Title
The Impact of Fetal Exposure to SIV on the Outcome of Neonatal Infection

Permalink
https://escholarship.org/uc/item/4pf7p02v

Author
Baker, Christopher Arthur Renfrew

Publication Date
2013

Peer reviewed|Thesis/dissertation
The Impact of Fetal Exposure to SIV on the Outcome of Neonatal Infection

By

Christopher Arthur Renfrew Baker

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Infectious Diseases and Immunity

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Laurent Coscoy, Chair
Professor Joseph McCune
Professor Russell Vance
Professor David Raulet

Fall 2013
Abstract

The Impact of Fetal Exposure to SIV on the Outcome of Neonatal Infection

By

Christopher Arthur Renfrew Baker

Doctor of Philosophy in Infectious Diseases and Immunity

University of California, Berkeley

Professor Laurent Coscoy, Chair

HIV kills more than 2 million people worldwide, annually. Progressive HIV disease is marked by the loss of peripheral CD4\(^+\) T cells, leading to immune dysfunction and leaving the host susceptible to opportunistic infections. Immune activation is thought to play a major role in T cell depletion and the ability of the host to control immune activation during chronic infection may enhance survival. How may a host learn to control immune activation? Pregnancy appears to be a time in which immune activation is tightly regulated as the immune systems of mom and her genetically non-identical fetus learn to become tolerant of one another’s tissues. The trafficking of cells from mom across the placenta and into the fetus during gestation, a concept known as “maternal microchimerism,” exposes the immune system of the developing fetus to maternal alloantigens and has been shown to promote tolerance by the fetal immune system to maternal cells. We hypothesized that if the fetus were exposed to antigens from HIV in utero that it might become tolerant to HIV antigens in a similar fashion to maternal cells and that this tolerance of HIV antigens would reduce the damage caused by immune activation if this fetus were infected with HIV later in life. To test this hypothesis, rhesus macaques (Macaca mulatta) were studied to determine if maternal microchimerism exists in this species and if exposure to SIV in fetal macaques can induce tolerance to the virus and reduce activation-induced damage after infection postnatally. Our data indicate that maternal microchimerism is detectable in low frequencies in fetal tissues and peripheral blood but can be found in relatively high frequencies in postnatal tissues. Fetal exposure to the nonpathogenic SIV clone, SIVmac1A11, was associated with reduced viral loads (p<0.02) and altered immunologic parameters after pathogenic SIVmac239 challenge. While we did not find evidence of immune tolerance to SIV antigens in these animals our data indicate that prenatal exposure to the virus has a lasting impact on the immune system that may allow for control of viral replication after pathogenic challenge. These data also indicate that the rhesus macaque is a tractable system for studying immunologic development in the context of prenatal infections that may be directly relevant to humans.
Dedication

I really want to thank everyone who has gotten me to this point in my life. First and foremost, I owe everything to my wife, Rachel. You have stood beside me and encouraged me every step of the way and sacrificed to allow me to pursue a dream. Since I am certain that I don’t say it enough, thank you! I have to thank my kids, Cameron and Norah, that will never understand how much of a relief it is to come home and forget about the day that you just had where it seemed that you couldn’t do anything right, by watching two things that you didn’t screw up, dance in the middle of the living room. To my family, thank you for never putting pressure on me, for giving me the freedom to figure it out on my own and being proud of me every step of the way. Finally to my PI, Mike McCune, thank you for your patience and mentorship throughout this process. I hope that I sufficiently “stayed the course.” This one’s for all of you.
CONTRIBUTIONS OF CO-AUTHORS TO THE PRESENTED WORK

Chapter II of this dissertation is in press at the journal *Chimerism* as

“Analysis of maternal microchimerism in rhesus monkeys (*Macaca mulatta*) using real-time quantitative PCR amplification of MHC polymorphisms.” The co-authors of this publication are Sonia Bakkour,1 Alice F. Tarantal,3 Li Wen,1 Michael P. Busch,1 Tzung-Hae Lee,1 and Joseph M. McCune2

1Blood Systems Research Institute, San Francisco, California, USA

2Division of Experimental Medicine, Department of Medicine, University of California San Francisco, San Francisco, California, USA

3California National Primate Research Center and Departments of Pediatrics and Cell Biology and Human Anatomy, University of California Davis, Davis, California, USA
# TABLE OF CONTENTS

## CHAPTER I: Introduction
- HIV and AIDS ........................................................................................................ 1
- HIV and immune activation ....................................................................................... 1
- SIV and immune activation ......................................................................................... 4
- *In utero* tolerance .................................................................................................... 3
- The role of regulatory T cells (Tregs) ......................................................................... 4

## CHAPTER II: Maternal microchimerism is detectable in fetal rhesus macaques and can persist in tissues postnatally
- Abstract .................................................................................................................. 6
- Introduction ................................................................................................................ 7
- Methods .................................................................................................................... 8
- Results ...................................................................................................................... 12
- Discussion ................................................................................................................ 14
- Figures ....................................................................................................................... 17

## CHAPTER III: Fetal injection with SIVmac1A11 reduces viral loads after postnatal challenge and is associated with altered immunologic parameters
- Abstract .................................................................................................................. 24
- Introduction .............................................................................................................. 25
- Methods .................................................................................................................... 28
- Results ...................................................................................................................... 32
- Discussion ................................................................................................................ 37
- Figures ....................................................................................................................... 41

## CHAPTER IV: Conclusions and future directions
- Summary ................................................................................................................... 53

References .................................................................................................................. 55
LIST OF FIGURES AND TABLES

CHAPTER II

| Figure 1 | Performance of Mamu-MHC allele-specific real-time PCR assays used to identify microchimerism | 17 |
| Figure 2 | Analysis of maternal microchimerism in fetal rhesus monkey samples using informative Mamu-MHC allele assays | 19 |
| Figure 3 | Maternal microchimerism levels in prenatal and postnatal blood samples | 21 |
| Table 1  | Real-time PCR primer sequences and characteristics of amplicons used to identify informative alleles for microchimerism | 22 |
| Table 2  | Maternal microchimerism detected in prenatal and postnatal tissues | 23 |

CHAPTER III

| Figure 1 | Study design | 41 |
| Figure 2 | Animals exposed to SIVmac1A11 in utero have increased CD8 counts and elicit humoral, but not cellular responses prior to oral challenge | 42 |
| Figure 3 | Animals exposed to SIV in utero have lower RNA viral loads in association with various immunologic parameters | 43 |
| Figure 4 | Fetal exposure to SIVmac1A11 does not impact peripheral blood counts post-infection | 44 |
| Figure 5 | Animals exposed to SIVmac1A11 in utero have increased Th17 cells but not Tregs post-infection | 45 |
| Figure 6 | Ki67 expression patterns are altered on animals exposed to SIVmac1A11 in utero | 46 |
| Figure 7 | Ki67 intermediate cells are strongly inversely correlated with viral load at multiple time points post-infection | 48 |
| Figure 8 | Animals exposed to SIVmac1A11 in utero have reduced humoral but increased cellular responses post-infection | 50 |
| Figure 9 | Splenocytes from animals exposed to SIVmac1A11 in utero have lower expression of senescence markers associated with lower viral loads | 51 |
CHAPTER I: Introduction

HIV/AIDS

HIV/AIDS is a pandemic that is currently responsible for more than 2.5 million new infections and 2 million deaths per year, worldwide ("Progress Report 2011: Global HIV/AIDS Response," 2011). The most recent World Health Organization (WHO) data estimate that more than 33 million people are currently infected with HIV ("Progress Report 2011: Global HIV/AIDS Response," 2011). A hallmark of HIV disease is the progressive loss of peripheral CD4+ T cells, leading to a deficiency in T cell help and an inability of the host to mount effective immune responses, paving the way for opportunistic infection and significant morbidity and mortality.

HIV and immune activation

Activation of adaptive immune cells in response to many viral infections will lead to suppression of viral replication, elimination of infected host cells and eventual clearance of infection. In the context of HIV disease, it appears that instead the virus can use this response to propagate itself. In vitro studies indicate that while cellular activation is not required for initial infection of CD4+ T cells, the primary target of infection, activation is required for efficient integration of proviral DNA into the host chromosome and greatly increases viral production (Stevenson et al. 1990; Kinoshita et al. 1997). Studies of infected individuals have recapitulated this data in vivo, showing increased viral production in HIV-infected patients after routine vaccinations, presumably due to activation of adaptive immune cells in response to the vaccine (Ostrowski et al. 1997, 1998; Stanley et al. 1996). The persistence of antigen in infected individuals can drive the maturation of naïve T cells into an activated memory pool. As naïve cells begin to mature into memory cells they begin to express the chemokine receptor, CCR5, a co-receptor for HIV fusion and entry into target cells. Thus, immune activation can increase the pool of target cells for HIV infection (Lederman et al. 2006). Additionally, chronic activation of immune cells may increase their destruction at a rate that exceeds that of hematopoiesis, leading to a net loss of circulating cells available to control infection (McCune 2001; Jenkins et al. 1998; Marandin et al. 1996; Sodora and Silvestri 2008).

Chronic infection with HIV or SIV infection is also associated with damage to peripheral lymphoid organs. In particular, peripheral lymph nodes become small and fibrotic as T cell zones become filled with collagen and elastin fiber deposits (Estes 2013). Zheng et al., found that Tregs were partially to blame for this phenomenon as production of the cytokine, TGF-β1 by neighboring Tregs induced a signaling cascade that triggered the production of collagen from lymph node tissue fibroblasts. The authors propose a cycle in which chronic immune activation promotes an increase in the frequency of Tregs, which drives the deposition of collagen in lymph nodes, disrupts the fibroblastic reticular cell (FRC) network, and reduces IL-7 production from the FRC. Without access to the homeostatic cytokine, IL-7, naïve T cells die through apoptosis (Zeng et al. 2011). In support of this are reports of inverse associations between the amount of LN fibrosis and naïve CD4+ T cell frequencies (Schacker et al. 2006). Depletion of the pool of naïve T cells, both CD4+ and CD8+, may reduce the ability of the host to mount effective immune responses against opportunistic pathogens (Roederer et al. 1995).
Immune exhaustion is another consequence of chronic immune activation. Persistent stimulation of adaptive immune cells with circulating antigen can ultimately lead to their dysfunction in chronic infection. This is perhaps best demonstrated in mouse models of lymphocytic choriomeningitis virus (LCMV) where two strains of virus, differing by only a few amino acids, give rise to two distinct diseases. One (the Armstrong stain) is cleared within a week while another strain (clone 13) can establish a more persistent infection, lasting more than three months. Given the antigenic similarity of the two viruses, antigen-specific CD8\(^+\) T cell responses can be tracked using the same major histocompatibility complex (MHC) class I tetramer. In the acute model (Armstrong), antigen-specific CD8\(^+\) T cells can produce cytokines and proliferate rapidly in response to antigen stimulation in vitro. After two months of clone 13 infection antigen-specific CD8\(^+\) T cells do not proliferate well, produce significantly less cytokine in response to antigen stimulation, and are considered to be functionally “exhausted.”

The Programmed Death-1 (PD-1) receptor, is highly expressed on these exhausted cells and can be used as a marker of this phenotype (Allie et al. 2011; Barber et al. 2006; Blackburn et al. 2010; Duraiswamy et al. 2011). Other markers, including the T cell Immunoglobulin and Mucin domain containing 3 (Tim-3), and the lymphocyte-activation gene 3 (Lag 3), have been associated with an exhausted phenotype as well (Blackburn et al. 2009; Jin et al. 2010). Exhausted cells with similar phenotypes have been described during the chronic stages of HIV and SIV infection (Quigley et al. 2010; Trautmann et al. 2006). Importantly, interruption of signaling through these molecules has been shown to reverse the exhaustion observed in these cells and can restore their function (Dyavar Shetty et al. 2012; Jin et al. 2010; Trautmann et al. 2006; Trautmann, Chomont and Sékaly 2007).

Although the specific mechanism by which HIV may induce immune activation is not yet clear, many possibilities have been put forth. The persistence of high levels of circulating antigen is one potential explanation, as innate immune cells such as plasmacytoid dendritic cells (pDC) likely encounter and phagocytize many viral particles. Recognition of viral RNA by TLR7 in pDCs triggers the stimulation of adaptive immune responses and the release of pro-inflammatory cytokines such as IFN\(\alpha\) (O’Brien, Manches and Bhardwaj 2013). Other explanations include direct interaction of viral proteins with host machinery, as the HIV-1 protein Tat is known to bind NF-kB enhancer sequences, or indirect interactions such as recognition of viral proteins promoting the secretion of inflammatory mediators from neighboring immune cells (Sodora and Silvestri 2008; Dandekar, Ganesh and Mitra 2004).

Human cohort studies have demonstrated that untreated, HIV-infected individuals achieve a relatively constant level of immune activation early in infection and that this level of immune activation (the “activation set point”) can predict the rate of decline in CD4\(^+\) T cells independent of viral load (Deeks et al. 2004). Furthermore, this state of chronic immune activation is maintained in infected hosts despite effective suppression of viral replication via antiretroviral therapy and is inversely associated with CD4 count (Hunt et al. 2008). Using a transgenic mouse model in which B cells were generated to overexpress CD70, a ligand for the T cell costimulatory molecule CD27, Tesselaar et al. were able to establish a persistent state of T cell activation, which eventually lead to immunodeficiency and death from opportunistic infections, mirroring an AIDS-like syndrome in the absence of retroviral infection (Tesselaar et al. 2003). The role of cellular activation in HIV pathogenesis gained further attention in 2007 after the pharmaceutical company Merck halted a Phase IIb trial of a vaccine candidate.
engineered to elicit a robust T cell response to HIV-infected cells (Steinbrook 2007). Preliminary results indicated a higher infection rate in vaccinees than in the placebo arm, a finding that has been attributed to a stronger inflammatory response and higher levels of cellular activation in those receiving the vaccine, relative to controls (Pantaleo 2008).

SIV and immune activation

Reciprocally, studies of SIV, the most recent ancestor of HIV, in naturally infected African green monkeys (AGM) and sooty mangabeys (SM) have shown that these animals have low levels of cellular activation, can maintain CD4+ T cell counts and do not develop simian AIDS, despite relatively high viral loads (Silvestri 2008). In stark contrast to AGM and SM, rhesus macaques (RM) and pigtail macaques, two species not naturally infected with SIV, show a disease course very similar to that of humans with HIV infection, with high levels of cellular activation and progressive loss of CD4+ T cells (Brenchley, Silvestri and Douek 2010; Favre et al. 2009; Pandrea et al. 2007). Given these observations, it appears that the ability of the host to control activation during chronic infection may help preserve the integrity of the immune system and thus enhance the chances for survival, even in the face of ongoing viral replication.

