, CD45RO-APC, CD27-PE-Cy7 (ebiosciences), and p24-FITC (Beckman Coulter, gift of Dr. Helene Bernstein). For analysis of activation markers on isolated total CD3^+CD4^+ memory cells, the following antibodies were used: CD4-PE, CD45RO-APC, CD27-FITC, CD25- APC-eFluor780, HLA-DR-PE-Cy7 (ebiosciences); or CD4-Alexa700, CD45RO-APC, CD27-FITC, CCR5-PE (ebiosciences); Samples were processed (50,000 events) on a BD LSRII flow
cytometer and data analyzed with FlowJo software (Tree Star, Inc). Gating was determined by fluorescence minus one (FMO) staining and compensation calculated using Comp Beads (BD Biosciences) and compensation platform in FlowJo. Frequency was determined as percentage of cells with fluorescence greater than FMO gate while geometric mean fluorescence intensity (geoMFI) was used to evaluate expression levels. Integrated mean fluorescent indices (iMFI) were calculated as the product of percent positive cells and the geoMFI of the positive cells. Statistical comparisons of iMFI were performed using Student’s t test.

Host gene expression determination by PCR array in CD3⁺CD4⁺CD45RO⁺ cells

The total memory CD3⁺CD4⁺CD45RO⁺ subset was isolated from four samples each of Kenyan HIV susceptible, Kenyan HIV not susceptible, and North American samples for which greater than 120x10⁶ cryopreserved CBMC were available using the Memory CD4⁺ T cell Isolation Kit (Miltenyi Biotech), following manufacturer’s instructions. Flow cyotmetric analysis of isolated cells consistently demonstrated >93% purity as determined by CD4 and CD45RO staining. RNA was isolated from the CD4⁺CD45RO⁺ subset using the Qiagen RNeasy mini prep kit according to the recommended protocol, and purity and quantity was determined spectrophotometrically. HIV Infection and Host Response PCR Array (SA Biosciences/Qiagen) was used according to manufacturer’s instructions. Relative gene expression was calculated by ΔΔCt.
method. Real-time PCR quantification was performed using Applied Biosystems 7300 Real Time PCR System.
Results

_HIV_{BaL} enters only CD3^{+}CD4^{+} T cells_

We have previously shown that fifty percent of CBMC samples from infants demonstrating prenatal immune sensitization to malaria antigens were capable of sustaining viral replication following virus exposure in the absence of any exogenous mitogenic stimulation (129). These samples capable of sustaining viral replication have been termed “HIV susceptible” while those in which HIV replication was not detected have been termed “HIV not susceptible.” Further, we have previously shown that CD3^{+}CD4^{+} T cells from HIV susceptible samples represent the primary reservoir of replicating virus at day four post exposure to HIV_{BaL} as determined by flow cytometric detection of p24 (129). In this model it is possible that HIV_{BaL} could infect non-CD3^{+}CD4^{+} T cells but not lead to sufficient viral replication to be detectable by flow cytometric methods.

To determine whether HIV_{BaL} invades CD3^{+}CD4^{+} T cells only or also other cell types, frozen aliquots of CBMC from HIV susceptible samples were thawed and exposed to HIV_{BaL} at the same multiplicity of infection (MOI) as previously described (129). The CD3^{+}CD4^{+} and non-CD3^{+}CD4^{+} cellular subsets were subsequently isolated twenty-four hours after virus exposure. Virus entered and initiated reverse transcription only in CD3^{+}CD4^{+} T cells based on the detection of HIV minus strand strong stop DNA (HIV-ssDNA) in this cellular compartment and its absence in the non-CD3^{+}CD4^{+} compartment in all samples tested (Figure 3-1). HIV_{BaL} entered unstimulated CD3^{+}CD4^{+} T cells similarly compared to total CBMC that had been cultured in the presence of PHA for three days prior to virus
exposure, providing further evidence that virus preferentially enters CD3⁺CD4⁺ T cells in this in vitro model system.

**Kinetics of HIV gene transcription using EGFP containing virus**

To determine the frequency of infection events and the kinetics of viral spread CBMC from HIV susceptible and North American samples were exposed to the mutant virus HIV-1 p83-10-EGFP modified with a Yu envelope (Yu-GFP) which uses CCR5 as coreceptor for entry and contains the enhanced green fluorescent protein (EGFP) gene. EGFP production served as a marker of proviral gene transcription and translation and provided a means to visually detect productively infected cells. No EGFP-expressing cells were detected in two of the North American samples and a maximum of three EGFP-expressing cells was observed at 120 hours post infection (Figure 3-2, A). In contrast, EGFP-expressing cells were observed in CBMC from all HIV susceptible samples tested. EGFP-expressing cells were first observed forty-eight hours following virus exposure, though the frequency was low (median = 2 EGFP⁺ cells/well, Figure 3-2, B). The frequency of EGFP-expressing cells increased steadily in HIV susceptible samples at seventy-two (median = 11 EGFP⁺ cells/well) and 120 hours (median = 44 EGFP⁺ cells/well) following virus exposure. These observations suggested that either cells initially infected progressed to proviral gene transcription at varying rates, or that viral infection spread quickly from few initial infection events. However, this technique
Figure 3-1: HIV$_{\text{Bal}}$ minus strand strong-stop DNA (−ssDNA) is detectable in CD3$^+$CD4$^+$ cells and absent in non CD3$^+$CD4$^+$ cells twenty-four hours after virus exposure. HIV −ssDNA normalized to β-globin. Specific conditions indicated in the figure: i) CBMC cultured without virus, ii) Non CD3$^+$CD4$^+$ and iii) CD3$^+$CD4$^+$ conditions represent cell fractions following magnetic bead separation twenty-four hours after whole CBMC were exposed to virus, iv) PHA+Virus condition represents whole CBMC cultured in the presence of PHA for three days prior to virus exposure. N=8 HIV susceptible samples. Lines represent median for each condition.
Figure 3-2: Expression of virally encoded EGFP as a marker of HIV gene transcription and translation at early time points. CBMC from North American and HIV susceptible samples were exposed to a mutant strain of HIV which included a gene for EGFP. Entire wells were thoroughly scanned at 20X power and all EGFP-expressing cells were counted at indicated time points after virus exposure.  

A) CBMC from North American infants, N=5.  

B) CBMC from HIV susceptible infants, N=5. Lines represent mean count.
was not capable of discriminating between these possibilities nor was further phenotypic characterization of initial viral target cells possible.

