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Humanized Mice as a Model to Study Human Viral Pathogenesis and Novel Antiviral Drugs

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ABSTRACT

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and Novel Antiviral Drugs

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Animal models have greatly contributed to the understanding of different aspects of human biology, as well as a variety of human-related pathogens and diseases. In order to study them, humanized mice susceptible to pathogens that replicate in human immune cells have been developed (e.g., humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mice). These animals are engrafted with human hematopoietic stem cells (HSCs), resulting in the *de novo* development and maturation of the major functional components of the human adaptive immune system and the production of a variety of human cell types. Primary and secondary lymphoid organs in the mouse are populated with human cells, and animals have long term engraftment. These features make humanized mice an excellent in vivo model to study pathogenesis of human-specific viruses in the context of a human antiviral immune response. In addition, humanized mice have been shown to be useful preclinical models for the development and validation of antiviral therapeutics. In the present study, we aimed to successfully re-establish the humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mouse model using cord blood-derived HSCs in our laboratory. We have shown that these mice sustain long term engraftment and systemic expansion of human cells, including the major targets of Kaposi's sarcoma Herpesvirus (KSHV) and Human immunodeficiency virus type 1 (HIV-1), in peripheral blood and different lymphoid organs. Further, we have begun to evaluate the susceptibility of the humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mouse model to infection with KSHV. We demonstrate that human lymphocytes differentiated in reconstituted $Rag2^{-/-}$ $\gamma c^{-/-}$ mice are permissive to KSHV infection *ex vivo*. This finding was corroborated by detection of KSHV mRNA expression in the spleen of a humanized mouse at 6 months post infection. In a different study, we tested the in vivo antiviral efficacy of a novel HIV-1 fusion inhibitor (chol-PIE12-trimer) in humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mice. We have determined the half-life of chol-PIE12-trimer in mouse plasma. Furthermore, the administration of chol-PIE12-trimer to HIV-1 infected humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mice prevents depletion of $CD4^{+}$ T cells in blood, thus it may be useful to prevent AIDS in human patients.

Keywords: Humanized mice, $Rag2^{-/-}$ $\gamma c^{-/-}$ mice, Hematopoietic stem cells, KSHV, HIV-1-associated lymphomas, HIV-1, AIDS pathogenesis, HIV-1 entry inhibitors, D-peptide inhibitors.

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Table of Contents

List of Tables viii

List of Figures viii

CHAPTER 1 Characterization of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice in our laboratory 1

INTRODUCTION 1

MATERIALS AND METHODS 6

Human CD34^{+} cells 6

Generation of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice 6

Flow cytometry 7

Immunohistochemistry 7

RESULTS 9

Efficient engraftment of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice transplanted with human hematopoietic cells 9

CHAPTER 2 Humanized Rag2$^{-/-}$ γc$^{-/-}$ mice for the study of Kaposi's Sarcoma Herpesvirus Infection 14

INTRODUCTION 14

Kaposi's sarcoma-associated herpesvirus 14

KSHV epidemiology and associated diseases 17

Models to study KSHV infection and pathogenesis 22

RESEARCH OBJECTIVES 23

MATERIALS AND METHODS 24

Preparation and titration of KSHV 24

Infection of humanized Rag2$^{-/-}$ γc$^{-/-}$ mouse-derived human leukocytes with rKSHV.219 25
Infection of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice with rKSHV.219 25
Flow cytometry 25
In situ hybridization for KSHV LANA gene expression 26
RESULTS AND DISCUSSION 29
Humanized Rag2$^{-/-}$ γc$^{-/-}$ mice-derived human leukocytes are susceptible to rKSHV.219 infection 29
Humanized Rag2$^{-/-}$ γc$^{-/-}$ mice are permissive to KSHV infection 29
CONCLUSION 34
CHAPTER 3 Antiviral efficacy of the D-peptide HIV-1 Entry Inhibitor chol-PIE12-trimer in humanized Rag2$^{-/-}$ γc$^{-/-}$ mice 36
INTRODUCTION 36
Human immunodeficiency virus type 1: Virus structure, genomic organization, and protein function 36
HIV-1 life cycle 45
HIV-1 transmission 51
HIV-1 infection and pathogenesis 53
Antiretroviral therapy for the treatment of HIV-1 infection 54
HIV-1 entry inhibitors 56
Animal models to study HIV-1 infection and pathogenesis 60
RESEARCH OBJECTIVES 66
MATERIALS AND METHODS 66

Generation of Rag2$^{-/-}$ γc$^{-/-}$ mice 66

chol-PIE12-trimer IC_{50} calculation 67

chol-PIE12-trimer dosing and pharmacokinetic analysis in mice 68

Antiviral efficacy of chol-PIE12-trimer in Rag2$^{-/-}$ γc$^{-/-}$ mice.....68

RESULTS AND DISCUSSION 72

Determination of IC50 of PIE12-trimer against HIV-1_{Ba-L} 72

Pharmacokinetics of chol-PIE12-trimer in humanized Rag2$^{-/-}$γc$^{-/-}$ mice 72

Antiviral efficacy of chol-PIE12-trimer in humanized Rag2$^{-/-}$γc$^{-/-}$ mice 72

CONCLUSIONS 75

BIBLIOGRAPHY 78

List of Tables

Table 1. Summary of the representative characteristics of humanized mice models 5

List of Figures

Figure 1. Production of humanized Rag2-/- γc-/- mice. 8
Figure 2. FACS analysis of human hematopoietic cell engraftment in peripheral blood of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice mice..... 11
Figure 3. Lymphoid tissues of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice are populated by human hematopoietic cells.. 12
Figure 4. Immunohistochemical analysis of human leukocytes in lymphoid organs of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice..... 13
Figure 5. KSHV genome organizaton..... 16
Figure 6. Genomic organization of rKSHV.219..... 26
Figure 7. Production of infectious rKSHV.219 from latently infected Vero.219 cells 27
Figure 8. Titering of rKSHV.219 on 293T cells. 28
Figure 9. Human cells differentiated in humanized Rag2$^{-/-}$ γc$^{-/-}$ mice are susceptible to infection with rKSHV.219 ex vivo..... 31
Figure 10. Suceptibility of humanized mouse-derived B and T lymphocytes to rKSHV.219 ex vivo. 32
Figure 11. Detection of LANA mRNA expression in the spleen of a infected humanized Rag2$^{-/-}$ γc$^{-/-}$ mouse by in situ hybridization..... 33
Figure 12. HIV-1 genome with encoded proteins. 42
Figure 13. Schematic representation of a mature HIV-1 virion..... 43
Figure 14. The life cycle of HIV-1. 48
Figure 15. Schematic representation of the steps of HIV-1 attachment and entry into a target cell.. 59
Figure 16. IC_{50} curve for chol-PIE12-trimer against Hiv-$1_{Ba-L.}$..... 70
Figure 17. Experimental design for the evaluation of the antiviral efficacy of chol-PIE12-trimer D-peptide in Rag2-/- γc -/- mice. 71

Figure 18. Effect of chol-PIE12-trimer on plasma viral load of infected humanized Rag2$^{-/-}$γc$^{-/-}$ mice 76
Figure 19. Effect of chol-PIE12-trimer on CD4+ T cell levels of infected humanized Rag2-/-γc-/- mice 77

CHAPTER 1

Characterization of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice in our laboratory

INTRODUCTION

The genetic and physiological similarities between mice and humans, the experimental feasibility, and the low cost maintenance of mice are among the reasons that make these animals a valuable in vivo model for the study of different human biological processes. Despite the many genetic traits conserved in mice and humans, species-specific differences exist, including susceptibility and response to pathogens. Often these differences make difficult the translation of significant preclinical achievements from rodents to humans. Thus, over the past two decades, efforts have been made to develop effective mouse models that more accurately resemble the complexity of human biology. One such development has been the "humanization" of mice.

Humanized mice are described as immune-compromised mice capable of transgenically expressing human genes or capable of being engrafted with hematopoietic stem cells (HSCs), peripheral-blood mononuclear cells (PBMCs), and /or tissues of human origin. These animals have been invaluable for the past two decades as in vivo models in preclinical studies. Humanized mice have generated important results that are of relevance for different fundamental aspects of human biology and immunology, including human hemato-lymphopoiesis, innate and adaptive immune responses, autoimmune diseases, infectious diseases, cancer, etc (Shultz, Ishikawa et al. 2007).

This overview focuses on the three major advances accomplished in the generation of humanized mice since their inception. The first step in the progressive attempts of murine humanization began with the discovery of the severe combined immunodeficiency (SCID) mutation (*Prkdc*^{scid}) in mice (C.B-17 SCID mice) (Bosma, Custer et al. 1983). This mutation

severely impairs the development of functional B and T lymphocytes. Using SCID mice, Mosier and McCune demonstrated for the first time that human PBMCs and human fetal hematopoietic tissues respectively can engraft in such mice, thus developing the SCID-hu-PBL and SCID-hu thy/liv chimeric human/ mouse models (Table 1) (McCune, Namikawa et al. 1988; Mosier, Gulizia et al. 1988). Despite the many contributions these models have provided to the study of some aspects of hematolymphopoiesis and adaptive immune responses (Shultz, Ishikawa et al. 2007; Pearson, Greiner et al. 2008), they pose important limitations. Among them, poor engraftment due to the high levels of host natural killer (NK) cell activity and the spontaneous generation of mouse B and T cells (leakiness), and limited primary human immune responses. Additionally, Implantation of human fetal thymus and liver tissues into the SCID-hu thy/liv model requires a surgical procedure that is arduous and cost intensive (Legrand, Weijer et al. 2006; Shultz, Ishikawa et al. 2007).

In an attempt to overcome the limitations present in the SCID model, additional genetic modifications capable of decreasing the engraftment barriers were necessary. Thus, the second main advance was the development of the non-obese diabetic NOD/SCID strain. Mice carrying the NOD mutation exhibit a reduced NK cell activity, are defective in the production of complement and, because of the SCID mutation, these mice do not produce functional B and T lymphocytes (Table 1) (Shultz, Schweitzer et al. 1995; Pflumio, Izac et al. 1996; Greiner, Hesselton et al. 1998). To further enhance the levels of engraftment and to reinforce the deficiencies in the mouse immune system, this model has been improved by the addition of new genetic variations. For instance, NK cell depletion was accomplished by the introduction of the β2 microglobulin mutation (NOD/SCIDβ2m$^{-/-}$ mice) (Christianson, Greiner et al. 1997; Kollet, Peled et al. 2000); however, the appearance of spontaneous thymic lymphomas (a problem found

in all NOD/SCID mice) shortens the life span of this strain. Other mutations combined with this model include the recombination activating gene1 (Rag1) locus, and the interleukin-2 receptor (IL-2R) common gamma chain (γc) locus (NOD/Rag1$^{-/-}$ γc$^{-/-}$, NOD/SCIDγc$^{-/-}$) (Table 1).

Mice deficient in Rag1 or Rag2 are unable to generate T and B cells expressing antigen-specific receptors, and leakiness is not observed in these animals, although they maintain normal levels of NK cells. In addition, Rag-deficient mice are more resistant to irradiation than SCID mice (Table 1) (Pearson, Greiner et al. 2008; Pearson, Shultz et al. 2008). The γc$^{-/-}$ mutation contributes to a significant reduction of both innate and adaptive immune responses manifested by the complete depletion of NK cells and the severe impairment of functional B and T cells. Consequently, transplanted γc$^{-/-}$ mice sustain a higher engraftment of HSCs, PBMC, or fetal tissue (Table 1) (Ishikawa, Yasukawa et al. 2005). Another advantage observed in γc$^{-/-}$ deficient mice is the development, post-transplantation, of human B and T cells, monocytes /macrophages, dendritic cells in the periphery and in lymphoid organs such as bone marrow, spleen, thymus, and lymph nodes. The different mouse strains originated from the NOD/SCID mice have been employed in different areas of biomedicine and have contributed to the elucidation of problems associated, for instance, with HSC function and infection diseases such as AIDS (Shultz, Ishikawa et al. 2007; Berges and Rowan 2011). Nevertheless, the absence of a fully functional human immune system and the short life span are among the factors that have made this model remain limited in effectiveness.

The experience accumulated over the past 10 years in the production of humanized mice has led to the exploration of alternative mouse strain combinations. Thus, the most recent innovation in the humanization of mice was achieved by crossing homozygous Rag2 mice with homozygous γc mice (Rag2$^{-/-}$ γc$^{-/-}$) (Table 1). These mice support greatly increased engraftment

compared with SCID and NOD/SCID humanized mouse models. Different studies have demonstrated that the secondary lymphoid tissues in these animals are substantially and sustainably populated throughout the mouse lifespan with different cellular compartments, including T and B lymphocytes, monocytes/macrophages, and dendritic cells (Table 1). Furthermore, $Rag2^{-/-}$ $\gamma c^{-/-}$ mice do not require the addition of exogenous factors (e.g. cytokines) to stimulate development of such cellular compartments. In addition, they do not exhibit spontaneous lymphoma formation and are capable of producing primary human adaptive immune responses (Goldman, Blundell et al. 1998; Mazurier, Fontanellas et al. 1999; Traggiai, Chicha et al. 2004; Chicha, Tussiwand et al. 2005). Together, all these features have made $Rag2^{-/-}$ $\gamma c^{-/-}$ mice one of the most versatile and preferred mouse models to date. Reviews of how and in which areas of preclinical research $Rag2^{-/-}$ $\gamma c^{-/-}$ mice are being used are available (Shultz, Ishikawa et al. 2007; Goldstein 2008; Pearson, Greiner et al. 2008; Berges and Rowan 2011).

Table 1. Summary of the representative characteristics of humanized mice models

Common mouse strain (genetic mutation)	Mouse phenotype	Sours of human cells	Site of reconstitution	Human leucocytes present
hu-PBL-SCID (Prkdc^{scid})	Absence of mature T and B cells. High levels of NK cells.	Human peripheral blood mononuclear cells (PBMCs).	Peripheral blood and spleen.	B and T cells
SCID-hu (Prkdc^{scid})	Absence of mature T and B cells. High levels of NK cells.	Fetal thymus and liver.	mainly the thymic organoid.	Predominantly T cells
Rag1$^{-/-}$ or Rag2$^{-/-}$	Lack of functional T and B cells Residual NK cells	Hematopoietic stem cells (HSCs)	Peripheral blood, spleen, and lymph nodes	B and T cells
NOD-SCID	No mature T and B lymphocytes. Reduced NK cell activity	PBMCs / HSCs	Peripheral blood, spleen, bone marrow, and lymph nodes	B and T cells
NOD-SCID γc$^{-/-}$ (Prkdc^{scid} / IL2r γc$^{-/-}$)	Impaired T and B cell development. Complete absence of NK cells	HSCs	Peripheral blood, spleen, thymus, bone marrow, lymph nodes, intestines	B and T lymphocytes DCs, monocytes/macrophages
Balb/c-Rag2$^{-/-}$ γc$^{-/-}$	No mature T and B cells. NK cells absent	HSCs	Peripheral blood, spleen, thymus, bone marrow, lymph nodes, intestines, rectal and vaginal mucosa	B and T cells, monocytes/macrophages, DCs,
hu-BLT model in - NOD-SCID and - NOD-SCID γc$^{-/-}$	No mature T and B cells. NK cells absent	Fetal liver/ thymus, HSCs	Peripheral blood, spleen, thymus, bone marrow, lymph nodes, intestines, liver, lung, male and female reproductive tract	B and T cells, monocytes/macrophages, DCs, NK cells

MATERIALS AND METHODS

Human $CD34^+$ cells

Human cord blood-derived $CD34^+$ cells were isolated by magnetic separation using the Human Cord Blood CD34 Positive Selection Kit (STEMCELL technologies, Vancouver, Canada) according to the manufacturer's protocol. Purified $CD34^+$ cells were then cultured for two days in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 ug/ml) and containing IL-3, IL-6, and SCF as described previously (Akkina, Rosenblatt et al. 1994). Quantity and purity of isolated $CD34^+$ cells were evaluated by FACS using antibodies specific for hCD45 and hCD34. Cells were either maintained in liquid nitrogen or immediately transplanted into newborn mice.

Generation of humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mice

Humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mice were prepared as described by Berges et al (Berges, Wheat et al. 2006) with the exception that human cord blood-derived $CD34^+$ cells were used for engraftment. 2-5 day old neonatal mice were conditioned by irradiation at 350 rads and then injected intrahepatically with 0.2-0.5 x 10^6 $CD34^+$ cells. Approximately 8 weeks post-reconstitution, transplanted mice were screened for human cell engraftment. Peripheral blood was collected by tail bleed and red blood cells were lysed. The white cell fraction was stained with species-specific antibodies for the pan-leukocyte markers mCD45 and hCD45 (eBioscience). Samples were then FACS analyzed to determine the levels of human cell engraftment (Fig.1).

