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Enhancement of HIV-1 pathogenesis by plasmodium falciparum: mechanisms and clinical applications

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Enhancement of HIV-1 Pathogenesis by Plasmodium falciparum; Mechanisms and Clinical Applications

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences by Marika Orlov

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2012
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Co-Chair

Chair

University of California, San Diego
2012
DEDICATION

For Bella, Boris, and Nich

In loving memory of Anna and my father,

Michael
EPIGRAPH

Not all those who wander are lost.

*J. R. R. Tolkien*
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature Page</td>
<td>iii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Epigraph</td>
<td>v</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>viii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>ix</td>
</tr>
<tr>
<td>Vita</td>
<td>x</td>
</tr>
<tr>
<td>Abstract of the Dissertation</td>
<td>xi</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Chapter 1: <em>P. falciparum</em> Enhances HIV Replication in an Experimental Malaria Challenge System</td>
<td>12</td>
</tr>
<tr>
<td>Chapter 2: Phagocytic, Antigen Presenting Cells Ingest Malaria Parasites and Cause an Increase in HIV Replication in a Contact- and Cytokine-Dependent Manner</td>
<td>37</td>
</tr>
<tr>
<td>Chapter 3: Subpatent Parasitemia is not Associated with Development of Clinical Malaria Among Study Participants in Malawi from the PEARLS/ACTG 5175 Randomized Clinical Trial</td>
<td>62</td>
</tr>
<tr>
<td>Discussion</td>
<td>84</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1.1: Schematic representation of controlled *P. falciparum* infection........ 27
Figure 1.2: *In vitro* Co-Culture System ........................................................................ 28
Figure 1.3: *Plasmodium falciparum* Enhances HIV-1 Production .................................. 29
Figure 1.4: Activation of Memory and Total CD4/8 T-cells by iRBCs in D56 Post Malaria Challenge PBMCs ............................................................................................................. 30
Figure 1.5: *Plasmodium falciparum* Stimulates Enhanced Secretion of TNF-α, IFN-γ, and MIP-1α, but not IL-6 ................................................................................................................ 32
Figure 1.6: General Inflammation in the Periphery does not Correlate to HIV Production in Co-Cultures .................................................................................................................. 33
Figure 2.1: iRBCs Activate CD4+ T-cells, but not CD8+ T-cells, in Co-Cultures ..... 52
Figure 2.2: Increase in TNF-α, IFN-γ, and MIP-1α Secretion in iRBC Co-Cultures... 53
Figure 2.3: TNF-α, but not IFN-γ, Neutralizing Antibodies Inhibit HIV Production in Co-Cultures .................................................................................................................. 54
Figure 2.4: Phagocytic, Antigen Presenting Cells are Necessary for Increased HIV Production in Co-Cultures ........................................................................................................... 55
Figure 2.5: Macrophages and Dendritic Cells Need to be in Contact with both iRBCs and the Rest of the PBMCs for Maximal HIV Production .................................................. 56
Figure 2.6: Macrophages and Dendritic Cells Pre-Treated with iRBCs are Sufficient for Inducing HIV Production ............................................................................................... 57
Figure 2.7: Proposed Mechanism for HIV-1/*P. falciparum* Interactions ................. 59
Figure 3.1: Detection of Subpatent Parasitemia does not Predict Development of Clinical Disease .................................................................................................................... 76
Figure 3.2: Prevalence of Subpatent Parasitemia Over the Course of the Study 77
Figure D.1: Diagrammatic Illustration of CD4+ T-cell Counts and Plasma HIV-1 RNA Levels in a Co-Infected Individual .......................................................................................... 90
Figure D.2: Conceptual Illustration of Erosion of Malarial Immunity in an HIV-1 Infected Individual .................................................................................................................... 91
LIST OF TABLES

Table 3.1: Correlates of Baseline Subpatent Malaria (PCR+) ........................................ 78
Table 3.2: Positive Predictive Value of Subpatent Parasitemia for Development of Clinical Disease ........................................................................................................ 79
Table 3.3: Persistence of Subpatent Parasitemia ............................................................. 80
Table 3.4: Correlates of Probable and Confirmed Clinical Malaria ............................. 81
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Chapter 2, in part is currently being prepared for submission for publication of the material. Orlov, M; Vaida, F; Deng, Q; Smith, DM; Williamson, K; Duffy, PE; and Schooley, RT. The dissertation author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

Enhancement of HIV-1 Pathogenesis by *Plasmodium falciparum*; Mechanisms and Clinical Applications

by

Marika Orlov

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2012

Professor Robert Schooley, Chair
Professor Victor Nizet, Co-Chair

HIV-1 and *Plasmodium falciparum* malaria cause substantial morbidity in Sub-Saharan Africa, especially as co-infesting pathogens. However, many aspects of this interaction have not been fully delineated. We hypothesize that dual infection causes increased HIV expression and more rapid HIV disease progression and that activation of malaria-specific CD4+ T-cells in the presence of uncontrolled HIV replication leads to depletion of malaria-specific immunity. To better understand the effects of *P. falciparum*
on HIV, we studied the interaction ex vivo using peripheral blood mononuclear cells (PBMCs) from human malaria naïve volunteers experimentally infected with *P. falciparum* in a malaria challenge trial and we used an *in vitro* co-culture system to study the mechanisms contributing to increased viral replication. To assess the effects of HIV on *P. falciparum*, we took advantage of a recently completed clinical trial of antiretroviral therapy to study the relationship between subpatent parasitemia (detection of parasites by methods more sensitive than blood smears, but without clinical malaria) and clinical disease and attempted to assess whether the HIV-1 protease inhibitor (PI) atazanavir reduced the rate of detection of plasma *P. falciparum* DNA. The ex vivo experiments showed an increase in HIV production and pro-inflammatory cytokine secretion that occurred after the parasitemia and generalized immune activation resolved, suggesting that enhanced HIV production is related to the development of anti-malaria immunity and may be mediated by pro-inflammatory cytokines. We also demonstrated that phagocytic, antigen presenting cells ingest *P. falciparum* infected red blood cells and stimulate CD4+ T-cells to produce HIV-1 in a cytokine- and contact-dependent manner. Lastly, subpatent parasitemia was not significantly associated with clinical malaria and HIV PI therapy did not decrease the prevalence of parasitemia. Taken together, our results suggest that while subpatent parasitemia may not lead to clinical disease, it may contribute to an increase in plasma HIV-1 RNA levels in untreated patients, especially upon repeat encounters with malaria parasites. It is plausible that any level of parasitemia may contribute to CD4+ T-cell activation, even in the presence of anti-retroviral therapy. Thus, it may be important to suppress parasitemia in HIV seropositive individuals, even if they are asymptomatic.
Introduction

Despite the discovery of multiple promising vaccine candidates in mice, development of novel anti-malarial agents (artemisin), and efforts to eradicate the Anopheles mosquito vector, malaria still causes 881,000 deaths annually, 91% of which occur in Africa [1]. Most deaths from malaria are in children under 5 years of age, pregnant women and immunocompromised individuals [1].

Adaptive immune responses developed during repetitive episodes of malaria protect individuals from severe disease. Sterilizing immunity to malaria never fully develops as individuals remain susceptible to both infections with P. falciparum and to clinical disease. In malaria endemic regions, many individuals harbor malaria parasites within peripheral blood erythrocytes without discernible clinical symptoms [2]. An effective immune response that can control parasitemia consists of an initial phase of immune activation, marked by secretion of inflammatory cytokines such as interferon (INF)-γ and tumor necrosis factor (TNF)-α, which is followed by a phase of immune deactivation, marked by secretion of anti-inflammatory cytokines such as tumor growth factor (TGF)-β and interleukin (IL)-10. While early secretion of activating cytokines is linked to parasite clearance, there is a distinction between cellular immunity to parasites and resistance to clinical disease because as levels of pro-inflammatory cytokines increase in the circulation, especially IFN-γ, there is an increased risk of developing severe clinical symptoms, despite the ability to clear parasites. One of the characteristics of semi-immunity is that peripheral blood mononuclear cells (PBMCs) taken from persons living in malaria endemic areas secrete lower levels of IFN-γ upon exposure to malarial antigens in vitro than do PBMCs isolated from malaria naïve donors [3]. This might explain the observation that people living in malaria endemic regions can have detectable
parasites in their peripheral blood without experiencing clinical symptoms. These states are called subclinical (if the parasites are detectable in a blood smear) or subpatent/sub-microscopic (if the parasites are detectable by more sensitive methods, such as PCR) parasitemia. It remains unclear, however, whether subclinical or subpatent parasitemia predisposes to clinical malaria, whether they are indeed markers of partial immunity, thereby signifying a lower probability of contracting clinical disease, or they are unrelated disease states altogether.

According to UNAIDS, an estimated 33 million people were living with HIV at the end of 2009 and approximately 2.6 million additional people were infected with HIV during that year [4]. There are three countries in southern Africa (Botswana, Lesotho, and Swaziland) where more than 20% of the population is infected, and nearly 18% of the population is infected in South Africa [4]. HIV-1 vaccine development is at an impasse. Major advances in antiretroviral therapy have resulted in substantial reductions in morbidity and mortality – especially in resource rich settings. Although access to contemporary HIV therapy has increased substantially in resource limited settings, millions who need treatment do not receive it – a condition that is likely to persist for at least the next decade or more.

HIV infects T helper cells using the CD4 molecule as the entry receptor. CD4 memory T cells that express CCR5 (a marker for memory cells) have been shown to be most efficiently infected and subsequently depleted during acute infection since HIV uses CCR5 as a co-receptor for viral entry [5]. Memory CD4 T cells can be broken down into two distinct subsets: central memory cells (CD45RO+CD62L+) and effector memory cells (CD45RO+CD62L-). Effector memory cells are short lived and generally infiltrate the site of infection, these are the cells that express high levels of CCR5 and are quickly depleted in an acute HIV infection. Central memory cells express less CCR5 receptor but are still targeted in an acute infection, albeit to a lesser degree. They are progressively
depleted throughout the course of chronic HIV infection and this depletion has been noted to correlate with HIV disease progression. During chronic HIV infection, overstimulation of the immune system contributes to progressive generalized loss of these cells as a result of a lowered apoptotic threshold. HIV-1 preferentially infects CD4 T-cells that have become activated in response to pathogen specific antigens. This both acutely impairs pathogen-specific immune responses and, over time, results in erosion of the cellular immune response to chronically or repetitively encountered antigens. Indeed, in the case of HIV-1 specific immunity, the selective depletion of HIV-1 directed CD4 cells is believed to be one of the pathogen’s most effective approaches to immune evasion. Stimulation of memory cells by a specific antigen related to another disease also leads to depletion. In addition, it has been shown that in vitro HIV preferentially replicates in the memory cell (CD45RO+) subset of primary CD4+ T-cells [6].

Although it was not initially fully appreciated [7, 8], HIV-1 and malaria likely interact in individuals and in populations. During acute bouts of clinical malaria, plasma HIV-1 RNA levels rise [9-11], and CD4 cells decline by approximately 40 cells/µL/year with each malaria episode [12] compared to the rate of decline in individuals without clinical malaria episodes. Conversely, in regions of unstable malaria transmission, HIV infection is associated with increased malaria disease severity and death [13]. Recently, a mathematical model further explored the potential importance of the malaria/HIV interaction. Based on this model, in an area of Kenya, with an adult population of roughly 200,000 that has been exposed to both pathogens since 1980, the interaction of the two diseases may have caused 8,500 excess HIV infections and 980,000 excess malaria episodes [14]. Supporting this mathematical model, a study that examined geographical overlap of the two pathogens in East Africa found that those who live in areas of high P. falciparum incidence have about twice the risk of being HIV infected compared to individuals who live in areas of low incidence [15].
The host immune response to *Plasmodium* infection includes the rapid release of IFN-γ by natural killer (NK) cells and TNF by macrophages, while cells of the adaptive immune system release TNF, IL-12, and IFN-γ in response to parasitized erythrocytes [16]. Several cytokines that are elaborated during acute bouts of malaria can cause stimulation of HIV-1 replication. IL-1, IL-2, IL-3, IL-6, IL-12, granulocyte macrophage-colony stimulating factor (GM-CSF), and TNF-α/β have been identified as inducers of viral expression [17], prompting speculation that cytokines involved in the control and clearance of a *Plasmodium* infection, especially TNF-a, may contribute to the increase in HIV replication [11]. But the exact mechanism that is responsible for the increase in HIV production and the specific cell types that are involved has not yet been fully elucidated.

While it has been demonstrated that humoral immunity can be elicited or maintained with sub-microscopic parasitemias [18], there is no published evidence so far that persistent subpatent or subclinical parasitemia results in chronic immune activation. This might be hard to determine, however, since large differences in baseline activation exist among individuals and within the same individual over time because of other intercurrent immune modulating events, resulting in the need to follow participants longitudinally before, during, and after bouts of subclinical/subpatent parasitemias. An alternative would be to compare those with subpatent or subclinical parasitemia to those without any evidence of parasitemia, but such a comparison would likely require a large number of study participants and would require careful consideration be given to factors other than subpatent parasitemia that might cause differences between the groups. Malaria parasites are immune activating, suggesting that even at persistent subpatent levels, there would be some level of ongoing chronic immune activation, which in HIV-untreated co-infected populations, could cause enough CD4+ T-cell stimulation to drive small increases in viral loads chronically. Even in individuals taking
anti-retroviral therapy, chronic subpatent parasitemia could cause persistent stimulation of CD8+ T-cells. It has been shown that there are detectable increases in CD8+ T-cell activation due to bacterial translocation in the gut of individuals on anti-retroviral therapy and that this increased CD8+ T-cell activation is associated with a worse HIV prognosis [19]. A similar chronic increase in activation in CD8+ T-cells due to subpatent parasitemia could be deleterious to HIV prognosis as well.

There have only been a handful of studies that have examined mechanisms involved in malaria/HIV interactions. These studies, which have been conducted both in vitro and in murine model systems have yielded a number of contradictory findings. A major challenge facing those interested in examining malaria/HIV interactions is that there is no good working animal model for either of these pathogens. Major inter patient variability complicates human studies requiring careful matching of patient and disease-specific parameters as well as relatively large sample sizes for robust observations.

Xiao, et al, reported that *Plasmodium falciparum* antigen-induced HIV-1 replication is mediated through the induction of TNF-α. HIV and malaria naïve, CD8+ T-cell depleted PBMCs from healthy donors were infected with HIV and co-cultured either in the presence or absence of malarial antigens, which were prepared by isolating merozoites and soluble antigens after removing erythrocytes. The presence of malarial antigens greatly increased HIV-1 replication, and this increase was not dependent on the presence of antigen presenting cells in culture. Addition of a TNF-α neutralizing monoclonal antibody to the cultures resulted in a reduction of HIV-1 replication by more than 60%. In contrast, an antibody directed at IL-6 did not affect HIV-1 replication. Their conclusion was that the increase in HIV-1 replication was dependent on the increased secretion of TNF-α by activated T cells [20]. In this study, CD8+ T-cell depleted PBMCs from HIV uninfected donors were infected with a laboratory strain of HIV and exposed to only merozoites or hemozoin. It is not clear how well this system mimics the situation in
HIV-1 infected persons in which patient-adapted strains of HIV-1 infect cells of an immune system that has both been partially depleted and dysregulated by HIV-1 infection and in which HIV-1 and malaria specific immunologic effector cells are present. Immune cells in the peripheral blood of a person who is infected with *P. falciparum* are exposed to merozoites for only a brief period of time and rarely to free hemoglobin. Most malaria antigens are contained within infected red blood cells and need to be processed by phagocytic, antigen presenting cells for CD4+ T-cells to be exposed to malaria antigens, other than those expressed on the surface of the iRBC.

