Host T Cell Responses to Malaria, Acquired Immunodeficiency Virus, and *Listeria* Infections

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THESIS
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This work is dedicated to my daughter, Rosalyn Marie Payseur, in the hopes that she will never feel her dreams are out of her reach.

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LIST OF ABBREVIATIONS

Ab – Antibody

Ag – Antigen

AID_{50} – Animal infectious dose 50%

AIDS – Acquired immunodeficiency syndrome

APC – Antigen presenting cell

BALF – Bronchioalveolar lavage fluid

CFU – Colony forming unit

CTL – Cytotoxic lymphocyte

DC – Dendritic cell

DNA – Deoxyribonucleic acid

EDTA - Ethylenediaminetetraacetic acid

ELISA – Enzyme linked immunosorbent assay

ER – Endoplasmic reticulum

FITC – Fluorescein isothiocyanate

FIV – Feline immunodeficiency virus

GM-CSF – Granulocyte Macrophage- Colony Stimulating Factor

HIV – Human immunodeficiency virus

HMBPP - (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate

IACUC – Institutional animal care and use committee

ICS – Intracellular cytokine staining

IFNγ – Interferon γ

IL - Interleukin

i.m. – Intramuscular

IPP – Isopentanyl pyrophosphate

i.v. - Intravenous

LTR – Long terminal repeat

mAb – Monoclonal antibody

MACS – Magnetic activated cell sorting

MHC – Major histocompatability complex

PBL – Peripheral blood lymphocytes

PE – Phycoerythrin

RNA – Ribonucleic acid

RT-PCR – Reverse transcriptase polymerase chain reaction

SHIV – Simian/human immunodeficiency virus

SIV- Simian immunodeficiency virus

TCR – T cell receptor

TGFβ – Transforming growth factor β

TNFα – Tumor necrosis factor α

SUMMARY

T cells are an important part of the acquired immune system. They provide immunity against a variety of different pathogens, including both extracellular and intracellular pathogens. T cells utilize many mechanisms to fight infections. CD4+ helper T cells produce cytokines to activate effector immune cells, such as CD8+ T cells, macrophages, B cells, and neutrophils. CD8+ T cells can directly kill infected cells. Vγ2Vδ2 T cells provide both helper and cytolytic functions. Although there are innate immune cells that can provide many of these functions, T cells provide the benefit of memory. When antigen is initially encountered, naïve T cells that recognize that antigen are activated and proliferate. After the infection is cleared, the majority of the activated T cells die, but a few remain and become memory cells. Upon re-infection, memory T cells quickly respond and are able to fight off the secondary infection more rapidly. The adaptation of an acquired immune response has been an important part of protection against serious infections.

Although a great deal of research has been performed studying T cells, there are still many questions regarding their function in various infections. For example, CD4+ T cells are important for controlling malaria parasite levels, as inhibition of T cell responses in humans has been associated with severe disease. However, a lack of regulatory T cells has also been associated with severe disease. This evidence suggests that a proper balance of activated CD4+ T cells is crucial during infection, but the specifics of this balance, such as what types of T cells must be activated and when, are not fully understood. This balance becomes even more difficult to discern during coinfection. Malaria and HIV have significant geographic overlap, and coinfection is very common. As HIV infects CD4+ T cells and has its own immunopathological consequences, the immune interactions of these two pathogens would be very complex. It is possible that HIV-induced CD4+ T cell death would result in increased malaria disease. On the other hand, it is also possible that malaria-induced CD4+ T cell activation would result in increased HIV replication. Several studies have been performed looking at these possibilities, but there is little information available regarding the immune responses involved in this. In our studies, we

found that malaria disease and AIDS progression are both significantly enhanced during coinfection of naïve subjects. However, during malaria coinfection of chronic-AIDS-virus infected subjects, malaria and AIDS-disease were only moderately enhanced. The different outcomes of disease appeared to be due to differences in the type of CD4+ T cell responses elicited during infection.

Vγ2Vδ2 T cells are a specific type of γδ T cell that are only found in primates. They are unique from other types of γδ T cells in that they recognize small phosphorylated molecules termed phosphoantigens. They therefore can respond to a variety of pathogens and even certain tumor cells. Vγ2Vδ2 T cells, for example, have been shown to expand in malaria infection of humans, and *L. monocytogenes* infection *in vitro*. They produce cytokines such as IFNγ, perforin, and IL-4. However, the exact role of Vγ2Vδ2 T cells during infection is still poorly elucidated. We found that Vγ2Vδ2 T cells were activated *in vivo* by *L.* monocytogenes infection, but not *P. fragile* malaria infection. Activated Vγ2Vδ2 T cells could co-produce different types of cytokines, and were able to directly lyse infected cells.

CHAPTER 1- Introduction

A. T cells

1. T cell biology

T cells are lymphocytes that develop in the thymus, and play an important role in immunity against pathogens. Because many T cells develop memory, they are able to initiate rapid and potent responses upon secondary or later infections. There are several types and subtypes of T cells, which all have different, specific roles in immunity. T cells recognize antigen via the T cell receptor (TCR) (Krogsgaard and Davis 2005; Smith-Garvin, Koretzky, Jordan 2009). The TCR can be composed of either an α and β chain (Hedrick et al 1984; Malissen and others 1984; Yanagi et al 1984) or a γ and δ chain (Brenner et al 1986; Hedrick et al 1984; Yanagi et al 1984), and the chain expressed on the cell is determined during thymic development (Ciofani and Zuniga-Pflucker 2010). αβ T cells are considered conventional T cells. They generally express either the CD4 or CD8 co-receptor molecule, recognize peptide antigen presented by major histocompatibilty complex (MHC) molecules, and are considered part of the adaptive immune system (Smith-Garvin, Koretzky, Jordan 2009). On the other hand, murine γδ T cells often do not coexpress CD4 or CD8, though some primate γδ T cells may express the CD8 coreceptor (Ali et al 2007). γδ T cells recognize a variety of microbial antigens including glycolipids (O'Brien et al 2007) and small phosphorylated molecules termed phosphoantigens (Hintz et al 2001; Wang et al 2003). γδ T cells are mostly considered to be either part of the innate immune system, or a bridge between innate and adaptive immunity (Chen and Letvin 2003).

When a T cell is activated by antigen, a complex signaling cascade begins, which results in development of effector functions (Smith-Garvin, Koretzky, Jordan 2009). The cytokines present during activation, the presence of CD4 or CD8, and whether the T cell has an αβ or γδ TCR all influence the effector function a T cell will possess after activation.

2. CD4+ T cells

T cells bearing the CD4 coreceptor are generally considered helper T cells, which are vital for the proper activation and function of innate immune cells, B cells, and CD8+ T cells. CD4+ T cells recognize antigen presented by professional antigen presenting cells (APCs). APCs ingest antigenic particles present the antigen to CD4+ T cells via MHC class II molecules. CD4+ T cells are divided into different subtypes, including T helper 1 (Th1), Th2, Th17, and regulatory T cells (Treg), all with different effector functions. The effector function of a CD4+ T cell is determined during development and activation. Th1 cells, under control of the transcription factor T-bet, are generally induced by IL-12 and are proinflammatory, producing interferon-γ (IFNγ) and tumor necrosis factor α (TNFα) (**Table I**, Reiner 2007; Zhu and Paul 2010; Zhu, Yamane, Paul 2010). Th1 cells assist in cell-mediated immunity against intracellular pathogens by activating CD8+ T cells and macrophages. Over-activation of Th1 cells can result in immunopathology and autoimmune disease. Excess Th1 activation has been shown to be involved in immunopathogenesis in malaria (Good et al 2005) as well as human immunodeficiency virus (HIV) infection (Douek, Roederer, Koup 2008; Margolick et al 1987). In the case of malaria, excess inflammation leads to tissue damage (Good et al 2005) and causes symptoms such as fever. During HIV infection, activation of Th1 cells actually increases viral replication via activation of the 5' Long Terminal Repeat (Douek, Roederer, Koup 2008; Margolick et al 1987).

Table I

	Induced by	Major Transcription Factor	Produces	Function	Inhibited by
Th1	IL-12	T-bet, STAT1	IFNγ, TNFα	Intracellular pathogens	IL-17, IL-4
Th2	IL-4, IL-10	GATA-3	IL-10	Extracellular pathogens	T-bet
Th17/Th22	IL-23, TGFβ, IL-6	RORγt	IL-17, IL-22, IL-23	Extracellular pathogens	IFNγ
Treg	IL-2, TGFβ	FOXP3	IL-10, TGFβ	Regulation of T cell response	IFNγ

IL-17- and IL-22-producing T cells have recently received a great deal of attention. In mice, IL-17 and IL-22 are produced by the same cell population (Martin et al 2001; Zhu and Paul 2010; Zhu, Yamane, Paul 2010), whereas in humans these cytokines are produced in separate cell populations (Trifari et al 2009; Yao et al 2010). Th17 cells are induced by TGF-β, IL-6, and IL-23 in humans, and controlled by the transcription factor RORγt (**Table I**, Zhu and Paul 2010; Zhu, Yamane, Paul 2010). Like Th1 cells, Th17 and Th22 cells are considered proinflammatory, and have been implicated in a variety of autoimmune diseases (Iwakura et al 2011). However, they have important protective roles during microbial infection as well. Depletion of IL-17-producing T cells during early AIDS-virus infection has been shown to be a part of viral pathogenesis (Favre et al 2009); loss of Th17 cells permitted microbial translocation across gut mucosae, which is a cause of inflammation and immunopathogenesis (Raffatellu et al 2008). In addition, IL-17-producing T cells have protective roles in toxoplasmosis (Kelly et al 2005), *L. monocytogenes* infection (Conti et al 2009), and *Klebsiella pneumoniae* infection (Curtis and Way 2009; Hamada et al 2008; Zheng et al 2008). IL-17 targets epithelial cells, keratinocytes, T cells, B cells, and macrophages, inducing production of

proinflammatory cytokines and chemokines, neutrophil recruitment, production of antimicrobial peptides, osteoclastogenesis, angiogenesis, and promotes T cell priming and antibody production (Iwakura et al 2011). IL-22 is suspected of being able to help protect epithelial barriers by inducing tissue repair and production of matrix metalloproteinases (Sonnenberg, Fouser, Artis 2011; Zelante et al 2011).

The Treg phenotype is induced by IL-10 and TGF-β (Zhu and Paul 2010; Zhu, Yamane, Paul 2010). These cells help regulate immune responses by inhibiting replication of CD4+ and CD8+ T cells (Gondek et al 2005; Sakaguchi et al 2006). Th2 cells are induced by and produce primarily IL-4. They activate B cells to produce antibody (Zhu and Paul 2010; Zhu, Yamane, Paul 2010).

Once a CD4+ T cell has committed to a lineage, it produces cytokines that promote commitment to that subtype, as well as cytokines that inhibit development of other subtypes (**Table 1**). Th1-produced IFNγ inhibits GATA-3, the main transcription factor responsible for development of Th2 cells, as well as RORγt, and thereby development of Th17 (Yang et al 2011) and Th22 cells (Yao et al 2010). Th2 cells counter-regulate proliferation of Th1 cells, as IL-4 inhibits signaling that promotes a type 1 phenotype (Hegazy et al 2010; Zhou, Chong, Littman 2009). IL-17 and IL-22 appear to inhibit IFNγ-producing Th1 cells (Yao et al 2010).

3. CD8+ T cells

CD8+ T cells, also called cytolytic T lymphocytes (CTL), recognize peptide antigen from intracellular pathogens, including bacteria, viruses, and protists. Antigen, produced within the infected cell then process by the proteasome, is presented to CD8+ T cells by MHC class I molecules, which are present on all nucleated cells (Harty, Tvinnereim, White 2000; Zhu and Paul 2010; Zhu, Yamane, Paul 2010). CD8+ T cells can kill infected cells either by production of cytolytic cytokines, such as perforin and granulysin, which create holes in the cell membrane, or by upregulation of Fas Ligand (CD95L),

which will lead to apoptosis of the infected cell upon binding to Fas (CD95, Harty, Tvinnereim, White 2000). In addition, CD8+ T cells produce cytokines such as IFNγ and TNFα, and chemokines (Harty, Tvinnereim, White 2000). Cytokines and chemokines produced by CD8+ T cells would have roles similar to cytokines produced by CD4+ T cells, such as recruitment of phacoytes and activation of effector cells.

4. γδ T cells

T cells expressing the Vγ2 (also called Vγ9) and Vδ2 T cells are the predominant subset of circulating γδ T cells in humans and nonhuman primates. Vγ2Vδ2 T cells recognize and respond to small, phosphorylated metabolic intermediates of isoprenoid biosynthesis termed phosphoantigens (Ali et al 2007; Hintz et al 2001). Because only primates, such as humans and monkeys, have phosphoantigen-specific γδ T cells, mice and other small laboratory animals cannot used to be study this cell type. Though the molecule used to present phosphoantigen to Vγ2Vδ2 T cells is not known, there is evidence suggesting that F1-ATPase may be involved (Scotet et al 2005), and cell-to-cell contact is required for activation (Morita et al 2001; O'Brien et al 2007; Wei et al 2008). Isoprenoid biosynthesis occurs by one of two pathways: the classical, mevalonate pathway, found in higher eukaryotes, and the alternative, non-mevalonate pathway, found in prokaryotes such as Gram+ bacteria and the plastids of eukaryotes (Hintz et al 2001). Due to their recognition of phosphoantigens, Vγ2Vδ2 T cells are activated in response to a wide variety of microbes, including *Listeria monocytogenes* (Begley et al 2004), mycobacteria (Shen et al 2002), malaria (Dieli et al 2001), and smallpox (Shao et al 2009), as well as tumor cells (Scotet et al 2005). While most evidence suggests that the foreign phosphoantigen (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) is the primary activating molecule of Vγ2Vδ2 T cells during infection (Hintz et al 2001), some evidence suggests that accumulation of host-produced

isopentenyl pyrophosphate (IPP) may activate Vγ2Vδ2 T cells instead (Kistowska et al 2008). Activation of Vγ2Vδ2 T cells by viral infection (Shao et al 2009) indeed supports the role of IPP in the activation of Vγ2Vδ2 T cells during microbial infection. IPP has been used for *in vitro* activation and proliferation of Vγ2Vδ2 T cells, however it requires a 1000-fold higher concentration compared to HMBPP for similar bioactivity (Hintz et al 2001). This difference in bioactivity between foreign HMBPP and endogenous IPP may be a method for Vγ2Vδ2 T cells to differentiate between host cells and foreign or altered self cells.

Upon *in vivo* activation with phosphoantigen, Vγ2Vδ2 T cells proliferate rapidly, comprising up to 70% of total T cells (Ali et al 2007). Unlike most γδ T cell subsets, which are considered innate, Vγ2Vδ2 T cells display memory properties, including more rapid recall response to infection after previous priming with a similar microbe (Shao et al 2009; Shen et al 2002). In addition, Vγ2Vδ2 T cells have a wide range of effector functions. These include production of IFNγ (Ali et al 2007; Shao et al 2009; Shen et al 2002); lysis of infected cells via production of cytolytic cytokines (Ali et al 2007; Dieli et al 2001; Martino et al 2007; Troye-Blomberg et al 1999); and provide helper type effects on CD4+ and CD8+ T cell and antibody responses (Ali et al 2009). Vγ2Vδ2 T cells can also counter-regulate regulatory T cells (Gong et al 2009) and IL-22-producing T cells (Yao et al 2010). All of these characteristics make Vγ2Vδ2 T cells an interesting and versatile vaccine target.

B. Malaria

1. Life cycle of *Plasmodium* species

Plasmodium species, the causative agents of malaria, have a complex life cycle involving both an insect vector and a mammalian host. Female *Anopheles* mosquitoes ingest malaria parasite gametocytes when taking a blood meal from an infected host. The gametocytes then develop into gametes, and undergo sexual reproduction within the mosquito, turning into sporozoites (Aly, Vaughan, Kappe 2009). Sporozoites are injected into the mammalian host during the next blood meal. Sporozoites move from the blood to the liver, and develop into merozoites, which then infect red blood cells. The merozoites within the red blood cell develop into a schizont, which then releases 16 new merozoites. Rupture of the infected erythrocyte causes malarial paroxysms, which include fever, headaches, and malaise. It is the erythrocytic stage of infection that is responsible for disease in humans (Miller, Good, Milon 1994).

2. Disease prevalence and severity

Malaria in humans is caused by five species of *Plasmodium* parasites: *P. falciparum* (Miller, Good, Milon 1994)), *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* (Cox-Singh et al 2008; Pain et al 2008). The most significant species for human infection is *P. falciparum* as it is responsible for most cases of severe malaria and death (Miller, Good, Milon 1994; Miller et al 2002). Annually, there are approximately 150 million cases of malaria, with approximately 1-3 million deaths. Most malaria deaths occur in young children, who have not developed protective immunity (Miller, Good, Milon 1994; Miller et al 2002). Malaria is highly prevalent in Sub-Saharan Africa, as well as parts of Central and South America and Southeast Asia (Slutsker and Marston 2007). In many areas, malaria is endemic year round, while in other areas, malaria comes and goes with the rainy seasons.

P. falciparum is well suited to cause lethal infections due to many factors. First, it can infect all types of red blood cells, including reticulocytes and mature erythrocytes. Second, it has multiple

redundant entry pathways, so it is not restricted to infecting cells of a specific blood type (ie A, B, or O). Knob protrusions from the infected erythrocyte cause adherence to endothelial cells lining the blood vessel. This causes sequestration of the parasite within the capillaries. If this occurs within cerebral capillaries, it causes a life threatening condition known as cerebral malaria (Miller, Good, Milon 1994; Miller et al 2002). Sequestration of infected erythrocytes in capillaries is also responsible for tissue damage in other parts of the body.

Four of the 5 malaria-causing parasites do not infect Old World Monkeys (Collins 1992); *P. knowlesi* is the only parasite that infects both humans and Old World Monkeys (Cox-Singh et al 2008; Pain et al 2008). In order to study malaria pathogenesis and immune responses in the context of AIDS-virus coinfection and Vγ2Vδ2 T cell responses, we utilized *P. fragile* infection of rhesus macaques. *P. fragile* causes *falciparum*-like malaria in macaques (Collins et al 2006), including formation of knobs and cerebral malaria (Fujioka et al 1994).

3. T cell responses to malaria infection

While antibody is considered the primary source of immunity during blood-stage malaria, T cell responses have an important role as well (Good and Doolan 1999; Good et al 2005). CD4+ and CD8+ T cells have both been shown to be activated and proliferate during blood-stage malaria infection. The role of CD8+ T cells during malaria infection is not clear (Lundie et al 2008; Miyakoda et al 2008), as red blood cells do not express MHC I and therefore do not normally activate CD8+ T cells. However, there is evidence that CD8+ T cells play a role in development of cerebral malaria, possibly by killing endothelial cells (Nitcheu et al 2003). CD4+ T cells are more significant than CD8+ T cells during malaria blood stage infection (Good et al 2005).

Malaria infection generally activates Th1 responses, with T cells producing IFNγ, TNFα, and Lymphotoxin α (Engwerda et al 2002; Good et al 2005; Ramharter et al 2003; Todryk et al 2009).

Interestingly, malaria antigens can activate CD4+ T cells from naïve patients who have not been previously exposed to malaria, which seems to indicate that activation of CD4+ T cells during malaria infection is TCR independent (Dick et al 1996). Th1 responses during malaria have been shown to be both beneficial and pathogenic. CD4+ T cells are able to prevent growth of blood-stage parasites in mice unable to produce antibodies (Good et al 2005). Any such protection generated by CD4+ T cells likely occurs in the spleen or lymph node, and any such protection would be limited without concurrent antibody response (Good et al 2005). In contrast, uncontrolled Th1 responses can result in immunopathology, resulting in cerebral malaria, anemia, and weight loss. An excessive Th1 response activates expression of adhesion molecules on endothelial cells, allowing more infected red blood cells to adhere, and causing inflammation and tissue damage (Good et al 2005; Schofield and Grau 2005; Schofield 2007). TNFα, which is produced in part by Th1 cells, is considered the causative agent of fever during malaria paroxysms (Karunaweera et al 1992).

In addition to upregulation of Th1 responses, highly pathogenic malaria strains can upregulate Tregs (Walther et al 2005). *P. falciparum* infected erythrocytes can suppress production of IFNγ in a variety of innate and adaptive immune cells, and this suppression correlates with increased parasite growth (Couper et al 2008; D'Ombrain et al 2007; Hisaeda et al 2004; Walther et al 2005), emphasizing the protective role of proinflammatory Th1 responses discussed in the previous section. Immune suppression is associated with IL-10 and TGF-β (Couper et al 2008; Walther et al 2006). However, the development of Treg responses may actually help prevent severe disease, including cerebral malaria, by preventing excessive inflammation and tissue damage (Nie et al 2007). While this data may seem contradictory, it merely indicates that a balanced, well-controlled T cell response is most beneficial during malaria infection, as it is in any infection. Antibody, IL-4, and IFNγ work in concert to control parasite replication and limit tissue damage (Petritus and Burns 2008). Understanding of this balance is essential for development of an effective anti-malarial vaccine.