Flow cytometry

FACS analysis was performed to evaluate human cell engraftment in peripheral blood and in different lymphoid organs of reconstituted $Rag2^{-/-}$ $\gamma c^{-/-}$ mice. Whole blood was collected in heparinized capillary tubes and cellular and plasma fractions were separated by centrifugation. Erythrocytes were lysed as indicated above. White blood cells were stained with hCD45 (leukocytes), hCD3 (T cells), hCD4 (helper T cells), hCD8 (cytotoxic T cells), hCD14 (monocytes/macrophages), hCD19 (B cells), hCD20 (B cells), and hCD123 (dendritic cells). To analyze the presence of human cells in different mouse lymphoid organs, single cell suspensions were made from spleen, liver and bone marrow. Cell suspensions were stained with different antibodies and analyzed on a BD FACS Canto analyzer. White blood cells from unengrafted mice were routinely stained with each of the above 'human-specific' antibodies to confirm that there was no binding to a pure population of mouse cells. These experiments confirmed that the antibodies used herein were truly human-specific (data not shown).

Immunohistochemistry

4-μm tissue sections frozen in OCT compound from spleen, lymph nodes, and thymus of mice transplanted with human cord blood-derived $CD34^{+}$ cells were stained with antibodies for detection of human leukocytes. The primary antibodies used were human monoclonal antibodies specific for CD45, CD3, CD4, CD8, CD20, and CD68. Staining was performed as described previously (Berges et al., 2006).

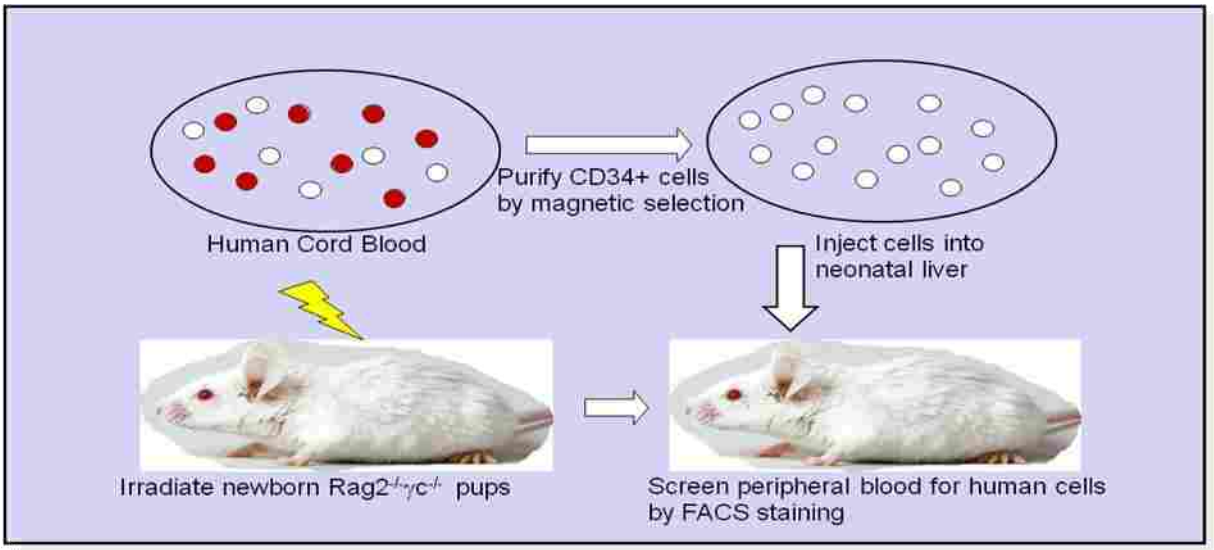

Figure 1. Production of humanized Rag2-/- γc-/- mice.

RESULTS

Efficient engraftment of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice transplanted with human hematopoietic cells

Neonatal BALB/c-Rag2$^{-/-}$ γc$^{-/-}$ mice were transplanted with human umbilical cord blood-derived CD34^{+} cells by intrahepatic injection. Eight to ten weeks post-transplantation, mice were monitored by FACS analysis for human cell engraftment in peripheral blood and lymphoid organs. *Engraftment in peripheral blood*: Cells stained positive for the human CD45 marker, indicated the presence of human leukocytes (Fig. 2A). Within the leukocyte population, a variety of human immune cells, including those susceptible to KSHV and HIV-1 infection (i.e. B and T lymphocytes, and monocytes/ macrophages) were detected in blood following engraftment. Human B cells were recognized by the positive staining for CD19 and CD20 (Fig. 2B) and human monocytes displaying the characteristic marker CD14 were also identified (Fig. 2C). The recognition of human T lymphocytes was assessed by positive staining for CD3 (Fig. 2D). Furthermore, the presence of CD4 and CD8 markers which correspond to helper and cytotoxic T lymphocytes, respectively, was established in peripheral blood, and the ratio between these sub-populations was determined (Fig. 2E). Finally, circulating myeloid (CD11c) and plasmacytoid (CD123) dendritic cells were found as part of the subset of leukocytes monitored by FACS in the blood of reconstituted mice (Fig. 2F).

Human hematopoietic cells in lymphoid organs: The presence of lymphoid and myeloid cells in different organs of transplanted Rag2$^{-/-}$ γc$^{-/-}$ mice was analyzed by FACS (Fig. 3) and immunohistochemical staining (Fig. 4). Engrafted mice were sacrificed and cell suspensions from bone marrow, spleen, and liver were stained with a panel of anti-human antibodies specific for the surface markers CD11c, CD14, CD19, CD20, and CD123. Human B cells and dendritic

cells were identified in bone marrow. In addition, human B cells were also detected in the spleen of transplanted mice (Fig. 3A-3D). In addition to FACS analysis, immunohistochemistry was performed to evaluate the human cell types present in lymphoid organs. Tissue sections obtained from spleen, lymph nodes, and thymus were immune-stained and the presence of human leukocytes was revealed in all three organs (Fig.4). Human macrophages (CD68) were seen mostly in spleen. Human T lymphocytes including the subset of CD4 and CD8 cells were also detected in all three samples, especially with a high concentration in the thymus and lymph nodes. The positive staining for hCD20 confirmed that human B cells were also populating the analyzed tissues. The presence of human leukocytes in circulating peripheral blood and in different lymphoid organs of transplanted $Rag2^{-/-}$ $\gamma c^{-/-}$ mice evidences the satisfactory reconstitution of a human lymphoid system in this animal model.

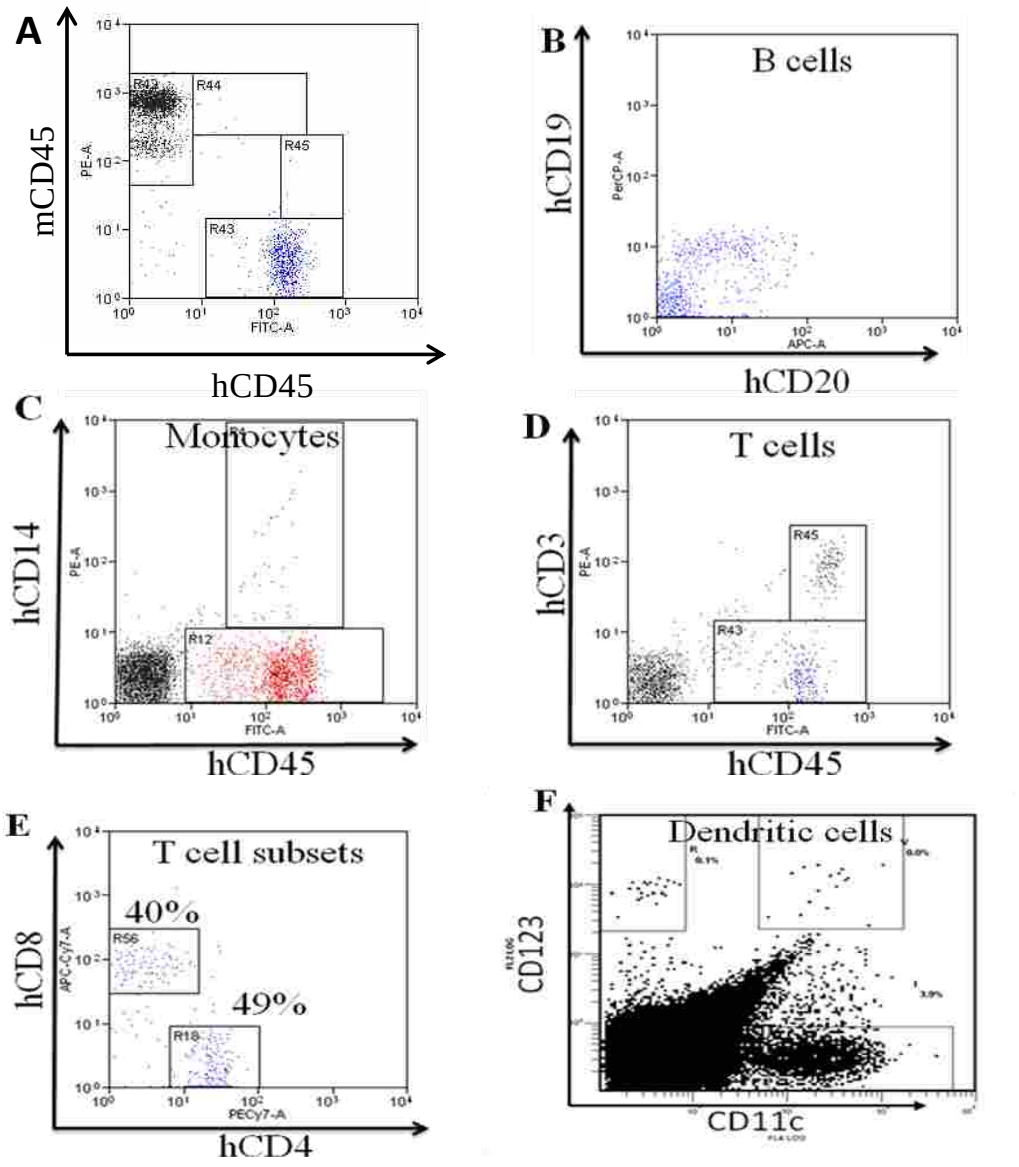

Figure 2. FACS analysis of human hematopoietic cell engraftment in peripheral blood of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice mice. Irradiated neonatal mice were transplanted with human CD34^{+} cells by intrahepatic injection. At 8-10 weeks post-transplantation, mice were bled and peripheral blood cells were FACS stained and evaluated for human cell engraftment. (A) Cells stained positively for the human marker CD45 indicate levels of engraftment by human leukocytes in transplanted mice. (B) Cells stained with anti-human CD19 and CD20 show successful engraftment with human B cells. (C) Cells positive for antibodies against the human monocyte marker CD14. (D) Detection of hCD3^{+} T cells in peripheral blood. (E) Helper (CD4) and cytotoxic (CD8) human T lymphocytes. (F) Positive stained cells for anti-human CD11c and CD123 depict the presence of dendritic cells in blood.

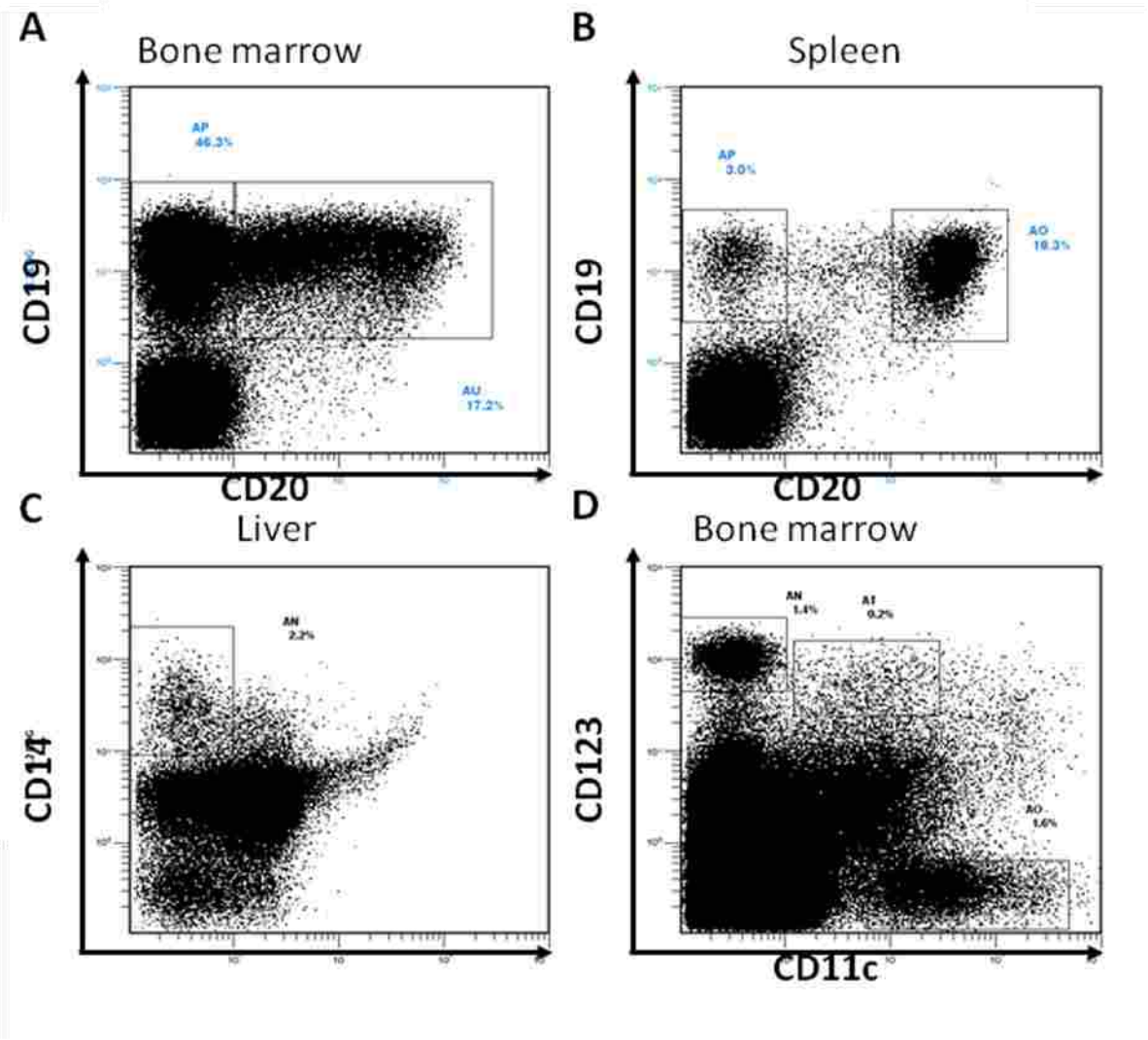

Figure 3. Lymphoid tissues of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice are populated by human hematopoietic cells. Reconstituted mice were sacrificed and cell suspensions from bone marrow, spleen, and liver were obtained and FACS stained to assess the presence of human leukocytes. Two subsets of B cells (CD19, and CD20) were localized in bone marrow and spleen (A and B). CD14 expressing macrophages were also detected (C). Myeloid and plasmacytoid dendritic cells (CD11c and CD123 respectively) were present in lymphoid organs of engrafted mice (D).