In utero tolerance

How might control over immune activation arise? It appears that pregnancy is predominantly a period of immunosuppression. In 1911, the noted embryologist, Frank Lillie, reported odd sex ratios in non-identical twin cattle in which male/male twins were overrepresented relative to female/female twins. He found that the fusion of placental vasculature between heterosexual (male/female) twins caused the female to develop masculine-like gonads, and thus the pair were recorded as male/male twins (Lillie 1916). This fueled the study of “freemartin” cattle, which led, thirty years later, to the publication of a paper by Owen describing how this phenomenon of vascular fusion can have immunologic consequences. Owen found occasions in cattle in which bulls fathered offspring expressing antigens in their blood that bore little genetic similarity to antigens found in the blood of the bull. He went on to find that these bulls developed as non-identical twins and that antigens in the blood of the bull were identical to those found in the bull’s twin. He went on to propose that what had happened was that while the bull was in utero he and his twin had a vascular fusion event, of the kind described by Lillie, and that cells (probably hematopoietic stem cells) had been transferred from his twin, engrafted, and given rise to a blood system that was of a different genetic background to his own (Owen 1945). Even though these blood cells were not antigenically identical to his somatic cells, they were tolerated without consequence for the entirety of the bull’s life.

This idea, that non-identical cells could be tolerated if introduced early enough in gestation, set the stage for the seminal paper by Billingham, Brent and Medawar less than a decade later. The authors were able to demonstrate that mice of one strain (CBA) rapidly rejected a skin allograft from donors of a different genetic background (strain A) with a median time of rejection of about 11 days. If another graft from the A strain was attempted in the same CBA mouse within two months, this time of rejection decreased to less than six days. If, however, the experimenters injected A strain cells into a developing CBA fetus, when that CBA mouse grew up he was much more likely to accept skin allografts from an A strain donor. Importantly, these mice were still able to reject a skin graft from a third donor (AU strain) indicating that these mice were not simply globally immunosuppressed, but rather that antigen-specific tolerance had
developed in the CBA mouse towards A strain tissue (Billingham, Brent and Medawar 1953). A similar experiment was conducted in humans in 1959 whereby a pair of 21 year-old heterosexual twins was found to have cells circulating in their bloodstream from one another. It was reported that of the red blood cells circulating in the male twin’s bloodstream, 14% were of his sister’s predominate blood type. Additionally, he had a proportion of circulating leukocytes that bore only female sex chromosomes. The sister carried only about 1% red blood cells from her brother. Surprisingly, they each agreed to donate a skin graft to the other. Over the course of a year of follow-up neither graft showed signs of rejection (Woodruff and Lennox 1959).

Given that a developing fetus shares only half of its genetic identity with its mother, the fetus is akin to a transplanted semi-allograft in its mom. Similar to the observations of the Billingham experiment, this allograft can be well maintained. While the fetus and its mother are not vascullary fused, as described in twins by Lillie, we now know that both maternal and fetal cells are able to pass through the placental barrier and engraft into the tissues of the other where they can be well tolerated and persist for years; a phenomenon know as microchimerism (Bianchi et al. 1996; Jonsson et al. 2008; Lo et al. 1996; Maloney et al. 1999; Mikhail et al. 2008). The transit of fetal cells into maternal circulation is known as “fetal microchimerism” and the transit of maternal cells into fetal circulation is known as “maternal microchimerism.”

The role of Tregs

While tolerance is thought to be due, in part, to the elimination of alloreactive T and B cells by central deletion in the thymus and bone marrow, it appears that CD4+ Tregs are also essential. Tregs are central regulators of the immune system and have been described as a “rheostat” that controls the balance between an inflammatory response to foreign substances and protection from autoimmune diseases (Burt 2013). Tregs can functionally suppress effector immune responses through various mechanisms. Among these are: disrupting the interactions of conventional T cell (Tconv) priming by dendritic cells (DCs), starving Tconv of IL-2 and the essential amino acid tryptophan, and production of the immunosuppressive cytokine, IL-10 (Yamaguchi, Wing and Sakaguchi 2011). Expression of the transcription factor, FoxP3, has become both a phenotypic marker of these cells and has proven essential to their development and function (Ohkura, Kitagawa and Sakaguchi 2013). Defects in the expression of FoxP3 are associated with the development of the inflammatory syndrome, immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) that is fatal in human newborns (Powell, Buist and Stenzel 1982).

Tregs appear to also be essential for the maintenance of the fetus during pregnancy. In humans, Treg proportions increase during the late follicular phase of the menstrual cycle, peaking around the time of ovulation and implantation (Arruvito et al. 2007). Functionally suppressive Tregs in maternal circulation increase during the first two trimesters of pregnancy and decline in the third trimester (Somerset et al. 2004). Additionally, maternal Tregs have been shown to increase in abundance at the maternal-fetal interface during pregnancy (Schumacher et al. 2009). The essential role of Tregs during healthy pregnancy is perhaps best demonstrated by the fact that any disruption of this regulatory system can have serious or fatal consequences for the developing fetus. Reduced Treg frequencies or reduced Treg function have been associated with preeclampsia, placental lesions, and spontaneous abortion (Quinn and Parast 2013; Sasaki et al. 2004; Somerset et al. 2004; Steinborn et al. 2008).
This state of immunosuppression is mirrored on the fetal side as well (Michaëllson et al. 2006; Leslie 2008). Previous work from the McCune lab has demonstrated an active mechanism of antigen-specific suppression that acts to dampen fetal reactivity to maternal alloantigens (Mold et al. 2008). It was observed that transit of maternal cells into fetal circulation ("maternal microchimerism") was a relatively common occurrence (detectable in 15/18 fetal samples tested) and was important for inducing a population of fetal CD4⁺CD25⁺FoxP3⁺ Tregs that actively suppress fetal responses to non-inherited maternal alloantigens (NIMA).

The role of Tregs in HIV or SIV infection is not entirely clear, as data conflicts about their frequencies and contributions to disease, but it may be that these cells have a “split personality,” depending on the stage of disease. Early in infection, Tregs may be able to suppress immune activation, thus controlling the pool of activated, CCR5-expressing target cells, an hypothesis supported by the observation that highly exposed seronegative (HESN) sex-workers have higher Treg frequencies and lower levels of immune activation than seronegative controls (Chevalier and Weiss 2013). In more advanced disease, Treg frequencies may be insufficient to control activation and may instead contribute to pathology, by reducing antigen-specific antiviral responses and contributing to lymph node fibrosis, as previously discussed. In the context of maternal HIV infection, it is reasonable to hypothesize that the trafficking of HIV antigens (and even of HIV virions) into the fetal circulation would likewise induce an antigen-specific Treg response. Preliminary studies examining neonates who were exposed to HIV in utero but who remain uninfected revealed the presence of HIV-specific CD4⁺ and CD8⁺ T cells under the suppressive control of Tregs (Legrand et al. 2006). Should such Treg activity be confirmed, the induction of tolerance to HIV antigens could serve to suppress HIV-specific T and B cell responses, thereby dampening immune activation and inflammation, and reducing both viral replication rates and the immunopathology associated with chronic activation discussed above.

This line of thinking could potentially provide a novel strategy for vaccine design: whereas current vaccine candidates attempt to induce a sufficiently strong adaptive immune response to clear infection, this alternative approach would seek to suppress immunologic responses in a virus-specific manner, thereby preventing inflammation and immune activation that would otherwise drive viral replication and spread. In fact, a recent report of a tolerogenic vaccine in rhesus macaques demonstrated just this effect. By simultaneously administering an oral vaccine made up of whole, inactivated SIV in combination with a preparation of a bacterial strain (L. plantarum) that had previously been associated with tolerance induction, Lu et al. demonstrated reduced antigen-specific cellular responses to SIV antigens in vitro and complete protection from oral SIV challenge in 15 of 16 animals tested (Lu et al. 2012). While many questions still remain as to the mechanism of this protection, it remains an intriguing example of the role that tolerance might play in vaccinations.

The goals of this dissertation were two-fold: 1) to study maternal-fetal interactions in rhesus macaques and to see if mechanisms shown to influence maternal-fetal tolerance in humans (i.e. microchimerism) also exist in macaques, and 2) to study the impact of fetal exposure to virus in utero on the outcome of SIV infection. These goals are evaluated in the following chapters.
Chapter II: Maternal microchimerism is detectable in fetal rhesus macaques and can persist in tissues postnatally

Abstract

Although pregnancy-associated microchimerism is known to exist in humans, its clinical significance remains unclear. Fetal microchimerism has been documented in rhesus monkeys, but the trafficking and persistence of maternal cells in the monkey fetus and infant have not been fully explored. To investigate the frequency of maternal microchimerism in the rhesus monkey (Macaca mulatta), a real-time polymerase chain reaction (PCR) strategy was developed and validated targeting polymorphic major histocompatibility complex (MHC) gene sequences. Informative PCR assays were identified for 19 of 25 dams and their respective offspring. Analyses were performed on tissues (thymus, liver, spleen, lymph nodes and bone marrow) and peripheral blood mononuclear cell (PBMCs) samples collected prenatally and postnatally in a subset of animals. 7 of 19 monkeys had detectable maternal microchimerism in at least one compartment (range: 0.001-1.9% chimeric cells). In tissues, maternal microchimerism was found in 2 of 7 fetuses and 3 of 12 juveniles (1-1.5 years of age), and most of the animals that were positive had microchimeric cells in more than one tissue. Maternal microchimerism was detected in all fetal PBMC samples (4 of 4). These observations suggest that maternal microchimerism occurs in the rhesus monkey fetus and can persist in the tissues of some offspring after birth.
Introduction

During pregnancy, cells traffic bi-directionally by transplacental transport between the mother and the fetus (Gammmill and Nelson 2010). In humans, a small population of maternal cells can be detected in fetuses, representing maternal microchimerism (Hall et al. 1995; Maloney et al. 1999; Saadai et al. 2012). Conversely, the presence of fetal cells in the mother is known as fetal microchimerism (Schröder 1975; Herzenberg et al. 1979). It has been suggested that trafficking of fetal antigens in the maternal circulation is important for the establishment of maternal-fetal tolerance, thus ensuring full term pregnancies without rejection of the fetus (Erlebacher 2013). Fetal T cells are specifically nonresponsive to non-inherited maternal alloantigens through the action of Tregs (Mold et al. 2008). However, central tolerance mechanisms may also be involved in the non-responsiveness to non-inherited maternal alloantigens in chimeric subjects (Yunis et al. 2007). The bi-directional exchange of cells during pregnancy can have a lasting impact on tolerance, which has been shown in transplantation studies (Dutta and Burlingham 2011). Conversely, fetal microchimerism in humans has been associated with the development of autoimmune diseases such as scleroderma (Gammmill and Nelson 2010). Thus, the ability to accurately detect and quantify microchimeric cells may be informative for investigations of the biological significance of microchimerism from a developmental and transplantation perspective.

Detection of microchimerism has typically relied on assays that distinguish between the genetic material of maternal and fetal cells, e.g., sex chromosome-specific assays to allow discrimination between mothers and male offspring (Ariga et al. 2001; Bianchi et al. 1990; Gammmill and Nelson 2010). Alternatively, assays specific for polymorphic alleles in the major histocompatibility complex (MHC) or insertion-deletion (In-Del) polymorphisms have been used to detect microchimerism (Lambert et al. 2004; Lee et al. 1999, 2006). Techniques such as fluorescence *in-situ* hybridization, flow cytometry, or quantitative polymerase chain reaction (qPCR) allow for detection and measurement of microchimeric populations by cell counting and/or by comparison against known standards, with qPCR offering the advantage of higher throughput, better sensitivity, and reagent versatility.

Fetal microchimerism (the presence of fetal cells in maternal circulation) has been extensively described in humans and in rhesus monkeys (Jimenez and Tarantal 2003a, 2003b; Jimenez et al. 2005). Previous reports have clearly demonstrated the importance of rhesus monkeys for these studies because they directly parallel findings in humans and also provide an important translational model (Lo 2003). While the use of sensitive assays targeting the Y chromosome have proven highly informative for the assessment of fetal microchimerism and to confirm engraftment of cells post-transplantation, (Reitsma, Harrison and Pallavicini 1993; Zhao et al. 1999) there is a need for additional assays that can target a larger population of animals regardless of sex. Given the association between microchimerism and immune tolerance as well as the importance of the rhesus monkey as a model of human health and disease, we sought to develop a technique capable of detecting and quantifying maternal microchimerism in the rhesus monkey. In this study, a panel of qPCR assays was designed and validated based on genetic polymorphisms in rhesus monkey MHC loci.
Methods
Animals

All animal procedures conformed to the requirements of the Animal Welfare Act and protocols were approved prior to implementation by the Institutional Animal Care and Use Committee at the University of California, Davis. Normally cycling, adult female rhesus monkeys (Macaca mulatta) (N=25) with a history of prior pregnancy were selected based on negative testing for Mamu-A*01, A*02, B*08, and B*17 alleles, and bred with males that were positive for Mamu-A*01 and A*02 alleles and negative for B*08 and B*17 alleles. Pregnancies were time-mated using established protocols and detected by ultrasound (Tarantal 2005). Pregnancy in the rhesus monkey is divided into trimesters by 55-day increments, with 0-55 days gestation representing the first trimester, 56-110 days gestation representing the second trimester, and 111-165 days gestation the third trimester (term 165±10 days) (Tarantal and Gargosky 1995). Activities related to animal care (e.g., diet and housing) were performed according to California National Primate Research Center standard operating procedures (SOPs). Fetal growth and development were monitored by ultrasound, fetal blood was collected under ultrasound guidance, (Tarantal 2005) and maternal blood samples were collected during gestation and at pregnancy termination. Hysterotomies were performed (N=10) to collect fetal tissues, using established protocols (Jimenez et al. 2005) based on study assignment, and included controls (N=4) or fetuses of dams that had been administered nonpathogenic SIVmac1A11 intravenously in the late first trimester (N=6). Fetal body weights and measures were assessed after the collection of fetal blood, then all organs including the brain, lung, heart, thymus, spleen, liver, lymph nodes (axillary, inguinal, mesenteric), pancreas, adrenals, kidneys, reproductive tract including gonads, gastrointestinal tract (stomach, duodenum, jejunum, ileum, colon), skin, muscle, and bone marrow were removed and select organs weighed. A placental evaluation was also performed, and sections of the umbilical cord, membranes, decidua, and placenta obtained. Cell suspensions from thymus, liver, spleen, lymph nodes, and bone marrow were prepared according to established protocols (Jimenez et al. 2005).