Malaria infection of humans also induces proliferation of Vγ2Vδ2 T cells (Behr et al 1996; Dieli et al 2001; Goerlich et al 1991). Vγ2Vδ2 T cells have also been to shown to lyse malaria-infected erythrocytes (Troye-Blomberg et al 1999). *P. falciparum* only contains genes for the non-classical isoprenoid biosynthesis pathway (Wiesner and Jomaa 2007), and thus produces the Vγ2Vδ2 T cell antigen HMBPP. As with many infections, the *in vivo* significance of activated Vγ2Vδ2 T cells is not fully understood, as this can only be studied in humans and non-human primates, which are difficult and costly to perform.

C. HIV/AIDS

1. Introduction to HIV/AIDS

Acquired immunodeficiency syndrome (AIDS) is caused in humans and nonhuman primates by a lentivirus, a type of retrovirus. HIV-1 and HIV-2 infect and cause AIDS in humans, but generally not other species, due to the inhibition of HIV-1 infection by the antiretroviral component TRIM5α in macaques (Stremlau et al 2004). Therefore, a variety of animal models have been developed to study AIDS pathogenesis. While FIV study of cats provides many parallels to HIV infection (Elder et al 2010), rhesus macaques are the preferred model for studying AIDS infection (Baroncelli et al 2008; Shedlock, Silvestri, Weiner 2009). Macaque simian immunodeficiency virus (SIVmac) and simian-human immunodeficiency virus (SHIV), a chimera of SIV and HIV, are used to study AIDS in rhesus macaques (Baroncelli et al 2008; Reimann et al 1996; Reimann et al 2005; Shedlock, Silvestri, Weiner 2009). Both types of virus cause rapid and permanent decline in CD4+ T cell levels, and result in immunodeficiency, when infecting the proper host. SIV naturally infects African monkeys, but does not cause AIDS. However, in Asian macaques, which are not natural hosts, SIV is pathogenic and infection results in immunodeficiency disease (Baroncelli et al 2008). This dichotomy between natural and laboratory hosts has been useful in determining potential mechanisms of immune pathogenesis by comparing immune responses in natural and experimental hosts.

HIV and SIV are retroviruses; RNA is reverse-transcribed into DNA, which is then integrated into the host cell genome. HIV and SIV infect cells expressing the CD4 receptor and the CXCR4 or CCR5 co-receptors, including CD4+ T cells, dendritic cells, and macrophages (Stevenson 2003). Resting T cells are less susceptible to viral infection and replication than activated T cells (Chou, Ramilo, Vitetta 1997; Tang, Patterson, Levy 1995). Activated T cells are more permissive to viral replication, due to activation of the 5'LTR of HIV/SIV by proinflammatory immune factors, such as TNF (Nti et al 2005).

2. Immunity and immunopathogenesis

CD8+ cytolytic T cells are the major players in cellular anti-HIV immunity, which has been demonstrated by depletion of CD8+ T cells using a neutralizing antibody that results in increased viral loads in infected macaques (Jin et al 1999). They presumably kill virus-infected CD4+ T cells using the perforin-granulysin pathway. Interestingly, the magnitude of the CD8+ T cell response does not correlate with virus load, which suggests that the effector functions of activated CD8+ T cells are more important than the number of activated CD8+ T cells during AIDS-virus infection (Douek, Roederer, Koup 2008). However, CD8+ T cells are not sufficient to eliminate the virus, since infected individuals retain some viral load for life. This may be due to either defects in the host immune response, such as insufficient helper T cell activity or CD8+ T cell anergy; or due to viral causes, such as epitope mutation and down regulation of MHC (Benito, Lopez, Soriano 2004). Because HIV integrates into the host cell genome, the virus is able to remain in a latent state for an indefinite amount of time, making elimination difficult.

CD4+ T cells, expressing either the CXCR4 coreceptor or CCR5 coreceptor, are the main targets of viral infection. As activated CD4+ T cells are more easily infected than resting targets, immune activation therefore can increase AIDS pathogenesis (Pandrea et al 2008). Immune activation can be caused by a variety of sources, including coinfections (Douek, Roederer, Koup 2008; Shen et al 2004; Slutsker and Marston 2007) and microbial translocation, when microbes move across the gut mucosal barrier (Brenchley et al 2006). Increased immune activation has many detrimental consequences, including increased viral replication, T cell exhaustion, and a decreased pool of resting T cells (Douek, Roederer, Koup 2008).

Th17 cells also seem to play an important role in immune protection during HIV infection. Long-term non-progressors, individuals infected with virus but with no detectable viral replication even without anti-retroviral therapy, were shown to have an overall higher percentage of IL-17-producing CD4+ T cells than typical progressors (Salgado et al 2011). Loss of IL-17-producing T cells has been

suggested to play a part in immunopathogenesis of AIDS virus infection, as depletion of Th17 cells was only noted in monkeys with pathogenic SIV infection (Cecchinato et al 2008; Favre et al 2009), but not in monkeys with non-pathogenic SIV infection. Though the specific role of Th17 cells is unclear, it appears to be due to protection of mucosal surfaces from pathogens, as absence of IL-17 permitted enhanced microbial translocation of *Salmonella* from the gut (Raffatellu et al 2008). By preventing microbial translocation, a major potential source of immune activation is halted, thereby restricting viral replication. There are some conflicting reports, however, that associate IL-17 with increased AIDS virus pathogenesis (Campillo-Gimenez et al 2010). The reasons for these conflicting results are unclear, but may stem from variances in infection route, dosage, viral strain, and species studied.

D. Malaria/AIDS coinfection

Malaria and HIV have a significant geographical overlap; both are highly prevalent in Sub-Saharan Africa, Southeast Asia, and parts of Central and South America (Slutsker and Marston 2007). As a result, a number of studies have been conducted looking at pathologic interactions during coinfection of malaria and HIV. Although early studies failed to find increased pathogenesis of either disease during coinfection, more recent studies using vigorous parameters have found interactions. HIV+ patients had a higher incidence of malaria infection than HIV- patients in one longitudinal study (Patnaik et al 2005). Malaria was also more severe in HIV+ patients, including complications such as cerebral malaria, coma, and death (Chalwe et al 2009; Cohen et al 2005; French et al 2001; Grimwade et al 2004; Laufer et al 2006; Malamba et al 2007; Mouala et al 2009; Whitworth et al 2000). Interestingly, most of these studies did not detect a significant difference in malaria parasite levels in HIV+ patients. Only a handful of studies linked low CD4+ T cell counts due to HIV disease with higher parasite levels (Laufer et al 2006; Whitworth et al 2000). This is in an indication of the complex nature of malaria and HIV coinfection, and raises many questions regarding the immunopathogenesis of coinfection.

Malaria coinfection can exacerbate HIV disease. Several studies have demonstrated transient viral load increases during malaria coinfection of HIV+ patients (Hoffman et al 1999; Kublin et al 2005; Tatfeng et al 2007; Van Geertruyden et al 2006; Van Geertruyden et al 2009). *In vitro* and mouse studies have shown that *P. falciparum* antigens can increase replication of HIV-1 in culture cells and transgenic mice, respectively (Ayouba et al 2008; Freitag et al 2001). The increased viral replication was shown to be caused by TNFα production in response to malaria hemozoin, and was more prevalent in peripheral blood mononuclear cells (PBMC) from rhesus macaques with advanced SIV disease compared to those with less severe disease (Nti et al 2005; Xiao et al 1998).

The conflicting results between studies may have been due to differences in study design, including patient recruitment, geographic area, malaria and HIV prevalence, and quality of care. An appropriate animal model could circumvent these limitations to study malaria and AIDS virus coinfection.

Despite the knowledge that malaria and AIDS-virus coinfection can lead to increased severity of both diseases, few studies have been performed looking at in depth immune mechanisms during coinfection, likely due to the difficulty of such studies in human subjects. A recent study in rhesus macaques coinfected with the relapsing malaria parasite *P. cynomolgi* and SIV did demonstrate alterations in the immune response. Pre-existing malaria infection resulted in more rapid SIV progression, including decreased survival (Koehler et al 2009). However, the immune interactions, including induction of cytokine-producing T cells and histological examination of lymphoid tissue were not described. Determining immune factors during malaria and AIDS-virus coinfection is important, as disease interaction has increased the spread of both diseases in Sub-Saharan Africa (Abu-Raddad, Patnaik, Kublin 2006), and understanding of these interactions could be crucial for vaccine and immunotherapeutic development.

E. *Listeria monocytogenes* infection and γδ T cell activation

1. Isoprenoid biosynthesis in *L. monocytogenes* and activation of Vγ2Vδ2 T cells

The Gram+ bacterium *L. monocytogenes* is the only pathogen known to utilize both the classical and alternative pathways of isoprenoid biosynthesis (Begley et al 2004). By removing one or both pathways, *L. monocytogenes* can be used as a tool to study activation of Vγ2Vδ2 T cells, and be used to determine whether microbial HMBPP or endogenous IPP is more significant for activation of Vγ2Vδ2 T cells during infection.

In vitro activation of Vγ2Vδ2 T cells has been previously shown (Begley et al 2004); however, *in vivo* activation has not been demonstrated. In addition, no functional studies of Vγ2Vδ2 T cells upon activation with *L. monocytognes* have been performed aside from production of IFNγ. Many mouse studies have been performed to study γδ T cells during *L. monocytogenes* infection; however, since mice do not have phosphoantigen-specific γδ T cells, these studies are irrelevant to human biology.

2. PrfA* ΔActA mutant strains

In the following studies, we utilized a strain of *L. monocytogenes* with a PrfA* mutation and an ActA deletion (Yan et al 2008). The PrfA* mutant has a highly constitutively active form of PrfA, a regulatory factor that promotes production of a variety of *L. monocytogenes* virulence factors (Shetron-Rama et al 2003). The ΔActA mutation removes the ActA protein, which is required for actin polymerization and cell-to-cell spread of the bacterium (Ramaswamy et al 2007). PrfA*ΔActA mutant *L. monocytogenes* strains are both safe and highly immunogenic, making them good vaccine candidates (Yan et al . Immunogens are inserted into the PrfA*ΔActA strain using the pPL6 plasmid (Yan et al 2008), a derivative of the pPL2 plasmid (Lauer et al 2002). Immunogens are under control of the Hly promoter, which is activated by PrfA (Lauer et al 2002; Yan et al 2008). We utilized this particular PrfA*ΔActA strain, NF974, as we have previously shown its ability to induce robust T cell responses in

mice (Yan et al 2008). A systemic infection was used in order to generate strong Vγ2Vδ2 T cell proliferation, as we have previously shown that mucosal mycobacterial infection is not sufficient to induce systemic Vγ2Vδ2 T cell proliferation (Huang et al 2008).

CHAPTER 2- Materials and Methods

A. Rationale for infectious agents

The purpose of the following studies was to investigate the interaction of specific T cell subtypes during *in vivo* infections of malaria, SHIV, and *L. monocytogenes*, as well as malaria and SHIV coinfection. Our goal was to identify activation of T cell subsets and protective efficacy during infection, which could potentially help in rational vaccine design.

We first focused on coinfection of malaria and AIDS-virus, as these are two of the top causes of death due to infectious disease worldwide (http://apps.who.int/malaria, http://www.who.int/hiv/data/en). In addition, the geographic overlap of malaria and AIDS-virus is substantial, and coinfection is very common (Slutsker and Marston 2007). As there is no appropriate model for HIV infection in either small animals or New World monkeys, we used Chinese-origin rhesus macaques. SHIV89.6p is a chimeric virus that causes rapid and permanent depletion of CD4+ T cells in macaques (Ali et al 2009; Reimann et al 1996). SIVmac is less pathogenic in Chinese-origin rhesus macaques than Indian-origin rhesus macaques (Reimann et al 2005), and therefore was not appropriate for our studies.

We chose to utilize *P. fragile* infection of rhesus macaques as a model of blood-stage *falciparum*-like malaria. Human malaria parasites, such as *P. falciparum*, infect New World monkeys, but not Old World monkeys such as macaques. *P. fragile* has been shown to cause *falciparum*-like malaria in macaques (Collins et al 2006), and has also been used as a model of cerebral malaria (Fujioka et al 1994). We utilized a blood-stage infection model, as this is the stage of malaria responsible for disease (Miller, Good, Milon 1994), and many T cell responses can be detected in peripheral blood during blood-stage malaria infection (Dick et al 1996; Good and Doolan 1999; Good et al 2005; Karunaweera et al 1992; Langhorne 1994; Ramharter et al 2003; Schofield and Grau 2005; Todryk et al 2009).

We also studied immune responses to an attenuated strain of *L. monocytogenes*, which is a potential vaccine vector candidate (Yan et al 2008). *L. monocytogenes* infects macrophages (Gray, Freitag, Boor 2006), delivering antigens intracellularly and thus eliciting a CD8+ T cell response (Tvinnereim, Hamilton, Harty 2002). *L. monocytogenes* also produces HMBPP, the phosphoantigen believed to be important for activation of Vγ2Vδ2 T cells (Begley et al 2004). Importantly, attenuation of *L. monocytogenes* does not inhibit immunogenicity (Starks et al 2004; Yan et al 2008), making attenuated *L. monocytogenes* strains suitable for immunocompromised patients. In addition, pre-existing anti-vector immunity does not seem to interfere with establishment of vaccine-induced immunity (Starks et al 2004). In the current studies, we utilized an attenuated, recombinant *L. monocytogenes* strain with a constitutively active *prfA* mutation that we have shown enhances production of foreign immunogens and development of vaccine-elicited immune response (Shetron-Rama et al 2003; Yan et al 2008). Due to the deletion of the *actA* gene, the strain used is unable to polymerize actin, and thus unable to spread cell-to-cell. In order to both generate strong peripheral blood Vγ2Vδ2 T cell activation, and to assess the utility of *L. monocytogenes* as a systemic vaccine candidate, we vaccinated rhesus macaques via i.m. or i.v. injection.

B. Malaria and SHIV coinfection studies

1. Animals

Eighteen Chinese rhesus macaques (4-8 years old), free of simian retrovirus, simian T-lymphotrophic virus type 1, and SIV infection were used. Macaques' ages were relevant to young adult or adult humans (**Table I**). Four macaques were simultaneously coinfected with SHIV/malaria; five chronically-SHIV-infected macaques were coinfected with malaria; four were infected with malaria; six were infected with SHIV alone. All animals were maintained and used in accordance with the guidelines of the Institutional Animal Care and Use Committee under an approved protocol. Chloroquine (West-ward Pharmaceutical, Eatontown, NJ) was given (37.5mg/kg by stomach gavage) to control life-threatening malaria (including high parasite levels or low hematocrit), or to cure malaria (two doses given two days apart) at the endpoint. Animals infected with SHIV were humanely euthanized at the end of the study, or earlier if deemed necessary by veterinary staff.

Table II

Group	Malaria infection	SHIV infection
Malaria-only	10^4 pRBC i.v. D0	None
SHIV-only	None	1000 MID_{50} SHIV89.6p i.v. D0
Malaria/acute SHIV	10^4 pRBC i.v. D0	1000 MID_{50} SHIV89.6p i.v. D0
Malaria/chronic SHIV	10^4 pRBC i.v. D296	1000 MID_{50} SHIV89.6p i.v. D0

2. Infections

Animals were inoculated intravenously with 1000AID_{50} SHIV89.6P and/or 10^4 *P. fragile*-parasitized red blood cells (pRBC). pRBC were propagated in a donor rhesus macaque, stored in liquid nitrogen until use, thawed, and washed in a series of saline washes, as previously described (Malaria Research and Reference Reagent Resource Center 2004). SHIV89.6P was stored at -20°C, washed, and resuspended in RPMI-1040 + 10% heat-inactivated FBS (Ali et al 2009). For chronic-SHIV-coinfection, animals had been infected with SHIV89.6P for 296 days before coinfection with malaria. For SHIV-only infection, macaques had been infected with the same lot, dose, and route of SHIV89.6P approximately one year earlier for use in another study (Ali et al 2009). As chronic SHIV-only controls, data were collected from 16 weeks through 24 weeks after SHIV infection.

3. Estimation of parasitemia

Thin blood smears were made by expressing a drop of blood from a tail prick of malaria-infected monkeys. Smears were air dried, and stained using the DipQuick stain kit (Jorgenson Laboratories, Loveland, CO) according to manufacturer's instructions. Parasitemia was estimated by comparing parasitized and total erythrocytes in blood film. Data was expressed as percentage of pRBC in total RBC.

4. Quantitative measurement of SHIV RNA

Plasma SHIV RNA was assessed using real-time RT-PCR as previously described (Ali et al 2009; Amara et al 2001; Cline et al 2005).

5. Histology

Tissue samples obtained from necropsy were formalin fixed (Protocol Formalin, Kalamazoo, MI), embedded in paraffin, and sectioned at 5μm for routine staining with hematoxylin and eosin (Huang et al 2009).

6. Isolation of lymphocytes from peripheral blood

PBL were isolated from freshly collected EDTA blood by Ficoll-Paque Plus (Amersham, Piscataway, NJ) density gradient centrifugation before analysis.

7. Immunofluorescent staining and flow cytometric analysis

For cell-surface staining, PBL were stained with up to 5 Abs (conjugated to FITC, PE, allophycocyanin, pacific blue, and PE-Cy7) for 15min. After staining, cells were fixed with 2% formaldehyde-PBS prior to analysis on a CyAn ADP flow cytometer (DakoCytomation, Carpinteria, CA). Lymphocytes were gated based on forward- and side-scatters, and pulse-width and ~40,000 gated events were generally analyzed using Summit Data Acquisition and Analysis Software (DakoCytomation).

The following Abs were used: IL-17 (eBio64CAP17) [eBioscience, San Diego, CA]; IL-22-biotin (anti-human) [R&D Systems, Minneapolis, MN]; Streptavidin-Pacific Blue [Invitrogen, Carlsbad, CA]; CD3 (SP34-2), CD4 (L200), CD8 (RPA-T8), IFNγ (4S.B3), TNFα (mAb11) [BD Pharmingen, San Diego CA].

8. Intracellular cytokine staining

Modified intracellular cytokine staining (ICS) without *in vitro* Ag stimulation was adopted as recently described (Yao et al 2010). Briefly, 0.5×10^6 PBL plus mAbs α-CD28 (1μg/ml) and α-CD49d (1μg/ml) were incubated in 100μl final volume for 1h at 37°C, 5% CO_2 followed by an additional 5h incubation with brefeldin A (GolgiPlug, BD). After staining for cell-surface markers (CD3, CD4, CD8) for 15min, cells were permeabilized for 45min (Cytofix/cytoperm, BD) and stained 45min for IFNγ, TNFα, IL-17, or IL-22 before fixation in formalin.

As *in vivo* control experiments, longitudinal analyses during acute- and chronic-SHIV-infection of four macaques did not have measurable increases in IFNγ-producing T-cells in PBL (Ali et al 2009). In addition, we did not detect increased levels of IL-17- or IL-22-producing T-cells during acute- and chronic-SHIV-infection. Control data was obtained using CD28 and CD49d mAbs (without antigen restimulation) as described above.

9. Determination of plasma cytokine levels

Cytokine levels were determined using Monkey 5-plex Cytokine Kit (Invitrogen), read on a Bio-Rad Bio-Plex machine (Bio-Rad, Hercules, CA), and analyzed using Bio-Plex Manager 5.0 software (Bio-Rad) according to manufacturers' instructions.

10. Enzyme linked immunosorbent assay (ELISA)

ELISA was performed as previously described (Ali et al 2009). Plasma was heat inactivated for 30 min at 56°C before analysis. MSP-1 (19kDa fragment) antigen was obtained through Malaria Research and Reference Reagent Resource center (MRA-47), deposited by DC Kaslow. Antibody was

detected using anti-monkey IgG and IgM antibodies (KPL, Gaithersburg, MA) and OD_{405} was determined on an ELISA plate reader (Bio-rad model 550).

11. Statistical analysis

Statistical analysis was done using paired or unpaired two-tailed Student's *t* test (Shen et al 2002) using Graphpad software (Prism, La Jolla, CA). Similar trends were seen using non-parametric analysis. Data compared were based on percentage, unless otherwise stated.

C. *Listeria monocytogenes* studies

1. Animals

Five Chinese-origin rhesus macaques, ages 5-11, were used in this study (**Table II**). All animals were housed and used in accordance with the guidelines of the IACUC under an approved protocol. Two macaques were vaccinated at day 0 with 10^8 CFU attenuated recombinant *L. monocytogenes* i.m. and boosted on day 35 with 10^8 CFU i.v. Three macaques were vaccinated at day 0 with 10^6 CFU i.v. and boosted on day 51 with 10^8 CFU i.v. Day 0 blood was drawn immediately before vaccination. Rectal biopsies and BAL fluid were collected as previously described (Ali et al 2007). Briefly, for rectal biopsies, animals were restrained in ventral recumbency with the pelvis elevated 4-5 inches. Four or five pellets were collected using 2x3mm forceps. For BALF collection, a pediatric feeding tube was inserted into the trachea to the level of the carina. Ten mL of saline was instilled and immediately withdrawn and repeated to collect 12-15mL BALF. Animals were anesthetized with 10mg/kg Ketamine HCL (Fort Dodge Animal Health) prior to all procedures.