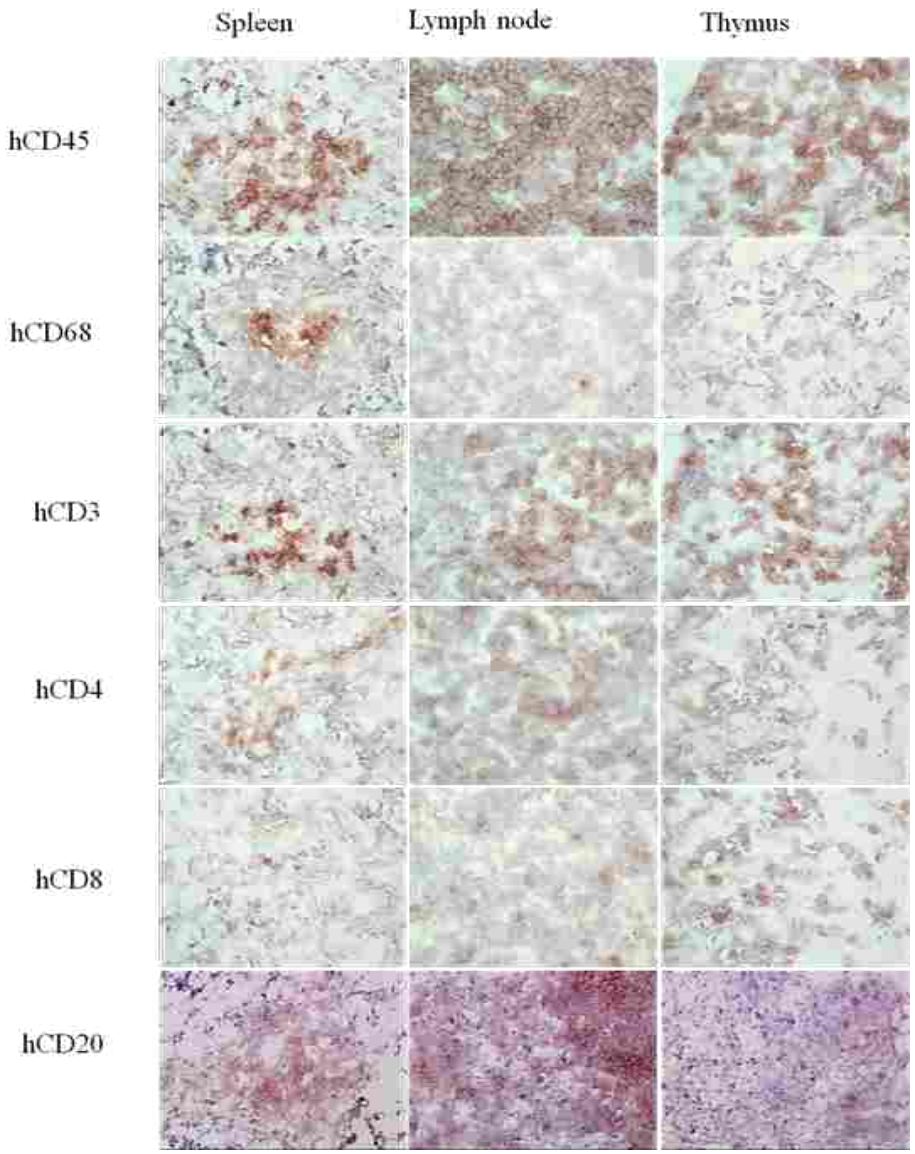

Figure 4. Immunohistochemical analysis of human leukocytes in lymphoid organs of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice. Tissue sections obtained from spleen, lymph nodes, and thymus from engrafted mice were immunostained to evaluate the expression of human immune cell markers. Human leukocytes (hCD45^{+}) are present in all three organs. Spleen was highly positive for human macrophages (CD68). Human T lymphocytes (hCD3) including the CD4 and CD8 subsets were localized in all three organs. Spleen and Lymph node were also positively stained with hCD20 B cells.

CHAPTER 2

Humanized Rag2$^{-/-}$ γc$^{-/-}$ mice for the study of Kaposi's Sarcoma Herpesvirus Infection

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV8), is a member of the Herpesviridae family and together with EBV belongs to the subfamily Gammaherpesvirinae. KSHV is the etiological agent of Kaposi's sarcoma (KS), the most common cancer detected in HIV-infected patients, and the lymphomas multicentric Castleman's disease (MCD) and primary effusion lymphoma (PEL) (Chang, Cesarman et al. 1994; Cesarman, Chang et al. 1995). These malignancies are most frequently observed among HIV^{+} patients. However, they have also been diagnosed in people receiving organ transplants and immunocompromised individuals. KSHV contains a 170 kb double-stranded DNA genome enclosed in an icosahedral capsid which in turn is surrounded by a tegument layer and a lipid bilayer envelope. The KSHV genome is composed of a long unique region (LUR) containing approximately 95 genes (Fig. 5) (Renne, Lagunoff et al. 1996; Neipel, Albrecht et al. 1998). Roughly 25% of the viral genome comprises viral homologues of human genes. They are associated with functions such as immune modulation, inhibition of apoptosis, and cell cycle regulation. KSHV also contains genes that are conserved in other herpesviruses, as well as genes that are unique to the virus (Russo, Bohenzky et al. 1996; Neipel, Albrecht et al. 1997).

In vitro, KSHV infection has been localized to human B lymphocytes, macrophages, dendritic cells, epithelial cells, endothelial cells, fibroblasts, and mesenchymal stem cells (Schulz, Sheldon et al. 2002; Chandran 2010). It has also been shown that KSHV can establish latent infection in several cell lines, among them human embryonic kidney cells (293 cells),

human cervical carcinoma cells (HeLa cells), baby hamster kidney cells (BHK cells), green monkey fibroblasts cells (Vero cells), and murine fibroblast cells (3T3 cells) (Bechtel, Liang et al. 2003; Greene, Kuhne et al. 2007). In vivo, the principal target cells infected by KSHV include B cells, monocyte /macrophages, endothelial cells, and epithelial cells (Blackbourn, Lennette et al. 2000). Additional evidence has shown that KSHV can also be detected in human hematopoietic progenitor cells and fetal mesenchymal stem cells (Parsons, Szomju et al. 2004; Wu, Vieira et al. 2006), suggesting that these reservoirs may act as a continuous source of virus dissemination in vivo.

Similar to other herpesviruses, KSHV alternates between two states of infection as part of its life cycle. In vitro and in vivo, KSHV remains predominantly in a latent phase. During this phase the virus resides as an episome in the nucleus of infected cells and expresses a limited number of genes necessary for dissemination of viral nucleic acids within the host via cell division. The genes expressed during latent infection include the Latency Associated Nuclear Antigen (LANA/ORF73), v-cyclin (ORF 72), and v-FLIP (ORF 71), which are adjacent to one another within the viral genome. In such position, they constitute a multicistronic transcriptional unit (Parravicini, Chandran et al. 2000). No infectious viral particles are produced during latency.

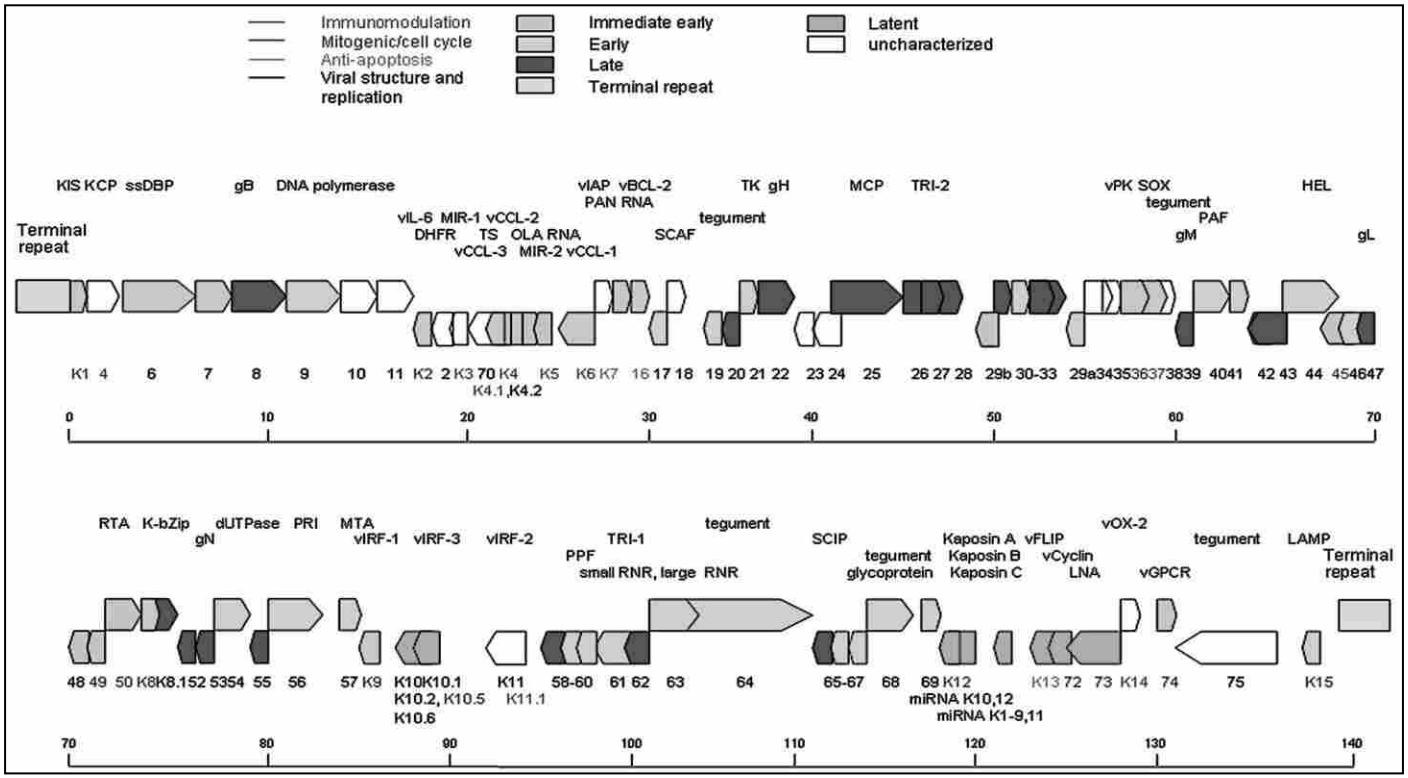

Figure 5. KSHV genome organization (Greene et al. 2007)

The lytic cycle of KSHV is characterized by the expression of a wide range of gene products; they are grouped in three categories as immediate early, early, and late genes produced at different times during the replication cycle. The general contribution of these genes is to facilitate the production of mature virions by assisting KSHV in the replication of viral genome, viral assembly and budding.

The switch from latency to lytic replication of KSHV is initiated by a number of stimuli that induce the expression of the lytic switch protein, Replication and Transcription Activator (RTA), encoded by the viral ORF 50 (Lukac, Kirshner et al. 1999). It has been observed that in KS and PEL, approximately 95% of the infected cells contain latent virus, while in MCD the most predominant form of KSHV replication is lytic (Greene, Kuhne et al. 2007).

KSHV epidemiology and associated diseases

It is estimated that several million people are infected with KSHV worldwide with the majority of them living in Africa (Plancoulaine and Gessain 2005). The prevalence of KSHV infection differs among populations around the world. It has been documented that the homosexual population exhibits higher prevalence (20-30% are seropositive) than the general population (Engels, Atkinson et al. 2007). In the general population, the distribution of KSHV infection in northern Europe, North America, and Asia is less than 10 %. In Italy and other areas of the Mediterranean, South America and west Africa, the prevalence is relatively moderate (10 to 30 %). Certain regions in sub-Saharan Africa and Native American tribes in the Amazon basin show the highest seropositivity observed among the different regions worldwide (up to 60 %) (Dukers and Rezza 2003; Plancoulaine and Gessain 2005).

The mechanisms by which KSHV is transmitted between hosts are not well understood. However, there is increased evidence indicating that saliva is the major route of transmission

(due to the higher viral concentration compared with other body fluids), similar to what is observed in most human herpesviruses (Pauk, Huang et al. 2000). Heterosexual transmission remains low; thus, it is not considered a main route for spreading KSHV (Smith, Sabin et al. 1999). Due to the high incidence of KSHV infection in men who have sex with men, it is suggested that homosexual contact is an important form of virus transmission (Blackbourn, Lennette et al. 1998; Engels, Atkinson et al. 2007). Blood or blood products can potentially transmit KSHV, although the risk is generally estimated to be low. Additionally, organ transplantation has been listed among the means through which virus transmission can occur (Regamey, Tamm et al. 1998).

Since its discovery, extensive studies have linked KSHV with cancer because the virus was originally isolated from KS lesions. KS is the fourth most common cancer caused by an infectious agent, and is the most common neoplasm associated with AIDS (Chang, Cesarman et al. 1994; Lebbe, de Cremoux et al. 1995; Chang, Ziegler et al. 1996). As noticed earlier, KSHV can also cause lymphoproliferative disorders, affecting especially B lymphocytes. Thus this herpesvirus has been implicated in the pathogenesis of PEL and MCD. Although these lymphomas are less common than KS, they are associated with a higher rate of mortality, especially in immunocompromised individuals. (Cesarman, Chang et al. 1995; Soulier, Grollet et al. 1995). The probability for HIV^+ patients to develop lymphomas each year is between 1.6-8.0 %, and approximately half of $KSHV^+$ transplant recipients develop a lymphoproliferative-related disease (Regamey, Tamm et al. 1998). Because PEL and MCD are rare complications of KSHV infection, there is not yet an established treatment regimen for these diseases.

Kaposi's sarcoma: This multifocal neoplasm was first described in 1875 as an "idiopathic multiple pigmented sarcoma of the skin" by the Hungarian dermatologist Moritz

Kaposi (Kaposi 1872). However, it was not until the AIDS pandemic arose in the 1980s that the development of KS was linked to an infectious agent, as a result of the high incidence of KS among HIV^+ individuals. Subsequently in 1994, with the use of representational difference analysis, Chang and colleges identified a novel herpesvirus (named KSHV) in DNA fragments from Kaposi's sarcoma tissues from HIV-infected patients. Thus, a strong association was established between the newly discovered virus and KS (Chang, Cesarman et al. 1994).

As mentioned earlier, KS is the most frequent malignancy found in AIDS patients. KS is a highly vascular tumor of endothelial origin characterized by the proliferation of so called spindle-shaped cells which are poorly differentiated and mainly latently infected with KSHV. Only a small percentage of these cells support viral lytic replication. The latent gene LANA is predominately expressed in the nucleus of infected cells. Additionally, high levels of certain proinflamatory cytokines and growth factors have been detected in the serum of KS patients (e.g. IL-6, TNF-α, TGF-β, IFN-γ, etc.), which are considered to induce angiogenesis and proliferation of KS tumor cells (Ensoli, Nakamura et al. 1989; Gasperini, Sakakibara et al. 2008). KS tumors also contain abnormal blood vessels. Although skin lesions are the most common manifestations of KS, it may also involve the lymph nodes, oral mucosa, respiratory tract, and visceral organs (Dupin, Fisher et al. 1999; Greene, Kuhne et al. 2007). According to clinical and epidemiological studies, KS is currently classified into four different subtypes: Classic (sporadic), endemic (African), epidemic (AIDS-related), and iatrogenic (post transplant-associated).

Classic KS (CKS) is a non-aggressive tumor that usually affects elderly men. It is most commonly found in Mediterranean countries and Eastern Europe. The lesions present in CKS are usually localized in the upper and/or lower extremities (Wahman, Melnick et al. 1991). *Endemic KS (EKS)* is common in Central Africa, affecting predominantly women, young adults, and

children. EKS can be indolent, similar to what is observed in CKS. However, there are many reports indicating that EKS can also be associated with HIV infection, in which case the lesions are more aggressive (Edelman 2005; Greene, Kuhne et al. 2007). *Epidemic KS (AIDS-KS)* is the most common and aggressive form of KS, encountered most frequently among homosexual individuals infected with HIV-1. Unlike CKS lesions, AIDS-KS lesions are multifocal; they tend to affect many parts of the body (e.g. face, oral cavity, extremities, etc.) as well as different internal organs, including the visceral organs, and lymph nodes (Goedert 2000; Hengge, Ruzicka et al. 2002; Greene, Kuhne et al. 2007). *Iatrogenic KS (IKS)* is developed by organ-transplant recipients (especially renal transplant patients) under immunosuppressive therapy (Edelman 2005; Greene, Kuhne et al. 2007).

Primary effusion lymphoma: PEL, also referred to as body-cavity-based lymphoma, is a rare malignancy commonly found in immunosupressed patients, particularly in HIV-infected males. It accounts for approximately 4% of all AIDS-associated lymphomas with a mean survival time of about 6 months. PEL is characterized by the presence of lymphomatous effusions in the peritoneal, pleural, and pericardial cavities, usually in the absence of solid tumors. However, there are some studies reporting the presence of solid mass in lymph nodes and other organs (extra-cavity PEL) (Greene, Kuhne et al. 2007; Gasperini, Sakakibara et al. 2008). The lymphoma cells in PEL are commonly monoclonal and derived from post-germinal center B-lymphocytes. The immunophenotype of PEL cells is undetermined. These lymphomas lack B or T cell-associated antigens, but they typically express the surface markers corresponding to leukocytes (CD45) and plasma cells (CD138). KSHV is invariably present in PEL; it has been observed that KSHV DNA can be found at a high copy number in PEL cells (50-150 copies/cell). Thus, the detection of the viral genome in this malignancy is considered a

hallmark for the diagnosis of this lymphoma. The persistence of KSHV in PEL is predominantly latent with the majority of cells expressing the viral latent proteins LANA-1, vFLIP, vCyclin, and Kaposin (Carbone and Gloghini 2005; Edelman 2005).

Multicentric Castleman's disease: MCD is a rare systemic lymphoproliferative disorder most commonly encountered in HIV-infected individuals and transplant recipients. In the context of AIDS, MCD is highly associated with KSHV infection (90% of the cases). MCD also can occur in HIV-seronegative patients, in which case the prevalence of KSHV infection is approximately 40%. MCD is characterized by lymphadenopathy with expansion of B cells (germinal center expansion) and vascular proliferation within the compromised lymph nodes (Hengge, Ruzicka et al. 2002; Edelman 2005). B lymphocytes are the main targets of KSHV infection in MCD; they adopt a plasmablastic morphology accompanied with a phenotype observed in plasma cells (CD27, CD38, and CD78). In lymph nodes, the cells harboring KSHV are mainly located in the mantle zone of the follicle. Although immunohistochemical analyses have shown that the infected cells in MCD are positive for LANA-1, there is also evidence of abundant lytic infection associated with this malignancy. Additionally, the high levels in serum of certain inflammatory cytokines such as IL-6 and IL-10 link these molecules with the pathogenesis of MCD. Furthermore, KSHV encodes a viral homologue of hIL-6 (vIL-6) which has been shown to stimulate the hIL-6 signaling pathway, promoting the proliferation and survival of plasma cells as well as tumor angiogenesis. Thus, the high levels of inflammatory cytokines together with viral load can contribute with the progression of a more aggressive form of MCD, leading to fatality (Carbone and Gloghini 2005; Gasperini, Sakakibara et al. 2008).