Freitag, *et al.*, showed that malaria infection induces HIV-1 expression by CD4 T-cell dependent immune activation using a transgenic *in vivo* murine model, but obtained slightly different results. They used a transgenic mouse that harbors multiple copies of the HIV-1 genome integrated into its DNA under the control of the HIV promoter (tat). Since mice lack the functional receptors and co-receptors for viral entry, these animals are not immunocompromised and cannot serve as a model for HIV pathogenesis. They can, however, be used to monitor HIV expression in response to other stimuli. These transgenic mice were infected with *Plasmodium chabaudi chabaudi* clone AS, which produces an acute, nonlethal parasitemia controlled by T lymphocyte-dependent cellular and humoral mechanisms. Using this model system, the authors reported that *P. chabaudi* infection induced HIV-1 expression *in vivo*. This induction was limited to the initial acute parasitemia and did not occur upon recrudescence or after secondary infection. In this system the increase in expression was not dependent on cytokine production, but was the result of increased viral production by antigen presenting cells in a CD4 T cell-dependent manner [21]. Since HIV-1 neither enters nor productively infects murine cells, this murine transgenic model is limited to the assessment of induction of expression of latent HIV sequences and does not result in the amplification of viral infection that occurs when infectious virions secondarily infect
activated lymphocytes. Thus, this murine model is far removed from HIV-1 infection in man in which activation from latency often occurs in the presence of an expansion of the number of cells that are permissive to viral infection.

Recently, Diou, et al, provided another insight into the mechanism driving HIV production in the presence of *P. falciparum* that disputes both of the previously published papers. They showed that hemozoin, a byproduct of *P. falciparum* heme breakdown, on its own caused enhanced HIV production *in vitro* [22]. While Xiao, et al, showed that antigen presenting cells were not required for the increase they observed in HIV production, this paper argues that monocytes matured in the presence of hemozoin developed into immature dendritic cells that were capable of increased HIV production by both enhancing dendritic cell mediated transfer of HIV-1 between CD4+ T-cells and increasing HIV production when HIV-infected CD4+ T-cells were co-cultured with hemozoin treated dendritic cells. Their findings, as well as those of others, also dispute the Frietag, et al, results that plasma HIV-1 RNA levels increase due to increased HIV replication within the antigen presenting cells, as hemozoin crystals have been shown to inhibit replication of HIV in macrophages [23] and dendritic cells [22].

Even without knowing the exact mechanism of interaction, several groups have hypothesized that HIV/Plasmodium interactions could be minimized due to overlap in drug susceptibility. HIV protease inhibitors are in wide use for treatment of HIV infection in the US and Europe. Although used less frequently in resource limited settings, protease inhibitors are routinely used following failure of first-line regimens. HIV protease inhibitors target the viral aspartyl protease and inhibit replication of *P. falciparum* in tissue culture [24]. It has been suggested that this effect is related to the structural similarity of the aspartyl proteases of *P. falciparum* (collectively known as plasmepsins) to the aspartyl protease of HIV-1, against which these agents were initially developed. Despite *in vitro*
and murine model data, the activity of HIV protease inhibitors against *P. falciparum* in vivo has not been reported.

We outline below our efforts to study HIV-1/*P. falciparum* interactions, using a spectrum of ex vivo, *in vitro*, and *in vivo* approaches. First, we took PBMCs from healthy human donors that were experimentally infected with malaria and placed them into a co-culture system where we exposed the PBMCs to *P. falciparum* infected red blood cells (iRBCs) and infected them with HIV-1 ex vivo to study the effect of a repeat malaria exposure on HIV production. We then used this co-culture system to examine the mechanism responsible for the increases seen in HIV production *in vitro* by studying the cytokines and cell types that are involved in the interaction. Lastly, we worked with samples from a cohort of HIV-1/*P. falciparum* infected individuals in Malawi to investigate whether HIV protease inhibitors reduced *P. falciparum* parasite burden and to see if subpatent parasitemias were predictive of or protective from development of clinical disease. In this series of bench-to-bedside studies, we sought to better understand the pathogenesis of both diseases and the mechanisms responsible for their interactions at the individual and population levels.
References:


Chapter 1:

*P. falciparum* Enhances HIV Replication in an Experimental Malaria Challenge System

Introduction.

To evaluate interactions between *P. falciparum* and HIV-1 ex vivo, we developed a culture system using PBMCs collected from humans experimentally infected with *P. falciparum* under carefully controlled conditions in the context of a malaria human challenge trial (Talley, AK, manuscript in preparation). Using this model system, we were able to further evaluate the mechanisms responsible for the deleterious interactions between HIV-1 and *P. falciparum*, and to determine whether these interactions are affected by prior exposure to *P. falciparum*. 
Results.

Study Population: Healthy HIV- and malaria-naive adult volunteers were infected with *Plasmodium falciparum* sporozoites from bites of five *P. falciparum*-infected *A. stephensi* mosquitoes under controlled conditions. Blood sampling for isolation of PBMCs and plasma (Figure 1.1, below; outlined in more detail in methods section) occurred prior to challenge (baseline) and at regular intervals after challenge, including day 5 (corresponding to the liver stage of parasite development), the day of the first positive blood smear (corresponding to the blood stage), and in the post-treatment period approximately 35, 56, and 90 days following mosquito bites. Volunteers were closely monitored in the post-challenge period and treated with standard doses of chloroquine upon diagnosis of parasitemia by positive thick blood films. After day 90 PBMCs were collected and cryopreserved, co-cultures were set up using PBMCs from all time points for a given donor in a single experiment. Figure 1.2 (below) depicts the co-culture system used for these and subsequent studies.

*P. falciparum*-infected red blood cells (iRBCs) stimulate more HIV-1 p24 Ag production than uRBCs: Whole PBMCs isolated from HIV uninfected, malaria naïve donors produced significantly more HIV when co-cultured with iRBCs than when co-cultured with uRBCs (Figure 1.3B (below), p=0.0045, area under the curve comparison). The increase in HIV-1 production was evident by day 8 in culture. By day 10, HIV production in the iRBC co-cultures was increased about 2.5-fold over parallel co-cultures with uRBCs (Figure 1.3A).

Repeat exposure to *P. falciparum* enhances stimulation of HIV production by iRBCs: To study the effects of acute *P. falciparum* infection on HIV-1 p24 Ag production by PBMCs after *in vitro* infection with HIV-1, we used PBMCs isolated from the controlled human malaria challenge participants. As noted above, all samples from any given study
participant were thawed and studied in the same experiment. Sufficient PBMCs were available from 5 of the 6 malaria challenge trial participants. Figure 1.3C (below) depicts HIV production in the iRBC/uRBC co-cultures for the 5 participants at the 6 different visits. For all of the participants at the baseline, liver stage, and blood stage visits, the amount of HIV produced from the PBMCs co-cultured with iRBCs (pink lines) was about 2 fold higher than the amount of HIV produced from the PBMCs co-cultured with uRBCs (grey lines). For all participants at the convalescent visits (day 35, 56, and 90 post malaria exposure time points), HIV production from the iRBC co-cultures (burgundy lines) was about 3 fold higher than that from the respective PBMCs co-cultured with uRBCs (black lines).

In order to further explore the relationship between P. falciparum stimulation and HIV-1 production by cells collected from malaria-exposed volunteers, we performed an area-under-the-curve calculation of p24 Ag production *in vitro* at each time point under each condition and evaluated serial differences for each patient. The amount of HIV produced from the uRBC co-cultures (black line) was unchanged across all the visits (Figure 1.3D). In contrast, we observed a significant increase in HIV production when iRBCs were co-cultured with PBMCs collected at the day 35 (p<0.001), day 56 (p=0.004), and day 90 (p=0.017) post-exposure visits, compared to iRBCs co-cultured with PBMCs collected at earlier time points. Because the enhanced HIV production occurred after chloroquine-mediated clearance of parasitemia in the study participants, our results suggest that development of malaria specific cellular immunity, rather than activated effector cells, are responsible for the heightened HIV production. In order to determine if CD4+ memory T-cells were indeed responsible for the increase we saw in HIV production, we compared surface activation markers on total CD4+ T-cells, memory (CD45RO+) CD4+ T-cells, as well as total CD8+ T-cells and memory (CD45RO+) CD8+ T-cells in PBMCs obtained on Day 56 post-malaria exposure compared to surface activation markers
expressed by these cell types when obtained from malaria naïve donors. Limitations in the number of available baseline PBMC samples from the malaria exposed participants precluded a matched longitudinal study to examine differences in memory cell activation to malaria antigens. In these experiments, cells were analyzed at 48, 72, and 96 hours post co-culture with either iRBCs or uRBCs for surface expression of cellular activation markers (Figure 1.4). We observed a general trend toward increased CD4 memory T-cell activation in the Day 56 malaria exposed donor PBMCs compared to naïve control PBMCs after 72 and 96 hours (Figure 1.4B). Statistical significance was noted with the CD25 (at 72 hours, p=0.046), CD69 (at 48 hours, p=0.008; at 72 hours, p=0.034) and CD38 (at 72 hours, p=0.011; at 96 hours, p=0.003) markers but not with the HLA-DR marker or among HLA-DR/CD38 double positive cells. Within the total CD4 cell population, we noted only upregulation of CD25 (p=0.018) and CD69 (p=0.027) in the P. falciparum exposed donor PBMCs compared to naïve control PBMCs at the 72 hour time point (Figure 1.4A).

Increase in TNF-α, IFN-γ, and MIP-1α secretion is enhanced in iRBC co-cultures following in vivo infection with P. falciparum: We then measured cytokine production in co-culture supernatants to examine relationships between HIV production and cytokine secretion. We measured the secretion of 7 different cytokines: IL-4, IL-6, IL-10, IL-17, TNF-α, IFN-γ, and MIP-1α. The levels of IL-4, IL-10, and IL-17 secretion were below the limit of detection of the BioPlex assay (data not shown). Increases in the secretion of TNF-α, IFN-γ, and MIP-1α were observed in the co-cultures that had been established with iRBCs compared to uRBCs. Production of these cytokines was further increased in PBMCs obtained 35, 56, and 90 days post malaria challenge compared to those obtained at baseline or at early stages of P. falciparum infection (Figure 1.5A-C) (below). TNF-α: day 35 p=0.013, day 56 p=0.29, day 90 p=0.19; IFN-γ: day 35 p<0.001, day 56 p=0.019, day 90 p<0.001; MIP-1α:
day 35 \( p<0.001 \), day 56 \( p=0.002 \), day 90 \( p<0.001 \). These cytokine profiles closely mirror the HIV production profiles in Figure 1.3D. IL-6 secretion was similar in iRBC and uRBC co-cultures (Figure 1.5D).

**Systemic inflammation does not correlate with increased HIV production in the co-cultures:** In order to evaluate whether increases in HIV production at the later time points might be related to generalized immune activation following recovery from acute *P. falciparum*, we measured levels of C-Reactive Protein (CRP) in plasma obtained at the time of PBMC isolation. For all but one participant (011-3), levels of CRP appear to be higher during the early time points than at the later time points (Figure 1.6, below). Thus, CRP levels were generally higher during active infection compared to convalescent samples, where HIV-1 was seen to increase. In fact, a spike in the CRP levels was noted in two participants (018-4 and 016-6) at the blood stage time point of the active malaria infection, but there was no corresponding increase in HIV production at the same time point. This supports the concept that increased HIV production during the convalescent phase is not the result of systemic inflammation.
Discussion.

Using PBMCs and plasma collected from donors before, during and after controlled infection with *P. falciparum*, we explored interactions between HIV-1 and *P. falciparum*. We confirm that iRBCs co-cultured with PBMCs from malaria naïve donors enhance replication of HIV-1, and show that HIV-1 production is further enhanced in PBMCs collected in the convalescent period after experimental infection with *P. falciparum*. HIV-1 replication after exposure to *P. falciparum* infected erythrocytes was accompanied by increased secretion of pro-inflammatory cytokines TNF-α, IFN-γ, and MIP-1α, which was also enhanced after experimental *P. falciparum* infection. In addition, we noted a trend toward increased memory CD4+ T-cell activation in response to iRBCs during the convalescent period. Enhanced HIV production *in vitro* following controlled human infection with *P. falciparum* was not associated with systemic inflammation *in vivo* as assessed by plasma C-reactive protein levels.

Under the controlled conditions of the standard human challenge model [1-3], subjects are diagnosed and treated at low parasite density, often prior to the onset of clinical symptoms and likely prior to the development of the cytokine storm observed in severe bouts of clinical malaria in non-immune individuals [4]. Despite the limited duration of parasitemia in the experimental malaria infection, production of HIV-1 by *P. falciparum*-exposed PBMCs was substantially enhanced following treatment and resolution of the blood stage of infection. Although not examined in these initial experiments, the changes we observed might have been more pronounced had study participants progressed to clinical disease.

The role of cytokines during malaria has been extensively studied. In controlled *Plasmodium* infections of malaria-naïve subjects, serum levels of pro-inflammatory cytokines, including TNF-α, IL-6, IFN-γ, and IL-12p40, increase at the time that parasites
emerge from the liver and at the first appearance of parasitized erythrocytes [5]. McCall, et al, reported that PBMCs isolated from participants who were infected with *P. falciparum* in a controlled human malaria infection and exposed to cryopreserved iRBCs secreted higher levels of IFN-γ by NK cells than naïve PBMCs, and that this response was dependent on CD4 help [6]. In semi-immune individuals living in malaria endemic areas, the amount of IFN-γ secreted is lower upon repeat exposure to malaria antigens than in malaria naïve individuals, and malaria episodes are less severe [7]. Separately, several TNF-α alleles have been correlated to increased plasma TNF levels, increased susceptibility to severe malaria [8], and increased susceptibility to cerebral malaria [9].

Higher levels of circulating TNF-α were found in adults and children with severe malaria compared to both uncomplicated malaria cases and healthy individuals [8, 10]. While IFN-γ is a pro-inflammatory cytokine, it inhibits HIV infection *in vitro* and has been used in patients with advanced AIDS to reduce the number of opportunistic infections [11, 12]. Thus, even though both TNF-α and IFN-γ are classified as pro-inflammatory cytokines and even though both increase during acute bouts of clinical malaria, TNF-α is a better candidate cytokine than IFN-γ to stimulate HIV production during *P. falciparum* infection. Furthermore, peak plasma levels of TNF-α do not decrease with repeat *P. falciparum* infections while those of IFN-γ decline with repeated infection.

Our data extend those of Xiao et al., who employed a similar *in vitro* system to examine the effects of *P. falciparum* on HIV-1 production by CD8+ T-cell depleted PBMCs from malaria-naïve individuals [13]. In their hands, exposure to *P. falciparum* merozoites and hemozoin increased HIV-1 replication [13]. Our studies more closely mimic a natural malarial infection by stimulating unfractionated PBMCs with *P. falciparum*-infected red blood cells rather than with merozoites or hemozoin. In the model system reported by Xiao, HIV-1 replication was the result of increased TNF-α production, but not the result of IL-6 production, demonstrated by the use of TNF-α and IL-6 blocking antibodies. We also
observed that TNF-α increased in iRBC co-cultures compared to uRBC co-cultures and in post-exposure iRBC cultures compared to the early cultures, while IL-6 production did not change significantly in any of these conditions. Although there are clear-cut differences between the experimental systems and neither system fully captures all aspects of natural infection, HIV-1 production was enhanced by *P. falciparum* exposure under both sets of experimental conditions.