Cesarean-sections were also performed at term (N=15) using established methods and included collection of umbilical cord blood and a placental evaluation (Tarantal et al. 2000). Newborns were raised in the nursery according to established SOPs, with blood samples collected from a peripheral vessel at defined time points (weekly or monthly) under ketamine sedation (10 mg/kg) (Tarantal et al. 2005). PBMCs were purified from whole blood (dam, fetus, infant/juvenile) by density centrifugation over a Histopaque gradient (Sigma, St Louis, MO). Isolated cells were subsequently cryopreserved in fetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO) prior to DNA extraction. Of these 15 animals, seven were fetal controls and received either pathogenic SIVmac239 or SIVmac251 postnatally (as part of a parallel series of studies). For five, either the dam or the fetus was administered nonpathogenic SIVmac1A11 in the late first trimester, and three were administered a lentiviral vector prenatally and SIVmac251 postnatally. Tissue harvests were performed according to established protocols at ~1 to 1.5 years postnatal age and five collected tissues were analyzed from each animal (thymus, liver, spleen, mesenteric lymph nodes, bone marrow). Tissues were collected and weighed as described above, and cell suspensions prepared according to established protocols as noted.
DNA extraction for PCR genotyping

An estimated $5 \times 10^6$ cells were washed in 1 ml PBS and digested in 500 µl of PCR solution (100 mM KCl, 10 mM Tris pH 8.3, 2.5 mM MgCl$_2$, 1% Tween-20, 1% NP40) and 62.5 µg of proteinase K (Qiagen Inc., Valencia, CA) at 60°C for 1.5 hours. Proteinase K was inactivated by heating at 95°C for 30 minutes. For typing reactions, genomic DNA was diluted 1:50 in PCR solution to a concentration of approximately 1000 genomic equivalents/5 µl.

Design of Mamu-MHC allele-specific real-time PCR assays

Four A locus primers were designed based on polymorphic regions in published Mamu-A gene sequences (Lobashevsky and Thomas 2000). Likewise, 10 B locus primers were designed based on polymorphic regions in Mamu-B gene sequences included within the Immuno Polymorphism Database (IPD-MHC Non-Human Primates (NHP): the Immuno Polymorphism Database. 2010 ; Robinson et al. 2013). All A and B locus primers are located within the highly variable second and third exons. The A locus primers were combined to form two assays and the B locus primers were combined to form six assays, each of which targets a single MHC class I allele. The real-time PCR primer sequences and amplicon characteristics (e.g., size and dissociation temperature) are shown in Table 1. An additional four assays based on previously published reports were included to form a screening panel consisting of 12 MHC allele-specific qPCR assays and one GAPDH reference assay. In addition to the screening panel, we performed sequence-based typing of Mamu-DQB for each animal in these studies and designed qPCR assays specific for the non-inherited DQB maternal allele for 12 maternal-fetal pairs (Table 1).

Identification of informative alleles for maternal microchimerism by real-time PCR

To determine which assays were informative for detection of maternal microchimerism, the MHC allele-specific qPCR panel was used to genotype samples from mother-fetal rhesus monkey pairs. 5 µl of the diluted DNA lysate were added to 10 µl of PCR buffer containing 5 mM MgCl$_2$, 1 mM dNTPs (Bioline USA Inc., Taunton, MA), 1 µM of each primer (Integrated DNA Technologies, Coralville, IA), 0.25X SYBR Green I (Invitrogen, Carlsbad, CA), and 0.7 U FastStart Taq (Roche Applied Science, Indianapolis, IN). As described below in the Results section, eight Mamu-A and Mamu-B assays and 12 Mamu-DQB assays were designed. In addition, we also used techniques kindly provided by Dr. David Watkins, Department of Pathology, University of Miami Miller School of Medicine, as well as published assays (Marcondes et al. 2007; Otting et al. 2005; Vigon and Sauermann 2002). GAPDH primers were used as a loading control reference. Real-time PCR was performed in a 384-well format on a sequence detection system (LightCycler 480 II, Roche) with the following cycle conditions: 1 minute at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 45 seconds at 72°C, with a melting curve analysis at the end of the reaction. Once informative alleles were identified, 10-fold serial dilutions of the DNA lysate were amplified in a 96-well format using different annealing temperatures ranging from 56-70°C. Optimal annealing temperatures were chosen for each assay based on increased specificity at higher temperatures without a loss in sensitivity.

Identification of informative alleles for maternal microchimerism

To determine which assays were informative for detection of maternal microchimerism, the MHC allele-specific qPCR screening panel was used to genotype samples from mother-fetus rhesus monkey pairs. Any weak maternal-specific signal detected in the fetus was considered as
possibly derived from microchimeric cells that crossed the placenta from the dam into the fetus. An assay was defined as “informative” for maternal microchimerism if a Ct between 20 and 30 was detected in the maternal sample and if the Ct of the fetal sample was either undetectable or at least five cycles higher than that of the maternal sample for equivalent maternal and fetal DNA input. Examples of informative and non-informative alleles identified by qPCR genotyping are presented in Figure 1Ci-ii, respectively.

**PCR analysis and quantification**

To quantify the level of maternal microchimerism, fetal DNA and 10-fold serial dilutions of maternal DNA samples diluted in PCR solution were amplified with primers for informative allele(s) and GAPDH. Quantification of maternal microchimeric cells in fetal DNA was calculated using the ΔCt method with GAPDH as a reference gene. The specificity of the amplified products in the fetal samples was determined by melting curve analysis and gel electrophoresis in comparison to amplified products in the maternal samples.

**Specificity and sensitivity of Mamu-MHC assays**

To determine whether microchimerism could be detected by the Mamu-MHC assays, the primers were used to screen rhesus monkey DNA from at least five different animals. As shown by the melting curve analysis from one representative assay for Mamu-A1*003 (Figure 1A), the primer pairs were informative for distinguishing between rhesus monkey samples known to be positive or negative for this allele. The predicted sizes of the amplified products were verified on an ethidium-stained agarose gel (data not shown). Amplification of serial dilutions of DNA with the Mamu-MHC allele-specific assays showed linearity over at least four orders of magnitude. The linear dynamic range for the reference assay for GAPDH and a representative allele-specific MHC PCR assay (designed to target B*047) are shown in Figure 1B. To determine the limit of detection of maternal DNA in a fetal sample, serial dilutions of rhesus macaque DNA positive for the targeted MHC allele were spiked into a fixed amount of DNA negative for the targeted allele. The sensitivity of the Mamu-MHC allele-specific PCR assays ranged from 0.001% to 0.05% (data not shown).

**Sequencing of PCR products**

35 µl of the PCR product were electrophoresed in a 2% agarose gel containing ethidium bromide. The band was excised and purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA). The purified PCR product was eluted into 30 µl of water and 20 µl was submitted with the 5’ and 3’ primers used during amplification for automated sequencing (MCLAB, South San Francisco, CA). Alternatively, the PCR product was directly purified using a QIAquick PCR purification kit prior to sequencing with the amplification primers. For amplicons shorter than 100 bp, the PCR product was cloned into pCR™4-TOPO® using the TOPO® TA Cloning® kit for sequencing (Life Technologies, Carlsbad, CA) and for transformation of One Shot® TOP10 chemically competent E. coli. Transformants were analyzed by PCR using M13 Forward(-20) and M13 Reverse primers and visualized by agarose gel electrophoresis. Amplicons of the correct size were purified using a QIAquick PCR purification kit prior to sequencing with the M13 primers. Sequence-based typing of Mamu-DQB1 was performed by amplifying exon 2 (270 bp) using the primers DQBF (5’-TCCCCGCAGAGGATTTGCTG-3’) and DQB1*18R (5’-CGCTCACCCTGCCTGCTGCA-3’) and sequencing the PCR products using the amplification primers. Sequence analysis was performed using Sequencher® version 5.0 software (Gene Codes
Corporation, Ann Arbor, MI). Mamu-DQB sequences for each maternal-fetal pair were aligned and primers specific for the non-inherited maternal DQB allele were designed such that there was a 1-3 nucleotide mismatch at each primer 3’ end relative to the fetal DQB sequences.
**Results**

**Analysis of maternal microchimerism levels using Mamu-MHC allele-specific qPCR assays**

A total of 25 monkeys were initially selected for maternal microchimerism testing. Six animals were excluded from analysis because maternal allele-specific qPCR assays could not be identified. Microchimerism analysis was done on DNA extracted from tissues (thymus, liver, spleen, lymph nodes and bone marrow) collected prenatally for seven animals and postnatally for 12 animals. In the latter group, testing was also done on DNA extracted from PBMCs collected both prenatally and postnatally for four animals, whereas for the remaining eight animals, testing was done on DNA extracted from PBMCs collected only at or after birth.

Each maternal-fetal pair was first genotyped using a panel of Mamu-MHC allele-specific qPCR assays (see Methods) to identify assays informative for investigating maternal microchimerism. Subsequently, the fetal DNA sample (containing at least $10^5$ genomic equivalents of input DNA) and three 10-fold serial dilutions of maternal DNA were amplified in parallel using the assays for the informative MHC alleles and for GAPDH. Melting curve analysis and gel electrophoresis were used to verify that any signal detected was specific in both fetal and maternal DNA samples, and not due to nonspecific products or primer dimer artifacts. Results for a representative assay from one pair are shown in Figure 2A. The GAPDH assay Ct for each maternal sample dilution was plotted against the MHC informative allele assay Ct. A standard curve was generated and analyzed by performing linear regression. For the example shown in Figure 2B, the frequency of the maternal allele within the fetal sample, calculated using the $\Delta$Ct method, was $2^{10.6}$ or 0.06%. To further verify specificity of the signal detected in the fetal sample, the PCR products amplified from the maternal and fetal DNA samples were purified and sequenced. The sequence obtained from the fetal sample was identical to that from the maternal sample (Figure 2C).

**Detection of maternal microchimerism in fetal rhesus monkey tissues**

Ten maternal-fetal pairs were screened to identify informative alleles to quantify the level of maternal microchimerism in DNA extracted from fetal tissues, as described above. Three pairs were excluded from analysis because an informative allele assay could not be identified. The remaining seven animals were tested for maternal microchimerism in the thymus, spleen, liver, mesenteric lymph nodes, and bone marrow. In some cases, more than one informative allele was identified. In each case where maternal microchimerism was detected, direct sequencing of the PCR product (for informative allele assays yielding a product longer than 100 bp) or cloning followed by sequencing (for short amplicon assays) was used to confirm the 100% identity of the PCR product detected in the fetal sample relative to the product amplified from the maternal sample. Furthermore, in some cases, paternal DNA was also amplified and sequenced in parallel using the informative allele assays to rule out the possibility that weak amplification of the inherited paternal allele in the fetal DNA was misinterpreted as maternal microchimerism. In those cases, paternal alleles were clearly distinguishable from maternal alleles by at least six nucleotide mismatches (data not shown).

A summary of maternal microchimerism levels in fetal tissues is presented in Table 2. Two of seven animals tested had detectable levels of microchimerism in at least one of the tissues analyzed (Table 2): in one animal (#2), microchimerism was detected in all five tissues with a range of 0.005% to 0.07%; in another, (#6) a low-level positive result near the limit of
detection was identified in the bone marrow in one of three independent experiments, each using two different assays, suggesting that a single copy or small number of copies of the target sequence was present in the aliquots tested for that animal. Within the limits of detection of the assays (ranging from 0.001% to 0.05%), no maternal microchimerism was detected in five animals. Importantly, a similar number of cells were tested for each sample. Several of the informative allele-specific PCR assays demonstrated cross-amplification to a minor extent of MHC alleles in the fetal DNA other than the targeted non-inherited maternal alleles, despite amplification at relatively high annealing temperatures (Table 2). Sequencing of the amplified products allowed discrimination between maternal-specific DNA and weak cross-reactivity to fetal DNA.

**Transient maternal microchimerism levels in rhesus monkey PBMCs**

Fifteen rhesus monkey maternal-infant/juvenile pairs were screened for informative alleles that could allow quantification of maternal microchimerism in DNA extracted from PBMCs. After exclusion of three pairs where an informative allele assay could not be identified, a total of 12 animals were tested for maternal microchimerism in PBMCs at different time points. Fetal blood collected under ultrasound guidance in the early third trimester was included from four animals for microchimerism analysis. At this time point, maternal microchimerism ranging from 0.001% to 0.04% was detected in PBMCs of all fetuses (Figure 3). No microchimerism was detected in any of these animals at birth in PBMCs from umbilical cord blood or postnatally. Eight animals were tested for maternal microchimerism in PBMCs at birth and subsequent postnatal time points. No microchimerism was detected in these eight animals at any of the time points assessed (data not shown).

**Persistence of maternal microchimerism in rhesus monkey tissues postnatally**

Twelve animals assessed for microchimerism in PBMCs were also analyzed for maternal microchimerism in tissues at ~1 to 1.5 years of age (i.e., thymus, spleen, liver, mesenteric lymph nodes, and bone marrow). Maternal microchimerism was detected in at least one tissue in three out of 12 animals assessed, ranging from 0.004% to 1.9% (Table 2). Microchimerism levels and tissue distribution varied widely between each of the three animals.
Discussion

The results of this study suggest that it is possible to successfully quantify maternal microchimerism via an MHC allele-specific qPCR assay in rhesus monkeys. In many cases, multiple informative alleles were identified, increasing the likelihood of identifying primer pairs capable of detecting low levels of microchimerism with a high level of confidence.

In humans, microchimerism analysis based on HLA assays rely on initial genotyping of family members to identify non-inherited informative alleles, often using well-characterized sequence-specific oligonucleotide methods on commercial platforms (Chan et al. 2012). In rhesus monkeys, there are as many as 22 active MHC class I genes (compared to six in humans) and 10-fold higher sequence divergence in MHC relative to humans (Daza-Vamenta et al. 2004). This complexity renders MHC typing of rhesus monkeys difficult with current methodologies. A recent report described the use of massively parallel pyrosequencing for macaque MHC genotyping (Wiseman et al. 2009) although the widespread adoption of this technique may be limited because of the current cost. Despite the complexity of the macaque MHC, this study shows that a panel of 24 real-time allele-specific PCR assays targeting rhesus monkey MHC can be applied to analyze maternal microchimerism in this species.

In some cases, the qPCR assays used to analyze microchimerism could cross-react with an inherited allele in the fetal sample at low efficiency, leading to misinterpretation of the level of microchimerism if the cross-reactive product had the same dissociation curve profile as the targeted non-inherited maternal allele. These cases could be ruled out as bona fide microchimerism by DNA sequencing. Each informative allele-specific qPCR assay was tested at several annealing temperatures to identify the optimal conditions for increased specificity at higher temperatures without a loss in sensitivity. While specificity could be further enhanced by introduction of an internal probe within the PCR product, in this study SYBR Green chemistry was included due to cost considerations.