To date, different aspects of KSHV-associated pathogenesis are poorly understood, due in part to the lack of a suitable in vivo model that supports productive infection and disease. A

clearer understanding of the mechanisms of pathogenesis will facilitate the improvement of the treatments currently applied for KS, PEL, and MCD (e. g., antiretroviral therapies, chemotherapy), as well as the development of novel therapies for the treatment and prevention of KSHV-associated malignancies.

Models to study KSHV infection and pathogenesis

Because KSHV can efficiently establish a latent infection in many cell types in vitro, it provides a valuable system for the study of latent infection. However, it appears that the virus tends to initially infect immature cells in vivo and establish a latent infection, followed by a lytic cycle after maturation of the target cell and other external stimuli. Since the technology to allow cells to mature in culture (especially immune cells) is poorly understood, it is not likely that a cell culture model will be available in the near future.

Animal models (i.e. non-human primates and mice) have been used for the study of KSHV infection and related diseases, but with limited success due to the specificity that KSHV presents for human cells. The development of humanized immune deficient mice has provided a small animal model capable of being infected by KSHV, allowing the study of its associated pathogenesis (Greene, Kuhne et al. 2007). In one such study, KSHV-transformed cells (BCBL-1 cells) were injected alone or together with human PBMCs into SCID mice (Picchio, Sabbe et al. 1997). One of the goals of this study was to demonstrate transmission of KSHV from BCBL-1 cells to PBMCs grafts with the resulting transformation of new B cells. However the results did not resolve this issue. The lymphomas observed in the mice appeared to derive from the injected tumor cells and not as a result of transmission. In a different report, NOD/SCID mice were successfully infected intravenously with purified KSHV (Parsons, Adang et al. 2006). Virion production and increasing levels of viral DNA were documented in this model. Additionally, in

the same study latent and lytic viral transcripts were measured in different organs over a period of four months. However, there was no evidence of lymphoproliferation.

One of the limitations of the NOD/SCID mouse model is its attenuated lifespan as a result of a high incidence of thymic lymphomas. Finally, the low levels of engraftment make this model incapable of mounting an effective adaptive immune response against viral infection. In a study conducted by Wu and colleagues, human hematopoietic stem cells were infected with KSHV in culture and then transplanted into NOD/SCID mice. It was observed that human B cells and monocytes/macrophages were the major infected human cell types in the spleen and bone marrow. Furthermore, viral gene expression was detected in the same organs (Wu, Vieira et al. 2006). Although these murine models are valuable and provide considerable information for the study of certain aspects of KSHV biology, they are not completely adequate due to the poor production of the major target cells of KSHV.

Currently, there are no studies reporting the use of humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mice to investigate KSHV infection and pathogenesis, although successful EBV infection has been reported in this model with a predominantly latent infection. This model also provided evidence of the presence of proliferation of human B cells similar to that observed in MCD and PEL lesions in immunocompromised patients (Traggiai, Chicha et al. 2004; Cocco, Bellan et al. 2008; Yajima, Imadome et al. 2008).

RESEARCH OBJECTIVES

The overall objective of this study was to assess the suitability of humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mice for KSHV infection and to characterize basic aspects of the infection. This research

specifically addressed the cellular and organ tropism of KSHV, the state of infection (latent/or lytic), and the patterns of viral gene expression.

Specific Aims:

1. Demonstrate successful ex vivo replication of KSHV in human leukocytes isolated from humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mice.
2. Detection of viral gene expression in lymphoid organs of humanized mice.

MATERIALS AND METHODS

Preparation and titration of KSHV

To facilitate the identification of infected cell as well as to evaluate the state of infection (latent or lytic) in specific cell types, a recombinant strain of KSHV (rKSHV.219) was used for in vivo and ex vivo infection. rKSHV.219 expresses the green fluorescent protein (GFP) from the cellular promoter EF-1α in all infected cells and selectively expresses the red fluorescent protein (RFP) in lytically infected cells due to the placement of the RFP gene under the control of the KSHV lytic PAN promoter. Additionally, puromycin resistance is used to maintain latent genomes (Fig. 6). Preparation of rKSHV.219 was performed as previously described (Vieira and O'Hearn 2004). Briefly, latently infected Vero cells (Vero.219) were expanded in the presence of puromycin. For induction of lytic replication, 80-90 % confluent Vero.219 cells were infected with a recombinant Baculovirus (BacK50), expressing KSHV RTA (MOI of 1,000). Two hours post infection, Vero.219 cells were cultured in fresh medium with 2.5 mM sodium butyrate. 72 hours post-induction, rKSHV.219 was collected and concentrated 10-fold by ultracentrifugation (Fig. 7). Infectious units (IU) of rKSHV.219 were determined by titering the virus by FACS analysis of GFP^+ 293T cells at 48 hours post infection (Fig. 8).

Infection of humanized Rag2$^{-/-}$ γc$^{-/-}$ mouse-derived human leukocytes with rKSHV.219

Animals with approximately 70% engraftment were sacrificed at 10 weeks post reconstitution. Lymphoid organs, namely bone marrow, spleen, and thymus, were collected and single cell suspensions were made and cultured in IMDM with 10% FBS, except that no cytokines were added. Cell suspensions were divided into 2 groups for ex vivo rKSHV infection or mock infection. Cells were infected with rKSHV.219 at different MOIs (0.04 for bone marrow, 0.05 for spleen, and 0.02 for thymus) and in the presence of 4 µg/ml polybrene.

Infection of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice with rKSHV.219

Mice with at least 8 weeks of engraftment were infected with 3.6 x 10^4 I.U. of rKSHV.219 in a volume of 200 µl via intraperitoneal injection. Control animals were mock infected using 100 µl of the original medium (DMEM) used to re-suspend the virus stocks. Mice were monitored daily and blood samples were collected at different time points for detection of viremia.

Flow cytometry

FACS analysis was performed to assess the presence of KSHV-infected human leukocytes ex vivo and to identify the specific phenotype of such cellular populations. One portion of the single cell suspensions (infected ex vivo with rKSHV.219) obtained from bone marrow, spleen, and thymus of humanized Rag2$^{-/-}$ γc$^{-/-}$ mice was stained with hCD45-PECy7 antibody to detect the presence of GFP$^+$ cells (indicative of infection). The remaining portion was stained with human specific antibodies to determine the identity of the infected cells. To accomplish this, the following antibodies were used: hCD45-PECy7, hCD3-PECy5, hCD19-

APC AF 750, hCD20-APC, and hCD138-PECy5.5. Human blood was used to establish FACS settings and unengrafted mouse blood was used to verify human specificity.

In situ hybridization for KSHV LANA gene expression

In situ hybridization was performed on 20-μm tissue sections frozen in OCT compound from spleens of KSHV infected and uninfected humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mice. BCBL-1 tumors, previously shown to be latently infected with KSHV, were used as a positive control. At least five sections were evaluated. Hybridization was performed using digoxigenin-labeled antisense and sense riboprobes specific for KSHV LANA (from nt 3096 to 3120) and detected using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/ BCIP), as reported previously (Bergesetal.,2006)

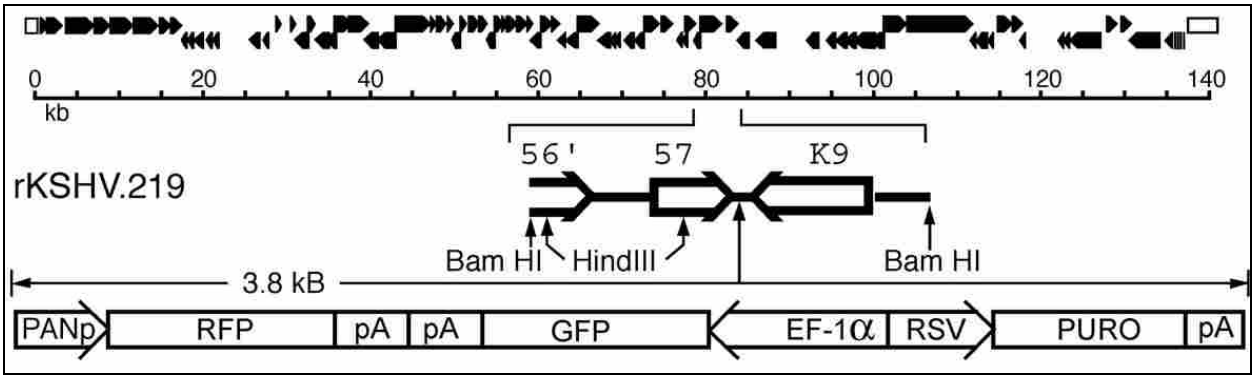

Figure 6. Genomic organization of rKSHV.219 (Vieira and O' Hearn. 2004)

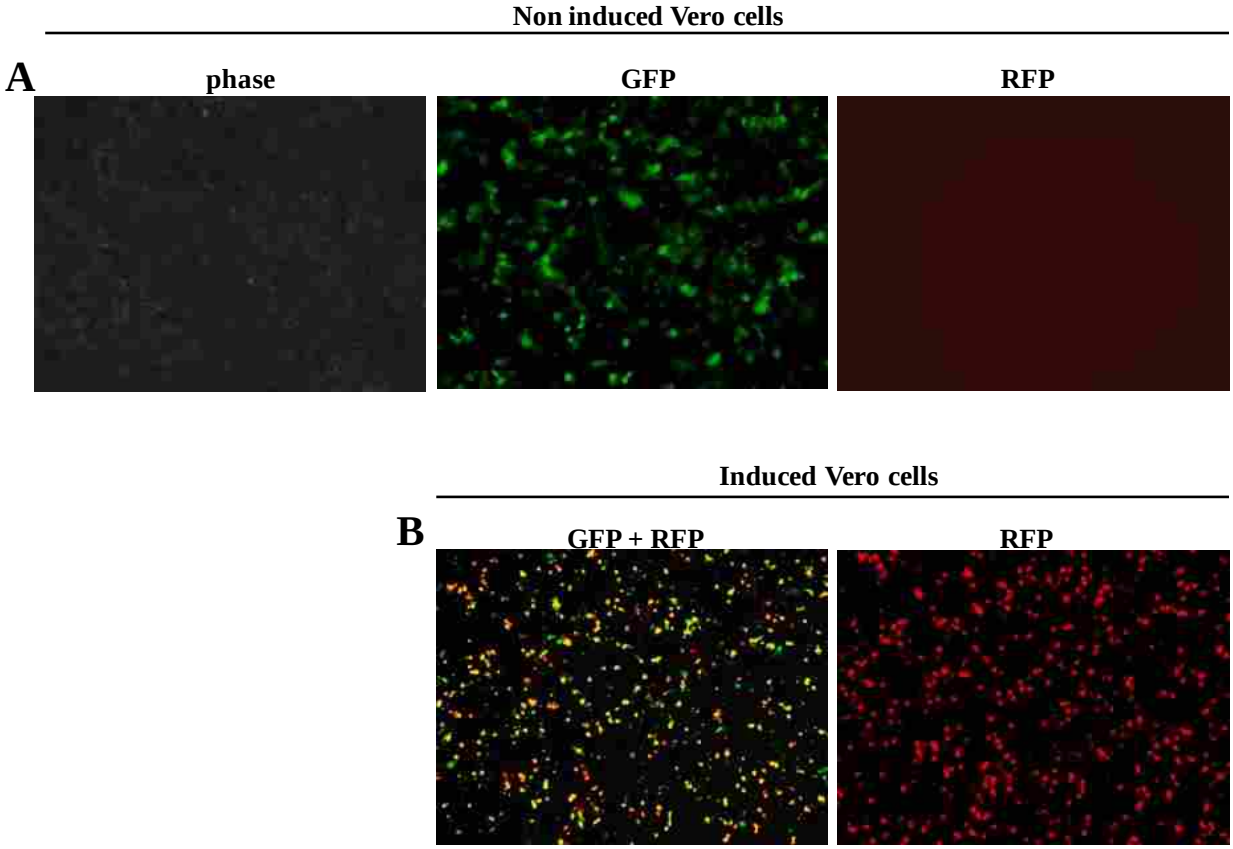

Figure 7. Production of infectious rKSHV.219 from latently infected Vero.219 cells. (A) Vero.219 cells harboring rKSHV.219 (GFP) with not activation (none induced with BacK50) showing a lack of lytic gene expression (RFP). (B) Vero.219 cells 2 days post induction with BacK50 and sodium butyrate showing the overlay of GFP and RFP fluorescence, and RFP fluorescence indicating lytic gene expression.

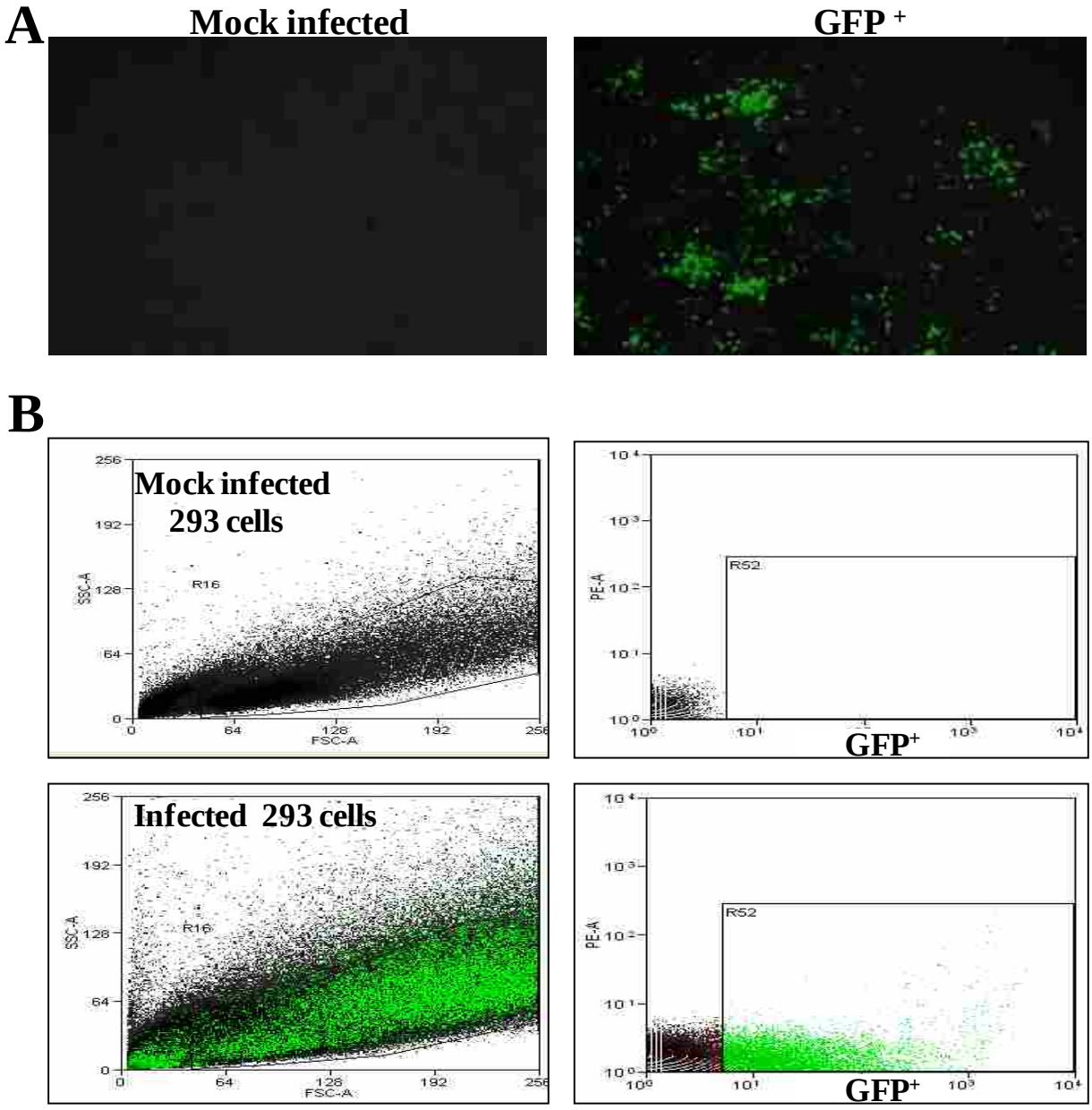

Figure 8. Titering of rKSHV.219 on 293T cells. (A) Mock infected and infected 293T cells, the latter showing GFP expression 2 days post infection with 0.2 ml of cell free medium harvested from Vero cells containing infectious rKSHV.219. (B) FACS analysis showing GFP^+ cells indicative of infection. Mock infected cells did not present any GFP background. The titer of rKSHV.219 was 1.8 x 10^5 IU/ml.