Table III

Group	Primary Infection	Secondary Infection
1	10^8 CFU rLM i.m. D0	10^8 CFU rLM i.v. D35
2	10^6 CFU rLM i.v. D0	10^8 CFU rLM i.v. D51

2. *L. monocytogenes* strains and infection

L. monocytogenes strain NF-L974 was obtained from Nancy Freitag. Attenuated bacteria were stored in aliquots at -80°C in 20% glycerol (Fisher Chemical, Fairlawn, NJ). Bacteria were thawed, washed 2x with Brain Heart Infusion broth (BD, Franklin Lakes, NJ), and resuspended in 1ml sterile PBS (Gibco, Invitrogen, Carlsbad, CA) for injection.

3. Isolation of lymphocytes from peripheral blood, BAL fluid, and rectal mucosae

PBL were isolated from freshly collected EDTA blood by Ficoll-Paque Plus (Amersham, Piscataway, NJ) density gradient centrifugation before analysis. Lymphocytes from BAL fluid and rectal mucosae were isolated as previously described (Ali et al 2007).

4. Immunofluorescent staining and flow cytometric analysis

Cell staining was performed as previously described (Ali et al 2009). For cell-surface staining, PBL were stained with up to 5 Abs (conjugated to FITC, PE, allophycocyanin, pacific blue, and PE-Cy7) for 15min. After staining, cells were fixed with 2% formaldehyde-PBS prior to analysis on a CyAn ADP flow cytometer (DakoCytomation, Carpinteria, CA). Lymphocytes were gated based on forward- and side-scatters, and pulse-width and at least 40,000 gated events were generally analyzed using Summit Data Acquisition and Analysis Software (DakoCytomation).

The following mouse Abs were used: IL-17 (eBio64CAP17) [eBioscience, San Diego, CA]; Streptavidin-Pacific Blue [Invitrogen]; Vγ9 (7A5), Vδ2 (15D) [Pierce}; goat-anti-mouse IgG (H+L)PE [Beckman Coulter, Brea, CA]; CD3 (SP34-2), IFNγ (4S.B3), IL-4 (8D4-8), TNFα (MAb11) [BD Pharmingen, San Diego CA]; perforin (Pf-344) [Mabtech, Mariemont, OH].

5. Intracellular cytokine staining

Intracellular cytokine staining (ICS) was performed as previously described (Ali et al 2009). Briefly, 0.5×10^6 PBL plus mAbs CD28 (1μg/ml) and CD49d (1μg/ml) were incubated with or without 50ng/mL HMBPP in 100μl final volume for 1h at 37°C, 5% CO_2 followed by an additional 5h incubation in the presence of brefeldin A (GolgiPlug, BD). After staining for cell-surface markers (CD3, Vγ2) for 15 min, cells were permeabilized for 45 min (Cytofix/cytoperm, BD) and stained 45 min for IFNγ, IL-4, IL-17, perforin, and TNFα before fixation in formalin.

6. Cytotoxicity assay

PBL were incubated 3 hours at 37°C in a 10ml flask. Non-adherent cells (lymphocytes) were separated from adherent cells (monocytes). Adherent cells were released from the flask using ice cold PBS (Gibco), then incubated with 50ng/ml GM-CSF (Sigma, St. Louis, MO) and 20ng/ml IL-4 (Sigma) for three days at 37°C to differentiate into DCs. DCs were then infected with recombinant attenuated *L. monocytogenes* at an MOI of 5. The cells were treated with 30μg/ml gentamycin sulfate (Gibco) after two hours to kill non-internalized bacteria, then incubated an additional two days. Non-adherent cells were isolated into Vγ2+ cells and CD20+ B cells (eBioscience, clone 2H7) using MACS (Miltenyi Biotech, Auburn, CA). Effector and Target cells were incubated at a ratio of 5:1 for 14-16 hours, then stained with CD11c (clone 3.9, BioLegend, San Diego, CA) and propidium iodide (Invitrogen) and analyzed by flow cytometry.

7. Statistical analysis

Statistical analysis was done using paired two-tailed Student's *t* test (Shen et al 2002) using Graphpad software (Prism, La Jolla, CA).

CHAPTER 3- Virus Infection Stages and Distinct Th1 or Th17/Th22 T-cell Responses in Malaria/SHIV-Coinfection Correlate with Different Outcomes of Disease

A. Introduction

Worldwide, there are approximately 300-500 million malaria infections annually, causing 1-3 million deaths (http://apps.who.int/malaria). The most clinically relevant human malaria parasite is *P. falciparum*, which causes severe anemia and cerebral malaria (Miller, Good, Milon 1994). In addition to the burden of malaria, there are over 30 million people infected with HIV worldwide (http://www.who.int/hiv/data/en). Because both diseases are highly prevalent in overlapping geographic areas, there is a high probability of coinfection (Slutsker et al 2007), and mathematical modeling has demonstrated that disease interaction during coinfection has increased the spread of both HIV and malaria in Sub-Saharan Africa (Abu-Raddad, Patnaik, Kublin 2006).

Studies of the mutual impacts of HIV and malaria have yielded conflicting results. Early human studies failed to show a correlation between HIV infection and enhanced malaria disease (Butcher 2005). More recently, vigorous analyses of both HIV status (i.e. viremia, CD4 count) and malaria status (i.e. fever, parasite density) have shown mutual impact on both diseases. HIV viral load can increase by approximately 1 log during malaria coinfection, but then returns to normal after treatment (Hoffman et al 1999; Kublin et al 2005). Human cohort studies have demonstrated an increased risk of contracting severe malaria in HIV infected patients (Cohen et al 2005; Grimwade et al 2004; Kublin et al 2005; Patnaik et al 2005). However, different groups reported discrepant effects of HIV infection on parasite levels. Several studies have found no correlation between HIV status and parasitemia (French et al 2001; Grimwade et al 2004; Patnaik et al 2005), while others have shown that lower CD4+ T cell levels correlate with higher parasitemia (Laufer et al 2006; Whitworth et al 2000). These different findings are possibly due to the differences in malaria transmission and patient care between various geographic

locations. In order to properly compare data between various human studies, one must evaluate or control the rate of malaria and HIV transmission, the stage of HIV infection, and the age and immune status of the patient. All of these variables could be adequately controlled for by utilizing an animal model of coinfection. Importantly, in-depth mechanistic studies of HIV/malaria coinfection have not been done. In this context, immune regulation or immunity against the coinfection remains largely unknown, and studies cannot be done in small lab animals due to their resistance to HIV/AIDS. Utilizing nonhuman primate models of malaria and AIDS-virus coinfection is therefore of central importance for circumventing the limitations of human studies, and for enhancing our understanding of malaria-HIV interaction and disease pathogenesis.

In the current studies, we utilized our decades-long SIV/mycobacterium-coinfection expertise and utilized *P. fragile* and SHIV89.6P to develop models of malaria/acute-SHIV-coinfection and malaria/chronic-SHIV-coinfection in Chinese-origin rhesus macaques. *P. fragile* causes malaria pathologically similar to *P. falciparum* in macaques (Collins et al 2006; Fujioka et al 1994), whereas SHIV89.6P resembles HIV by infecting CD4+ T-cells and macrophages and inducing simian-AIDS as HIV does in humans (Ali et al 2009). These features suggest that co-infection with *P. fragile* and SHIV is relevant to co-infection with *P. falciparum* and HIV. In these proof-of-concept studies, SHIV89.6P was used to optimally determine clinical outcome during highly pathogenic AIDS-virus coinfection, as Chinese rhesus are somewhat resistant to SIVmac. The nonhuman primate models of malaria and AIDS-virus coinfection allowed us to illustrate the reciprocal effects of malaria and AIDS-virus infections and potential immune mechanisms underlying coinfection-enhanced viral pathogenicity/malaria disease and immunity. We made novel observations that virus infection status and distinct Th1 or Th17/IL-22+ immune responses after malaria-coinfection of acutely- or chronically-SHIV-infected macaques correlate with different clinical outcomes of virus infection and virus-associated malaria.

B. Results

1. Malaria-coinfection of acutely-SHIV-infected macaques led to fatal virus-associated malaria

We presumed that mutual enhancement of malaria and HIV/AIDS could be best demonstrated in naïve individuals who have not developed anti-malaria or anti-HIV immune responses. Thus, a group of naïve macaques was coinfected simultaneously with malaria and SHIV89.6P (malaria/acute-SHIV). Additionally, chronically-SHIV-infected macaques were similarly coinfected with malaria (malaria/chronic-SHIV). As controls, a group of macaques was infected with malaria-only, and another with SHIV-only. The malaria-only group developed uncomplicated malaria, manifesting transient low hematocrits, and low (<5.26%) but measurable parasitemia starting from day 19 and fluctuating through day 54 (**Figure 1a**). In contrast, malaria/acute-SHIV-coinfected macaques developed fatal malaria, exhibiting sharp increases in parasitemia to >70% at day 20 (**Figure 1a**, p=0.0001, D19, between groups). All acutely-coinfected macaques manifested severe anemia (pale mucosae and **Figure 1b**), shivering, and lethargy/coma-like signs 2-3 weeks post-coinfection, and received chloroquine treatment. Despite treatment, three (7282, 7289, 7344) of these coinfected macaques became moribund and were euthanized by D22 post-coinfection (**Figure 1c**). The fourth (7284) recovered from life-threatening malaria after chloroquine treatment. These results demonstrated profound impact of SHIV on malaria, as malaria/acute-SHIV-coinfection led to high-level parasitemia and rapidly progressed to fatal virus-associated malaria. The data also indicate that an active, progressive malaria/AIDS-virus coinfection model can be developed by simultaneous SHIV and *P. fragile* coinfection of macaques.

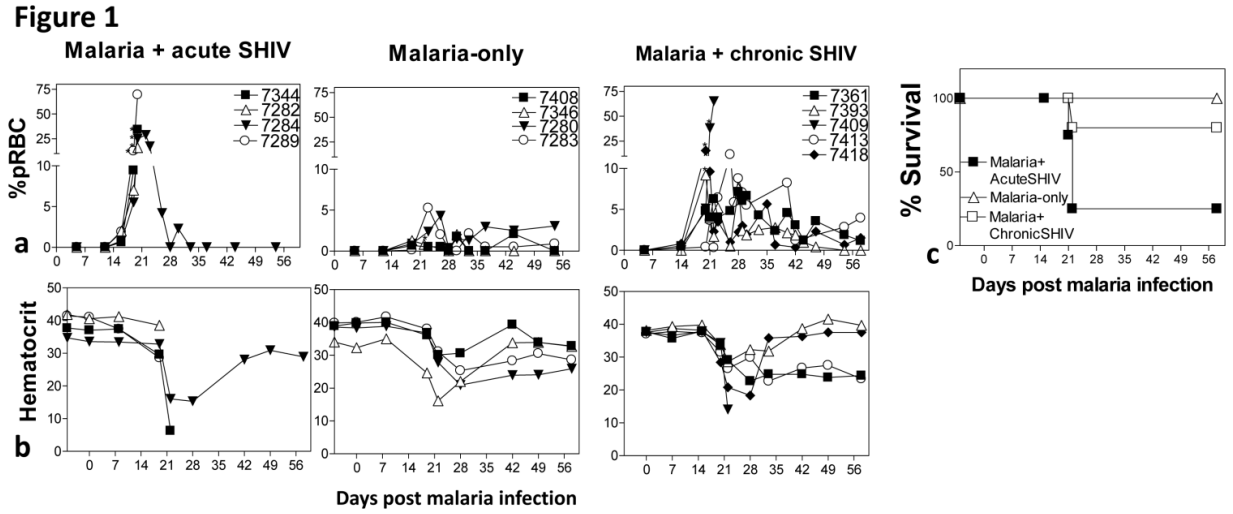


Figure 1. Malaria-coinfection of acutely-SHIV-infected macaques led to fatal virus-associated malaria; chronically-SHIV-infected macaques developed moderately-enhanced parasitemia without fatal outcome after malaria-coinfection. **1a.** Malaria/acute-SHIV-coinfected macaques (left) had significantly higher parasite levels than malaria-only controls (center, p=0.0004 at Day 20). All acutely-SHIV-infected macaques developed life-threatening malaria, and all but one (7284) became moribund despite receiving chloroquine treatment. Malaria/chronic-SHIV-coinfected macaques without AIDS (right) had higher parasitemia than malaria-only controls (p=0.0245 at Day 20), but much lower parasitemia than malaria/acute-SHIV-coinfected macaques (p=0.0034). * indicates chloroquine treatment. **1b.** Hematocrit data for macaques as indicated on the top of the panels. Note that the macaques with fatal malaria had extremely low levels of hematocrit despite chloroquine treatment. All moribund macaques had clinical signs of severe anemia although some were not tested for hematocrit at endpoints. **1c.** Survival curves for the malaria/acute-SHIV, malaria-only, and malaria/chronic-SHIV groups. Only one macaque (7284) in the malaria/acute-SHIV group survived from life-threatening malaria after chloroquine treatment. All macaques without AIDS in the malaria/chronic-SHIV group survived, with two (7393 and 7418) treated with chloroquine at day 20. The single macaque with prior AIDS (7409) in the malaria/chronic-SHIV group did not survive despite chloroquine treatment at day 19, and was euthanized on day 22.

2. Malaria-coinfection of acutely-SHIV-infected macaques appeared to accelerate SHIV disease

We then sought to determine whether malaria-coinfection could reciprocally enhance pathogenicity and accelerate SHIV disease. Acutely-coinfected macaques appeared to have faster progressing SHIV disease than SHIV-only controls (**Figure 2-3**). Remarkable lymphoid destruction/depletion was found in both lymph nodes and spleen tissues collected at necropsy from malaria/acute-SHIV-coinfected macaques 21 days post-coinfection (**Figure 2a-b**). No or very few lymphocytes were detectable in T-cell zones of lymph nodes, with apparent disappearance of germinal centers and T-cell zones. Lymphoid structures were destroyed with notable necrosis in the lymph nodes (**Figure 2a**). Similarly, lymphoid destruction/depletion and necrosis were seen in spleen tissues (**Figure 2b**). In contrast, such profound destruction/depletion and necrosis in lymphoid tissues were not seen in lymph nodes and spleens from SHIV-only control macaques in acute-SHIV infection or 1.5 years after SHIV infection (**Figure 2a-b**). More apparent lymphoid depletion in rectal mucosa was detected 21 days post-malaria/acute-SHIV-coinfection compared to SHIV-only controls (**Figure 2c**). Blood CD4+ T-cell counts sharply declined to 54.9±66.2/μl 19 days post-coinfection, whereas the SHIV-only group had CD4+ T-cell counts 362.67±217.78/μl at day 21-22 (**Figure 3**, $p=0.0154$). The acutely-coinfected macaques exhibited high-level plasma SHIV mRNA (**Figure 3**). Due to early chloroquine treatment and deaths of the macaques, a full comparison of peak viremia and set-points between the acute-coinfected and SHIV-only groups could not be done. Despite this, it was surprising that profound destruction/depletion and necrosis in lymphoid tissues with sharp decline of blood CD4+ T-cell counts occurred in acutely-coinfected macaques, as such severe pathogenic events would normally not be detected until very end-stage AIDS in humans and nonhuman primates (Shimada et al 2003; van Grevenynghe et al 2008). These results suggested that malaria-coinfection remarkably enhanced acute-SHIV disease.

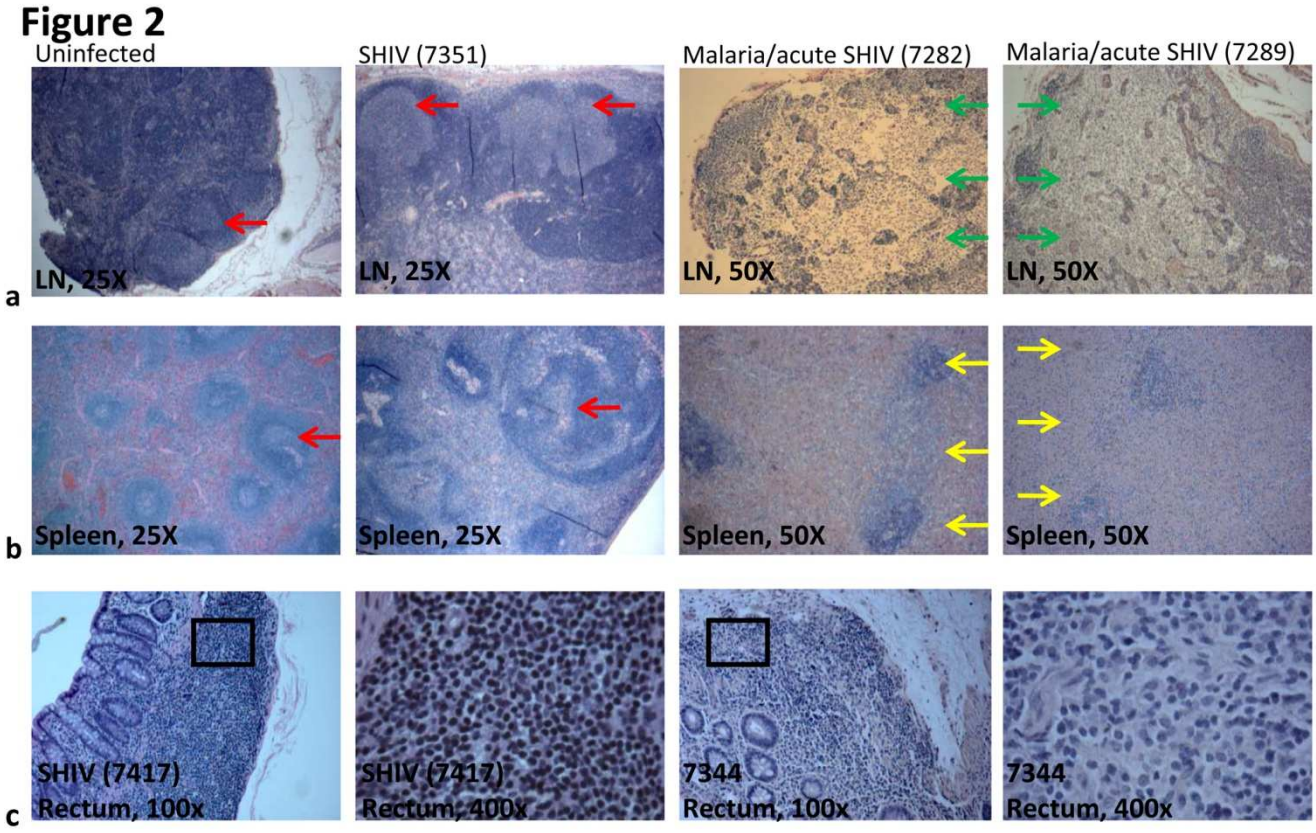


Figure 2. Malaria-coinfection of acutely-SHIV infected animals resulted in severe and rapid lymphoid depletion in the lymph nodes, spleen, and rectal mucosa. **2a-b.** Histopathology images indicating lymphoid depletion/destruction in lymph nodes (top, 50x magnification) and spleens (bottom, 50x) of two representative macaques (7282, 7289) in the malaria/acutely-SHIV-coinfected group. Note the destruction of lymphoid structures, the disappearance of both germinal centers and T-cell zones, and apparent necrosis in lymph nodes and spleens collected at necropsy at day 21 after the coinfection. Two sets of control images (25x) are shown on the left. The first set from a representative uninfected macaque indicated normal structures of T-cell zones/germinal centers in the lymph node and white/red pulps in the spleen. The other set from SHIV-only macaque (7351, necropsied on day 627 post SHIV) showed enlarged germinal centers, but no lymphoid destruction or necrosis. Similar finding was also seen in acute SHIV-only controls. Of note, acute SHIV-infection of macaques did not cause lymphoid destruction, necrosis or disappearances of germinal centers despite the minor decreases in cell density in T-cell zones and subtle paracortical hyperplasia were seen in lymph nodes collected one month after SHIV infection(data not shown). All were formalin-fixed sections stained by hematoxylin and eosin (HE). Red arrows indicate germinal centers; green arrows indicate necrotic regions without germinal centers/T-cell zones or normal structures; and yellow arrows indicate depleted germinal centers and necrosis in spleen. **2c.** Histopathology images of rectal mucosa collected at day 21 after SHIV from representative SHIV infected control (left two panels) and malaria/acute-SHIV-coinfected macaques (right two panels). Malaria/acute-SHIV-coinfected macaques shows decreased densities of lymphoid cells in gut mucosa overall, with fewer lymphocytes seen (400x).