Memory CD4+ T-cells have been an active area of research in the malaria field. Some have proposed that memory responses to malaria are very short lived and that malaria specific CD4+ T-cells are deleted, resulting in very little memory against future infection [14, 15]. Others have proposed that the apoptosis of antigen-specific CD4 cells would be expected following resolution of acute infection [16]. Animal models of malaria have shown that fully functional memory CD4+ T-cells are maintained for prolonged periods of time [17]. Recently, Wipasa, *et al.*, showed that malaria specific CD4+ effector memory responses decay with a half-life of about 3 years and that malaria specific CD4+ central memory responses are maintained for at least six years after the last documented clinical episode of malaria in humans [18]. If the enhanced production of HIV-1 observed in our system is truly attributable to the development of adaptive immune responses to *P. falciparum* antigens, our results suggest that memory CD4+ T-cells also develop in association with subclinical malarial infection and that they persist for at least 90 days post-exposure.

Our study examined *in vitro* HIV-1 replication induced by exposure to *P. falciparum* using samples derived from human volunteers experimentally infected with malaria for the first time. As others have demonstrated, malarial antigens increase HIV-1 replication *in vitro* prior to experience with the parasite *in vivo*. We have demonstrated that this increase is further enhanced following an experimental infection with *P. falciparum*. These findings provide experimental support to the clinical observations that
plasma levels of HIV-1 RNA rise during clinical bouts of malaria [19-21]. Although our study examined an initial exposure to *P. falciparum*, persons living in regions endemic for *P. falciparum* undergo repeated exposures to the parasite and serial bouts of clinical illness. In the course of these repeated infections, *P. falciparum*-specific CD4+ T-cells develop and play a critical role in ameliorating morbidity and mortality [18, 22, 23]. In the setting of HIV-1 infection it might be expected that pathogen specific CD4+ cells would be activated by malarial infection and, thus, would be likely to be especially vulnerable to HIV-1 infection. Such an interaction would be expected both to erode malaria-specific immunity and to contribute to enhanced replication of HIV-1 as has been demonstrated in the case of HIV-1 and *M. tuberculosis*-specific immunity [24]. This unfortunate immunopathogenic interaction would then form, at least in part, the basis for a progressively more deleterious bidirectional clinical interaction between these two pathogens with advancing HIV-1 associated immunodeficiency.

Further studies are needed to more fully examine the interactions between HIV-1 and *P. falciparum* infection both in the clinic and in *in vitro* model systems. We believe that our studies lend further support to the emerging evidence that the interactions of these pathogens are critical to the outcome of both infections. This experimental culture system provides a convenient platform in which to more carefully examine the bidirectional interactions between these pathogens both in patients with recurrent bouts of clinical malaria and in those with a subclinical infection.
**Materials and Methods.**

**PBMC collection and isolation:** PBMCs were isolated from human subjects enrolled in an experimental malaria challenge trial (protocol MC-001) at the Malaria Clinical Trials Center at Seattle Biomedical Research Institute (Talley, AK, manuscript in preparation). The experimental infection of human subjects was conducted according to standard procedures as previously described [25]. Briefly, healthy malaria-naïve adult volunteers were infected with *Plasmodium falciparum* sporozoites from bites of five *P. falciparum*-infected *A. stephensi* mosquitoes under controlled conditions. Volunteers were closely monitored in the post-challenge period and treated with standard doses of chloroquine phosphate (CQ) upon diagnosis of parasitemia by positive thick blood films. Blood sampling for isolation of PBMCs occurred prior to challenge (baseline) and at regular intervals after challenge (Figure 1) including day 5 (corresponding to the liver stage of parasite development), the day of the first positive blood smear (corresponding to the blood stage), and in the post-treatment period approximately 35, 56, and 90 days following mosquito bites. Cryopreserved PBMCs were thus available prior to the challenge, during the liver and blood stage parasitemia, and at three time points during convalescence.

PBMCs were isolated and cryopreserved according to standard methods and frozen within 8 hours of venipuncture to ensure optimal viability [26] under Good Clinical Laboratory Practices (GCLP). Fresh PBMCs were isolated by Histopaque 1077 centrifugation and cultured overnight at 37°C in 5% CO₂ in IL-2/PHA free media (10% FBS, 1% Pen-Strep in RPMI 1640) and used the following day in co-cultures. Frozen PBMCs were isolated at each designated collection point and cryopreserved in 10% DMSO/90% FBS and stored at -80°C until use in the designated assay. Fresh PBMCs were obtained from malaria naïve donors enrolled in a separate protocol (HS103) at Seattle Biomedical
Research Institute. Both protocols were approved by the Western Institutional Review Board.

**Co-cultures:** On the day of study, PBMCs from all time points from a given volunteer were thawed and placed in R10 media overnight (10% FBS, 1% Pen-Strep, 1% L-Glutamine, RPMI 1640). The day following the thaw, PBMCs were placed in 96-well plates (2x10^5 cells/well/200µL) and infected with HIV-1 (MOI=25) without exogenous mitogens or cytokines in R20 media (20% FBS, 1% Pen-Strep). Red blood cells (either *P. falciparum* infected or uninfected) were then added to selected wells in a 10:1 ratio of RBCs to PBMCs (2x10^6 RBCs/well/200µL). All conditions were run in triplicate. After 22 hours, the entire 200µL of media was collected and replaced with new media. 100µL of the culture supernatants was collected at days 4, 6, 8 and 10 and replaced with fresh medium. These supernatants were frozen at -80°C and used to determine HIV p24Ag and cytokine levels. Viral production was quantified by determining amount of p24 antigen in the culture supernatants by p24 Antigen capture ELISA (Perkin Elmer) by the UCSD CFAR Translational Virology Core.

**Plasmodium falciparum culture:** *P. falciparum* NF54 parasites were grown in type O human RBCs in RPMI 1640 (Invitrogen) with 5g/L albumax (Invitrogen), 2g/L dextrose (Fisher), 50mg/L hypoxanthine (Sigma), 2.25g/L sodium bicarbonate (Sigma), 11mg/L gentamycin (Invitrogen), and 5% pooled human AB serum (Valley Biomedical). Parasite chambers were gassed with 5% O2/5% CO2/90% N2 and incubated at 37°C. Parasite cultures were maintained continuously and split 1-2 days prior to setting up co-cultures. iRBCs were used once 6-7% of the RBCs in the culture were parasitized, as assessed by
light microscopy. Cultures were routinely monitored for mycoplasma contamination by PCR (Takara) and shown to be mycoplasma free.

**Cytokine Quantification:** Cytokine levels were measured in culture supernatants using a BioPlex platform (BioRad). A 6-plex kit containing IL-10, IL-17, IL-6, TNF-α, IFN-γ and MIP-1α or a 5-plex kit containing IL-4, IL-6, TNF-α, IFN-γ and MIP-1α was used according to the manufacturer’s protocol. Briefly, the antibody coupled beads were mixed with 50µL of the collected supernatants, which were diluted 1:2 with media and incubated on a shaker for 30 minutes. After three washes, the detection antibodies were added and the plate was incubated on the shaker for 30 minutes. After another three washes, Streptavidin-PE was added to each well and plates were incubated on the shaker for 10 minutes. The beads were resuspended in 1% formaldehyde in assay buffer and after a 30 second shake the plate was read on a Bioplex200 (BioRad). An 8-point standard curve was used to determine cytokine concentrations using a 5 parameter logistic regression curve. Detection limits for cytokines are as follows: IL-4, 0.7 pg/mL; IL-6, 2.6 pg/mL; IL-10, 0.3 pg/mL; IL-17, 3.3 pg/mL; IFN-γ, 6.4 pg/mL; TNF-α, 6.0 pg/mL; MIP-1α, 1.6 pg/mL.

**CRP ELISA:** CRP Quantikine Kit (R&D) was used to quantify the CRP in the plasma of the participants. Briefly, plasma (diluted 1:100) was added to a 96 well plate pre-coated with anti-human CRP antibodies and incubated at room temperature for 2 hours. The plate was washed 4 times with wash buffer. A horseradish peroxidase-conjugated CRP antibody was added and incubated at room temperature for 2 hours. Followed by another 4 washes with wash buffer, color solutions A and B were added in equal amounts and were allowed to incubate at room temperature for 30 minutes at which point a stop solution was added. The plate was read at 450nm and a plate correction
was read at 540nm. The log$_{10}$ of the concentration for the standards was plotted against the log$_{10}$ of the respective OD values and a regression line was obtained that was used to calculate the CRP concentrations for the obtained OD values for the unknowns.

**Flow cytometry:** PBMCs were collected, frozen, and then thawed as described above. PBMCs from day 56 post malaria challenge and from malaria naïve donors were plated (2x10$^5$ PBMCs/well) in a 96 well plate and co-cultured with either iRBCs or uRBCs (2x10$^6$ RBCs/well) in triplicate. Cultures were incubated at 37°C, 95% O$_2$ for 48, 72, or 96 hours. At the indicated time point, all three wells for a given condition were combined into a single FACS tube. Cells were washed once in FACS buffer (PBS + 2% FBS), resuspended in 50µL of Live/dead – Aqua stain (Invitrogen) and stained with either CD3-FITC (eBioscience), CD4-Pacific Blue (eBioscience), CD8-APC (BD Pharmingen), CD45RO-PE (BD Pharmingen), HLA-DR-PerCP (Biolegend), and CD38-PE-Cy5.5 (BD Pharmingen) or CD3-FITC, CD4-Pacific Blue, CD8-APC, CD45RO-PE, CD69-PerCP-Cy7 (eBioscience), and CD25-PE-Cy5.5 (eBioscience). Cells were stained at room temperature for 20 minutes and washed twice with FACS buffer. The red blood cells were then lysed using 120µL of BD FacsLyse at room temperature for 15 minutes, washed once with PBS and resuspended in 130uL of 2% formaldehyde in distilled H$_2$O. Samples were subjected to flow cytometric analysis within 18 hours of fixing on the BD LSRII. All data were analyzed using FlowJo (Treestar).

**Statistical Methods:** Total HIV-1 p24Ag and cytokine production were calculated over the 10 days in culture using a trapezoidal method (area under the curve). The log-transformed HIV p24Ag and cytokine production were compared between the iRBC and uRBC groups at each time point following experimental HIV infection in co-cultures to
which iRBCs or uRBCs had been added, and between earlier and later time points following exposure, using a normal mixed-effects profile analysis method. Specifically, different mean values were allowed at each time point and for each of the two groups for the log$_{10}$ p24 AUC values, while correlation of data points measured for the same subject was modeled by a normally-distributed random intercept. The main comparisons of interest between the iRBC and uRBC groups were of the change in mean log$_{10}$ p24 AUC between baseline and later stages. These were evaluated by the Wald test, with a Bonferonni correction for multiple comparisons. Graphs were made in GraphPad Prism Software.

**Ethics statement:** Human studies undertaken at the Seattle Biomedical Research Institute were reviewed and approved by the Western Institutional Review Board. Studies undertaken at UCSD were reviewed and approved by the UCSD Human Research Protections Program. Study participants provided written informed consent prior to their participation in the study.
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Figure 1.1: Schematic representation of controlled *P. falciparum* infection.

Visits (blue arrows): Baseline was pre-malaria challenge; Liver Stage was 5 days post-exposure; Blood stage was at the time of 2 parasites in 100+ thick smear fields, 9-14 days post exposure; ~day 35 post-exposure; ~day 56 post-exposure; and ~day 90 post-exposure. Chloroquine treatment was initiated directly following the blood stage blood draw and was administered until parasitemia resolved (about 3 days later).
Figure 1.2: In vitro co-culture system.

PBMCs from any single donor were infected with HIV-1 and co-cultured with either iRBCs or uRBCs. Supernatants were replaced after 22 hours and then half the co-culture supernatants were collected on days 4, 6, 8, and 10, stored at -80°C and subsequently used to measure p24Ag and cytokine concentrations. Neither IL2 nor PHA was added to the co-cultures. iRBCs (parasitemia 6-8%) or uRBCs were added in a 10:1 RBC:PBMC ratio. Parasites survived and continued to invade for ~4 days post-initiation of co-cultures as determined by Giemsa stains and light microscopy.
Figure 1.3: *Plasmodium falciparum* enhances HIV-1 production.

A. HIV production, as measured by p24 antigen concentration in the supernatants from PBMCs (from malaria naïve donors) co-cultured with *P. falciparum* infected (iRBC, red line) or uninfected red blood cells (uRBC, black line). B. Area under the curve (AUC) comparisons of the amount of HIV p24 Ag produced in the iRBC versus uRBC co-cultures for 6 naïve donors averaged in the graph (Error bars represent SEM, p-value was determined with a paired two-tailed T-test). C. HIV production from PBMCs that were isolated at the 6 different visits from study participants and placed into co-culture with either iRBCs or uRBCs. Pink and grey lines represent the means for the 5 participants at the three earliest time points (D0, D5, day of first parasitemia) and the burgundy and black lines represent the means of the convalescent time points (~D35, D56, D90), error bars represent the SEM. D. AUC comparisons of HIV production from the iRBC (red) and uRBC (black) co-cultures at all 6 visits. HIV production in the iRBC co-cultures is greater than the HIV production in the uRBC co-cultures at each visit; HIV produced in iRBC post-exposure co-cultures is greater than that produced in the iRBC co-cultures at the 3 early visits (p<0.001 day 35, p=0.004 day 56, and p=0.017 day 90 post-exposure). Each point is the mean for the 5 donors at that visit; the error bars represent the 95% confidence interval. p-values were determined using a repeated measures ANOVA, corrected for multiple comparisons.
Figure 1.4: Activation of memory and total CD4/8 T-cells by iRBCs in D56 post malaria challenge PBMCs.

PBMCs were cultured with iRBCs or uRBCs (without HIV) for 48, 72 or 96 hours. Cells were stained with either CD3-FITC, CD4-Pacific Blue, CD8-APC, CD45RO-PE, HLA-DR-PerCP, and CD38-PE-Cy5.5 or CD3-FITC, CD4-Pacific Blue, CD8-APC, CD45RO-PE, CD69-PerCP-Cy7, and CD25-PE-Cy5.5 and acquired using an LSRII. The percent of cells activated by iRBCs was normalized to the amount of activation due to uRBCs; this value was then logged and plotted. Line and error bars represent the mean and standard error of the mean. All values above the dotted line represent stimulation due to iRBCs. p-values were determined using an unpaired, two-tailed T-test, not corrected for multiple comparisons. A. The Day 56 post malaria challenge total CD4 cells only show increased expression of CD25 (p=0.018) and CD69 (p=0.027) at 72 hours and there are no obvious trends toward increased activation in the malaria exposed PBMCs. B. While expression of CD 69 at 48 hours (p=0.008), CD38 (p=0.011), CD25 (p=0.046), and CD69 (p=0.034) at 72 hours and CD38 (p=0.003) at 96 hours are significantly increased in CD4+CD45RO+D56 malaria exposed PBMCs compared to naive controls at 72 hours, there is also a trend toward increased activation in the HLA-DR/CD38 double positive cells in the D56 post-exposure PBMCs compared to naive controls at both 72 and 96 hours post co-culture in the memory CD4 compartment.
Figure 1.4: Activation of memory and total CD4/8 T-cells by iRBCs in D56 post malaria challenge PBMCs, continued.

