Dissertation

Host Cell Antigen and T-Lymphocyte Subset Contribution to Simian Immunodeficiency Virus Pathogenicity

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DEBORA SHAWN STUMP ENTITLED HOST CELL ANTIGEN AND T-LYMPHOCYTE SUBSET CONTRIBUTION TO SIMIAN IMMUNODEFICIENCY VIRUS PATHOGENICITY BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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Abstract of Dissertation

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The continuity of the host cell plasma membrane and the simian immunodeficiency virus (SIV) envelope at the time of budding results in the incorporation of host membrane antigens. Of these host antigens, major histocompatibility complex class II (MHCII), is abundantly represented on the virion surface. In Chapter 1, the investigation the potential of antibodies specific for MHCII to block viral infection by binding viral envelope MHCII *in vitro* is presented. Our results did not demonstrate viral neutralization associated with anti-MHCII antibodies but illustrate that viral infectivity is influenced by target cell membrane and immunological signaling characteristics. In Chapter 2 we investigated the utility of alloimmunization of genetically divergent rhesus macaques in eliciting immune responses specific for host cell antigens capable of limiting SIV infectivity *in vivo*. Our results suggest that alloimmune responses are not sufficient to protect animals from SIV challenge.

We were also able to assess differences in response to pathogenic SIV infection in rhesus macaques of Chinese origin (ChRh) compared to Indian origin (InRh) in Chapter 3. ChRh in our study were better able to control viral replication and resist disease progression compared to InRh. Peripheral immunocyte kinetics were

evaluated using four color flow cytometry in order to define parameters of the differential immune response. No consistent differences were evident, demonstrating that peripheral immune correlates of viral control and disease progression remain unknown.

Natural SIV infection has been identified exclusively in primate species inhabiting continental Africa. Serological evidence of exogenous lentiviral infection has been noted in wild lemurs in Madagascar. In Chapter 4, we investigated evidence of a naturally occurring lentivirus, possibly related to African SIVs, in samples from a captive population of *L. catta* at the Indianapolis Zoo. We show confirmatory serological reactivity to diverse lentiviral antigens but failed to amplify lentiviral specific sequences using established degenerate primer sets.

In total, this work represents investigations that interrogate important aspects of nonhuman primate lentiviral pathogenicity. While results were primarily negative in nature, these studies provide important new information and point to additional studies required that will continue investigations into the complex nature of lentiviral host: virus relationships.

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Introduction

Host Cell Antigen and T-lymphocyte Subset Contribution to Simian Immunodeficiency Virus Pathogenicity

SIV is member of Retroviridae, Genus Lentivirus:

Simian immunodeficiency virus (SIV) is a member of the large and diverse retrovirus family in the genus lentivirus. This genus includes five broad species that infects a) monkeys (13), apes, humans (6), b) horses (25), c) cattle (56), d) sheep, goats (52), and e) felidae (44). Lentiviruses are classified based on morphogenesis, morphology, and viral genomic relatedness. The viral genome is a dimer within the viral capsid consisting of linear, positive sense, single strand RNA coding for Gag, Pol, and Env proteins along with at least three additional auxiliary proteins. Primate lentiviruses contain some combination of the accessory genes *vif*, *vpu*, *vpr*, *vpx*, *tat*, *rev*, and *nef* whose expression contribute to the complexity of the viral lifecycle (33, 58).

Following binding of viral envelope protein to its receptor and subsequent interaction with the required co-receptor, membrane fusion occurs and the nucleoid enters the target cell cytoplasm. Subsequent to uncoating, viral reverse transcriptase creates double-stranded linear DNA by reverse transcription of the RNA genome.

Double stranded DNA becomes part of an integration complex with the ability to enter the nucleus and integrate into the host cell genome thus becoming provirus. Transcription of proviral DNA results in both spliced RNAs transcribed into viral protein and full length genomic RNAs for packaging into newly formed viral particles. Following assembly, budding occurs at the plasma membrane without a preformed nucleoid followed by maturation to a cone or rod-shaped nucleoid (33).

Discovery and characterization of primate lentiviruses:

Human immunodeficiency virus (HIV) was first cultivated in 1983 following investigation of unusual case reports of previously healthy patients presenting with a syndrome characterized by lymphadenopathy, *Pneumocystis carinii* pneumonia, cytomegalovirus-associated retinitis, cryptococcal meningitis, non-Hodgkin's lymphoma, and Kaposi's sarcoma (3, 6, 15). HIV-1 is most closely related to the SIVs infecting chimpanzees and gorillas (SIVcpz and SIVgor) and is now known to be the result of a cross-species transmission from chimpanzees to humans (57). Similarly, SIV sooty mangabey (SIVsmm) is the origin of HIV-2, endemic to Western Africa. Despite the emergence of HIV from SIVs, the capacity for cross-species transmission appears to be rare (3, 58).

To date, 41 full primate lentiviral genomes representing 23 types have been sequenced. Partial sequences are available for 11 others, while additional distinct phylogenetic groupings almost certainly remain to be discovered (7, 9, 58). Natural host-adapted SIVs do not cause appreciable disease in their African hosts (1, 42); however, SIV sooty mangabey (SIVsmm) and SIV African green monkey (SIVagm)

cause disease in Asian macaque species following experimental inoculation (2, 19, 29, 35). Asian macaque species do not support a species specific SIV and are considered a non-adapted host resulting in susceptibility to viral pathogenesis.

Chapter Summaries

Chapter 1: Characterization of cellular proteins in SIV-Env

Studies have estimated that the number of MHCII molecules acquired during budding of HIV and SIV virions from the cell surface may surpass the number of virally-encoded gp120 molecules (5, 26). The MHCII isotype HLA-DR is preferentially incorporated into virions over the DP and DQ isotype, HIV Gag assists trafficking of MHC II to the cell surface, and viral Env is required to package MHCII into the budding virion (17, 46) supporting the hypothesis that an active mechanism selectively directs MHCII to be 'captured' by HIV during virion maturation (12,14). MHC class II is upregulated on activated T cells infected with HIV. Class II transactivator (CIITA), which stabilizes MHCII subunit binding and is expressed after CD4+ T cell activation is known to directly increase HIV replication (50, 51). Thus, multiple lines of evidence suggest that incorporation of MHC molecules may well be a consequence of evolutionary pressure conferring an advantage by enhancing the efficiency of viral infectivity, and MHC molecules may represent an important target present on the viral envelope that is not subject to viral immune evasion mechanisms.

Monoclonal antibodies against a number of membrane proteins have been shown to block retroviral induced syncytium formation (22). Monoclonal antibodies specific for distinct antigens expressed at the surface of CD4+ T cells including CD4, CD3, CD7, LFA1/CD11a-CD18, and HLA class I and II (MHCI and II) have been reported to interfere with HIV infectivity (8, 38, 40). Additionally, mature viruses are lysed by anti-sera against the same cells from which the virus was derived (27).

Based on these important findings, we hypothesized that monoclonal antibodies specific for MHCII would bind the envelope of SIV produced from MHCII positive cells and inhibit viral infectivity. We, therefore, assessed the ability of monoclonal antibodies specific for MHCII to block infectivity of SIV *in vitro* to further define the basis of previous observations. Our results did not replicate the viral inhibition associated with anti-MHCII antibodies and illustrate that viral infectivity is strongly influenced by factors unique to the cell culture system and immunocyte signaling/cell membrane protein characteristics.

Chapter 2: Allogeneic immunity as an adjunct to lentiviral vaccine efficacy

A commercial whole inactivated viral (WIV) vaccine has been shown to protect cats from Feline Immunodeficiency Virus (FIV) infection (37, 47), and WIV SIV preparations have been shown to protect macaques challenged with SIV, providing evidence that immune responses to some vaccine preparations are capable of protecting against lentiviral infections (20, 21, 23, 31, 32, 48). Of the 17 studies in which macaques were challenged with human grown SIV, seven showed complete protection of all vaccinated animals, seven showed protection of $\geq$ 50% vaccinated

animals, and two showed protection from increased viral loads compared to controls (59). Hopes for an effective HIV vaccine using standard WIV immunogen technology were dampened, however, when protected macaques re-challenged with SIV grown in macaque PBMC did not resist infection (10, 18, 39). Further investigation of this phenomenon indicated that the basis of protection against SIV grown in human cells in early trials was at least partially attributable to an anti-human xenogeneic response, versus anti-SIV adaptive immunity (11). Consequently, optimism about use of WIV immunization as a strategy for HIV vaccination waned significantly. However, the observations that: 1) the majority of SIV vaccine trials using WIV preparations afforded substantial protection, and, 2) anti-host immune response may contribute to efficacy, have not been fully exploited. We therefore developed a nonhuman primate (NHP) vaccine model to assess novel paradigms of host-cell induced immunity that evaluated the utility of anti-host cell responses noted in initial WIV vaccine trials.

Alloimmune responses to non-self polymorphic membrane proteins, most notably the major histocompatibility complex I and II proteins (MHCI and MHCII), are among the most potent immune responses known (4, 54). Studies involving the use of sera from patients receiving multiple transfusions (60) or alloimmunization (28) to neutralize HIV *in vitro* illustrate the utility of anti-cellular responses in limiting HIV infectivity. Contradictory to these results, however, are two studies that failed to show efficacy of 1) vaccination of rhesus macaques with fixed allogeneic B cells (45) and 2) vaccination of cats with allogeneic dendritic cells (49) followed by virus challenge. Our study design modeled human alloimmunization studies shown to

produce immune responses capable of neutralizing both laboratory and wild type heterologous HIV *in vitro*. We hypothesized that the generation of a strong alloresponse would be able to limit the initial infectivity of pathogenic SIV that has incorporated matching alloantigens into the viral envelope. Rhesus macaques with divergent genotypes were utilized in order to maximize an alloimmune response. Rhesus macaques of Chinese origin (ChRh) and of Indian origin (InRh) were immunized with Liposome Antigen Nucleic Acid Complex (LANAC) adjuvanted ChRh PBMC and boosted with the same prior to challenge with ChRh passaged SIVmac239. Although stimulation indices, cellular immune activation markers, serum neutralization titers, and specific cell membrane antibody production indicated vaccinated animals raised specific immune responses to alloimmunization, animals were neither protected from viral challenge nor afforded clinical benefit. These results suggest that allogeneic immune responses do not provide the same level of protection as xenogeneic immune responses, and further studies to define the mechanism responsible for this stark difference continue to be important.

Chapter 3: Immunocyte kinetics during pathogenic vs. apathogenic SIV infection

It is well documented that InRh and ChRh differ in response to pathogenic SIV infection; ChRh have significantly lower set point viral loads and slower progression to disease (12, 30, 34, 55). The decline in HIV and SIV viral replication following acute infection is thought to be related to virus-specific immune responses and exhaustion of activated CCR5+ CD4+ T cells (14, 36). Both direct effects of viral replication and indirect effects of host response contribute to the chronic loss of

CD4+ T lymphocytes, a key predictor of progression to AIDS (24). Importantly, chronic immune activation is also closely correlated with disease progression as studies of apathogenic SIV infection of natural hosts suggest (41, 43). Factors contributing to significant viral load reductions and a more favorable clinical response to infection in ChRh are not well characterized. As a correlate to observations made in the vaccine studies described in Chapter 2, four color flow cytometry was used to assess peripheral immunocyte kinetics in order to elucidate differential immune mechanisms of viral control and resistance to disease progression. We hypothesized that the magnitude of peripheral cell responses to SIVmac239 infection in ChRh would be significantly different compared to InRh and will correlate to more effective viral control. Although key immunocyte subsets have been correlated with viral control and improved clinical outcome, no consistent differences were evident in our study animals. This suggests that peripheral immune correlates (humoral, CD8+ and CD4+ T cell responses or natural immunity) of lower plasma viral load (VL) in experimental infection are yet to be defined—reiterating the tremendous challenge that has thus far thwarted development of an effective HIV vaccine.

Chapter 4: Evaluation of retroviral infection in L. catta

Natural SIV infection is exclusive to Old World primates native to continental Africa. Lemurs have evolved in near isolation for more than 60 million years on Madagascar but have ancestral ties to Africa (16). During a health screening of ring-tailed lemurs (*Lemur catta*) captured in the Beza Mahafaly Reserve in Madagascar,

samples tested positive for seroreactivity to multiple lentiviral antigens (53). This broad seroreactivity was suggestive of infection with an uncharacterized exogenous lentivirus. A lemur-specific exogenous lentivirus would be of great interest with regards to the elucidation of the origin of primate lentiviruses and ongoing wild lemur conservation efforts. We followed this interesting finding with investigations of a captive population of *L. catta* housed at the Indianapolis Zoo. As with wild lemurs, captive lemurs showed broad seroreactivity to a panel of divergent lentiviral antigens and cross-reactivity to SIV p28 capsid antigens. However, SIV, FIV, and universal degenerate primer sets failed to amplify a lentiviral specific sequence.

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Chapter 1

MHC class II mAb inhibition of SIV *in vitro*

Abstract

To mechanistically define the ability of monoclonal antibodies specific for MHCII to bind host cell derived viral envelope MHCII and limit SIV infectivity *in vitro,* we exposed SIV to α-MHCII antibody prior to infection of susceptible cell lines. Inhibition with blockers of cell surface FcReceptors demonstrated that *in vitro* antibody binding may have indirect effects on viral entry or replication. While we demonstrated only modest inhibition of SIV by MHCII specific antibodies, our results suggest that in some cell culture systems, antibody interaction with the target cell membrane antigens complicates interpretation of traditional indicators of infection kinetics.

Introduction

Human Immunodeficiency Virus (HIV) and Simian Immunodeficiency Virus (SIV) are enveloped viruses that bud from host cells during the viral replication cycle. A number of host cell membrane proteins, including CD3, CD4, CD5, CD11a, CD18, CD25, CD30, CD43, CD54, CD63, CD71, HLA class I (MHCI), and HLA class II (MHCII) have been demonstrated to be acquired by the viral envelope together with the envelope glycoprotein, gp41/gp120 (18). The composition of host membrane derived proteins varies depending on cellular origin of the virus particle, and the resulting viral envelope is thus chimeric for both viral and host cell proteins. This observation has implications for viral pathogenesis and immune protection. Alloimmune responses to haplotype mismatched MHCI and MHCII are known to limit HIV infection. For example, sera from alloimmunized women and polytransfused patients are able to neutralize HIV *in vitro* (13, 26, 27). Experiments to determine the mechanism of this inhibition more precisely demonstrated that monoclonal antibodies specific for MHCI and II were able to neutralize viral infectivity. There is also epidemiologic evidence suggesting a role for alloimmune responses in limiting HIV transmission. Kenyan 'exposed seronegative' sexworkers are able to generate strong alloimmune responses likely to contribute to seronegative status (4). Conversely, uniparous women and HLA concordant mother and child pairs have been found to be at increased risk for perinatal HIV-1 transmission (11,

14). Perhaps most remarkably, xenogeneic immune responses in macaques to human cell membrane proteins associated with SIV vaccine preparation were shown to be sufficient for protection against challenge with SIV propagated in human cell culture (32).

MHCII has been shown to be incorporated into both experimental and primary isolates of HIV and SIV (1, 3, 10, 15, 22) and incorporation is conserved across viral subtypes (20). MHCII molecules may out number HIV-1 gp41/gp120 on the virion envelope (1). The HLA-DR isoform of the MHCII gene family is preferentially included in HIV virions due to interactions with the cytoplasmic tail of gp41 (7, 19). As noted above, Arthur et al. showed that vaccination with purified HLA-DR resulted in sterilizing immunity in macaques challenge with human cell derived SIV; however, the mechanism of viral interference is not known. A mechanistic explanation for this observed virus neutralization includes neutralizing anti-MHCII antibody binding to virion MHCII resulting in steric hindrance, or protein crowding that prevents envelope glycoprotein binding or fusion events.

Studies described here were conducted to further define the mechanism by which anti-MHCII is able to reduce SIV infection *in vitro*. While an *in vitro* culture system allows more specific control of variables, antibody neutralization studies using anti-cell membrane antibodies can be confounded by indirect effects of antibody binding to immunocyte cellular antigens and resultant post-binding events on immunocytes. A series of experiments was designed to examine both the consequence of virion-incorporated MHCII and the effects of α-MHCII antibody on the kinetics of SIV infection of T cells. The results of these studies demonstrate that negative regulation

exerted by antibody binding to cell membrane MHCII is likely a contributory mechanism in addition to steric hindrance imposed by α-MHCII antibody bound to virions underlying viral inhibition. FcReceptor enhancement of viral infection was also suggested and has implications for interpreting *in vitro* inhibition and potential mechanisms mediating anti-allotype viral infection interference *in vivo*.

Methods and Materials

Cell lines:

Sources of all cells obtained for these studies are noted in Table 1.1B. The CEMX174.T1 (CEM.T1) cell line was created by fusion between human B-cell line 721.174 and T-cell lymphoma line CEM. These cells are readily infected with SIV (25). Cells were grown in complete media containing penicillin-streptomycin 100U/ml, 9mg/ml D-glucose (Sigma), sodium bicarbonate (Gibco) 0.75% final volume, sodium pyruvate 1mM final volume, non-essential amino acids (Gibco) 0.1 mM final volume, and β_2 mercaptoethanol (Gibco) 5.5 X 10^{-5} M final volume.

The CEMX174.T2 (CEM.T2) cell line is a variant of the T1 (174 X CEM.T1) cell line (ATCC CRL-1991) produced by selection with the anti HLA-DR monomorphic antibody SFR-MI.3 and complement. This hybrid does not express MHCII DR antigen and is class II negative (24). Cells were grown in complete media as above.

The cell line CMMT-CD4-LTR-β-Gal (sMAGI, Dr. Julie Overbaugh; [5]) is derived from CMMT, a rhesus macaque mammary tumor adherent line, infected with

the retroviral vector LT4SN expressing human CD4 and stably transfected with a plasmid containing the HIV-1 LTR fused to the β-galactosidase gene. sMAGI allows quantification of infectious SIV. Cells were grown in propagation media for the infection assay containing DMEM supplemented with 100U/ml penicillin, 100 μg/ml streptomycin, 300 μg/ml L-glutamine, 0.2 mg/ml G418, and 50 U/ml hygromycin, and 10% fetal bovine serum.

Table 1.1 **Cell lines used during *in vitro* infection and inhibition experiments.**

	Description	Experimental Use	Reference/Source
Cells			
CEM.T1	Fusion product of human B-cell line & Human T-cell lymphoma line CEMX174.T1	Expansion of MHCII+ SIVsmmPbj & MHCII+ SIVmac239; target cell line for α-MHCII & α-TCR inhibition	NIH AIDS Research and Reference Reagent Program, Rockville, MD (25)
CEM.T2	CEMX174.T1 variant, MHCII DR negative	Expansion of MHCII- SIVmac239	American Type Tissue Culture (ATTC), Rockville, MD (24)
sMAGI	Rh mammary tumor line; CMMT-CD4-LTR-β-Gal	Target cell line for α-MHCII inhibition	NIH AIDS Research and Reference Reagent Program, Rockville, MD (5)

Viruses:

Sources of all viruses obtained for these studies are noted in Table 1.1A. SIVsmmPBj14 bcl3 non-filtered, clarified supernatant grown in pig-tailed macaque (Mne) PBMC was diluted 1:100 in RPMI as originally prepared for in vivo inoculation and titrated on CEMX174.T1. The stock titer was $10^{4.4}$ $TCID_{50}$ /100 μl. SIVmac239 non-filtered, clarified supernatants grown in rhesus macaque PBMC was expanded on CEM.T1 and CEM.T2. The stock titers were $10^{3.6}$ $TCID_{50}$/100 μl and $10^{3.4}$ $TCID_{50}$/100 μl respectively.

Table 1.2 **Virus stocks used during *in vitro* infection and inhibition experiments.**

	$TCID_{50}$/ml	Days in Culture	RT Activity	SIV p28 pg/ml	Passage History/ Source
Viral Stock					
SIVsmmPBj	$10^{4.4}$	17	13058	Not Done	Mne PBMC SIVsmmPBjbcl3 (Dr. P. Fultz) (17) Grown in CEM.T1
SIVmac239:					
MHCII+	$10^{3.6}$	29	3226	34,729	Rh PBMC SIVmac239 (Dr. C. Apetrei) Grown in CEM.T1
MHCII-	$10^{3.4}$	12	2174	36,597	Rh PBMC SIVmac239 (Dr. C. Apetrei) Passaged 2 times in CEM.T2

Phenotype analysis

1.1 *CEM.T1 and CEM.T2 characterization:*

Cells were used to produce viral stocks and as cell targets of SIV infection (Table 1.1). Expression of CD4, CD8, MHCII, CD45, CCR5, CXCR4, and T cell Receptor (TCR) was determined by flow cytometry using monoclonal antibodies to human cell membrane antigens (Table 1.1). Briefly, 1.5 $X10^5$ cells were incubated in the dark for 30 minutes at room temperature with 5 μg/ml antibody in flow buffer (1X PBS containing 2% FBS, and 0.2% sodium azide). Cells were then washed twice in flow buffer. Cells labeled with CD45 antibodies were resuspended in 100 μl fluorescein-labeled sheep anti mouse IgG (Sigma, St. Louis, MO) at 10 μg/ml in flow buffer and incubated in the dark for 30 minutes at room temperature and washed as described. Cells were analyzed using a Cyan flow cytometer (Dako Cytomation, Glostrup, Denmark) and list mode files analyzed with Summit software (Dako Cytomation).