**CD3+CD4+CD45RO+CD27- effector memory cells are the initial target of infection**

To further specify the initial cellular targets of HIVBaL infection in our system, flow cytometric phenotyping of p24 expressing cells was performed four days after infection. Cell surface markers CD45RO and CD27 were used to divide the CD3+CD4+ T cell population into naïve (CD45RO-CD27+), central memory (CD45RO+CD27+, TCM) and effector memory (CD45RO+CD27-, TEM) subsets, as previously described (Figure 3-3) (138–140). Though the naïve subset consistently represented greater than 92% of CD3+CD4+ T cells, HIV p24 was not detectable in this compartment (Table III). In contrast, p24 was detectable in both the central memory (median of 4.7% of CD45RO+CD27+ cells) and effector memory (median of 10.6% of CD45RO+CD27- cells) compartments (Table III). Therefore both central and effector memory CD3+CD4+ cells are capable of sustaining HIVBaL replication at day four in our model system, but naïve CD4+ T cells are not.

To determine which memory CD3+CD4+ T cell subset was the initial target of infection, experiments were performed in which the protease inhibitor saquinavir was present throughout the four days of cell culture. Saquinavir does not interfere with first round viral infection events detectable by p24 expression, but prevents subsequent spreading and second round infection. In the presence of saquinavir, p24 expression in the CD45RO+CD27+ central memory
Figure 3-3: Expression of p24 as a marker of HIV<sub>BaL</sub> replication in naïve, central memory (T<sub>CM</sub>), and effector memory (T<sub>EM</sub>) CD4<sup>+</sup> cells in the absence or presence of saquinavir. CD3<sup>+</CD4<sup>+</sup> T lymphocytes were gated into naïve (CD45RO<sup>-</sup>CD27<sup>+</sup>, top row), T<sub>CM</sub> (CD45RO<sup>+</sup>CD27<sup>+</sup>, middle row), and T<sub>EM</sub> (CD45RO<sup>+</sup>CD27<sup>-</sup>, bottom row). p24 expression in each subset was determined in the absence (middle column) or presence (right column) of the protease inhibitor saquinavir.
**Table III:** Frequency of p24 expression in CD3⁺CD4⁺ T lymphocytes 96 hours after HIV<sub>BaL</sub> exposure and in the presence or absence of protease inhibitor saquinavir.

<table>
<thead>
<tr>
<th>HIV susceptible samples</th>
<th>p24 expression in CD45RO⁺CD27⁺ (naïve)</th>
<th>p24 expression in CD45RO⁺CD27⁺ (T&lt;sub&gt;CM&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>p24 expression in CD45RO⁺CD27⁻ (T&lt;sub&gt;EM&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- saquinavir</td>
<td>+ saquinavir</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>3.2</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>4.7</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>4.8</td>
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<tr>
<td>5</td>
<td>0</td>
<td>5.1</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> central memory  
<sup>b</sup> effector memory
compartment was never detected while the frequency of p24 expressing CD45RO⁺CD27⁻ effector memory cells was half of that detected in the absence of saquinavir (Figure 3-3 and Table III). There was no difference in the frequency of T_CM and T_EM among CBMC of HIV susceptible, HIV not susceptible, and North American subjects (Figure 3-4), excluding the possibility that differences in frequency of these memory cell subsets could account for differences in susceptibility to HIV_BaL infection. Thus T_EM appear to be the primary initial targets of HIV_BaL infection with subsequent spread to both T_CM and additional T_EM.

*Expression of activation markers on memory cells correlates with HIV susceptibility*

Expression of CD25 and HLA-DR were evaluated to determine if CD3⁺CD4⁺ T_CM and T_EM from HIV susceptible samples showed increased activation compared to those from HIV not susceptible samples. Frequency and expression of CD25 were increased on both CD45RO⁺CD27⁻ T_EM (iMFI= 21,843 versus 3,600, p=0.05) and CD45RO⁺CD27⁺ T_CM (iMFI=6,715 versus 1,676, p=0.02) from HIV susceptible compared to HIV not susceptible samples respectively (Figure 3-5). Similarly, HIV susceptible samples demonstrated higher frequency and expression of HLA-DR for both T_EM (iMFI=563 versus 346 for T_EM, p= 0.05) and T_CM (iMFI=408 versus 250 for T_CM, p=0.05). CCR5 expression did not differ between HIV susceptible and not susceptible samples.
Figure 3-4: Frequency of central memory or effector memory CD4\(^+\) cell subsets does not significantly vary between HIV susceptible, HIV not susceptible, and North American samples. Frequency of CD45RO\(^+\)CD27\(^+\) (central memory) and CD45RO\(^+\)CD27\(^-\) (effector memory) cells were determined after gating on viable CD3\(^+\)CD4\(^+\) lymphocytes. Lines represent median values. N=8 for each group.
Figure 3-5: Expression of activation markers on effector memory (T<sub>EM</sub>) and central memory (T<sub>CM</sub>) CD<sup>4</sup><sup>+</sup> T cells correlates with HIV susceptibility. Integrated mean fluorescent indices (iMFI) for CD25 (top) and HLA-DR (bottom) gated on viable CD3<sup>+</sup>CD4<sup>+</sup> lymphocytes and appropriate CD45RO and CD27 expression. N=6 for each group. Lines indicate geometric mean of iMFI. Statistics calculated by t test.
for either $T_{EM}$ (IMFI=1686 versus 1583, $p=.14$) or $T_{CM}$ (IMFI= 875 versus 824, $p=0.62$). CD25 and HLA-DR expression were similar for CD45RO-CD27+ naïve cells from HIV susceptible versus HIV not susceptible samples.

_HIV entry does not account for differences in HIV susceptibility_

To determine whether differing susceptibility to HIV infection was due to differences in the ability of HIV to enter cells or a later viral life cycle event, DNA and RNA were isolated from HIV susceptible and HIV not susceptible CBMC twenty-four hours after virus exposure. Quantitative PCR (qPCR) for HIV minus strand strong-stop DNA (-ssDNA) and RT-qPCR for *gag/pol* RNA were performed. HIV –ssDNA was detected in all HIV susceptible samples with greater median magnitude of expression compared to HIV not susceptible samples (Figure 3-6). However, HIV –ssDNA was able to be amplified in 27 of 31 (87%) HIV not susceptible samples with the range of detectable expression levels similar to that of the HIV susceptible group (3100-1.07x10⁶ vs. 2000-9.28x10⁵, respectively). In contrast, *gag/pol* RNA was detected in all HIV susceptible samples analyzed, but in none of the HIV not susceptible samples. These results suggest that, while HIV is capable of entering both HIV susceptible and not susceptible CBMC in vitro, only in HIV susceptible CBMC does the viral life cycle progress to proviral gene transcription.
Figure 3-6: Detection of HIV minus strand strong-stop DNA (-ssDNA) is similar between HIV susceptible and HIV not susceptible CBMC twenty-four hours post virus exposure, but gag/pol RNA is only detected in HIV susceptible CBMC. 

A distinct set of genes relevant to proviral gene transcription are differentially expressed in memory T cells and correlate with HIV susceptibility in vitro

To further address possible differences between HIV susceptible and not susceptible memory T cells, gene expression levels of a subset of host genes relevant to HIV infection were assessed using the HIV Infection and Host Response PCR array (SA Biosciences/Qiagen). This array analyzes the expression of eighty-four host genes selected for their described role in HIV viral entry, reverse transcription, integration, proviral gene transcription, and virion assembly as well as host immune responses to infection. This targeted array system was chosen because we wished to specifically determine expression profiles in the CD4⁺CD45RO⁺ memory subset and limited cell numbers made whole genome analysis impossible.