Reports of chimerism in the setting of transfusion or transplantation rely on testing of a sample derived from the patient prior to treatment (Lee et al. 1999) in order to demonstrate specificity through the absence of signal amplified using the informative assay. Unfortunately, this type of control sample does not exist for analysis of maternal microchimerism. Instead, in some cases, paternal DNA was used as a control to demonstrate specificity of the assays for detecting maternal microchimerism. The use of this control was only informative for cases where the paternal DNA did not share the targeted non-inherited maternal allele. In other cases, where misinterpretation of maternal microchimerism could be due to weak cross-amplification of the inherited paternal or maternal allele, an appropriate control could not be identified. Furthermore, studies have failed to identify tissues that are consistently negative for microchimerism(Su et al. 2008), precluding their use as a control for specificity. In fact, due to the persistence and variety of cell types identified as microchimeric in many studies (Bianchi et al. 1996; Lee et al. 1999; Maloney et al. 1999) it is likely that cell trafficking between mother and fetus includes stem and progenitor cells that can populate many different tissues. One strategy to obtain an animal-specific negative control sample for maternal microchimerism may be to clone population of fetal cells in tissue culture by limiting dilution, although this approach is labor-intensive and exceeds the level of confidence described in most maternal microchimerism studies. Alternatively, droplet-based digital PCR technology may allow discrimination of maternal
microchimeric DNA from background fetal DNA and accurate quantification of microchimerism.

A limitation of our method is that microchimerism can only be detected in the setting of maternal disparity at one or more target alleles. This method, by its very nature of employing non-inherited MHC polymorphisms, cannot detect microchimerism if the dam is homozygous at the targeted MHC locus or if the maternal-fetal histocompatibility relationship is isogenic. Since the MHC plays a critical role in regulating immune tolerance, it seems likely that the potential for microchimeric cells to engraft could be influenced by the context of the MHC relationship shared between mother and fetus. In fact, studies in mice have shown that the pattern of maternal-fetal histocompatibility was associated with differences in the microchimerism levels detected (Bonney and Matzinger 1997; Kaplan and Land 2005).

Analysis of microchimerism in mice has been facilitated by the availability of transgenic strains whose cells can easily be tracked in a large background of wild-type cells by amplification of the transgene, such as green fluorescent protein (GFP), (Khosrotehrani et al. 2005) neomycin resistance, (Kaplan and Land 2005) or luciferase (Su et al. 2008). This approach, however, is not currently feasible in the rhesus monkey model. Non-MHC polymorphisms have been used to identify chimerism in humans, such as the use of insertion-deletion (Alizadeh et al. 2002) and single-nucleotide polymorphisms, (Lo et al. 1996; Maas et al. 2003) and Y-chromosome assays (Bianchi et al. 1996). The sequencing of the rhesus macaque genome and development of databases for genomic data analysis (Gibbs et al. 2007; Malhi et al. 2007) provide valuable resources for future design of non-MHC based PCR assays informative for detection of microchimerism in monkeys.

Previous work in humans and animal models has shown that pregnancy-associated microchimerism is a common event. In humans, maternal microchimerism was observed in 15 of 18 fetal lymph node samples tested (Mold et al. 2008) while in mice, maternal microchimerism was seen in 51 of 60 animals in various organs within three weeks after birth (Su et al. 2008). It is important to note that the mouse has a yolk sac placenta which differs from the placental structure of human and nonhuman primates (Ramsey, Houston and Harris 1976). In our study, 7 of 19 monkeys with informative alleles were positive for maternal microchimerism in at least one of the compartments tested, a considerably lower fraction. Human data also suggests that maternal cells may be found in a variety of fetal tissues (Jonsson et al. 2008). Of the two rhesus monkey fetuses that had detectable microchimerism, one showed the presence of maternal DNA in 5/5 tissues evaluated, indicating wide distribution.

It is possible that the assays employed may be underestimating the frequency of maternal microchimerism in rhesus monkeys due to the difficulty of detecting microchimerism using MHC assays as a result of the relative complexity of the MHC locus in macaques as compared to humans. Another possibility is that the number of cells used or the time points selected for screening in this study limits the ability to detect more prevalent microchimerism in monkeys. As for the level of maternal microchimerism previously reported within a sample, it was shown to range from 0.0035-0.83% in human fetal lymph nodes (Mold et al. 2008) and averaged around 0.16% in mice (Su et al. 2008). Furthermore, in rhesus monkeys, fetal cells have been detected in the CD34+ fraction of maternal blood at an average level of 0.009% (Jimenez et al. 2005). The
level of maternal microchimerism detected in the current study falls within the lower end of the range reported in these prior studies in regard to fetal monkey tissues, although results were higher in postnatal monkey tissues. It is possible that exposure to SIV in utero (described in the Methods) may have skewed the measurement of microchimerism reported here by altering the relative abundance of immune cells in response to circulating virus. A couple of observations refute this: 1) CBC results from infected dams did not indicate any alterations in peripheral blood counts (data not shown), 2) animal #9 was not administered virus in utero yet had maternal microchimerism measurements in peripheral blood within the range of animals that were exposed (0.0153% vs 0.0011-0.0389%, respectively). This implies that fetal exposure to virus in utero did not impact the levels of microchimerism, a finding consistent with a study of maternal infection in pigs (Karniyuchuk et al. 2012). Indeed, it would have been interesting to examine the impact of a retroviral infection on the transfer of cells across the placenta. Unfortunately, the relatively low number of animals with detectable microchimerism did not allow us to draw a conclusion regarding the impact of maternal or fetal SIV on the levels of chimeric cells.

Finally, it has been shown that maternal microchimeric cells can engraft into fetal tissues and persist for decades in humans (Maloney et al. 1999). Additionally, fetal progenitor cells have been shown to persist in the tissues of female rhesus monkeys for many years post-delivery (Jimenez et al. 2005). Our study suggests that maternal microchimerism in peripheral blood of rhesus monkeys is may only be detectable during gestation, comparable to fetal microchimerism. Tissue analysis confirmed that maternal microchimerism does persist.

In conclusion, these studies have demonstrated that a panel of MHC sequence-specific qPCR assays can be readily applied to the detection of pregnancy-associated maternal microchimerism in rhesus monkeys, and that maternal microchimerism can be detected in postnatal tissues up to ~1.5 years of age. This method expands the current toolkit available for studies of maternal-fetal cell trafficking in this important translational model of human health and disease.
Figure 1. Performance of Mamu-MHC allele-specific real-time PCR assays used to identify microchimerism. (A) Samples from five different monkeys were amplified using A03-113F + A03-249R as a representative Mamu-MHC assay. Dissociation curves are shown for one animal positive for the target sequence (solid black curve) and one animal negative for the target sequence (dashed gray curve). (B) Four 10-fold serial dilutions of rhesus monkey DNA were spiked in PCR buffer (for GAPDH amplification) or in background rhesus monkey DNA negative for the target sequence (for MHC amplification). Standard curves are shown for GAPDH (used to control for total genomic input) and a representative Mamu-MHC assay. Amplification efficiency (Eff) was calculated based on the slope of the standard curve. (C) DNA from maternal-fetal monkey pairs was amplified with the assays shown in Table 1. Amplification curves are shown for GAPDH (in gray) to control for genomic input. Maternal samples are shown by solid lines and fetal samples are indicated by dashed lines. Examples of informative (Ci) and non-informative (Cii) allele assays for detection of maternal microchimerism are shown.
in black. The maternal sample is positive for the target sequence whereas the fetal sample may be positive for maternal microchimerism (Ci) or for the target sequence (Cii).
Figure 2. Analysis of maternal microchimerism in fetal rhesus monkey samples using informative Mamu-MHC allele assays. (A) Three 10-fold serial dilutions of maternal DNA (solid lines) were amplified with the informative alleles identified during the initial screening. GAPDH was amplified to control for genomic input. In parallel, a concentration of fetal DNA equivalent to that used in the intermediate diluted maternal sample (dashed line) was amplified using the same assays. The specificity of the maternal microchimeric signal detected in the fetal sample was verified by dissociation analysis (right panels). (B) The Ct values from amplification of the maternal dilutions with the informative allele and GAPDH assays (circles) were plotted to create a standard curve used to calculate the level of maternal microchimerism in the fetal sample (square). Here there is a 10.6 cycle difference between the informative allele signal detected in the fetal sample and the extrapolated signal for an equivalent genomic input of maternal sample. The calculated maternal microchimerism level is $2^{-10.6} = 0.06\%$. (C) The products amplified from maternal and fetal DNA were purified and sequenced using the
amplification primers. The sequence obtained from the fetal sample was identical to the sequence obtained from the maternal sample.
Figure 3. Maternal microchimerism levels in prenatal and postnatal blood samples. Blood samples collected from four fetuses during the third trimester and at select postnatal time points were tested by real-time PCR assays for non-inherited maternal MHC alleles. The dashed horizontal line indicates that no maternal microchimerism was detected. The dashed vertical line represents birth. At least two independent experiments were performed for each sample. Results are shown as the mean; error bars indicate the standard deviation. When more than one informative assay was identified, the maternal microchimerism values were averaged across assays for each sample.
Table 1. Real-time PCR primer sequences and characteristics of amplicons used to identify informative alleles for microchimerism

<table>
<thead>
<tr>
<th>Real-time PCR assay</th>
<th>Forward primer sequence (5'→3')</th>
<th>Reverse primer sequence (5'→3')</th>
<th>Amplicon size, bp</th>
<th>Dissociation temp, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC Class I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A03</td>
<td>MHC Class I A03-113F + A03-249R</td>
<td>GCGACGCCGAGAGTCCGAGAGA</td>
<td>137</td>
<td>92.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCCGCGCAGGTTCCGCAGGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A04</td>
<td>MHC Class I A04-271F + A02-487R</td>
<td>GGGTCTCACACCTACCAGGT</td>
<td>217</td>
<td>93.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CGCCCTCCAGGTAGGTTCTGTGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B42</td>
<td>MHC Class I B42-206F + B30-255R</td>
<td>GACGCCGAGAGTCCGAGGAT</td>
<td>88</td>
<td>9.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCGTGTTCGTGTCTGCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B0</td>
<td>MHC Class I B0-206F + B0-278R</td>
<td>GACGCCGCGAGTCCGAGAGA</td>
<td>111</td>
<td>90.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCGGTCAGTCTGTGCCTGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B46</td>
<td>MHC Class I B46-414F + B24-536R</td>
<td>GCCTCCTCCGCGGGTACCGG</td>
<td>161</td>
<td>92.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTCCAGGTAGGCTCTGAACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B24</td>
<td>MHC Class I B24-540F + B28-600R</td>
<td>ACCGTTATGCGGAGCGGTTC</td>
<td>80</td>
<td>88.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTTCCCGTTCTCCAGGTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A4<em>14F + A4</em>14R</td>
<td></td>
<td>GGGAACCCGACGGGCGCCTCCAA</td>
<td>179</td>
<td>92.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGCCCTCCAGGTAGACTCTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHC Class II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPB1<em>06F + DPB1</em>06R</td>
<td></td>
<td>ATCTACAATCGGGAAGAGTACGT</td>
<td>172</td>
<td>90.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCTCGTCCAGTTCGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTTGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For1 + rev51</td>
<td></td>
<td>CCTGTGCTACTTCACCAACG</td>
<td>252</td>
<td>92.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACCTCGTAGTTGTGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For1 + 1802rev1</td>
<td></td>
<td>CCTGTGCTACTTCACCAACG</td>
<td>267</td>
<td>92.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGTCGTGCGGAGCTCTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1*06:01 F154 + R285</td>
<td></td>
<td>TGGACGGAGCGCGTGCGTTA</td>
<td>132</td>
<td>90.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCTGTTCCAGTACTCGGCGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1*06:01 F278 + R379</td>
<td></td>
<td>GGAACAGCCAGAAGGACGTG</td>
<td>102</td>
<td>88.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTCTCCTCTGCAAGATCCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1*06:02 F241 + R374</td>
<td></td>
<td>GCGGTGACGCCGCAGGGGCG</td>
<td>134</td>
<td>91.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTCTGCAAGATCCGGCGGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1*15:01 F1 + R2</td>
<td></td>
<td>GAGCGCGTGTGGAGTGTAGA</td>
<td>124</td>
<td>90.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGTTCCAGTACTCGGCACTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1*16:03 F41 + R99</td>
<td></td>
<td>AGAGCGCGTGCGGTTAGTAG</td>
<td>59</td>
<td>86.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CGAAGCGCGCGAACTCTTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1*18:01 F2 + R2</td>
<td></td>
<td>GCGGTGACGCCGCAGGGGCA</td>
<td>98</td>
<td>89.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTATGCACACCGTGTCCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1*18:02 F208 + R285</td>
<td></td>
<td>GTGCGCTTCGACAGCGACTG</td>
<td>78</td>
<td>92.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCTGTTCCAGTACTCTGCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1*18:04 F172 + R268</td>
<td></td>
<td>GTTGTGACCAGACACATTTC</td>
<td>97</td>
<td>89.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CGTCAGGTCGCCCCAGCGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1*18:11 F248 + R335</td>
<td></td>
<td>CGCCGCTGGGGCGGTCTTGG</td>
<td>88</td>
<td>88.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTGCACACCGTGTCCACCGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1*18:17 F1 + R2</td>
<td></td>
<td>GTCTTGTGACCAGACACGTT</td>
<td>166</td>
<td>92.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTGCACACCGTGTCCACCGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1*18:17 F45 + R172</td>
<td></td>
<td>GGGACGGAGCGCGTGCGTCT</td>
<td>128</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTCCAGTACTCGGCGCTAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQB1*18:22 F192 + R270</td>
<td></td>
<td>GAGAGCACCCGGGCGGAGGC</td>
<td>79</td>
<td>89.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTCGCCGCTGCAACGTCGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH + GAPDH</td>
<td></td>
<td>TATCGACCGGCCGCACGGT</td>
<td>105</td>
<td>86.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GACGAGCACTTGGCGTGGATACG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The sequences of these primers were taken from Otting et al. (Otting et al. 2005)
†The sequences of these primers were provided by Dr. David Watkins
‡The sequences of these primers were taken from Vigon and Sauermann (Vigon and Sauermann 2002)
§The sequences of these primers were taken from Marcondes et al. (Marcondes et al. 2007)
Table 2. Maternal microchimerism detected in prenatal and postnatal tissues

<table>
<thead>
<tr>
<th>Animal #</th>
<th>Sample</th>
<th>Informative allele assay</th>
<th>Maternal microchimerism, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BM</td>
<td>LIV</td>
<td>0.0683 ±0.0459</td>
</tr>
<tr>
<td>2</td>
<td>BM</td>
<td>LN</td>
<td>0.0683 ±0.0459</td>
</tr>
<tr>
<td>3</td>
<td>BM</td>
<td>SP</td>
<td>0.0384 ±0.02</td>
</tr>
<tr>
<td>4</td>
<td>BM</td>
<td>LN</td>
<td>0.0078 ±0.0056</td>
</tr>
<tr>
<td>5</td>
<td>BM</td>
<td>SP</td>
<td>0.0046 ±0.0015</td>
</tr>
<tr>
<td>6</td>
<td>BM</td>
<td>LN</td>
<td>0.0042 ±0.0015</td>
</tr>
<tr>
<td>7</td>
<td>BM</td>
<td>LN</td>
<td>0.0042 ±0.0015</td>
</tr>
<tr>
<td>8</td>
<td>BM</td>
<td>LN</td>
<td>0.0042 ±0.0015</td>
</tr>
<tr>
<td>9</td>
<td>BM</td>
<td>LN</td>
<td>0.0042 ±0.0015</td>
</tr>
<tr>
<td>10</td>
<td>BM</td>
<td>LN</td>
<td>0.0042 ±0.0015</td>
</tr>
<tr>
<td>11</td>
<td>BM</td>
<td>SP</td>
<td>0.0042 ±0.0015</td>
</tr>
</tbody>
</table>

BM=bone marrow; LIV=liver; THY=thymus; LN=mesenteric lymph nodes; Sp=spleen; NS=non-specific; and ND=not determined.