C – D. For the total and memory CD8 T-cells, there is increased activation in the Day 56 post malaria challenge PBMCs at 72 and 96 hours compared to naïve controls. For total CD8 cells at 72 hours, there is increased expression of CD38 (p=0.016) and CD69 (p=0.001); at 96 hours, there is increased expression of HLA-DR/CD38 double positives (p=0.025) and CD38 alone (p=0.015). For memory CD8 cells at 72 hours, there is increased expression of CD38 (0.036) and CD69 (0.001); at 96 hours, there is increased expression of CD38 alone (p=0.0003).
Figure 1.5: *Plasmodium falciparum* stimulates enhanced secretion of TNF-α, IFN-γ, and MIP-1α, but not IL-6.

PBMCs from the malaria challenge trial participants were infected with HIV and co-cultured with iRBCs and uRBCs at the 6 visits as described earlier. Cytokine secretion in the iRBC (red lines) and uRBC (black lines) co-culture supernatants was measured using the BioPlex platform. Cytokine production was measured at days 1, 4, 6, and 8 post initiation of co-culture and the area under the curve was calculated for each participant at each visit and plotted in A – D as the average for the 5 participants, error bars represent the 95% CI. Repeated measures ANOVA was used to determine significance in the difference in amount of cytokines secreted (iRBC-uRBC) at the post-exposure time points compared to the difference in amount of cytokine secreted (iRBC-uRBC) at baseline. p-values have been corrected for multiple comparisons: TNF-α day 35 p=0.013, day 56 p=0.29, day 90 p=0.19; IFN-γ day 35 p<0.001, day 56 p=0.019, day 90 p<0.001; MIP-1α day 35 p<0.001, day 56 p=0.002, day 90 p<0.001. There was an increase in TNF-α, IFN-γ, and MIP-1α (A – C) secretion in the iRBC co-cultures compared to the uRBC co-cultures at all time points and an enhanced secretion at the convalescent time points. There was no difference in IL-6 secretion (D).
Figure 1.6: General inflammation in the periphery does not correlate to HIV production in co-cultures.

Generalized immunological activation as assessed by C-Reactive Protein (CRP) levels in the plasma collected at the time of PBMC isolation in the five malaria challenge participants. CRP was detected by ELISA. The light pink bars correspond to the early visits (baseline, liver stage, time of first parasitemia) and the burgundy bars correspond to the three post-exposure time points (day 35, day 56, and day 90). The bars show the average CRP levels in the plasma at the time of PBMC isolation for the 5 participants. Error bars represent the SEM for the 5 participants. Levels of CRP in the plasma did not increase at the post-exposure time points when the increase in HIV production was observed, suggesting that the increase in HIV production was specific to malaria antigen re-exposure and not a result of generalized immune activation.
References.


Chapter 2:

Phagocytic, Antigen Presenting Cells Ingest Malaria Parasites and Cause an Increase in HIV Replication in a Contact- and Cytokine-Dependent Manner

Introduction.

Monocytes, macrophages, and dendritic cells are all antigen presenting, phagocytic cells that are capable of ingesting parasite-infected red blood cells (iRBCs) and activating T– and B– cells to help clear the infection. Since HIV resides in immune cells (mainly in CD4+ T-cells, but also in macrophages and dendritic cells) and its replication is enhanced in an activated immune system, it seems likely that pro-inflamatory cytokines that are being secreted in response to malaria parasites could result in increased plasma HIV-1 RNA levels that are seen in HIV-1 patients who are experiencing a clinical malaria episode [1]. However, viral loads stay elevated long after the parasitemia has been treated and cleared, and thus it is likely that there are other factors at play.

Using an in vitro co-culture system (Orlov, M, et al, manuscript submitted), we examined which cytokines and cell types were involved in the interaction. Also, we demonstrated that physical contact among the participating immune cell types and *P. falciparum* infected blood cells was important within our experimental system.
Results.

**iRBCs activate CD4+ T-cells and cause an increase in HIV production:** We have previously shown that when PBMCs are co-cultured with iRBCs, in the absence of any activating cytokines/mitogens, HIV production increases significantly compared to uRBC co-cultures (Orlov, M, et al, manuscript submitted. (Figure 1.3A, B, above)). Because HIV replicates best in activated CD4+ T-cells, we evaluated whether the increase in HIV production in the iRBC co-cultures could be explained by increased CD4+ T-cell activation. Flow cytometry was used to assess the expression of surface activation markers on CD4+ and CD8+ T-cells 48 and 72 hours after initiation of co-cultures (Figure 2.1, below). All conditions (iRBC, uRBC, CD3/CD28 antibodies) were standardized to baseline activation in the media only controls at the same 48 or 72 hour time point. There was a significant increase in HLA-DR expression (p=0.008) and in expression of both HLA-DR and CD38 antigens (HLA-DR/CD38 double positive cells, p=0.001) on CD4+ T-cells after 48 hours of co-cultivation with iRBCs compared to when uRBCs were used (Figure 1A). These changes were less apparent at 72 hours (Figure 2.1B). We saw no increase in expression of CD38, CD25 or CD69 expression in the iRBC co-cultures compared to the uRBC co-cultures. In order to assess the extent to which CD4 cells were activated by iRBCs compared to when CD4+ T-cells were more broadly activated, we also measured cell surface expression of markers of activation after ligation of CD3 and CD28 with monoclonal antibodies. The increased expression of all markers due to CD3/CD28 stimulatory antibodies was significantly greater compared to iRBC stimulation (Figure 2.1 A, B. 48 hours: HLADR/CD38: p<0.001, HLADR: p=0.003, CD38: p=0.028, CD25: p<0.001, CD69: p<0.001; 72 hours: HLADR/CD38: p=0.001, HLADR: p=0.001, CD38: p=0.027, CD25: p<0.001, CD69: p<0.001). CD8+ T-cells did not show increased activation due to iRBCs compared to uRBCs for any of the markers at either time point (Figure 2.1 C, D). However, there was significant stimulation of the CD8+ T-cells due to the CD3/CD28
antibodies compared to the iRBC co-cultures for all markers at 48 hours and for all markers except CD69 at 72 hours (Figure 2.1 C, D 48 hours: HLADR/CD38: p<0.001, HLADR: p<0.001, CD38: p<0.001, CD25: p<0.001, CD69: p<0.001; 72 hours: HLADR/CD38: p<0.001, HLADR: p<0.001, CD38: p=0.001, CD25: p<0.001, CD69: p=0.13). Thus iRBC mediated T-cell activation was restricted exclusively to the CD4+ T-cell subset.

**The Increase in HIV production due to iRBC stimulation is TNF-α dependent:** To assess which cytokines are involved in the increased HIV production due to iRBC stimulation, the BioPlex platform was used to assay TNF-α, IFN-γ, MIP-1α, IL-4, IL-6, IL-10, and IL-17 secretion in the co-culture supernatants. IL-4, IL-10, and IL-17 production were at or below the limit of detection of our assay (data not shown). There was an increase in production of TNF-α, IFN-γ, and MIP-1α in the iRBC co-cultures compared to the uRBC co-cultures (Figure 2.2A-C, below). In contrast, IL-6 production did not increase in the iRBC co-cultures (Figure 2.2D, below). TNF-α and IFN-γ are both pro-inflammatory cytokines that could potentially cause the increase in HIV production seen in the iRBC co-cultures. In order to test their roles individually in HIV production, TNF-α or IFN-γ neutralizing antibodies were added to the co-cultures (Figure 2.3, below). Addition of the TNF-α neutralizing antibody significantly decreased the amount of HIV produced compared to control co-cultures (Figure 2.3A, Figure 2.3B p=0.001). However, addition of the IFN-γ neutralizing antibody did not change the overall amount of HIV produced in the presence of iRBCs (p=0.61), which was still significantly greater than the amount produced in the presence of uRBCs (p<0.001, Figure 2.3A). There was no difference in the amount of HIV produced in the control iRBC condition versus the iRBC condition when the IFN-γ antibody was present (p=0.53, Figure 2.3B).
**HIV production is dependent on macrophages and dendritic cells:** PBMCs are a mixture of immune cell types, all of which have complex roles in the innate and/or adaptive immune response. In order to determine which cell types in the PBMC mixture contribute to the increase in HIV production in the co-cultures, we depleted different cell types from total PBMCs prior to setting up co-cultures. First we depleted either all cell types except CD3+ T-cells or all cell types except CD4+ T-cells to determine whether CD3+ or CD4+ T-cells alone were sufficient for the increase in HIV production due to iRBC stimulation (Figure 2.4, below). When only CD3+ T-cells (99.4% CD3+, data not shown) or only CD4+ T-cells (99.5% CD4+, data not shown) remained in the co-cultures, no HIV production was detected in the iRBC co-cultures (Figure 2.4A, C). Next we depleted macrophages and dendritic cells, the main antigen presenting leukocytes from the co-cultures. When only the macrophages and dendritic cells were depleted (30-40% reduction in CD14+/CD11c+ cells, data not shown), HIV production was also significantly reduced (p<0.001, Figure 2.4D). Since only 30-40% of all CD14+/CD11c+ cells were depleted, the co-cultures likely still contained sufficient macrophages and dendritic cells to increase HIV production in the “macrophage/dendritic cell depleted” iRBC co-cultures compared to parallel uRBC co-cultures (p<0.001), although maximal HIV production only occurred when all macrophages and dendritic cells were present.

**HIV production is contact dependent:** Next we tested if HIV production was dependent on iRBC:PBMC contact or if iRBCs secreted soluble factors that could stimulate HIV production. Co-cultures were set up in transwell plates with i/uRBCs in the top wells and unfractionated PBMCs in the bottom wells (control co-cultures were set up in the transwell plate where PBMCs and i/uRBCs were both placed in the bottom well). The PBMCs co-cultured with iRBCs in the control condition produced more HIV than those co-cultured with uRBCs (p=0.05). There was less HIV production when the iRBCs were placed
into the top wells and the PBMCs were placed into the bottom wells compared to the wells were iRBCs and PBMCs were co-cultured together in the bottom wells of the transwell plate (did not reach statistical significance \( p=0.14 \), \( N=3 \) for controls and \( N=5 \) for transwell condition, Figure 2.5 A, B, below). We then determined whether contact between macrophages/dendritic cells and iRBCs would be sufficient to induce HIV production or if the macrophages/dendritic cells also required contact with the rest of the PBMCs to induce HIV replication. Macrophages and dendritic cells were mixed either with iRBCs or uRBCs and placed in the top well of a transwell plate and unfractionated PBMCs were placed in the bottom well. Preliminary data \( (N=2) \) suggest that maximal HIV production takes place when the iRBCs, macrophages/dendritic cells and the rest of the PBMCs are all in contact and not separated by membranes, suggesting that soluble factors are not sufficient to induce maximal HIV production (Figure 2.5A red line vs. green line).

**HIV production can be induced by incubating PBMCs with macrophages/dendritic cells that have been pre-treated with iRBCs, and is TNF-\( \alpha \) dependent:** To determine if macrophages/dendritic cells (MP/DC) that have ingested ruptured iRBC cell debris or whole iRBCs are necessary and sufficient for HIV production following iRBC contact, MP/DCs were pre-treated with either iRBCs or uRBCs for 48 hours and then added to autologous PBMCs and infected with HIV (no RBCs were added to these co-cultures, \( N=2 \)). Parallel control co-cultures with the same PBMCs were set up with iRBCs/uRBCs. Giemsa smears were done on the MP/DCs after 48 hours of incubation with iRBCs or uRBCs (Figure 2.6 A, B, respectively, below). The MP/DCs that had been co-cultured with iRBCs were full of hemozoin crystals (Figure 2.6A arrows) while those that were incubated with uRBCs had no evidence of hemozoin deposits. The amount of HIV produced from
the co-cultures in which only iRBC pre-treated MP/DC cells were added was close to the amount of HIV produced from the co-cultures in which iRBCs were added (p=0.14, Figure 2.6 C, D). Cytokine secretion in these co-cultures was determined using the same BioPlex assay mentioned above. TNF-α was the only cytokine that increased following exposure of iRBC pre-treated MP/DCs to PBMCs (Figure 2.6E, below). IFN-γ and MIP-1α patterns of secretion resembled the uRBC co-cultures in these experiments (Figure 6 F, G). Thus phagocytic, antigen presenting cells are both necessary and sufficient to induce HIV replication in the presence of malaria parasites in a TNF-α dependent manner.
**Discussion.**

Using an *in vitro* co-culture system, we were able to show that iRBCs activate CD4+ T-cells in unfractionated healthy donor PBMCs more effectively than uRBCs, but not as substantially as antibodies that activate CD4+ T-cells through ligation of the T-cell receptor (CD3+/CD28+ antibodies). There was increased secretion of some pro-inflammatory cytokines (TNF-α, IFN-γ, and MIP-1α) in the supernatants from PBMCs co-cultured with iRBCs (compared to those cultured with uRBCs), but not others (IL-6). We confirm that TNF-α likely plays an important role in the increased HIV production seen in the co-cultures as HIV production was significantly reduced in the presence of a TNF-α neutralizing antibody. We also show that phagocytic, antigen-presenting cells, specifically macrophages and dendritic cells, must be present and in physical contact with both iRBCs and the rest of the PBMCs (presumably CD4+ T-cells) for the observed increase in HIV production. When macrophages and dendritic cells were pre-treated with iRBCs for 48 hours and then added (without iRBCs) to autologous unfractionated PBMCs, we showed that HIV production levels were equivalent to co-cultures which contained iRBCs and that among the pro-inflammatory cytokines only TNF-α levels were elevated in the supernatants of these co-cultures, further supporting the premise that TNF-α is the primary cytokine involved in driving the HIV-1/P. falciparum interaction. One limitation of the co-culture studies is that with the labor intensity of these experiments, only a limited number of individual donors were studied.

To obtain nutrients once inside red blood cells, *P. falciparum* parasites break down hemoglobin and produce hemozoin crystals as a byproduct in order to avoid the toxic effects of free heme build up. When merozoites break out of iRBCs, the hemozoin crystals are released. Phagocytic cells ingest the free floating hemozoin as well as iRBCs and build up hemozoin deposits inside their cytoplasm [2], which can persist in macrophages for several months [3, 4]. Despite the fact that it has been reported that
heavily hemozoin-laden macrophages have impaired activation and function [5], that hemozoin crystals hinder the maturation process of monocytes [6] and inhibit replication of HIV in macrophages [7] and dendritic cells [6]; hemozoin has none the less also been implicated in the deleterious interaction between HIV and *P. falciparum*. Hemozoin has been reported to enhance dendritic cell-mediated transfer of HIV to CD4+ T-cells [6] and increase HIV-1 production by CD4+ T-cells that are in contact with hemozoin loaded dendritic cells [6]. In pregnant woman, TNF-α and IFN-γ production tended to increase in intervillious blood mononuclear cells isolated from HIV seropositive women with increasing levels of hemozoin crystals, while in HIV-1 seronegative women, a suppressive effect of hemozoin was seen on the production of TNF-α, IFN-γ, and IL-10 [8]. In PBMCs isolated from SIV infected animals during AIDS, hemozoin increased viral replication and TNF-α production [9]. Separately, natural killer cells of the innate immune system have been shown to be both contact dependent and require cytokine-mediated signals from myeloid accessory cells in order to optimally respond to *P. falciparum* parasites [10], suggesting that soluble factors are sometimes not sufficient for full activation unless there is simultaneous contact of the necessary cells. In addition to the effects hemozoin has on macrophages and dendritic cells, it has also been reported that *P. falciparum* glycophosphatidylinositol (GPI) fragments released when the iRBC ruptures are capable of activating macrophages to secrete TNF-α [11] and that *Plasmodium* CpG containing DNA is capable of activating dendritic cells to secrete IFN-γ and TNF-α [12].