Of the eighty-four genes analyzed, eight demonstrated greater than two-fold differences in expression between at least two of the comparison groups (Figure 3-7) while seventy-six genes showed no difference between groups (Table IV). Notably, mRNA of proinflammatory cytokines TNF and IFNG were elevated in HIV susceptible samples as were the transcription factors and regulators NFATc1, IRF1, FOS and the cellular cofactor PPIA compared to HIV not susceptible and North American CBMC (Fig. 3-7, A & C). In contrast, the mRNA expression of YY1 and TFCP2, two transcription factors that have been associated with suppression of HIV viral replication, were 9-fold lower in HIV susceptible memory T cells compared to both HIV not susceptible and North American memory T cells. There was little difference in gene expression
Figure 3-7: Differential expression of host genes in total memory CD3^+CD4^+ cells from HIV susceptible, HIV not susceptible, and North American CBMC.  
Table IV: Analyzed host genes not demonstrating differential expression in total memory CD3^+CD4^+ cells from HIV susceptible, HIV not susceptible, and North American CBMC.

<table>
<thead>
<tr>
<th>GeneBank</th>
<th>Symbol</th>
<th>Description</th>
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</thead>
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<tr>
<td>NM_080649</td>
<td>APOBEC3F</td>
<td>Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3F</td>
</tr>
<tr>
<td>NM_091822</td>
<td>APOBEC3G</td>
<td>Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G</td>
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<tr>
<td>NM_04322</td>
<td>BAD</td>
<td>BCL2-associated agonist of cell death</td>
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<tr>
<td>NM_03860</td>
<td>BANF1</td>
<td>Barrier to autointegration factor 1</td>
</tr>
<tr>
<td>NM_04324</td>
<td>BAX</td>
<td>BCL2-associated X protein</td>
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<td>NM_02898</td>
<td>BCL11B</td>
<td>B-cell CLL/lymphoma 11B (zinc finger protein)</td>
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<tr>
<td>NM_00633</td>
<td>BCL2</td>
<td>B-cell CLL/lymphoma 2</td>
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<td>BTRC</td>
<td>Beta-transducin repeat containing</td>
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<td>Caspase 3, apoptosis-related cysteine peptidase</td>
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<tr>
<td>NM_01228</td>
<td>CASP8</td>
<td>Caspase 8, apoptosis-related cysteine peptidase</td>
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<tr>
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<td>CBX5</td>
<td>Chromobox homolog 5 (HP1 alpha homolog, Drosophila)</td>
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<tr>
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<td>Chemokine (C-C motif) ligand 2</td>
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<td>CCL4</td>
<td>Chemokine (C-C motif) ligand 4</td>
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<td>CCNT1</td>
<td>Cyclin T1</td>
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<td>CCR2</td>
<td>Chemokine (C-C motif) receptor 2</td>
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<td>CCR3</td>
<td>Chemokine (C-C motif) receptor 3</td>
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<td>CD209</td>
<td>CD209 molecule</td>
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<td>Cyclin-dependent kinase 9</td>
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<td>CDKN1A</td>
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<td>COP56</td>
<td>COP9 constitutive photomorphogenic homolog subunit 6 (Arabidopsis)</td>
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<td>CREBBP</td>
<td>CREB binding protein</td>
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<tr>
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<td>CXCL12</td>
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<td>CXCR4</td>
<td>Chemokine (C-X-C motif) receptor 4</td>
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<td>E1A binding protein p300</td>
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<td>FCAR</td>
<td>Fc fragment of IgA, receptor for</td>
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<td>GADD45A</td>
<td>Growth arrest and DNA-damage-inducible, alpha</td>
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<td>NM_002110</td>
<td>HCK</td>
<td>Hemopoietic cell kinase</td>
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<tr>
<td>NM_145904</td>
<td>HMGA1</td>
<td>High mobility group AT-hook 1</td>
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Table IV (continued): Analyzed host genes not demonstrating differential expression in total memory CD3^+CD4^+ cells from HIV susceptible, HIV not susceptible, and North American CBMC.

<table>
<thead>
<tr>
<th>Gene Accession</th>
<th>Gene Symbol</th>
<th>Description</th>
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<tr>
<td>NM_014500</td>
<td>HTATSF1</td>
<td>HIV-1 Tat specific factor 1</td>
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<td>NM_024013</td>
<td>IFNA1</td>
<td>Interferon, alpha 1</td>
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<td>NM_002176</td>
<td>IFNB1</td>
<td>Interferon, beta 1, fibroblast</td>
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<td>NM_000572</td>
<td>IL10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>NM_002187</td>
<td>IL12B</td>
<td>Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)</td>
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<td>IL16</td>
<td>Interleukin 16 (lymphocyte chemoattractant factor)</td>
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<td>LTBR</td>
<td>Lymphotoxin beta receptor (TNFR superfamily, member 3)</td>
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<td>NM_003400</td>
<td>XPO1</td>
<td>Exportin 1 (CRM1 homolog, yeast)</td>
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between HIV not susceptible and North American CBMC (Fig. 3-7, B). These results suggest that HIV susceptible memory T cells not only express greater levels of genes that facilitate HIV infection and replication, but they also express less of two important suppressors of the viral life cycle.
Discussion

Our previous study established that CBMC from fifty percent of malaria sensitized Kenyan infants were capable of being productively infected with HIV in vitro without prior exogenous stimulation (129). This provided a relevant model that may better recapitulate host susceptibility factors to HIV in vivo. Here we extend these observations to demonstrate that CD3⁺CD4⁺ cells are the only cellular compartment in which HIVBaL enters and initiates reverse transcription. Further, while both central (T_CM) and effector (T_EM) memory T cells are capable of sustaining detectable viral replication following virus exposure in this in vitro model of CBMC infection, naïve CD4⁺ T cells are not. By utilizing a protease inhibitor to pharmacologically prevent second-round infection, we demonstrate that the initial cellular target of HIV in our model system is the T_EM with rapid spread to T_CM. Both T_EM and T_CM from HIV susceptible CBMC demonstrated evidence of increased cellular activation as indicated by increased frequency and expression of surface CD25 and HLA-DR compared to HIV not susceptible samples. Analysis of gene expression profiles for eighty-four host genes relevant to HIV infection within the CD4⁺CD45RO⁺ total memory subset revealed enhanced gene expression of TNF, IFNG, NFATc1, IRF1, FOS, and PPIA while expression of YY1 and TFCP2 were markedly decreased in HIV susceptible samples. Interestingly, no difference was observed in HIV –ssDNA expression between HIV susceptible and HIV not susceptible CBMC, suggesting that cellular activation is not necessary for HIV entry. In contrast, detection of HIV gag/pol RNA was exclusively detected in HIV susceptible CBMC. Together, these
findings support a model in which HIV binding and invasion of CBMC is not the primary difference between HIV susceptible and not susceptible CBMC. Rather, differential cellular activation and expression of host genes that influence HIV gene transcription enhances viral replication and spread within memory CD4⁺CD45RO⁺ T cells, initially in T_EM and subsequently in T_CM.