†Limit of detection 0.05% calculated by spiking 10-fold serial dilutions of maternal DNA in background fetal DNA negative for the target allele.

‡Non-specific signal detected, determined by sequencing PCR products from maternal and fetal DNA. At least two independent experiments were performed for each sample. Results are shown as mean ± standard deviation.

§Limit of detection 0.005%.

║Limit of detection 0.03%.

¶Limit of detection 0.003%.

#Limit of detection 0.02%.

**Detectable in 1 of 3 independent experiments (and shown by sequencing to be a true positive,+).

††Limit of detection 0.001%.
CHAPTER III: Fetal injection with SIVmac1A11 reduces viral loads after postnatal challenge and is associated with altered immunologic parameters

Abstract

HIV disease appears to be driven by chronic immune activation. Given observations that fetal exposure to infectious pathogens in utero can lead to reduced antigen-specific responses, or tolerance, to those pathogens postnatally, we hypothesized that fetal exposure to HIV may tolerize the fetus to the virus, thus reducing damage caused by immune activation after exposure later in life. To test this hypothesis, fetal rhesus macaque (Macaca mulatta) were injected with the attenuated virus SIVmac1A11 during the late first trimester of gestation. Animals were then challenged with pathogenic SIV one year after birth. SIVmac1A11 injected animals had significantly reduced viral loads (p<0.02) up to 33 weeks post-infection. Differences in viral load were correlated against a panel of immunologic parameters. Animals with reduced viral loads appeared to have enhanced, not suppressed, SIV-specific cellular responses, expressed less PD-1 on CD8+ T cells, had more circulating Th17 cells, and had more circulating CD4+ T cells with a unique Ki67 intermediate phenotype. While we were not able to demonstrate the development of immune tolerance, these data indicate that monkeys exposed to SIVmac1A11 in utero may have altered immune responses that control viral replication after challenge with pathogenic SIV postnatally.
Introduction

The most recent World Health Organization report estimates that roughly 1.5 million children were born to HIV-infected mothers in 2009. ("Progress Report 2011: Global HIV/AIDS Response," 2011) Left untreated, mother-to-child-transmission (MTCT) of HIV occurs in about 30-45% of cases. Of these, only 5-10% of transmissions occur in utero (Lehman and Farquhar 2007; Scarlatti 1996). Given a 9-month gestation in a mother with a moderate HIV viral load (e.g., 100,000 copies/ml), an estimated $10^{14}$ viral particles could come in contact with the placental bed by the time the fetus reaches full term. Mounting evidence indicates that the placenta is a partially permeable barrier, allowing the transit of maternal cells and DNA into fetal circulation during gestation (Jonsson et al. 2008; Gammill and Nelson 2010; Mold et al. 2008). This would indicate that a large proportion of infants born to HIV-infected mothers may have encountered antigen in utero but remain uninfected. The question becomes: what is the immunologic consequence of fetal exposure to virus in the absence of productive infection?

Results from studies of HIV exposed/uninfected (EU) children vary in their findings of the impact of maternal HIV infection on the health of uninfected newborns. Nielsen et al. found reduced thymic output in cultures derived from infants born to HIV-infected mothers (Nielsen et al. 2001). However, this defect appears to be transient as the same group reported reduced thymic mass but no impact on thymic output in 15 month-old HIV EU children (Kolte et al. 2011). Relative to unexposed children, HIV EU kids have been documented to have “stunted” growth that does not appear to be reversible with nutritional supplementation (Filteau et al. 2011; McGrath et al. 2012). Other data indicate that HIV EU children have altered immune responses as well. Thus, CD4$^+$ lymphocytes from children born to HIV-infected mothers have been shown to make less IL-2 after polyclonal stimulation than do cells from children born to uninfected mothers (Rich et al. 1997). Other groups have reported increased production of IFN$\gamma$ and IL-10 and decreased production of IL-12, as well as decreased ability for NK cells to release perforin in HIV EU children (Chougnet et al. 2000; Kuhn et al. 2001; Slyker et al. 2012). Alterations in circulating antibody titers have also been shown. Reduced levels of IgG to tetanus, measles, Streptococcus pneumoniae, polio, pertussis, and Haemophilus influenzae type b have all been reported in HIV EU newborns, although most of these antibodies are likely maternally-derived (de Moraes-Pinto et al. 1996; Jones et al. 2011; Sanz-Ramos et al. 2013).

Unfortunately, studies of the impact of maternal HIV infection on the development of the newborn tend to be confounded by a variety of factors, including the health of the mother and the ability of an unhealthy mother to care for her newborn (Filteau 2009). Maternal health can have a large impact on humoral responses as high maternal viral loads have been shown to reduce the transplacental transfer of IgG from mother to fetus in utero (Farquhar et al. 2005). While reduced transfer of maternal antibodies can put a newborn at risk for infection, the absence of circulating IgG may increase the availability of vaccine antigens to the fetal immune system and is associated with increased antibody production in HIV EU after routine vaccination (Jones et al. 2011). Additionally, HIV-infected mothers are encouraged not to breastfeed in order to prevent the transfer of virus to the newborn via breast milk. The decision not to breastfeed may have independent detrimental impacts on the newborn as indicated by the fact that reduced breastfeeding is associated with the poor development of antibody titers against the polio vaccine and increased risk for gastrointestinal infections (Sanz-Ramos et al. 2013; Singh et al. 2011). Not least, the use of antiretroviral drugs (ARVs) by the mother during pregnancy has been associated
with decreased IgG production in neonates and increased risk for the development of chromosomal abnormalities (André-Schmutz et al. 2013; Bunders et al. 2010). These data indicate that understanding the impact of fetal exposure to HIV on the developing immune system may be quite complex and require more controlled study.

Cohorts of EU children have been reported for a variety of other infectious diseases as well (Dauby et al. 2012). In studies of filarial infections during pregnancy, children born to infected mothers with a high worm burden during pregnancy had reduced cellular responses to filarial antigens and were at higher risk of filarial infection during childhood (Malhotra et al. 2009). Similarly, studies of children born to mothers that had placental malaria infection during pregnancy have found increased frequencies of Tregs associated with decreased malaria-specific cytokine production by T cells from children born to infected mothers (Mackroth et al. 2011). HIV EU children have been shown to have detectable levels of T cell responses to HIV antigens after in vitro stimulation (Kuhn et al. 2002; Rich et al. 1997). These data were corroborated in a study by Legrand et al., where researchers found a population of antigen-specific T cells in cord blood from HIV EU children. Importantly, this study also found that removal of CD4+ CD25+ Tregs resulted in a significant increase in antigen-specific CD4+ and CD8+ T cell responses, revealing the presence of a circulating population of Tregs that suppress HIV-specific immune responses (Legrand et al. 2006).

These data indicate that prenatal exposure can lead to the induction of tolerance to infectious pathogens in neonates. However, it remains unclear how immunologic tolerance to HIV may influence the outcome of subsequent HIV infection. Human cohort studies are lacking and would prove difficult, as HIV EU children would have to be followed to the time of infection, which may be decades from the time of birth. We turned to a rhesus macaque model of SIV infection to address this question.

SIV infection of rhesus macaques is a widely accepted model for studying HIV infection (Hirsch and Lifson 2000). A species not naturally infected with SIV in the wild, their use as a model for HIV infection probably came about by accident, as a consequence of co-housing with naturally infected sooty mangabees (SM) in captive animal colonies (Hirsch and Johnson 1994). SM and African green monkeys (AGM), two species naturally infected with SIV, appear to have no clinical complications from SIV infection, do not develop simian AIDS, and live a normal lifespan. Rhesus macaques, however, develop high viral loads, have progressive loss of peripheral CD4+ T cells, and eventually develop secondary infections associated with progression to AIDS (Silvestri 2008). Experimental models in which rhesus macaques are infected with SIV isolated from healthy AGM show that macaques develop AIDS-like illness and succumb to infection within a year, while their AGM counterparts remain healthy (Hirsch et al. 1995). These data indicate two things: 1) rhesus macaques develop a disease course very similar to that which is seen in HIV infection, and 2) it is the host response to viral infection, rather than an intrinsic feature of the virus itself, that dictates the outcome of infection.

Similarly, studies of MTCT of SIV in SM and AGM indicate that vertical transmission of virus is quite rare in these species, while in rhesus macaques MTCT has been documented in frequencies similar to those found in humans (McClure et al. 1992; Chahroudi et al. 2011). When infected, SIV disease progresses rapidly in newborn macaques (Abel et al. 2006; Davison-
While data on the impact of maternal infection on the uninfected primate fetus are lacking, Tarantal et al. have shown that direct fetal infection restricted the growth of the developing fetus, associated with reductions in insulin-like growth factor (IGF) proteins. This study also noted that the virulence of the viral clone and the duration of infection (i.e., infection during the first trimester) strongly impacted the severity of the growth defects (Tarantal et al. 1995). Animals injected with the attenuated SIV isolate, SIVmac1A11, had normal growth, no alterations in IGF levels, and no clinical signs of disease. SIVmac1A11 is attenuated due to a mutation in the transmembrane domain of the envelope protein, and truncation mutations in the SIV accessory protein, vpr. It is poorly replicative and in infection models is cleared in a period of 6 weeks (Luciw et al. 1998). In a small study, Otsyula et al. have shown that fetal or neonatal injection with SIVmac1A11 followed by oral challenge with pathogenic SIVmac251 at one year of age resulted in the absence of detectable virus in two of five animal and reduced viral load, relative to controls, in a third (Otsyula et al. 1996a). The ability of this isolate to replicate, albeit at low levels, without altering fetal development and the intriguing results from Otsyula et al. made SIVmac1A11 an ideal candidate for fetal injections in our study.

In this study, fetal macaques were directly injected with SIVmac1A11 or phosphate buffered saline (PBS) during the late first trimester of gestation. Both groups of macaques (PBS or SIVmac1A11) were subsequently challenged first orally and then intravenously (IV) with a pathogenic clone of SIV (SIVmac239) at one year of age. Animals directly injected with SIVmac1A11 in utero had reduced viral loads following pathogenic SIV challenge (p<0.02). Linear regression models were used to identify immunologic parameters associated with or predictive of changes in viral load, and longitudinal analyses were used to assess persistent difference between groups. SIV-exposed animals had no evidence of Treg induction, more Th17 cells, reduced markers of immune exhaustion, and higher frequencies of SIV-specific T cell responses. We also found an intriguing population of Ki67 intermediate cells that was strongly inversely correlated with viral load at multiple time points post-infection. Although we could find no evidence that SIVmac1A11 exposure in utero led to immunologic tolerance to the virus, the improvement in viral control based on such an exposure has interesting implications for HIV EU children and demands further study.
**Methods**

*Rhesus monkeys.* All animal procedures conformed to the requirements of the Animal Welfare Act, and protocols were approved prior to implementation by the Institutional Animal Care and Use Committee at the University of California, Davis, CA. Female rhesus macaques (n=14) negative for the MHC alleles Mamu-A*01 and Mamu-A*02 as well as for Mamu alleles associated with “elite control” of SIV viremia (e.g., Mamu-B*08 and Mamu-B*17) (Loffredo et al. 2007) were time-mated with males who are heterozygous for Mamu-A*01 and Mamu-A*02. Animals were identified as pregnant by ultrasound, using established methods (Tarantal 2005). Activities related to animal care were performed as per California National Primate Research Center (CNPRC) standard operating procedures. Newborns were delivered by cesarean section at term (160 ± 2 days gestation; term 165±10 days) using standardized protocols then nursery-reared up through 3 months postnatal age. Infant health, food intake, and body weights were recorded daily in the nursery and then on a regular basis when moved to juvenile housing and according to established protocols. Blood samples (~3-6 ml, dependent on age) were collected from a peripheral vessel to monitor complete blood counts, clinical chemistry panels, and viral loads post-inoculation. At defined time points an aliquot (1-3 ml) was also sent to UCSF for immunologic assays. An outline of the study is shown in Figure 1A.

**SIV Antigens.** Lentiviral vectors expressing the ectodomain of the SIV envelope protein (gp140) were produced using plasmids by the Penn Vector Core, University of Pennsylvania, Philadelphia, PA. A second plasmid containing a gene insert for SIV gp140 was used for naked DNA injections (SIV DNA). *In vitro* assays stimulated with whole, aldritiol-2 (AT-2) inactivated SIVmac239 or microvesicle control (MV), a kind gift from Jeff Lifson (NIH/NCI), or SIVmac239 15-mer peptide pools corresponding to p27 (Gag) or gp120 (Env) proteins (NIH AIDS Reagent Resource Program).

**Immunizations.** In the late first trimester, eight fetuses were injected using an intraperitoneal approach with nonpathogenic SIVmac1A11 (100 TCID\textsubscript{50}), under ultrasound guidance. Controls were administered phosphate buffered saline (PBS). Beginning at 4 weeks postnatal age four infants that had received SIVmac1A11 prenatally and three macaques that had received PBS were immunized with SIV plasmid DNA (1.4 mg) IM. At 8 weeks and at 12 weeks they were administered a lentiviral vector (LV#1, LV#2) expressing SIV proteins (LVenv) (~10\textsuperscript{7} infectious units) (Figure 1A). A summary of the treatment groups is shown in Figure 1B.