Increasingly, it appears that the mechanism responsible for the increase in plasma HIV-1 RNA levels that is observed during an episode of clinical malaria involves phagocytic, antigen presenting cells ingesting free hemozoin, malaria derived GPls, CpG containing DNA, or iRBCs, traveling to tissues (macrophages) or lymph nodes (dendritic cells), and activating CD4+ T-cells in a TNF-α and contact dependent manner. In co-infected populations, it has been shown that plasma HIV RNA levels remain elevated
from 4 weeks [13] to 9 weeks [1] after an episode of malaria has resolved. If hemozoin does persist in macrophages and dendritic for several months, then these antigen presenting cells can continue to stimulate CD4+ T-cells to produce HIV long after all parasites have been eradicated from the blood stream. Our results showing increased HIV production from PBMCs co-cultured with iRBC pre-treated macrophages and dendritic cells in the absence of iRBCs support this mechanism. Figure 2.7 (below) summarizes our proposed mechanism for the *Plasmodium falciparum* induced increase in HIV-1 production, highlighting the importance of TNF-α, hemozoin, and the close interaction needed between dendritic cells/macrophages and CD4+ T-cells for maximal, prolonged viral induction.
Methods.

PBMC collection and isolation: PBMCs were isolated by Histopaque 1077 centrifugation and cultured overnight at 37°C in 5% CO₂ in IL-2/PHA free R20 media (20% FBS, 1% Pen-Strep in RPMI 1640) and used the following day in co-cultures. PBMCs were obtained from malaria naïve donors enrolled in the blood draw program (protocol HS103) at the Seattle Biomedical Research Institute, which was approved by the Western Institutional Review Board.

Plasmodium falciparum culture: P. falciparum NF54 parasites were grown in type O human RBCs in RPMI 1640 (Invitrogen) with 5g/L albumax (Invitrogen), 2g/L dextrose (Fisher), 50mg/L hypoxanthine (Sigma), 2.25g/L sodium bicarbonate (Sigma), 11mg/L gentamycin (Invitrogen), and 5% pooled human AB serum (Valley Biomedical). Parasite chambers were gassed with 5% O₂/5% CO₂/90% N₂ and incubated at 37°C. Parasite cultures were maintained continuously and split 1-2 days prior to setting up co-cultures. iRBCs were used once 6-7% of the RBCs in the culture were parasitized, as assessed by light microscopy. Cultures were routinely monitored for mycoplasma contamination by PCR (Takara) and shown to be mycoplasma free.

Co-cultures: The day following isolation, PBMCs were placed in 96-well plates (2x10⁵ cells/well/200µL) and infected with HIV-1 (MOI=25) without exogenous mitogens or cytokines in R20 media. Red blood cells (either P. falciparum infected or uninfected) were then added to selected wells in a 10:1 ratio of RBCs to PBMCs (2x10⁶ RBCs/well/200µL). All conditions were run in triplicate. After 22 hours, the entire 200µL of media was collected and replaced with new media. 100µL of the culture supernatants was collected at days 4, 6, 8 and 10 and replaced with fresh medium. These supernatants were frozen at -80°C and later used to determine HIV p24Ag and cytokine
levels. Viral production was quantified by determining amount of p24 antigen in the culture supernatants by p24 Antigen capture ELISA (Perkin Elmer) by the UCSD CFAR Translational Virology Core.

For the macrophage/dendritic cell depletion experiments, PBMCs were isolated as described above and allowed to culture overnight in TC-treated flasks. The next day, only the cells that did not attach to the plastic were collected and used for experiments. For the CD3+/CD4+ T-cell enrichment experiments, PBMCs were isolated as described above, allowed to rest overnight, and then either the CD3+ T-cell enrichment kit or the CD4+ T-cell enrichment kit (StemCell Technologies) was used to isolate only CD3+ cells or only CD4+ T-cells, respectively from recovered cells. Unfractionated PBMC co-cultures were always set up in parallel as a control. For the TNF-α and IFN-γ neutralizing antibody co-culture experiments, co-cultures were set up as described above and either TNF-α (500pg/mL) or IFN-γ (300pg/ml) were added at the time of set up (keeping the volume at 200µL). The same concentration of antibody was added 22 hours later when media was changed and half of the concentration of antibody was added at days 4, 6, and 8 when 100µL of the supernatant was collected and replaced with fresh media.

Transwell cultures were set up in 96-well transwell plates that had a membrane pore size of 0.4µm (Coming). Control cultures had 2x10^5 PBMCs and 2x10^6 RBCs in the bottom well in 230µL media and only media in the top well (70µL). In other cultures, the RBCs were placed in the top well (2x10^6 in 70µL of media) and PBMCs were placed in the bottom well (2x10^5 in 230µL media). For the co-cultures where RBCs were mixed with macrophages/dendritic cells in the top well, 2x10^6 RBCs were mixed with 1x10^4 macrophages/dendritic cells (isolated only cells that attached to plastic O/N) in the top well in 70µL of media, and 2x10^5 PBMCs were placed in the bottom well in 230µL media. PHA control cultures: PHA was added to the top well (5ug/mL) and 2x10^5 PBMCs were added to the bottom well. The HIV was always added to the bottom well and all
conditions were set up in triplicate, avoiding all outer edge wells to minimize evaporation.

For the pre-treatment experiments, PBMCs were isolated (day -3) as described above and allowed to rest overnight in TC-treated culture flasks. The next day (day -2), all cells that did not attach were discarded and only those that attached to the flask after 2 washes with PBS remained (mainly macrophages and dendritic cells, ~20-30% CD4+ T-cells, as determined by flow cytometry, data not shown). The cells that attached were re-plated in 12 well plates (3x10^5 cells/well in 1mL of media) and RBCs were added in a 40:1 ratio of RBCs:attached cells (1.2x10^7 RBCs/well) and co-cultured for 48 hours. On day -1, whole PBMCs were isolated from the same donor(s) as on day -3 and allowed to rest overnight. On day 0, whole PBMCs were plated in 96 well plates (2x10^5, as described above) in triplicate. Either RBCs (2x10^6) or the pre-treated attached cells (2x10^4) were added to the plated PBMCs. All 200µLs of media was changed on day 1 and 100µL of supernatant was collected on days 4, 6, 8, and 10 post-initiation of co-cultures.

**Cytokine Quantification:** Cytokine levels were measured in culture supernatants using a BioPlex platform (BioRad). A 5-plex kit containing IL-10, IL-6, TNF-α, IFN-γ and MIP-1α or IL-4, IL-6, TNF-α, IFN-γ and MIP-1α was used according to the manufacturer’s protocol (BioRad). Briefly, antibody coupled beads were mixed with 50µL of collected supernatants, which were diluted 1:2 with media and incubated on a shaker for 30 minutes. After three washes, the detection antibodies were added and the plate was incubated on the shaker for 30 minutes. After another three washes, Streptavidin-PE was added to each well and plates were incubated on the shaker for 10 minutes. The beads were resuspended in 1% formaldehyde in assay buffer and after a 30 second shake the plate was read on a Bioplex200 (BioRad). An 8-point standard curve was used to
determine cytokine concentrations using a 5 parameter logistic regression curve. Detection limits for cytokines are as follows: IL-4, 0.7 pg/mL; IL-6, 2.6 pg/mL; IL-10, 0.3 pg/mL; IFN-γ, 6.4 pg/mL; TNF-α, 6.0 pg/mL; MIP-1α, 1.6 pg/mL.

**Flow cytometry:** PBMCs were isolated as above and the next day were plated (2x10^5 PBMCs/well) in a 96 well plate and co-cultured with CD3/CD28 antibodies, iRBCs, uRBCs (2x10^6 RBCs/well), or media only in triplicate. CD3 antibodies (BD) were used at a concentration of 1 µg/mL and CD28 antibodies (BD) were used at a concentration of 2 µg/mL. Cultures were incubated at 37°C, 95% O₂ for 48 or 72. At the indicated time point, all three wells for a given condition were combined into a single FACS tube. Cells were washed once in FACS buffer (PBS + 2% FBS), resuspended in 50µL of Live/dead – Aqua stain (Invitrogen) and stained with either CD3-FITC (eBioscience), CD4-Pacific Blue (eBioscience), CD8-APC (BD Pharmingen), HLA-DR-PerCP (Biolegend), and CD38-PE-Cy5.5 (BD Pharmingen) or CD3-FITC, CD4-Pacific Blue, CD8-APC, CD45RO-PE, CD69-PerCP-Cy7 (eBioscience), and CD25-PE-Cy5.5 (eBioscience). Cells were stained at room temperature for 20 minutes and washed twice with FACS buffer. The red blood cells were then lysed using 120µL of BD FacsLyse at room temperature for 10 - 15 minutes, washed once with PBS and resuspended in 130uL of 2% formaldehyde in distilled H₂O. Samples were subjected to flow cytometric analysis within 18 hours of fixing on the BD LSRII. All data were analyzed using FlowJo (Treestar).

**Statistical Methods:** For the flow cytometry experiments, we fit a mixed-effects linear model (repeated measures ANOVA) to the log10-transformed biomarker response as a function of the four conditions. The within-subject correlations are modeled via a normal random effect term (random intercept). We computed point estimates, 95% confidence intervals, and p-values for the two contrasts of interest: iRBC versus uRBC, and CD3/CD28
versus iRBC. A significantly positive difference for both contrasts shows a significant ranking of responses as uRBC < iRBC < CD3/CD28. The p-values are based on the Wald test of the repeated measures ANOVA model. The p-values were not corrected for multiple comparisons. p-values < 0.05 are considered statistically significant. All models were fit using the nlme package in the R statistical program [14]. For the AUC calculations, total HIV-1 p24Ag and cytokine production were calculated over the 10 days in culture using a trapezoidal method (area under the curve) and were computed and analyzed for seven different types of biomarkers (conditions): macrophage/dendritic cell depletion, CD4+ T-cell enrichment, T-cell enrichment, TNF-α neutralizing antibody, INF-γ neutralizing antibody, MP/DC-pretreatment, and the transwell condition. For each biomarker the AUC biomarkers were computed under four conditions: iRBC, uRBC, iRBC+b biomarker, uRBC+b biomarker, in a 2×2, factorial, repeated-measures design (4 data points per subject). For each biomarker we fit the log-transformed AUC values using a mixed-effects linear model (repeated measures 1-way ANOVA). The within subject correlations were modeled via a subject-specific normal random effect (random intercept). In each case, we examined (i) the interaction effect between the presence of the biomarker and the malaria infection status, examining whether the effect of malaria infection is different in the biomarker cell types than in the controls; (ii) the separate contribution (additive effect) of malaria infection (i.e., iRBC versus uRBC) and presence of the biomarker (i.e., macrophages depleted versus controls, etc.). The p-values were computed based on the Wald test and were not adjusted for multiple comparisons. p-values below 0.05 were deemed statistically significant.
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Figure 2.1: IRBCs activate CD4+ T-cells, but not CD8+ T-cells, in co-cultures.

PBMCS were cultured with IRBCs or uRBCs (without HIV) for 48 or 72 hours. Cells were stained with either CD3-FITC, CD4-Pacific Blue, CD8-APC, HLA-DR-PerCP, and CD38-PE-Cy5.5 or CD3-FITC, CD4-Pacific Blue, CD8-APC, CD69-PerCP-Cy7, and CD25-PE-Cy5.5 and acquired using an LSRII. The percent of cells activated by IRBCs, uRBCs, and CD3/CD28 antibodies was normalized to the amount of baseline activation (media only); this value was then logged and plotted. Line and error bars represent the mean and standard error of the mean. A. There was increased activation of CD4+ T-cells (CD38/HLA-DR double positive cells) at 48 hours compared to uRBCs. Activation due to IRBCs was less than that due to CD3/CD28 antibodies (all markers except CD38). B. Activation due to IRBCs decreased by 72 hours. C. and D. For CD8+ T-cells, there was little activation by IRBCs compared to uRBCs, but there was significant activation due to CD3/CD28 antibodies compared to IRBCs and uRBCs.
Figure 2.2: Increase in TNF-α, IFN-γ, and MIP-1α secretion in iRBC co-cultures.

PBMCs from malaria naïve, healthy donors were infected with HIV and co-cultured with iRBCs or uRBCs. Cytokine secretion in the iRBC (red lines) and uRBC (black lines) co-culture supernatants was measured using the BioPlex platform. Cytokine production was measured at days 1, 4, 6, and 8 post initiation of co-culture. Each dot represents the average cytokine secretion from 5 donors; the error bars represent the standard error of the mean. iRBCs stimulated increased secretion of pro-inflammatory cytokines TNF-α (A), IFN-γ (B), and MIP-1α (C), but not IL-6 (D).
Figure 2.3: TNF-α, but not IFN-γ, neutralizing antibodies inhibit HIV production in co-cultures.

Co-cultures were set up in the presence of either a TNF-α neutralizing antibody (500ng/mL, N=4) or an IFN-γ neutralizing antibody (300ng/mL, N=3). A. p24 was measured in the supernatants at days 4, 6, 8, and 10 post-initiation of co-cultures. The dots are the average of all donors and the error bars represent the standard error of the mean. B. The p24 concentration was adjusted for serial sampling dilutions, logged (Log10,) and the area under the curve was calculated for each donor for each condition. The AUC values were used to generate the box plots, p-values were determined using a repeated measures 1-way ANOVA and computed based on the Wald test, they have not been corrected for multiple comparisons. There is significantly less HIV produced in the presence of the TNF-α neutralizing antibody (p=0.001). C. There is no change in the amount of HIV produced in the presence of the IFN-γ neutralizing antibody (p=0.53).
Figure 2.4: Phagocytic, antigen presenting cells are necessary for increased HIV production in co-cultures.

The day after PBMC isolation, enrichment kits were used to isolate either only CD3+ T-cells or only CD4+ T-cells and used for experiments. For macrophage and dendritic cell depletions, the day after PBMC isolation, all the cells that did not attach to the plastic were collected and used for the experiment. The cells that did attach were discarded. A. HIV production curves for co-cultures that were set up with only CD4+ T-cells (blue: iRBC; black triangle: uRBC), only CD3+ T-cells (purple: iRBC; black circle: uRBC), or whole, unfractionated PBMCs (red: iRBC; black square: uRBC). B. HIV production curves for co-cultures that were set up with macrophage/dendritic cell depleted PBMCs (dashed red line: iRBC; dashed black line: uRBC) or whole, unfractionated PBMCs (solid red line: iRBC; solid black line: uRBC). C-E. The p24 concentration was adjusted for serial sampling dilutions, logged (Log10), and the area under the curve was calculated for each donor for each condition. The AUC values were used to generate the box plots, p-values were determined using a repeated measures 1-way ANOVA and computed based on the Wald test, they have not been corrected for multiple comparisons. E. For maximal HIV production in the co-cultures, macrophages and dendritic cells need to be present in the co-cultures as HIV production was significantly decreased when they were depleted (p<0.001), and neither CD4+ T-cells nor CD3+ T-cells were sufficient on their own to induce much HIV production.
Figure 2.5: Macrophages and dendritic cells need to be in contact with both iRBCs and the rest of the PBMCs for maximal HIV production.