Other studies have examined mechanisms of susceptibility of CBMC to HIV in infection in vitro. These studies have only examined differences in susceptibility to HIV in vitro between CBMC and adult peripheral blood mononuclear cells (PBMC) (93, 135, 136). They have also required pre-activation to allow HIV infection in vitro. Interestingly, these studies demonstrated enhanced HIV replication in monocytes/macrophages and total T lymphocytes, as well as more specifically in both CD4⁺CD45RA⁺ naïve T cell and CD4⁺CD45RO⁺ memory T cell subsets from CBMC compared to PBMC (93, 135). This is in contrast to our observations in which HIV –ssDNA is only detected in CD3⁺CD4⁺ T cells twenty-four hours after virus exposure and further only CD4⁺CD45RO⁺ memory cells exhibit productive viral replication as determined by flow cytometric detection of p24. The required prestimulation used in other studies may have artificially enhanced viral replication in cells that may not have been susceptible in vivo, thereby explaining the more restricted profile of HIV susceptible cells in our model system. In support of our observations, previous studies have demonstrated both predominant HIV infection of CD4⁺CD45RO⁺ T cells in infants as well as preferential replication of HIV in the memory T cell subset (141–143).
We demonstrate that TEM are the primary initial target of infection in our model system. This observation is consistent with several other studies that have shown initial HIV infection of TEM in diverse settings such as cervico-vaginal tissue, dendritic cell mediated transmission, and a humanized mouse model system (144–146). Preferential depletion of the TEM compartment is a characteristic feature of early infection in both human HIV and SIV model systems, particularly in mucosal sites such as the gut associated lymphoid tissue (147–149). Importantly, while CD4+CD45RO−CD27+ naïve cells are not an important source of replicating virus in our model system, this does not preclude infection of naïve T cells as HIV infection progresses and spreads. Indeed, HIV proviral transcripts may be detected in naïve as well as TCM and TEM cells from patients with established HIV infection (150). Further, though virus does not seem to infect and replicate within non-CD3+CD4+ cells, we cannot exclude the possibility that such cells may not be productively infected yet facilitate invasion of other target cells. Dendritic cells comprise 0.3-0.4% of CBMC and are capable of transferring intact, infectious HIV to target cells in a replication independent manner termed trans-infection (151–154). Separation of CBMC into non-CD3+CD4+ and CD3+CD4+ compartments prior to exposure to HIV may define the contribution, if any, of trans-infection by dendritic cells in our in vitro infection model.

It has been hypothesized that effector memory CD4+ T cells are more easily infected due to increased cell surface expression of CCR5, the primary co-receptor mediating infection of R5 type HIV (90, 144). Interestingly, we did not
observe any difference in surface CCR5 expression on memory T cells from HIV susceptible compared to not susceptible samples. We did, however, observe increased expression of the cell surface activation markers CD25 and HLA-DR on both T_{EM} and T_{CM} from HIV susceptible samples. Further, total memory CD3^{+}CD4^{+} cells from HIV susceptible samples had increased mRNA expression of genes associated with cellular activation. These included TNF, which enhances HIV viral replication through downstream effects of NF-κB, and IFNG, which has been shown to possess both inhibitory and activating effects on HIV replication (155–157). Transcription regulators FOS, NFATc1, and IRF1 were also upregulated in memory T cells from HIV susceptible samples. FOS, a component of the transcription factor AP-1, and NFATc1 have been described to enhance HIV proviral gene transcription directly by binding to sites in both the HIV long terminal repeat (LTR) region and intragenic regions as well as by synergizing with NF-κB pathways (158–163). IRF1 binds directly to target sites within the HIV sequence and forms a functional complex with NF-κB at the LTR kappa sites thereby enhancing viral gene transcription and viral replication (164–166). Interestingly, the expression of transcriptional repressors YY1 and TFCP2 were both dramatically decreased in CD4^{+}CD45RO^{+} memory CBMC from HIV susceptible samples compared to HIV not susceptible and North American samples. TFCP2 recruits YY1 to the HIV LTR where they cooperatively repress proviral gene transcription by recruitment of histone deacetylase to the viral promoter region resulting in alteration of the chromatin structure to an inactive state (101, 102). Recently, decreased YY1 expression has been observed and
correlated with increased HIV susceptibility in CBMC compared to adult PBMC, further supporting a potentially important role of these repressive transcription factors in modulating viral replication in CBMC (136).

In conclusion, this study demonstrates that CD3+CD4+CD45RO−CD27− T_{EM} cells are initial targets of HIV_{BaL} in this in vitro model system with rapid spread to other memory cells including those of the CD3+CD4+CD45RO+CD27+ T_{CM} phenotype. HIV susceptibility was further correlated with increased immune activation as measured by both cell surface activation marker expression and differing gene expression profiles within the CD4+CD45RO+ compartment. While this study does not specifically address the cause of this observed increase in immune activation in HIV susceptible samples, we have previously demonstrated that CBMC from infants primed in utero to malaria antigens were more susceptible to in vitro HIV infection (129). Therefore we propose that prevention of malaria and other parasitic infections in pregnant women may decrease the activation of highly susceptible fetal memory T cells and thereby decrease risk of MTCT of HIV to their offspring in utero or perinatally. Further, the model system of HIV infection used here is unique in that we observe differences between CBMC samples in susceptibility to HIV at low MOI and in the absence of exogenous stimulation which may allow further studies to reveal novel pathways mediating susceptibility to HIV infection.
CHAPTER 4

Discussion and Future Directions
This work has provided greater insight into the impact of prenatal sensitization to parasitic antigens on MTCT of HIV and has developed a model with which to address some of the molecular processes critical in understanding the dynamics of MTCT of HIV in this setting. These advances were possible due to unique access to a population of pregnant women and their offspring prenatally exposed to malaria, the technical and laboratory experience to identify this prenatal sensitization, facilities and personnel capable of collecting and cryopreserving CBMC, and access to expertise and reagents necessary for in vitro HIV infection and monitoring. Together these provided a unique opportunity to ask new questions and gain new insights.