**Virus Preparations.** All virus stocks were obtained through the UC Davis National Primate Research Center Analytical and Resource Core. Stocks were prepared and titered by endpoint dilution in CEMX174 cells according to standard protocols (Marthas et al. 1993), and stored frozen at ≤-135°. Aliquots were thawed immediately prior to inoculation.

**Virus Challenge.** At 16 weeks of age all groups were challenged orally with 1ml of SIVmac239 (10\textsuperscript{5} TCID\textsubscript{50}). Only one animal (Group B2) had detectable viral loads post-infection. Consequently, all remaining animals were injected IV with SIVmac239 (100 TCID\textsubscript{50}).

**Sample preparation.** Freshly isolated peripheral blood was sent by same day courier from UC Davis to UCSF within hours of collection. Sample tubes were spun at 1000g in a benchtop centrifuge to separate cells from plasma. The plasma fraction was removed, spun again at 1000g
to pellet any contaminating cells, placed in aliquots, and frozen (at ≤-80°C). PBMCs were isolated from the cellular fraction by diluting samples 1:2 in PBS then layered onto Ficoll-Hypaque (Sigma) and centrifuged at 1000g for 20 minutes. The leukocyte layer was removed by pipette, diluted in PBS containing 2% Fetal Bovine Serum (FBS), and cells pelletted by centrifugation at 1000g for 5 minutes. Cells were washed twice with PBS containing 2% FBS and resuspended in fresh RPMI 1640 supplemented with 10% FBS, 2nM L-glutamine, and 100 U/ml penicillin and streptomycin (R10), and left overnight at 4°C.

**Cytokine Flow Cytometry.** PBMCs were washed in PBS supplemented with 2% FBS resuspended in fresh R10 media at 5x10^5 cells/ml. 100ul (5x10^5 cells) was added to individual wells in a 96-well round bottom plates and cells were stimulated with AT-2 SIV (10ug capsid/ml), MV (100ug/ml), p27 peptides (2ug/ml) or gp120 peptides (2ug/ml), along with the co-stimulatory antibodies, CD28 (BD Biosciences, 4ug/ml) and CD49d (BD Biosciences, 4ug/ml). A mixture of PMA (50ng/ml) and ionomycin (1ug/ml) was used as a positive control. Plates were then incubated at 37°C and 5% CO2 for 2 hours and then treated with a 1:1000 dilution of a cellular protein transport inhibitor (Golgi Plug, BD Biosciences). Plates were then returned to the incubator at 37°C and 5% CO2 for another 16 hours. Cells were harvested by centrifugation, washed in PBS-2% FBS and surface stained with the viability dye Aqua Amine Reactive Dye (Invitrogen), as well as monoclonal antibodies directed against CD4 (NIH-NHP Reagent Resource Program) and CD8 (Clone 3B5, Invitrogen) for 20 minutes at room temperature. Cells were washed in PBS-2%FBS and permeabilized with BD Cytofix/Cytoperm reagents (BD Biosciences) as per manufacturer’s instructions. Permeabilized cells were then stained intracellularly for CD3 (Clone SP 34-2, BD Biosciences), IL-17a (Clone eBio64DEC17, eBiosciences), IL-4 (Clone 7A3-3, eBiosciences), IL-2 (Clone MQ1-17H12, BD Biosciences), IFNγ (Clone B27, BD Biosciences), and TNFα (Clone MAb11, BD Biosciences) for 30 minutes at 4°C. Cells were washed in PBS-2%FBS and analyzed by flow cytometry. A minimum of 150,000 events were acquired on a BD LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.).

**Elispot.** Elispot plates (Millipore) were washed with PBS and coated with anti-IFNγ capture antibody (Clone GZ-4, Mabtech, 16ug/ml) for 1 hour at room temperature. Plates were washed 4 times with PBS and blocked with R10 media for 1 hour at 37°C and 5% CO2. AT-2 SIV (10ug capsid/ml), MV (100ug/ml), p27 peptides (10ug/ml), or gp120 peptides (10ug/ml), along with the co-stimulatory antibodies, CD28 (BD Biosciences, 4ug/ml) and CD49d (BD Biosciences, 4ug/ml) were added in triplicate wells. 1x10^5 cells/well in R10 medium were added and plates were incubated at 37°C and 5% CO2 for 16-18 hours. A mixture of PMA (50ng/ml) and ionomycin (1ug/ml) was used as a positive control. Plates were then washed twice in PBS, washed two more times in PBS with 0.05% Tween-20 (PBST) and incubated with a biotinylated anti-IFNγ secondary antibody (Clone 7B6-1, Mabtech, 1ug/ml) and incubated at for 1 hour at 37°C and 5% CO2. Plates were next washed twice in PBST and incubated with streptavidin-Alkaline Phosphatase for 1 hour at room temperature, washed with PBST and soaked in bath of PBST for 1 hour at room temperature. PBST was then removed and spots were developed with Vector Blue substrate in the dark for 5-15 minutes, and the reaction was stopped by rinsing plates with water. When plates were dry, spots were counted using an S5 Analyser (CTL, LLC, Shaker Heights, OH). Results were reported as spot forming cells (SFC) per million PBMCs after subtraction from background (DMSO only wells).
**Phenotypic analysis of lymphocyte populations.** Freshly isolated PBMCs (5 x 10^5 cells) were surface stained with the viability dye Aqua Amine Reactive Dye (Invitrogen), as well as monoclonal antibodies directed against CD3 (Clone SP 34-2, BD Biosciences), CD4 (NIH-NHP Reagent Resource Program), CD8 (Clone 3B5, Invitrogen), CD25 (Clone M-A251, BD Biosciences) or CD25 (Clone 4E3, Miltenyi), CD95 (Clone DX2, BD Biosciences) or CD45RA (Clone 2H4, Beckman Coulter), CD27 (Clone M-T271, BD Biosciences) or CD28 (Clone CD28.2, BD Biosciences), and CD127 (Clone hIL-7R-M21, BD Biosciences) for 20 minutes at room temperature, fixed and then permeabilized with Affymetrix FoxP3 Fix/Perm Buffers (Affymetrix) per manufacturer’s instructions. Permeabilized cells were then stained intracellularly for Ki67 (Clone B56, BD Biosciences) and FoxP3 (Clone PCH101, Affymetrix) for 30 minutes at 4°C, washed in PBS-2%FBS and analyzed by flow cytometry. A minimum of 150,000 events were acquired on a BD LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.).

**Treg depletion assays**
Treg were depleted from PBMC cultures using anti-CD25- labeled paramagnetic microbeads and MS columns, according to the manufacturer’s instructions (Miltenyi Biotec).

**Proliferation assays**
Freshly isolated PBMCs were washed once with PBS containing 2% FBS and pelleted by centrifugation for labeling with CFSE. CFSE stock was diluted in PBS containing 2% FBS to a concentration of 15μM and 1mL of diluted CFSE was used to resuspend cells (0.5 – 10x10^6 cells per ml), and the cell suspension was inverted several times to mix. The cells were incubated for 5 min at room temperature, and 9mL of RPMI 1640 supplemented with 10% FBS, 2nM L-glutamine, and 100 U/ml penicillin and streptomycin (R10) was added to quench the reaction. Cells were then washed at least three times in 5ml of R10 resuspended at 3x10^6 cells per ml in R10. 100ul (3x10^5) cells were then added to individual wells in a 96 well plate and stimulated with AT-2 SIV (2ug capsid/ml), MV (20ug/ml), p27 peptides (3ug/ml), or gp120 peptides (3ug/ml) and cultured for 6 days at 37°C and 5% CO2. The mitogen concanavalin A (ConA, 2.5ug/ml) was used as a positive control. Cells were then harvested, stained with cell surface markers: Aqua Amine Reactive Dye (Invitrogen), as well as monoclonal antibodies directed against CD3 (Clone SP 34-2, BD Biosciences), CD4 (Clone L200, BD Biosciences), and CD8 (Clone 3B5, Invitrogen) for 20 minutes at room temperature. Cells were then washed in PBS-2%FBS, fixed with 1% paraformaldehyde and analyzed by flow cytometry. A minimum of 150,000 events were acquired on a BD LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.). Proliferation was reported as the percentage of cells (CD4 or CD8) dividing more than once (based on positive control) after subtraction from background (DMSO only wells).

**Antibody production.** Antigen-specific antibody production was measured by ELISA. 96-well ELISA plates (Nunc, Inc) were coated overnight with AT-2 SIV (1ug capsid/ml) or PBS. Plates were then washed three times with PBS containing 0.05% Tween (PBST) and blocked with blocking buffer (PBS containing 2.5% BSA) for one hour at room temperature. Frozen plasma samples were thawed and eight 4-fold serial dilutions were made in blocking buffer. 100ul of diluted plasma was then added to duplicate wells and incubated at room temperature for 2 hours.
Wells were washed 3 times in PBST and incubated with an anti-NHP IgG secondary antibody conjugated to horseradish peroxidase (HRP) (12.5µg/ml, Rockland Immunochemicals). Plates were then incubated for 1 hour at room temperature, developed using a TMB substrate kit (BD Biosciences) and the reaction stopped with dilute sulfuric acid (2N). Plates were read at 450nm on an ELISA plate reader and at 690nm for the background. Midpoint titers were calculated from sigmoidal dilution curves using Prism Software (Graph Pad, Inc.).

**Measurement of tryptophan and kynurenine concentrations in plasma.** Indolamine 2,3-dioxygenase (IDO) activity was measured as previously reported (Huang et al. 2013). Briefly, tryptophan and kynurenine concentrations in plasma were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Plasma samples (100 µl) were added to 100 µl of internal standard, Trp-d5 (3.5 µg/ml) and Kyn-d4 (1.1 µg/ml), and mixed for 30 seconds. Trifluoroacetic acid (20 µl) was added to precipitate the proteins. After vortexing for 1 min, the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was transferred to autosampler vial, and 5 µl was injected to the LC-MS/MS system. The mass detector was a Micromass Quattro Ultima using electrospray positive ionization mode. The column was Synergi Polar RP (4.6 × 75 mm, 4-mm particle size) with mobile phase consisting of 2% acetonitrile, 5.2% methanol, and 0.1% formic acid. The flow rate was 1.0 ml/min. A standard curve was generated by adding serial dilutions of tryptophan and kynurenine standard solutions to water, treated in the same way as the plasma samples.

**Statistical analyses.** Statistical analyses were performed using R software (http://CRAN.R-project.org). Longitudinal analyses were generated using generalized estimating equations (http://cran.r-project.org/web/packages/gee/index.html). Linear regression models were generated using ln commands.
**Results**

**Study Design**

A diagram of the study timeline as described in the Methods section is shown in Figure 1A. Given differences in exposures *in utero* (Group A injected with PBS and Group B injected with SIVmac1A11) or after birth (Group 1 not immunized with SIV antigens and Group 2 immunized with SIV antigens), four groups of animals were generated, a summary of which is shown in Figure 1B. For the purposes of clarity, the following data presentation focuses on differences observed between groups based on *in utero* exposure alone (PBS or SIVmac1A11). In those few cases in which significant differences were found between groups based on neonatal immunizations as well, these are also discussed.

**Cell Counts Prior to Oral Challenge**

To monitor cell distributions, complete blood counts (CBC) were performed at each immunization and two weeks prior to oral challenge. No differences in absolute CD4 counts were found between the groups prior to oral challenge (Figure 2A). Animals exposed to SIVmac1A11 did have significantly higher numbers of CD8+ T cells (p=0.00445) (Figure 2B). As a consequence of the increase in CD8 counts, animals from the SIVmac1A11 group had a slightly (although not significant) lower CD4:CD8 ratio prior to oral challenge (p=0.0515) (Figure 2C).

**Immune Responses Prior to Oral Challenge**

To assess antigen-specific tolerance, PBMCs from 12 week-old animals were assayed for immune responses to SIV antigens *in vitro*. Had tolerance been induced upon exposure to SIVmac1A11 *in utero*, we would expect decreased antigen-specific immune responses in these animals. No significant differences were found in proliferative responses to *in vitro* stimulation with aldrithiol-2-inactivated SIV (AT-2 SIV) (Figure 2D, solid bars) or SIV peptides (data not shown). To assess the role of Tregs in antigen-specific responses, CD25+ cells were removed from PBMCs by magnetic depletion. CD25 depletion had no impact on proliferative responses, implying that Tregs were not involved in suppressing these responses in either group (Figure 2D, hatched bars). Cytokine production was assessed by culturing PBMCs in the presence of SIV antigens *in vitro*. No significant differences in antigen-specific cytokine responses were found (data not shown). Plasma samples taken from animals at 9 weeks of age were assayed for IgG directed against AT-2 SIV. Animals that had not received antigen up to this point (group A1) had no detectable IgG titers against AT-2 SIV (Figure 2E). However, animals that had only received virus *in utero* without neonatal immunization (group B1) had antibody titers equal to animals that had received no virus *in utero* but were immunized as neonates (group A1). Animals receiving both *in utero* SIV and neonatal immunizations (group B2) did not have a significant increase in antibody titers relative to those that had received only one or the other immunization. These data indicate that *in utero* exposure had no measurable impact on tolerance prior to oral challenge.

**Oral SIVmac239 Challenge**

At 16 weeks of age animals were administered an oral dose of SIVmac239 (10^5 TCID₅₀). Viral load measurements post challenge revealed that only one animal was productively infected. The remaining 13 animals were repeatedly challenged orally and, for reasons that are unclear
(but possibly related to the challenge dose of SIV that was used), none developed productive infection.

**Intravenous SIVmac239 Challenge**

The 13 animals that remained SIV-negative from oral challenges were subsequently infected IV with SIVmac239 (100 TCID₅₀). Animals ranged in age at the time of infection from 37-57 weeks. Following IV challenge, all animals were productively infected. Animals exposed to SIVmac1A11 in utero had significantly lower RNA viral loads over the course of study (p<0.02) (Figure 3A). Intriguingly, one animal from the SIV-exposed group was able to control viral replication to undetectable levels at 16 and 20 weeks post-infection. DNA viral loads were not significantly different between the groups (Figure 3B).