Transwell plates (with 0.4µm pore membrane) were used to set up co-cultures. For the control co-cultures, RBCs and PBMCs were plated together in the bottom wells and media only in the top well. For the transwell co-cultures, RBCs were placed in the top well and PBMCs were placed in the bottom well. For the RBC + MP/DC co-cultures, RBCs were mixed with macrophages and dendritic cells in the top well and unfractionated PBMCs were placed in the bottom well. A. HIV production curves for control (red: iRBC; black uRBC), transwell (iRBC: purple; uRBC: pink), and RBC + MP/DC (iRBC: green; uRBC: back circle) co-cultures. B. The p24 concentration was adjusted for serial sampling dilutions, logged (Log10,) and the area under the curve was calculated for each donor for each condition. The AUC values were used to generate the box plots, p-values were determined using a repeated measures 1-way ANOVA and computed based on the Wald test, they have not been corrected for multiple comparisons. There is significantly less HIV produced in the iRBC co-cultures compared to the uRBC co-cultures (p=0.05). While it appears that the difference in HIV production in the transwell condition is less than that in the control (A, purple line vs. red line), the p-value did not reach statistical significance (N=3 for controls, N=5 for transwell condition, p=0.14). Also, it appears that there is less HIV produced when the iRBCs are in contact with macrophages and dendritic cells, but not the rest of the PBMCs (A, green line vs. red line).
Macrophages and dendritic cells were isolated from whole PBMCs and co-cultured with either iRBCs or uRBCs for 48 hours. After 48 hours, red blood cells were washed away and the macrophages and dendritic cells were added to whole, unfractionated PBMCs and infected with HIV. A. Giemsa smears of macrophages and dendritic cells that have been co-cultured with iRBCs for 48 hours. Arrows point to hemozoin deposits in the cytoplasm of the leukocytes. B. Giemsa smears of macrophages and dendritic cells that have been in co-culture with uRBCs for 48 hours. No evidence of hemozoin deposits in all cells visualized. C. HIV production curves for control co-cultures (PBMCs co-cultured with iRBC, red; or uRBCs, black) and MP/DC pre-treated co-cultures (PBMCs co-cultured with iRBC pre-treated MP/DC, purple; or uRBC pre-treated MP/DC, grey). D. There was no difference in the amount of HIV produced from the iRBC control co-cultures compared to the MP/DC pre-treat co-cultures (p=0.14).
Figure 2.6: Macrophages and dendritic cells pre-treated with iRBCs are sufficient for inducing HIV production, continued.

E-H. Cytokine secretion in the control (iRBC, red lines; and uRBC, black lines) and MP/DC pre-treated (iRBC, purple lines; and uRBC grey lines) co-culture supernatants were measured using the BioPlex platform. Cytokine production was measured at days 1, 4, 6, and 8 post initiation of co-culture. Each dot represents the average cytokine secretion from 2 donors; the error bars represent the standard error of the mean. TNF-α is the only cytokine whose secretion increased in the presence of iRBC pre-treated macrophages and dendritic cells. Hemozoin loaded macrophages and dendritic cells are sufficient to cause an increase in HIV production via TNF-α in the absence of iRBCs.
Figure 2.7: Proposed mechanism for HIV-1/Plasmodium falciparum interactions.

Whole iRBCs and debris from ruptured iRBCs is ingested by monocytes in the peripheral blood. Monocytes are activated as hemozoin crystals accumulate in their cytoplasm and as malaria GPI fragments and malaria DNA fragments containing CpG motifs are ingested, causing a release of TNF-α. Once monocytes leave the blood stream, they differentiate into either macrophages or dendritic cells and migrate either to tissues or lymph nodes, respectively. HIV-infected dendritic cells aid in the transfer of HIV to uninfected CD4+ T-cells and both macrophages and dendritic cells cause an increase in CD4+ T-cell activation, which causes an increase in HIV production. Even after the malaria episode has resolved, hemozoin crystals persist in the macrophages and presumably dendritic cells and continue to drive the increase in HIV production.
References.


Chapter 3:

Subpatent parasitemia is not associated with development of clinical malaria among study participants in Malawi from the PEARLS/ACTG 5175 randomized controlled clinical trial.

Introduction:

To study HIV/P. falciparum interactions in vivo, we took advantage of a recently completed clinical trial of antiretroviral therapy that included two clinical trials sites in Malawi. We used stored plasma samples to study the relationship between subpatent parasitemia and clinical disease and attempted to assess whether the HIV-1 protease inhibitor, atazanavir, reduced the rate of detection of plasma P. falciparum DNA by nested polymerase chain reaction (PCR).
Results.

Study Population: The PEARLS (Prospective Evaluation of Antiretrovirals in Resource limited settings, also known as ACTG 5175) study was a randomized clinical trial of three antiretroviral regimens (two NNRTI and one PI regimen) in treatment-naïve individuals (Campbell, et al, IAS 2008 and CROI 2011, manuscript in preparation). The study recruited 1571 study participants in 9 countries and included sites in Blantyre and Lilongwe, Malawi. During follow-up of participants (median of three years), all but one of the bouts of clinical malaria were reported from the two sites in Malawi, where 221 of the study participants were enrolled and 124 of those participants had a clinical malaria diagnosis during follow-up. Plasma samples were obtained prior to study entry and at scheduled intervals during follow-up and were used to detect the presence of P. falciparum DNA.

Subpatent Parasitemia: Of the 221 participants enrolled at the two Malawi sites of the clinical trial, 149 (67%) were female and 72 (33%) were male and the mean age was 33 (range 18-65). 217 study participants had a baseline plasma sample available for analysis for P. falciparum DNA. Subpatent parasitemia was observed in 20 (9.2%, 95% CI 5.7 to 13.9%) of the baseline samples. Study participants were more likely to have P. falciparum DNA in their baseline plasma sample if it had been obtained during the rainy season (OR 2.6, 95% CI 0.96 – 7.07, p=0.049). Those with lower BMI (15% increase in odds per BMI unit decrease) and lower hemoglobin (30% increase in odds per g/dL decrease) were also more likely to be positive for plasma P. falciparum DNA at baseline. None of the 37 participants with a prior tuberculosis diagnosis were positive for P. falciparum at baseline (p=0.023). PCR positivity for parasite DNA at baseline was not significantly associated with early cotrimoxazole use, age, gender, CD4+ cell count or plasma HIV-1 RNA level (Table 3.1, below).
Of the 221 participants enrolled in the study, 57% of them (124) went on to have probable or confirmed clinical malaria. We found that neither subpatent parasitemia at baseline (p=0.24) (at the time participants entered the antiretroviral trial) nor during follow-up visits (p=1.0) (during the course of the study) was significantly associated with the subsequent development of clinical malaria (confirmed or probable cases) over the course of the study. Having subpatent parasitemia at baseline had a positive predictive value (PPV) for developing confirmed or probable clinical malaria over the course of the trial of 70% (CI 45.7 – 88.1, not a much higher predictive value for participants developing clinical malaria than the actual value of 57%) and a negative predictive value (NPV) of 55.3% (CI 48.1 – 62.4). If the analysis was restricted to when the parasitemia was detected in the first available plasma sample prior to developing malaria (on trial “follow-up” sample), the PPV of developing subsequent confirmed or probable clinical malaria was 53.8% (CI 25.1 – 80.8) and the NPV was 53.7% (CI 46 – 61.2). If the analysis was further restricted to confirmed malaria cases alone, the PPV dropped to 65% (CI 40.8 – 84.6) and the NPV dropped to 42.1% (CI 35.1 – 49.4). Subpatent parasitemia after study entry had a PPV for confirmed clinical malaria of only 46.2% (CI 19.2 – 74.9) and an NPV of 40.7% (CI 33.4 – 48.3). Further, if we restricted the analysis to assess the PPV and NPV of subpatent parasitemia at baseline for developing disease within 24 weeks after sampling rather than over the course of the three year study, the PPV dropped to 55% (CI 31.5 – 76.9) and the NPV dropped to 23.9% (18.1 – 30.4) (Table 3.2, below).

Among the 102 cases of malaria for whom plasma was available for PCR preceding diagnosis, parasitemia was detected in only 7 (6.9%). Among 88 controls who were selected because they did not experience subsequent clinical malaria, parasitemia was detected in only 6 (6.8%) (Figure 3.1, below). Follow-up subpatent parasitemia was not associated with length of ARV use (p=0.7) or amount of time elapsed between plasma sampling and clinical diagnosis among those with a clinical
disease (p=0.6). Randomization to the atazanavir arm was not significantly associated with a decrease in subpatent parasitemia compared to those assigned to the efavirenz arm (OR for participants developing subpatent parasitemia assigned to atazanavir relative to either NNRTI arm is 1.04, 95% CI 0.3 to 3.2). As described in the methods section, the number of samples that we tested for the presence of malaria parasite DNA was determined by the number of available baseline samples, the number of participants that presented with malaria episodes, and by the number of negative controls needed for our comparisons. In our sample population, we saw that the prevalence of parasitemia, as defined by PCR, was relatively stable over the course of the 3 year study (5-10%) (Figure 3.2, below). Although we tested a total of 422 plasma samples, the subset of specimens subjected to PCR for *P. falciparum* was not randomly selected and therefore not necessarily fully representative of the entire sample set. Nonetheless, we did not see strong evidence that the prevalence of subpatent parasitemia was significantly affected by antiretroviral therapy.

We also measured the persistence of subpatent parasitemia in the participants assigned to the PI arm versus the NNRTI arms to assess whether treatment with PIs would alter the length of time that parasites could be detected in the periphery. Of the 20 positive PCR results at baseline, 19 follow-up samples were available to test for persistence of the positive result. If the follow-up test remained positive, an additional third sample was requested. Of the 19 participants with follow-up results, 6 (32%) had no persistence of parasitemia past the baseline sample. 9 of the participants (47%) remained PCR positive during the second plasma sampling and the remaining 4 participants (21%) had persistent parasitemia for all three samplings. The patterns of PCR persistence were not associated with NNRTI use (p=0.8, Table 3.3, below) or early co-trimoxazole use (p=0.7). Also, persistence of PCR positivity was not associated with clinical malaria (confirmed, p=0.1; probable and confirmed p=0.2).
Clinical Malaria: One hundred twenty four of the 221 participants (57%) had at least one probable or confirmed malaria diagnosis during the course of the three year study, for a total of 244 clinical events. Because of the way in which clinical events were collected during the parent antiretroviral study, it is possible that on occasion ongoing bouts of malaria might have been coded as separate events, although almost all of these clinical events were separate and distinct episodes. Clinical malaria (confirmed or probable) was observed more frequently in those with history of tuberculosis at study entry (OR 2.85, 95% CI 1.28 – 6.38, p=0.007) and with a lower body mass index (OR 0.89, 95% CI 0.82 – 0.96/BMI unit increase, p=0.003, 11% higher odds of clinical malaria per each unit decrease in BMI). Those with cotrimoxazole use in the first 8 weeks were more likely to develop malaria (OR 1.82, 95% CI 1.02 – 3.25, p=0.041) but this association did not persist once adjusted for baseline BMI and TB history. Other baseline characteristics including CD4 cell count, plasma HIV-1 RNA level and hemoglobin levels were not significantly associated with development of clinical malaria during the study (Table 3.4, below). Confirmed clinical malaria was only associated with baseline BMI (OR 0.89, 95% CI 0.82 – 0.97/BMI unit increase, p=0.007). Assignment to the atazanavir arm of the study was not significantly associated with the outcome of clinical malaria (OR= 1.1, 0.6 to 2.0).
Discussion.

In this cohort of HIV-1 infected study participants we did not find that identification of *P. falciparum* DNA in the plasma was a significant predictor of subsequent clinical disease. We did not note a significant reduction in either subpatent parasitemia or clinical disease among those randomized to unboosted atazanavir. Subpatent parasitemia at study entry was more common in the rainy season, in those with lower BMI or hemoglobin levels and in those with no prior history of TB. A prior study from Brazil demonstrated that subpatent parasitemia was a risk factor for developing anemia [1]. The resulting anemia is likely due to anemia of chronic disease or as a residual effect of prior bouts of clinical malaria. Although both the number of participants with a history of TB (37) and the number of participants with baseline subpatent parasitemia was small (20), we noted that subpatent parasitemia was not observed among the 37 with prior tuberculosis. Study participants with a lower BMI or with a history of tuberculosis at study entry were also more likely to develop clinical malaria during the study. When we restricted the analysis to a smaller number of those with confirmed cases of clinical malaria, the negative association with prior tuberculosis was not observed (perhaps reflecting the smaller number of those in the analysis). In contrast, lower pre-entry CD4 cell counts and higher plasma HIV-1 RNA levels at study entry did not predict the development of clinical malaria.

There are a number of potential limitations of this study. Firstly, it was performed retrospectively using available samples from a previously completed antiretroviral drug study. Although many cases of malaria were reported among study participants enrolled in Malawi; neither blood nor plasma samples were routinely collected and stored at the time of the malaria episode. The samples available to us were ones that were collected at study entry and at regular intervals during the study for the purpose of assessing the efficacy of antiretroviral therapy. The samples were collected, on average,
31 days prior to the malaria diagnosis and some of the samples were collected as many as 60 days prior to the malaria episode, although we did not see an association between parasitemia and the distance in time previous to the malaria diagnosis \((p=0.6)\). Furthermore, the samples that were banked were plasma samples rather than whole blood or dried blood spots. While it has been previously reported that parasite DNA can be found in the plasma of patients with malaria \([2, 3]\), quantitative and qualitative relationships between levels of detection of \(P. falciparum\) DNA in blood and plasma have not yet been systematically evaluated. Another current limitation of this study is that the PI arm was terminated early due to poor HIV outcomes. All the participants on the PI arm were placed on one of the two NNRTI treatments. In addition, when individual patients on an NNRTI or a PI-based treatment arm developed antiretroviral treatment failure, a second-line regimen was constructed based on a switch of the NNRTI to a PI or vice versa. The analyses presented here were performed as intention-to-treat analyses based on the originally assigned treatment regimen. We are currently conducting a treatment analysis based on the drug regimen taken at the time samples were obtained to more directly examine whether atazanivir affected the subpatent parasitemia rate.

The prevalence of subclinical and subpatent parasitemias increase with age, while the incidence of severe malaria episodes decrease with age. It has been proposed that the ability to maintain subpatent or subclinical parasitemia is a manifestation of the development of partially effective immunity \([4]\). Semi-immune individuals may have sufficiently effective adaptive immune responses to maintain parasitemia at a level below a threshold at which the pro-inflammatory cytokines associated with bouts of malaria induce clinical symptomatology \([5]\).