Figure 3

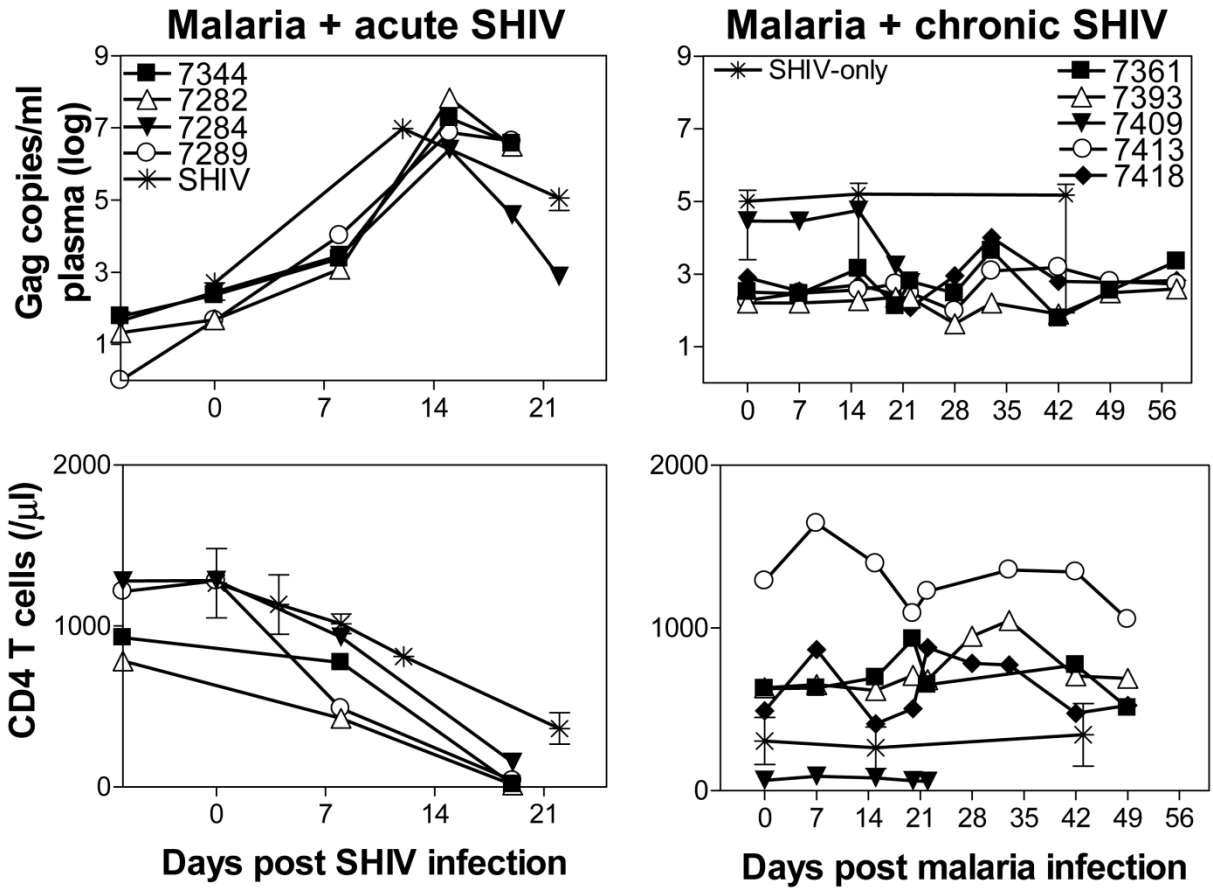


Figure 3. Malaria-coinfection of acutely-SHIV-infected macaques enhanced SHIV disease; malaria-coinfection of chronically-SHIV-infected macaques only induced a transient increase in viral loads with stable CD4+ T-cell counts. (Top) Plasma SHIV RNA levels detected in individual malaria/acute-SHIV macaques (left, n=4), individual malaria/chronic-SHIV macaques (n=5), and the SHIV-only group (means ±SD, n=6). (Bottom) Numbers of CD4+ T cells in malaria/acute-SHIV and malaria/chronic-SHIV macaques, and the SHIV-only group (means± SD, n=6). Data for the chronic SHIV-only group were derived from a 6-week period of chronic infection. The transient ~1 log increase in SHIV during the chronic SHIV co-infection was not statistically significant when compared to baseline. Absolute CD4+ T-cell counts in malaria/acute-SHIV-coinfected macaques appeared to reach a lower level than those in SHIV-only controls. p=0.0154 day 19 malaria/acute-SHIV vs day 22 SHIV-only (a progressive decline of CD4 T-cell counts usually is most striking at weeks 3-5 after SHIV89.6P). No significant declines of CD4+ T-cell counts in malaria/chronic-SHIV-coinfected macaques were noted.

3. Malaria-coinfection during chronic-SHIV-infection caused moderately-enhanced malaria, with stable CD4 counts and transiently increased viral loads.

Next, we sought to determine whether malaria coinfection of chronically-SHIV-infected macaques led to similar or different clinical outcomes of SHIV disease and malaria compared to malaria/acute-SHIV-coinfection. Chronically-SHIV-infected macaques without AIDS did not develop clinical signs of fatal malaria or accelerated SHIV disease after malaria-coinfection. While macaque #7409 with AIDS (CD4+ count <200/μl) exhibited rapidly-increased parasitemia, and deteriorated to moribund status despite chloroquine treatment (**Figure 1a-c**), other coinfected macaques remained clinically stable, despite exhibiting moderately-increased malaria parasitemia (up to 15% pRBC) compared to malaria-only controls (**Figure 1a, 1c**).

The malaria impact on chronic-SHIV-coinfection was much less dramatic than on acute-SHIV-coinfection. Malaria-coinfection of chronically-SHIV-infected macaques led to transient ~1 log increase in plasma viral RNA (**Figure 3**). Malaria/chronic-SHIV-coinfected monkeys did not have significant changes in CD4+ T-cell counts (**Figure 3**). These results demonstrated that malaria-coinfection in chronically-SHIV-infected macaques without AIDS led to uncomplicated, moderately-enhanced malaria disease, with stable CD4 counts and transient/subtle increases in viral loads.

4. Hyper-activation and expansion of Th1 effector cells and high-level proinflammatory cytokines during malaria/acute-SHIV-coinfection coincided with accelerated SHIV-disease and fatal AIDS-associated malaria.

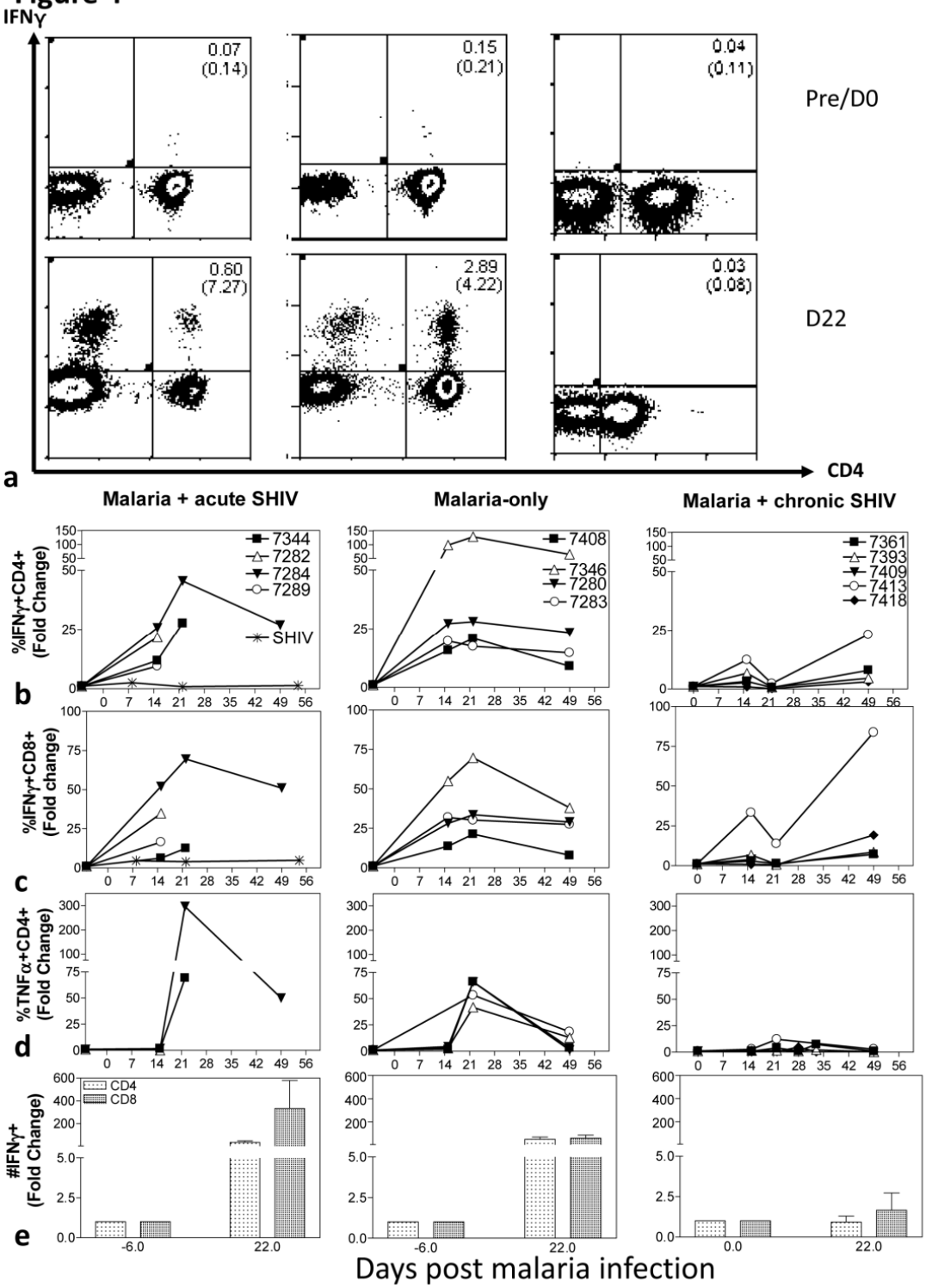
We then undertook mechanistic studies to determine if different host factors or immune responses after malaria/SHIV-coinfection correlated with different outcomes of disease. This compelling question has not been addressed despite decade-long studies of malaria/HIV-coinfection. We first

explored whether AIDS-related fatal malaria resulted from SHIV-mediated inhibition of anti-malaria T-cell responses or parasite-mediated hyper-proinflammatory responses in malaria/acute-SHIV-coinfection. We employed our novel methods for detecting activated/differentiated T-cells capable of *de novo* production of anti-microbial cytokines without *in vitro* antigen re-stimulation (**Figure 4a** and (Yao et al 2010)). We focused on CD4+ and CD8+ T effector cells producing IFNγ, TNFα or IL-17/IL-22, as IFNγ or Th1/Th17 cells may have a role in both malaria protection and pathology (Good et al 2005; Stephens et al 2005), and TNFα is likely involved in proinflammatory responses during malaria (Karunaweera et al 1992). Malaria-only controls showed remarkable increases in IFNγ-producing CD4+ and CD8+ T effectors, which emerged at week 2, peaked at week 3 and sustained at least through week 7 after infection (**Figure 4a-e,** see p values in legend). TNFα-producing CD4+PBL were also increased (**Figure 4d**).

Interestingly, malaria/acute-SHIV-coinfected macaques exhibited hyper-activation and expansion of Th1 cytokine-producing T effectors, rather than suppression. Malaria/acute-SHIV-coinfected macaques with fatal malaria and profound AIDS showed 50-70-fold increases in CD4+ and CD8+ T effectors producing IFNγ *de novo* (**Figure 4a-e**, see p values in legend), and up to 300-fold increases in TNFα-producing T effectors (**Figure 4d**). It was noteworthy that despite a marked loss of CD4+ T-cells (**Figure 3**), increased IFNγ- and TNFα-producing CD4+ T effectors were detected in malaria/acute-SHIV-coinfected macaques (**Figure 4a-e**). In contrast, SHIV-only controls did not show any detectable increases in the number of IFNγ-producing CD4+ or CD8+ T effector cells during acute and chronic stages of SHIV infection, as no or very few IFNγ-producing CD4+ or CD8+ T cells were detected by direct ICS without in vitro Ag re-stimulation before and after SHIV infection (**Figure 4b-d**, and (Ali et al 2009). This suggested that marked increases in CD4+ and CD8+ T effectors were driven by malaria.

The remarkable increases in Th1 cells were consistent with high plasma levels of Th1 cytokine IFNγ but not Th2 cytokine IL-4 in malaria/acute-SHIV-coinfected macaques (**Figure 5**). Interestingly, IFNγ levels in plasma of malaria/acute-SHIV-coinfected macaques were higher than those of the malaria-only controls (**Figure 5a**), although the frequencies of increased Th1 cells were comparable (**Figure 4**). These results suggested that hyper-activation/expansion of Th1 cytokine-producing CD4+ and CD8+ T effectors and high-level plasma Th1 proinflammatory cytokines during malaria/acute-SHIV-coinfection correlated with accelerated SHIV disease and fatal AIDS-associated malaria. We also noted small increases of IL-17-producing CD4+ T effectors, but not IL-22-producing CD4+ T effectors (**Figure 6**). Of note, no or very few IL-22+ and IL-17+ T effectors were detected in SHIV-only controls during acute and chronic phase infections (**Figure 6a** and (Yao et al 2010)). The marked increases in IFNγ+CD4+ and CD8+ T effectors, but small changes in IL-17+ and IL-22+ T effectors, were associated with enhanced CD4+ T-cell depletion and lymphoid destruction/necrosis (**Figure 7),** suggesting malaria-activated CD4+ T-cells served as productive SHIV reservoirs leading to accelerated SHIV/AIDS. Reciprocally, increased Th1 effector cells also coincided with SHIV-associated increases in parasitemia in malaria/acute-SHIV-coinfected macaques (**Figure 7**), implicating a role of these effector cells in development of fatal virus-associated malaria.

Figure 4


IFNγ
0.07
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Malaria + chronic SHIV
%IFNγ+CD4+ (Fold Change)
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7409
7413
7418
b
%IFNγ+CD8+ (Fold change)
c
%TNFα+CD4+ (Fold Change)
d
#IFNγ+ (Fold Change)
CD4
CD8
e
Days post malaria infection

Figure 4. Hyper-activation/expansion of Th1 effector cells during malaria/acute-SHIV-coinfection coincided with accelerated SHIV disease and fatal AIDS-associated malaria; suppressed Th1 T effector function associated with uncomplicated malaria and 'stabilized' SHIV infection in malaria/chronic-SHIV-coinfection. **4a.** Representative histograms showing increases in CD4+ and CD4-(CD8+) T effector cells producing IFNγ at day 22 post infection in malaria/acute-SHIV-coinfected macaque (left) and malaria-only control (center), but not malaria/chronic-SHIV-coinfected macaque (right). Data were gated on CD3. Numbers in upper right and brackets were percentages of CD3+ T-cells and of CD4+ T-cells, respectively. **4b.** Hyper-activation/expansion of IFNγ+CD4+ T-effector cells in malaria/acute-SHIV-coinfected macaques (left; coinfected, p=0.0096, baseline vs. day 15, n=4; p=0.0665, baseline vs. day 22, n=2) and malaria-only controls (center, n=4, p<0.0001, baseline vs. day 15; p=0.0018, baseline vs. day 22). Almost no CD4+ T effectors were detected in malaria/chronic-SHIV-coinfected macaque (right, p=0.0008 compared to malaria/acute-SHIV group, and p=0.0004 compared to malaria-only group at day 22, n=5). SHIV-only controls had few or no IFNγ+ CD4+ T effector cells measured by ICS without Ag stimulation (n=6, p=0.0001 at day 22 compared to malaria/acute-SHIV-coinfected group). Data were fold increase % IFNγ+ cells among CD4+ T-cells. **4c.** Hyper-activation/expansion of IFNγ+CD8+ T effectors (CD8 Ab used for ICS) in malaria/acute-SHIV-coinfected and malaria-only macaques (p=0.0099 for malaria/acute-SHIV-coinfected macaques, baseline vs day 15, n=4; p<0.0001 for malaria-only infected macaques, baseline vs. day 15, n=2). Much lower increases of CD8+ T effectors were detected in malaria/chronic-SHIV-coinfected macaques (right, p=0.0002 compared to malaria/acute-SHIV group, and p =0.0015 compared to malaria-only group at day 22). SHIV-only controls (n=6) showed very few or no IFNγ+ CD8+ T effector cells (p=0.0004 at day 22, compared to malaria/acute-SHIV-coinfected group). **4d.** Hyper-activation/expansion of TNFα+CD4+ T effectors in malaria/acute-SHIV-coinfected and malaria-only macaques (p=0.0100, baseline vs. day 22; malaria/acute-SHIV n=2 at day22, malaria-only n=4). However, few TNFα+CD4+ T effectors were detected in malaria/chronic-SHIV (n=5 at day 22) group (right, p=0.005 compared to malaria/acute-SHIV group, and p=0.0030 compared to malaria-only group at D22). **4e.** The absolute number of IFNγ+CD4+ and IFNγ+CD8+ T effectors increased 50-100-fold and 100-300 fold, respectively, in malaria/acute-SHIV coinfected macaques and malaria-only infected macaques by day 21. The fold-increase in malaria/chronic-SHIV coinfected macaques was much less significant.

Figure 5

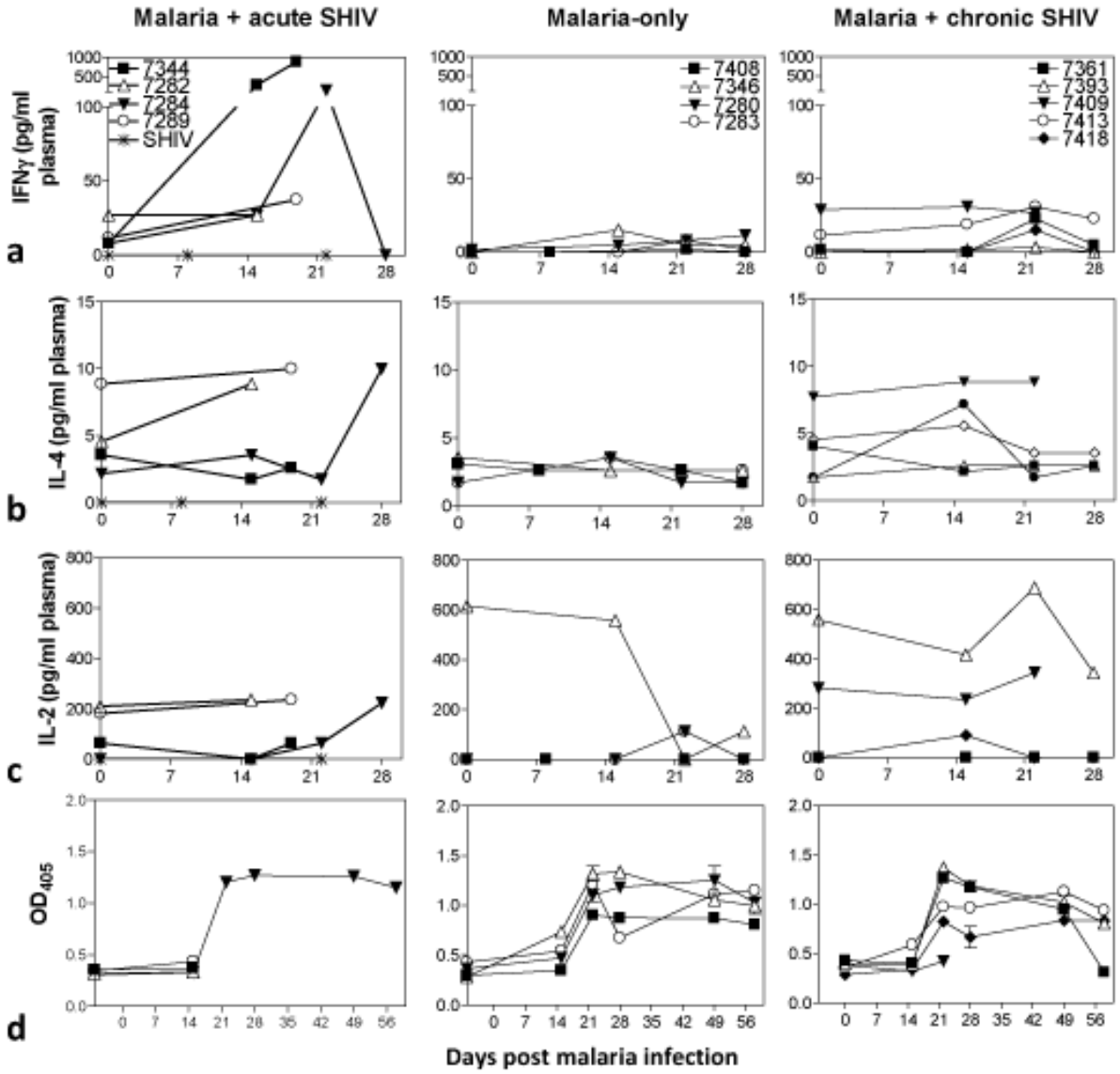
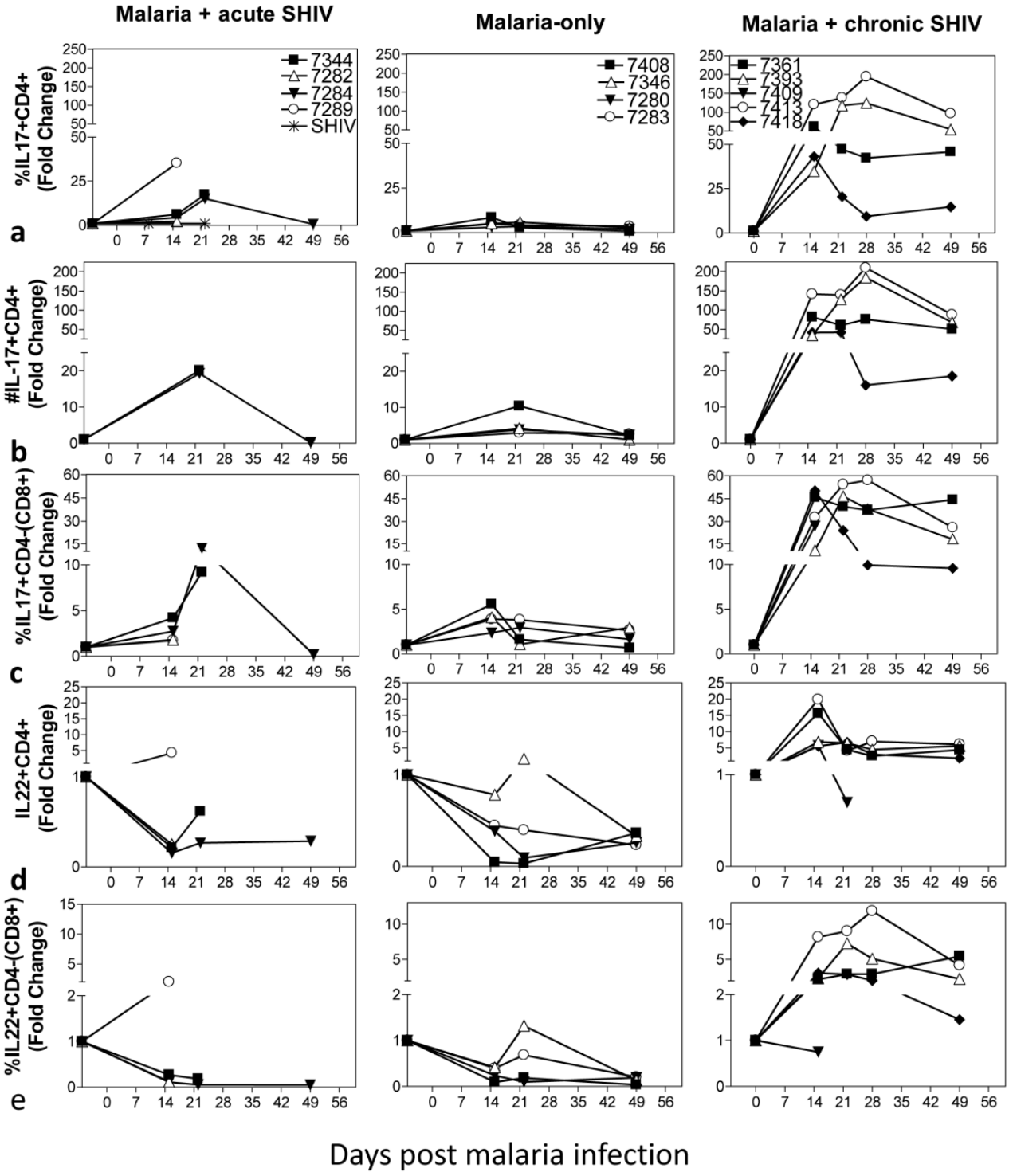