RESULTS AND DISCUSSION

Humanized Rag2$^{-/-}$ γc$^{-/-}$ mice-derived human leukocytes are susceptible to rKSHV.219 infection

Prior to evaluating if humanized Rag2$^{-/-}$ γc$^{-/-}$ mice are permissive to KSHV infection, we first examined if the human hematopoietic cells differentiated in these mice were susceptible to viral infection ex vivo. To accomplish this, cells obtained from bone marrow, spleen and thymus of humanized mice were cultured in vitro and infected with rKSHV.219. FACS analysis showed detectable GFP expression at 42 hours post infection in rKSHV.219 infected cells derived from all three organs, thus indicating successful infection (Fig. 9). The phenotypic identification of the human cells susceptible to viral infection was carried out by FACS using specific anti-human antibodies (Fig.10). KSHV infected cells were predominantly B lymphocytes, evidenced by the positive staining of human CD20 and CD138/GFP^{+} cells detected mainly in bone marrow. It is worth noting that B lymphocytes are the main targets of KSHV in MCD and PEL. Interestingly, CD3/GFP^{+} cells were found in thymic samples. Rare macrophages (CD14) and human stem cells (CD34) were also positive stained in bone marrow (not shown). No GFP^{+} cells were present in samples from a mock infected humanized mouse. These data demonstrate that humanized Rag2$^{-/-}$ γc$^{-/-}$ mice produce human cells susceptible to KSHV infection in multiple organs.

Humanized Rag2$^{-/-}$ γc$^{-/-}$ mice are permissive to KSHV infection

Following the identification in humanized mice of differentiated human cells susceptible to KSHV infection; we next proceeded to analyze the feasibility of successful viral replication in vivo. Mice were infected intraperitoneally with rKSHV.219 and evaluated for viral infection in lymphoid tissue. Tissue sections of spleen obtained from a mouse infected for 6 months were subjected to RNA in situ hybridization to monitor the expression of KSHV-LANA mRNA.

Infected cells were readily detected 6 months post infection, as were cells from positive control tissues (Fig. 11A and D). As expected, the uninfected sections were negative for the presence of LANA expression (Fig.11 C).

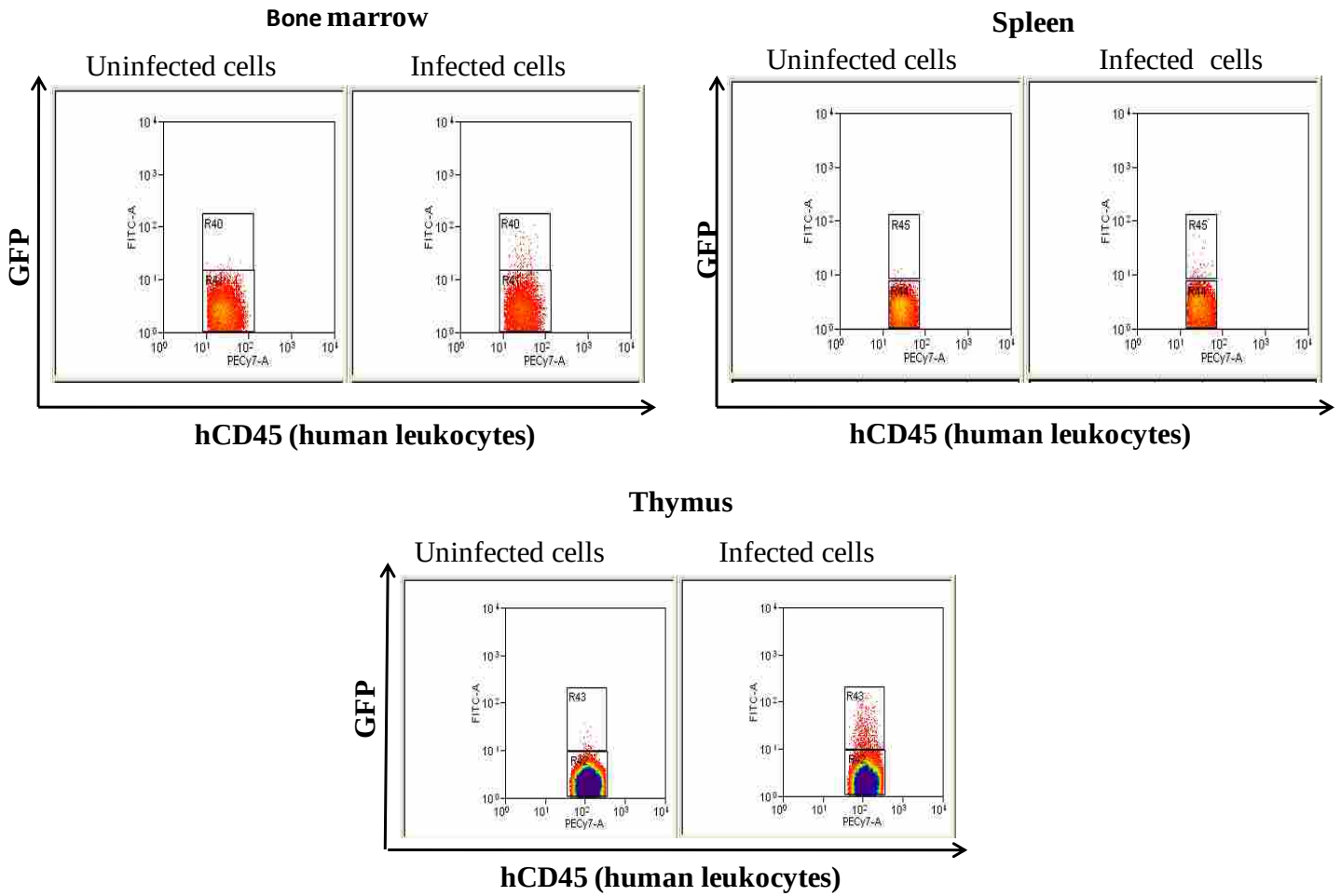

Figure 9. Human cells differentiated in humanized Rag2$^{-/-}$ γc$^{-/-}$ mice are susceptible to infection with rKSHV.219 ex vivo. One reconstituted mouse was sacrificed 10 weeks post transplantation and cell suspensions from bone marrow, spleen, and thymus were collected. Humanized Rag2$^{-/-}$ γc$^{-/-}$ mouse-derived human leukocytes (hCD45^{+}) were challenged with rKSHV, and examined for GFP expression (indicative of successful infection) by flow cytometric analysis. A representative FACS analysis indicates the presence of GFP^{+} cells in infected samples from all three organs. Samples from an uninfected mouse (control) do not display positively stained GFP cells.

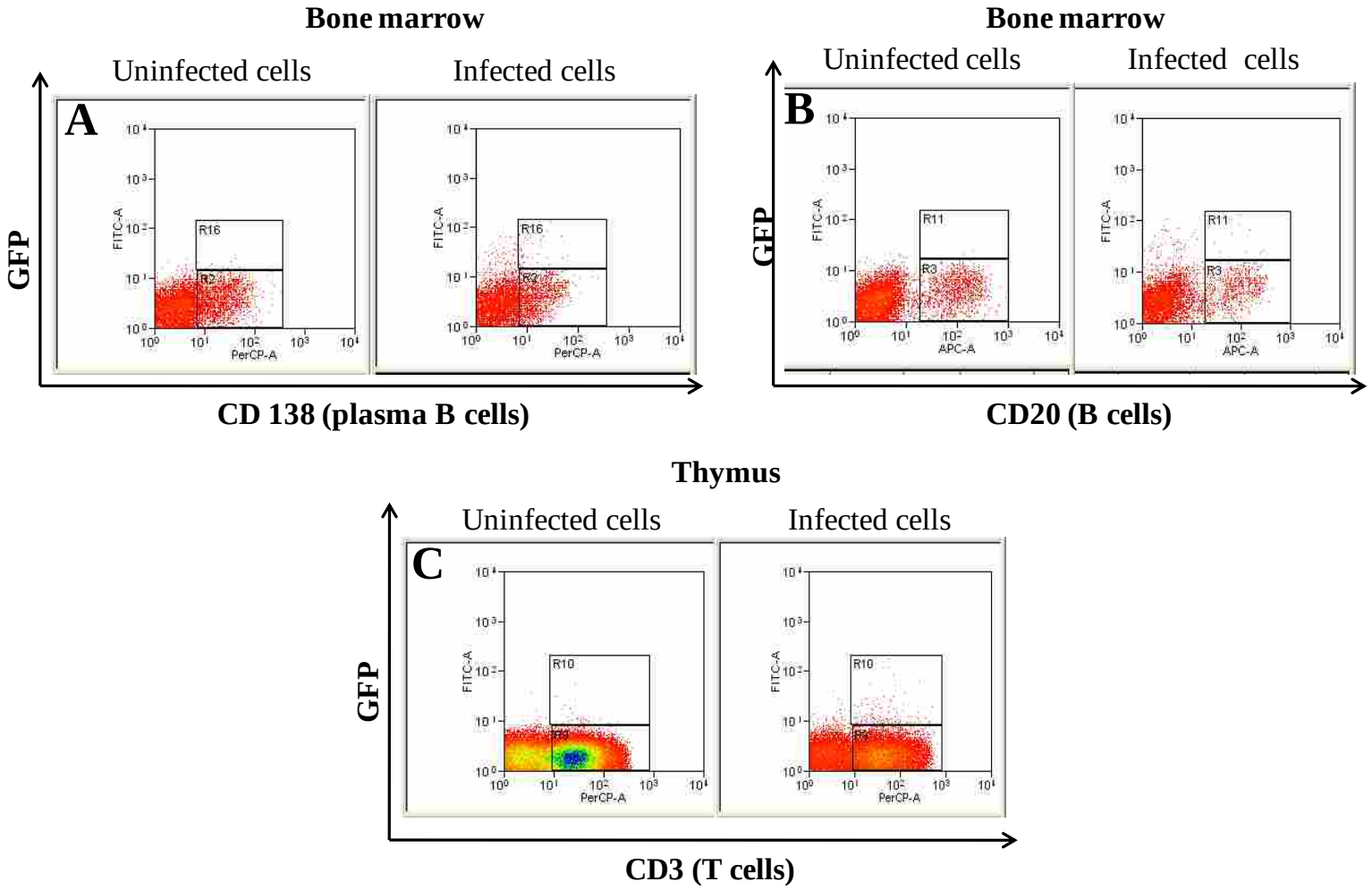

Figure 10. Suceptibility of humanized mouse-derived B and T lymphocytes to rKSHV.219 ex vivo. Cell suspensions from bone marrow, spleen, and thymus were exposed to rKSHV and analyzed by FACS staining to determine the phenotype of susceptible cells. Representative FACS plots of cells positively labeled with antibodies directed against CD20 and CD138 (A and B). Representative scatter plot of GFP^{+} cells versus T cells(CD3)(C).

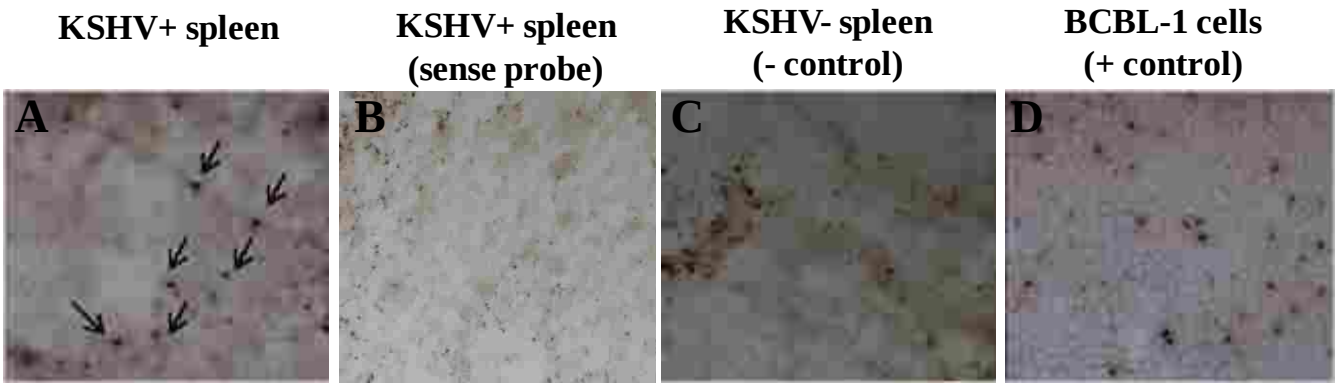

Figure 11. Detection of LANA mRNA expression in the spleen of a infected humanized Rag2$^{-/-}$ γc$^{-/-}$ mouse by in situ hybridization. (A) KSHV infected mouse was sacrificed 6 months post infection and spleen was collected. Sectioned tissue samples were probed for KSHV-LANA using digoxigenin-labeled antisense probes by in situ hybridization. Arrows point to positively stained cells, indicative of LANA mRNA expression. (B) Infected tissue section probed with a sense probe is shown as a negative control. (C) Uninfected tissue sections are shown as a negative control. (D) KSHV-positive human tumors (BCBL1) were used as a positive control for the expression of LANA transcripts.

CONCLUSION

Previous attempts to study KSHV infection and pathogenesis in humanized mice have shown limited success. SCID-hu-PBL mice showed propagation of KSHV-transformed cells with the consequent formation of tumors, but these mice are not susceptible to in vivo infection (Staudt, Kanan et al. 2004). The absence of infection may be due to the lack of human B cells in this model. KSHV inoculation of SCID-hu Thy/Liv mice can initiate a productive infection (Dittmer, Stoddart et al. 1999). Latent and lytic KSHV transcripts were detected in human B lymphocytes in these mice, although lymphoid depletion or lymphoproliferation were difficult to monitor because of the rarity of the B cell population in this model. Additionally, the deficiency of human B lymphocytes makes this model incapable of mounting an effective adaptive immune response against viral infection.

In this study, we report the use for the first time of $Rag2^{-/-}$ $\gamma c^{-/-}$ mice reconstituted with human $CD34^{+}$ cell as a model for the study of KSHV infection. The results presented here show that these mice are susceptible to KSHV infection ex vivo and in vivo, indicated by the following factors. 1) The expression of GFP in rKSHV.219 infected cells obtained from bone marrow, spleen, and thymus of reconstituted $Rag2^{-/-}$ $\gamma c^{-/-}$ mice. KSHV infected cells were predominantly B lymphocytes, similar to what is observed in the human tropism of KSHV. 2) The detection by in situ hybridization of KSHV mRNA in the spleen of a humanized mouse 6 months post infection, accompanied by detection of viral genome, at 9 months post-infection (data not shown), in spleen demonstrates that humanized mice are fully capable of supporting persistent KSHV infection faithfully recapitulating what is seen in $KSHV^{+}$ patients.

As previously mentioned, in KSHV infection, latently infected cells represent a reservoir from which progeny virus is constantly disseminated and transmitted between hosts. An ideal

animal model will recapitulate a persistent infection, which is required for development of KSHV-associated cancers. Currently, there is not enough information about KSHV persistence in vivo including the cell types acting as reservoirs of viral infection. In addition, it is know that in vivo lytic replication in KSHV is mediated by the expression of the immediate early gene RTA; however the cellular stimuli responsible of lytic reactivation are not well understood. Finally, antiviral drugs designed to target KSHV replication are not capable of eliminating persistent KSHV. Thus, the observation that KSHV-infected humanized Rag2$^{-/-}$ $\gamma c^{-/-}$ mice develop a long-term infection can help to discover the molecular mechanisms leading to KSHV persistence. This will greatly contribute in finding ways to revert latent infection, facilitating the prevention or treatment of KSHV-associated diseases.

We are currently establishing various additional experiments to further confirm the results described in this study and to more precisely evaluate the susceptibility of Rag2$^{-/-}$ $\gamma c^{-/-}$ mice to KSHV infection as well as the state of infection. A larger sample of Rag2$^{-/-}$ $\gamma c^{-/-}$ mice including the appropriated control groups will be inoculated with rKSHV. Quantitative PCR (Q-PCR) will be included as part of the assays for detection and quantification of KSHV DNA in peripheral blood and lymphoid tissues. Also, lymph nodes, and bone marrow will be analyzed by in situ hybridization and immunohistochemistry for the expression of KSHV latent and lytic transcripts, and protein patterns, respectively.

CHAPTER 3

Antiviral efficacy of the D-peptide HIV-1 Entry Inhibitor chol-PIE12-trimer in humanized Rag2$^{-/-}$ γc$^{-/-}$ mice

INTRODUCTION

Human immunodeficiency virus type 1: Virus structure, genomic organization, and protein function

Human Immunodeficiency Virus Type 1 (HIV-1), the causative agent of Acquired Immune Deficiency Syndrome (AIDS), is a member of the family Retroviridae, genus Lentivirus. Retroviruses utilize a virus-encoded enzyme, the reverse transcriptase, to convert viral RNA into linear double stranded DNA (proviral DNA) which later during the virus life cycle is integrated in the host genome (Goff 1990). HIV-1 is an enveloped virus with a diploid single-stranded RNA (5' capped and 3' polyadenylated) genome of approximately 9.7 Kb in length. Both ends of the viral genome are flanked by regulatory regions known as long terminal repeats, which are important for transcription initiation and polyadenylation (Figure 12). HIV-1 contains nine genes encoding fifteen proteins. Three of these genes are referred to as structural genes (*Gag, Pol, Env*), two are known as regulatory (*Tat* and *Rev*) and the other four (*Nef, Vpr, Vpu, Vif*) are designated as accessory genes (Figure 12) (Sierra, Kupfer et al. 2005).