There is a lack of studies that definitively conclude whether subpatent or subclinical parasitemias are protective of or predictive of clinical malaria in HIV uninfected populations, and a very limited number of studies have been done in HIV
infected individuals. None of these studies used plasma DNA detection as a marker for subpatent parasitemia, as we have done. All the studies used whole blood samples, usually in the form of dried blood spots or smears, as their starting material for malaria diagnosis. In one study conducted in Mozambique, *P. falciparum* DNA was detected more frequently in rural study participants with HIV-1 infection than in contemporaneous study participants without HIV-1 infection. It is possible that the higher prevalence of subclinical *P. falciparum* DNA detection in the HIV-1 infected population is a reflection of compromised parasite-directed immune responses in this population (Noormahomed, EV, *et al.*, in review). A study in Uganda also found that asymptomatic malaria infection, as defined by rapid malaria antigen tests, in HIV positive patients on HAART did not lead to development of clinical malaria, although the number of malaria positive asymptomatic individuals in their study was quite small [6]. Among HIV uninfected participants, there have been a number of inconclusive studies performed mainly on the African continent. In one study conducted in Brazil, patent parasitemia (parasites detectable by peripheral blood smear, with or without clinical symptoms) was predictive of clinical disease but subpatent parasitemia was not [1]. There were two studies conducted in the same two villages in Senegal that attempted to correlate asymptomatic malaria in children with clinical disease. One study found that short term asymptomatic malaria carriage (diagnosed with thick smears) was predictive of mild clinical disease [7], while the other study found that long term asymptomatic malaria carriage (diagnosed with thick smears and some PCR) was protective from clinical disease [8]. In Northern Tanzania, it was reported that sub-microscopic infections were able to elicit or maintain humoral (antibody) immune responses, but they did not look to see if these immune response were protective from clinical disease [9]. It is clear that despite repeated attempts to understand persistent parasitemias, no consensus has been reached, perhaps owning to the fact that terminology is not consistent in the
malaria field. It is still important to know if subclinical or subpatent parasitemias develop into clinical malaria because when patients present with a fever and parasites are found on a smear, it is presumed that the fever is the result of malaria and patients are treated accordingly. With the high prevalence of subclinical parasitemias that exist in malaria endemic regions and the high incidence of other febrile diseases, it is quite possible that febrile illnesses due to other etiologies are misdiagnosed as malaria because of the demonstration of malarial parasites in peripheral blood that are not necessarily driving the acute illness. With the increasing prevalence of drug resistant strains of malaria, physicians in malaria endemic areas should be vigilant about over-diagnosis of malaria on the basis of clinical presentation even when low level parasitemia is demonstrated on blood smears.

Andrews, et al, showed that a number of PIs, including atazanavir, reach concentrations in the plasma that exceed the EC50 for anti-malaria activity in vitro and some of these PIs have in vivo activity against murine strains of *Plasmodium* [10]. It has also been shown that sera from subjects taking PIs has antimalarial activity in vitro and that some PIs potentiate the action of chloroquine, an anti-malaria agent [11, 12]. Another potential limitation of this study is that the protease component of the regimen administered was unboosted atazanavir. Atazanavir blood levels were measured in the parent antiretroviral drug study and found to be 28nM – 14µM (Adriana Andrade, G Baheti, et al, CROI 2011). Although these atazanavir blood levels overlap those required to inhibit parasite growth in vitro (2.5 – 11.6 µM) [13], the blood levels of atazanavir in some patients in the trial would have been lower than those one might have expected to suppress malarial parasites. Whether ritonavir boosting of atazanavir or of other HIV-1 protease inhibitors might be associated with more consistent plasma levels sufficient to inhibit *P. falciparum* in HIV-1 infected individuals receiving antiretroviral therapy could not be evaluated in this study.
In conclusion, we did not find that the detection of *P. falciparum* DNA in the plasma of our population of HIV-1 infected persons was significantly predictive of subsequent clinical disease. We also did not demonstrate that the ability to maintain *P. falciparum* at low levels was predictive of protection from clinical illness. Prior studies that have addressed the impact of *P. falciparum* infection on HIV-1 and vice versa have been conducted using clinical evidence of malaria as a marker for *P. falciparum*. Larger scale longitudinal studies in regions of varying malaria endemicity in patient populations with and without HIV-1 infection are needed to more conclusively examine the clinical implications of subpatent parasitemia and of the impact of subpatent parasitemia on the natural history of HIV-1 infection. While atazanavir was not associated with decreased parasitemias in our study, it would be of interest to perform a more focused evaluation of a ritonavir-boosted HIV-1 protease inhibitor to further evaluate this issue. Such data might have implications for antiretroviral treatment decisions for pregnant women and/or children in areas where malaria causes substantial morbidity and mortality in these HIV-1 infected populations.
Methods.

Patient population and sampling: PEARLS (the Prospective Evaluation of Antiretrovirals in Resource limited settings, also known as ACTG 5175) was a randomized clinical trial of three antiretroviral regimens (zidovudine/lamivudine/efavirenz; tenofovir/emtricitabine/efavirenz; or ddI/emtricitabine/atazanavir) in treatment-naïve individuals (Campbell, et al, IAS 2008 and CROI 2011, manuscript in preparation). The study recruited 1571 study participants in 9 countries and included sites in Blantyre and Lilongwe, Malawi. During follow-up of participants (median of three years), all but one of the bouts of clinical malaria were reported from the two sites in Malawi where 221 of the study participants were enrolled. Cases of malaria were classified as confirmed malaria when study participants presented for clinical care with a compatible clinical syndrome and were found to have *Plasmodium* sp. parasites on a peripheral blood smear and as probable malaria when study participants presented with a compatible clinical syndrome and specific treatment for *P. falciparum* was initiated or recommended. Plasma samples were obtained prior to study entry and at scheduled intervals during follow-up; they were stored at study sites at -70°C.

To choose baseline samples for PCR testing, two sampling approaches were used. For those who either were on co-trimaxazole during the first 8 weeks of study, or who had a clinical malaria diagnosis within the first 12 weeks of follow up, a sample strictly prior to treatment initiation was used. For the remaining participants, any sample up to week 8 might also be tested and be considered “baseline” for this study. Among those whose baseline samples were positive, another sample (distinct from the follow-up sample below) approximately 4-12 weeks later was requested to test persistence of subpatent parasitemia. For those 124 participants who had a clinical malaria diagnosis during follow-up, the sample selected for PCR testing for subpatent parasitemia was the closest available specimen prior to the first diagnosis of clinical malaria (the sample was
drawn an average of 31 days (range 1 – 60 days) prior to the malaria diagnosis. For those who never had a clinical malaria diagnosis (controls), a 2-stage restricted sampling design was used to attempt to control for 2 potential confounding factors: time of year (high malaria incidence season versus low) and exposure time to ARVs. Time since ARV initiation had 4 stratus levels: <12 weeks, 12 – 24 weeks, 24 - < 96 weeks, and 96+ weeks. All available stored plasma from the control group was considered for sampling. Samples identified for this study were shipped on dry ice to San Diego where they were tested for *P. falciparum* DNA.

Subpatent parasitemia is defined as having parasites in the periphery detectable with more sensitive methods, such as PCR, and without evidence of clinical malaria. In this study, we are defining subpatent parasitemia as having evidence of parasite DNA in the plasma. The malaria “high” season was defined as the months of November through May (rainy season), and “low” season was the months of June – October. “Early” co-trimoxazole use refers to administration in the first 8 weeks of study.

**DNA extraction and PCR:** 200µL of plasma were used to extract DNA using Qiagen Mini Blood Kit (Qiagen) as per manufacturer’s instructions. The PCR for *P. falciparum* detects a 205bp portion of the 18s rRNA gene. A nested PCR reaction was performed on 40µL of supernatant from the plasma DNA extraction in a 50µL PCR reaction (2.5 U Taq polymerase (Invitrogen), 3mM MgCl₂, 500nM dNTPs (Fermentas), and 50 pmol of each primer (IDT)). In the first round of the nested PCR, the gene was amplified using primers fPLU: 5’- TTA AAA TTG TTG CAG TTA AAA CG-3’, and rPLU: 5’- CCT GTT GTT GCC TTA AAC TTC-3’. For the PCR, denaturation was performed at 94°C for 3 minutes, flowed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 1 minute, and elongation at 72°C for 2 minutes, with a final extension at 72°C for 5 minutes. 2µL of the outer PCR product was used as the template for the inner PCR reaction using primers
fFAL: 5’-TAAACTGGTTGGGAAAAACCAAATATATT-3’ and rFAL: 5’-ACACAAATGAACTCAATCATGACTACCCGTC-3’. For the PCR, denaturation was performed at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 1 minute, and elongation at 72°C for 1 minute, with a final extension at 72°C for 5 minutes. The PCR products from the inner PCR were run on a 1% agarose gel at 175V for 30 minutes and stained with ethidium bromide for visualization. The PCR result was qualitative and could detect parasite DNA in the plasma down to 1.8 parasite genomes/5μL of extracted DNA.

**Statistical Analysis:** Categorical variables were compared by Fisher’s exact test and continuous variables were compared with Wilcoxon RankSum Test. Univariable logistic regression examined the association between subpatent malaria status at baseline (or during follow-up) and outcome of malaria diagnosis during study follow-up. To control for confounders, effect modifiers, and other prognostic factors, multi-variable logistic regression was used including covariates identified as associated with either development of clinical malaria during follow-up, or with subpatent status. Adding subpatent status tested for independent association provided by a plasma DNA finding at baseline.
Acknowledgements.

We would like to thank all the participants of the PEARLS/ACTG A5175 study. Chapter 3, in part is currently being prepared for submission for publication of the material. Orlov, M; Smeaton, LM; Kumwenda, J; Hosseinipour, MC; Smith, DM; Campbell, TB; and Schooley, RT. The dissertation author was the primary investigator and author of this paper.
Figure 3.1: Detection of Subpatent Parasitemia does not predict development of clinical disease.

Subpatent parasitemia is not a good predictor of development of clinical disease. Among the 102 cases of malaria for whom plasma was available for PCR preceding diagnosis, parasitemia was detected in only 7 (6.9%). Among 88 controls without subsequent clinical malaria, 6 (6.8%) had parasitemia detected. Levels of subpatent parasitemia were equivalent in the participants that went on to develop clinical disease to those that never developed clinical malaria over the course of the study.
Figure 3.2: Prevalence of Subpatent Parasitemia Over the Course of the Study.

We analyzed 217 baseline plasma sample, the closest available plasma sample prior to the first malaria diagnosis, and a stratified random sample from the 97 study participants who never had a malaria diagnosis. PCR positivity remained relatively stable (5 – 10%) over the course of the study in the samples that we assayed.
Table 3.1: Correlates of Baseline Subpatent Malaria (PCR+).

Study participants were more likely to have subpatent parasitemia (*P. falciparum* DNA in the plasma) during the rainy season or if they had lower BMI or hemoglobin levels. A prior diagnosis of tuberculosis was associated with being negative for *P. falciparum* at study entry. PCR positivity at study entry was not significantly affected by co-trimoxazole use in the first 8 weeks, age, gender, CD4 cell count or HIV-1 RNA level. Randomization to the atazanavir (PI) arm was not associated with a decrease in subpatent parasitemia compared to those assigned to the efavirenz (NNRTI) arms.

<table>
<thead>
<tr>
<th></th>
<th>Negative (N=197)</th>
<th>Positive (N=20)</th>
<th>OR* (95% CI; p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaria Season of Sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High (Rainy)</td>
<td>93 (87%)</td>
<td>14 (13%)</td>
<td>2.61 (0.97 – 7.07; p=0.049)</td>
</tr>
<tr>
<td>Low (Dry)</td>
<td>104 (95%)</td>
<td>6 (5%)</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (s.d.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22 (4)</td>
<td>21 (3)</td>
<td>0.88 (0.74 – 1.04; p=0.103)</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin Value (g/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (s.d.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 (2)</td>
<td>11.2 (1.7)</td>
<td>0.8 (0.63 – 1.02; p=0.066)</td>
<td></td>
</tr>
<tr>
<td>Any History of TB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>160 (89%)</td>
<td>20 (11%)</td>
<td>0.10 (0.01 – 1.83; p=0.023)</td>
</tr>
<tr>
<td>Yes</td>
<td>37 (100%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>CD4+ Cell Count</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (s.d.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>169 (79)</td>
<td>185 (62)</td>
<td>1 (1 – 1.01; p=0.379)</td>
<td></td>
</tr>
<tr>
<td>Plasma HIV-1 viral load</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (s.d.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 (1)</td>
<td>5.0 (1)</td>
<td>1.05 (0.53 – 2.09; p=0.879)</td>
<td></td>
</tr>
<tr>
<td>(log10 copies/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (s.d.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33 (8)</td>
<td>32 (8)</td>
<td>0.98 (0.92 – 1.04; p=0.557)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>64 (90%)</td>
<td>7 (10%)</td>
<td>0.89 (0.34 – 2.35; p=0.820)</td>
</tr>
<tr>
<td>Female</td>
<td>133 (91%)</td>
<td>13 (9%)</td>
<td></td>
</tr>
<tr>
<td>Early TMP/SMX use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>24 (92%)</td>
<td>2 (8%)</td>
<td>1.39 (0.54, 3.56; p=0.502)</td>
</tr>
<tr>
<td>No</td>
<td>173 (91%)</td>
<td>18 (9%)</td>
<td></td>
</tr>
<tr>
<td>Randomization to NNRTI arm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NNRTI</td>
<td>130 (91%)</td>
<td>13 (9%)</td>
<td>0.96 (0.36 – 2.51; p=0.929)</td>
</tr>
<tr>
<td>PI</td>
<td>67 (91%)</td>
<td>7 (9%)</td>
<td></td>
</tr>
</tbody>
</table>

*OR values are not adjusted.
Table 3.2: Positive Predictive Value of Subpatent Parasitemia for Development of Clinical Disease.

To determine if subpatent parasitemia is a good predictor of clinical disease, we analyzed positive predictive value (PPV) when restricted to different lengths of follow up or by looking at only confirmed malaria cases as opposed to all confirmed plus probable cases. We also took into account if the positive PCR occurred at baseline or during follow-up. When the analysis was restricted to the first 24 weeks of follow up after the plasma samples were taken or when we looked only at confirmed malaria cases, the PPV decreased suggesting that subpatent parasitemia is not a good predictor of clinical disease.

<table>
<thead>
<tr>
<th></th>
<th>PPV Confirmed Malaria All follow-up</th>
<th>PPV Confirmed + Probable All follow-up</th>
<th>PPV Confirmed malaria 24 week follow-up</th>
<th>PPV Confirmed + Probable 24 week follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (CI)</td>
<td>65% (40.8 – 88.1)</td>
<td>70% (45.7 – 88.1)</td>
<td>50% (27.2 – 72.8)</td>
<td>55% (31.5 – 76.9)</td>
</tr>
<tr>
<td>Follow-up (CI)</td>
<td>46.2% (19.2 – 74.9)</td>
<td>53.8% (25.1 – 80.8)</td>
<td>28.6%* (3.7 – 71)</td>
<td>28.6%* (3.7 – 71)</td>
</tr>
</tbody>
</table>

*Sample size was very low; these values may not be representative.
Table 3.3: Persistence of Subpatent Parasitemia.

Of the 20 baseline samples that tested positive for subpatent parasitemia, 19 of them had a follow-up sample available. Follow-up samples were also tested for presence of malaria DNA, if the test came back positive, a third sample was tested. Persistence of subpatent parasitemia was not associated with NNRTI use (p=0.8).