Figure 5. Malaria induced high-level Th1 proinflammatory cytokines in acute-SHIV-coinfection, whereas Th1 cytokine responses were suppressed in chronic-SHIV-coinfection. **5a.** Plasma IFNγ increased in malaria/acute-SHIV-coinfected macaques (left), but not malaria-only controls (middle) or malaria/chronic-SHIV-coinfected macaques (right). IFNγ was not detectable in acute SHIV-only controls. **5b.** There was no increase in plasma IL-4 in malaria/acute-SHIV-coinfected macaques except the chloroquine-treated survivor (left) or malaria-only controls (middle) or malaria/chronic-SHIV-coinfected macaques (right). **5c.** Overall, there was no significant increase in plasma IL-2 in malaria/acute-SHIV (left), malaria-only (middle), or malaria/chronic-SHIV groups of macaques (right) detected. **5d.** There was no significant difference in the development of malaria–specific Ab responses between malaria/acute-SHIV (left), malaria-only (middle), and malaria/chronic-SHIV groups of macaques (right). Shown were the data of MSP-1-specific IgG and IgM Ab levels (OD values) derived from ELISA using anti-monkey IgG and IgM as second Ab. A lack of differences in total Abs between groups suggests that humoral immune responses did not appear to correlate with clinical outcomes of malaria coinfection.

Figure 6


Malaria + acute SHIV
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Malaria + chronic SHIV
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7409
7413
7418
%IL17+CD4+ (Fold Change)
a
#IL-17+CD4+ (Fold Change)
b
%IL17+CD4-(CD8+) (Fold Change)
c
IL22+CD4+ (Fold Change)
d
%IL22+CD4-(CD8+) (Fold Change)
e
Days post malaria infection

Figure 6. Remarkable expansion of Th17 cells and unique increases in IL-22+ T effector cells coincided with the absence of fatal malaria and enhanced SHIV disease in malaria/chronic-SHIV-coinfection. **6a.** IL-17-producing CD4+ T effector cells increased remarkably up to 200 folds during malaria/chronic-SHIV-coinfection (right; p=0.0167 at D15, n=6; p=0.0021 at D22, n=5), and the magnitude was much greater than that in malaria-only group (p=0.0181 at day 15; p=0.0173 at day 22, using fold-change data) or malaria/acute-SHIV group (p = 0.0294 at day 15; p=0.0757 at day 22, using fold-change data). SHIV-only infected animals did not show any detectable IL-17- or IL-22-producing T-cells during the acute and chronic SHIV infection(Yao et al 2010, and data not shown). **6b.** The absolute number of IL-17+CD4+ T cells increased less than 25-fold in malaria/acute-SHIV coinfected macaques and malaria-only infected macaques. In contrast, the absolute number increased up to 200-fold in malaria/chronic-SHIV coinfected macaques. **6c**. Malaria/chronic-SHIV-coinfection macaques also developed marked increases in IL-17-producing CD3+CD4-(CD8+) T effectors (p=0.0181, baseline vs day 15, n=5; p=0.0081 baseline vs day 22, n=5), and the expansion magnitude was significantly greater than that of malaria-only group (p=0.0218 at day 15, p=0.0338 at day 22, using fold change data) and malaria/acute-SHIV-coinfection (p=0.0440 at day 15; p=0.1990 at day 22, using fold change data). **6d.** IL-22-producing CD4+ T-cells are increased significantly up to 22 folds from baseline after malaria infection of chronically-SHIV infected macaques (p=0.0038 at D15, n=5; p= 0.035 at D22, n=5). In contrast, the frequency of IL-22-producing CD4+ T effector cells actually decreased after malaria infection of acutely-SHIV-infected or naïve macaques. Baseline IL-22-producing T effector cells were low in chronic-SHIV-infected macaques, a finding similarly seen in SIV-infected macaques (Raffatellu,M. et al 2008). No detectable IL-22-producing T-cells during the chronic SHIV-only infection without malaria coinfection (Yao et al 2010, and data not shown). **6e.** IL-22-producing CD3+CD4-(CD8+) T-cells increased during malaria infection of chronically-SHIV-infected macaques (p=0.0260 at D15, n=5; p=0.0097 at D22, n=5). Conversely, IL-22-producing CD3+CD4-(CD8+) T effector cells did not significantly increase during malaria/acute-SHIV-coinfection, and actually decreased during malaria-only infection (p=0.0323 at D15, n=4). Similar trends of fold-changes and p values were seen by comparative analyses of absolute numbers of T effectors.

Figure 7

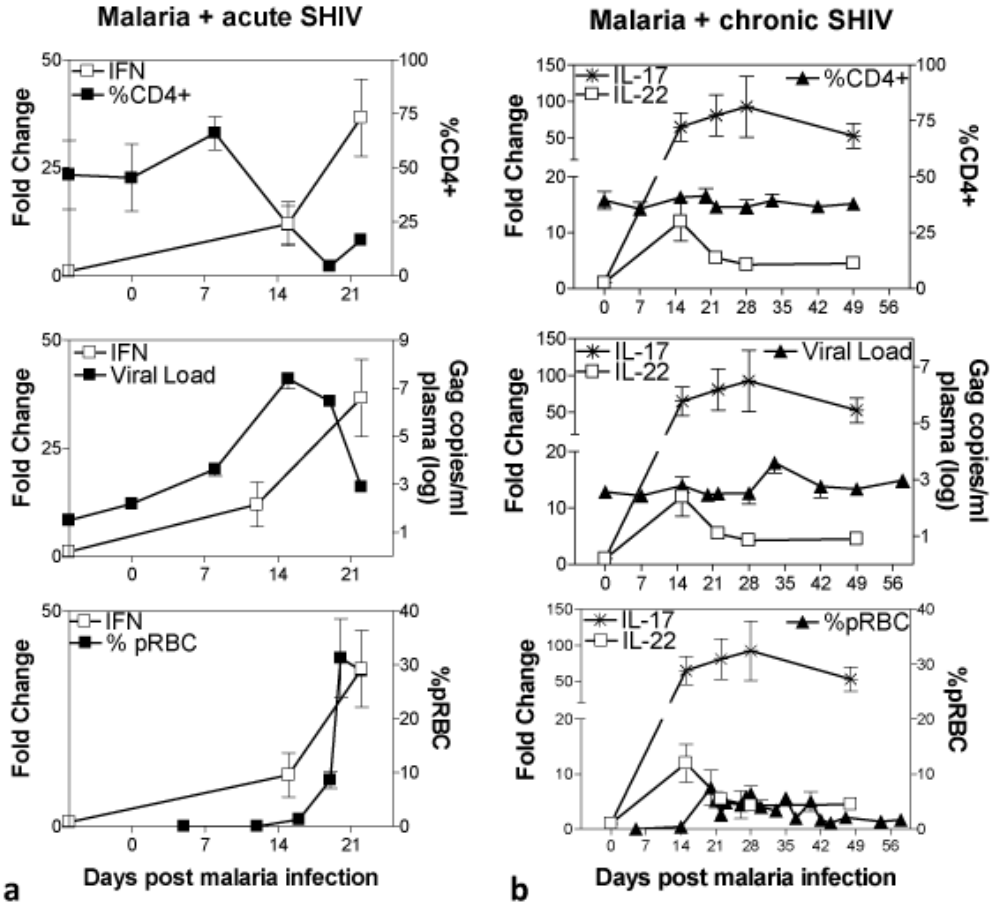


Figure 7. Over-reactive Th1 responses coincided with progressive SHIV disease(lymphoid depletion/destruction/necrosis) and fatal AIDS-associated malaria, whereas marked expansion of Th17 cells and unique increases in IL-22+ T-effector cells with Th1 suppression correlated with uncomplicated malaria and stable SHIV infection. **7a.** Spiking IFNγ-producing CD4+ T-cells representing hyper-activation of T-cells correlated with CD4+ T-cell depletion (top), bursting SHIV viral replication (middle), and high parasitemia (bottom) in malaria/acute-SHIV-coinfected macaques. Increases in SHIV virus loads were seen earlier than those in detectable expansion of CD4+ T effector cells. This might be due to the fact that virus replication was enhanced dramatically upon cellular activation and that CD4+ T cells underwent activation events before they proliferated to a detectable expansion. Note that at week 3 after malaria-coinfection of acutely-SHIV-infected macaques, lymphoid depletion and destruction/necrosis occurred (Fig.2). **7b.** High frequency Th17 cells and IL-22-producing CD4+ T effector cells correlated with stable CD4+ T-cell counts (top), subtle change in plasma SHIV RNA (middle), and moderate parasitemia in malaria/chronic-SHIV-coinfected macaques. No or few Th1 cells also coincided with the uncomplicated SHIV/malaria diseases (Fig.3).

5. Remarkable expansion of IL-17+ T effectors, unique increases in IL-22+ T effectors and suppression of over-reacting Th1 effectors coincided with stable SHIV infection and absence of fatal malaria during malaria/chronic-SHIV-coinfection.

Since malaria-coinfection of chronic-SHIV-infected macaques without AIDS did not lead to fatal malaria or rapid progression of SHIV disease, we examined if different immune responses correlated with the favorable clinical outcome. Despite moderately-enhanced parasitemia, these coinfected survivors developed few CD4+ and CD8+ T effectors producing IFNγ or TNFα *de novo*, indicating profound suppression of Th1 effectors compared to malaria/acute-SHIV-coinfected macaques and malaria-only controls (**Figure 4**; $p<0.0001$ malaria/chronic-SHIV vs malaria/acute-SHIV; $p=0.0007$ malaria/chronic-SHIV vs malaria-only at D22). Consistently, plasma IFNγ levels were much lower in malaria/chronic-SHIV-coinfected macaques than malaria/acute-SHIV-coinfected macaques (**Figure 5**). Interestingly, plasma IL-4 and anti-malaria Abs were not significantly different between malaria/chronic-SHIV-coinfected, malaria/acute-SHIV-coinfected, and malaria-only groups (**Figure 5b, 5d**).

Surprisingly, IL-17-producing CD4+ T effectors were remarkably increased in malaria/chronic-SHIV-coinfected macaques, undergoing ~200-fold expansion after coinfection (**Figure 6a, 6b**; $p=0.0021$ baseline vs D22). IL-17-producing CD4- (presumed CD8+) T-cells were also increased during malaria/chronic-SHIV-coinfection (**Figure 6c**). In addition, malaria/chronic-SHIV-coinfected macaques developed unique 10-20-fold expansion of IL-22-producing CD4+ T effectors after malaria-coinfection (**Figure 6d**). We also detected increases in IL-22-producing CD3+CD4-(CD8+) T effectors in malaria/chronic-SHIV-coinfected macaques (**Figure 6e**). Importantly, IL-17+ and IL-22+ T effectors were detected without additional *in vitro* Ag re-stimulation, implicating previous *in vivo* activation and maturation. In contrast, malaria/acute-SHIV-coinfected macaques exhibited decreases in IL-22+CD4+ T effectors (**Figure 6**), a down-regulation as described in SIV-infected macaques (Raffatellu et al 2008).

In SHIV-only controls, no or very few IL-17+ or IL22+ T-cells were detected in acute or chronic SHIV infection (Yao et al 2010). As reported by us and others, IL-17-producing and IL-22-producing T effectors were distinct populations (data not shown and (Trifari et al 2009; Yao et al 2010)). Notably, the remarkable expansion of IL-17+ and IL-22+ T effectors coincided with controllable parasitemia and absence of fatal malaria, with stable CD4+ T-cell counts and transient minor increase in SHIV viremia (**Figure 7b**). Thus, remarkable expansion of IL-17+ T effectors, unique increases in IL-22+ T effectors and suppression of over-reacting Th1 effectors correlated with absence of fatal malaria in malaria/chronic-SHIV-coinfected macaques without AIDS.

C. Discussion

The proof-of-concept studies in macaque models of malaria/SHIV-coinfection allowed us to make the novel observation that AIDS-virus infection stages dictate coinfection-induced immune responses and that distinct Th1 or Th17/IL-22+ immune responses correlate with different outcomes of SHIV disease and virus-associated malaria. Our studies extend human HIV/malaria-coinfection studies (Butcher 2005; Cohen et al 2005; French et al 2001; Grimwade et al 2004; Hoffman et al 1999; Kublin et al 2005; Laufer et al 2006; Patnaik et al 2005; Renia and Potter 2006; Troye-Blomberg and Berzins 2008; Whitworth et al 2000), and provide new information regarding pathogenesis of AIDS-virus and malaria-coinfection. Our findings also suggest that macaque models of SHIV and *P. fragile* can be useful for studying pathologic and immunologic events in human patients coinfected with HIV and *P. falciparum*.

The macaque model of *P. fragile*/acute-SHIV-coinfection suggests that malaria-coinfection during acute-AIDS-virus-infection can induce fatal virus-associated malaria, characterized by bursting parasitemia and severe anemia. We temporarily use the term ‘fatal virus-associated malaria’, since SHIV89.6P infection usually does not induce acutely fatal disease within a month after infection. Moreover, earlier reports and the current study demonstrated that *P. fragile* infection of naïve macaques often does not induce acutely fatal malaria, although some individuals may develop complications due to unusually high-level parasitemia and associated anemia (Collins et al 2006). The extraordinary effects of acute HIV or SIVmac infection on malaria-coinfection in naïve individuals have not been reported in humans or nonhuman primates, perhaps due to difficulty to recruit and study newly-coinfected patients or because of previous exposures to malaria in the acutely-AIDS-virus-infected individual (Cohen et al 2005; French et al 2001; Grimwade et al 2004; Hisaeda et al 2004; Hoffman et al 1999; Koehler et al 2009; Kublin et al 2005; Laufer et al 2006; Patnaik et al 2005; Whitworth et al 2000).

Fatal virus-associated malaria and rapidly-accelerated AIDS go hand-in-hand during *P. fragile*/acute-SHIV-coinfection. CD4+ T-cell depletion appeared more profound in acutely-coinfected macaques than SHIV-only controls. More importantly, dramatic lymphoid destruction/depletion and necrosis in lymph nodes and spleens were seen in malaria/acute-SHIV-coinfected monkeys as early as 21 days post-infection. Significant lymphoid depletion was also seen in gut mucosae. The rapid and profound lymphoid destruction/depletion and necrosis (temporarily defined as AIDS in the study) are attributed to malaria-coinfection of acutely-SHIV-infected macaques, since such fulminating changes can rarely be seen even in very advanced or end stages of AIDS induced by HIV, SIVmac or SHIV. Such lymphoid destruction/necrosis might potentially occur in acute malaria/HIV coinfection, as *P. fragile* causes *P. falciparum*-like malaria (Collins et al 2006; Fujioka et al 1994), and as SHIV induces simian AIDS like HIV (Ali et al 2009).

The hyper-activation/expansion of IFNγ- and TNFα-producing CD4+ and CD8+ T effectors after malaria-coinfection of acutely-SHIV-infected macaques appears to be the mechanism underlying accelerated AIDS. This hyper-activation appeared to be driven mainly by malaria, because similar magnitudes of T effectors are detected in malaria-only controls, and SHIV-only control macaques did not develop detectable IFNγ-, IL-17- or IL-22-producing CD4+ or CD8+ T effectors in acute (3-4 weeks) and chronic SHIV infection (**Fig. 3** and (Ali et al 2009)). Notably, due to SHIV-coinfection, peak IFNγ plasma levels in malaria/acute-SHIV-coinfected macaques were almost 100-fold higher than in malaria-only controls. Hyper-activation of the immune system can readily transactivate the HIV 5' LTR (Nti et al 2005; Xiao et al 1998) for massive viral replication, provide more CD4+ T-cell or macrophage sources for productive virus infection and make infected CD4+ T-cells more susceptible to virus-mediated destruction (Douek, Roederer, Koup 2008; Margolick et al 1987). We did not detect much higher SHIV viral loads 2-3 weeks after acute-SHIV/malaria-coinfection than SHIV-only perhaps

due to extremely high turnover of virions in plasma during acute-coinfection. The malaria/SHIV-driven hyper-immune activation might contribute to the profound lymphoid destruction/depletion and necrosis.

Due to acutely bursting SHIV replication, malaria-driven hyper-activation/expansion of Th1 effectors and over-production of proinflammatory cytokines IFNγ and TNFα in malaria/acute-SHIV-coinfected macaques might compromise anti-malaria immune responses and lead to high-level parasitemia and fatal malaria. The extremely-high-level Th1 cytokines in AIDS-virus coinfection may not be protective against malaria, as IFNγ has been shown to have a role in both protection and inflammation/pathology in malaria (Good et al 2005). Conversely, the suppression or disruption of CD4+ T cell-mediated anti-malaria immunity may have taken place as a result of profound lymphoid destruction/depletion and necrosis. Because cell-mediated immunity is believed to occur primarily in the spleen for blood stage malaria (Good et al 2005), profound lymphoid depletion in the spleen would lead to a loss of anti-malaria effector function. Furthermore, the disappearance of germinal centers and lymphoid depletion of CD4+ T-cells certainly would block early development of anti-malaria Ab responses.

One of the novel findings in the current study is that chronically-SHIV-infected macaques employed a different defense mechanism to respond to malaria-coinfection. In sharp contrast to acutely-SHIV-infected macaques, chronically-SHIV-infected macaques without AIDS showed marked suppression of Th1 responses after malaria-coinfection. Rather, they mounted 50-200-fold expansion of IL-17-producing CD4+ T effectors as well as unique, potent IL-22 responses. Unique expansion of IL-22-producing T effectors with Th1 suppression in malaria/chronic-SHIV-coinfection is consistent with our recent finding that IFNγ networking pathways can down-regulate IL-22+ T effector cells in tuberculosis (Yao et al 2010). By mounting IL-17+ and IL-22+ T-cell responses and suppressing over-reacting Th1 responses to malaria-coinfection, chronically-SHIV-infected macaques exhibit only transient, low-magnitude increases in viremia, maintain stable CD4+ T-cell counts, and avoid rapid

progression to AIDS. In addition, marked expansion of Th17 cells and unique increase in IL-22+ T effectors might act in concert with Ab and other immune components to attenuate moderately-enhanced malaria in a timely manner, therefore avoiding fatal virus-associated malaria. IL-17 and IL-22 primarily act by recruitment of neutrophils and induction of anti-microbial peptide production by responder cells, and have been shown to have protective roles in infections of various pathogens, including toxoplasmosis (Kelly et al 2005), candidiasis (Conti et al 2009), *Listeria* (Hamada et al 2008), *Klebsiella pneumonia* (Aujla et al 2008; Zheng et al 2008) and others (reviewed in (O'Connor, Zenewicz, Flavell 2010)). It would be interesting to see if these responses play a role in resistance to multiple malaria exposures in future studies.

Thus, our proof-of-concept study demonstrates for the first time that virus infection status and distinct Th1 or Th17/IL-22 responses after malaria-coinfection of AIDS-virus-infected individuals correlate with different clinical outcomes. As far as we know, this is also the first illustration of the dichotomy of Th1 and Th17/IL-22 responses during infection/coinfection of higher primates. The findings may be potentially useful in the development of AIDS and malaria vaccines and immunotherapeutics.