Inhibition and infection experiments

1.2 *SIVsmmPBj inhibition by α-MHCII* **:**

$1X10^5$ CEM.T1 (Table 1.1) were infected with $10^{4.4}$ $TCID_{50}$ /100µl SIVsmmPBj (Table1.2) pre-incubated with 10 µg/ml α- MHCII (Table 1.3), α-CD45 (Table 1.3), or no antibody and culture supernatants harvested at 10 dpi. Culture supernatant reverse transcriptase activity determined as previously described (29).

Table 1.3 **Reagents used during in vitro infection and inhibition experiments.**

	Description	Experimental Use	Reference/Source
Antibodies			
α-MHCII	Mouse anti-human specific for HLA-DR&DP (clone HL-40)	Phenotyping CEMX.T1 &CEM.T2; α-MHCII inhibition	Monosan, Caltag Laboratories, Burlingame, CA
Mouse Isotype	MsIgG2a (clone HOPC-1)	Control antibody for α-MHCII inhibition	Southern Biotech, Birmingham, AL
α-TCR	Mouse anti-human FITC-labeled T cell receptor alpha/beta	α-TCR inhibition	Serotec, USA, Raleigh, NC
α-CD8	Mouse anti-human PE-labeled CD8 beta	Phenotyping CEM.T1	Imunotech, Quebec
α-CD4	Mouse anti-human FITC-labeled	Phenotyping CEM.T1	Pharmingen, San Diego, CA
α-CD45	Mouse anti-human (clone MEM-28)	Phenotyping CEM.T1; control antibody for α-MHCII inhibition	Monosan, Caltag Laboratories, Burlingame, CA
α-MHCII (LB3.1)	Mouse anti-human Specific for HLA-DR	α-MHCII inhibition	Hybridoma, ATTC, Rockville, MD
Reagents			
FcReceptor blocking reagent	Human IgG, blocks non-specific FcReceptor binding	α-MHCII inhibition in combination with FcReceptor blockage of the target cell CEM.T1	Miltenyi Biotec, Auburn, CA

1.3 MHCII+ or MHCII- SIVmac239 infection in the presence of α-MHCII:

Three hundred microliters of MHCII+ SIV239 or MHC II- SIVmac239 (Table 1.2) were pre-incubated for 1.5 hrs at 37° C and 5% CO_2 with 33 μg/ml or 17 μg/ml α-MHCII, α-CD45, Mouse isotype (Table 1.3), or no antibody. Following incubation, 10μl of each SIV preparation was used to infect $1X10^5$ CEM.T1 cells in 100 μl complete media. Cultures were refed at 5 days and frozen at 8 days post infection. The remaining SIV preparation was used to resuspend $3X10^5$ CEM.T1 cells. Resuspended cells were incubated 1 hour at 37° C and 5% CO_2, washed 2 times in 200 μl cold 1X PBS, moved to new plate in 100 μl 1XPBS, and frozen at -20° C. One hundred microliters of cell supernatant from infected cultures and washed cell suspensions were assayed for SIV p28 capsid by ELISA (AIDS Vaccine Program, NCI-Frederick Cancer Research and Development Center).

1.4 *CEM.T1 pretreatment with FcReceptor blocking reagent prior to MHCII+ or MHCII- SIVmac239 infection in the presence of α-MHCII:*

One hundred microliters of 1:100 MHCII+ SIVmac239 or MHCII- SIVmac239 were incubated with 20 μg/ml α-MHCII, α-CD45, or no antibody at 4°C for 2 hours. CEM.T1 cells, ($1X10^5$), were incubated with FcReceptor blocking reagent (Miltenyi Biotec) at 40 μl/ $1X10^7$ cells for 30 minutes at 37° C at 5 % CO_2. SIV preparations were added to pre-treated cells, final culture volumes adjusted to 200 μl, and cells cultured for 8 days. Supernatant, (100 μl), was removed and cells refed fresh media every 2 days. SIV capsid concentration was determined from 100 μl day 8

supernatant by SIV p28 ELISA (AIDS Vaccine Program, NCI-Frederick Cancer Research and Development Center).

1.5 *Adsorption of MHCII-bearing virions and antibody prior to infection of CEM.T1:*

One hundred microliters of SIVsmmPBj 1:1 in PBS was incubated with 20μg/ml α-MHCII or α-CD45 at 4°C overnight. Twenty μl Immobilized protein A 6% beaded agarose (Pierce Rockford, IL) or no beads were added followed by incubation at room temperature on a rocker for 3 hours. SIV preparations were then centrifuged at 2500 rpm for 15 minutes to remove beads. Supernatant was used to infect $1X10^5$ CEM.T1 cells and 100 μl fresh media added to all wells after 4 hours. Supernatant, (100 μl), was removed and replaced with fresh media every 3 days. At 7 days post infection, 100 μl supernatant was assayed for SIV p28 capsid concentration by ELISA (AIDS Vaccine Program, NCI-Frederick Cancer Research and Development Center).

1.6 *MHCII+ or MHCII- SIVmac239 infection of sMAGI cells in the presence of α-MHCII:*

Three hundred thousand sMAGI cells/well were seeded into a 96 well plated and incubated for 24 hours at 37° C at 5 % CO_2. One hundred microliters MHCII+ SIV or MHCII- SIV diluted 1:10 in media were incubated with 10 μg/ml α-MHCII, α-CD45, or no antibody at 37° C at 5 % CO_2 for 1 hour. Media was removed from plated sMAGI cells and SIV preparations added followed by incubation for 2 hours at 37° C at 5 % CO_2. Two hundred microliters additional sMAGI complete media was added to each well and the plate returned to 37° C at 5 % CO_2 for 3 days. The cells were

then fixed, stained, and infectious centers quantified as follows: media was removed, and wells washed two times with 100µl 1X PBS, and 200 µl fixative (1% formaldehyde, 0.2% gluteraldehyde) per well added; fixative was removed after 5 minutes and wells washed with 100µl 1X PBS two times followed by the addition of 200 µl staining solution (0.02% 0.2M potassium ferrocyanide, 0.02% 0.2 M potassium ferricyanide, 0.001% 2.0 M Mg_2Cl, 0.01% 40mg/ml X-Gal in 1X PBS) and incubation at 37° C with no carbon dioxide for 50 minutes; the staining solution was then removed, cells washed with 100 µl 1X PBS two times, and blue nuclei counted via light microscopy.

Statistical analysis:

The Anderson-Darling test was used to determine data normality. The Student's *t*-test was used to determine statistical significance. All analyses were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA) or MINITAB statistical software (State College, PA).

Results

Characterization of CEM cell phenotype.

A high percentage of CEM.T1 cells expressed surface MHCII, CD4, CD45, moderate numbers expressed T-cell receptor (TCR), and low numbers expressed the viral coreceptors CCR5 and CXCR4. CD8 was not expressed (Fig. 1.1 a-g).

The daughter cell line, CEM.T2, was used to produce a MHCII- SIV stock and was confirmed to be MHCII negative in addition to expressing identical attributes to

the parent cell line using flow cytometry (Fig. 1.2). Based upon these findings in experiment 1.1, it was assumed viral particles produced by each cell type would have different host cell Env protein composition.

Fig. 1.1

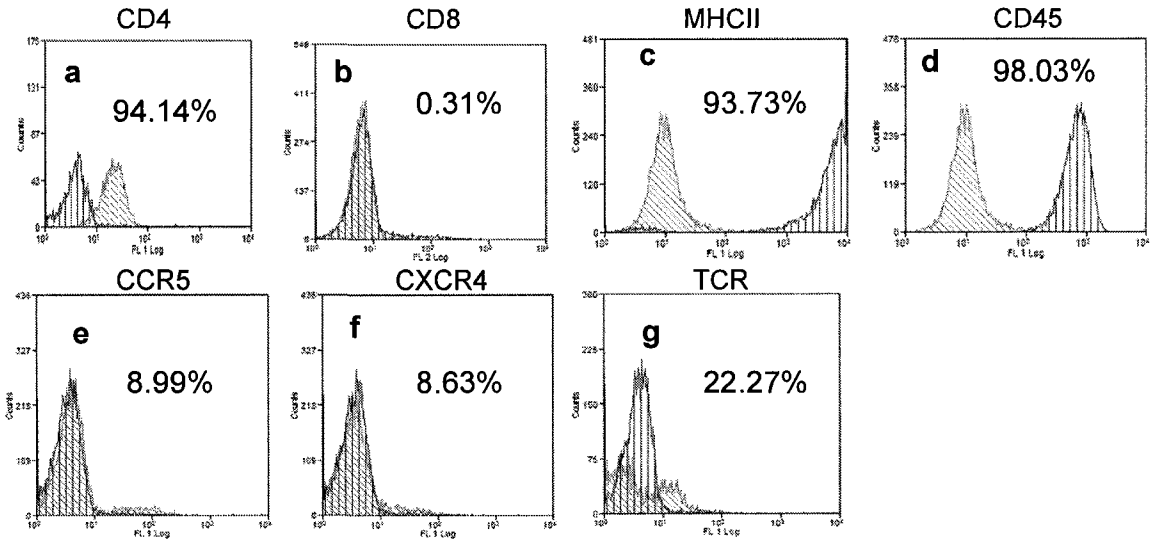

Figure 1.1
Flow cytometric phenotype characterization of the CEM.T1 cell line. Over 90% of cells express CD4, MHCII, and CD45 (a,c,d), approximately 22% express TCR (h), and less than 10% express CCR5, and CXCR4 (e,f). Fewer than 1% of cells labeled with CD8 (b).

Fig. 1.2

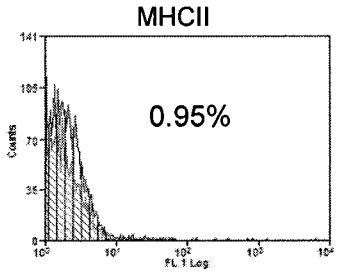

Figure 1.2
Flow cytometric analysis of the CEM.T2 cell line. Cells had identical labeling attributes to parental cell line, but were MHCII negative.

Virus neutralization by α-MHCII.

Previous studies have demonstrated that mAb specific for MHCII is able to inhibit HIV-1 replication *in vitro* in lymphoid cell lines and in primary PBMC (1, 13). These results suggest inhibition is a consequence of antibody binding directly to MHCII incorporated into the viral envelope which inhibits viral entry into target cells via mechanisms analogous to antibody neutralization. An experiment was designed to demonstrate pre-exposure of cell-free MHCII+ SIVsmmPBj to α-MHCII would result in reduction in culture virus production (described above, expt. 1.2).

Culture reverse transcriptase activity 10 days post-infection of MHCII+ SIVsmmPBj pre-incubated with α-MHCII was lower compared to controls but the differences were not statistically significant ($p > .05$, Fig. 1.3). The control antibody used in this experiment, α-CD45, is specific for a human cell membrane protein known to be excluded from the SIV virion envelope but present on the target cell membrane (30). The standard deviation (σ) of cultures inoculated with SIV pre-incubated with α-CD45 or no antibody were within 10% of the means. This compared to σ greater than 30% in cultures inoculated with virus pre-incubated with α-MHCII.

Statistically significant interference with viral production by α-MHCII may have been masked by confounding factors unique to CEM.T1 culture. One mechanism that could relate is antibody dependent FcReceptor enhancement of infection related to the expression of functional Fc Receptors on CEM.T1 cells (assessed in expt. 1.4). The huFcγRIIB expressed on B cells and has been shown to be capable of internalization of IgG coated pathogens (16). Although FcReceptor expression on CEM.T1 cells has

not been described, these cells are a hybrid T/B cell line (Table 1.1) that may have retained the expression and function of this receptor. Another possibility is that there was initial inhibition of viral binding, but by 10 days pi viral replication has outpaced the initial steric hindrance. It is also possible that the antibody concentration was not optimal to inhibit infection to a statistically significant level.

Fig. 1.3

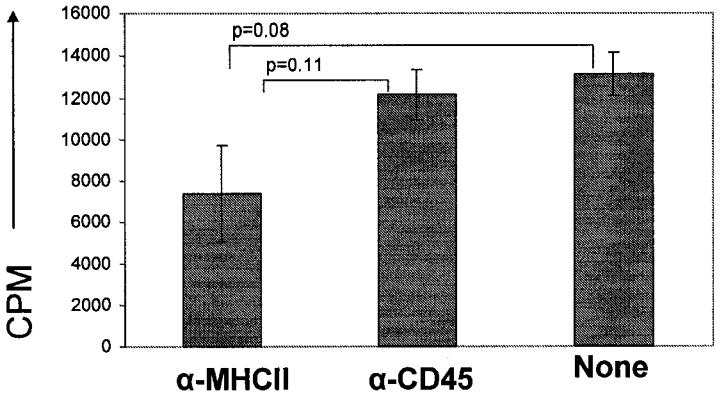

Figure 1.3
MHCII+ SIVsmmPBj growth in the presence of α-MHCII. CEM.T1 cells were exposed to MHC+ SIVsmmPBj pre-incubated with α-MHCII, α-CD45, or no antibody and culture supernatant reverse transcriptase activity determined at 10 days post inoculation. Data shown represents the means of triplicate samples.

Inhibition of viral attachment or entry by α-MHCII.

To test the possibility that inhibition of early attachment and entry events may be masked following infection and multiple rounds of viral replication experiment 1.3 was conducted. MHCII+ SIVmac239 or MHCII- SIVmac239 was pre-incubated with α-MHCII, mouse isotype control, or no antibody for 1.5 hours at 37° C with 5% CO_2 then plated on CEM.T1 cells. Cells were washed post-exposure and harvested. Following cell lysis, cell-associated SIV concentrations were determined by SIV p28 ELISA (21). Significantly less (p=0.005) MHC II+ SIV was recovered in the

presence of α-MHCII compared to mouse isotype. No significant differences were seen between MHCII- SIV treatments (Fig. 1.4 A). This suggests that α-MHCII is capable of inhibiting early viral attachment/entry events and is dependent on the incorporation of MHCII into the viral envelope.

In the second part of this experiment, viral preparations from the above experiment were used to inoculate CEM.T1 cultures and replication was allowed to proceed for 8 days in order to compare mAb inhibition of attachment with overall inhibition of culture infectivity. In agreement with experiment 1.2, there were no significant differences observed in SIV capsid production in supernatants of MHCII+ SIV regardless of antibody pre-treatment, suggesting that attachment inhibition does not persist following multiple cycles of viral replication in culture. However, in this trial, significantly less viral production was detected in SIV MHCII- cultures pre-incubated with α-MHCII compared to no antibody ($p=0.037$; Fig. 1.4B).

Fig. 1.4

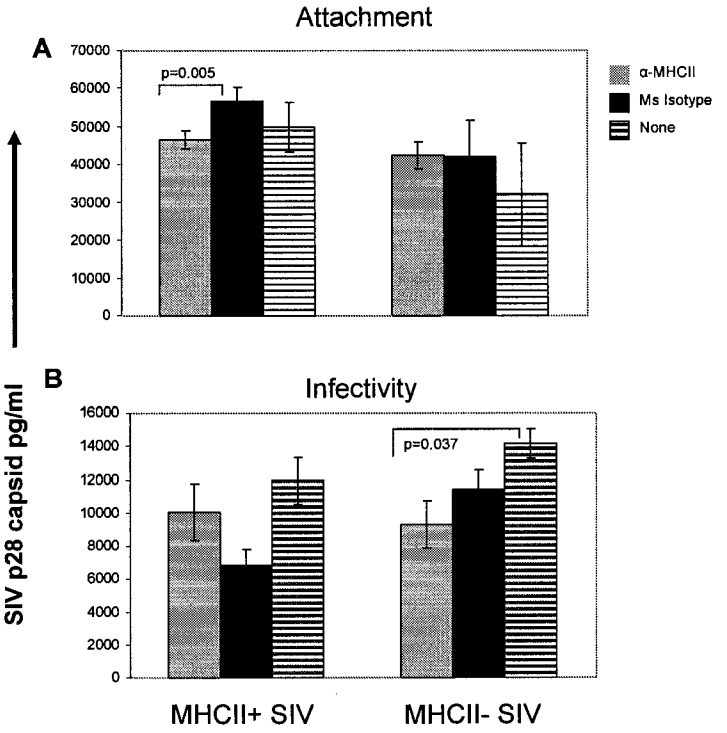

Figure 1.4
Inhibition of early SIVmac239 attachment and infectivity (Expt 1.3). (A) Significantly less cell-associated MHCII+ SIV was detected in cell lysates 3 hours pi ($p=0.005$) in the presence of α-MHCII (grey bar) compared to mouse isotype (black bar). This difference is not evident when viral supernatant is assayed 8 days pi. (B) Growth of MHCII- SIV, however is significantly lower when α-MHCII is present compared to no antibody (striped bar) 8 days pi ($p=0.037$). Data shown represents the means of triplicate samples.

Blocking cellular Fc Receptor prior to infection with SIV in the presence of α-MHCII.

Because the target cell line CEM.T1 is a T-B cell hybrid and may express functional surface FcReceptors, antibody bound virions could be internalized and contribute to contradictory enhancement of viral infectivity noted in Fig 1.4B. Surface expression of FcReceptors on this cell line has not been described in the literature. In order to assess the possible contribution of this mechanism, target cells

were pre-treated with FcReceptor blocking reagent (Table 1.3) prior to inoculation with SIV preparations. As in previous experiments, MHC II+ SIV and MHC- SIV supernatants were pretreated with α-MHCII, mouse isoptype, or no antibody.

A statistically significant decrease in SIV capsid production occurred in cultures inoculated with MHCII+ SIV in the presence of α-MHCII compared to mouse isotype ($p=0.016$) or no antibody ($p=0.006$) 8 days post-infection when FcReceptors have been blocked. However, no significant differences were noted in cultures inoculated with MHCII- SIV in the presence of α-MHCII, mouse isotype, or no antibody in the presence of FcReceptor inhibitor (Fig. 1.5) as would be predicted since these virions would not be sterically inhibited by bound antibody. These results support the conclusion that initial viral-MHCII/α-MHCII interactions were facilitated by interaction with CEM FcReceptors.

Fig. 1.5

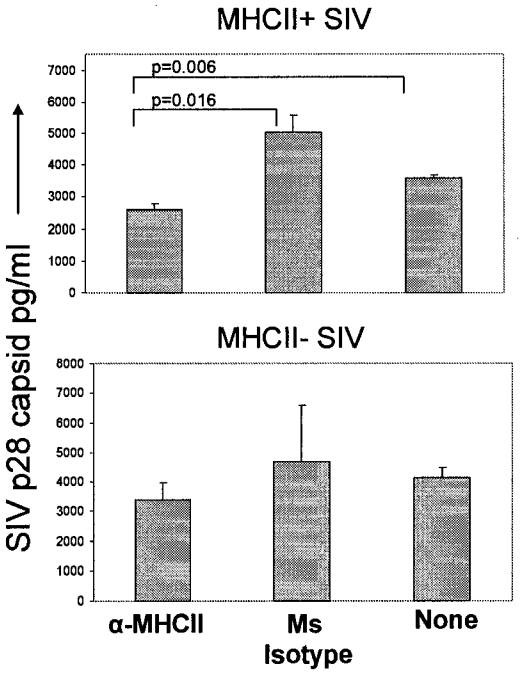

Figure 1.5
Blocking cellular FcReceptors prior to infection with SIV in the presence of absence of α-MHCII (expt. 1.4). SIV p28 capsid production is significantly decreased when MHCII+ SIV inoculums are pre-treated with α-MHCII compared to inoculums incubated with irrelevant mAb (p=0.016) and no antibody (p=0.006). There are no significant differences in capsid production in cultures infected with similarly treated MHCII- SIV inoculums. All target cells were pretreated with FcReceptor blocking reagent as described. Data shown represents the means of triplicate samples.

Adsporption of MHCII-bearing virions and antibody prior to infection of CEM.T1 cells.