The first notable discovery was that CBMC from Kenyan newborns demonstrated increased susceptibility to in vitro HIV infection compared to North American CBMC. This was initially observed in developing a dose-response curve of varying MOI exposure. Once an optimal MOI was determined, this observation was fully explored and confirmed using an expanded sample size (n=60, Kenyan, n=30 North American). Stratification of Kenyan CBMC into malaria sensitized and malaria not sensitized groups, based upon recall responses to malaria antigens, demonstrated that malaria sensitized CBMC were significantly more susceptible to in vitro HIV infection compared to malaria not sensitized or North American CBMC. Culture in the presence of dominant malaria peptides prior to exposure to HIV further enhanced susceptibility of malaria sensitized CBMC, but not those from North Americans. This implies that the effect is not nonspecific, but rather that prenatal exposure and development
of a specific immune response contributed to the observed enhancement of susceptibility to HIV. When precultured with malaria peptides, some CBMC classified as malaria not sensitized also demonstrated increased susceptibility to HIV infection, likely due a misclassification of these samples as not sensitized because of the stringent classification criteria and limited panel of antigens used to elicit a recall response. Usage of additional *P. falciparum* antigens such as recombinant protein or peptides from not only MSP-1 and PfP0, but also region II of erythrocyte binding antigen-175 (EBA-175) and/or apical membrane antigen 1 (AMA1) would perhaps allow greater precision in classification of malaria sensitization. However this greater precision would require additional CBMC for the initial sensitization screen and further reduce the available cryopreserved CBMC available for subsequent study. Further observations determined that the initial productive HIV infection events were rare and confined exclusively to the CD4⁺CD45RO⁺ compartment. HIVBaL was able to enter and initiate reverse transcription in both HIV susceptible and not susceptible CBMC, but proviral gene transcription was only found in HIV susceptible samples, demonstrating the importance of late life cycle events. Susceptibility to HIV correlated with evidence of cellular activation both by cell surface activation marker expression and gene expression analysis which demonstrated differential expression of several genes associated with control of the HIV viral life cycle. Based upon these results, a schematic model of prenatal sensitization to malaria and its impact on HIV susceptibility may be constructed (Figure 4-1).
Maternal circulation
Placental barrier
Fetal circulation

Figure 4-1: Model of prenatal sensitization to malaria and increased susceptibility to HIV. Fetal naïve T cells (T\textsubscript{n}) are exposed to parasitized maternal red blood cells (pRBC), soluble malaria antigens (sMal Ag), or malaria antigens in immune complexes resulting in the formation of effector memory (T\textsubscript{EM}) and central memory (T\textsubscript{CM}) cells. The memory cell populations have increased activation and altered gene expression profiles. When exposed to HIV, T\textsubscript{EM} cells are the initial targets of infection (iT\textsubscript{EM}) with rapid spread to T\textsubscript{CM} (iT\textsubscript{CM}).
Taken together, these observations suggest that in utero immune activation due to exposure to a specific pathogen or antigens derived thereof is capable of altering the susceptibility of CBMC to HIV infection. Importantly, while this current work focused entirely on prenatal sensitization to malaria, it is unlikely that malaria is unique in its ability to enhance susceptibility to HIV. Though generally population based and retrospective, other studies have demonstrated increased in vivo MTCT in the setting of a variety of maternal co-infections including helminthes, tuberculosis, bacterial chorioamnionitis, and sexually transmitted infections (39, 67, 80, 130, 167–171). It may be hypothesized that if similar in vitro studies were to be performed stratifying groups based on evidence of prenatal sensitization to these and other maternal coinfections, a similar differentiation of CBMC susceptibility to HIV may be observed.

The observation that prenatal sensitization of the fetal immune system to malaria antigens increased susceptibility of CBMC to in vitro HIV infection provides insight into a broader question that has remained in the field of MTCT. Namely, how does transmission of HIV occur when most studies have found CBMC to be broadly resistant to productive HIV infection in the absence of exogenous mitogenic stimulation? Early studies on in vitro infection of CBMC with HIV demonstrated that, while the virus was capable of entering and initiating reverse transcription, it was incapable of completing reverse transcription and incomplete, partial DNA transcripts accumulated and were detectable (95). Further, partially reverse transcribed HIV genome has been detected in CBMC
from in vivo HIV exposed, uninfected infants (172). These observations were consistent with similar studies performed using quiescent adult peripheral blood mononuclear cells, thus the lack of successful completion of the HIV lifecycle in CBMC was attributed to the quiescent nature of the overwhelmingly naïve fetal CD4+ T cell compartment (95–97). Indeed, most researchers studying HIV infection in CBMC utilize in vitro stimulation prior to virus exposure to enhance and facilitate infection (40, 93, 95, 135, 136, 173, 174). Together with the observation that the majority of MTCT events seem to occur during the peripartum period – the time when extensive lymphocyte activation is presumed to first occur – these experimental results have led some to hypothesize that HIV may enter a fetus but not successfully complete reverse transcription and integrate, thereby failing to establish productive infection until appropriate and sufficient lymphocyte activation occurs (6, 40, 175). While this proposed framework helps explain why the majority of transmission events in non-breastfeeding populations are termed peri- or intrapartum based on the timing of PCR detection of infection, it fails to account for the approximately 20% of MTCT events that occur prior to 36 weeks of gestation, a time period presumed to be relatively devoid of lymphocyte activation (5–7).

Our present work both affirms the importance of lymphocyte activation in the fetus/neonate for MTCT of HIV as well as to extend the current conceptual framework by correlating in utero immune activation, specifically prenatal sensitization to malaria antigens, with increased susceptibility to in vitro HIV infection. While the timing of prenatal sensitization to malaria in the present
study population cannot be determined precisely and is likely to vary considerably, because sensitization is defined as recall responses to malaria antigens by CBMC collected at delivery, it may be presumed that the initial immune activation event was not intrapartum, but rather occurred in utero, perhaps as early as the second trimester. Though dependent upon development of the fetal thymus which becomes functional between the seventh and sixteenth week of gestation, both CD4\(^+\) and CD8\(^+\) T lymphocytes are detectable in fetal lymphoid tissues by gestational week twenty (176–179). Though widely believed to be entirely inactive, recently fetal T cells have been shown to exist in a dynamic balance, controlled largely by the influence of CD4\(^+\)CD25\(^+\) regulatory T cells, between states of activation and quiescence (177). Provided appropriately stimulating conditions, activation of fetal T lymphocytes capable of driving the HIV viral life cycle to completion may be hypothesized to occur as early as week twenty of gestation. Our results show that in utero exposure to malaria antigens is one potential cause of prenatal immune activation sufficient to render fetal T lymphocytes susceptible to productive HIV infection and provides evidence that prenatal immune activation is an important variable not only for peri- and intrapartum MTCT, but also in utero transmission.