**Correlates of Viral Loads**

To investigate what might be contributing to the observed differences in viral load, a series of linear regression models were used to identify significant associations between viral load at specific time points and a panel of various immunologic parameters measured at different time points. A panel of 118 immunologic parameters, each measured at multiple time points were compared with 4 independent viral load measurements: the peak of viral load (2 weeks post-infection), the set-point (4 weeks post-infection), the point of greatest variance (16 weeks post-infection), and the time point of least variance (tissue harvest). Correlations were deemed to be warrant further investigation if an explanatory variable (immunologic parameter) was significantly associated with (p<0.05) and was measured at the same time or before the outcome variable (viral load) and if independent measurements of an explanatory variable at different time points correlated well with independent measurements of the outcome variable. Significant associations were then plotted individually to assess the validity of the association (distribution, skewness, outliers) and the direction of the association (positive or negative) was assessed. Parameters were excluded if the association was driven by a single outlier. Remaining parameters were then given a sign (positive or negative) corresponding to the direction of the association and a heatmap was generated comparing the strength of the associations (p-value) for each variable with each viral load time point (Figure 3C).

This strategy revealed that, amongst all of the immune parameters measured, a small subset was predictive of viral load at various time points: CD4 count during acute infection, Th17 cells during the later stages of infection, and CD4⁺ cells with an unusual phenotype (intermediate in expression of the cell cycle marker, Ki67). Conversely, a number of immune parameters previously associated with viral load (e.g., Tregs, CD4 counts at later stages of infection, CD8 counts, and antibody titers) were found not to be predictive. Finally, some parameters were either not associated with viral load or were only significant at one time point, but were differentially expressed between the groups: e.g., cells expressing the senescence markers PD-1 or Tim-3, and antigen-specific cytokine responses. Each of these will be discussed in turn below.

**Peripheral immune cells post-infection**

Peripheral loss of CD4⁺ T cells is a hallmark of pathogenic HIV/SIV infection. Loss of CD4⁺ T cells is usually accompanied by an expansion of CD8⁺ T cells and an inversion of the normal CD4:CD8 ratio, and the CD4:CD8 ratio has been reported to be an independent predictor
of disease progression (Margolick et al. 2006). Despite significant positive associations between CD4 count and viral load during the acute stages of infection, this association became negative by 16 weeks post-infection. CD8+ central memory (CM) T cells were also significantly positively associated with viral load in the acute stages of infection and did not appear in our associations after 4 weeks post-infection. These findings are consistent with published reports of T cell expansion in the early stages of SIV infection in neonatal and adult macaques (Veazey et al. 2003; Kaur et al. 2000). No significant difference was found in CD4, CD8, or the CD4:CD8 ratio between the two groups (Figure 4A-C).

Different CD4+ T cell subsets have also been recognized as important predictors of disease progression. Among these, CD4+ Tregs and CD4+ IL-17 producing T cells (Th17) appear to be particularly important. CD4+ T cells producing the cytokine IL-17 are important for the maintenance of gut barrier integrity (Cecchinato and Franchini 2010). Studies in rhesus macaques reveal that the levels of Th17 and Treg cells, and the relative ratio of the two, can distinguish pathogenic from non-pathogenic SIV infection and may predict disease progression (Favre et al. 2009; Hartigan-O'Connor et al. 2012; Favre et al. 2010). Had tolerance been induced in this study, we might have predicted an increase in peripheral Tregs in animals exposed to SIV in utero. A flow cytometry panel was developed to detect Treg cells by co-expression of CD25, the transcription factor FoxP3, and low level expression of the IL-7 receptor, CD127, as has been described previously (Anderson et al. 2008; Hartigan-O'Connor, Abel and McCune 2007; Legrand et al. 2006; Wang et al. 2013). A representative plot is shown in Figure 5A (left panel). No association was found between the frequency of Tregs in peripheral blood and plasma viral load at any time points by our regression model. Also, no difference in Treg frequency was found between the two groups post-infection (Figure 5A, right panel).

Th17 cell frequencies were measured in PBMCs after polyclonal stimulation by intracellular staining for the cytokine, IL-17 (representative plot- Figure 5B, left panel). The frequency of Th17 cells measured at multiple time points post-infection was significantly negatively correlated with viral load at 16 weeks post-infection as well as at the time of tissue harvest. Animals exposed to SIV in utero had significantly higher frequencies of circulating Th17 cells in peripheral blood (Figure 5B, right panel) a phenotype associated with improved outcomes in lentiviral infections (Kanwar, Favre and McCune 2010).

If tolerance were induced, the hypothesis would be that tolerance it might reduce the magnitude of T cell activation that has been reported to drive disease progression. Ki67, a nuclear protein associated with progression through the cell cycle, has been used previously as a marker for immune activation and has been shown to be predictive of disease progression in HIV (Dang et al. 2012; Ganesan et al. 2010). Ki67 expression on CD4+ T cells was significantly negatively associated with viral load at nearly every time point measured in our regression model. In contrast, Ki67 on CD8+ T cells was only significantly associated with viral load at the time of tissue harvest. Ki67 was significantly increased in CD4+ T cells in macaques exposed to SIV in utero, although there was no difference in CD8+ T cells (Figure 6A and B). Upon closer inspection we noted the presence of two distinct Ki67+ populations among memory (CD45RA-) CD4+ T cells (Figure 6C). One population clearly expressed a high level of Ki67 (Ki67hi) while the other expressed an intermediate level. When assessed independently, Ki67 intermediate cells were found to be more abundant in animals that had received SIVmac1A11 in utero, whereas
Ki67hi cell frequencies were not different. Ki67 intermediate cells did not appear to be differentially expressed at the time of infection but became significantly more abundant after infection in the SIV exposed animals whereas the Ki67hi population was not (Figure 6D).

Including Ki67hi and Ki67 intermediate CD4+ and CD8+ T cells into the linear regression model showed that Ki67 intermediate cells were significantly inversely associated with viral load at all but the last time point measured (Figure 7A). In comparison, the frequency of Ki67hi cells had no association with viral load except at 2 weeks post-infection, the time point of peak viral load (Figure 7B).

Antiviral responses post-infection

Following infection, a model of immune tolerance might predict an absence or a reduction in immune responses to viral antigens. To test this hypothesis, plasma samples were assayed for IgG titers against whole, aldrithiol-2 inactivated SIV (AT-2 SIV). Macaques from group A1 (that had no exposure to SIVmac1A11 in utero and that did not receive any neonatal vaccinations) did not develop detectable antibody titers until after the peak of viremia (2 weeks post-infection). We were not able to assess antibody titers between 2 and 16 weeks post-infection, so we cannot determine at what point titers from group A1 became detectable within this range. All other groups had detectable titers by 2 weeks post-infection, with only a single animal having detectable antibody titers on the day of infection (Figure 8A). Interestingly, this animal (#27) had relatively high antibody titers at 9 weeks of age, and was the same animal that controlled viral replication to undetectable levels at 16 and 20 weeks post-infection. Aside from this anecdote, no association between antibody titers and viral load at any time point was found from our regression model.

To test for cellular responses against SIV antigens, cryopreserved PBMCs from four selected time points were thawed and stimulated with AT-2 SIV or with peptide pools corresponding to the SIV p27 (Gag) or gp120 (Env) proteins in cytokine flow cytometry (CFC) (Figure 8B) or IFNγ-capture Elispot assays. Animals exposed to SIV in utero had significantly higher responses to SIV antigens by CFC and Elispot assays (Figure 8C and D). Although the variance was high, as not every animal responded to these stimulations, the results were consistent within a given animal and increased over the course of disease in both assays. SIV-specific responses were not associated with viral load at any time point in our regression model. This may be due to the relatively small number of animals mounting detectable responses. Collectively, these data indicate that exposure to SIVmac1A11 in utero resulted in T and B cell responses against SIV that appear qualitatively different than those found in control animals.

T cell exhaustion

Chronic stimulation can contribute to the dysfunction or “exhaustion” of immune cells making them less able to respond to stimuli. Exhausted CD8+ T cells have been shown to lose the ability to proliferate, degranulate and produce cytokines (Day et al. 2006; El-Far et al. 2008; Jin et al. 2010; Sakhdari et al. 2012; Zhang et al. 2007). Numerous markers have been used to phenotype these cells, including PD-1 and Tim-3. In HIV infection, PD-1 has been found to be present on HIV-specific but not CMV-specific CD8+ T cells, implying that the unique nature of HIV infection is what drives dysfunction (Trautmann et al. 2006). We sought to investigate the
expression of these markers on PBMCs from our animals. Due to a lack of available cells, we chose to look at splenocytes cryopreserved from the day of tissue harvest.

Applying our regression model, we found multiple significant associations between viral load and PD-1 and/or Tim-3 expressing CD4 and CD8 subsets at the time of tissue harvest. These included positive associations between CD4 central memory (CM) cells expressing PD-1, but not Tim-3, and negative associations with CD4 CM cells expressing neither marker. The model also found positive associations between CD8 CM cells expressing Tim-3 alone, or Tim-3 and PD-1 co-expressing cells and viral load. Animals exposed to SIV in utero had significantly fewer CD8+ T cells expressing PD-1 than control animals (p=0.03) (Figure 9A). By co-staining for proteins shown to define memory subsets in macaques (Pitcher et al. 2002) we were able to measure PD-1 expression in naïve, central memory (CM) and effector memory (EM) subsets. Animals exposed to SIVmac1A11 in utero had significantly fewer PD-1 expressing central memory cells (p<0.003)(Figure 9B). Tim-3 expression, with or without co-expression of PD-1, was not significantly different between these groups (data not shown). We found high levels of PD-1 positive CD4+ T cells as well (Figure 9C). Interestingly, despite not finding a statistically significant difference between the frequencies of PD-1 expressing CD4+ T cells, we found a fairly robust positive association between PD-1 expressing CD4+ T cells at tissue harvest and viral load (Figure 9D), a finding consistent with previous reports in HIV infection (Day et al. 2006). Although these data were collected from cells at the time of tissue harvest, a time when viral loads have re-converged and are no longer different between the groups, they may be reflective of what was happening at earlier time points. If so, exhaustion of CD4+ and CD8+ T cells may play a role in the differences observed in viral load between our groups.
Discussion

These data show that exposure to SIVmac1A11 \textit{in utero} was associated with lower viral loads for a period of 28 weeks after pathogenic challenge. In turn, these lower viral loads were significantly associated with or predicted by a higher fraction of Th17 cells and of CD4$^+$ T cells expressing intermediate levels of Ki67. They were also associated with increased antigen-specific cytokine production and reduced markers of immune exhaustion. Finally, animals exposed to virus \textit{in utero} had humoral responses to viral antigens prior to infection and during the acute phase of infection that were on par with animals receiving neonatal vaccinations. These animals also had a population of Ki67 intermediate CD4$^+$ T cells that were consistently inversely associated with viral load.

The presence of detectable antibody titers at 9 weeks of age in animals who had only received SIVmac1A11 \textit{in utero} (group B1) indicated that fetal exposure to SIV had taken place and that a fetal response had developed. It remains a possibility that these antibodies, being an IgG isotype, were maternal antibodies that had been transplacentally transferred to the fetus \textit{in utero}. While maternal IgG has been shown to persist for period of up to twelve months in humans, studies in the rhesus macaque indicate that maternal IgG may be cleared in less than two months, although part of this difference may be driven by differences in antigen exposure between the two studies (Eitzman 1970; Leuridan and Van Damme 2007). Importantly, previous studies of maternal SIVmac1A11 infection in macaques have found rapid clearance of SIV-specific maternal IgG, with a half-life of 4-9 days (Van Rompay et al. 1996). Given the rapid decay of maternal IgG and the finding that titers in animals from the B1 group were comparable to those in the vaccinated groups (A2 and B2), it seems unlikely that these titers were maternally derived, although we cannot rule out the possibility. Detection of other isotypes in newborns such as IgM (which cannot cross the placenta), or quantification of titers in maternal serum may help resolve this. While the serum titers were transient in most animals and became undetectable by the time of IV infection, they increased rapidly during the acute phase of infection in all groups except animals whom had not received any immunizations (group A1). This finding indicates that there may have been an expansion of antigen-specific B cells in the antigen-experienced groups, but not in the unimmunized group, an hypothesis that demands further investigation.

Animals exposed to SIV \textit{in utero} also had an expansion of CD8$^+$ T cells prior to infection that was greater than that of control animals. We were not able to detect antigen-specific cellular responses in animals of this young age so it is difficult to determine the specificity of these cells, a finding consistent with previous reports in neonatal macaques (Van Rompay et al. 2003). Complicating matters was the fact that animals were not derived from timed pregnancies and so, although every attempt was made to control as many assay variables as possible (internal controls, fluorescence target values, etc.), inter-assay variability may have drowned out any differences that may have existed between these groups. However, antigen-specific responses were found at later time points (weeks 16 and 24) post-infection. While modest, these responses were consistent within a given animal and were higher in animals exposed to SIVmac1A11 \textit{in utero}. These data support those found by Otsyula et al. (Otsyula et al. 1996a) who showed that fetal or neonatal infection with SIVmac1A11 induced a small but detectable CTL response in cells from SIVmac1A11- exposed animals stimulated \textit{in vitro}. However, our regression model showed no association between these antigen-specific immune responses and viral load. This
finding may be due to insufficient sampling, as plasma samples were not available between week 2 and week 16, a time when the viral load declines rapidly. Additionally, antigen-specific cellular responses were not assessed at more acute stages of infection due to insufficient cell numbers (as these animals became lymphopenic).

While antibody titers were transient in most animals and became undetectable by the time of birth, titers persisted in a single animal (#27). This animal also had consistently the highest proportion of CD4+ Ki67 intermediate cells over the course of infection and was the same animal that was able to suppress viral replication to undetectable levels for a stretch of 4 weeks (weeks 16 to 20), before rebounding. This animal also had the lowest levels of PD-1 or co-expression of PD-1 and Tim-3 on central memory (CM) CD8s of either group and, though its CD8+ T cells did not produce much cytokine in response to SIV antigens, it was one of the strongest producers of cytokines (TNF and IFNγ) after polyclonal stimulation, implying intact effector responses.

The failure of the oral challenge was surprising, as this was a route of infection that had been well established at the UC Davis National Primate Research Center. Of the 14 animals that were challenged, only one animal became infected (#30). This animal was assigned to the B2 group and thus had received SIVmac1A11 in utero and SIV antigens after birth. On the whole, #30 looked very similar to the rest of the animals in the cohort. Antibody titers were within the range of other animals and CD4 and CD8 counts seemed quite normal. The only significant findings were that #30 had a spike in neutrophil counts about 4 weeks prior to infection and a spike in NK cells on the day of challenge. We can speculate that this animal may have been battling some other type of infection just prior challenge, perhaps making it more susceptible. It is also worth noting that when oral challenges did not infect, they were repeated. As a consequence some animals were given more challenge inoculations than others. PBS and SIVmac1A11 animals received an average of three oral challenges but individual animals ranged from 1-5 challenges. While we have no direct evidence that the oral challenges had an immunologic impact, this possibility cannot be ruled out.