The *Gag* gene gives rise to the polyprotein precursor p55. Shortly after virus budding, p55 is cleaved by the viral protease into the mature structural proteins: matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7), and p6 (Fig. 12). These proteins constitute the viral nucleocapsid or core (Henderson, Sowder et al. 1990; Freed 1998; Adamson and Freed 2007). In the mature viral particle, MA remains associated to the inner surface of the viral membrane, providing stability to the virus (Fig.13). During the viral life cycle, MA participates in important steps in virus replication. For instance, MA is involved in the incorporation of the

envelope glycoprotein into virions, in virus assembly and budding, and in the nuclear transport of the viral genome. This last feature allows HIV infection of nondividing cells (Bukrinskaya 2007). The CA protein forms the core of viral particles and protects the genomic RNA (Fig.13). CA promotes virus assembly and maturation. It has been observed that CA mediates the incorporation of the host protein Cyclophilin A into HIV particles, which may assist in proper folding or disassembly (Freed 1998). NC (Fig.13) mediates the encapsidation of the genomic RNA into virions, thus protecting it from nucleases. NC has chaperone activity that facilitates efficient reverse transcription. It has been determined that NC also plays a role in *Gag* multimerization (Frankel and Young 1998). The p6 polypeptide region of the p55 (Fig.13) is important for the efficient release of budding virions from infected cells. It also mediates the internalization of the accessory protein Vpr into the assembled virion (Frankel and Young 1998).

The *pol* gene corresponds to the genomic region encoding the viral enzymes protease (PR), reverse transcriptase (RT), and integrase (IN) which are encapsulated within the viral particle (Fig.13). These enzymes are the product of the Pol domain within the Gag-Pol polyprotein (Fig. 12), which during viral maturation is processed by the viral PR (Darke, Nutt et al. 1998; Louis, Weber et al. 2000). RT has RNA-dependant and DNA-dependant polymerase activity. Early during the infection process, RT catalyzes the synthesis of a double-stranded DNA from the single-stranded genomic RNA. RT has an RNase domain that degrades the RNA strand of the RNA-DNA hybrid generated during reverse transcription. HIV-1 replication is error-prone due to the absence of proof-reading activity by RT; consequently, the rate of variation among HIV-1 populations is high (Kati, Johnson et al. 1992). IN mediates the integration of the HIV proviral DNA into the host chromosome. IN has exonuclease activity, it removes two nucleotides from the 3’ ends of the linear double-stranded viral DNA. Additionally,

IN cleaves via endonuclease activity the cellular DNA at the integration site, and the 3' processed ends of the viral DNA are covalently joined to the cleaved 5' end of the cellular chromosome (Delelis, Carayon et al. 2008). The integrated provirus is maintained within the host genome for the life time of the infected cell, serving as a template for further production of new viruses.

Mature HIV virions are surrounded by a lipid membrane of cellular origin that includes approximately 72 glycoprotein complexes. Each complex of glycoprotein is constituted of non-covalently associated trimers of the surface glycoprotein gp120 and the transmembrane glycoprotein gp41 (Fig. 13). These complexes are encoded by the *Env* gene as the precursor gp160 (Fig.12) (Zhu, Chertova et al. 2003). gp120 mediates the interaction between HIV and the cell receptor CD4 and the coreceptor CCR5 or CXCR4, whereas, gp41 promotes the fusion of the viral envelope and the cell plasma membrane.

In addition to the structural proteins, the genome of HIV-1 codes for regulatory and accessory proteins, which are important during the early stages of infection and are vital for effective viral replication. The regulatory proteins Tat and Rev (Fig.12) are RNA binding proteins localized in the nucleus of infected cells. Within the nucleus, Tat and Rev interact with determined regions of the viral RNA required for transcription and translation. Tat is a transcriptional transactivator that binds to a stem loop structure known as TAR (transactivation-response elements) located within the 5' end of all viral transcripts (Knipe and Howley 2007). It has been demonstrated that Tat enhances the elongation phase during transcription at least a hundred times, thus helping in the production of full length transcripts. In the absence of Tat, the level of progeny virus is undetectable due to the generation by HIV of only premature transcripts (Feinberg, Baltimore et al. 1991). The second regulatory protein, Rev, binds to the RRE (Rev

Response Element) structure found in all incompletely spliced viral transcripts (unspliced and partially spliced mRNAs). Through this interaction, Rev assists in the nuclear export of unspliced (Gag and Pol) and partially spliced (Env, Vpr, Vpu, and Vif) viral mRNA to the cytoplasm where they are translated into viral proteins necessary for the formation of mature viral particles (Emerman and Malim 1998; Pollard and Malim 1998; Kimura, Hashimoto et al. 2000).

Several studies in tissue culture and in vivo have shown that the HIV-1 accessory proteins (Vif, Vpr, Vpu, and Nef) (Fig.12) have critical roles in the virus life cycle and are required for evasion of antiviral defense, and disease progression in vivo (Knipe and Howley 2007). The virus infectivity factor (Vif) protein is best known for inhibiting the antiviral activity of the intracellular protein apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (APOBEC3G), a member of a family of intracellular enzymes with cytidine deaminase activity (Mangeat, Turelli et al. 2003; Sheehy, Gaddis et al. 2002). This observation came from the finding that Vif-deficient viruses produced from non-permissive cells were non-infectious, suggesting that non-permissive cells contained a cellular factor capable of blocking productive infection in the absence of Vif (Simon, Gaddis et al. 1998; Madini and Kabat 2000). The conclusion of the antagonizing activity of Vif against an antiviral cellular factor was also supported by studies showing that Vif function from different lentiviruses is species-specific.

For instance, HIV-1 Vif does not have inhibitory activity against APOBEC3G from murine cells (Madini and Kabat 2000). The inhibition of APOBEC3G antiviral activity is mediated through direct interaction with Vif, resulting in the degradation of APOBEC3G by the ubiquitin-proteosome system. This mechanism of viral inhibition prevents the incorporation of

APOBEC3G into newly formed viral particles, which facilitate the synthesis of proviral DNA in subsequently infected cells (Sheehy, Gaddis et al. 2003).

Vpr is a small protein (14KDa) that is incorporated into mature viral particles released from infected cells. Thus, Vpr is present during the early steps of infection. However, it is also expressed late during the virus life cycle. Early during infection, Vpr participates in the nuclear import of the viral pre integration complex (PIC) in non-dividing cells (e.g., resting T cells and differentiated macrophages) (Heinzinger, Bukinsky et al. 1994; Fassati 2006). Additionally, different studies have indicated that Vpr plays a potential function in the initial steps of the reverse transcription process, as well as in the modulation of virus mutation rate, by influencing the fidelity of the reverse transcription process (Frankel and Young 1998; Stark and Hay 1998; Le Rouzic and Benichou 2005). A major biological function of Vpr is its ability to induce cell cycle arrest of infected cells during the G2 phase (Zhao, Mukherjee et al. 1994). Since the levels of transcription and translation of viral genes are high during the G2 phase, it has been proposed that the mechanism of cell arrest by Vpr may contribute to the efficient transcription of HIV-1 and the enhancement of virus production (Emerman and Malim 1998; Goh, Rogel et al. 1998; Le Rouzic and Benichou 2005). In addition to G2 arrest, Vpr also induces apoptosis and the eventual depletion of CD4 T cells.

Vpu is a membrane-associated phosphoprotein expressed late during virus replication and found in the endoplasmic reticulum (ER). In vitro replication studies using HIV-1 clones lacking a functional vpu gene revealed two main functions for Vpu. It is important for modulation of CD4 expression on the surface of infected cells and efficient virus release. In the ER, Vpu interacts with newly synthesized CD4 which is then targeted for ubiquitin-proteasome mediated degradation. This mechanism facilitates the transit of Env to the cell surface for assembly into

HIV-1 virions. In addition to its role in the downregulation of CD4, Vpu is involved in virus particle release through the formation of ion channels in the plasma membrane (Knipe and Howley 2007).

Nef was initially defined as negative factor because of the assumption that it was involved in inhibition of viral replication, although extensive studies have indicated that Nef is an important determinant for effective HIV-1 replication and pathogenesis in vivo. One such study demonstrated that individuals infected with an HIV-1 strain carrying a deletion in the Nef gene do not show the classic manifestations of AIDS, and only a slight reduction in the levels of CD4 was observed several years post infection in such patients (Learmont, Geczy et al. 1999).

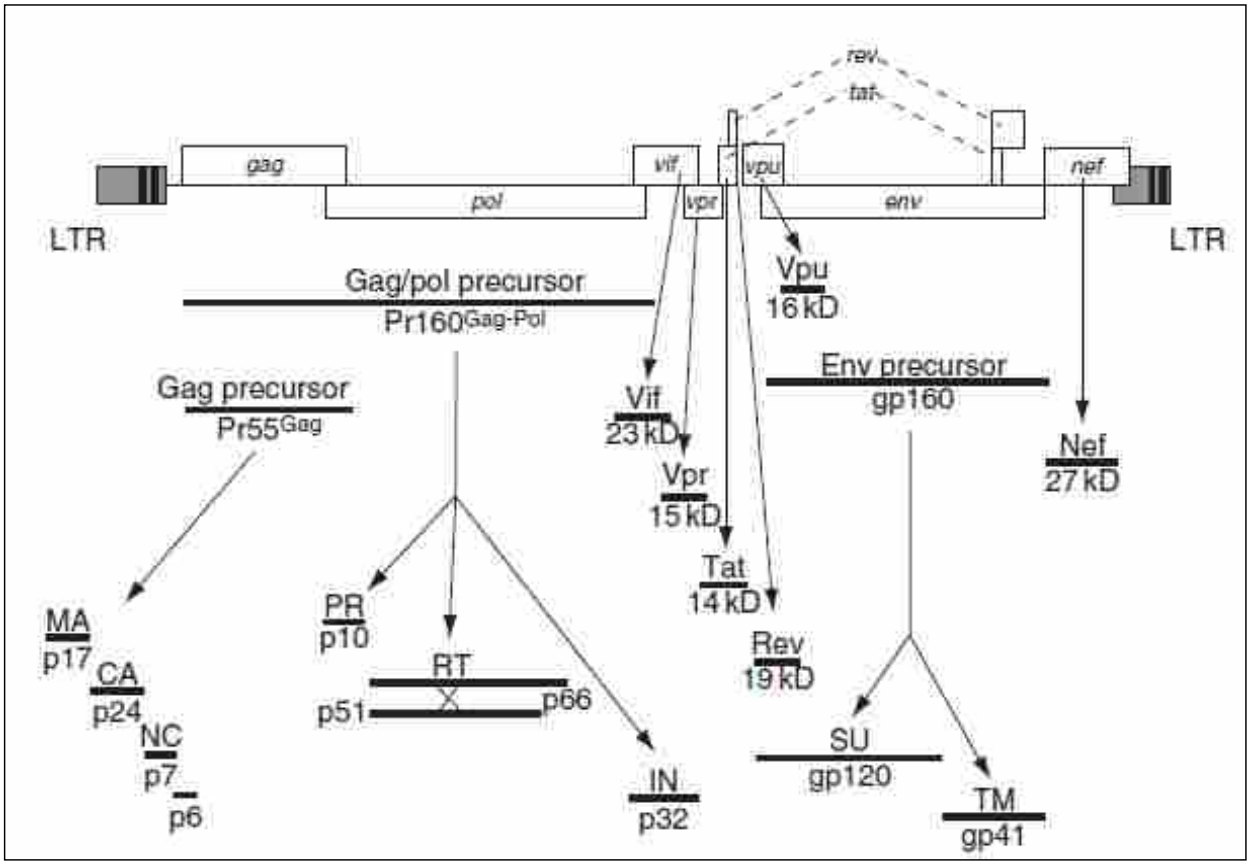

Figure 12. HIV-1 genome with encoded proteins.

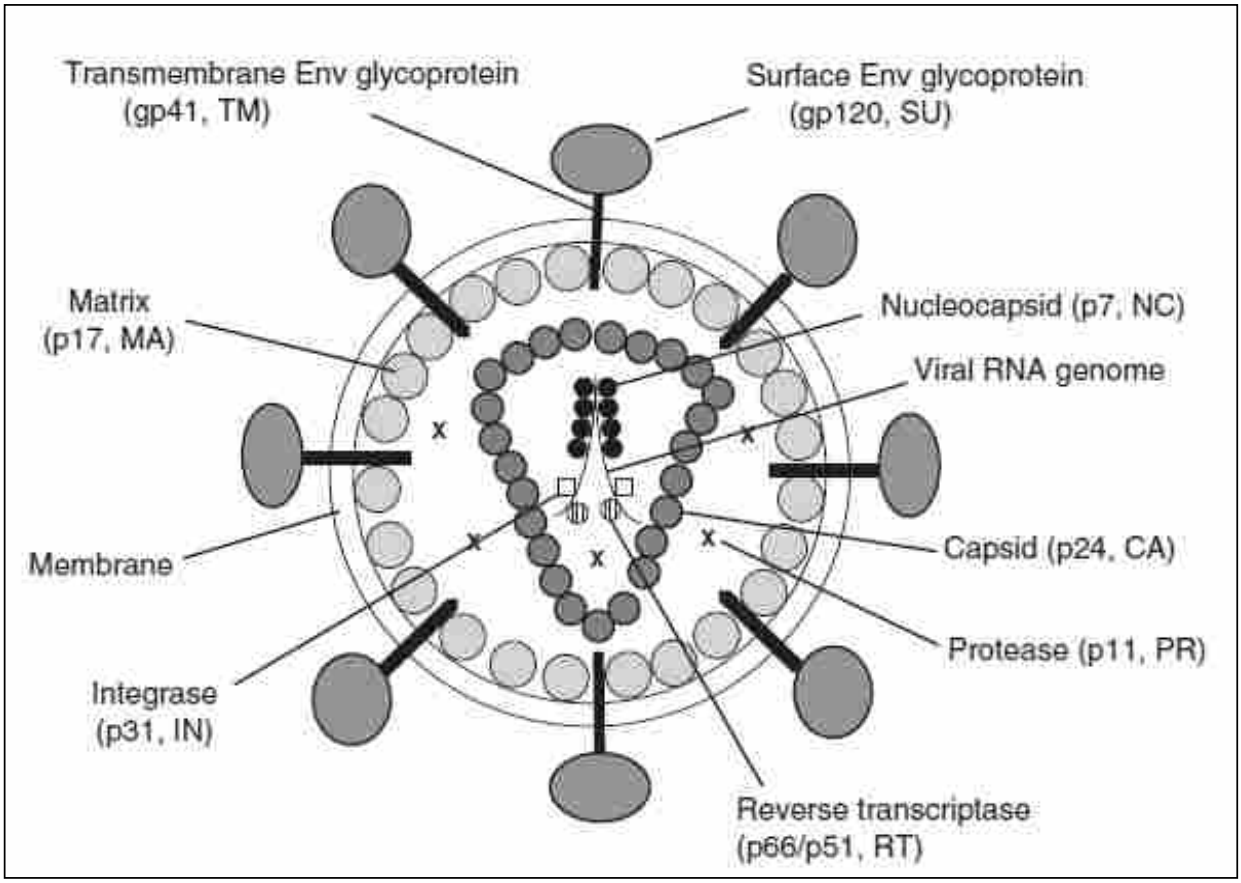

Figure 13 Schematic representation of a mature HIV-1 virion (Freed. 2002).

One of the most significant ways in which Nef contributes to HIV-1 pathogenesis is the downregulation of CD4 and MHC class I (MHC-I) from the cell surface of infected cells. Even though CD4 is important for HIV-1 entry into target cells, the continued expression of this receptor can also interfere with proper viral budding and release. Thus, HIV-1 Nef mediates CD4

degradation into lysosomes, ensuring a controlled and productive infection (Lindwasser, Chaudhuri et al. 2007). A strategy used by HIV-1 to prevent recognition and lysis of infected cells by cytotoxic T lymphocytes is the reduction of cell surface expression of MHC-I. At the same time, to protect infected cells from NK cells-mediated lysis, Nef specifically affects a subset of MHC-I molecules (i.e. HLA-A and HLA-B) (Swigut, Alexander et al. 2004; Carl, Greenough et al. 2001). In addition to the biological functions mentioned above, Nef also stimulates the activation of T lymphocytes, making them more susceptible to infection. Additionally, Nef prevent apoptosis by interrupting the p53-mediated apoptosis pathway (Knipe and Howley 2007).