Both MHCII and CD45 are expressed on the target cell surface (Fig. 1.1). Anti-MHCII added to cell supernatant has been shown to alter cell physiology resulting in reduced viral production (23) by a mechanism unrelated to steric inhibition of gp41/gp120/CD4 interactions. Thus, removal of α-MHCII and α-MHCII –bound virions prior to exposure to target cells would serve to both: (1) decrease viral

inoculum, thus demonstrating α-MHCII –specific viral interactions, and (2) remove α-MHCII from cell supernatant.

An experiment was therefore conducted to remove free antibody and antibody bound virions by subjecting α-MHCII treated viral preparations to protein A agarose bead immunoprecipitation. MHC II+ SIVsmmPBj was pre-incubated with α-MHCII or α-CD45 followed by the addition of protein A agarose beads. Protein A beads were removed by centrifugation and culture supernatants were inoculated on CEM.T1 cells.

A statistically significant decrease in viral capsid production occurred in cultures infected with MHCII+ SIVsmmPBj in the presence of α-MHC II vs. α-CD45 mAb ($p=.001$). However, this was not altered by immunoprecipitation. There is no difference in SIV capsid concentration in cultures infected with viral inoculum in which α-CD45 was removed or allowed to remain (Fig. 1.6). SIV production was significantly increased 8 days pi when α-MHC II was removed from the viral preparations prior to infection vs. cultures in which α-MHC II remained ($p=0.001$, Fig 1.6).

The implications of these results are that α-MHCII may result in inhibition of viral production when present at the time of viral attachment/entry but does not exert significant inhibition by binding directly to the virion prior to infection. This effect may be masked in previous experiments by competing events, i.e., inhibition of viral replication via entry inhibition (demonstrated in Expt 1.3) concurrent with FcReceptor dependent viral uptake in the same cell culture (illustrated in Expt 1.4), and further complicated by the effects exerted by α-MHCII binding to cellular MHCII

(demonstrated in Expt 1.5). Anti-MHCII immunoprecipitation following incubation with virus had no demonstrated effect on infection suggesting that viral- α-MHCII interactions are weak, and/or molar excess of cell membrane MHCII out competed viral particles bound MHCII for α-MHCII.

Fig. 1.6

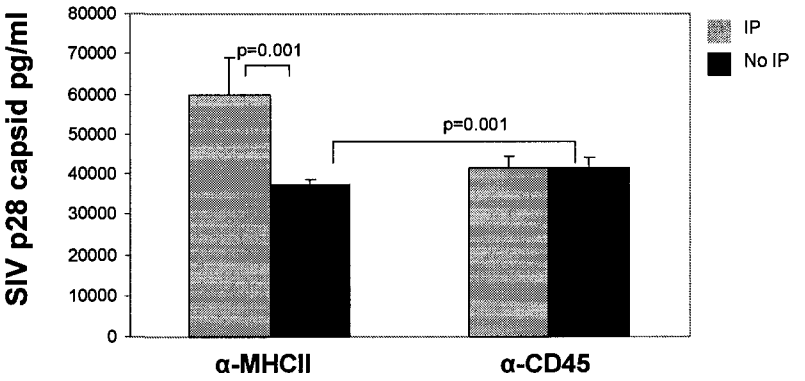

Figure 1.6
Adsorption of MHCII-bearing virions and antibody prior to infection of CEM.T1 cells (expt. 1.5). MHCII+ SIV was pre-incubated with α-MHCII or α-CD45 followed by protein A immunoprecipitation (grey bar) or no immunoprecipitation (black bar). SIV p28 capsid concentration from infected CEM.T1 culture supernatants was determined by ELISA. SIV capsid production is significantly increased (p=0.001) when α-MHCII is subjected to IP. SIV capsid production was significantly decreased where α-MHCII remained with the SIV inoculums compared to cultures in which α-CD45 remained with inoculums (p=0.001). There is no significant difference in cultures where α-CD45 was removed or allowed to remain with SIV inoculums. Data shown represents the means of six reactions.

Inhibition of viral entry by α-MHCII using the sMAGI cell line.

MHCII+ or MHCII- SIVmac239 infection in the presence of α-MHCII using an indicator cell line

Experiments 1.2, 1.3, 1.4, and 1.5 demonstrate complexities in interpretation of inhibition experiments because of the myriad effects α-MHCII has on SIV infection in a T cell/B cell hybrid target cell line. Because the sMAGI cell line does not

express T cell receptor (TCR), FcReceptor, CD45, or MHCII, yet is capable of indicating a single round of SIV replication which can be recognized colorimetrically, experiments were conducted to further investigate inhibition of early SIV infection by α-MHCII. MHC II+ SIV and MHCII- SIV were pretreated with α-MHC II, α-CD45, or no antibody, plated on sMAGI, and blue nuclei counted as described (5).

There were no significant differences in viral infectivity relative to antibody treatment for either viral stock (Fig. 1.7). In agreement with previous data, these experiments suggest that antibody bound to MHCII incorporated into the SIV envelope may not interfere with CD4/gp41/gp120 interactions strongly enough to significantly neutralize attachment/ entry in this culture system. Experiments 1.3, 1.4, 1.5 suggest, however, that this interference may be target cell dependent. MHCII- SIV was significantly more infectious vs. MHCII+ SIV ($p=0.019$) on sMAGI cells. This confirms that virally-incorporated MHCII may not strongly enhance viral infectivity.

Evaluation of α-MHCII (LB3.1) recognizing a different DR epitope than α-MHCII.

LB3.1 recognizes a determinant formed by the association of α and β chains of HLA- DR proteins (8, 9) while α-MHCII (HL-40) recognizes a determinant exclusive to the β chains of HLA-DR and –DP (9). When MHCII+ SIV was pre-incubated with α-MHCII (LB3.1; Table 1.1C) or no antibody, significant inhibition of infection was demonstrated ($p=0.022$; Fig. 1.7B). A control hybridoma antibody was not tested, so the contribution of non-commercial antibody purification techniques to diminution of infection in this culture system was not assessed. This experiment suggests that

antibody specificity is yet another factor that may influence antibody mediated inhibition of viral MHCII infection.

Fig. 1.7

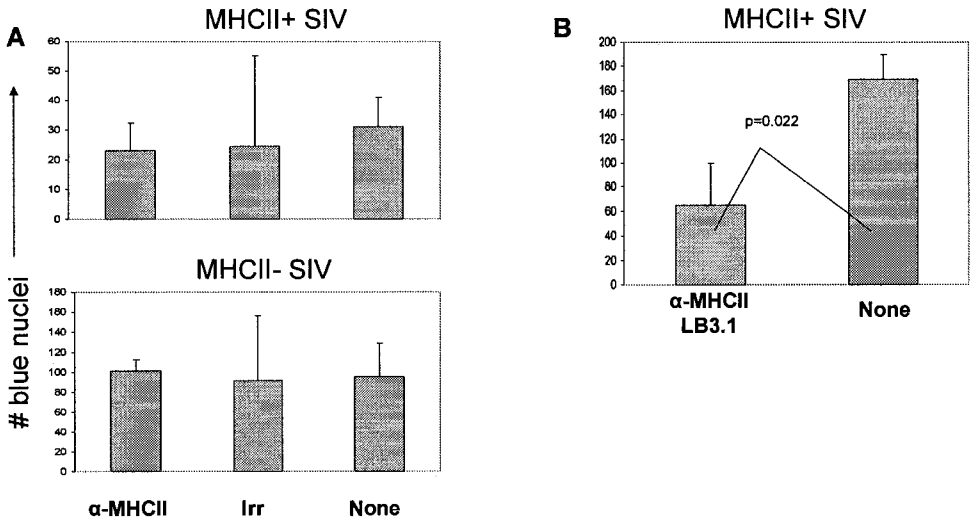

Figure 1.7
MHCII+ or MHCII- SIVmac239 infection of sMAGI indicator cell line (Expt. 1.6). (A) Virus was pre-incubated with two α-MHCII (α-MHCII and α-MHCII (LB3.1) α-CD45, or no antibody. Blue nuclei indicate cell infection. No significant differences were seen when MHCII+ or – SIV is pre-incubated with α-MHCII compared to α-CD45, or no antibody. (B)There is a statistically significant difference (p=0.022) in the number of infected cells when SIV239 is pre-incubated with α-MHCII (LB3.1) compared to no antibody. Data shown represents the means of triplicate samples.

Discussion

Significant inhibition of HIV and SIV infection has been demonstrated *in vitro* and *in vivo* as a result of monoclonal or polyclonal anti-human MHC class II antibodies. In fact, xenogeneic responses resulting from cross-species exposure to human cellular antigens, MHC II in particular, were sufficient to confer sterilizing

immunity to macaques challenged with SIV grown in human cells (2, 28). Allogeneic responses induced by alloimmunization or multiple transfusions have been shown to limit the infectivity of HIV-1 *in vitro* (13, 33). Monoclonal anti-MHC II limited the infectivity of clinical HIV-1 isolates in a dose dependent manner (13).

It has been suggested that the mechanism for the observed inhibition of viral replication is steric hindrance of viral envelope gp120/gp41 (viral Env) binding to its primary receptor CD4 on the target cell. Viral Env co-localizes with MHC class I and class II molecules in detergent insoluble lipid rafts (33). HIV-1 Gag can restore defective transport of HLA-DR to the cell surface suggesting they may share the same specific transport pathway (7). Both the matrix protein and cytoplasmic tail of gp41 are thought to interact with MHC II (6). There is evidence the CD4+ T cells exhibit low avidity exploratory adhesive interactions with MHC II molecules present on the surface of antigen presenting cells. These interactions may hasten the encounter of viral Env with CD4 (31). The interference of this process by anti-MHCII viral binding may also be responsible for the observed restriction of viral production.

The studies presented here were designed to more clearly define the mechanism of inhibition using human cell grown SIV and monoclonal antibodies specific for MHC II. Although a significant inhibition of initial viral attachment was demonstrated when MHCII positive virus was preincubated with mAb compared with MHCII negative virus, this inhibition did not predictably result in the reduction of viral production from downstream cultures (Fig. 1.4).

The reduction in viral production in cell culture assays including α-MHCII may be the result of negative regulatory events in target cells subsequent to antibody binding. This is supported by experiments in which target cells were pre-treated with FcReceptor blocking reagent (Fig. 1.5). It is also possible that antibody affinity and specificity were not sufficient to block infection (Fig. 1.7), and that post-blocking entry and replication eventually masked mild steric inhibition (Fig. 1.3, 1.4B).

The inhibition in these and other studies when demonstrated has been modest, and increased infectivity of viruses that are MHCII positive vs. MHC negative was not evident. Detailed electron microscopy studies have demonstrated substantial differences in the numbers of viral Env spikes per virion. Although spikes did not show evidence of being able to freely diffuse, local spike density varied allowing for clustering (34). It is probable that these properties vary among experimental and primary viral isolates, resulting in MHCII being positioned outside local concentrations of viral Env spikes reducing any effect of antibody binding. It is also possible, as Lakhashe *et al.* propose, that the combined size of a MHC II/antibody complex (≈260 kDa) is not large enough to inhibit interactions by the viral Env trimers (≈483 kDa)(12). Finally, it is possible that polyclonal high titer antibody concentrations generated *in vivo* are required for effective inhibition, in contrast to low titer monoclonal inhibition attempted in these studies.

Since stronger inhibition was found using polytransfused patient anti-HLA antibodies and in xenoantibody studies performed by others, future studies should more closely evaluate which xenogeneic or allogeneic antibody specificities may be more relevant to neutralization of viral activity, and should include controls that

measure effects of FcReceptor-immunocyte interactions inherent in a tissue culture system. The use of a Rh macaque model to test alloimmunization as a component of SIV vaccination was pursued in order to test the capacity of whole animal polyclonal immunity to overcome challenges noted in this series of *in vitro* experiments.

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Chapter 2

Lack of protection against SIV239 challenge in alloimmunized Chinese or Indian-origin rhesus macaques

Abstract

Previous studies have indicated that sterilizing immunity can be achieved in rhesus macaques by immunization with whole inactivated SIV propagated in human cell lines. In most instances where this has been observed, the protective immune responses achieved are thought to have been directed primarily against human cellular antigens rather than against SIV antigens. This study addressed the potential for alloimmune responses to generate protection against SIV infection in rhesus macaques as a model of HIV infection. We immunized rhesus macaques of two different strains (n=3 animals per group) with allogeneic PBMC. Control animals were immunized with autologous PBMC. Immunized animals developed allo-specific immunity, and serum from immunized animals neutralized virus infectivity *in vitro*. However, when animals were challenged with SIVmac239 propagated in rhesus allotype matched PBMC, all animals became infected to an equivalent degree. These results suggest that alloimmunization alone is not sufficient for induction of protective immunity against transmission of SIV and HIV.

Introduction

Interrogation of *ex vivo* demonstrations of host cell contributions to SIV infection are limited in their ability to mimic the complexity of host: viral interactions in a whole animal model. Simian immunodeficiency virus (SIV) infection of rhesus macaques (Rh) is the most widely utilized model of HIV infection and is of great importance in HIV vaccine development (14). We therefore chose this model to test the hypothesis that allogeneic immune responses would include anti-cell membrane specificities able to neutralize SIVmac239 challenge *in vivo*. Early vaccine studies utilizing whole inactivated SIV (WIV) resulted in substantial protection against challenge with virus grown in human cell culture (32). Despite initial optimism about these results, Stott *et al.* reported that Rh immunized with uninfected human cells alone were also protected from infection with SIVmac251, and anti-cell titers were correlated with protection (29), suggesting xenogeneic immunization was a key component of sterilizing immunity. Further, anti-human cell antibodies have been consistently identified in the sera of Rh immunized with WIV (17). The mechanism assumed to be responsible for these observations is the inclusion of host cell membrane proteins, especially MHC molecules, into the HIV and SIV viral envelope during the budding process which is neutralized by antibodies directed against these host antigens (3, 5, 6).

Studies demonstrating that allogeneic immunization could similarly contribute to vaccinal immunity against SIV have not been reported, though studies involving sera from polytransfused patients, alloimmunized patients, and patients with the autoimmune disease systemic lupus erythematosus demonstrate the interesting possibility that humoral responses to cell membrane antigens, most importantly MHC molecules, can limit HIV infectivity (18, 25, 28, 33).

Simultaneously, MHCII is upregulated on activated CD4+ T-cells and appears to be 'preferentially' incorporated into HIV Env during budding. HIV interacts specifically at several points of viral replication with MHCII expression on the cell surface. The class II transactivator (CIITA) directly increases HIV replication, providing one explanation for higher HIV replication in activated vs. quiescent T cells (23, 24). Also, HIV Gag facilitates trafficking of MHCII to the cell surface, and viral Env is required for packaging MHCII into the budding virion (10, 22). MHCII is the most commonly identified and most abundant host membrane protein associated with the viral envelope (2, 16). The observation that the MHCII isotype DR is preferentially incorporated over the DP and DQ isotype supports the idea that there is an active mechanism not yet defined that directs MHCII incorporation into the virus (2, 26). Taken together, MHCII incorporation likely represents a mechanism important for viral infection and persistence, making it an ideal target for anti-viral vaccine and therapeutic strategies. Therefore, alloimmunity raised against a non-self haplotype has been proposed as a potential mechanism to augment vaccine efficacy. Further, proof of this phenomenon provides interesting information relevant to lentiviral biology and natural transmission.

Rh of Indian (InRh) and Chinese (ChRh) lineages are allogeneically distinct (19), and therefore represent an opportunity to maximize an experimentally induced alloresponse in a well-established animal model of HIV infection. This study was designed to prime and boost a strong alloresponse followed by challenge with virus presenting the same Env alloantigen—providing a platform for comparison of the strength of alloresponse and its effects on infection with pathogenic SIVmac239. Our results demonstrated that: 1) immunization with freshly isolated allogeneic PBMC in conjunction with cationic liposome DNA adjuvant is immunostimulatory; and, 2) alloresponses could be measured post-immunization and serum from vaccinated macaques neutralized corresponding allo-SIV *in vitro*. However, protection against intravenous challenge with pathogenic SIVmac239 was not observed in alloimmunized animals. Further, as previously reported, ChRh had significantly lower viral load (VL) by day 45 post infection than InRh regardless of immunization status. We conclude that, while immune responses resulting from this alloimmunization protocol were induced, the response was not robust enough to protect Rh against parenteral challenge with pathogenic SIV. It is possible that protection might be afforded against a less pathogenic virus, a relevant mucosal challenge, or could significantly augment a vaccine immunity against viral Env. Our results suggest, however, an alloimmunization vaccine strategy for SIV has significantly less potential to enhance vaccine efficacy than xenoimmunization.

Methods and Materials

Animals:

Eight adult male Rh (*Macaca mulatta*), three ChRh and five InRh, ranging in weight from 7-12 kg were selected from animals born at the Tulane National Primate Research Center. The ChRh colony has been maintained as a separate and unique breeding colony with no IndRh introduced since 1987 (19). Animals were housed in AAALAC-international accredited facilities. All animal work was reviewed and approved by the TNPRC animal care and use committee prior to initiation. Animals were haplotyped for w201, 0401/06, and DPB1*06 alleles. Results for InRh showed them to be closely related while primers failed to type any of the ChRh.

Viral stock for virus neutralization and T cell proliferation assays and challenge:

SIVmac239 generated from an *in vivo* passage was propagated on CEM.T1 (presented in Chapter 1, Table 1.1) cells. Virus, ($10^{4.9}$ $TCID_{50}$) was added to $1X10^7$ PBMC from each Rh in individual cultures supplemented with IL-2 100U/ml (Chemicon International, Temecula, CA) and concanavalin A 5μg/ml (Sigma, St. Louis, MO). Stocks (presented in chapter 3, Table 3.2) were collected bi-weekly from each of the eight cultures and quantitated by SIVp28 ELISA (SAIC-Frederick, Inc.). Viral supernatants grown from ChRh PBMC were combined to generate a virus pool containing equal capsid concentrations from each animal; this stock was titrated on CEM.T1 cells. Four hundred $TCID_{50}$ as calculated by the Spearman-Karber method was used to challenge all animals (9).

Immunization of Rh with allogeneic or autologous PBMC:

Based on genetic divergence and pre-vaccination stimulation indices, PBMC from three ChRh and two InRh were isolated using a Histopaque® density gradient (Sigma, St. Louis, MO), suspended in culture media overnight, washed in 1X PBS two times, and resuspended in sterile 1X PBS for initial immunization and boosts. Animals were anesthetized with ketamine hydrochloride (10mg/kg) intramuscular (i.m.) and three ChRh and three InRh were vaccinated with $1X10^7$ pooled ChRh PBMC. The two remaining InRh were immunized with $1X10^7$ autologous PBMC. Vaccines were administered as follows: $5X10^6$ cells were delivered intravenous (i.v.) and $5X10^6$ cells equally divided between three intradermal (i.d.) sites. Thilsl strategy was employed to mimic alloimmunization protocols in human clinical settings (18). The cationic liposome DNA adjuvant, LANAC, dosed at 200 µl per $5X10^6$ cells (3mg/ml DNA), was mixed with PBMC immediately prior to administration (35). Animals were boosted twice at six week intervals using the same protocol (Fig. 2.1).

Table 2.1 **Alloimmunization study design.**

Origin	Immunization/ Boost	Number of Animals	Challenge Virus
Indian (InSelf)	Autologous PBMC	2	ChSIVmac239
Chinese (ChAllo)	Allogeneic (Ch) PBMC	3	ChSIVmac239
Indian (InAllo)	Allogeneic (Ch) PBMC	3	ChSIVmac239

Table 2.1: Allogeneic immunization scheme with gradient purified autologous (self) or pooled Chinese macaque PBMCs. Ten million PBMC where injected i.v. in 3 intradermal sites. Animals were challenged i.v. with pooled 400 $TCID_{50}$ SIVmac239 grown in stimulated PBMC from each of the study animals of Chinese origin.

Fig. 2.1

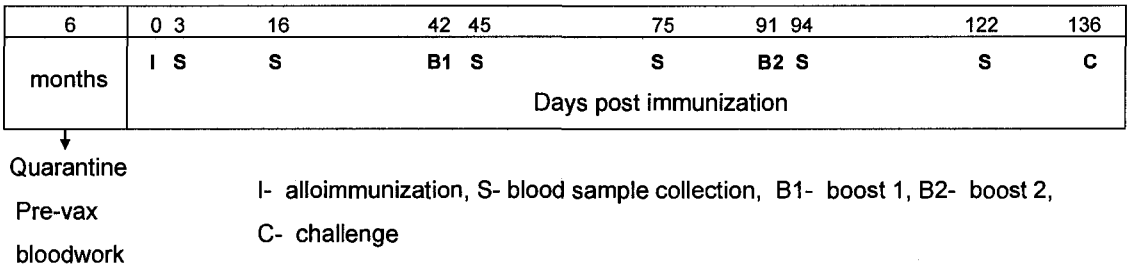

Figure 2.1: Timeline for alloimmunization, boost and challenge protocols. Early samples were taken to evaluate acute changes and 4 week post-treatment samples to evaluate more persistent changes associated with the immunization and subsequent boosts.