CHAPTER 4- γδ T Cell Responses During *P. fragile* and *L. monocytogenes* Infections

A. Introduction

Accumulating evidence suggests that Vγ2Vδ2 T cells may be a useful target for immunotherapeutics and vaccine-induced protection against a variety of pathogens, due to their diverse effector functions. Vγ2Vδ2 T cells produce IFNγ (Ali et al 2007; Shao et al 2009; Shen et al 2002), lyse cells by producing the cytolytic cytokines granulysin and perforin (Ali et al 2007; Dieli et al 2001; Martino et al 2007; Troye-Blomberg et al 1999), and have adjuvant effects of CD4+ and CD8+ T cell and antibody responses (Ali et al 2009). In addition, Vγ2Vδ2 T cells have been shown to proliferate extensively and provide protection against a variety of different microorganisms (Begley et al 2004; Huang et al 2009; Martino et al 2007; Meraviglia et al 2010; Shao et al 2009; Shen et al 2002) due to recognition of metabolites from isoprenoid synthesis, such as isopentenyl pyrophosphate (IPP) and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) (Hintz et al 2001; Tanaka et al 1994), which are referred to as phosphoantigens. Isoprenoids are produced via two major pathways: the classical, mevalonate pathway, or the alternative, non-mevalonate pathway (Begley et al 2004; Hintz et al 2001). IPP is an intermediate in both pathways, while HMBPP is only found in the non-mevalonate pathway. While both IPP and HMBPP can activate Vγ2Vδ2 T cells, HMBPP requires an approximately 1000-fold lower concentration than IPP (Hintz et al 2001). The non-mevalonate pathway is utilized by many Gram+ bacteria, and the plastids of some eukaryotes, including the parasites that cause malaria. *Listeria monocytogenes*, a Gram-positive, intracellular baceterium that can induce systemic infection causing spontaneous abortion, septicemia, and meningitis, is the only pathogen known to possess both the mevalonate and non-mevalonate pathways of isoprenoid biosynthesis (Begley et al 2004), making it an ideal candidate to study Vγ2Vδ2 T cells.

Human studies have shown proliferation of Vγ2Vδ2 T cells during malaria infection (Dieli et al 2001), but phosphoantigen-specific γδ T cells response to *Listeria* has not been reported *in vivo* (Ladel,

Blum, Kaufmann 1996; O'Brien et al 2000; Stavru, Archambaud, Cossart 2011; Tramonti et al 2008), and *in vivo* studies cannot be done in mice or other small lab animals as HMBPP-specific γδ T cells exist only in humans and nonhuman primates. In the current studies, we utilized *P. fragile* malaria infection and an attenuated *L. monocytogenes* strain (Shetron-Rama et al 2003; Yan et al 2008). In order to generate strong peripheral blood Vγ2Vδ2 T cell activation, we infected rhesus macaques via i.m. or i.v. injection.

B. Vγ2Vδ2 T cell responses during *P. fragile* infection

Vγ2Vδ2 T cells have been shown to proliferate in humans infected with malaria (Behr et al 1996; Goerlich et al 1991), and can even lyse malaria-infected red blood cells *in vitro* (Farouk et al 2004). As malaria parasites only contain the non-mevalonate pathway of isoprenoid biosynthesis (Wiesner and Jomaa 2007), it was therefore surprising that Vγ2Vδ2 T cells did not expand in rhesus macaques after infection with *P. fragile* malaria (**Figure 1a**). Only two animals had very minor increases in the number of Vγ2Vδ2 T cells, and this occurred late in infection, after parasitemia came under control. There were also very minor increases in the number of Vγ2Vδ2 T cells capable of producing IFNγ (**Figure 1b**), TNFα (**Figure 1c**), or IL-17 (**Figure 1d**), indicating that Vγ2Vδ2 T cells which were present during malaria infection were not activated by malaria infection.

Figure 1

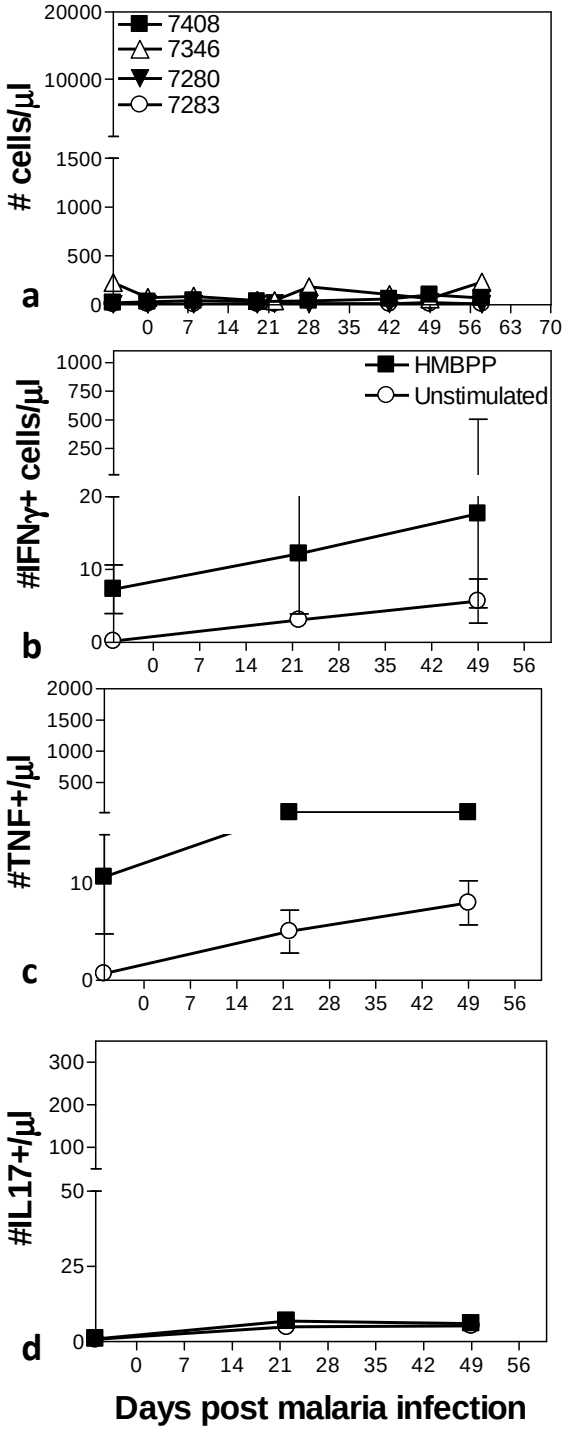


Figure 1. *P. fragile* malaria infection of macaques did not induce Vγ2Vδ2 proliferation (a), or production of cytokines IFNγ (b), TNFα (c), or IL-17 (d).

C. Vγ2Vδ2 T cell responses during *L. monocytogenes* infection

1. Transient systemic *Listeria* infections could uncover the ability of HMBPP-specific Vγ2Vδ2 T cells to mount adaptive immune responses.

γδ T cells appear to possess both innate and adaptive immune features (Chen 2011). However, little is known about whether microbial and/or host factors can dictate the development of innate versus adaptive γδ T-cell responses. While it is well known that *Listeria monocytogenes* can produce large amounts of HMBPP activating Vγ2Vδ2 T cells *in vitro*, it remains unknown whether active infection of individuals with *L. monocytogenes* could induce adaptive immune responses of HMBPP-specific Vγ2Vδ2 T cells. As a proof-of-concept study, two groups of five total rhesus macaques were infected and re-infected systemically with *L. monocytogenes* Δ*actA prfA** mutant. The justification for introducing attenuated *L. monocytogenes* infection by systemic route is to prove a concept and to optimize detection of *L. monocytogenes*-induced immune responses of Vγ2Vδ2 T cells. Also, extremely-attenuated *L. monocytogenes* Δ*actA prfA** mutant is unable to induce active infection after mucosal inoculation due to *actA* deletion, and no *L. monocytogenes* bacteria can be isolated even after intravenous inoculation with this strain (Qiu et al 2011; Yan et al 2008). We presumed that systemic inoculation of macaques with *L. monocytogenes* Δ*actA prfA** mutant would induce very transient systemic *L. monocytogenes* infection, which might be somehow relevant to human listeriosis (systemic infection) after wild-type virulent *L. monocytogenes* infection (Allerberger and Wagner 2010; Bakardjiev, Theriot, Portnoy 2006; Stavru, Archambaud, Cossart 2011).

The first group of macaques was inoculated i.m. with10^8 CFU of attenuated *L. monocytogenes* at day 0, and at day 35, inoculated again i.v. with 10^8 CFU *L. monocytogenes*. While primary *L. monocytogenes* infection induced detectable increases in percentage and absolute numbers of Vγ2Vδ2 T cells, the secondary *L. monocytogenes* infection led to 5-10 fold greater expansion of Vγ2Vδ2 T cells that sustained at least 3-4 weeks (**Figure 2a-b)**. This initial finding suggested that *L. monocytogenes*

infection could induce an adaptive or recall-like response of Vγ2Vδ2 T cells. Given the possibility that low-dose initial infection could drive optimal recall-like response of Vγ2Vδ2 T cells in subsequent microbial exposure (Shao et al 2009), we infected the second group of macaques by inoculating 10^6 CFU *L. monocytogenes* Δ*actA prfA** i.v. day 0 and 10^8 CFU i.v. on day 51. Interestingly, while one of three macaques in this group had a similar recall-like γδ T-cell response as the group one animals, the other two animals developed a much greater magnitude of recall-like response of Vγ2Vδ2 T cells after the second infection (**Figure 2a, 2c**). Only one week after the second infection, Vγ2Vδ2 T cells proliferated and increased percentage numbers from <2% up to 60% of total T cells, or absolute numbers from <50 cells/μl up to ~13,000 cells/μl of blood (**Figure 2a, 2c**). However, no live bacteria could be isolated from the blood one week after the first and secondary *L. monocytogenes* inoculations. Thus, transient systemic *L. monocytogenes* infections could uncover the ability of HMBPP-specific Vγ2Vδ2 T cells to mount adaptive immune responses.

Figure 2

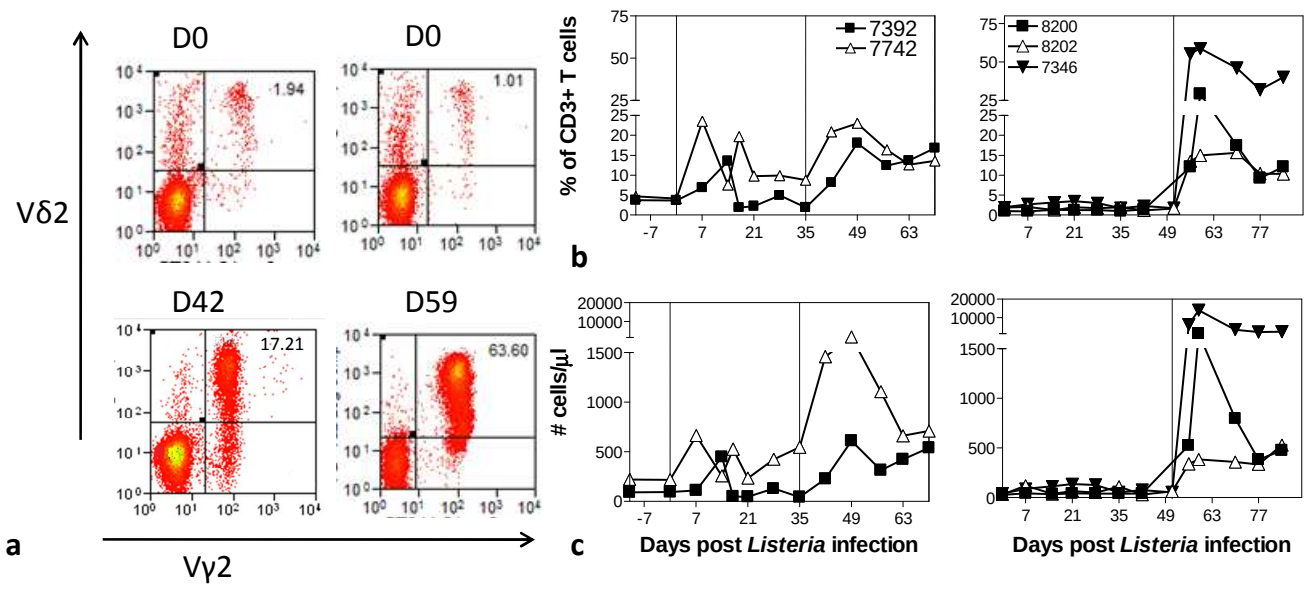


Figure 2 Expansion and re-expansion of Vγ2Vδ2 T cells after infection with rLM. 2a. Representative histograms showing Vγ2+ Vδ2+ T cells on day 0 (top) and day 65 (bottom). Gated on CD3+ lymphocytes. 2b. Vγ2Vδ2 T cells, expressed as percent of CD3+ T cells, expanded after primary and secondary infection in group 1 (left), and demonstrated recall expansion after secondary infection in group 2 (right). 2c. Absolute number of Vγ2Vδ2 T cells showed a similar pattern of expansion in group 1 (left) and recall expansion in group 2 (left).

2. Expanded Vγ2Vδ2 T cells trafficked to and accumulated in pulmonary compartment and intestinal mucosa after primary and secondary *Listeria* infections

We then sought to determine if Vγ2Vδ2 T cells activated by systemic *L. monocytogenes* infection were able to traffic to mucosa/tissue sites for potential mucosal immunity (Ali et al 2007). To test this function, we examined the accumulation of Vγ2Vδ2 T cells in pulmonary compartment and intestinal mucosa. Consistent with major expansion of Vγ2Vδ2 T cells in the blood, up to10-fold increases in numbers of Vγ2Vδ2 T cells were detected in bronchioalveolar lavage fluid (BALF) after both the primary and secondary *L. monocytogenes* infections in both groups when compared to controls ($p=0.0067$, baseline vs. 2 weeks post primary infection; $p=0.0024$ baseline vs. 1-2 weeks post secondary infection; **Figure 3a-e**). Particularly, much greater increases in Vγ2Vδ2 T cells in BALF from the group 2 macaques were seen after the secondary *L. monocytogenes* infection (**Figure 3**). These results implicated that Vγ2Vδ2 T cells underwent airway trafficking after cellular expansion during *L. monocytogenes* infections. To examine if Vγ2Vδ2 T cells could traffic to intestinal muosae, we performed rectal mucosal biopsy to isolate lymphocytes from the biopsies, and analyzed percentage numbers of Vγ2Vδ2 T cells in total mucosal T cells as we previously did (Ali et al 2009). Similarly, we found ~10-fold increases in numbers of Vγ2Vδ2 T cells in the rectal mucosae after primary and secondary *L. monocytogenes* infections of the macaques, when compared to controls ($p=0.0288$, baseline vs. 1-2 weeks post secondary infection; **Figure 3d**). These results therefore demonstrated that expanded Vγ2Vδ2 T cells could traffic to and accumulate in pulmonary compartment and intestinal mucosa after primary and secondary *L. monocytogenes* infections.

Figure 3

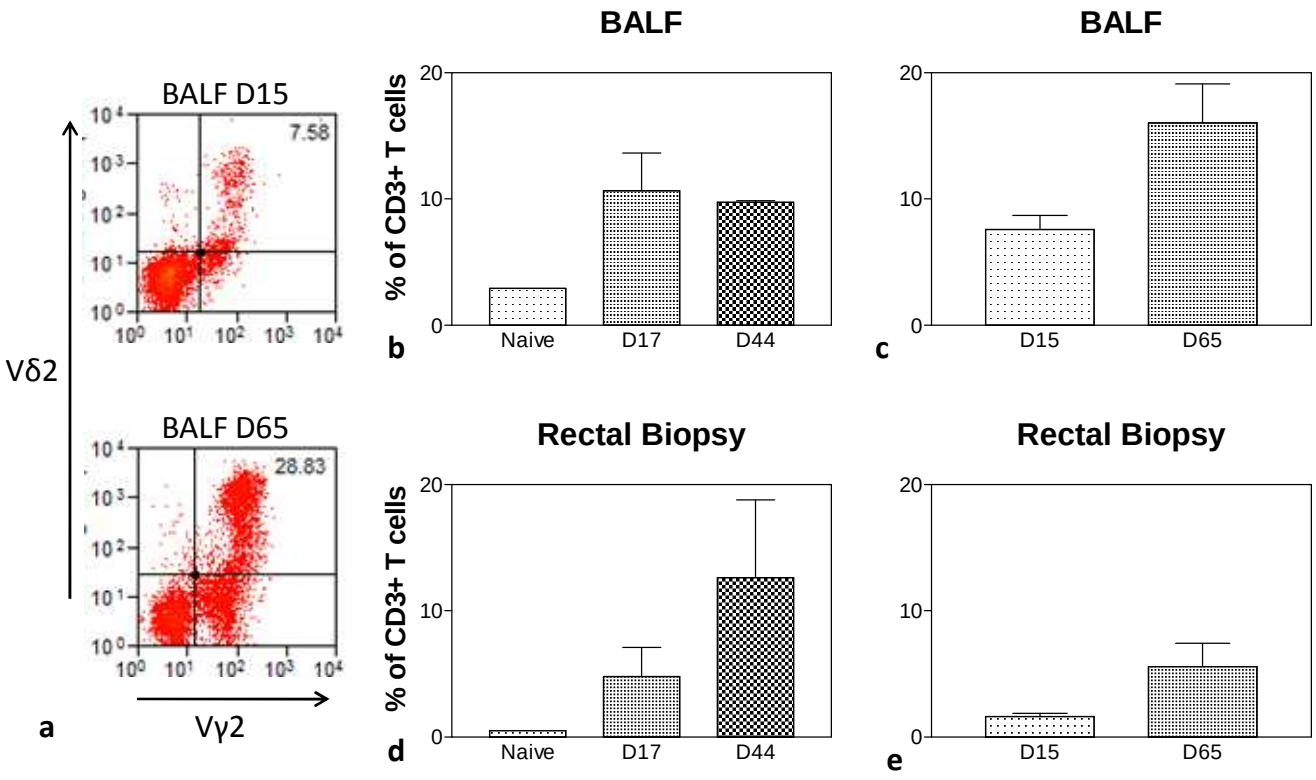


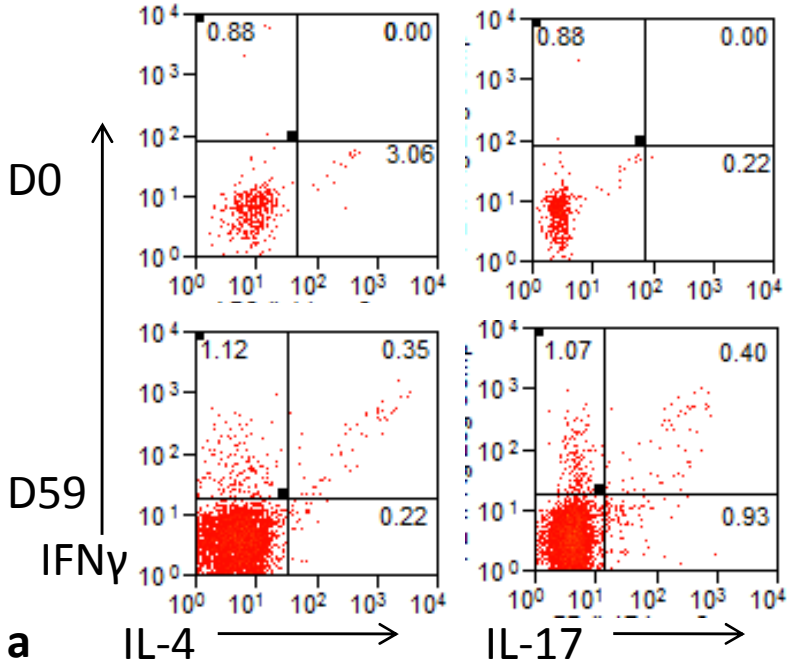
Figure 3 Mucosal accumulation of Vγ2Vδ2 T cells. 3a. Representative histograms of Vγ2+ Vδ2+ T cell accumulation in bronchioalveolar lavage fluid on day 0 (top) and day 65 (bottom). Gated on CD3+ lymphocytes. 3b. Vγ2Vδ2 T cell levels peaked at approximately 10% of all CD3+ T cells by day 17 post infection in group 1. They remained at a similar level after boost infection. 3c. Two weeks after primary infection in group 2, Vγ2Vδ2 T cells reached ~10%. One week after the boost infection, Vγ2Vδ2 T cells increased to almost 20% of all T cells. P=0.0067, baseline vs. 2 weeks post primary infection; p=0.0024, baseline vs. 1-2 weeks post secondary infection (both groups combined). 3d. At day 17 after primary infection, Vγ2Vδ2 T cells in rectal mucosa were at a level of ~5%. One week post boost, they had increased further to 15-20% of all CD3+ T cells. 3e. Vγ2Vδ2 T cell levels remained low in group 2 after both prime and boost infections. P=0.0288, baseline vs. 1-2 weeks post secondary infection (both groups combined).