Given this evidence that prenatal sensitization to malaria increases susceptibility to HIV infection, it may be expected that that the presence of placental malaria at the time of delivery might be an independent risk factor for MTCT. Several studies attempting to elucidate this interaction have resulted in conflicting observations, some finding increased MTCT with placental malaria,
some finding no impact or even a protective effect of placental malaria, and one study finding a complex situation in which high density placental malaria increased MTCT transmission risk while a low density placental parasitemia conferred a protective effect (42, 44, 63, 64, 66, 68, 69, 116). An alternative approach to address this question of the impact of maternal malaria on MTCT is to attempt to eliminate and actively prevent malaria infection during gestation. Consistent use of long-lasting insecticide-treated bednets coupled with an intermittent presumptive treatment protocol (IPTp) consisting of at least two doses of efficacious anti-malarial drugs given at least one month apart beginning in the second trimester has been shown to dramatically reduce the burden of maternal malaria infection when properly implemented (52, 69, 180–183). Therefore one might hypothesize that implementation of such anti-malarial interventions among HIV positive pregnant women would decrease the risk of MTCT by reducing the likelihood of fetal sensitization to malaria. While not assessing prenatal malaria sensitization, the single study performed to date described a very slight, though not statistically significant decrease in MTCT associated with IPTp which led the authors to conclude that active malaria prevention during pregnancy had no effect on MTCT (69).

It is important to note, however, in some of the studies that failed to demonstrate an increased risk of MTCT with maternal malaria infection, the enrolled women and their offspring were provided with anti-retroviral therapy according to then current standards of care for the country in which the study took place (66, 68, 69). Given the magnitude of the impact of such anti-retroviral
therapies in decreasing the rate of MTCT, it may not be surprising that malaria infection or its active prevention during pregnancy did not have a statistically significant impact on risk of MTCT. Indeed the impact of malaria may be expected to be small in comparison to such other highly efficacious, direct interventions to prevent MTCT. However, several recently published studies using data collected prior to the introduction of anti-retroviral therapy have demonstrated statistically significant increased risk of MTCT with placental malaria overall and particularly among primigravid women (64, 65). Moreover, prenatal sensitization does not always correlate with reported maternal malaria parasitemia and the data presented here demonstrate increased susceptibility to HIV of malaria sensitized CBMC and not simply those from newborns of malaria infected women (70, 73, 74, 79, 129, 184). To date, only one study has sought to correlate prenatal sensitization to parasitic antigens with in vivo MTCT, and while limited by sample size, a trend toward increased MTCT with sensitization to helminth antigens was found (80).

Clinically, the results of the current study suggest that prevention of malaria and other parasitic infections in pregnant women in order to prevent development and activation of highly susceptible fetal memory T cells may synergize with other interventions aimed at reducing the risk of MTCT. The most recent recommendations of the World Health Organization consist of the initiation of anti-retroviral prophylaxis as early as fourteen weeks of gestation for HIV positive pregnant women not needing anti-retroviral therapy for their own health (185). However, implementation of this recommendation has been slow and
many pregnant women in sub-Saharan Africa receive anti-retrovirals much later in gestation or not at all (186–191). Therefore, as prenatal sensitization to malaria can increase susceptibility of fetal cells to HIV infection, this sensitization may occur as early as week twenty of gestation, and implementation of anti-retroviral therapy protocols for the prevention of MTCT often are not initiated until late in gestation, it may be argued that aggressive prevention of malaria during pregnancy may have a small but important impact on preventing MTCT, particularly in settings of poor anti-retroviral access and coverage.

Finally, several recent studies have suggested that placental malaria may increase risk of MTCT when maternal viral loads are low (64, 68). Though not demonstrating an impact of placental malaria on overall MTCT, Msamanga et al. demonstrated a strong trend toward increased risk of MTCT in women with low viral loads and placental malaria (68). Brahmbhatt et al. showed that placental malaria is a significant risk factor for MTCT at both high and low viral loads compared to the absence of placental malaria (64). Malaria infection has generally been associated with elevated plasma viral loads in both pregnant and non-pregnant adults, which has provided a conceptual framework for the hypothesis that malaria in pregnancy may increase the risk of MTCT (43, 45, 46, 114, 115, 192). However, MTCT still occurs, albeit at a low rate, in the context of low maternal viral loads occurring with or without active suppression with anti-retroviral therapy (34).

In this current work, a marked dose response effect was noted where all CBMC tested were productively infected in vitro at high MOI (0.005), while only
CBMC from Kenyan infants demonstrated susceptibility at lower MOI (0.001, Figure 2-1). All further experiments exploring differential susceptibility to HIV between malaria sensitized, malaria not sensitized, and North American CBMC relied upon exposure to HIV at this lower MOI. Therefore the observations of this work are consistent with and provide a possible mechanism accounting for the epidemiologically observed increased risk of MTCT in the context of placental malaria and low maternal viral loads. Together these findings suggest that active prevention of malaria during pregnancy to reduce fetal immune sensitization may reduce the risk of MTCT in the context of suppressed maternal viral loads such as may occur with anti-retroviral therapy, an important hypothesis that warrants further investigation.

**Future directions**

While this work has provided insight into the impact of fetal immune activation due to maternal coinfections during pregnancy on susceptibility to HIV infection, in many ways this has simply established a foundation from which future directions of study may be envisioned.

**Prenatal sensitization to other pathogens and susceptibility to HIV**

Most broadly, this work has provided an experimental framework that may be utilized to study the impact of fetal immune sensitization to many other maternal coinfections on susceptibility to HIV. In addition to malaria responses, durable and experimentally detectable prenatal sensitization to several
helminthes, particularly schistosomiasis and lymphatic filariasis have been demonstrated in CBMC collected from newborns in the same geographic location as was utilized for this work in Coast Province, Kenya (80, 117, 193). It would be relatively straightforward, therefore, to repeat this study focusing on the impact of fetal immune sensitization to one or both of these helminth infections. Further, the prevalence of other geohelminthes such as *Entameoba histolytica*, hookworm, and *Giardia* species is high in this population (81, 117). While it would take significant investment of resources to develop assays capable of determining whether an infant has been prenatally sensitized to these pathogens, such studies are theoretically feasible. Similarly, though difficult to perform in our population due to low prevalence, other maternal coinfections that have been associated with increased MTCT in epidemiologic studies could be explored. Maternal infections such as syphilis, cytomegalovirus, and herpes simplex virus-2 have been consistently reported as risk factors for increased MTCT, although the mechanism of this increased risk has not been firmly established (38, 39, 67, 167, 168, 194). It may be hypothesized that one such contributing mechanism is sensitization and activation of the fetal immune system to antigens from these maternal coinfections, thereby increasing HIV susceptibility and replication within fetal lymphocytes. Similarly, a recent study has described increased risk of MTCT of HIV in the context of maternal coinfection with tuberculosis (TB) (130). While this study was purely epidemiologic, the authors proposed several hypotheses that could explain the increased risk of MTCT observed among TB infected women, including the possibility of alterations in the fetal immune
compartment (130). In light of our work, this hypothesis may be extended to suggest that priming of the fetal immune system to TB antigens could be a contributing factor in increasing the risk of MTCT in the context of maternal coinfection with TB.