Experiments demonstrating generation of an alloimmune response:

2.1 *Evaluation of cell surface lymphocyte activation markers*

Pre-challenge lymphocyte activation marker expression was determined by staining $2X10^5$ isolated PBMC from each animal on each sample date with monoclonal antibodies to human MHCII –DR FITC (Biolegend, San Diego, CA) and CD69 PE-Cy7 (BD Pharmigen, San Jose, CA) and analyzing samples with a Coulter EPICS XL MCL flow cytometer (Beckman Coulter, Miami, FL).

2.2 *Detection of alloantibodies specific for mixed ChRh lymphocyte membrane antigens.*

EDTA plasma was collected from each animal two weeks after the second boost of the vaccine protocol. Viably frozen PBMC, ($1X10^7$), from study animals were cultured in standard media (30) for 14 days. Equal numbers of cells from each of the 3 ChRh were combined, and $2.5X10^5$ total cells were incubated with EDTA plasma at

1:20 for 1 hour at room temperature. Cells were washed two times, incubated with Goat anti-monkey IgG: FITC (Serotec, Oxford, UK) at 1:20 for 45 minutes at room temperature, washed two times, and resuspended in 400µl flow buffer. Cultured cells, ($2.5X10^5$), from an autoimmunized InRh (InSelf2) were stained in the same way. Events were gated for lymphocyte parameters and 5000 cells analysed using the Cyan Flow Cytometer (Dako Cytomation, Glostrup, Denmark). List mode files were analyzed with Summit software (Dako Cytomation).

2.3 *Detection of alloantibodies capable of mixed ChRh SIV neutralization.*

A constant amount (50 $TCID_{50}$) of SIV stock generated from autologous or allogeneic PBMC was added to two-fold dilutions of plasma starting at 1:4 taken from animals pre- and post-immunization. Following 90 minute incubation at 37° C with 5% CO_2, 100µl of the plasma-virus mixture was added in triplicate to CEM.T1 target cells. Virus was detected by reverse transcriptase assay (34) after 11 days in culture at 37° C with 5% CO_2. Virus neutralization (VN) titers were calculated using the Spearman-Karber formula (9).

2.4 *Detection of alloantigen specific T cell responses by the mixed leukocyte reaction (MLR) and antigen stimulation/proliferation assay.*

Whole PBMC was prepared from pre- and post-immunized animals. One hundred thousand PBMC were plated in triplicate with the following additions: media, concanavalin A, autologous-cells, and 10^5 cells from each of the other seven animals. ^{3}H-Thymidine was added at day 3 and cells harvested for analysis of proliferation activity 18 hours later. Post-vaccination PBMC were exposed to irradiated autologous- or pooled allo-PBMC (ChRh), or irradiated viral preparations

for 5 days, exposed to ^{3}H-Thymidine, and harvested to evaluate proliferative activity 18 hours later.

Allotype-matched SIVmac239 challenge:

Animals were challenged six weeks after the second boost with 400 $TCID_{50}$ pooled ChRh SIVmac239 intravenously.

Quantification of post infection plasma viral load (VL) and CD4+ T cell numbers:

2.5 *VL quantification.*

EDTA blood was collected using EDTA as an anticoagulant at six time points post challenge and processed within 2 hours. Viral quantification was performed on plasma sample by the SIV bDNA method (Siemens Testing Laboratory, Emeryville, CA) and compared to an external standard curve.

2.6 *Determination of CD4+ T-cell dynamics.*

One hundred µl of whole blood was stained with CD4 PerCP, and CD3 PE (BD Pharmigen, San Jose, CA) monoclonal antibodies. Red blood cells were lysed with FACS lysing solution (Becton Dickinson, San Jose, CA), cells washed with PBS (400xg, 7 min), and resuspended in 2% paraformaldehyde. Samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences Immunocytotometry Systems,

San Jose, CA). All list mode files were analyzed with FlowJo software (Treestar, Inc., San Carlos, CA).

Statistical analysis:

The Anderson-Darling test was used to determine data normality. Statistical significance of group mean differences for stimulation indices were determined using the one-way ANOVA and the Dunnett's post test. The Student's *t*-test was used when appropriate. All analyses were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA), GraphPad Prism 4.0 (San Diego, CA), or MINITAB statistical software (State College, PA).

Results

Alloimmunization and autoimmunization resulted in a generalized increase in immune activation.

MHCII and CD69 are upregulated on peripheral lymphocytes upon immune activation (1, 27). In order to assay for immune responses to the alloimmunization protocol, surface expression of MHCII and CD69 on ficol gradient purified PBMC from study animals was determined by flow cytometric analysis (expt. 2.1). There were no consistent differences between animals correlating with either treatment group or animal origin at any time point. The trend line calculated from individual MHCII and CD69 mean expression shows an increase over the treatment period with

a correlation coefficient of 0.95 and .75, respectively (Fig. 2.2 A, B). The immunization protocol resulted in a sharper increase in MHCII expression compared to CD69 (Fig. 2.2, trend line slope of the mean=9.3929 and 1.7143 respectively). The LANAC adjuvant, comprised of cationic liposomes and bacterial DNA, has been shown to generate strong cellular and humoral immune responses in previous studies (8, 12, 15, 35). The fact that animals immunized with autologous PBMC demonstrated increases in activation marker expression comparable to alloimmunized animals suggests that the change is attributable to exposure to LANAC adjuvant and not to allogeneic PBMC *per se*.

Fig. 2.2

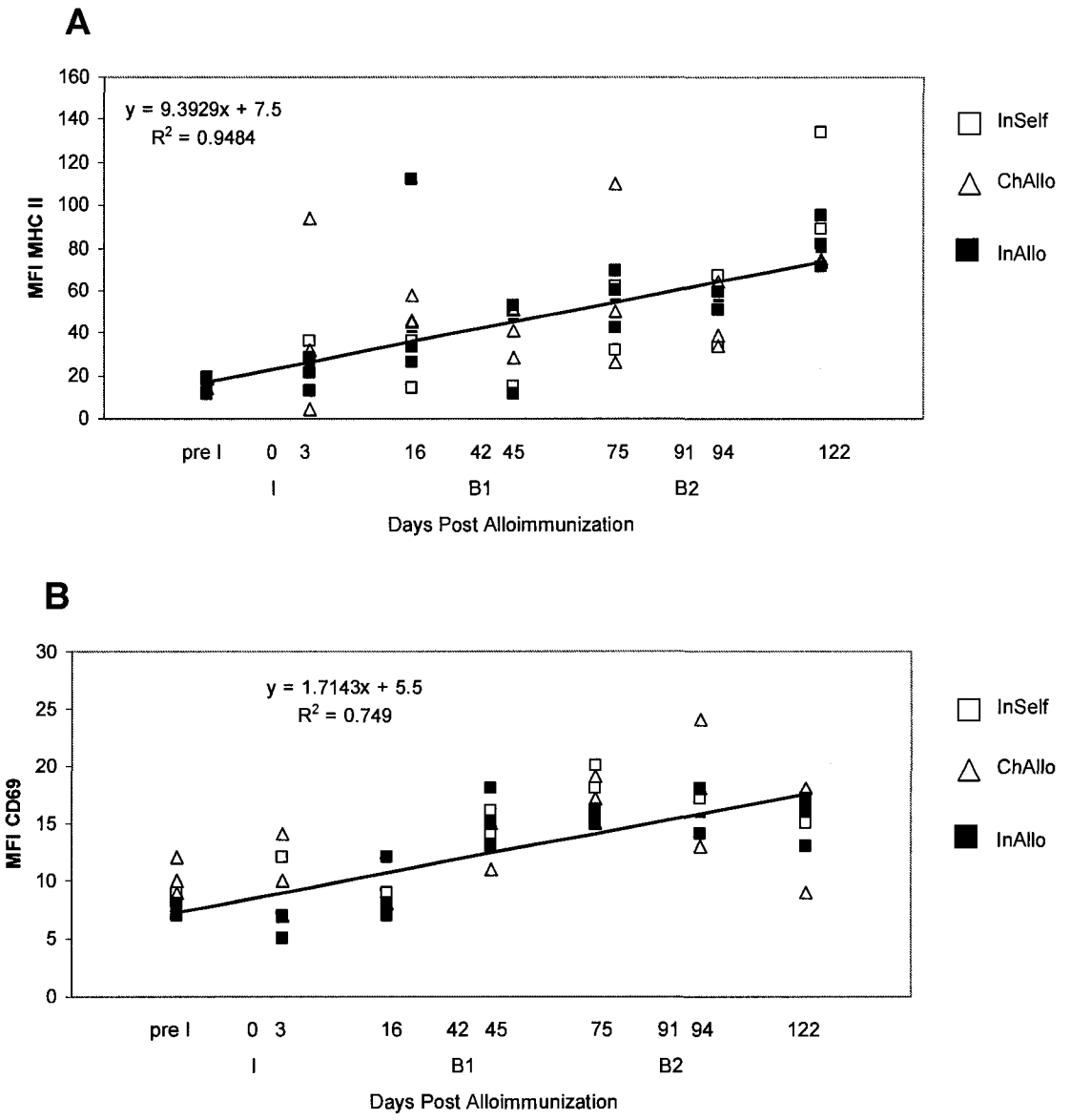

Figure 2.2: Pre-challenge mean fluorescence intensities of A) MHCII and B) CD69 isolated PBMC from adult male rhesus macaques immunized and boosted with adjuvanted self or allogeneic PBMC. □ InSelf animals (n=2) were immunized and boosted with LANAC adjuvanted autologous PBMC, Δ ChAllo (n=3) animals were macaques of Chinese origin immunized and boosted with LANAC adjuvanted mixed Chinese PBMC, and ■ InAllo (n=3) were macaques of Indian origin immunized and boosted with LANAC adjuvanted mixed Chinese PBMC. The trend line was calculated using the median value for all animals at each time point. The trend line for the median value of both the MFI of MHC II and CD69 have a positive slope (mean=9.3929 and 1.7143 respectively).

Plasma samples of alloimmunized Rh specifically bound to lymphocytes derived from ChRh.

Because inhibition of SIV challenge is thought to be alloantibody dependent, the production of specific alloantibodies was determined by the detection of plasma IgG staining of culture stimulated mixed ChRh or InRh lymphocytes followed by flow cytometric analysis (expt. 2.2). To assess anti-cell antibody production induced by alloimmunization, viably frozen cells from all ChRh and from one autoimmunized InRh were cultured and stained with anti-sera from alloimmunized and autoimmunized study animals and mean fluorescence intensity (MFI) was determined by flow cytometry (Fig. 2.5, Table 2.2). MFIs resulting from staining of cultured PBMC from mixed ChRh compared to autoimmunized InRh (InSelf2) with EDTA plasma from alloimmunized animals demonstrate significant differences ($p=0.001$) ranging from 27.37 to 162.24 (representative histograms Figure 2.5 a,b; Table 2.2). MFI differences for the autoimmunized animals measuring 11.32 and 14.86 were not significant ($p=0.086$, representative histogram Figure 2.3 c; Table 2.2).

Table 2.2

Plasma	Cultured PBMC			
	InSelf2	ChAllo1,2,3		
	MFI	**MFI**	**MFI Difference**	**p-value**
α-ChAllo1,2,3				
InAllo1	30.69	109.8	79.11	
InAllo2	25.38	131.32	105.95	
InAllo3	207.0	261.19	54.19	
ChAllo1	29.76	192.0	162.24	
ChAllo2	17.56	68.9	53.34	
ChAllo3	31.44	58.81	27.37	.001
No Alloantibody				
InSelf1	34.53	45.85	11.32	
InSelf2	19.13	33.99	14.86	.086

Table 2.2: MFI of alloantibody binding to lymphocytes matching alloimmunization immunogen. To demonstrate the production of alloantibodies by the alloimmunization protocol, post-boost plasma from alloimmunized and autoimmunized animals was used to stain either mixed ChAllo1,2, and 3 cultured PBMC or InSelf2 PBMC. Significantly greater staining of cells matching those used as alloimmunogen (ChAllo1,2,3) is seen in all alloimmunized animals compared to autoimmunized animals.

Fig. 2.3

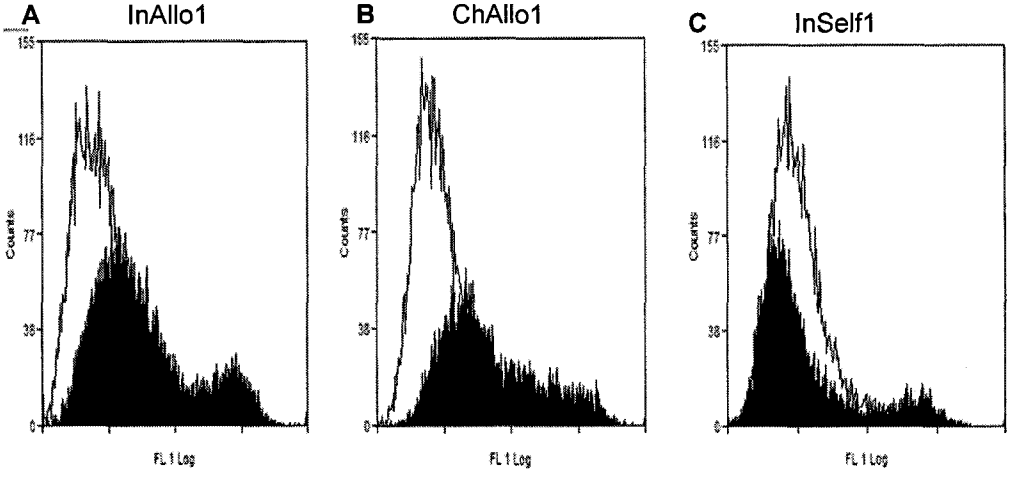

Figure 2.3: Representative histograms from an alloimmunized animal of Indian origin (InAllo1), an alloimmunized animal of Chinese origin (ChAllo1), and an autoimmunized animal of Indian origin (InSelf2). (A,B) Plasma from alloimmunized animals shows increased staining of mixed ChAllo cultured PBMC (solid black) when compared to staining of InSelf2 cultured PBMC (black line). (C) No significant differences are seen in staining with plasma from autoimmunized animals.

Virus neutralization of pre-vaccination compared to day of challenge sera in alloimmunized and autoimmunized animals.

There was no significant difference between pre-vaccination and post-vaccination neutralization titers against challenge SIV stock in the InRh vaccinated and boosted with autologous PMBC (Table 2.3). However, a highly significant difference was noted between pre-vaccination and post-vaccination neutralization titers in InRh and ChRh immunized with allogeneic PBMC ($p<0.01$), suggesting that a specific humoral response was generated by the vaccination protocol, and this response was capable of inhibiting viral infectivity *in vitro* (expt. 2.3).

Table 2.3

Animal ID	Pre-immunization	Post-immunization	
	SN Titer	SN Titer	Group p-value
Autoimmunized			
InSelf1	1:40	1:40	
InSelf2	1:25	1:50	0.5
Alloimmunized			
ChAllo1	1:25	1:40	
ChAllo2	1:50	1:79	
ChAllo3	1:22	1:50	
InAllo1	1:22	1:126	
InAllo2	1:40	1:50	
InAllo3	1:22	1:79	.00966

Table 2.3: Serum neutalization titers of day of challenge and pre-treatment sera against the challenge virus. P values of pre- vs. post treatment titers are calculated using the paired Student's *t*-test. The pre-treatment neutralization titers are not significantly different from the post-treatment titers determined on the day of challenge in the self PBMC immunized group. The neutralization titers are significantly different in the group immunized with allogeneic PBMC.

Mixed leukocyte reactions to allogeneic mixed ChRh PBMC.

The mixed leukocyte reaction using PBMC from alloimmunized or autoimmunized animals and irradiated mixed ChRh PBMCs was employed to determine if alloimmunized animals exhibited the expected specific heightened immune response 3 days following the final boost (expt. 2.4). When isolated PBMC from study animals were cultured with concanavalin A, stimulation indices were between 210 and 861 (not shown) demonstrating the ability of all individuals to proliferate strongly to a non-specific stimulus. The magnitude of the non-specific response was not associated with treatment group or animal origin. Stimulation indices calculated from PBMC cultured with irradiated mixed ChRh PBMC, however, demonstrated a statistically significant difference (p=.04) between SI values depending upon animal origin, (ie ChRh vs InRh, Fig. 2.4) but not alloimmunization.

Fig. 2.4

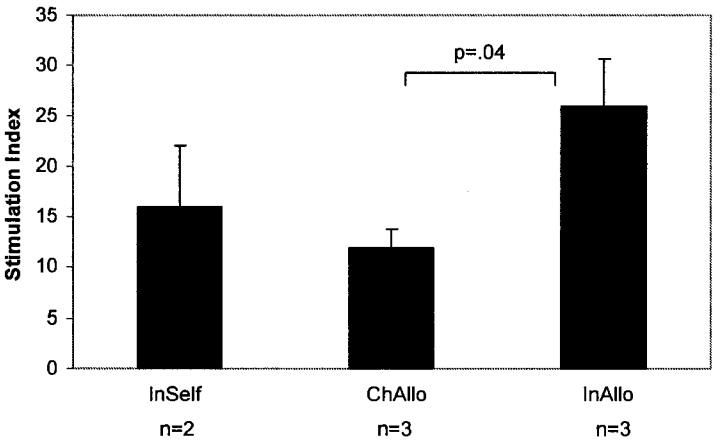

Figure 2.4: MLR of PBMC isolated 3 days post-boost 2 from all study animals and stimulated with irradiated mixed Chinese macaque PBMC matching that used in the alloimmunization protocol. SI are means of each treatment group separated according to animal origin.

Lymphocyte proliferation against inactivated SIV grown in matched allogeneic or xenogeneic cell culture.

SIV grown in human cell culture or in stimulated Rh PBMC share viral protein content but differ in cell membrane protein composition. In order to demostrate T cell proliferation specific to cell membrane alloantigens, PBMC from study animals were isolated 4 weeks post-boost 2 and cultured with irradiated SIVmac239 grown in the human cell line, CEMT.1, irradiated SIVmac239 grown in stimulated ChRh PBMC, or no stimulation (expt. 2.4; Figure 2.5). Seven of eight animals showed a higher stimulation index when exposed to SIVmac239 grown in allogeneic Rh cells than to SIVmac239 grown in human cells, demonstrating stronger immune recognition of Rh cell membrane antigens. The highest stimulation indices were generated in cultured PBMCs from alloimmunized animals, suggesting that the alloimmunization protocol resulted in specific recognition of alloantigens.

Fig. 2.5

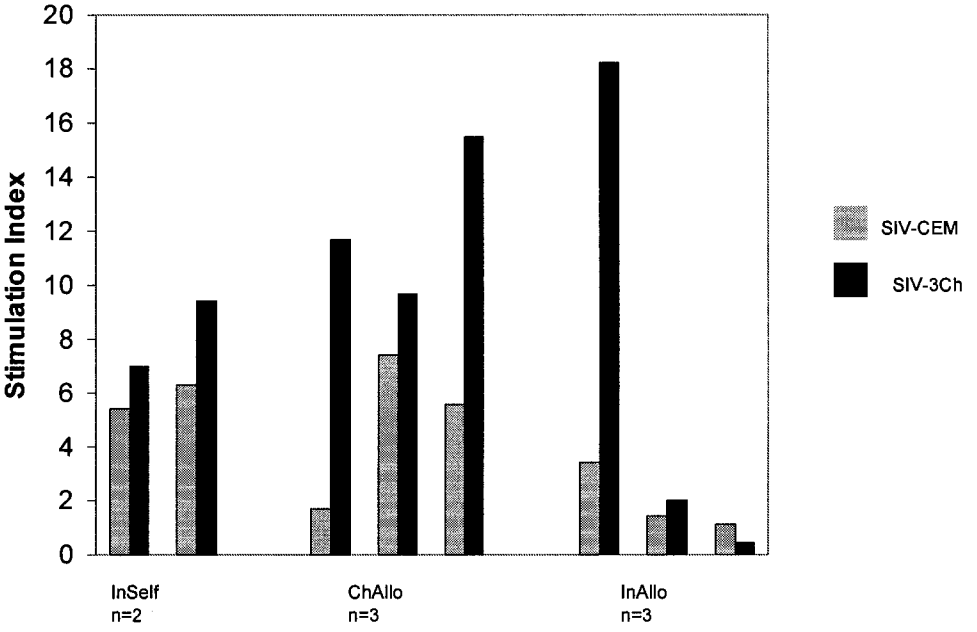

Figure 2.5: Lymphocyte blastogenesis in PBMC isolated 6 weeks post-boost 2 versus irradiated SIVmac239. Virus was grown in stimulated mixed ChRh PBMC (■SIV-3Ch), or human CEM.T1 cells (■SIV-CEM). Proliferation of 7/8 animals is greater against the ChRh PBMC grown virus and the highest values measured in alloimmunized animals.