3. Majority of expanded Vγ2Vδ2 T cells evolved into effector cells capable of de novo production of perforin, and some Vγ2Vδ2 T cells exhibited multiple effector functions producing or co-producing Th1, Th2, and Th17 cytokines

Little is known about whether Vγ2Vδ2 T cells expanded *in vivo* can mount multiple effector functions *de novo* producing different cytokines during infections, although they have been shown to be potent produces of inflammatory cytokines IFNγ and TNFα (Ali et al 2007; Eberl et al 2009; Shao et al 2009; Sicard et al 2005). In fact, the possibility that Vγ2Vδ2 T cells can simultaneously co-produce Th1, Th2, and Th17 cytokines has not been investigated (Caccamo et al 2011). We therefore assessed Vγ2Vδ2 T cells for effector functions producing multiple cytokines over time after primary and secondary *L. monocytogenes* infections using intracellular cytokine staining with mAbs against IFNγ, TNFα, IL-17/IL-22, IL-4, and perforin. While *L. monocytogenes* infections induced very few Vγ2Vδ2 T cells producing IL-22 (data not shown), there were increases in numbers of Vγ2Vδ2 T effector cells producing IFNγ, TNFα, IL-4, IL-17 or perforin after secondary *L. monocytogenes* infection (**Figure 4, 5**). Particularly, ~1000-fold increase in numbers of Vγ2Vδ2 T cells producing IFNγ *ex vivo* was seen in one of the infected macaques (**Figure 5**). Importantly, Vγ2Vδ2 T cells producing perforin *de novo* without *ex vivo* HMBPP stimulation remarkably increased from baseline of <5 cells/μl up to 400-1100 cells/μl after the second *L. monocytogenes* infection (**Figure 5**). Interestingly, we found that some of Vγ2Vδ2 T cells were able to simultaneously co-produce Th1 and Th2 or Th17 cytokines (**Figure 4, 6**). After secondary *L. monocytogenes* infection, Vγ2Vδ2 T cells were able to co-produce IFNγ and IL-17 or IL-4, a finding that has not been previously demonstrated (**Figure 6a, 6b**). Co-production of IFNγ and either IL-17 or IL-4 was dependent on *ex vivo* HMBPP restimulation. In addition, Vγ2+ T cells were able to co-produce TNFα and perforin after secondary *L. monocytogenes* infection, with or without HMBPP restimulation (**Figure 6c**).

Thus, the majority of expanded Vγ2Vδ2 T cells evolved into effector cells capable of *de novo* production of perforin, and some Vγ2Vδ2 T cells exhibited multiple effector functions producing or co-producing Th1, Th2, and Th17 cytokines. To our knowledge, this is the first description of multi-functional Vγ2Vδ2 T cell responses during microbial infection.

Figure 4



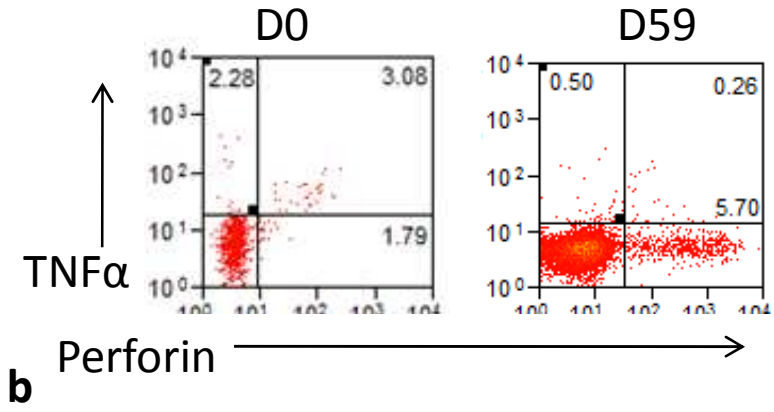


Figure 4 Histograms showing co-production of various cytokines by Vγ2Vδ2 T cells 4a. Representative histograms showing production of IFNγ and IL-17 (top 2 rows) or IFNγ and IL-4 (bottom 2 rows), either with *ex vivo* HMBPP restimulation (right columns) or without (left columns). 4b. Production of TNFα and Perforin either with HMBPP restimulation (right) or without (left).

Figure 5

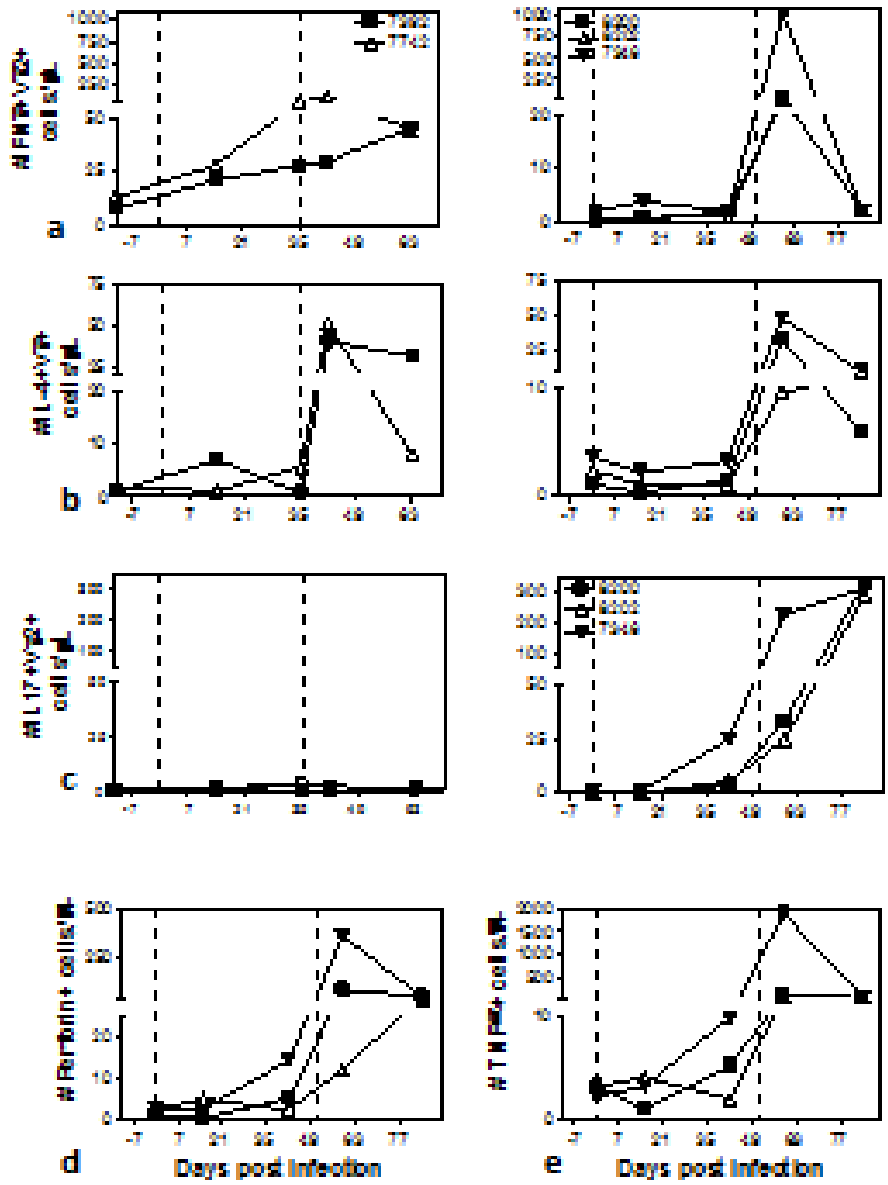


Figure 5 Production of cytokines by Vγ2+ T cells after *ex vivo* HMBPP re-stimulation. 5a.The absolute number of IFNγ-producing cytokines in Group 1 (left) increased steadily after primary and secondary infections. IFNγ-producing Vγ2+ T cells in Group 2 (right) did not expand significantly after primary infection. After secondary infection, however, IFNγ-producing Vγ2+ T cells expanded rapidly and profoundly, indicating memory. 5b. Vγ2+ T cells were able to produce IL-4 only after secondary infection in Group 1 (left) and Group 2 (right). 5c. IL-17 was not produced by Vγ2+ T cells in Group 1 (left), however it was produced by Vγ2+ T cells in Group 2 (right) after secondary infection. 5d. Absolute number of perforin-producing Vγ2+ T cells increased after secondary *Listeria* infection in Group 2. 5e. As with other cytokines, TNFα-producing effector Vγ2+ T cells increased after the secondary *Listeria* infection.

Figure 6

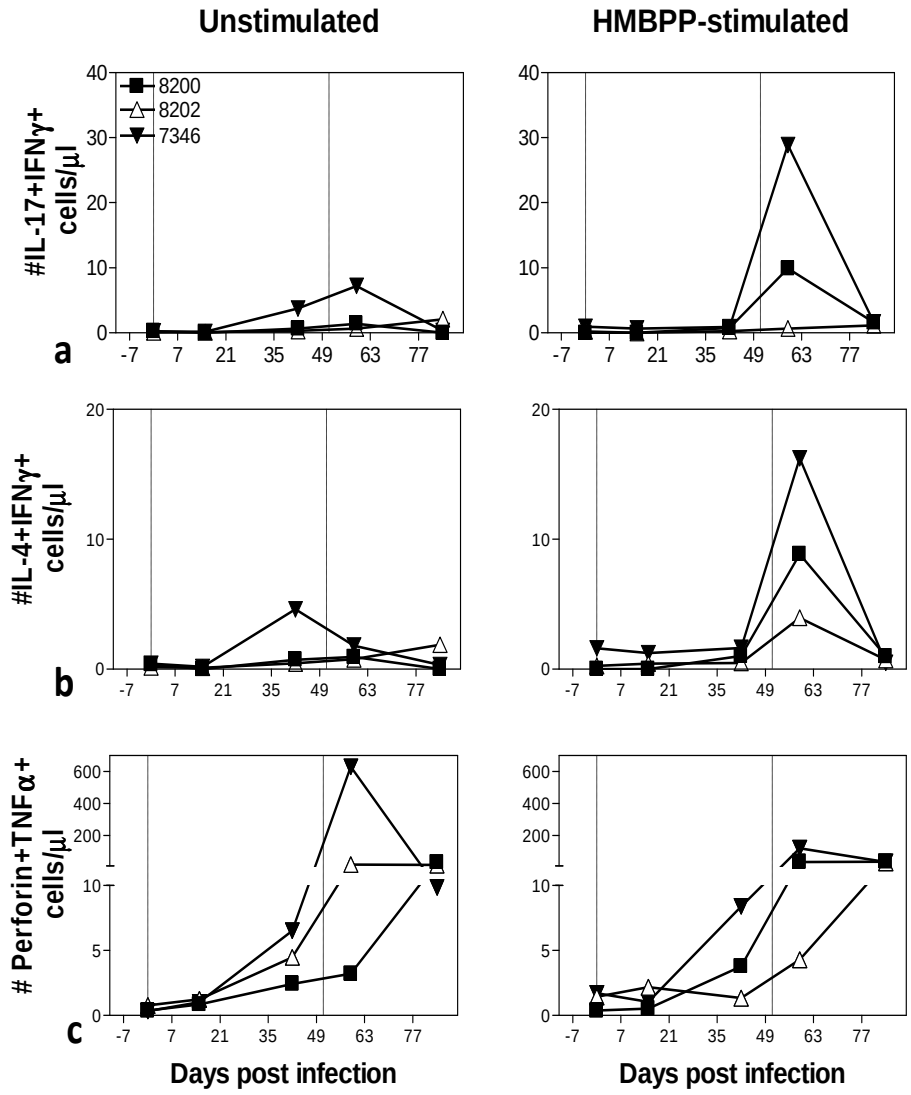


Figure 6 6a. Vγ2+ T cells co-produced IFNγ and IL-4 after secondary *Listeria* infection upon *ex vivo* HMBPP restimulation (right), but not without restimulation (left). 6b. IFNγ and IL-17 were also co-produced by HMBPP-stimulated Vγ2+ T cells after secondary *Listeria* infection (right), but not unstimulated cells (left). 6c. HMBPP was not required for *de novo* co-production of perforin and TNFα by Vγ2+ T effectors after secondary restimulation.

4. In vivo expanded-Vγ2Vδ2 T cells after Listeria infection could directly lyse Listeria-infected target cells

Emerging large numbers of Vγ2Vδ2 T cells *de novo* producing perforin raised a question as to whether these γδ T effector cells were able to exert cytotoxic killing of *L. monocytogenes*-infected target cells. Thus, we adapted a cytotoxicity assay testing the ability of Vγ2Vδ2 T cells to directly kill *L. monocytogenes*-infected autologous target cells. Since *L. monocytogenes* infections exclusively expanded Vγ2Vδ2 T cells but not other γδ T cells in blood, we purified *L. monocytogenes*-expanded Vγ2Vδ2 T cells directly from PBMC after *L. monocytogenes* infection using anti-Vγ2 mAb and immunobeads (MACS) as previously described (Zeng et al 2011). Purified Vγ2Vδ2 T cells were then co-cultured with *L. monocytogenes*-infected autologous monocyte-derived dendritic cells (DC), and assessed for cytotoxic killing of DCs. After the incubation, DCs were stained with anti-CD11c mAb and propidium iodide to measure cell death by flow cytometry analysis. Low-level Vg2Vd2 T cell expansion was detected, and a similar percentage of rLM-infected CD11c+ cells were dead or dying when incubated with Vγ2+ T cells, compared to rLM-infected CD11c+ cells incubated with CD20+ B cellsInterestingly, while low-level expansion of Vγ2Vδ2 T cells was seen at day 15 after *L. monocytogenes* infection (**Figure 2**), Vγ2+ T cells isolated at this time point did not mediate apparent cytotoxic killing of *L. monocytogenes*-infected DC (**Figure 7**). In contrast, Vγ2+ T cells purified at day 59, the peak expansion time point, could very efficiently lyse *L. monocytogenes*-infected DC (**Figure 7**). In effect, these Vγ2Vδ2 T cells isolated at peak expansion were able to kill or lyse almost 75% of *L. monocytogenes*-infected DC, when compared to the various controls (**Figure 7**). This potent cytotoxic killing of *L. monocytogenes*-infected DC appeared to be consistent with the remarkable increases in numbers of Vγ2Vδ2 T effector cells *de novo* producing perforin at day 59 after initial *L. monocytogenes* infection (**Figure 5d**). Thus, *in vivo* expanded-Vγ2Vδ2 T cells after *L. monocytogenes* infection could directly lyse *L. monocytogenes*-infected target cells.

Figure 7

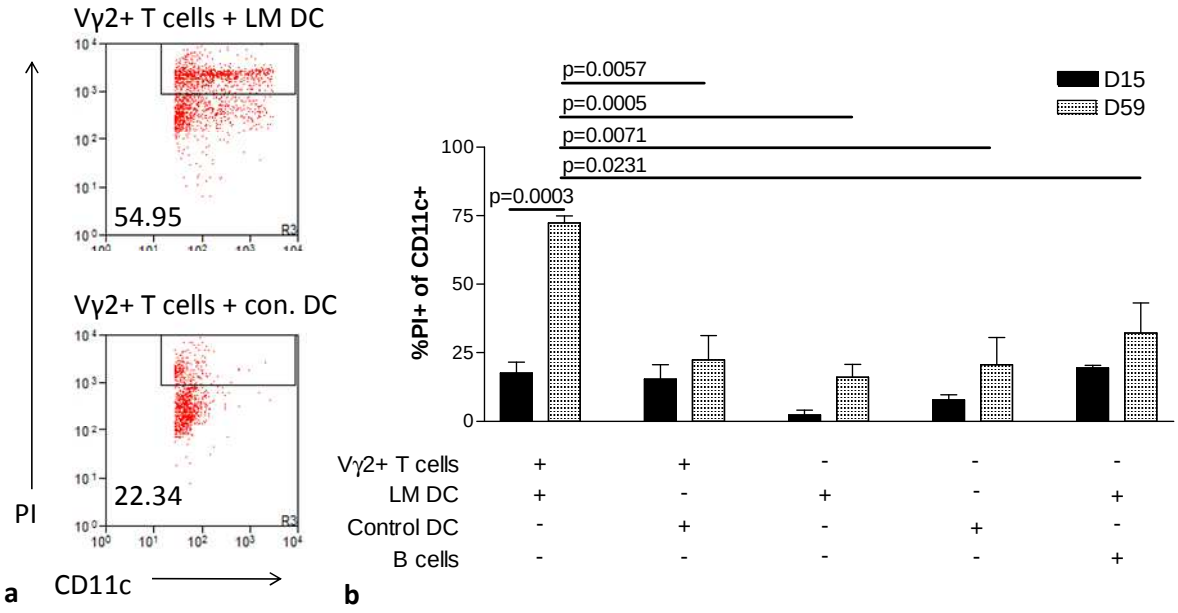


Figure 7 Direct lysis of CD11c+ DCs by Vγ2+ T cells. 7a. Representative histograms showing increased lysis of rLM-infected DCs compared to control DCs after incubation with Vγ2+ T cells. 7b. Cell lysis represented as %PI+ of CD11c+ DCs. D59 Vγ2+ T cells lysed more rLM-infected DCs than D0 Vγ2+ T cells (p=0.0003). D59 Vγ2+ T cells caused more lysis when incubated with rLM-infected DCs than control DCs (p=0.0057); Vγ2+ T cells only (p=0.0310); rLM-infected DCs only (p=0.0005); control DCs only (p=0.0071); or B cells incubated with rLM-infected DCs (p=0.0231).

D. Discussion

Although previous work in humans has shown increased numbers and percentages of Vγ2Vδ2 T cells, we did not have similar results during *P. fragile* infection of rhesus macaques (Behr et al 1996; Goerlich et al 1991) . It was unexpected that *P. fragile* infection did not activate Vγ2Vδ2 T cells. It is possible that prior infection with an HMBPP-producing organism is required to induce memory re-activation and proliferation of Vγ2Vδ2 T cells. We and others have shown that Vγ2Vδ2 T cells can respond to repeat infections with recall-like expansion (Abate et al 2005; Chen and Letvin 2003; Dieli et al 2003; Hoft, Brown, Roodman 1998; Morita, Mariuzza, Brenner 2000; Pitard et al 2008; Poccia et al 2006, Shao et al 2009, Shen et al 2002). It would be interesting to further explore if one HMBPP-producing pathogen, such as *L. monocytogenes*, can be used to prime Vγ2Vδ2 T cell responses for a second HMBPP-producing pathogen, such as malaria parasites.

The current study provided a first opportunity to examine *L. monocytogenes*-induced *in vivo* immune responses of HMBPP-specific Vγ2Vδ2 T cells during primary and secondary *L. monocytogenes* infections (Begley et al 2004). *L. monocytogenes* is unique for studies of *in vivo* HMBPP-specific Vγ2Vδ2 T-cell responses in that *L. monocytogenes* is the only pathogenic bacterium known to contain both mevalonate and non-mevalonate [2-C-methyl-D-erythritol 4-phosphate (MEP)] pathways of isoprenoid biosynthesis, concurrently producing metabolites such as HMBPP and IPP (Begley et al 2004). Our findings in macaques appear to be novel, as *L. monocytogenes* infections induce multi-functional immune responses of HMBPP-specific Vγ2Vδ2 T cells. The multi-functional immune responses of Vγ2Vδ2 T cells are characterized by the remarkable recall-like expansion, pulmonary or mucosal trafficking, broad effector functions producing or co-producing Th1 and Th2 or Th17 cytokines, and the dominant perforin-producing phenotype and direct lysis or killing of *L. monocytogenes*-infected target cells. To our knowledge, our work represents one of the significant *in*

vivo studies that elucidate broad anti-microbial functional aspects of HMBPP-specific Vγ2Vδ2 T cells in infection.

One of the interesting immune features for Vγ2Vδ2 T cells during infections with intracellular bacterial pathogens appears to mount remarkable adaptive immune responses. Clearly, absolute numbers of Vγ2Vδ2 T cells can increase from <50 cells/ul up to 15,000 cells/ul within a week after secondary *L. monocytogenes* inoculation. This extraordinary recall-like expansion is consistent with the adaptive immune response seen in macaques after *Mycobacterium bovis* BCG re-infection or BCG followed by *M. tuberculosis* infections (Shen et al 2002). These three bacterial pathogens share common capabilities to produce HMBPP and to intracellularly infect monocytes/macrophages/DC. However, in both BCG re-infection and *L. monocytogenes* re-infection, no or extremely low-level bacteria can be isolated from blood days or weeks after secondary bacterial inoculation despite major γδ T-cell expansion, suggesting that recall-like expansion of Vγ2Vδ2 T cells is driven by very transient and subtle systemic re-infection. Our results in the current study also consist with the scenario that low-dose primary infection could optimally prime Vγ2Vδ2 T cells and such primed γδ T cells could mount remarkable recall-like expansion in response to subsequent re-infection (Shao et al 2009). Data from human studies (Abate et al 2005; Chen and Letvin 2003; Dieli et al 2003; Hoft, Brown, Roodman 1998; Morita, Mariuzza, Brenner 2000; Pitard et al 2008; Poccia et al 2006) also support the notion that human Vγ2Vδ2 T cells can mount adaptive immune response in infections. These findings suggest that *in vivo* Vγ2Vδ2 T-cell responses to intracellular infectious agents may be different from the rapid Vγ2Vδ2 T-cell expansion after repeated phosphoantigen/IL-2 co-treatments (Ali et al 2007; Sicard et al 2005).