Following infection, Treg frequencies remained relatively similar between the groups but the increased number of Th17 cells post-infection could be important for mucosal health. While not directly measured in this study, we have previously proposed a model of chronic inflammation whereby the loss of Th17 cells, especially in the gut, compromises gut barrier integrity and can allow luminal contents, including microbial products, into the lamina propria or potentially into the bloodstream (Favre et al. 2010). This breakdown has been proposed to set off a cycle of inflammation where intestinal immune cells respond to microbial insult by producing pro-inflammatory cytokines (such as type I interferons) (Ancuta, Monteiro and Sekaly 2010; Elhed and Unutmaz 2010; Hunt 2010; Kim 2009; Paiardini 2010). Studies in humans and non-human primates have found elevated levels of lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, from blood and tissues during chronic HIV or SIV infection. While Th17 cells were measured in this study, assessments of gut barrier integrity, measurements of peripheral cytokine (IFNα) or markers of microbial translocation were not performed. Future studies should pay closer attention to the status of the mucosal immune system to make these conclusions.
It is certainly possible that there may be underlying inflammation in cells or tissues that we did not measure. As a marker for systemic inflammation, we chose to examine the activity of the enzyme, indolamine 2,3-dioxygenase (IDO), found mainly in macrophages and dendritic cells and that, in response to inflammatory cytokines such as IFNγ or cellular ligands like CTLA-4, can catabolize the amino acid tryptophan into kynurenines and other downstream metabolites. The catabolism of tryptophan can suppress proliferation of neighboring immune cells that are “starved” of the essential amino acid; downstream metabolites (e.g., 3-hydroxyanthranilic acid) can also modulate the polarization of T cells and reduce the Th17/Treg ratio, setting the stage for a chronic inflammatory process in the intestinal mucosa (Favre et al. 2010). Measurement of IDO activity then is enumerated as the relative ratio of the metabolite, kynurenine, to its parent molecule, tryptophan (a K:T ratio) by HPLC or LCMS/MS. We saw a peak in IDO activity at 2 weeks post-infection (the peak of viral load) but overall we saw no difference in this marker between the groups (data not shown).

A surprising finding from this study was the presence of CD4+ Ki67 intermediate cells. While we still do not have a good grasp as to what these cells are, they seem to be tightly and inversely linked to viral load. Previous reports of Ki67 turnover indicate that the protein has a half-life of 60-90 minutes, making it unlikely that we happened to catch a population of cells as they moved through the cell cycle (Scholzen and Gerdes 2000). One hypothesis is that these cells are arrested in one particular stage of the cell cycle. Studies of cell cycle using stage specific inhibitors in combination with Ki67 and a nuclear stain such as propidium iodide have been able to phenotype cell cycle stages (Tsurusawa and Fujimoto 1995). Upon inspection, one of the populations described in Tsurusawa et al. (1995) has Ki67 staining that is similar to the staining pattern observed for the Ki67 intermediate phenotype described in the current study. This phenotype was determined to describe cells in the G2/M phase of the cell cycle. Intriguingly, G2 cycle arrest is a property of one of the SIV accessory proteins, vpr, and has been suggested for another accessory protein, vpx. So what might be different about the in utero exposed animals? SIVmac1A11, while it is an attenuated virus, has a fully intact vpx, as well as the first 12 codons of vpr. Possibly, some of the virions injected in utero became integrated and may still be making some of these viral proteins. Another possibility is that these Ki67 intermediate cells may be infected and essentially sequestering virus, which may describe the inverse relationship between Ki67 intermediate cells and viral load. However the frequency of these cells (~10% of CD4+ T cells in some animals) would be very high in comparison with human studies (1:10,000 PBMCs), but not out of line with reports in SIV or SHIV-infected primates (Khatissian et al. 2003; Laforge et al. 2011; Nishimura et al. 2005, 2009; Simmonds et al. 1990). Sieg et al. have reported that cells from a subset of HIV-infected patients have an inability to upregulate Ki67 and progress through the cell cycle after TCR stimulation. Defects in IL-2 signaling were proposed to be playing a role, but the exact mechanism was unclear (Sieg, Bazdar and Lederman 2003). The Ki67 intermediate phenotype observed in this study may be a similar phenomenon. More investigation into the characteristics of this cell population (e.g., infection status, cell cycle analysis, and cytokine responsiveness) will need to be performed to test these hypotheses.

The measurement of PD-1 in tissues is intriguing. One potential model that arises from these data is that through some mechanism, possibly priming, animals exposed to SIV in utero expand CD8+ T cells (possibly antigen-specific), maintain gut health, control their levels of T cell exhaustion, mount better antiviral responses, and control viral replication better than
controls. Another possibility is that improved antigen-specific responses are an outcome of some other factor that reduces exhaustion and preserves immune responses. In fact, in a study of chronically infected rhesus macaques, Dyavar et al., found that blockade of the PD-1 pathway using a monoclonal antibody improved expression of proteins associated with improved gut barrier integrity, reduced levels of circulating lipopolysaccharide (LPS), reduced circulating IFNα levels, enhanced control of gut-resident bacteria, reduced opportunistic infections in the gut and significantly improved survival, demonstrating the importance of controlling immune exhaustion in overall health (Dyavar Shetty et al. 2012). Another possibility is that there is another intrinsic mechanism that is controlling viral replication, perhaps sequestration of virus within Ki67 intermediate cells, that independently lowers viral loads and the other data are a consequence of this. Further studies are being conducted to address these questions.
Figure 1: Study design. (A) The timeline of immunizations and virus challenges. Fetal macaques were injected with nonpathogenic SIVmac1A11 (intraperitoneal) or PBS during the late first trimester. Newborns were delivered by cesarean section according to established protocols at full term and immunized with or without SIV antigens as infants. At 16 weeks of age each animal was orally challenged with SIVmac239. Animals that did not demonstrate productive infection (13 of 14) were then challenged IV at 40 weeks of age with SIVmac239 and followed for disease progression. (B) Animal numbers and group assignments.
**FIGURE 2**

**A.** CD4 Count

**B.** CD8 Count

**C.** CD4:CD8 Ratio

**D.** Proliferation Week 12

**E.** Anti-SIV IgG Titers

Figure 2. Animals exposed to SIVmac1A11 *in utero* have increased CD8 counts and elicit humoral but not cellular responses prior to oral challenge. Absolute counts of peripheral blood (A) CD4⁺ and (B) CD8⁺ T cells, and (C) CD4:CD8 ratio prior to oral SIV challenge. (D) PBMCs from 12 week-old animals with and without depletion of CD25⁺ cells were CFSE-labeled and stimulated with AT-2 SIV for 6 days. The percentage of CD4⁺ T cells that have diluted CFSE are shown. (E) IgG mid-point titers against AT-2 SIV in plasma from 9 week-old animals.
Figure 3. Animals exposed to SIV in utero have lower RNA viral loads in association with various immunologic parameters. (A) RNA and (B) DNA viral loads after IV infection. (C) Heatmap of significant associations between a panel of immunologic parameters and log RNA viral load at specific time points post-infection: week 2 (peak viral load), week 4 (viral load setpoint), week 16 (the time of greatest variance) and tissue harvest (the time of least variance). Colors represent the strength of the association (p-value) and the sign (positive or negative) represents the direction of the association.
Figure 4. Fetal exposure to SIVmac1A11 does not impact peripheral blood counts post-infection. The percentage of (A) CD4\(^+\) and (B) CD8\(^+\) T cells, and (C) the ratio of CD4 to CD8 T cells over the course of infection. Percentages were calculated relative to the day of infection.
FIGURE 5

A. [Cytogram showing CD127low FoxP3+ Tregs in fresh rhesus peripheral blood and Treg frequencies post-infection.]

B. [Representative histogram of cryopreserved CD4+ T cells producing IL-17 after stimulation with PMA and ionomycin and Th17 cell percentages post-infection.]

Figure 5. Animals exposed to SIVmac1A11 in utero have increased Th17 cells but not Tregs post-infection. (A) (left panel) A representative cytogram of CD127low FoxP3+ (Tregs) in fresh rhesus peripheral blood and (right panel) Treg frequencies post-infection. (B) (left panel) Representative histogram of cryopreserved CD4+ T cells producing the cytokine IL-17 (Th17) after stimulation with PMA and ionomycin and (right panel) The percentages of Th17 cells were measured post-infection.
Figure 6. Ki67 expression patterns are altered on animals exposed to SIVmac1A11 in utero. Frequencies of total Ki67+ cells in (A) CD4 or (B) CD8+ T cells post-infection. (C) A representative cytogram of the distribution of Ki67 in memory (CD45RA-) CD4+ T cells found
in animals exposed to SIVmac1A11 \textit{in utero} (left panel) or PBS (right panel). (D) Frequencies of Ki67 intermediate (left panel) or Ki67 hi (right panel) cells over the course of infection.
**FIGURE 7**

A. **CD4 Ki67intermediate vs Log RNA Viral Load**  

B. **CD4 Ki67hi vs Log RNA Viral Load**  

Fetal Injection  
■ PBS  
■ SIVmac1A11
Figure 7. Ki67 intermediate cells are strongly inversely correlated with viral load at multiple time points post-infection. Linear regression analyses comparing the frequencies of CD4⁺ (A) Ki67 intermediate or (B) Ki67 hi cells (x-axes) with log SIV RNA viral loads (y-axes) at each time point over the course of disease. Numbers in grey bars represent weeks post-infection.
Figure 8. Animals exposed to SIVmac1A11 in utero have reduced humoral but increased cellular responses post-infection. (A) Elisa measurements of IgG titers from peripheral blood plasma. (B) Cryopreserved PBMCs were stimulated for 16-18 hours with an SIV p27 (Gag) peptide pool and assayed for cytokine production. (C) Data showing TNFα production from CD8+ T cells as assessed by flow cytometry and (D) IFNγ production measured by Elispot.
FIGURE 9

A. 

B. 

C. 

D. 

\[ \text{Adj R}^2 = 0.444 \]
\[ p = 0.00771 \]

*p-Mann-Whitney test

log RNA VL (copies/ml)

CD4+ PD-1+ CM Cells vs. Viral Load

Fetal Injection

PBS

SIV
Figure 9. Splenocytes from animals exposed to SIVmac1A11 in utero have lower expression of senescence markers associated with lower viral loads. (A) (left panel) Representative cytogram of PD-1 and Tim-3 expression on CD8+ T cells and (right panel) analysis of total PD-1 expression. (B) (left panel) Representative cytogram of CD8+ memory subsets and (right panel) analysis of PD-1 expression on CD8+ central memory (CM) cells. (C) (left panel) Representative cytogram of CD4+ memory subsets and (right panel) analysis of PD-1 expression on CD4+ central memory (CM) cells. (D) Linear regression analysis of PD-1 expression on CD4+ CM cells versus log RNA viral load.
CHAPTER IV: Conclusions and Future Directions

Summary

The work presented here focused on the potential for the development of tolerance to SIV in rhesus macaques. We found that macaques, like humans, exchange cells across the placenta between mother and fetus. In humans, this exchange of cells seems to be important for the development of tolerance of the fetus to maternal antigens. Our results of maternal microchimerism in fetal tissues were significantly lower than previous results from humans but it was quite interesting to find these cells in the peripheral circulation. As this was not a primary aim of this study, we only tested four animals, yet it was quite striking that we found detectable chimerism in every sample. Lots of interesting questions might be asked about this finding, such as: what cells were they? In humans, maternal microchimeric cells can be phenotyped by flow cytometry, using an HLA allele-specific antibody (such as those directed against HLA-A2) which is useful, if only for those occasions where mom is HLA-A2-positive and the baby is not. While these allele-specific antibodies are not available for rhesus macaques, another approach could be to sort different populations of hematopoietic cells using Fluorescence-Activated Cell Sorting (FACS) and then interrogate each of those populations for maternal DNA. If one cell population (say, CD4+ T cells) seems more enriched for maternal DNA than others, we may be able to infer that a larger proportion of chimeric cells are CD4+ T cells. We could then ask questions about how these proportions might change in different contexts, such as maternal SIV infection.

In experiments with fetal tissues, we were only able to detect maternal microchimeric cells in 2 of 7 animals assayed, so we were not able to make a determination regarding how maternal SIV infection might have altered the frequency of maternal microchimerism. As just described, we may be able to ask questions about what types of cells are chimeric. We may also be able to ask questions about how this may affect maternal-fetal tolerance. Is it possible that if the frequency or quality of chimerism is altered, does it also alter maternal-fetal tolerance? We are currently developing assays to test these questions. It was also quite striking to find that chimeric cells, which were undetectable in peripheral blood from our monkeys at birth and thereafter, were present in tissues more than one year later. This finding may enable us to ask questions about the persistence of tolerance as well, and how a chronic infection may disrupt this. Data from experiments such as these might be useful for transplantation studies, where long-term tolerance to foreign tissue is essential.

The data from the microchimerism study was encouraging in that it reinforced the model of the rhesus macaque as quite similar, although not identical, to humans in one of the systems that promotes the development of tolerance in utero. This has made the data from the fetal SIV exposures even more interesting as it implies that the macaque fetus has become tolerant of maternal antigen, given that maternal cells persist, yet we do not see any evidence of tolerance to SIV. Could it be a dose issue? Is it a problem of the persistence of antigen, given that SIVmac1A11 may not be replicating for very long enough, at high enough levels to induce tolerance? We are considering studies in which SIV antigens may be delivered in a manner that would allow for replication and antigen exposure over a longer course of gestation.
In studies that are still being currently analyzed, and thus did not make it into this dissertation, we tried to induce tolerance to SIV by delivery of antigen either through maternal infection with SIVmac1A11 or via direct injection of a fetus with a lentiviral vector expressing the SIV envelope protein. We did not see a reduction in viral loads in the experimental groups for either of these studies. In fact, for reasons that are not yet clear, the viral loads from these animals appear to be higher than their respective control groups and more experimental animals died prior to the end of the study than did their controls. It will be interesting to ask questions about tolerance in these animals and take the interesting data from the study presented here and see how it applies to these other groups. Do Ki67 intermediate cells exist in these animals? Is PD-1 expression altered? Are antigen-specific responses or Th17 cell frequencies any different? The data presented here give us a place to start.
REFERENCES


Nishimura, Yoshiaki, Charles R Brown, Joseph J Mattapallil, Tatsuhiko Igarashi, Alicia Buckler-White, Bernard A P Lafont, Vanessa M Hirsch, Mario Roederer, and Malcolm A Martin.


Tarantal, Alice F. 2005. Ultrasound imaging in rhesus (macaca mulatta) and long-tailed (macaca fascicularis) macaques: Reproductive and. *The Laboratory Primate*.