VLs and clinical disease post-challenge.

All animals became infected subsequent to challenge with 400 $TCID_{50}$ SIV239 produced in concanavalin A stimulated ChRh PBMC. Acute phase VLs range from $1X10^{4.7}$ to $1X10^{7}$ for InRh and from $1X10^{4.7}$ to $1X10^{6.9}$ for ChRh with no differences associated with treatment groups (Fig. 2.6A). One ChRh had comparatively lower VLs throughout the study period while all three ChRh demonstrated significantly reduced VLs on days 45 and 63 compared to InRh. ChRh experienced mild to

non-existent clinical disease all InRh animals all progressed to clinical illness and were euthanized by day 115.

Fig. 2.6A

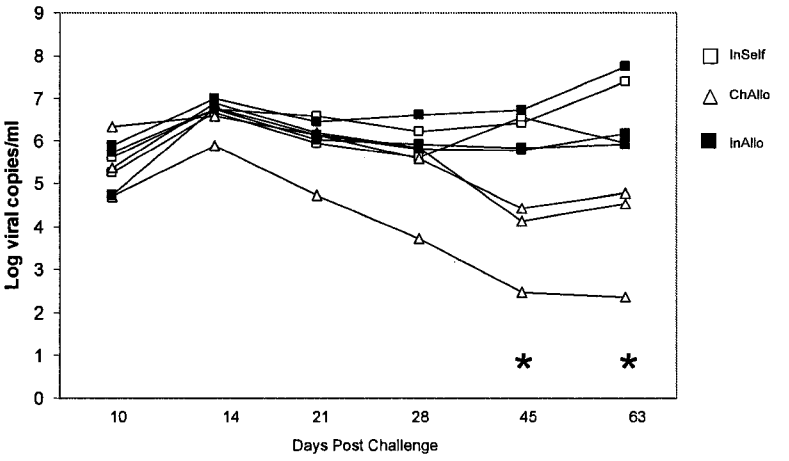

Fig. 2.6B

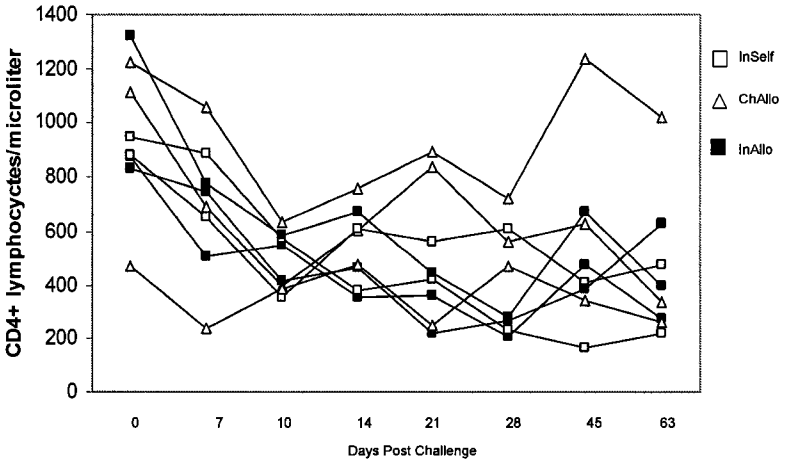

Figure 2.6: Viral loads (VLs) and CD4+ T lymphocyte counts after challenge in alloimmunized and self immunized groups. (A) Individual viral loads in autologous PBMC immunized animals of Indian origin (n=2, open boxes), in alloimmunized ChRh (n=3) (open triangles), and in alloimmunized InRh (n=3, black boxes). Peak viral loads of $1X10^6$ to $1X10^7$ viral RNA copies/ml measured in 7/8 animals. * denotes VLs statistically significantly lower ($p \leq 0.05$) in all ChRh. (B) Individual CD4+ cell depletion in autologous- immunized and alloimmunized animals.

CD4+ T cell kinetics.

SIV infection was associated with a sharp CD4+ T-cell decline in study animals with no differences associated with alloimmunization (Fig. 2.6B). The ChRh with the lowest viral titer was the only animal that recovered to pre-infection CD4+ T-cell numbers during the study period. The remaining two ChRh had low peripheral CD4+ T-cell counts on days 45 and 63 when corresponding VLs were significantly decreased.

Discussion

Studies have produced conflicting results concerning the ability of purified human alloantibodies to neutralize HIV-1 grown in PBMC with matching allotype. While Leith *et al.* were able to show significant neutralization of both a laboratory adapted and a primary strain with IgG from alloimmunized women (18), Lakhashe *et al.* were not able to replicate inhibition of infection using IgG from multiparous women using virus propagated in the partner's PBMC (16). Few studies, however, have explored the induction of anti-MHC humoral responses generated by alloimmunization as a mechanism to limit infection of macaques challenged with macaque passaged SIV (11, 21), despite the obvious utility and application of this approach given xenogeneic vaccination success. Polyanskaya *et al.* immunized a group of Rh with glutaraldehyde-fixed and β-propriolactone-inactivated transformed

B cells expressing high levels of MHCI and MHCII but were unable to demonstrate protection vs. 100 MID_{50} SIVmac220 (21). It is possible that the challenge dose overwhelmed the protective response or that the glutaraldehyde treatment of the cells altered the immunogenicity of the MHCII molecules masking important epitopes necessary for viral neutralization. The study described here follows the design of alloimmunization treatments in human patients and exploited the high degree of genetic diversity between macaques of Indian vs. Chinese origin to produce robust alloimmunity. Single-nucleotide polymorphism analysis of gene-linked regions suggests that the Chinese rhesus macaque subpopulation separated from the Indian population over 150 thousand years ago, and recurrent migration between populations has been minimal (13). The use of Indian rhesus specific primers for MHC haplotyping was unable to amplify relevant sequences in Chinese animals used in this study, supporting strong haplotype divergence.

Also as an extension of these earlier studies, and as a mechanism to extend *in vitro* studies presented in Chapter 1, we aimed to produce strong immune responses to allogeneic antigens expressed prior to challenge with allotype PBMC-generated SIVmac239. Vaccines were adjuvanted with LANAC and administered both intravenously and intradermally.

Results presented here demonstrate that immune activation was induced by re-exposure of InRh and ChRh to LANAC adjuvant. The magnitude of this increase may have possibly masked otherwise measurable differences in response to alloimmunization *vs.* autologous PBMC exposure (Fig. 2.2). However, expression of the activation markers MHCII and CD69 was not increased over baseline on the day

of challenge or during the early acute phase of SIV infection when adjuvant was not present (data not shown) suggesting that the nonspecific immunostimulatory effects of LANAC treatment did not influence infection kinetics.

As noted above, this study was designed to produce the strongest possible alloresponse to immunization by selecting animals of clearly divergent lineages to induce a robust allogeneic response. Alloantigen specific MLR responses were evident in that we witnessed a 3-5 fold increase in proliferation between pre-treatment and post-vaccination (Fig. 2.3; pre-treatment data not shown). Increased lymphocyte proliferation specific to alloantigen incorporated into inactivated SIVmac239 was also demonstrated in 7/8 animals, with the greatest responses measured in alloimmunized animals (Fig. 2.4). Further, anti-sera from alloimmunized animals was shown by flow cytometry to have specificity and modest affinity for the whole cell immunogen used in the alloimmunization protocol (Fig. 2.5, Table 2.2). Virus neutralizing titers in plasma of alloimmunized animals were increased over pre-treatment titers (Table 2.3). However, despite evidence that allogeneic recognition occurred and had some effect on limiting viral infection *in vitro*, animals were not protected against intravenous challenge with pathogenic SIVmac239 passaged in ChRh (Fig 2.6A).

These results are in agreement with *in vivo* macaque and *in vitro* human studies that failed to demonstrate a protective effect of specific anti-MHC antibody production (16, 21). Potential reasons for this vaccine failure include the possibility that the relatively high dose challenge that vaccinated animals were exposed to overwhelmed the ability of anti-cellular antibodies to neutralize infection, or, in fact,

allogeneic specificities are not capable of virus neutralization as apparently is possible when xenogeneic immune responses are mounted. This study was constrained to the use of low numbers of animals similar to initial WIV studies and in studies investigating anti-human cell membrane protein immunity contributing to viral neutralization ($n \leq 4$), making definitive interpretation difficult (29, 32). Interestingly, the two lowest VLs during initial infection were measured in a ChRh and an InRh that were alloimmunized (Fig. 2.6A), results that may have approached statistical significance had more animals been available.

We hypothesized that protection provided by alloimmunization would necessarily occur in the very early stage of infection by virus neutralization interference afforded by anti-self antibodies that bound host cell proteins in viral Env. An effective immunization would therefore limit the importance of variability in early and chronic stage VLs known to exist in ChRh vs. InRh. However, in this study we observed what has been related by others—that ChRh are able to control viral replication following SIV challenge more effectively than InRh.

Differences in VLs and SIV-induced disease have been strongly linked to the genetic origin of macaques, particularly in ChRh vs. InRh (4, 19, 20, 31), though VLs were approximately one log lower in this study compared to others (19). While one of the ChRh had significantly lower VLs on days 14, 21, and 28 post infection, the two other ChRh had VLs very similar to the five InRh on these days. All of the ChRh were able to significantly control viral replication by day 45 post infection while none of the InRh demonstrated viral control. These kinetics suggest that differences in immunization protocol were not responsible for the divergence in viral control seen in

the two lineages, and that it is more probable that this result is in line with observations described in trials without treatment intervention (7, 19, 31). This difference has been postulated to occur because ChRh may mount a more potent immune response to SIVmac (19), or may be innately resistant to infection due to cellular factors (19) or different viral tropism/compartmentalization (31). Immunocyte kinetics potentially predictive of, or resulting from these diverse clinical outcomes are examined in Chapter 3.

While it is disappointing that alloimmunization showed no influence on resistance to virulent SIV challenge, it is important for vaccine investigations to have determined that anti-cellular immune responses may have a negligible impact on vaccine efficacy, even in a system engineered to heighten anti-self adaptive immunity. Vaccine and therapeutic strategies aimed at preventing initial HIV infection continue to be the gold standard. Sterilizing immunity has only been consistently demonstrated in animals models based on WIV. Although safety concerns have necessarily limited study of this strategy in human trials, the role of anti-cell responses in this protection and in studies demonstrating HIV inhibition *in vitro* remains unclear. This study was unable to demonstrate a role for alloimmunization in providing vaccinal protection, but the implications of host cell proteins, particularly MHCII, incorporated into viral Env remains an intriguing phenomenon with potential implications for viral transmission and pathogenesis.

Acknowledgments

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Chapter 3

Similar peripheral immunocyte depletion in SIV infected Chinese and Indian macaques despite differences in capacity to control viral replication

Abstract

Rhesus macaques of Chinese and Indian origin differ in their ability to control viral replication during pathogenic SIV infection. As part of our study to assess alloimmunization as an HIV vaccine strategy, ChRh and InRh were challenged with SIVmac239. All animals became infected: however, ChRh showed significant reduction in viral loads by day 45 post infection compared to the Indian rhesus and did not exhibit rapid clinical disease. Conversely, all InRh were euthanized due to clinically apparent disease by day 115 post infection. Viral loads and peripheral immunocyte population dynamics were determined in order to find correlates for differential viral kinetics and host pathogenicity. We demonstrate that peripheral immune correlates do not consistently reflect differences in viral control or clinical outcome.

Introduction

Rhesus macaques of Chinese (ChRh) and Indian (InRh) origin were used in a novel SIV vaccine study presented in Chapter 2. Post challenge, differences in viral control and disease progression were strongly associated with animal genotype. Despite intense study, basic pathogenic mechanisms for SIV- and HIV-induced disease remain incompletely characterized, including: a) correlates of immune control, and drivers of b) viral production, c) immune cell loss, and d) immune failure. A clearer picture of the mechanisms responsible for these attributes of HIV infection is vital to the definition of specific prognostic and therapeutic indicators in HIV infected individuals.

SIV infection of Asian macaque species has been shown to result in disease closely resembling HIV infection and has been established as the experimental animal model of choice. Basic pathologic features of SIV infection of macaques, however, vary depending on viral strain, species, and individual genetic background. For example, rhesus macaques originating from the geographically distinct Indian and Chinese populations respond significantly differently to SIV infection with respect to viral loads and clinical disease progression (3, 14, 15, 31).

Here we evaluate peripheral immunocyte subset quantification post virulent SIV challenge in five InRh and three ChRh with markedly divergent clinical course of infection most likely unrelated to treatment group. These animals were subjects in an

allotypic vaccine strategy designed to evaluate anti-MHCII contribution to lentiviral immunity; however, vaccination failed to inhibit viral infection in any group and initial viral kinetics were very similar for all animals. Similar to other reports, at 45 days pi, InRh had significantly higher viral load than ChRh, and clinical course for InRh was accelerated with respect to ChRh. This study was conducted to evaluate immunophenotype kinetics in both groups of animals in an attempt to correlate appearance of specific leukocyte subsets with positive clinical outcomes, thus revealing new immune parameters associated with disease acceleration.

Table 3.1 Expected **immunocyte kinetics during controlled SIV infection in Rh.**

Cell type	Cell Surface Antigens	Predicted Role During Controlled SIV Infection	Expected Kinetics During Controlled SIV Infection
T cell	CD3+/CD4+	Support development of adaptive immune responses	Acute and chronic depletion
T cell	CD3+/CD8+	Cell mediated destruction of virally infected cells	Maturation of viral specific responses
Memory T cell Subsets:			
Naïve	CD3+/CD4+/CD28+/CD95-	Antigen inexperienced	Preservation
Central	CD3+/CD4+/CD28+/CD95+	Proliferation to replace effector site cell losses	Increased turn over, preservation
Effector	CD3+/CD4+/CD28-/CD95+	Central to virus specific responses	Dramatic cell depletion especially at mucosal sites (16)
Activated/Target	CD3+/CD4+/CCR2+	Unknown	Unknown
B cell	CD20+	Virus specific antibody production	Maturation of antibody response
Activated Monocyte	CD14+/CCR2+	Maturation to tissue Macrophages; phagocytosis/viral antigen presentation	Increased Activity

Immunocyte populations were evaluated based on their role in control of viral replication, susceptibility to virally mediated cell death, and contribution to clinical pathology and disease progression as outlined in Table 3.1. CD4+ T and CD8+ T cells and B cells are known to be important in initial control of viral replication following acute dissemination with control of viral load being associated with improved clinical outcome. Immune competence in the face of chronic viral infection has been associated with the ability of memory T cell subsets to respond and recover from virally induced cell losses. Additionally, markers of sustained immune activation have been associated with disease progression.

This study contrasts circulating immunocyte populations during apparent control of acute virus replication in rhesus macaques of Chinese origin (ChRh) with lack of control in animal of Indian origin (InRh). Animals from both groups experienced marked decline in CD4+ T cells, particularly of central memory phenotype. Additionally, both groups exhibited very similar upregulation of CCR2 expression on circulating T lymphocytes. Loss of CD4+ T cells, reduced regenerative capacity of central memory T cells, and a heightened immune activation state have all been associated with disease progression in HIV and SIV; however, here we demonstrate that these parameters do not consistently correlate with differential disease progression in animals with divergent genotypes. This demonstrates that immunophenotype measures on peripheral blood are not predictive of virulent SIV disease course, nor do they identify a correlate with immune control that provides a clue to mechanisms underlying this observation, ruling out these potential mechanisms for observed clinical differences.

Methods and Materials

Animals:

Eight adult male Rh (*Macaca mulatta*), three ChRh and five InRh, ranging in weight from 7-12 kg were selected from animals born at the Tulane National Primate Research Center. The ChRh colony has been maintained as a separate and unique breeding colony with no InRh introduced since 1987 (14). Animals were housed in AAALAC-international accredited facilities. All animal work was reviewed and approved by the TNPRC animal care and use committee prior to initiation. Animals were subjected to allogeneic vaccine strategies and blood collection protocols as described in the previous chapter.

Virus:

SIVmac239 generated from an *in vivo* passage was propagated on CEM.T1 cells as described previously. Stocks were collected bi-weekly from each of the eight cultures and quantitated by SIVp28 ELISA (AIDS Vaccine Program, NCI-Frederick Cancer Research and Development Center). Viral supernatants grown from ChRh PBMC were combined to generate a virus pool containing equal capsid concentrations from each animal; this stock was titrated on CEM.T1 cells. Animals were challenged intravenously with 400 $TCID_{50}$ as calculated by the Spearman-Karber method was used to challenge all animals (5).

Phenotype Analysis

3.1 *Flow cytometry*:

Cell subsets were prepared for flow cytometric analysis using four color staining combinations with monoclonal antibodies (mAb): CD3-fluorescein isothiocyanate (FITC), CD20-phycoerythrin (PE), CD8-peridinin chlorophyll A protein (PerCP), CD4allophycocyanin (APC), CCR5-PE, CD95-FITC, CD28 APC, CD25-PE, CCR2-PE, and CD14-FITC (BD Biosciences Pharmigen San Diego, CA). Cells were incubated with an excess amount of monoclonal antibodies as determined by titration at 4° C for 30 min. followed by a phosphate-buffered saline (PBS) wash (400Xg, 7 min.) and fixation in 2% paraformaldehyde. Whole blood was stained using the whole blood lysis technique as previously described.

Evaluation of SIV specific antibody response

3.2 *Western blot*:

Reverse transcriptase positive SIVmac239 was clarified the ultracentrifuged in a Beckman L70 ultracentrifuge using an SW28 rotor at 4° at 24,000 rpm for 2 hours. The pellet was resuspended in 1X PBS and protein concentration was determined using a commercial kit (BioRad BCA protein Assay, BioRad, Hercules, CA). Western blot assay was performed essentially as described (4). Fifty μg total protein was run on a 12% polyacrylamide gel and transferred to polyvinyl difluoride membrane using standard conditions. After blocking in 5% nonfat dry milk (BioRad) overnight, each membrane was cut into approximately 20 strips. Strips were incubated with 1:50 dilutions of serum from study animals taken on days 0, 28, and

45 pi in 1XPBS for 1 hour at room temperature, washed 3 times in 0.05% Tween20 in 1XPBS and labeled with 1:1000 protein A-HRP (Southern Biotech, Birmingham, AL) for 1 hour. Strips were developed in TMB peroxidase substrate system (KPL, Gaithersburg, MD) following the final wash. Responses to Env proteins only were graded as 1+, multiple bands as 2+, seroreactivity to capsid as 3+, and multiple bands with strong reactivity to capsid as 4+.

Evaluation of plasma IFNγ levels post SIVmac239 infection

3.3 *IFN γ:*

EDTA plasma, (100 µl), collected days 0, 28 and 45 pi was assayed for IFNγ concentration using Monkey IFN-γ ELISA kit according to manufacturer instructions (U-Cytech Biosciences , Utrecht, The Netherlands). IFNγ concentrations were determined by comparison to a standard curve generated using standards provided by the manufacturer.

Statistical Analysis:

The Anderson-Darling test was used to determine data normality. The Student's *t*-test was used to determine statistical significant differences between day 0 (baseline) and post infection cell subset numbers for individual animals and groups. Pearson correlation coefficients were determined to assess the linear relationship between viral loads and CD4+ T cell numbers or viral loads and plasma IFNγ levels. MINITAB statistical software (State College, PA) was used to perform all analysis.

Results

Viral kinetics and clinical disease course in SIVmac239 infected rhesus macaques.

As part of an experiment to study alloimmunization as a strategy to limit viral infection, 8 adult male rhesus macaques of Chinese (n=3) or Indian (n=5) origin were challenged with SIVmac239. Infection was established in all animals regardless of immunization status with peak viral loads of InRh being between 1 X $10^{6.6}$ and 1 X 10^{7} RNA copies per ml of plasma and remaining above 1 X $10^{5.6}$ RNA copies/ml until euthanasia due to clinical disease expression (Fig. 3.1 A). Peak viral loads for ChRh were similar, measuring between 1 X 10^{6} and 1 X 10^{7} RNA copies/ml prior to day 21. However, beginning on day 45 pi, viral loads of all ChRh were statistically significantly lower ($p=\leq 0.05$) than InRh (Fig. 3.1 A). All InRh (n=5) met the criteria of "rapid progressors" (10) and were euthanized due to the severity of clinical disease by 115 dpi. All ChRh were comparatively resistant to disease progression (n=3) (Fig. 3.1 B).