*Timing of prenatal malaria exposure, prenatal sensitization and HIV susceptibility*

There is significantly more work that may be done within the context of prenatal sensitization to malaria, particularly in regard to issues such as the impact of timing of fetal immune exposure to malaria during gestation and its impact on both development of sensitization and consequently susceptibility to HIV infection. A study is currently being undertaken in Papua New Guinea in which pregnant women visiting a prenatal clinic are randomly assigned to receive either the standard of care anti-malarial IPTp or a more intensive treatment regimen designed to completely eliminate malaria infection. Further, due to differences in when patients initially present to the prenatal clinic, women will begin treatment at different points during the second and third trimester. This combination of variable timing of enrollment and variable thoroughness of maternal malaria control will allow greater exploration of the timing of fetal exposure to malaria antigens in inducing prenatal sensitization. Additional in vitro HIV infection studies may be performed to determine if prenatal sensitization that occurs early (second trimester) or late (third trimester) is an important variable in inducing susceptibility to HIV. Given the differences in surface activation and gene expression among HIV susceptible CBMC observed in the
current study, it may be hypothesized that prenatal exposure to malaria later in gestation leading to immune activation detectable at delivery is more likely to increase in vitro HIV susceptibility compared to sensitization events that occur earlier in pregnancy. The results of these studies could further refine anti-malaria treatment strategies that could be utilized as an adjunct to anti-retroviral therapy in an effort to further reduce MTCT of HIV in malaria endemic, resource limited settings.

Malaria-specific memory T cells as targets of HIV infection

In addition to these broader studies, questions raised by this current work may be addressed further with the current in vitro HIV infection model. CD4+CD45RO+ cells represented the primary reservoir for replicating virus during early time points in our model system and memory cell activation but not frequency correlated with susceptibility to HIV infection. Malaria sensitized CMBC were more susceptible to HIV compared to malaria not sensitized and North American CBMC, an effect enhanced by prestimulation with malaria antigens. While these data are suggestive, it has not been demonstrated definitively that the memory CD4+ T cell targets of infection in our model system are malaria specific.

Ideally, malaria-specific CD4+ cells could be directly identified using class II major histocompatibility complex tetramers loaded with *P. falciparum* antigens. One published study to date utilized tetramers loaded with a peptide from the *P. falciparum* circumsporozoite protein to identify vaccine-induced responses in
human volunteers (195). However, such reagents are not widely available nor have MSP1 tetramers been created that correspond to the reagents used in our study to define sensitization. Therefore direct identification of malaria specific CD4+ T cells would be difficult. Indirect identification is feasible by performing experiments in which CBMC would be exposed to HIV in vitro following the standard protocol with subsequent culture in the presence of malaria antigens. Flow cytometric analysis of production of IFNγ or other cytokines and/or Ki67 (a marker of cells undergoing mitosis) in response to these antigens would identify malaria specific T cells and p24 expression would identify HIV infected cells. The addition of the protease inhibitor saquinavir would further identify whether malaria specific TEM CD4+ cells are the initial target of infection in our model system.

If p24 expressing cells are also producing cytokines and/or proliferating in response to malaria antigenic stimulation, this provides additional strong evidence that prenatal sensitization to malaria is a risk factor for MTCT of HIV. If p24 expressing cells do not appear to be specific or responsive to malaria antigens, interpretation will be more difficult. One possibility would be nonspecific activation of bystander cells via cytokines produced by malaria specific cells leading to enhanced susceptibility and replication of HIV in these bystander cells. Alternatively, memory T cells specific for other pathogens capable of sensitizing the prenatal immune system, such as helminthes, may serve as the initial targets in some samples and not require exogenous stimulation to sustain HIV replication. Finally, such nonspecific results could suggest that prenatal sensitization to any specific pathogen, such as malaria,
does not alter susceptibility to in vitro HIV infection. This interpretation would contradict many of the conclusions of the current work, would need to be critically viewed within the context of all the current data, and would warrant further detailed studies.

**HIV viral lifecycle studies**

Our model of in vitro HIV infection could also be used to study the viral lifecycle in CBMC in the absence of exogenous stimulation. Consistent with prior studies (95–97), we demonstrated that viral binding to target cells, entry, and initiation of reverse transcription did not differ between HIV susceptible and HIV not susceptible CBMC as indicated by HIV –ssDNA amplification. However, late HIV life cycle events, as detected by the presence of *gag/pol* RNA, were limited solely to HIV susceptible CBMC. These observations demonstrate a difference in progression of the viral life cycle between HIV susceptible and HIV not susceptible samples that could be further explored by utilizing qPCR techniques due to the highly ordered sequence of reverse transcription and integration. After the HIV genome is inserted into the host cell cytoplasm, the RNA sequence must be converted into double-stranded DNA (84–86). This conversion is mediated by the viral protein reverse transcriptase with complementary DNA strand production occurring in a well-defined pattern (86). Following completion of reverse transcription, the linear double-stranded DNA proviral genome must be transported into the nucleus for integration into the host cell genome (84, 86). Though linear duplex DNA is the precursor of integration, upon nuclear entry two
additional DNA intermediates may be formed: circles containing one or two LTR sequences (86). Importantly, the two-LTR containing circle is thought to occur solely within the nucleus and creates a unique LTR-LTR sequence that serves as a marker of nuclear entry of the viral genome (85, 93, 196, 197). This well-characterized sequence of events has been used to develop PCR amplification primers and techniques to monitor DNA intermediates and progression through reverse transcription and nuclear translocation (93, 95–97, 196, 197).

Given earlier studies indicating incomplete reverse transcription in quiescent CBMC, one hypothesis would be that late RT events – completion of reverse transcription, and nuclear importation – in addition to proviral gene transcription would only be detectable in HIV susceptible samples while HIV–ssDNA would remain detectable in both HIV susceptible and HIV not susceptible CBMC. This result would indicate a deficit in completion of reverse transcription, possibly due to low levels of free cytosolic nucleotides in fetal T cells and insufficient activation in T_EM from HIV not susceptible samples. Alternatively, it is possible no differences in reverse transcription completion or nuclear import may be observed with the only difference remaining in proviral gene transcription as indicated by detection of gag/pol RNA. This result would suggest that proviral gene transcription, an event heavily dependent upon and regulated by cellular activation, is the primary mediator of the difference in susceptibility to productive HIV infection and replication in our in vitro model system. In either case, whether the checkpoint in viral replication occurs early during reverse transcription or late through decreased proviral gene transcription,
HIV containing but non-productively infected cells may eventually be eliminated via directed killing by other immune cells or through apoptosis, thereby eliminating a source for spreading infection (97). Conversely, if cellular activation sufficient to drive the viral life cycle to completion occurs, productive HIV infection may be observed. This is consistent with our early observations that PHA stimulation of CBMC following HIV exposure led to an increased frequency of samples demonstrating detectable viral replication regardless of whether they were from malaria sensitized, malaria not sensitized, or North American infants (Figures 2-3, 2-5).