<table>
<thead>
<tr>
<th>Pattern of Persistence</th>
<th>NNRTI assigned</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Yes</td>
<td>Total</td>
</tr>
<tr>
<td>PN, no third test</td>
<td>2 (33%)</td>
<td>4 (67%)</td>
<td>6 (32%)</td>
</tr>
<tr>
<td>PPN</td>
<td>3 (33%)</td>
<td>6 (67%)</td>
<td>9 (47%)</td>
</tr>
<tr>
<td>PPP</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td>4 (21%)</td>
</tr>
<tr>
<td>Total</td>
<td>7 (37%)</td>
<td>12 (63%)</td>
<td>19</td>
</tr>
</tbody>
</table>
Table 3.4: Correlates of Probable and Confirmed Clinical Malaria.

Clinical malaria (confirmed or probable) was seen more frequently in those with a history of tuberculosis, a lower body mass index, and those with cotrimoxazole use in the first 8 weeks. The association with cotrimoxazole did not persist once adjusted for BMI and TB history. Other baseline characteristics including CD4 cell count, plasma HIV-1 RNA level and hemoglobin levels were not associated with development of clinical malaria during the study. Confirmed clinical malaria was only associated with baseline BMI. Assignment to the atazanavir (PI) arm of the study did not change the risk of clinical malaria.

<table>
<thead>
<tr>
<th></th>
<th>None (N=97)</th>
<th>Probable (N=28)</th>
<th>Confirmed (N=96)</th>
<th>OR* (95% CI; p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI Mean (s.d.)</td>
<td>23 (4)</td>
<td>22 (3)</td>
<td>21 (3)</td>
<td>0.89 (0.82 – 0.96; p=0.003)</td>
</tr>
<tr>
<td>Screening CD4+ Cell Count Mean (s.d.)</td>
<td>168 (78)</td>
<td>169 (83)</td>
<td>174 (77)</td>
<td>1.0 (1.0 – 1.0; p=0.675)</td>
</tr>
<tr>
<td>Plasma HIV-1 viral load (log10 copies/mL) Mean (s.d.)</td>
<td>5 (1)</td>
<td>5 (1)</td>
<td>5 (1)</td>
<td>1.15 (0.78 – 1.69; p=0.488)</td>
</tr>
<tr>
<td>Early TMP/SMX use No</td>
<td>72 (49%)</td>
<td>17 (11%)</td>
<td>59 (40%)</td>
<td>1.82 (1.02 – 3.25; p=0.041)</td>
</tr>
<tr>
<td>Any History of TB Yes</td>
<td>25 (34%)</td>
<td>11 (15%)</td>
<td>37 (51%)</td>
<td>2.85 (1.28 – 6.38; p=0.007)</td>
</tr>
<tr>
<td>Hemoglobin Value Mean (s.d.)</td>
<td>12 (2.1)</td>
<td>11.8 (1.8)</td>
<td>11.8 (1.9)</td>
<td>0.96 (0.84 – 1.11; p=0.599)</td>
</tr>
<tr>
<td>Randomized Treatment Group NNRTI PI</td>
<td>66 (45%)</td>
<td>22 (15%)</td>
<td>59 (40%)</td>
<td>0.88 (0.5 – 1.56; p=0.671)</td>
</tr>
</tbody>
</table>

*OR values are not adjusted.
References.


Discussion

We utilized \textit{ex vivo}, \textit{in vitro}, and \textit{in vivo} approaches to study HIV-1/\textit{Plasmodium falciparum} interactions in more depth. PBMCs from donors experimentally challenged with \textit{P. falciparum} were isolated and subsequently infected with HIV-1 in culture, showing for the first time the difference in effect of a first time encounter versus a repeat malaria exposure on HIV-1 production. Our results showed that while iRBCs do stimulate HIV production and pro-inflammatory cytokine (TNF-\(\alpha\), IFN-\(\gamma\), and MIP-1\(\alpha\)) secretion in PBMCs prior to the challenge (when they are malaria naïve), iRBCs cause even greater HIV production and cytokine secretion in PBMCs that have been isolated from participants after treatment and clearance of parasites, between 2-8 weeks after the blood stage infection. We also noted a trend toward increased memory CD4+ T-cell activation in the PBMCs isolated at Day 56 post challenge from the experimentally infected participants compared to malaria naïve donors when the PBMCs were co-cultured with iRBCs for 72-96 hours. Because the increase in HIV production occurred after parasitemia and generalized immune activation had resolved, our results suggest that enhanced HIV production is related to the development of anti-malaria immunity and may be mediated by pro-inflammatory cytokines. Next we explored the mechanism responsible for the increase in HIV production using the \textit{in vitro} co-culture system developed for the \textit{ex vivo} experiments. Our results suggest that phagocytic, antigen presenting cells ingest iRBCs and in a TNF-\(\alpha\) and contact dependent manner activate CD4+ T-cells to produce HIV. Lastly, plasma samples from a cohort of HIV-1 infected participants enrolled in a clinical trial in Malawi comparing different HIV-1 treatment regimens, including a protease inhibitor, who also presented with clinical malaria were tested for the presence of \textit{P. falciparum} DNA. Even though protease inhibitors were able to kill \textit{P. falciparum}
parasites in vitro, we did not see a reduction in parasitemias in the participants taking PIs versus those who were taking NNRTIs. Also, we did not see a significant association between having subpatent parasitemia and either progressing to, or being protected from, clinical disease.

It is not possible to accurately quantify the number of times individuals living in malaria endemic regions have been infected with malarial parasites. Also, as partial immunity develops, it becomes harder to define exactly what constitutes a malaria episode, as parasites can be found without symptoms or are found incidentally when symptoms of a different infection manifest and patients are treated for malaria. Therefore, without controlled infections of malaria naïve individuals, in malaria endemic regions, it is extremely difficult to study malaria's effects on HIV production during an individual’s first bout of malaria and to compare that response to those observed in subsequent episodes, after a malaria-specific immune response has developed. Obtaining cells from the Malaria Clinical Trials Center gave us the opportunity to directly study this issue. Even though the participants barely had time to develop any symptoms before they were treated with chloroquine, we saw a significant increase in HIV production during the repeat malaria exposure, suggesting that HIV seropositive individuals living in malaria endemic regions are at increased risk of faster HIV progression and erosion of malarial immunity with each subsequent malaria episode.

It would be of interest to test HIV production, in the presence of iRBCs, in PBMCs from semi-immune individuals living in areas of high malaria endemicity. While it has been shown that IFN-γ levels decrease in these individuals in response to malaria antigens, it has not been shown that semi-immunity has any effect on TNF-α levels. It would also be interesting to take PBMCs from HIV positive individuals that are either from areas of high or low malaria endemicity and assess HIV production once those PBMCs have been placed in co-culture with iRBCs. There have now been many studies looking
at the efficacy of the RTS, S malaria vaccine, as well as many studies looking at the efficacy of other malaria vaccine candidates. It would be interesting to use PBMCs isolated from these vaccine trial participants, infect the PBMCs with HIV \textit{in vitro} and place them into co-cultures with iRBCs to assess what happens to HIV replication if vaccine induced memory cells are present.

Prior studies provided a number of important insights into the potential mechanisms by which HIV-1 and malaria interact; there are, however, a number of apparent contradictions among the studies. The only concept upon which most investigators agree, whether \textit{in vivo} observations or \textit{in vitro} experiments, is that viral expression increases in the presence of malarial antigens. Our results confirm and extend this concept. As in the studies of Xiao, \textit{et al}, and in contrast to the Freitag observations, we demonstrated that TNF-\alpha plays a role in the increased production of HIV-1 \textit{in vitro}. Unlike Xiao, however, our results suggest that phagocytic, antigen presenting cells are necessary for maximal HIV production in response to iRBCs. In this instance, our results agree with Diou’s, who demonstrated that dendritic cells, matured in the presence of hemozoin, cause an increase in HIV trans-infection of CD4+ T-cells and activate CD4+ T-cells, resulting in increased HIV production. Taken together, our results lend increasing support to the theory that phagocytic, antigen presenting cells increase HIV production in a contact- and cytokine-dependent manner. It has been suggested that hemozoin remains inside macrophages for several months and coupled with the fact that HIV plasma viral loads stay elevated for weeks to months after a malaria episode, it stands to reason that phagocytic, antigen presenting cells remain in tissues where they can continue to activate CD4+ T-cells to produce HIV even after all detectable parasites have been cleared from the circulation.

Even though there are many pro-inflammatory cytokines that are secreted during a bout of clinical malaria, TNF-\alpha seems most likely be involved in the HIV/\textit{P. falciparum}
interaction that leads to increased levels of HIV in the plasma. Infliximab, a monoclonal antibody directed against TNF-α, is currently used to treat many different autoimmune diseases from Ankylosing spondylitis to Crohn’s disease. It would be interesting to see what effect Infliximab would have on HIV replication in response to malarial antigen stimulation. Also, we showed that iRBCs are able to activate CD4+ T-cells from malaria naïve, HIV-uninfected donors. It would be worthwhile to assess CD4+ T-cell activation in response to iRBCs in PBMCs isolated from HIV infected individuals both in the presence and in the absence of HAART.

While several promising studies were published showing a toxic effect of HIV-1 protease inhibitors on *P. falciparum* *in vitro*, to date, there have been no studies conducted that examine the effect of HIV-1 protease inhibitors on malaria parasites *in vivo*. We conducted a retrospective study in a large cohort of HIV-1 infected individuals in two locations in Malawi and found no difference in the occurrence of subpatent parasitemia or clinical malaria in the participants placed on atazanavir, an HIV-1 PI, to those placed on either one of two NNRTI regimens. One possible reason for the lack of anti-parasite activity that we saw *in vivo* has to do with the measured plasma levels of the drug (28nM – 14µM) reported by Andrade, *et al*; these levels are significantly below the published optimal concentration range to kill parasites *in vitro* (2.5 – 11.6 µM). Perhaps boosting with ritonavir could get atazanavir levels in all participants in the parasite killing range. We also took the opportunity to assess whether having malarial DNA in the plasma, or subpatent parasitemia, either at baseline or during follow-up could predict clinical disease. Our results suggest that subpatent parasitemia was not significantly predictive of subsequent clinical disease, at least in an endemic area for malaria where these studies were conducted. Our data agrees with a study conducted in Brazil in HIV uninfected participants, which showed that subpatent parasitemia does not progress to clinical disease and with a study done in Uganda on HIV seropositive
participants, which demonstrated that asymptomatic malaria infection, assessed by a rapid malaria antigen test, also did not develop into clinical disease.

Other HIV-1 protease inhibitors tested for activity against *P. falciparum* were shown to be active at lower concentrations than atazanavir. These lower concentrations would be easier to attain in the plasma of patients taking them, even without ritonavir boosting. It would be worthwhile to assess whether any of these other protease inhibitors, alone or in combination with ritonavir, would have an effect *in vivo* on parasite burden in people living in malaria endemic regions. It is also imperative to ascertain if subclinical parasitemias are predictive of clinical malaria episodes in both HIV positive and HIV negative populations in order to have a better understanding of when to treat individuals so as not to allow viral loads to increase, but at the same time, to prevent unnecessary treatment of malaria to limit spread of drug resistance. While our study and others have shown that subpatent parasitemia does not lead to clinical disease, the two previously published studies had fairly low numbers of subpatent individuals and our study only had plasma samples to test, not whole blood. Larger, more thorough studies are still needed to know for certain that subpatent parasitemias do not lead to clinical disease.

Since it is increasingly obvious that a harmful interaction between HIV and *P. falciparum* does exist, it is now a question of how do we stop the deleterious effects from occurring. Our data show that repeated episodes of malaria could well drive HIV-1 replication *in vivo*, that hemozoin loaded antigen presenting cells might be responsible for the prolonged increase in HIV production seen long after parasites are cleared, that atazanavir does not reduce incidence of subpatent parasitemia or clinical malaria, and that presence of parasite DNA in the plasma is not a good predictor of clinical disease. Figure D.1 (below) conceptually illustrates our hypothesis as to the deleterious effects that *Plasmodium falciparum* has on HIV-1 plasma RNA loads and CD4+ T-cell counts in an HIV-1 infected person who experiences repeat bouts of malaria and Figure D.2
(below) illustrates the effects that HIV-1 has on P. falciparum-specific immunity and disease severity in an individual who was born in a malaria endemic region acquires an HIV-1 infection at different points in their life.

Although progress has been made in understanding the pathogenesis and clinical implications of HIV-1/malarial co-infection, many questions remain. It is quite clear that acute bouts of malaria are more severe in those with untreated HIV-1 infection, especially in later stages of the illness, and that repeat acute bouts of malaria accelerate the natural history of HIV-1 disease. Although subpatent malaria is relatively common in HIV-1 infected persons living in areas that are endemic for malaria, the clinical implications of this interaction have not yet been fully delineated. Longer term studies that focus on this interaction, including those in which therapeutic interventions are employed to reduce the prevalence of subpatent parasitemia would be of interest and might provide further insights into the pathogenesis of both infections.
When a person who is chronically infected with HIV-1 has their first malaria episode, their viral load will increase and their CD4+ T-cell count will decrease. The viral load and CD4+ T-cell count will take a few weeks to months to rebound to pre-malaria levels. If that individual has a second (or subsequent) malaria infection before the baseline viral load or CD4+ T-cell count have had a chance to fully rebound, the viral load will increase even further and there will be a greater decrease in CD4+ T-cell count. If treated properly and no more malaria is encountered, eventually the CD4+ T-cell count will go up and the viral load will go down, although, with enough malaria episodes, there will not be a full CD4+ T-cell rebound. If HAART is initiated, the viral load will quickly drop to levels that are below detection, but the CD4+ T-cell count will take a while to go up. If this individual has a malaria episode while on HAART, there should be no increase in viral load, but there would still be a decline in CD4+ T-cell count that would take a while to rebound once treated for malaria. Even without an increase in HIV levels while on HAART, there could still be CD4+ T-cell activation and spread of the dormant virus, which is harmful to the individual even without the viral spike. If there is an interruption of HAART for any reason, the viral load will return to pre-treatment levels and the CD4+ T-cell count will begin to decline. If this person has a malaria episode after interruption of HAART, the viral load will spike even higher and the CD4+ T-cell count will drop more.

Figure D.1: Diagrammatic Illustration of CD4+ T-Cell Counts and Plasma HIV-1 RNA Levels in a Co-infected Individual
A. In a healthy individual living in a malaria endemic region, partial immunity to malaria is attained in the first 5 years of life with repeated bouts of clinical disease. As malaria immunity builds, the severity of disease declines. Healthy semi-immune adults seldom have severe clinical malaria, but may experience subpatent and subclinical parasitemia or mild clinical disease. B. If an individual is infected with HIV early in life, while still building immunity to malaria, we hypothesize that the level of malarial immunity may not reach the maximum potential since recurrent stimulation of malaria-specific cellular immunity in the presence of uncontrolled HIV replication may lead to the erosion of malaria immunity. Over time, adaptive immunity to malarial may decline and the risk of increased disease severity could increase. C. If an individual is infected with HIV-1 later in life, after semi-immunity to malaria has been established, a longer amount of time might be required to erode malaria-specific immunity. In this case disease severity would not increase until later in life. Whether initiation of antiretroviral therapy might allow restoration of malaria-specific immunity has not yet been evaluated.