Fig. 3.1

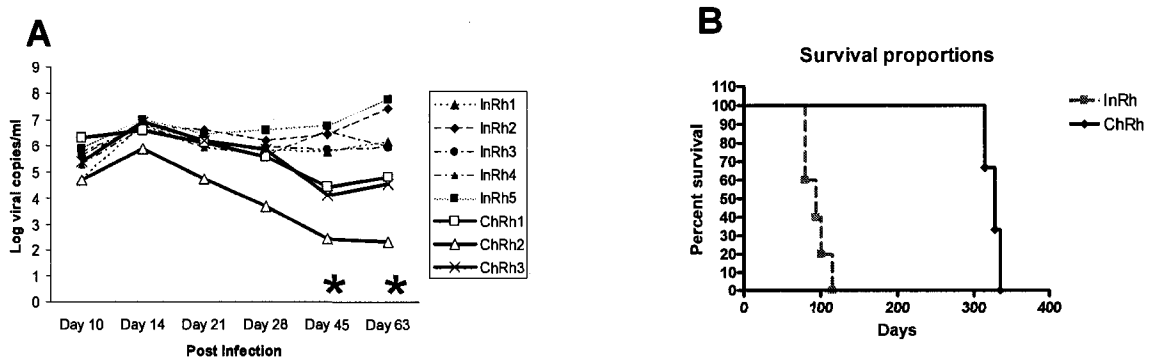

Figure 3.1 (A) Quantification of plasma viral loads in SIVmac239 infected macaques. * indicate statistically significant difference between the plasma viral loads of animals of Chinese origin (ChRh, n=3) compared to Indian origin (InRh, n=5) occurring 45 dpi and 65 dpi. (B) Kaplan-Meyer Survival Curve of all study animals. Infection related death of InRh (n=5) occurred between 80 and 115 days post infection ChRh (n=3) survived past 300 days post infection.

Ex vivo SIVmac239 production in stimulated PBMC culture.

Viral inoculums were generated in PBMC cultures from study animals and supernatant concentrations of virus determined by SIV p28 ELISA (Table 3.2). Similar quantities of virus were generated on In and Ch Rh cells, suggesting individual animal characteristics control viral production *in vitro.* The highest quantities of virus were produced by cultures of ChRh cells indicating that the lower *in vivo* viral loads measured in acute infection (Fig. 3.1 A) were not a result of intrinsic resistance to viral replication.

Table 3.2

SIVmac239	Stock 1	Stock 2
PBMC ID	µg/ml	µg/ml
InSelf1	14.2	12.6
InSelf2	13.7	12.0
ChAllo1	**22.6**	**21.6**
ChAllo2	**26.7**	**20.8**
ChAllo3	8.6	7.0
InAllo1	13.7	14.8
InAllo2	12.0	9.4
InAllo3	17.6	15.5

Table 3.2: Quantification of SIV capsid concentrations of viral stocks produced from individual animal PBMC (n=8) determined by SIV p28 ELISA. Concanavalin A stimulated cells isolated from ChRh produce the highest concentration of virus with each animal producing similar quantities on two separate culture dates.

Flow cytometric analysis of peripheral immunocytes (epxt. 3.1).

CD4+ T cells

Chronic progressive loss of CD4+ T cells is a feature of HIV and SIV infection and is the best predictor of disease progression in HIV infected individuals (2, 11, 20). CD4+ T cells isolated from peripheral blood from all animals on days 0, 7, 10, 14, 21, 28, 45, and 63 post infection (PI) were evaluated by flow cytometry (Fig. 3.3 A). All animals showed statistically significant losses ($p \leq 0.05$) compared to baseline at all time points PI. CD4+ cell numbers are statistically significant higher in ChRh ($p \leq 0.05$) on day 28 PI.

SIV plasma viral loads predict the rapidity of disease progression (13, 17, 33). In order to assess the predictive value of viral loads for CD4+ T cell loss, plasma viral loads were compared to CD4+ T cell numbers and while there was a correlation on days 14 ($p=0.057$) and 45 pi ($p=0.028$) this relationship was not consistent for all

study days (Fig. 3.3 B-E). One ChRh showed persistently low viral loads and comparatively high CD4+ T cell counts while the remaining animals demonstrated higher viral loads and marked CD4+ T cell losses. In two ChRh, the significant viral control evident at days 45 and 63 pi did not abrogate CD4+ cell losses.

Fig. 3.3

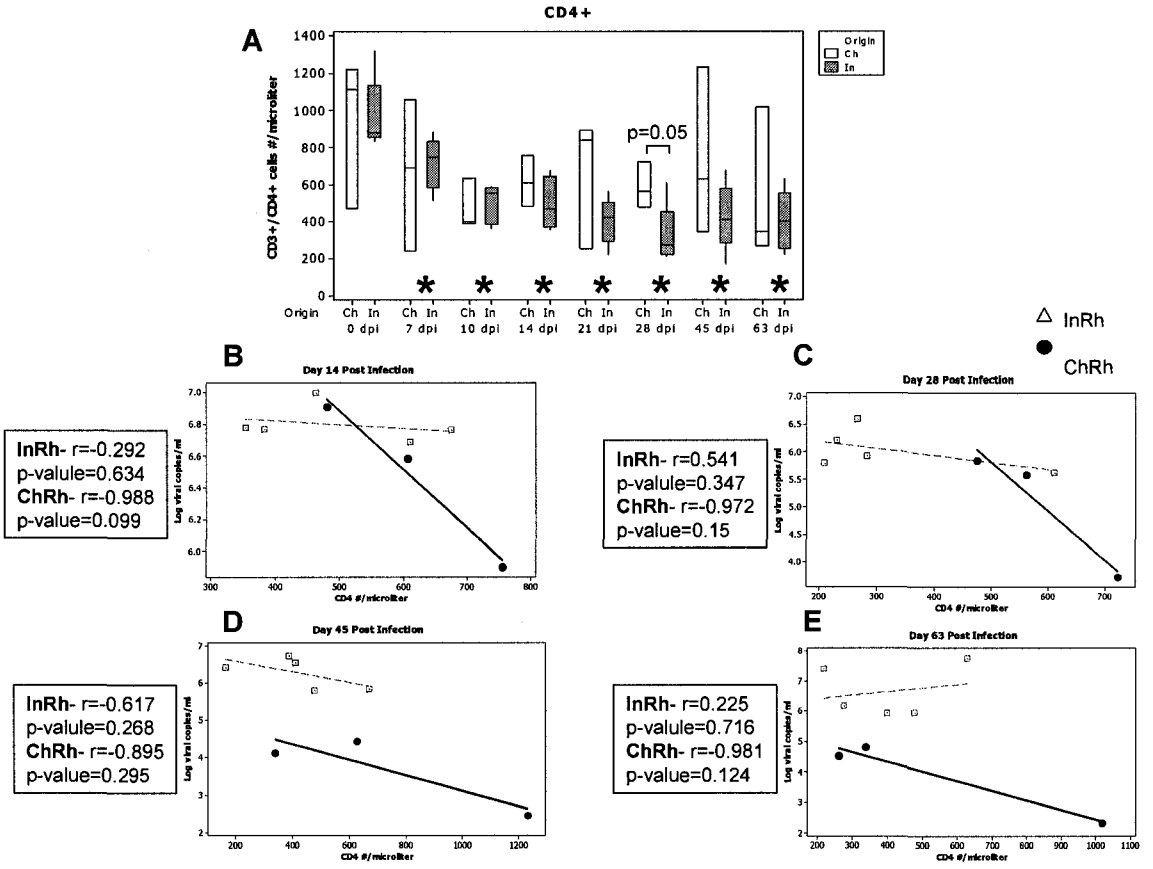

Figure 3.3 A) CD4+ T cell counts by animal origin measured on day of challenge and 7 post infection time points. * indicates statistically significant losses ($p \leq 0.05$) compared to day of challenge for all days post infection (dpi) for both InRh (n=5) and ChRh (n=3). InRh CD4+ T cell numbers were statistically significantly lower than those of ChRh 28 dpi (p=0.05). (B-E) Correlations between plasma viral RNA levels and CD4+ T cell numbers for InRh (dotted lines) and ChRh (black lines) on days 14 (B), 28 (C), 45 (D), and 63 (E) pi. No statistically significant trends are demonstrated for InRh or ChRh.

CD8+ T cells and plasma IFNγ levels

During acute infection, decline of viral load coincides with peak CD8+ T cell numbers (19). Effective CD8+ T cell responses have been shown to correlate with viral load reduction (32) and depletion of CD8+ T cells is typically associated with increases in viral load (9, 28). In this study, CD8+ T cells were quantified for all animals using flow cytometry. Statistically significant decreases relative to baseline were recorded in animals ($p \leq 0.05$) on days 7, 10, and 63 pi (Fig.3.4 A). CD8+ T cells were significantly higher ($p=0.038$) in ChRh on day 28 pi. Overall, there was no correlation between CD8+ cell numbers and viral load associated with either individual macaques or animals grouped by origin (data not shown).

In order to assess antiviral responses and state of immune activation post infection, plasma IFNγ levels were determined by ELISA on days 0, 28, and 45 pi (expt. 3.3). Although Plasma IFNγ concentrations increased modestly compared to day of challenge in one InRh (InRh5) and one ChRh (ChRh1) (Fig. 3.4 B), there was no correlation between IFNγ levels and viral load for individuals or animals grouped by origin for either date (Fig. 3.4 C,D). Interestingly, InRh2 and InRh5 showed relatively higher IFNγ concentrations (11.7 ng/ml and 12.2 ng/ml respectively) and high viral loads (Fig. 3.4 D) on day 45 pi and progressed most rapidly to clinical disease (data not shown). This is consistent with immune activation being an important component of pathogenesis, and does not suggest a defect in IFNγ production is responsible for rapid disease progression.

Fig. 3.4

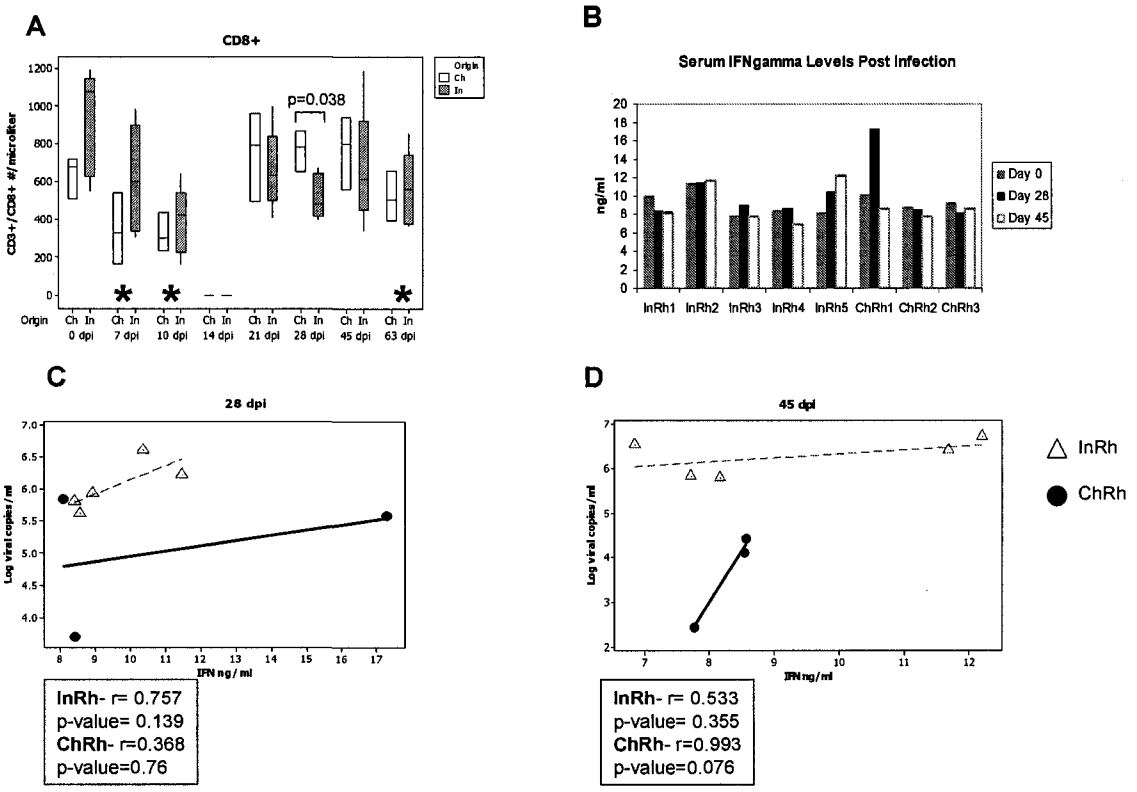

Figure 3.4 (A) CD8+ T cell counts by animal origin measured on day of challenge and 7 post infection time points. * indicates statistically significant losses ($p \leq 0.05$) compared to day of challenge for 7, 10, and 63 dpi for both InRh and ChRh. InRh CD8+ T cell numbers were significantly lower than those of ChRh 28 dpi. CD8+ T cell counts were not determined on 14 days PI. (B) Plasma Interferon γ concentrations. Concentrations measured on days 0, 28, 45 PI. There was no statistically significant correlation between plasma IFN γ levels and plasma viral load on 28 dpi (C) or 45 days PI (D). Trends for InRh are indicated by dotted lines and ChRh by black lines.

B cells

Although role of B cell responses in the control of primate lentiviral infections remains undefined, some studies have shown B cells to be necessary for control of viral replication (18). In this study, no statistically significant differences in B cell numbers were measured (Fig. 3.5). An antibody response as determined by western

blot (expt. 3.2), B cell numbers, and viral load comparison at 45 days PI suggest that, B cell responses are associated with a reduction in viral load for ChRh1 and ChRh2 (Table 3.3, Fig. 3.5).

Fig. 3.5

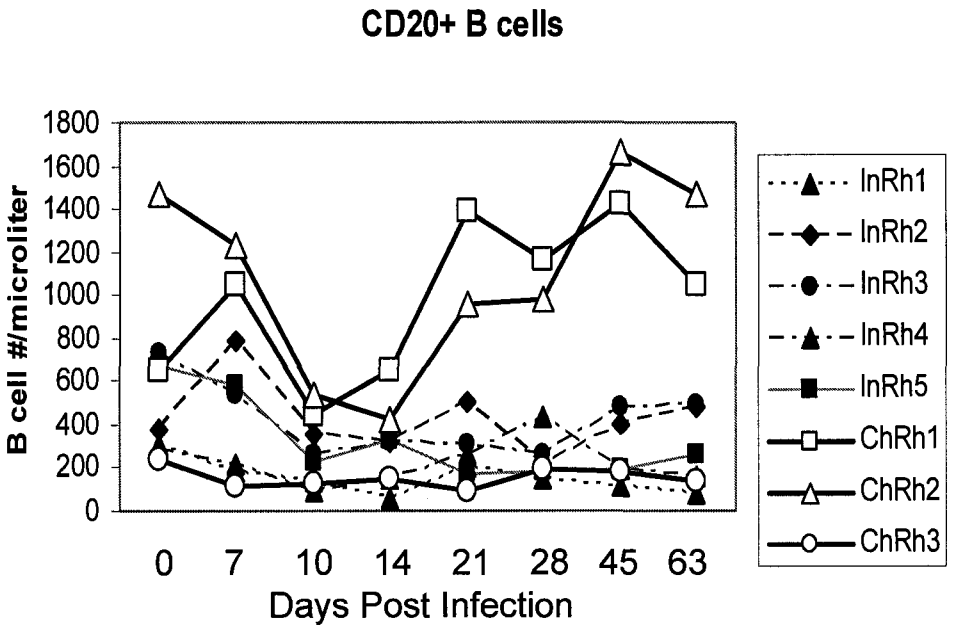

Figure 3.5 CD20+ B cell counts for all animals measured on day of challenge and 7 post infection time points.

Table 3.3

Day 28 Post Infection			
Animal	Ab Response	B cell #/microliter	Log viral copies/ml
InRh1	+	209	5.8
InRh2	+	231	6.2
InRh3	+	283	5.9
InRh4	++	610	5.6
InRh5	++	267	5.6
ChRh1	+++	561	5.6
ChRh2	+++	723	3.7
ChRh3	negligible	474	5.9
Day 45 Post Infection			
InRh1	+	117	5.8
InRh2	+	398	6.4
InRh3	+	479	5.8
InRh4	+	188	6.6
InRh5	+	194	6.7
ChRh1	++++	1424	4.4
ChRh2	++++	1665	2.5
ChRh3	negligible	182	4.1

Table 3.3 Comparison of antibody response determined by western blot, peripheral B cell counts, and viral loads for ChRh and InRh infected with SIVmac239 (n=8). Antibody responses were graded according to western blot reactivity from weak (1+) to strong peroxidase staining versus multiple antigens (4+).

Memory T cell subsets

The HIV/SIV coreceptor CCR5 is largely restricted to effector site homing memory T lymphocytes (26). There is increasing evidence that both the loss and diminished proliferative capacity of memory CD4+ T lymphocytes during acute infection is important in AIDS pathogenesis (7, 24, 26). To assess whether differences in memory T lymphocyte subset losses were associated with more rapid disease progression in InRh, peripheral CCR5+/CD4+, naive memory (CD4+/CD28+/CD95-), central memory (CD4+/CD28+/CD95+), and effector memory (CD4+/CD28-/CD95+) T lymphocyte subset numbers were determined using flow cytometry.

Similar population kinetics were evident in CCR5+/CD4+ T cells in both ChRh and InRh. Declines start 10 days PI and continuing through 21 days PI (Fig. 3.6 A). Populations rebound at day 28 or 45 PI in ChRh and InRh respectively, followed again by declines in both populations at day 63 PI. There was no correlation between CCR5+/CD4+ T cell numbers and viral load for either group at any of the time points (data not shown). Naive CD4+ memory cell numbers were statistically significantly decreased ($p \leq 0.05$) in ChRh and InRh on days 10, 28, and 63 PI with higher standard deviations in ChRh (Fig. 3.6 B). Similarly, central memory CD4+ T cells (CD28+/CD95+) were decreased ($p \leq 0.05$) on days 7, 10, 14, 21, 28, and 45 PI (Fig. 3.6 C) and effector memory CD4+ T cells (CD28-/CD95+) were decreased on days 14, 21, and 63 PI (Fig. 3.6 D). ChRh had significantly more effector memory cells ($p=0.008$) compared to InRh on day 28.