Phylogenetic studies of isolates collected from different locations around the world have permitted the classification of HIV-1 into three major groups: M (main), O (outlier/other), and N (non-M, non-O), with the M group being the most predominant worldwide (95% of the global virus isolates) and responsible for the majority of the HIV-1 infections (McCutchan 2000). HIV-1 M viruses are classified into 9 subtypes or clades (A-D, F-H, J, and K). Subtype B is dominant in the United States, Europe, and South America; subtype C is found throughout the world, especially in South Africa and India (Brodine, Mascola et al. 1995; Janini, Tanuri et al. 1998). Evidence derived from molecular epidemiologic data has demonstrated that HIV-1 was originally transmitted to humans from a chimpanzee virus closely related to HIV-1(simian immunodeficiency virus-SIV), presumably within the groups M and N. Viruses from these two

groups have been identified in chimpanzees in certain regions of Africa (Hahn, Shaw et al. 2000; Apetrei, Robertson et al. 2004)

HIV-1 life cycle

Virus Entry: Infection of target cells by HIV-1 begins with a multistep process consisting of virus attachment to cell membrane, coreceptor binding, and membrane fusion (Fig.14). Attachment of HIV-1 is mediated by the viral surface glycoprotein gp120, which interacts with the cell surface receptor CD4 (the major receptor of HIV-1), expressed mainly on T cells and monocytes /macrophages. Structural studies have determined that the region on gp120 responsible for binding to CD4 is located in its conserved domains C3 and C4, which constitute the core of gp120 (Sierra, Kupfer et al. 2005). This initial interaction between gp120 and CD4 triggers critical conformational changes in gp120, leading to the attachment of the viral glycoprotein with the host chemokine receptor CCR5 or CXCR4, the primary coreceptors for HIV-1. Binding of the HIV-1 envelope to the cellular co-receptor is mediated by the interaction of the variable domain 3 (V3-loop) of gp120 with the N-terminus of CCR5 or CXCR4 (Carter and Ehrlich 2008). Several observations have revealed that the conformational features of the V3-loop permit this domain to mimic the natural ligands of CCR5 and CXCR4, providing to HIV-1 the capacity for coreceptor choice and binding (Sharon, Kessler et al. 2003; Galanakis, Spyroulias et al. 2005; Sharon, Rosen et al. 2005).

Fusion of HIV-1 envelope with the cell membrane is mediated by the gp41 transmembrane subunit of the viral envelope. gp41 contains an ectodomain composed of a hydrophobic N-terminal fusion peptide and two heptad-repeat motifs. These regions are important for the proper fusion process. The binding of gp120 with cellular coreceptor promotes the exposure of the fusion peptide of gp41 and its insertion towards the cell membrane

(Markovic and Clouse 2004). Following penetration of the fusion peptide into the host membrane, the heptad-repeat domains of gp41 fold over onto each other in an antiparallel sense, forming a stable six-helix bundle structure. This conformational change permits the fusion of the viral and cell membranes, allowing the viral capsid to be delivered into the cytoplasm (Fig.14) (McManus and Doms 2000; Pierson and Doms 2003).

Reverse transcription: In the next stage of infection, the viral capsid is uncoated and the viral RNA genome is reverse-transcribed into a full-length double-stranded DNA by the virus-encoded reverse transcriptase (RT) (Fig.14). The HIV-1 RT has polymerase activity which allows the enzyme to synthesize copies of nucleic acid strands from either an RNA or DNA template. Additionally, RT poses RNase H activity that permits the degradation of the genomic RNA after DNA synthesis (Jonckheere, Anne et al. 2000). Similar to other DNA polymerases, HIV-1 RT requires a primer to achieve the synthesis of DNA. Thus, reverse transcription of the viral RNA genome is initiated by extending a tRNA primer provided by the host cell. The cellular tRNA is annealed to the primer binding site (PBS) located near the 5' end of the viral genome (Jiang, Mak et al. 1993). The PBS is an 18-nucleotide sequence complementary to the 3' end of the tRNA. Once RT binds to the tRNA, the synthesis of a minus-strand DNA continues toward the 5' end of the viral RNA genome, giving as a product an RNA/DNA hybrid. The RNA strand from the hybrid formed at the 5' end is later removed by the action of RNase H, generating a single stranded cDNA. This single-stranded DNA is then transferred from the 5' end of the viral genome to the 3' end where it binds to the repeat sequence R and the minus-strand DNA extends along the RNA template. As minus-strand DNA synthesis is completed, RNase H degrades the RNA strand (Sarafianos, Marchand et al. 2009).

Synthesis of the plus-strand DNA initiates near the 5' end of the minus-strand DNA. At this site, RT uses as primer for second DNA strand synthesis an RNA oligonucleotide sequence of approximately 10 nucleotides called the polypurine tract (PPT). This RNA region is a remnant of the viral RNA template that is resistant to RNase H degradation. Plus-strand DNA synthesis then continues to the end of the minus strand. At this stage of DNA synthesis, the newly synthesized fragment of plus-strand DNA "jumps" to the 3' end of the minus-strand DNA. There it binds to the PBS sequence which is complementary to the 3' end of the plus-strand DNA. Synthesis of plus-strand DNA is culminated and a full-length double-stranded viral DNA (proviral DNA) containing LTR regions at both ends is produced (Fig.14) (Sarafianos, Marchand et al. 2009).

Integration: After reverse transcription, the proviral DNA is transported to the nucleus of the infected cell where it is integrated into the host genome by the viral enzyme IN (Fig.14). The integration process is initiated by the removal of two or three nucleotides from the 3' end of both strands of the viral DNA. This initial step called 3' processing generates a double-stranded DNA molecule with 3' recessed ends (precursor ends for integration). Additionally, IN also processes the cellular DNA by cleaving it at the integration site. Thus, through a catalytic activity referred to as strand transfer, both ends of the viral DNA can be covalently joined to the cleaved 5' ends of the host genome. This integration process is finally completed when cellular enzymes repair the gaps originated between the integrated provirus and the host chromosome (Engelman, Mizuuchi et al. 1991; Delelis, Carayon et al. 2008). The integrated provirus is maintained in the host chromosome for the life-time of the infected cell. There it serves as a template for the generation of viral transcripts and the replication of viral RNA genome, making possible the production of new viruses.

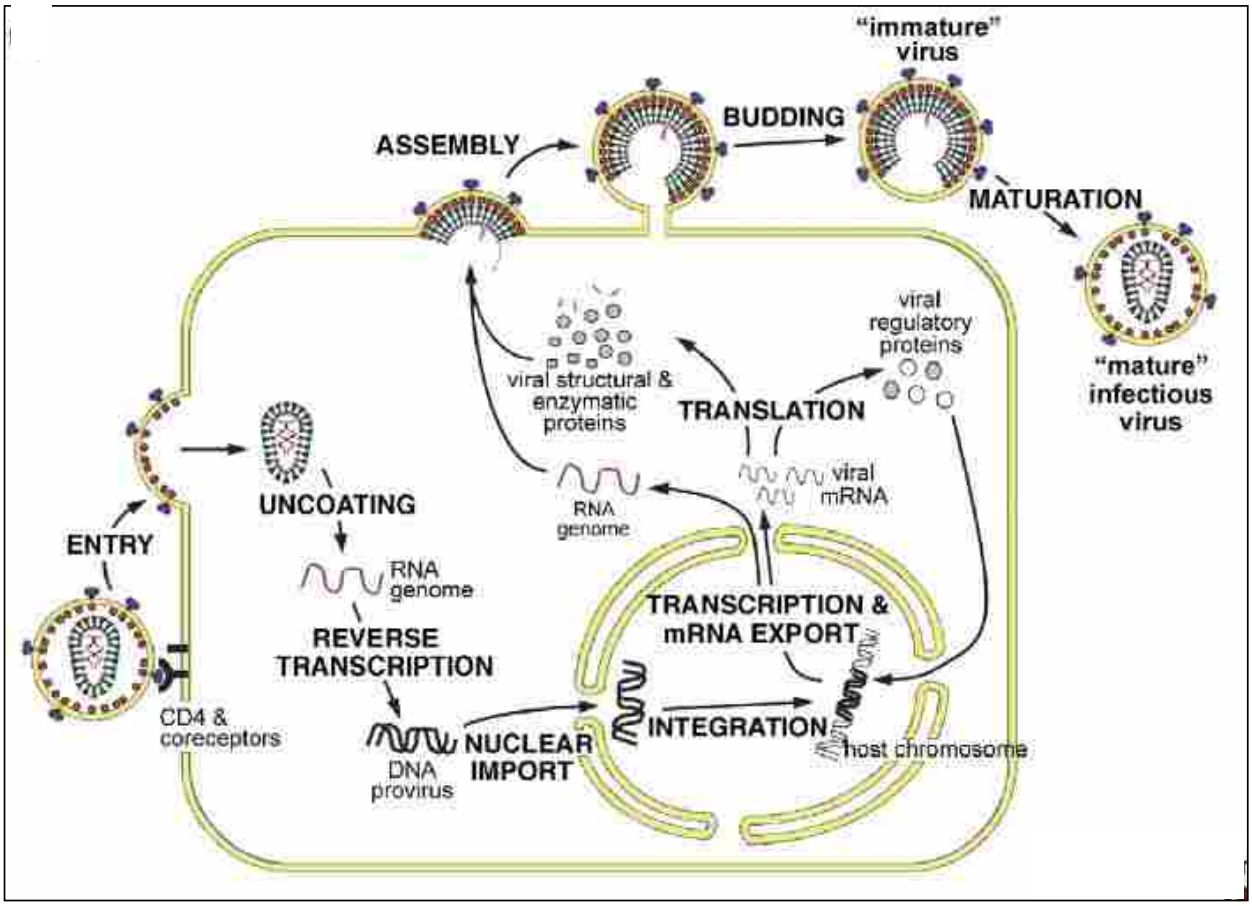

Figure 14. The life cycle of HIV-1 (Ganser-Pornillos et al. 2008).

Transcription and translation: In general, the initiation of proviral DNA transcription (Fig.14) is a process governed by host chromatin-associated proteins and viral proteins. The LTR promoter of the HIV provirus contains several regulatory elements (located within the U3 region) necessary for viral RNA synthesis by the host cell-encoded RNA polymerase II. The proper transcriptional activity is coordinated by the virus-encoded Tat protein. As mentioned previously, Tat stimulates transcription by interacting with the RNA sequence TAR present at the 5'end of all viral transcripts, thus enhancing the synthesis of viral RNA. (Marcello, Zoppe et al. 2001; Pumfery, Deng et al. 2003). Transcription of proviral DNA leads to the production of different viral transcripts which can be classified as completely spliced, partially spliced, and unspliced mRNAs.

Completely spliced mRNAs are processed before export to the cytoplasm, where they serve as template for the translation of the early regulatory proteins Tat and Rev and the accessory protein Nef. The partially spliced mRNAs encode the accessory proteins Vif, Vpr, Vpu, and the envelope glycoprotein precursor gp 160. Unspliced transcripts are templates for the synthesis of the Gag and Gag-Pol polyprotein precursors (Fig. 14). They can also be packaged into progeny viruses to be used as genomic RNA. The incompletely spliced viral mRNAs are exported to the cytoplasm with the assistance of the viral protein Rev, thus preventing nuclear retention and further degradation. Rev achieves the nuclear export of intron-containing viral transcripts by interacting with RRE, a secondary structure present within the 3' end of both partially and unspliced viral mRNAs (Pollard and Malim 1998; Kjems and Askjaer 2000). Thus, HIV gene expression includes an early step where the expression of completely spliced mRNAs stimulates a high level production of the structural proteins originated from partially and non-spliced transcripts. The second phase provides the structural proteins and viral enzymes required

for production of new viral particles. These viral proteins as well as full-length viral genomic RNA are taken to the plasma membrane for virus assembly (Fig.14).

Virus Assembly, release and maturation: Shortly after its synthesis, the HIV-1 structural precursor plyprotein Gag (Pr55) is conducted by its N-terminal (Matrix) domain to associate with the plasma membrane of the infected cell (Fig.14). For this interaction to take place, myristoylation of the matrix domain of Gag is necessary. At the membrane, Gag plays a central role in directing assembly of HIV-1 virions. This protein promotes viral core formation, genomic RNA encapsidation, associates with envelope glycoproteins during budding, and assists in the acquisition of a lipid bilayer for immature virions. These functions are mediated through important interactions (protein-protein, protein-RNA, protein-lipid interactions) between the different domains within Pr55. These interactions are essential for the proper assembly and maturation of HIV-1 (Freed 1998).

As noted above, the N-terminal domain of Gag is responsible for leading this polyprotein to the host cell membrane. Through a multimerization process, the central (capsid) domain of Gag facilitates Gag-Gag interactions important for particle morphology formation during virion assembly. The nucleocapsid (NC) domain of Pr55 is involved in the encapsidation of the full-length genomic RNA into virus particles. Packaging of viral RNA is mediated by direct interaction of NC with a cis-acting sequence (the packaging signal) located between the 5' LTR and the Gag gene. Finally, virus release from the plasma membrane is facilitated by the p6 domain of Gag located at its C-terminus. p6 stimulates virus release by interacting with host proteins responsible for the budding of vesicles into late endosomes (Adamson and Freed 2007).

After the assembly of newly produced viral proteins, genomic RNA, and lipids into immature noninfectious viral particles, HIV-1 virions bud from the surface of infected cells

(Fig.14). Later during these final steps of the HIV-1 replication cycle, the viral enzyme PR is activated and initiates the process of virion maturation (Fig.14). Activated PR cleaves the Gag and Gag-Pol polyprotein precursors into their corresponding domains. Subsequently, these processed proteins reassemble to form the different inner membranes present in a mature virus particle containing a condensed, conical core (Adamson and Freed 2007; Ganser-Pornillos, Yeager et al. 2008). Embedded in the envelope of infectious virus particles are the gp120/gp41 complexes responsible for cell attachment during the next round of infection.

The primary cellular targets of HIV-1 are $CD4^+$ T helper cells, monocytes, macrophages and dendritic cells. As noted earlier, these cells express the surface receptor CD4 and the co-receptor CCR5 and/or CXCR4 required by HIV-1 for effective fusion and entry into target cells. HIV-1 strains with tropism for CCR5 are designated as R5 viruses, while HIV-1 strains using CXCR4 are referred to as X4 viruses (Clapham and McKnight 2001). CCR5 is preferentially expressed on memory $CD4^+$ T cells (the preferred target of HIV), while CXCR4 is expressed on memory and naïve $CD4^+$ T cells. Only 5-10% of the helper T lymphocytes in the peripheral blood express CCR5, while between 80 and 90% express CXCR4 (Lee, Sharron et al. 1999; Moore, Kitchen et al. 2004). Viral transmission mostly occurs from R5 viruses and this phenotype remains during the acute phase of infection. However, during the chronic phase of infection, X4 strains can be encountered in approximately 40% of infected individuals (Connor, Sheridan et al. 1997).

HIV-1 transmission

The transmission of HIV occurs mainly by sexual contact when mucosal membranes are exposed to contaminated body fluids (i.e., blood, semen, vaginal secretions). This type of transmission can occur by either heterosexual or homosexual intercourse. According to

estimations by the United Nations for 2010, the sexual transmission of HIV-1 accounts for more than 80% of new HIV infections worldwide. Social and cultural behaviors can influence (enhance) sexual transmission. Such behaviors include: co-infection with other sexually transmitted viruses (e.g., herpes), unsafe sexual intercourse with an HIV-positive partner or a partner of unknown HIV serostatus, and mucosal trauma (Royce, Sena et al. 1997). It has also been determined that the risk of sexual transmission has a direct correlation with the viral load in peripheral blood. Transmission is less probable in persons with a viremia below 1,500 copies of viral RNA molecules per ml of blood (Quinn, Wawer et al. 2000).

Infants are at risk of contracting HIV through vertical transmission (i.e., before birth, during delivery, or after birth through breastfeeding) from infected mothers. Transmission from mother to infant accounts for 90% of all HIV-1 infections in children. Similar to what is observed in sexual transmission, there are different mother-related factors implicated in the increased possibilities for vertical transmission to occur. Among them are, high viral load, mode of delivery, advanced disease status, and drug abuse (Scarlatti 2004; Ahmad 2005). During the past decade, a great improvement has been made in reducing the rate of HIV-1 transmission from mother to child. This has been possible due to the opportune administration of combined antiretroviral therapies to HIV-1 positive pregnant women. Additionally, post exposure prophylaxis strategies have been implemented to assist the newborn infants infected with HIV-1 postpartum (Horvath, Madi et al. 2009; Sturt, Dokubo et al. 2010).

It is estimated that of the total HIV positive population, approximately 3 million people are injection drug users. This mode of transmission is greatly contributing to global mortality and morbidity due to HIV-1 infection. HIV-1 transmission among intravenous drug-users occurs mainly because of the exchange of contaminated blood when sharing needles and other injection

supplies. (Des Jarlais, Arasteh et al. 2009). Other modes through which HIV-1 is transmitted include needle sticks with a contaminated needle and transfusion with contaminated blood.