**Experimentally altered expression of relevant genes on HIV susceptibility**

Regulation of the HIV viral lifecycle is impacted by the activation state and expression of a variety of host cellular genes, many of which have been explored in peripheral blood mononuclear cells (PBMC) from adults (96, 97, 155, 164, 165, 198–201). However, similar studies on CBMC are lacking. Only one other study to date has sought to characterize host gene expression profiles within CBMC and correlate the findings with susceptibility to HIV infection (136). Importantly, those authors were primarily concerned with comparisons between CBMC and adult PBMC, both in terms of gene expression and susceptibility to in vitro HIV infection. In comparison to PBMC, CBMC had increased expression of mRNA encoding a variety of proteins including NF-κB, E2F, HAT-1, TFIIE, Cdk9, Cyclin T1, STAT3, STAT5a, IL-1β, IL-6, and IL-10, as well as decreased expression of YY1 (136). These differential gene expression patterns were further correlated
with enhanced HIV gene expression and replication in CBMC compared to PBMC (136). In addition to differing comparison groups (CBMC versus PBMC), preactivation of CBMC with mitogenic stimulation was used to facilitate productive viral infection. In contrast, our current work compared differences in gene expression between HIV susceptible and HIV not susceptible CBMC in the absence of any exogenous stimulation. Further, the data presented here focused solely on gene expression in CD4⁺CD45RO⁺ memory T cells that were demonstrated to be the primary initial targets of in vitro HIV infection.

Given these differences in experimental approach, it is perhaps not surprising that fewer and predominantly distinct differences in differential gene expression were found in our study compared to those described elsewhere (136). A notable similarity in findings is the marked decrease in expression of YY1 in both total CBMC compared to PBMC described by others as well as memory T cells from HIV susceptible samples compared to those from HIV not susceptible samples described here (136). As YY1 has been described to act in conjunction with TFCP2 in the cooperative suppression of HIV proviral gene transcription, the decreased expression of both genes in HIV susceptible memory T cells is notable (101–103). Together, these observations suggest that YY1, likely together with TFCP2, may play an important role in suppressing HIV proviral gene expression in CBMC with decreased expression facilitating increased viral replication. This hypothesis is consistent with our data suggesting that HIV is capable of entering and initiating reverse transcription similarly
between HIV susceptible and HIV not susceptible samples while proviral gene expression is solely detected in HIV susceptible samples.

Further characterization of the role for YY1 and TFCP2 in mediating CBMC susceptibility to in vitro HIV infection is warranted. Although performed in different model systems, expression and activity for YY1 and TFCP2 have been experimentally decreased using commercially available small interfering RNA (siRNA) and dominant negative constructs (101, 202–204). These reagents could be utilized to transfect and decrease expression of these transcription factors in either total CBMC or isolated CD4+CD45RO+ memory T cells from HIV not susceptible samples prior to exposure of these cells to HIV. Expression of p24 in HIV not susceptible cells transfected with active inhibitors or appropriate control constructs could be compared to p24 expression in equivalent cells from HIV susceptible samples transfected with control constructs. Conversely, transfection induced overexpression of YY1 and/or TFCP2 in CBMC from HIV susceptible samples could be performed to determine if susceptibility to HIV is reduced.

These experiments would serve to address the hypothesis that decreased YY1 expression, alone or in synergy with decreased TFCP2 expression, is sufficient to mediate increased HIV replication in otherwise resistant CBMC.
To date, nearly all in vitro studies investigating susceptibility of CBMC to HIV infection have been based upon exposure of target cells to free virus (93, 95, 129, 135, 136, 173). Indeed, from an epidemiologic standpoint, maternal HIV viral load at the time of delivery has been consistently described as a key independent risk factor with likelihood of transmission correlating with higher viral loads (2, 5–7, 32–34). This observation, coupled with relatively straightforward and well described protocols, has led to experimental systems relying upon exposure of CBMC to free virus to model MTCT. However, some have hypothesized that MTCT likely occurs not solely due to passage of free virus across the placental barrier, but rather may also occur via cell-to-cell transmission by maternal, HIV-infected cells that have gained access to the fetal circulation through disruption of the placental barrier (6, 28, 40). Two studies have demonstrated increased risk of MTCT in the context of maternal-fetal microtransfusions evidenced either by detection of the maternal enzyme placental alkaline phosphatase (PLAP) in cord blood or by direct PCR amplification of maternal DNA in cord blood (27, 28). These observations, together with the relative resistance of most CBMC samples to HIV infection with free virus in the absence of exogenous stimulation, have led some to suggest that direct maternal cell-to-fetal cell transmission of HIV may be an important mechanism of MTCT, particularly in utero (5, 6, 27, 28, 40). However, no studies to date have investigated this hypothesis in vitro.
Cell-to-cell transmission has become a recent area of interest in the HIV field, particularly in regard to evasion of antiretroviral therapies or host immune responses (205–207). A variety of experimental techniques and approaches have been described, though fewer have utilized a heterologous donor-target system. In the context of MTCT, it would be ideal to develop a model in which PBMC from a mother could be infected in vitro and subsequently co-cultured with CBMC from her offspring. However, standardizing and ensuring proper control experiments to allow meaningful interpretation across samples would likely prove difficult. An alternative, more feasible approach would be to use an HIV infected, human cell line as the donor to be co-cultured with CBMC. Jurkat and MOLT cell lines, both derived from human T cell leukemias, have been used experimentally to study cell-to-cell transmission of HIV to adult, primary T cells and the use of an infected cell line would allow greater standardization (207, 208). Fluorescent staining for p24 in addition to appropriately differentiating cell surface markers would allow discrimination of viral replication in donor versus target cells.

It could be hypothesized that CBMC from malaria sensitized samples demonstrating increased susceptibility to HIV infection with free virus would similarly show enhanced susceptibility to cell-to-cell transmission when compared to samples not susceptible to infection with free virus. Alternatively, the heterologous co-culture system may provide sufficient stimulation of otherwise HIV not susceptible CBMC to permit successful transmission to a similar degree as might be observed for free virus HIV susceptible samples. Regardless, it would be important to demonstrate that the transmission events
were occurring across the virologic synapse as opposed to transmission via free virus produced and released by the donor cells. This issue could be addressed by confocal microscopic techniques and by performing parallel transwell cultures in which the donor and target cells were physically separated.

In conclusion, this work has demonstrated that prenatal sensitization to malaria, evidenced by recall responses to parasitic antigens, increases the susceptibility of CBMC to HIV infection in vitro. This increased susceptibility was limited solely to CD3+CD4+ cells of which CD45RO+ memory T cells were the initial targets of infection. HIV gene transcription, but not viral entry and reverse transcription initiation, correlated with HIV susceptibility. Further, cell surface phenotype and host gene expression profile differences were observed between memory T cells from HIV susceptible versus HIV not susceptible samples. This study demonstrates that CBMC from newborns primed in utero to parasitic antigens are more likely to become infected with HIV. Therefore, by actively controlling malaria and other chronic parasitic infections in HIV positive pregnant women, prenatal sensitization to parasitic antigens may be prevented and the risk of MTCT reduced.
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