Fig. 3.6

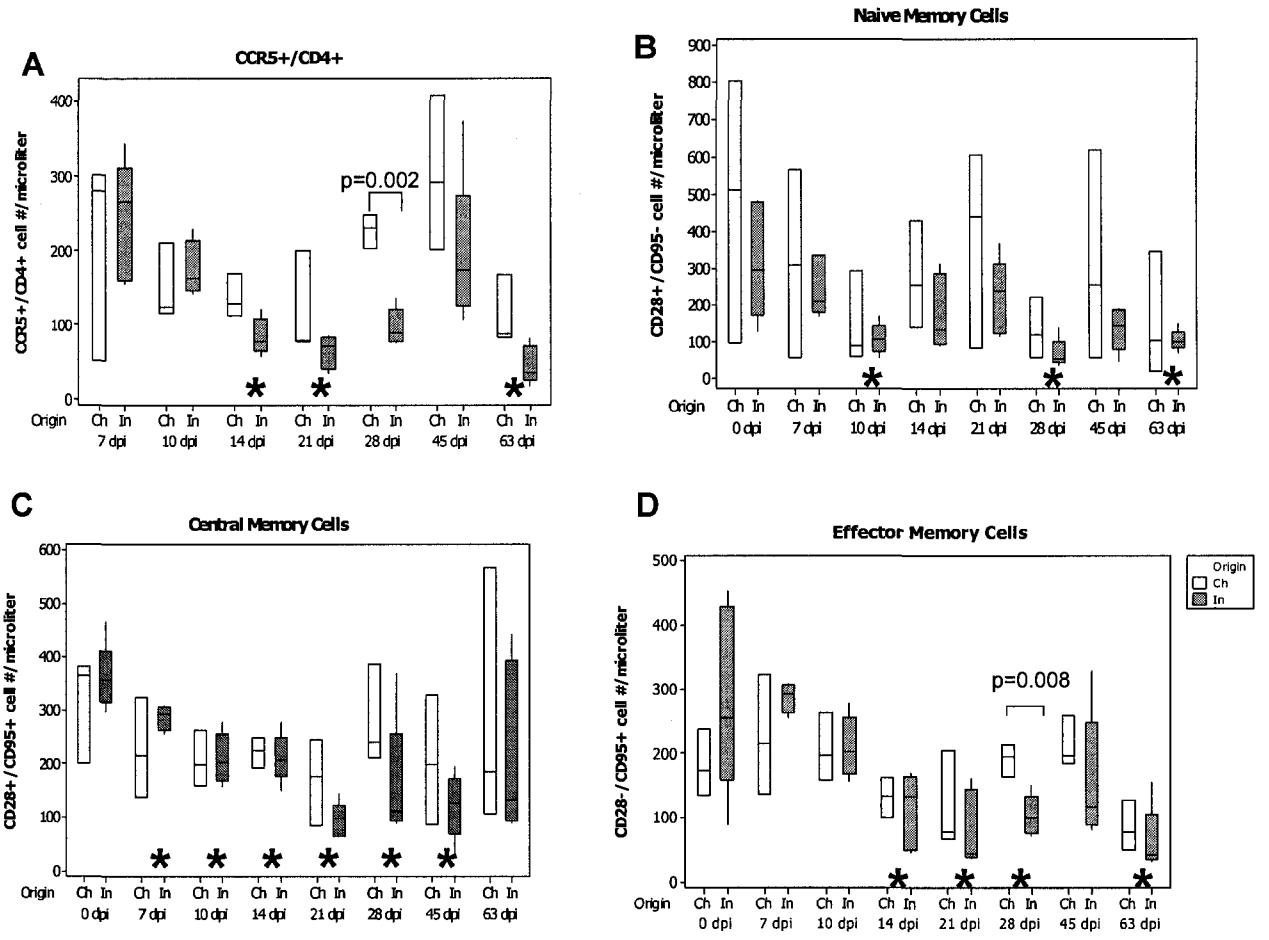

Figure 3.6 (A) CCR5+/CD4+ memory T cell counts by animal origin (InRh, n=5; ChRh, n=3) for 7 post infection time points. Day of challenge counts were not determined. * indicates statistically significant losses ($p \leq 0.05$) compared to day 7 pi for both InRh and ChRh. InRh CCR5+/ CD4+ T cell numbers were significantly lower than those of ChRh 28 dpi ($p=0.02$). (B-D) Peripheral CD4+ memory T cell subset counts by animal origin (InRh, n=5; ChRh, n=3) on day of challenge and 7 post infection time points. * indicates statistically significant losses ($p \leq 0.05$) compared to day of challenge for both InRh and ChRh. (B) Significant losses of naive memory cells (CD3+/CD4+/CD28+/CD95-) noted 10, 28, and 63 dpi. (C) Significant losses of central memory cells (CD3+/CD4+/CD28+/CD95+) noted on 7, 10, 14, 21, 28, and 45 dpi. (D) Significant losses of effector memory cells (CD3+/CD4+/CD28-/CD95+) noted on 14, 21, 28, and 63 dpi. InRh CD4+ effector memory T cell numbers were statistically significantly lower than those of ChRh 28 dpi ($p=0.008$).

CCR2+ CD4+ T cells

CCR2 is the chemokine receptor for human MCP-1 (monocyte chemotactic protein-1), MCP-2, MCP-3, and MCP-4 (21, 27) and is expressed on activated T lymphocytes, monocytes, and basophils (1). CCR2 has been functionally associated with the accumulation of T lymphocytes at sites of tissue inflammation and monocyte recruitment in acute inflammation (8, 30). A highly significant upregulation of CCR2 on T lymphocytes ($p \leq 0.005$) was measured in ChRh and InRh suggesting a state of immune activation, but this change did not predict viral load or disease severity (Fig. 3.7 A). Differences from baseline in CCR2 expression on circulating monocytes were not noted in any of animals (Fig. 3.7 B).

Fig. 3.7

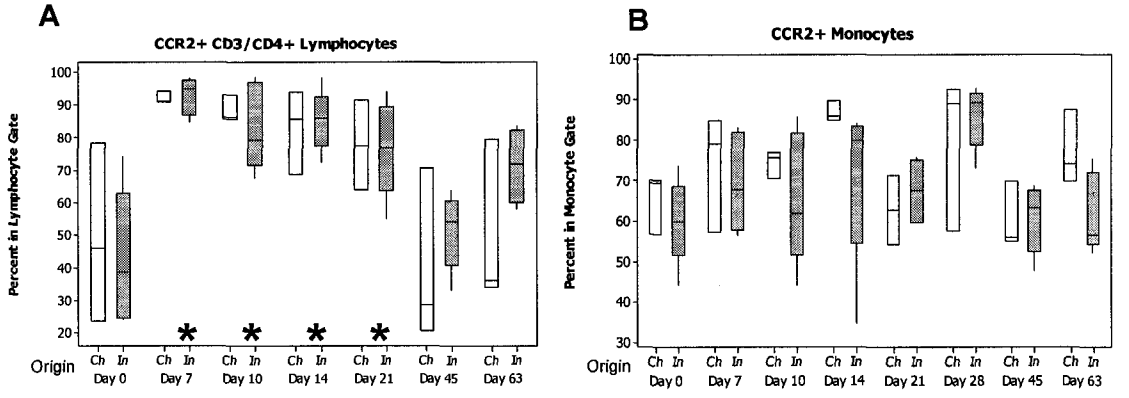

Figure 3.7 (A) CCR2+ expression on peripheral CD4+ T lymphocytes and (B) monocytes by animal origin (InRh, n=5; ChRh, n=3) on day of challenge and 7 post infection time points. * indicates significant increases ($p \leq 0.05$) compared to day of challenge for both InRh and ChRh for all dates post infection.

Discussion

In this study, pathogenic CCR5-tropic SIVmac239 passaged in ChRh PBMC was used to challenge InRh and ChRh. ChRh showed markedly better viral control and resistance to disease progression after day 45 PI as has been described in other studies (3, 14, 20). In order to more clearly define correlates of immune control of viral replication and resistance to clinical disease shown by ChRh, peripheral CD4+ T cells, CD8+ T cells, B cells, CCR2+ T cells, CCR2+ monocytes, antibody responses, and IFNγ were quantified. Although our data are in agreement with studies correlating decreased viral loads with better disease outcome, surprisingly, preservation of key CD4+ memory T lymphocyte populations was not associated with disease progression.

High viral loads are strongly associated with progression to AIDS in HIV infected humans and SIV infected macaques. Pathogenesis is thought to represent a complex interplay between virally-mediated destruction of cells and an unabated, highly activated immune response to chronic infection. The main immunophenotypic manifestation of HIV infection is a dramatic and sustained decrease in CD4+ T cells, the extent of which is positively correlated with progression to AIDS. In HIV and SIV, the CCR5+/ CD4+ memory T cell subset is targeted and rapidly depleted in acute infection. Increasing evidence that the loss of memory CD4+ T lymphocytes during acute infection is important in AIDS pathogenesis is illustrated by the clear survival advantage apparent for the monkeys with the preserved central memory T cell counts (12) . While others have observed preserved central memory T cells

subsets in the face of effector memory subset depletion as a factor in resistance to clinical progression (12, 23), in this study we observed early (7 dpi) and sustained declines in peripheral central memory cells (Fig. 8B) concurrent with intermittent effector memory declines (Fig 8C). Overall, both ChRh and InRh experienced similar dramatic CD4+ T cell declines while only ChRh were resistant to clinical disease. Possible mechanisms explaining this differential pathogenesis are: (1) ChRh are able to maintain immunological competence in the face of decreased cell numbers, (2) virological factors induce differential immune dysfunction in ChRh vs. InRh resulting immune deficiency in the latter only, or (3) ChRh possess an intrinsic resistance based upon other innate or adaptive factors not measured in this study that have eluded description in this and other SIV/HIV studies.

Uncontrolled immune activation has consistently been associated with pathogenic HIV and SIV disease (6, 29). Interestingly, the chemokine receptor CCR2+ is shown to be dramatically upregulated on circulating CD4+ T lymphocytes in both ChRh and InRh, suggesting a strong migratory potential in response to inflammation. Although Cumont *et al.* have demonstrated resistance to apoptosis in ChRh lymph nodes as a mechanism for resistance to disease progression (3), both ChRh and InRh appear to be equally susceptible to this virally-induced response to acute inflammation. In nonpathogenic SIVagm infections of African green monkeys, establishment of early anti-inflammatory responses have been associated with resistance to clinical disease (25). In this respect, Ch Rh do not seem to resist disease progression by the same mechanism.

CD8+ T cell dependent control of viral replication has been correlated with expansion of virus specific CD8+ T lymphocytes in HIV and SIV (22, 28). Additionally, elite viral control has been correlated with certain HLA alleles (HLA-B27, and-B57; Mamu A*01, B*17, and B*08 respectively) allowing for superior CD8+ T cell destruction of SIV-infected cells. A more effective CD8+ cytotoxic response may, therefore, underlie the superior viral control demonstrated by ChRh when clear differences in total CD8+ T cell numbers were not seen (Fig. 3.4A). No correlation was shown, however, between plasma IFNγ levels and viral load which does not support CD8+ production of IFNγ as a contributing mechanism (Fig. 3.4B). B cell responses have also been shown to be associated with reduced viral production. Although 2 of 3 ChRh show markedly increased B cell numbers (Fig. 3.5, Table 3.3) and a comparatively strong antibody response (Table 3.3), it is unlikely to have been the most important cause of the observed viral load reductions, considering one of the ChRh showed a negligible antibody response and had one of the lowest levels of B cells of all study animals. It is clear that neither differences in the magnitude of CD8+ T lymphocyte nor B cell responses, nor virus specific antibody responses were associated with the observed reduction in viral load in the ChRh. In summary, cell subsets thought to be key in associating disease control or reduced viral load do not differ between ChRh and InRh despite dramatic differences in disease outcome.

SIV infection of rhesus macaques is heavily utilized as a model for HIV vaccine development and in studies of viral pathogenesis. The ultimate goal of vaccine development is to lower peak and set point viral loads, but it is clearly important to develop therapeutics that address the negative immunological consequences of even

low levels of viral replication. Results of this study suggest that pathogenic SIVmac239 in InRh susceptible to rapid progression may better model indicators of immune failure while pathogenic SIVmac239 infection in ChRh may be a model of critical cell subset losses in the face of controlled viral replication. The mechanisms underlying clinical disparities were not elucidated despite evaluation of a variety of circulating immunocyte subsets thought to be key indicators of viral control, and are intriguing in terms of understanding of lentiviral-immunodeficiency inducing mechanisms that may reveal new concepts for AIDS therapies.

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Chapter 4

Investigation of a Putative Lemur-specific Lentivirus

Abstract

Ring-tailed lemurs (*L. catta*) endemic to Madagascar have been previously shown to exhibit seroreactivity to lentiviral antigens. SIV has been identified as a natural infection only in primate species of continental Africa. The identification of a lentivirus specific to lemurs would be of great interest with respect to conservation efforts as well as the elucidation of primate lentiviral origins. Our study extends this preliminary evidence of a lemur specific lentivirus with analysis of whole blood samples from from 16 *L. catta* at the Indianapolis Zoo. We demonstrated seroreactivity to SIV, LLV, PLV, and FIV antigens but were unable to amplify lentiviral sequences using a diverse set of degenerate primers.

Introduction

Lemurs are the most prevalent mammals on Madagascar, are endemic to this island, and have evolved in isolation for nearly 60 million years (7). The unique phylogeny of lemurs with respect to continental African primates, the only known natural hosts of SIV, provides a basis for the analysis of these species for potential lentiviral infections. Plasma obtained from ring-tailed lemurs (*Lemur catta*) captured during ongoing studies in the Beza Mahafaly Special Reserve on Madagascar were seroreactive against FIV and SIV Gag p25 antigen (11). Based on this lentiviral seroprevalence in wild ring-tailed lemurs, our lab began preliminary characterization of a putative lemur-specific lentivirus by culture and PCR analysis of samples from animals in the Indianapolis Zoo zoological collection.

We reasoned that a lemur-specific lentivirus might have minimal antigenic identity with African SIVs, and therefore used antigen panels for three FIV strains and one SIV to maximize our chances of detecting positive reactions and to assist us in determining true vs false positives by identifying samples with broad sero-reactivity. To identify nucleic acid sequences with homology to SIV, we utilized SIV, FIV and universal retroviral degenerate primer sets previously shown to successfully amplify unknown lentiviruses. The universal retroviral degenerate primers also amplify endogenous retroviral elements and it is likely that the sequences obtained using this methodology are retroviral elements related to

endogenous beta retrovirus and do not represent exogenous lentiviral sequences. Efforts to demonstrate reverse transcriptase activity in cultured lemur PBMC from captive animals were not conclusive; however, seroreactivity to lentiviral antigens and evidence of SIV p28 capsid cross-reactivity by ELISA further support the possibility of the existence of a putative lemur- specific lentivirus.

Methods and Materials

Animals:

Lemur catta (*L. catta*) EDTA plasma samples collected during routine health evaluations from 16 lemurs housed were provided by the Indianapolis Zoo. Buffy coat samples from wild *L. catta* were provided by scientists studying animals in the Beza Mahafaly Special Reserve on Madagascar.

Seroreactivity determined using the western blot assay:

We used a multiplex immunoblot assay employing both simian and felid lentiviral antigens to maximize chances of detecting seroreactivity. Antigens were prepared from culture supernatants containing SIVsmm(PBjBcl14), and three unique strains of FIV (FIV, strain B-2546; FIV-Pco, puma lentivirus, PLV-1695; FIV-Ple, lion lentivirus LLV-458). Antigen was clarified then ultracentrifuged in a Beckman L70 ultracentrifuge using an SW28 rotor at 4° at 24,000 rpm for 2 hours. The pellet was resuspended in 1X PBS and protein concentration was determined using a commercial kit (BioRad BCA protein Assay, BioRad, Hercules, CA). The western

blot assay was performed essentially as described (3) . Fifty μg total protein was run on a 12% polyacrylamide gel and transferred to polyvinyl difluoride membrane using standard conditions. After blocking in 5% nonfat dry milk (BioRad) overnight, each membrane was cut into approximately 20 strips. Strips were incubated with 1:50 dilutions of serum from Indianpolis Zoo lemurs in 1XPBS for 1 hour at room temperature, washed 3 times in 0.05% Tween20 in 1XPBS and labeled with 1:1000 protein A-HRP (Southern Biotech, Birmingham, AL) for 1 hour. Strips were developed in TMB peroxidase substrate system (KPL, Gaithersburg, MD) following the final wash.

Reverse transcriptase activity in L. catta culture supernatants:

PBMC were isolate from approximately 2 ml EDTA whole blood using a Histopaque (Sigma, St. Louis, MO) gradient according to the product insert. One million isolated PBMC were cultured in 5 ml standard media with the addition of interleukin-2 at 100U/ml (Invitrogen Life Sciences) and concanavalin A at and 9 g/liter glucose (Sigma). One-half of the supernatant was remove from cultures and replace with fresh media every three days. Supernatant assayed for reverse transcriptase activity in a microplate assay. Briefly, 15 μl sample in triplicate was incubated with 50 μl 0.05 M Tris(pH 7.8) with 75 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 2 mM dithiothreitol, 5 nM oligo(dT), 0.05% NP-40, poly(A) at 50 μg/ml, and ^{32}P at 20 μCi/ml for 90 minutes at 37° C. Aliquots of 2.5 μl of each reaction mixture were spotted onto a nylon filter (Wallac, Turku, Finland) and allowed to dry. Unincorporated label was washed away with five 15-minute washes with 0.03 M

sodium citrate, pH 7.0, in 0.3 M sodium chloride (SSC) buffer, and the membrane was then fixed in 100% ethanol. Counts per minute were measured using a Microbeta Counter (Wallac).

Viral RNA extraction from culture supernatants and PCR:

Because it is likely that a species-specific lemur lentivirus will be highly divergent from other previously genetically characterized lentiviruses, we attempted to amplify virus using: (1) degenerate primer sequences and nested PCR conditions developed from both a. SIV and b. FIV strains, and (2) non-nested PCR developed for divergent retroviruses which span approximately the same region of pol-RT. Four mls of lemur PBMC culture (from animals Meara, Sorcha, and Leadan) supernatants with low levels of SIV p28 ELISA reactivity was concentrated in a centricon 30 device (Millipore). RNA was then extracted using a commercially available kit (Qiamp Viral RNA Mini Kit, Qiagen Inc., Chatsworth, CA) according to manufacturer's instructions. RNA was transcribed to cDNA using Superscript II (Invitrogen). DNA was isolated from $1X10^7$ cultured PBMC or reverse-transcriptase using QIAamp blood (DNA) kit. All first round PCRs were performed using 100 ng cDNA or genomic DNA in 50 µl reactions using iQSupermix (BioRad). Nested and non-nested PCR reactions utilized primers designed from conserved reverse transcriptase region of *pol* to amplify SIV sequences with the primers listed in Table 4.2 using iQ Supermix and PCR conditions were as follows: 3 min. at 94 degrees C; 45 cycles of 15 s at 94 degrees, 30 s 50 degrees, 45 s at 72 degrees; 10 min. at 72 degrees in a Eppendorf Mastercycler. Second round PCRs were performed similarly

and product visualized with a 1.3% agarose gel. Gel bands of the expected product size were excised and extracted using QIAquick Gel extraction kit (Qiagen, Valencia, CA) prior to cloning and sequencing.

Cloning and Sequence analysis:

Extracted gel bands were cloned for sequencing using the TOPO TA Cloning Kit (with pCR2.1-TOPO vector) with One Shot TOP10 Chemically Competent E. coli (Invitrogen). Plasmid preparations with verified inserts were submitted to Macromolecular Resources for sequencing (Fort Collins, CO). Initial sequence tracings were evaluated for quality, compared to GenBank sequences using BLAST (WUblast2 nucleotide) to evaluate homologies to known viral sequences, and aligned to one another (ClustalW).

Results

Serology has been used as the basis for identification of previously uncharacterized lentiviruses. Antibodies binding to characterized lentiviral preparations of SIV, FIV, PLV, and LLV are suggestive of lentiviral infection in wild lemurs sampled in Madagascar (11). In order to survey a captive lemur population for comparison, EDTA plasma from 16 captive *L. catta* housed at the Indianapolis Zoo was tested for lentiviral seroreactivity to SIV, FIV, PLV, and LLV antigen preparations. Western blots demonstrated 100% positive for seroreactivity to SIV, 72% seroreactivity to two lentiviral antigens, 33% seroreactive to 3 lentiviral antigens (Table 4.1, Fig. 4.1). In agreement with data from wild lemur populations,

evidence of antibodies cross-reactive with lentiviral antigens suggests lemur specific lentiviral infection.

Figure 4.1

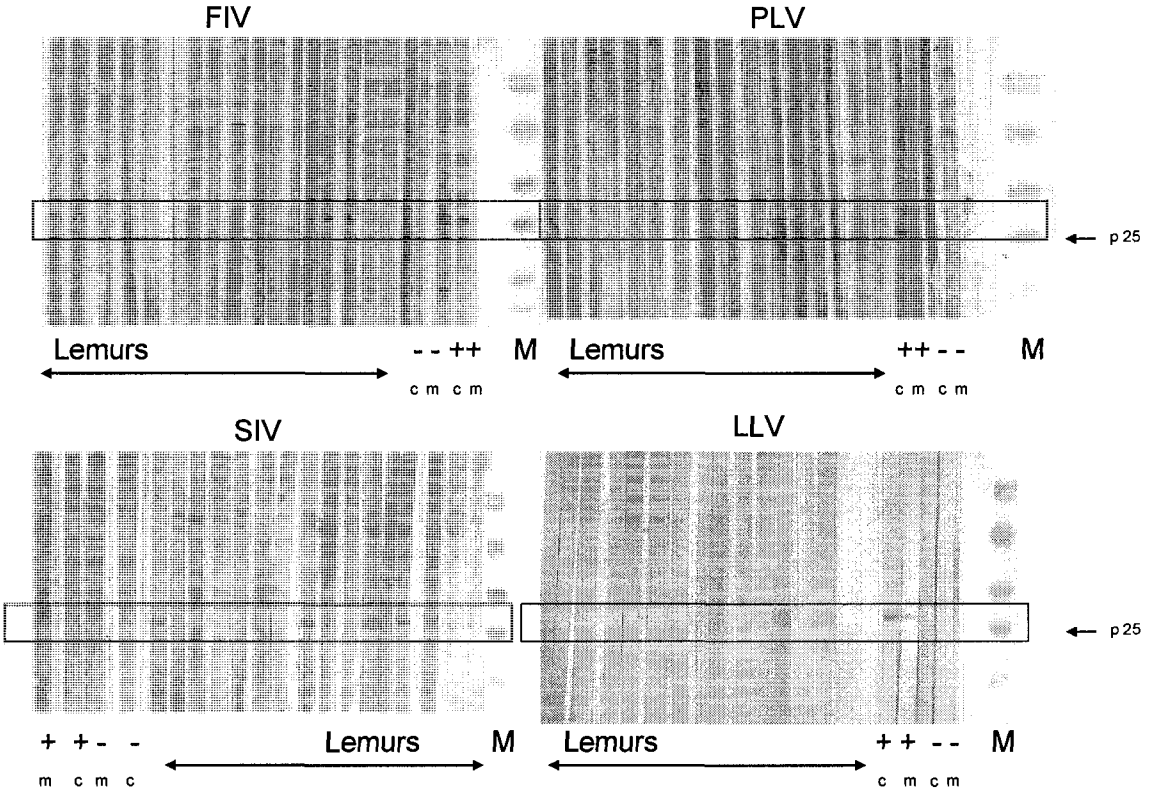

Figure 4.1 Seroreactivity of captive lemurs to the lentiviral antigens FIV, PLV, SIV, and LLV. Uninfected feline and macaque sera were used as negative controls (-c, -m) and chronically infected feline and macaque sera were uses as positive controls (+c, +m). Viral capsid antigen migrates with the 25kd molecular weight marker p25 (M).