L. monocytogenes infections also uncover pulmonary or intestinal mucosal migration characteristics of HMBPP-specific Vγ2Vδ2 T cells. This functional aspect is generally considered important for mucosal/pulmonary immunity as most microbial pathogens invade hosts at mucosal interface. Vγ2Vδ2 T cells appear to emerge as a dominant T-cell subpopulation among the T-cell pool in

airway and rectal mucosa after *L. monocytogenes* infections as they comprise up to 30% of total CD3+ T cells in those mucosae (**Figure 3**). Given the possibility that lymphoid tissues in small intestines are more enriched than in rectal mucosa, we anticipate that Vγ2Vδ2 T cells may more apparently traffic to and accumulate in the small intestinal mucosae. The trafficking and accumulation of Vγ2Vδ2 T cells in intestinal mucosae might underscore the importance of these cells for contributing to mucosal immunity against food-borne *Listerial* infection.

We previously demonstrated that expanded Vγ2Vδ2 T cells during BCG or *M. tuberculosis* infections or after HMBPP/IL-2 treatments could similarly undergo pulmonary/mucosal trafficking and accumulation (Ali et al 2007; Huang et al 2008; Huang et al 2009; Shen et al 2002). Thus, pulmonary and intestinal mucosal trafficking/accumulation appears to be a common biological feature for activated Vγ2Vδ2 T cells regardless of the stimuli.

It is surprising to see that Vγ2Vδ2 T cells can mount broad effector functions producing or co-producing Th1, Th2, Th17 and cytotoxic cytokines during *L. monocytogenes* infections. Whereas a number of studies are focused on innate production of cytokines by Vγ2Vδ2 T cells (Ali et al 2007; Sicard et al 2005; Wang et al 2001), the current study longitudinally follows up and reveals broad effector functions producing or co-producing cytokines by Vγ2Vδ2 T cells during adaptive immune response in infections. *L. monocytogenes*-expanded Vγ2Vδ2 T cells are able to produce not only IFNγ, IL-4, IL-17, and perforin, but also can co-produce Th1 and Th2 or Th17 cytokines. Simultaneous co-production of these cytokines by T helper cells appears to be a rare immune event as the development for each of T helper subsets producing Th1, Th2, and Th17 cytokines is controlled tightly by individual unique master transcriptional factors such as T-bet, GATA-3 and RORγt (Zhou, Chong, Littman 2009). One of the co-production patterns appeared to be the HMBPP-driven co-production of IFNγ and IL-17 or IL-4 by Vγ2Vδ2 T cells. 10-30% of Vγ2Vδ2 T cells coproduced both IFNγ and IL-17 in two of three animals, while 5-15% produced IFNγ and IL-4 in all three animals (**Figure 6**). This is in line with

recent evidence that IFNγ can regulate production of IL-17 (Yang et al 2011). Another co-production pattern seemed to be adaptive *de novo* co-production of IFNγ and IL-4 or IL-17. IL-4-producing (2 macaques) or IL-17-producing Vγ2Vδ2 T cells capable of producing IFNγ *ex vivo* could increase or sustain after primary *L. monocytogenes* infection, and even co-produce these cytokines without *ex vivo* HMBPP stimulation. IFNγ and IL-4 are generally thought to be produced by different cell populations (Zhou, Chong, Littman 2009), although may be coproduced in some instances (Hegazy et al 2010). Production or co-production of Th1, Th2 and Th17 cytokines by Vγ2Vδ2 T effector cells may play a role in activation of a variety of other immune cells such as macrophages, CD8+ T cells, and B cells, therefore may bridge innate and adaptive immunity. In addition, IFNγ and IL-17A can contribute to early protection against *L. monocytogenes* infection (Barry et al 2003; Hamada et al 2008). Although IL-4 may blunt anti-*Listeria* immune response (Haak-Frendscho et al 1992), IL-4-producing γδ T cells may facilitate vaccine-elicited Ab responses in attenuated *Listeria*-vaccinated individuals.

It is also surprising that most of *L. monocytogenes*-expanded Vγ2Vδ2 T cells evolve into effector cells capable of *de novo* production of perforin. While Vγ2Vδ2 T cells expand from baseline <50 cells/ul up to 15,000 cells/ul after secondary *L. monocytogenes* infections, ~70% of these expanded Vγ2Vδ2 T cells differentiate to effector cells producing perforin without the need for *ex vivo* HMBPP phosphoantigen stimulation. These Vγ2Vδ2 T effector cells capable of *de novo* production of perforin may play a role in protection against *L. monocytogenes* infection or listeriosis, as perforin expression in CD8+ αβ T cells has been shown to be important for protection against *L. monocytogenes* infection in mice (Messingham, Badovinac, Harty 2003).

Another novel finding from the current study is the demonstration of direct killing or lysis of *L. monocytogenes*-infected target cells by *in vivo* expanded Vγ2Vδ2 T cells. Lysis of mycobacterium-infected cells by *in vitro* activated Vγ2Vδ2 T cells has previously been demonstrated (Dieli et al 2000; Dieli et al 2001; Martino et al 2007). These studies showed the release of Cr^{51} or lactate dehydrogenase

by infected cells and decreased bacterial survival after co-culture with activated Vγ2Vδ2 T cells. In the current study, we directly detected membrane disruption of *L monocytogenes*-infected CD11c+ DCs after co-culture with *in vivo* expanded Vγ2Vδ2 T cells by utilizing flow cytometry analysis. To our knowledge, this is the first evidence showing direct lysis of microbe-infected cells by *in vivo* expanded Vγ2Vδ2 T cells. This unique finding appears to be linked to perforin production phenotype of Vγ2Vδ2 T cells, and most *in vivo* expanded Vγ2Vδ2 T cells produce perforin *de novo* without the need for ex vivo HMBPP stimulation.

Anti-*Listeria* immunity in humans remains largely unknown, although murine IFNγ and CD8+ T cells play a role in protection against *L. monocytogenes* infection (Berg et al 2005; Harty and Pamer 1995; Harty, Tvinnereim, White 2000). Studies of Vγ2Vδ2 T cells for anti-*Listeria* immunity cannot be done in mice or other small lab animals as γδ T cells in these lab animals do not recognize HMBPP or other phosphoantigens. Thus, our findings from the current study raise the possibility to conduct further *in vivo* studies and investigate the role of HMBPP-specific Vγ2Vδ2 T cells in controlling *L monocytogenes* infection or listeriosis.

Direct cell killing, coupled with cytokine production and activation of other effector cell types, makes Vγ2Vδ2 T cells a unique and versatile vaccine and immunotherapeutic target.

CHAPTER 5- Conclusion

A. Model of T cell Responses During Malaria and SHIV Coinfection

As demonstrated in Chapter 3, T cell responses appear to play an important role in disease progression during coinfection of malaria and AIDS-causing pathogens. Malaria and acute SHIV-coinfected macaques developed severe malaria, characterized by anemia, lethargy, and coma. These animals had hyper-activation of Th1 cells producing proinflammatory cytokines IFNγ and TNFα. In contrast, chronically SHIV-infected macaques did not develop severe malaria after coinfection with *P. fragile* parasites. Chronic SHIV and malaria coinfected macaques had significantly inhibited Th1 responses; however, they were able to mount potent and potentially protective Th17 and Th22 responses.

CD4+ T cells produce cytokines that stimulate development of a particular subset and inhibit development of other subsets. For example, IFNγ produced by Th1 cells inhibits IL-10, a cytokine involved in development of Th2 cells and Tregs, as well as STAT-3, an important transcription factor for Th17 cells (Hu and Ivashkiv 2009). A potential explanation for the different helper T cell responses developed during malaria and SHIV coinfections is outlined in Figure 1. Malaria induces proinflammatory T cell responses, and subsequent production of IFNγ (Engwerda et al 2002; Good et al 2005; Ramharter et al 2003). In addition, microbial translocation due to SHIV infection results in increased production of proinflammatory cytokines, including IFNγ (Brenchley et al 2006). The high-level IFNγ further promotes Th1 responses, by promoting T-bet expression and TNFα production (**Figure 1a**). TNFα production, in particular, enhances viral replication (Douek, Roederer, Koup 2008; Hu and Ivashkiv 2009). T cell activation and enhanced viral replication can both cause CD4+ T cell death, which occurred prominently and rapidly in lymph nodes and spleens (**Chapter 3**). Since the spleen is the primary location of T cell-mediated immunity against blood stage malaria (Good et al 2005), it follows that necrosis and lymphoid destruction of splenic lymphocytes would allow for

massive replication of malaria parasites, resulting in severe malaria of acutely SHIV coinfected macaques (**Chapter 3**).

During the chronic stage of SHIV infection, CD4+ T cells, particularly Th1 cells, have been depleted and are generally exhausted (Douek, Roederer, Koup 2008). Because Th1 activation is inhibited during the chronic stage of SHIV infection, TNFα production is not increased during malaria coinfection of chronically SHIV infected macaques. Viral replication is therefore more inhibited than during malaria/acute SHIV coinfection, so fewer T cells die in the periphery and lymphoid organs. The absence of a strong IFNγ response helps remove inhibitors of development of Th17 and Th22 response (**Figure 1b,** Hu and Ivashkiv 2009). Th17 cells appear to play a role in preventing pathogenesis early during AIDS infection (Favre et al 2009; Raffatellu et al 2008), but it is not clear what functions they may have during the chronic stage of AIDS-virus infection. Th17 cells may act by recruiting neutrophils to ingest malaria-infected RBCs (Iwakura et al 2011). IL-22 secretes matrix metalloproteinases, which are inhibited by IFNγ (Hu and Ivashkiv 2009). Matrix metalloproteinases may help repair endothelial tissue damaged during malaria infection, preventing immune-associated pathology (Sonnenberg, Fouser, Artis 2011; Zelante et al 2011).

Figure 1

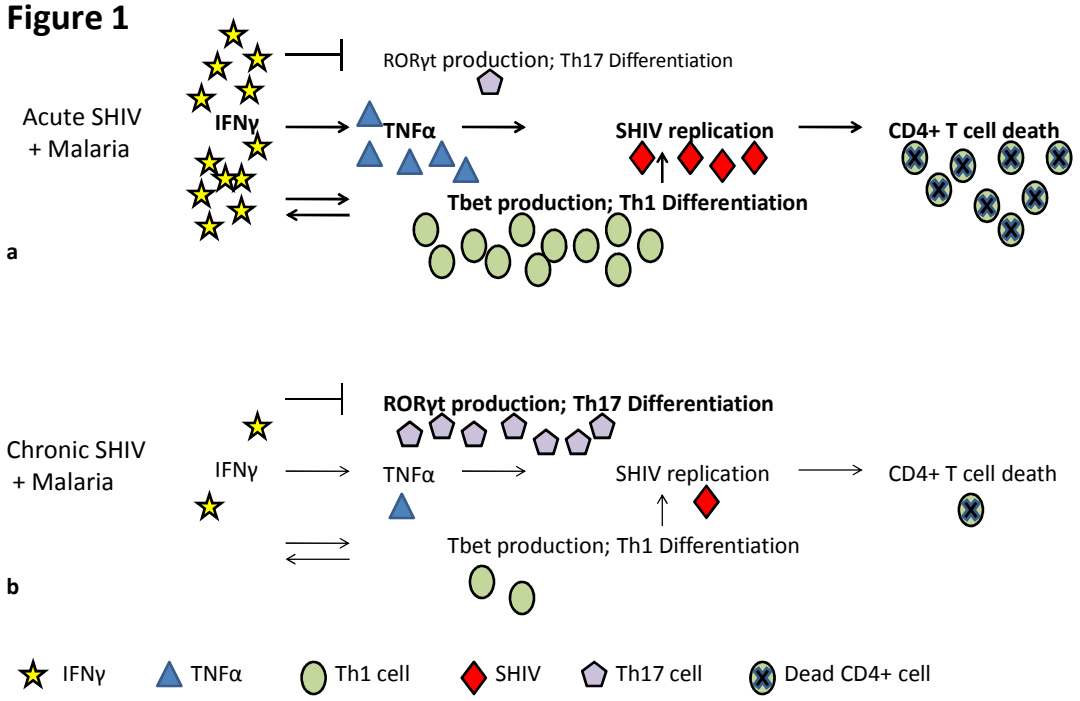


Figure 1 Model of cytokine production and T cell activation. 1a. Production of IFNγ during malaria/acute SHIV coinfection inhibits RORγt production and Th17 differentiation; TNFα replication is increased, which also increases SHIV replication and CD4+ T cell death. In addition, Tbet production is upregulated, promoting Th1 differentiation, continuing the cycle of T cell activation, SHIV replication, and cell death. 1b. Conversely, during chronic SHIV infection, IFNγ-producing T cells are not activated during malaria coinfection. This removes the block on Th177 differentiation, and inhibits viral replication.

B. γδ T Cell Responses to *P. fragile* and *L. monocytogenes*

Despite evidence showing that Vγ2Vδ2 T cells proliferate during human malaria infection (Dieli et al 2001), we did not detect expansion or activation of Vγ2Vδ2 T cells during *P. fragile* infection of rhesus macaques. We only noted minor increases in the absolute number of circulating Vγ2Vδ2 T cells late during malaria infection (weeks 6-7) in some, but not all, of the infected animals. As *P. falciparum* only contains genes for the non-mevalonate isoprenoid biosynthesis pathway (Wiesner and Jomaa 2007), it seems unlikely that *P. fragile* lacks the non-mevalonate isoprenoid biosynthesis pathway. Instead, *P. fragile* parasites may not have reached a high enough concentration or produced enough HMBPP in order to activate Vγ2Vδ2 T cells during infection of macaques. It is also possible that previous exposure to malaria or another HMBPP-producing pathogen is required to activate Vγ2Vδ2 during malaria infection, which would then be followed by a recall response. As the monkeys used have been isolated in controlled laboratory settings, it is possible that they have not previously been infected with such pathogens.

L. monocytogenes infection was able to activate Vγ2Vδ2 T cells, despite being an attenuated strain. It is noteworthy to point out that the most profound expansion of Vγ2Vδ2 T cells occurred after the secondary infection in animals that received a lower primary infection. This demonstrates a very clear and profound memory response. Vγ2Vδ2 T cells also migrated to the lung and gut mucosae. In addition to massive proliferation, activated Vγ2Vδ2 T cells were able to produce IFNγ, TNFα, IL-4, IL-17, and perforin. Importantly, they were able to co-produce IFNγ and IL-4 or IL-17, an observation not previously reported. These activated Vγ2Vδ2 T cells also had the ability to directly lyse *Listeria* infected macrophage-derived dendritic cells.

Despite being unable to detect Vγ2Vδ2 T cell activation during *P. fragile* infection, we did detect very significant activation during *L. monocytogenes* infection. The overall effector functions of Vγ2Vδ2 T cells have not been completely described during microbial infection. Our work provides new

evidence of the multiple effector potential of Vγ2Vδ2 T cells, which may provide as yet un-reported benefits during infection.

C. Future Directions

Because activation of CD4+ T cells was so rapid and profound, we hypothesize that T cell activation may be independent of TCR, and would like to further investigate these mechanisms. It has been shown the CD4+ T cells from naïve individuals can respond the malaria infection, which supports this hypothesis (Dick et al 1996). In regards to malaria and SHIV coinfection, it would be interesting to determine the possible roles of IL-17 and IL-22 in preventing severe disease. As mentioned above, recruitment of neutrophils and protection of endothelial tissue may be important consequences of IL-17 and IL-22 during coinfection.

It was unexpected that malaria infection did not activate or expand Vγ2Vδ2 T cells. It would be interesting to use serial malaria infections in order to determine if memory is required for activation of Vγ2Vδ2 T cells. We also are considering priming Vγ2Vδ2 T cells with *L. monocytogenes* infection and determining if Vγ2Vδ2 T cells are expanded during subsequent malaria infection. Previous studies utilizing *gcpE* deficient *L. monocytogenes* have shown that production of HMBPP is required for activation of Vγ2Vδ2 T cells *in vitro*. It would be interesting to see if this is also the case *in vivo*. In addition, we would like to utilize similar *lytB* mutant *L. monocytogenes* strains to determine if excess HMBPP can induce greater activation and proliferation of Vγ2Vδ2 T cells *in vivo*.

Lastly, *L. monocytogenes* is gaining interest as a vaccine vector (Yan et al 2008). We would like to use recombinant attenuated *L. monocytogenes* expressing SHIV proteins to determine if pre-existing anti-SHIV immunity can prevent severe disease during malaria and acute-SHIV coinfection by limiting viral replication.

Understanding host T cell immune responses is critical to the development of vaccines against AIDS and malaria. By learning how effector T cells function and provide protection during infection, we can rationally design vaccines to induce these T cell responses and prevent disease.

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APPENDIX

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

October 28, 2009

Zheng Chen
Microbiology & Immunology
M/C 790

Dear Dr. Chen:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **10/20/2009**. *The protocol was not initiated until final clarifications were reviewed and approved on* **10/27/2009**. *The protocol is approved for a period of* **3** years with annual continuation.

Title of Application: Malaria Vaccine in Healthy and SHIV-Infected Rhesus

ACC Number: 09-166

Condition of Approval: Prior to proceeding to Groups 2 and 3, the PI will submit a progress report to the ACC summarizes the results and data for review.

Initial Approval Period: 10/27/2009 to 10/20/2010

Current Funding: ***Currently protocol NOT matched to specific funding source. Modification will need to be submitted prior to Just in time or acceptance of award to match protocol to external funding source. All animal work proposed in the funding application must be covered by an approved protocol.***

UIC is the only performance site currently approved for this protocol.

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Richard D. Minshall

Richard D. Minshall, PhD
Chair, Animal Care Committee
RDM/*ss*
cc: BRL, ACC File

Phone (312) 996-1972 • Fax (312) 996-9088

UNIVERSITY OF ILLINOIS
AT CHICAGO

Office of Animal Care and Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

January 7, 2009

Zheng Chen
Microbiology & Immunology
M/C 790

Dear Dr. Chen:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **11/18/2008**. *The protocol was not initiated until final clarifications were reviewed and approved on* **12/11/2008**. *The protocol is approved for a period of* **3** years with annual continuation.

Title of Application: T-cell Responses in Rhesus Macaque Malaria Infection

ACC Number: 08-228

Initial Approval Period: 12/11/2008 to 11/18/2009

Current Funding: ***Currently protocol NOT matched to specific funding source. Modification will need to be submitted prior to Just in time or acceptance of award to match protocol to external funding source. All animal work proposed in the funding application must be covered by an approved protocol.***

UIC is the only performance site currently approved for this protocol.

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,
Mary B Bowman PhD

Mary B. Bowman, PhD
Director, Office of Animal Care and Institutional Biosafety
/Enclosure
MBB/*ss*
cc: BRL, ACC File, Bridgett Ryan-Payseur

VITA

Bridgett Ryan Payseur

Education:
PhD, Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, Chicago, IL. Expected completion date: Octobere 2011
Concentrations: Immunology, T cell biology
Thesis topic: Host T cell responses during malaria, Acquired immunodeficiency syndrome-virus, and *Listeria* infections

B.S., Molecular and Cellular Biology, University of Illinois at Urbana/Champaign, Urbana, ILMay 2006.

A.S., General Science, College of Lake County, Grayslake, IL May 2004.

Teaching Experience:
Teaching assistant, 2007-2008, University of Illinois at Chicago, College of Medicine.

Student Mentor, 2007-2011: Mentored graduate and medical students during laboratory rotations

Research experience:
Flow cytometry data analysis, T cell assays, Intracellular cytokine staining, ELISA, molecular biology techniques, microbiology techniques, statistical analysis

Publications:
Yan L, Qiu J, Chen J, Ryan-Payseur B, Huang D, Wang Y, Rong L, Melton-Witt JA, Freitag NE, Chen ZW. *Selected prfA* mutations in recombinant attenuated Listeria monocytogenes strains augment expression of foreign immunogens and enhance vaccine-elicited humoral and cellular immune responses.* Infection and Immunity, 2008.

Bridgett Ryan-Payseur, Zahida Ali, Dan Huang, Crystal Y. Chen, Lin Yan, Richard C. Wang, William E. Collins, Yunqi Wang, and Zheng W. Chen. *Virus Infection Stages and Distinct Th1 or Th17/Th22 T-cell Responses in Malaria/SHIV-Coinfection Correlate with Different Outcomes of Disease.* Manuscript in press.

Manuscripts:

Bridgett Ryan-Payseur, Zahida Ali, James Frencher, and Zheng W. Chen. Vγ2Vδ2 T cells display adaptive immune properties during *Listeria* monocytogenes infection of rhesus macaques. Manuscript in preparation.

Presentations and abstracts:

Yunqi Wang, Crystal Y. Chen, Bridgett Ryan-Payseur, Dan Huang, Yun Shen, Richard C. Wang, and Zheng W. Chen. *Microbial/host factors regulating adaptive immune features of Vγ2Vδ2 T cells in infections*. Presented at 4th International γδ T cell conference, 2010, Kiel, Germany by Zheng W. Chen.

Awards and honors:

Member of Phi Kappa Phi honor society since February 2008.
Member of Phi Theta Kappa honor society from 2002-2004.
Recipient of Illinois General Assembly Scholarship, August 2004-May2005.

Skills and Qualifications:

MS Word, Excel, Powerpoint.
GraphPad Prism data analysis software.
Flow cytometric analysis using Summit Software.