Table 4.1

Lemur ID	SIV	PLV	FIV	LLV
Leadan	3	0	2	0
Seiodin	3	0	1	0
Sorcha	1	0	0	0
Sona	2	0	0	0
Spike	2	0	0	0
Caera	2	0	0	0
Meara	2	1	0	0
Junior	3	0	2	0
Kate	3	0	2	2
Roy	1	0	0	0
Kim	3	0	0	2
Mick	2	0	2	1
Andy	3	0	3	1
Clarence	3	0	0	1
Cairrean	1	0	1	0
Keiran	1	0	2	0
Pos Mne	3	0	3	2
Neg Mne	0	0	0	0
Pos PLV cat	ND	3	ND	ND
Pos FIV cat	1	ND	3	3
Neg cat	0	0	0	0

Table 4.1 Seroreactivity of captive lemurs to an antigen panel of one SIV and three FIV strains. 1=weak, 2=moderate, 3=strong reactivity. Sera from a chronically SIV infected pig-tail macaque (Pos Mne), a PLV infected domestic cat (Pos PLV cat), and a FIV infected domestic cat (Pos FIV cat) served as positive controls. Sera from an uninfected pig-tail macaque (Neg Mne) and uninfected domestic cat (Neg cat) served at negative controls (ND=not done).

Reverse transcriptase assays were used to indicate the presence of a retroviral agent in lemur PBMC culture supernatants collected from separate cultures in 2006 and 2007. Reverse transcriptase activity in the supernatants of cultured lemur PBMC was 1.2X to 1.5X background values in 40% of the samples with the highest values occurring at 14 days in culture (Fig 4.2 A). SIV p28 ELISA was used to test for antigen cross-reactive with SIV capsid specific antibodies in supernatants from cultured lemur PBMC. All of the lemur culture supernatants tested from samples collected on day 14 were positive for SIV capsid by ELISA (Fig. 4.2B), though very low levels of antigen were detected relative to SIV controls (SIV p28 capsid standard curve was generated from serial dilutions measuring 1,000 to 500,000 pg/ml). These results suggested a lentivirus with low levels of sero-reactivity to known SIVs might be present, so additional tests were conducted as described below.

Polymerase chain reaction (PCR) to detect viral RNA in culture supernatants and provirus in genomic DNA were performed using SIV and FIV specific nested primers and universal retroviral primers (Table 4.2). There was no amplification of nested product from genomic DNA extracted from lemur cultured PBMC (data not shown). Universal retroviral primers amplified a band from viral RNA in culture supernatants of three lemurs selected based on SIV p28 capsid cross-reactivity (Fig. 4.2B). Clones of these extracted bands were sequenced and two sequences had significant homology to the beta retrovirus Simian Type D virus (Table 4.3). In order to further investigate this interesting retroviral element, specific primers (Table 4.4) were designed from the alignment of these sequences (Fig. 4.4). An approximately 100bp sequence was amplified from wild and captive lemur, but not from macaque or human genomic

DNA (Fig. 4.5). Overall these results suggest nested lentiviral degenerate primers lacked sensitivity to detect a lemur lentivirus, and universal retroviral primers detected what may be a lemur specific endogenous retroviral sequence.

Fig 4.2

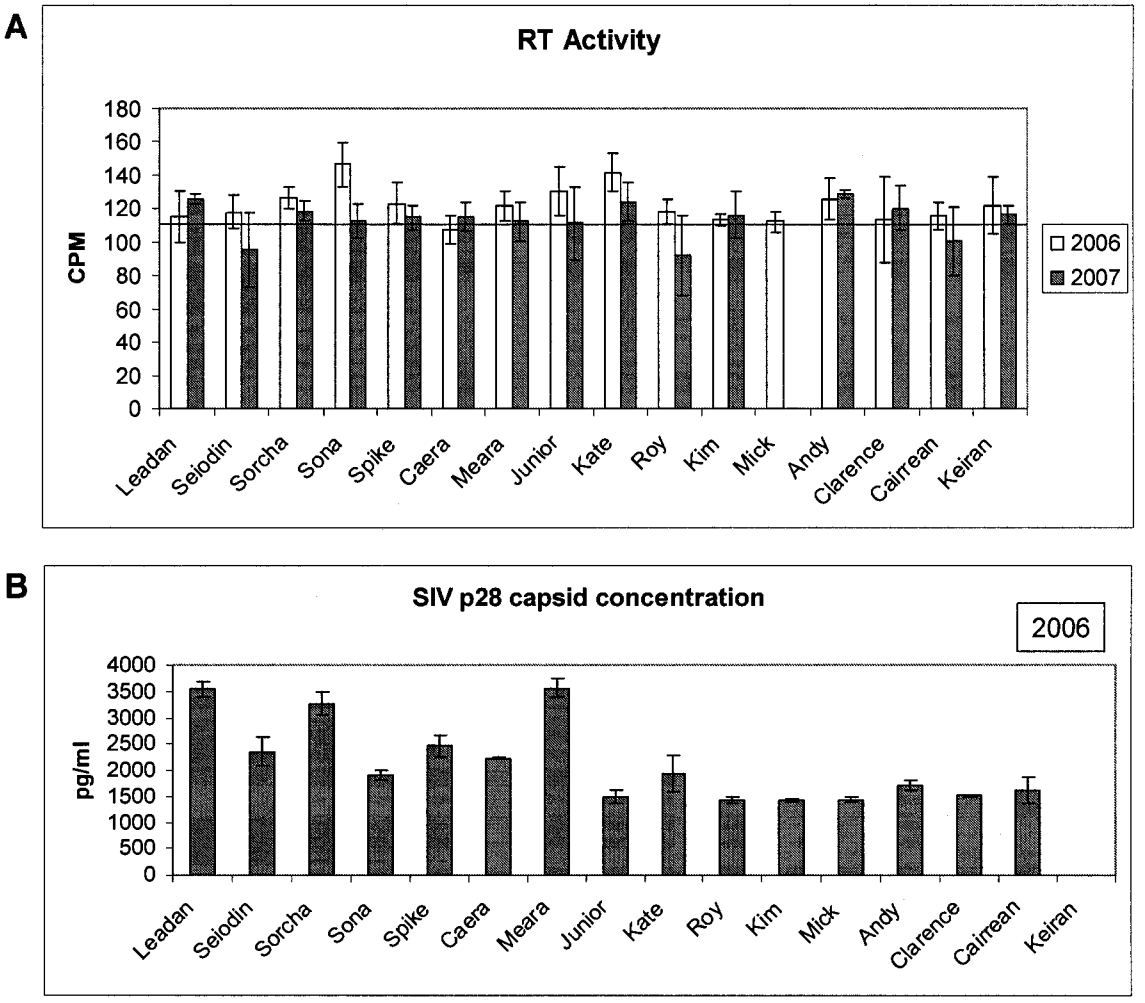

Figure 4.2
Reverse Transcriptase activity and SIV p28 capsid concentrations measured in PBMC culture supernatants after 14 days. Reverse transcriptase activity was very close to background level (black line) in both 2006 and 2007 samples (A). 2006 culture supernatants were positive for SIV p28 capsid by ELISA (B), though levels of antigen detected were low.

Table 4.2

Degenerate primers		**Ref.**	**Product**
SIV		(1, 2)	No
Outer forward: 5'TRCAYACAGGRGCWGAYGGA3'	Inner forward: 5'GGIATWCCICAYCCDGCAGG3'		
Outer reverse: 5'AIADRTCATCCATRTAYTG3'	Inner reverse: 5'GGIGAYCCYTTCCAYCCYTGHGG3'		
FIV		(12)	No
Outer forward: 5'TGGCCWYTAWCWAATGAAAARATWGAAGC3'	Inner forward: 5'TGAAAARATWGAAGCHTTAACAGAMATAG3'		
Outer reverse: 5'GTATTYTCTGCYTTTTTCTTYTGTCTA3'	Inner reverse: 5'GTAATTTRTCTTCHGGNGTYTCAAATCCCC3'		
Universal Retrovirus		(5)	Yes
Forward: 5'CTCGGATCCGTNYTNCCNCARGG3'			
Reverse: 5'CTCGTCGACRTCRTCCCATRTA3'			

Table 4.2 Degenerate primers used to amplify lemur lentivirus sequence from lemur culture supernatant viral RNA extractions. Products of the predicted size were not amplified using SIV or FIV nested primer sets but were obtained with universal retrovirus primers.

Fig. 4.3

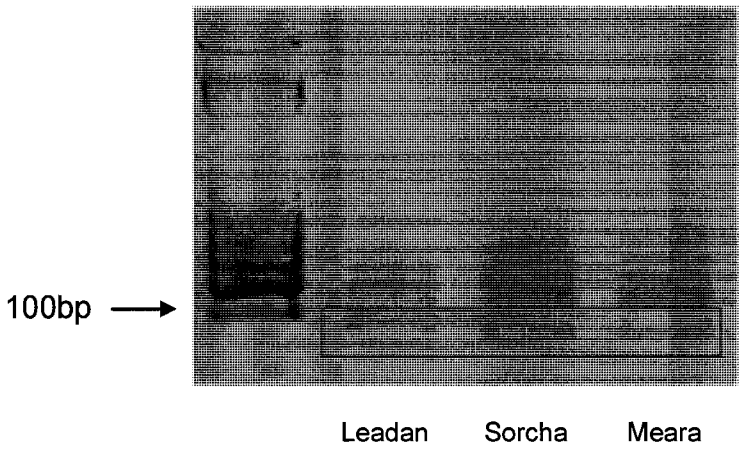

Figure 4.3 Universal degenerate primers amplifiy the expected 100bp product (black box) in viral RNA extracted from three lemur PBMC culture supernatants.

Table 4.3

Sequence	Data Base ID	Source	%Identity	E( )
Meara	EM_VI:S58806	pol=clone 2 [type D retrovirus, Genomic, 117 nt].	72	0.00084
Leadan	EM_VI:U85505	Simian type D virus 1, partial proviral genome.	68	0.0060

Table 4.3 Top BLAST results from cloned sequences of products amplified from viral RNA in lemur PBMC culture supernatants using universal retroviral degenerate primers.

Fig. 4.4

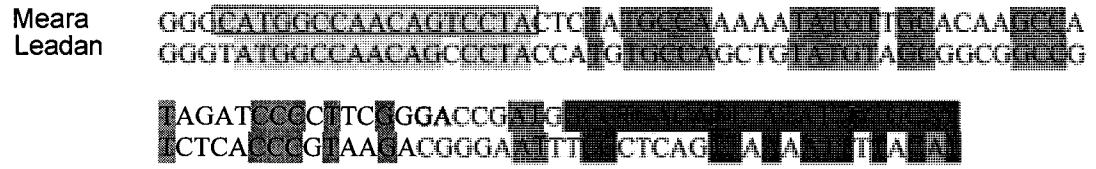

Figure 4.4 ClustalW alignment of cloned sequences of products amplified from viral RNA in lemur PBMC culture supernatants matching Simian type D retroviral element by BLAST search. The sequence used to design a specific forward primer (black box) is highlighted in light grey, the sequence used to design a specific reverse primer (black box) is highlighted in dark grey, and intervening alignments are highlighted in medium grey.

Table 4.4

Specific Primers	
Forward: 1F	CATGGCCAACAGTCCTA
Reverse: 1R	ATGCACAATATAAATGTGAGGC

Table 4.4 Specific primers designed from the alignment of lemur sequences with homology to Simian type D retrovirus.

Fig. 4.5

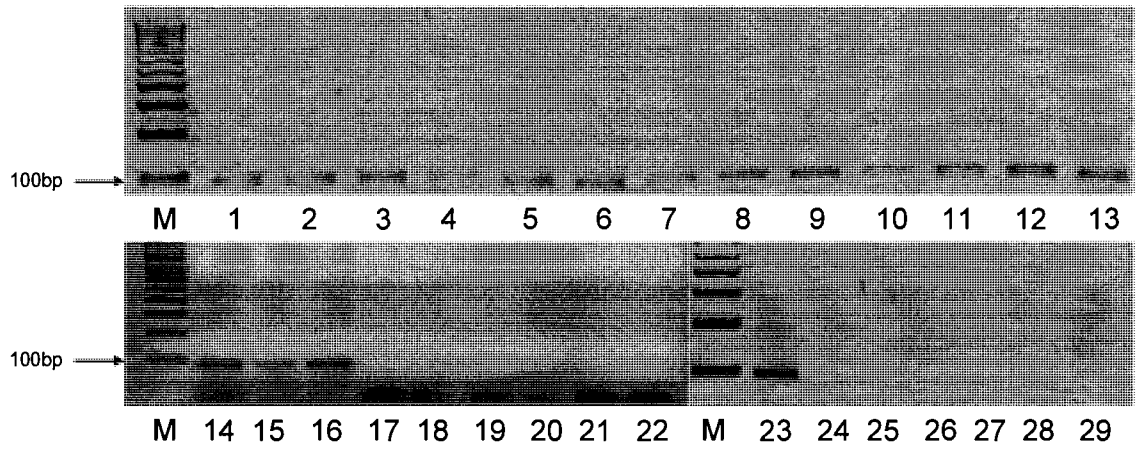

Figure 4.5 PCR amplification of genomic DNA from captive and wild ring-tailed lemurs, rhesus macaques and a human cell line using specific primers designed from sequences with homology to Simian type-D retrovirus. A band of the expected size (97 bp) was amplified from all captive (1-14, 23) and wild (15, 16) lemurs, but not rhesus macaque (17-21) or human (24-28) samples. Lanes 22 and 29 are water controls.

Discussion

Lentiviral seroreactivity, SIV p28 capsid ELISA cross-reactivity, and reverse transcriptase activity indicate that captive ring-tailed lemurs may be infected with a virus that is related to SIV, building upon earlier observations in Madagascar ring-tailed lemurs. Co-cultivation, cell line expansion, and electron microscopy have proven useful in characterization of previously unknown lentiviruses and these methodologies should be further explored with both wild and captive lemur samples.

As anticipated we found amplification of a lemur-specific lentivirus challenging likely due to the fact that, as with naturally-occuring lentiviral sequences, the proviral load may be as low as one proviral positive cell per 10,000 (6). In addition, endogenous retroviral (ERV) sequences are readily amplified by the universal

retroviral primer set used (5). *In Silico* characterization shows that *L. catta* ERV is closely related to the simian endogenous and type-D retroviruses found in several Old World Monkeys (Clarissa Dirks, unpublished). Similarly, lemur sequences amplified in this study using universal retroviral primers match to Simian type-D retrovirus sequences. Although most likely lentiviral sequences, ERV elements are of great interest since the diversity and distribution are the result of interactions between retroviruses and their hosts throughout evolution and ERVs generally cospeciate with host taxa (4, 10). ERVs can be used to detect viral transmission events as far back as 250 million years ago (8, 9). The very small size of the sequences amplified with retroviral degenerate primers make definitive identification impossible, however, and attempts to extend sequences using the specific primers designed from amplified sequences and a commercial gene walking kit have initially been unsuccessful. Further modifications to optimize degenerate primer PCR protocols may allow for both amplification of a lemur specific lentivirus and better characterization of important lemur specific ERVs.

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Conclusions

Chapter 1. *MHC class II mAb inhibition of SIV in vitro*

Preliminary studies in retroviruses suggested that monoclonal antibodies with specificity for cell membrane antigens incorporated into the viral envelope could diminish viral infectivity. MHC class II is consistently incorporated into laboratory and wild type HIV and SIV and has been shown to both enhance viral infectivity and serve as a target for antibody mediated viral interference. The mechanism responsible for viral interference has not been elucidated, though steric hindrance of viral binding/entry events has been proposed. Our studies involving preincubation of SIV grown from MHCII+, and MHCII- cells with α-MHCII, were not able to consistently replicate the diminution of viral replication demonstrated by others. We demonstrated that confounding factors involving interaction of mAb with cell membrane antigens present on the target cell influence viral replication independent of viral binding and entry events.

Future studies to extend our findings that MHCII epitope specificity may contribute to differential viral neutralization capacity could be carried out with a more extensive panel of α-MHCII antibodies. The polyclonal nature of antibody preparations used successfully with other HIV, SIV and other retroviruses also suggest that simultaneous multiple epitope specificities may contribute to better virus neutralization. The continued development of an *in vitro* viral infection model that

removes confounding factors resulting from identical cell membrane antigens on both the virus and the target cell will also be important.

Chapter 2. *Lack of protection against SIVmac239 challenge in alloimmunized Chinese or Indian-origin rhesus macaques*

Xenogeneic immune responses in macaques have been shown to successfully protect macaques against challenge from SIV grown in human cell culture. Additionally, alloimmunization of human patients has been successful in limiting HIV infection *in vitro*. We evaluated the ability of alloimmunization of Rhesus macaques of divergent genotype to elicit an immune response to host cell membrane antigens effective in protecting against a challenge with allotype matched SIV239. We were not able to demonstrate that an adjuvanted immunization and boost with allogeneic PBMC could provide resistance to a highly pathogenic SIV challenge.

Viral targets able to neutralize HIV infection continue to be elusive in ongoing vaccine development. Sterilizing immunity in animal models of HIV vaccine design has only been consistently demonstrated in whole inactivated preparations. Since the mechanism of viral neutralization appears to be exclusive of xenogeneic cell membrane antibody responses, it continues to be important to study the remarkable basis of protection demonstrated by these early successful studies. Characterization of this xenogeneic response may yield valuable information about the difference in outcome of allogeneic vs xenogeneic immunity. Alloimmune strategies may also augment vaccine candidates that include viral antigen components.

Chapter 3. *Similar peripheral immunocyte depletion in SIV infected Chinese and Indian macaques despite differences in capacity to control viral replication*

Rhesus macaques of Chinese origin have been shown to be more resistant to SIV infection induced viral pathology compared to animals of Indian origin. The ChRh in our study similarly demonstrated viral control and resistance to clinical disease progression in contrast to higher viral loads and rapid disease progression in InRh. Humoral responses and CD8+ and CD4+ T cell subsets as peripheral correlates of viral immunity were similarly affected in all animals, however.

As indicated in other studies, it is important to extend these investigations of differential control of viral replication and clinical disease by evaluating peripheral and central lymphoid compartments for differences in cell subset turnover, apoptotic indicators, and control of inflammation. Uncharacterized innate pathways should also be investigated as mechanisms for control of viral replication after initial viral dissemination. The marked variation in clinical disease progression that is apparently unrelated to peripheral immunocyte kinetics predicted to be related to disease reveals the need to develop new thought processes to understanding SIV and HIV pathogenesis and immunity.

Chapter 4. *Investigation of a putative lemur-specific lentivirus*

In agreement with studies of wild lemurs endemic to Madagascar, we demonstrated seroreactivity to diverse lentiviral antigens and weak cross reactivity to the African primate lentivirus SIV by ELISA. Although we were not able to amplify lentiviral specific sequences using standard lentiviral and retroviral degenerate primer sets, we were able to amplify and sequence a likely endogenous retrovirus related to Simian retrovirus D.

Further optimization of degenerate primer sets may lead to the amplification of a lentiviral sequence from stimulated cultured PBMC supernatants or genomic DNA. The development of a co-culture system or characterization of a susceptible cell line could enhance viral growth and aid in detection.

The extension of the putative endogenous retroviral element sequence in this study could be accomplished using the specific primers designed from this sequence to screen the available BAC library of *L. catta*. The sequence of the appropriate clone would allow for further primer design. Specific primers used to amplify wild and captive lemur genomic DNA could then be used for further phylogenetic analysis.

In total, these experiments have made measured progress in extending our understanding of SIV immunity and pathobiology, setting the stage for further informed studies to advance our knowledge of this highly relevant animal model for HIV/AIDS.