HIV-1 infection and pathogenesis

HIV establishes a productive and lifelong infection which, if not treated appropriately, leads to faster disease progression and the onset of AIDS (Pantaleo, Cohen et al. 1998). Plasma viral load, depletion of CD4 T lymphocytes (less than 200 cells/ µl of blood) and loss of effective immune responses reflected by the emergence of opportunistic infections and/or malignancies, have been defined as indicators of HIV infection and progression to AIDS in humans (Mellors, Munoz et al. 1997). During the first phase of viral infection (primary or acute infection), over 80% of infected individuals exhibit nonspecific symptoms that appear within the first three to six weeks post-infection. Symptoms are characterized by fever, headache, fatigue, malaise, myalgias, generalized lymphadenopathy, diarrhea, pharyngitis, and weight loss. This symptomatic time is followed by an asymptomatic period of several years. In primary infection, the levels of virus in peripheral blood and lymphoid tissue are very high. Viral load can go up to 10^6-10^7 RNA copies /ml of plasma. This high titer of viremia is accompanied by a decline in the number and function of $CD4^+$ T lymphocytes. Within the first 2 to 6 months of acute infection, the plasma viral load declines (a mean of 30,000 copies/ml), remaining on a relatively stable level. This reduction in viral load evidences the onset of an active immune response during the early phase of disease, which may be explained in part by the sudden increase of circulating cytotoxic T cells and the production of anti-HIV-1 neutralizing antibodies (Koup, Safrit et al. 1994; Musey, Hughes et al. 1997; Wei, Decker et al. 2003). The acute HIV infection is followed by a period of chronic disease, called clinical latency, which can persist for several years. Chronic infection is characterized by a progressive loss of CD4 lymphocytes (50-100 cells/µl), a

variety of systemic manifestations affecting almost every organ, and neurological disorders (Phillips., 1992; Iuliano et al., 1997). The last stage is characterized by the emergence of AIDS-associated diseases manifested by the development of opportunistic infections and HIV-associated malignancies in untreated patients, who will eventually die.

Almost 60 million people have been infected with HIV-1 and more than 25 million have died since the beginning of the AIDS pandemic almost three decades ago. Currently there are over 36 million people worldwide infected with HIV-1 and an estimated 2.7 million individuals newly infected each year, of which 95% live in developing countries (UNAIDS., 2010). The most affected region in the world is sub-Saharan Africa, with almost 22.5 million people living with HIV/AIDS. According to estimations of the United Nations Program on HIV/AIDS for 2010, in Eastern Europe there are 1.4 million infected people and the HIV/AIDS epidemic is currently increasing. In Asia and the Pacific region, an estimated 8 million people are HIV+. The estimated number of individuals infected with HIV-1 in Latin America and the Caribbean is 1.6 million. In North America the number of HIV-infected individuals is 1.5 million with approximately 50,000 new cases each year, from which 42 % are men who have sex with other men and 33% are individuals infected by heterosexual intercourse. Half of the new infections in U.S. are happening in people 25 years-old or younger (Cohen 2010).

Antiretroviral therapy for the treatment of HIV-1 infection

The development of a safe, protective, and affordable vaccine against HIV-1 offers a realistic strategy to bring the pandemic under control. However, despite intensive efforts over the past 25 years, such an accomplishment has proved to be an extremely challenging task. Meanwhile, different strategies exist to meet the urgent need to further prevent HIV-1 infection and the spread of AIDS. Among them, antiretroviral drugs (ARDs) capable of suppressing viral

replication and delaying progression to AIDS have been developed over the past two decades (Mehellou and De Clercq 2010). Currently, 31 ARDs have been approved by the FDA for clinical use. These drugs have been designed to target different steps of the HIV-1 life cycle (Fig.14); thus, they are classified as inhibitors of entry, reverse transcriptase, integrase, and protease (De Clercq 2009).

Current treatment options include the combination of several ARDs, referred to as highly active antiretroviral therapy (HAART). A typical HAART regimen usually includes the administration of three antiretroviral compounds consisting of two forms of inhibitors of reverse transcriptase and either a protease inhibitor or an inhibitor of viral integrase (Zolopa 2010). The main goal of HAART has been to permit a durable suppression of viral replication to undetectable levels (< 50 RNA copies/ml), as well as the recovery of the immune response reflected by the increase of $CD4^+$ T lymphocytes, and to decrease the emergence of HIV-1 drug-resistant strains. In fact, the application of antiretroviral therapy has led to a considerable reduction of morbidity and mortality, with a substantial improvement in life quality among infected patients (Sterne, Hernan et al. 2005; The-Antiretroviral-Therapy-Cohort-Collaboration 2008). Despite the important contributions of HAART in the control of the AIDS pandemic, the number of new infections worldwide continues to increase. Thus, it is evident that current anti-HIV-1 therapies face significant challenges, including the manifestation of long-term toxic side effects, poor adherence to drug regimens by patients, the occurrence and transmission of multidrug resistance viral strains, and limited financial resources to support life-long treatment. For these reasons, the identification of new and potent drugs that target additional steps of the virus life cycle is an urgent need for the treatment of the population suffering from HIV/AIDS.

HIV-1 entry inhibitors

The different proteins both in the virus and in the host cell implicated in the HIV-1 entry process (see above under virus entry) represent potential targets to prevent infection at the initial steps of the virus life cycle. The understanding gained over the past two decades regarding HIV-1 structure and the mechanisms by which the virus penetrates into the host cell has contributed to the development of new antiretroviral drugs, namely entry inhibitors.

HIV-1 entry into target cells is a process that requires attachment to the cell surface receptor CD4, binding to the coreceptor CCR5 or CXCR4, and membrane fusion (Fig.15A). Viral attachment to CD4 and coreceptor binding is mediated by the viral surface protein gp120, while the transmembrane protein gp41 drives membrane fusion. The initial interaction between HIV-1 and the target cell, mediated by gp120, triggers conformational changes in the viral envelope protein, resulting in the exposure of the N-terminal domain of gp41, which contains a fusion peptide that is inserted into the host membrane (Fig.15 A, B). At this point of viral entry, gp41 transiently adopts an extended conformation prehairpin intermediate, which is highly conserved and persists for approximately 15 minutes (Fig.15 C). Later, the N- and C-peptide regions (HR1 and HR2 helical domains respectively) of gp41 (Fig.15 B) reorganize and associate to form a very stable trimer-of-hairpin structure (six-helix bundle) and the prehairpin state formation ultimately collapses (Fig.15 C) (Eckert and Kim 2001). The six-helix bundle formation, which represents the fusion-active conformation of gp41, brings the viral and cellular membranes into close proximity and induces membrane fusion and the further internalization of the viral capsid into the target cell (Fig. 15 A, C).

The gp41 transient intermediate prehairpin conformation preceding membrane fusion is an attractive target for therapeutic intervention. It has been shown, for instance, that the

formation of the hairpin structure (fusion-active conformation) is prevented by the binding of peptides to the prehairpin intermediate. Thus, the steps that culminate in the fusion of viral and cellular membranes are interrupted (Jiang, Lin et al. 1993; Root, Kay et al. 2001). Several attributes have made the gp41 prehairpin intermediate an important inhibitory target. *1)* It has a highly conserved sequence (hydrophobic pocket) among diverse HIV-1 strains from both laboratory adapted and clinical isolates. The reason of this conservation is because the mRNA encoding the hydrophobic pocket is an integral part of the Rev-response element, which is important during viral transcription (see under transcription and translation) (Root, Kay et al. 2001). *2)* It plays an essential role in viral entry (Chan, Chutkowski et al. 1998) and *3)* the hydrophobic pocket within gp41 contains a compact binding surface, making this region a good target for small inhibitor molecules (Eckert and Kim 2001).

Several attempts have been made to produce potent fusion inhibitors targeting different sites within gp41. They include peptides derived from the heptad repeat regions of gp41 namely, N- and C-peptides (Weiss 2003), antibodies (Miller, Geleziunas et al. 2005; Nelson, Kinkead et al. 2008), and small molecules (Liu, Wu et al. 2007; Pan, Liu et al. 2010). The majority of HIV-1 entry inhibitors, including inhibitors of fusion, are under preclinical and clinical studies. To date, Enfuvirtide (also known as Fuzeon or T-20) is the only fusion inhibitor that has been approved for clinical use. Fuzeon is a 36 amino acid synthetic C-peptide that inhibits virus entry by binding to the HIV-1 HR1 domain (N-HR helical region) of gp41 (but not the hydrophobic pocket), preventing the interaction of the HR1 and HR2 domains, which is fundamental for virus-cell fusion (Wild, Shugars et al. 1994).

Although Fuzeon has been demonstrated to be effective in clinical trials, especially in the treatment of HIV-1 infected patients who have developed multidrug-resistance to HIV-1, it

presents several disadvantages that limit its large scale clinical use. Among them, the high daily dosing (90 mg twice a day by subcutaneous injection) and high cost (approximately $ 25,000 per year/patient). It is also susceptible to proteolytic degradation and the emergence of drug-resistant strains (Steffen and Pohlmann 2010). Thus, the development of new generation peptide fusion inhibitors with an improved potency and stability remains an urgent task in drug development.

Recently, new promising fusion inhibitory peptides have emerged that overcome the constraints faced by previously developed peptide compounds, including Fuzeon. One such alternative is the generation of D-peptides that specifically target the hydrophobic pocket of gp41; referred to as pocket-specific inhibitors of entry (PIE). Contrary to C-peptides, D-peptides (composed of D-amino acids) are resistant to natural proteases (increased stability/half-life in serum) and have the potential for oral bioavailability (Eckert, Malashkevich et al. 1999; Steffen and Pohlmann 2010). Recently, Welch and co-workers have reported the development of a third generation D-peptide namely, chol-PIE12-trimer. When tested in HIV-1 entry assays, chol-PIE12-trimer showed a strong antiviral potency against a wide range of primary HIV-1 isolates including Fuzeon-resistant HIV-1 strains. With an IC_{50} in the 0.1-5 nM range (mean = 1.25nM), chol-PIE12-trimer showed to be 100-fold more potent than Fuzeon, which in the same study (used as a control), recorded an IC_{50} in the 2-1,000 nM range (mean = 128.5nM) (Welch, Francis et al. 2010).

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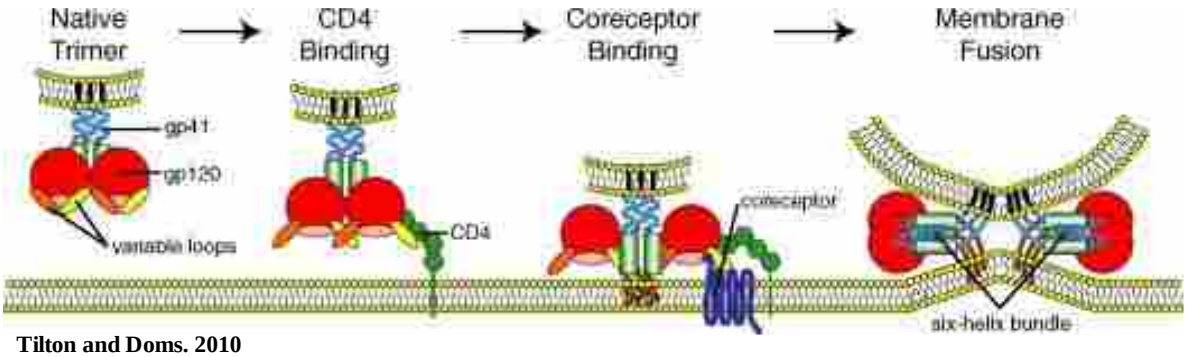

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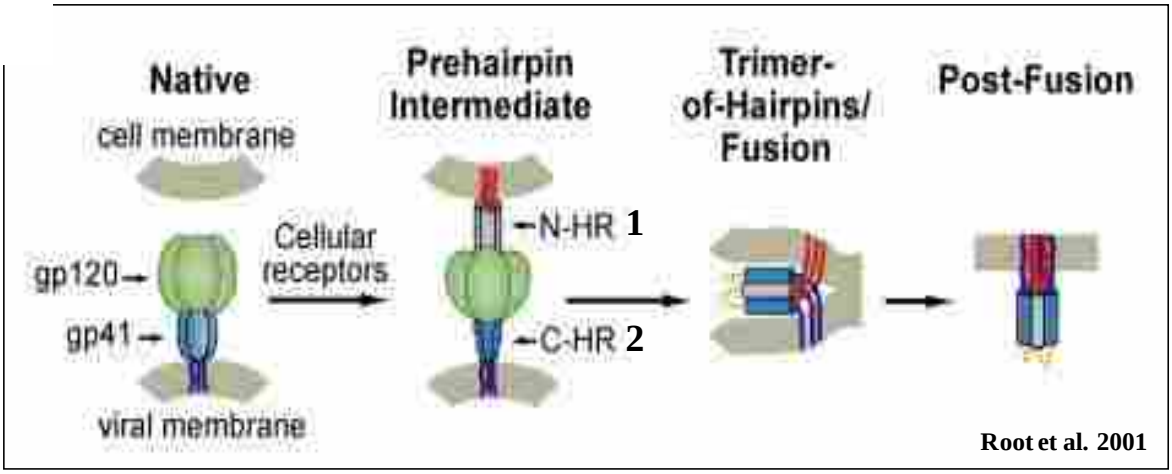

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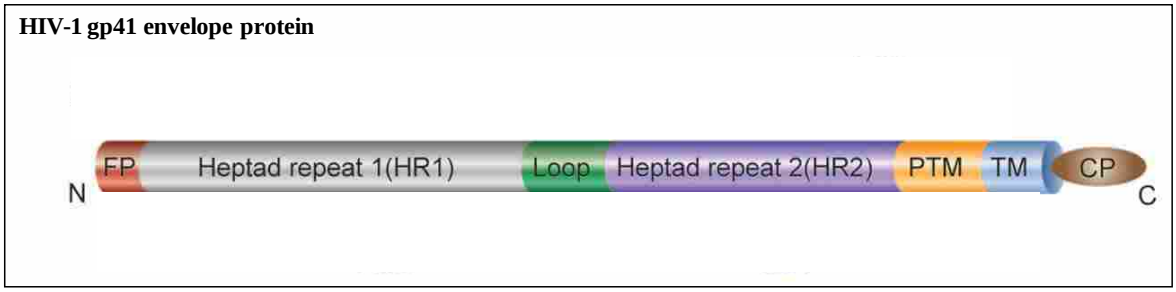

Figure 15. A) Schematic representation of the steps of HIV-1 attachment and entry into a target cell. B) Molecular structure of the HIV-1 gp41 envelope protein. gp41 consists of *1)* An extracellular domain containing a fusion peptide (FP), two heptad-repeat (N-HR1 and C-HR2) domains, a loop region, and a proximal transmembrane region (PTM). *2)* A transmembrane domain (TM). *3)* A cytoplasmic domain. C) Model of HIV-1 membrane fusion.

chol-PIE12-trimer also evidenced a high binding affinity (>100,000-fold higher than previously reported D-peptides) for the gp41 pocket, thus conferring a strong resistance capacity to the emergence of drug-resistant HIV-1 viruses (Welch, Francis et al. 2010). It is important to note that the inhibitory mechanism of chol-PIE12-trimer is similar to that of Fuzeon with the exception that Fuzeon binds to the N-HR helical region of gp41, near the pocket, while chol-PIE12-trimer specifically targets the hydrophobic pocket. These properties make PIE12-trimer an attractive HIV-1 entry (fusion) inhibitor candidate that could be used as either a therapeutic or prophylactic compound in the treatment of HIV/AIDS. To corroborate the potent antiviral activity that this novel pocket-specific D-peptide inhibitor shows in vitro, we sought to evaluate the pharmacokinetics and antiviral efficacy in vivo using humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mice.

Animal models to study HIV-1 infection and pathogenesis

During the past two decades, considerable efforts have been made to generate animal models to elucidate different aspects of HIV-1 infection and pathogenesis and to test antiretroviral drugs and vaccine efficacy. One such model is nonhuman primates (NHPs). Due to the many similarities (e.g., physiology and immunology) shared between humans and NHPs, these animals were among the first models to be used in the study of HIV-1 biology. Because NHPs such as chimpanzees and macaques are susceptible to HIV-1 and simian immunodeficiency virus (SIV) infection respectively, they have provided many valuable insights about the negative effects of HIV-1 infection and AIDS. It is important to mention that infection in chimpanzees is not pathogenic (no development of AIDS). Macaques infected with molecular clones of SIV are capable of developing AIDS-like disease, thus allowing the study of disease progression, virus transmission, and the function of certain genes that are common in humans and macaques. Furthermore, NHPs have played an important role in vaccine safety and efficacy

studies (Van Rompay 2005). Nevertheless, HIV-1 and SIV have certain differences in terms of genetic organization and disease course (Fultz 1993; Nath, Schumann et al. 2000). Likewise, the limited availability of these animals due to ethical issues and the high expense involved in their maintenance have made NHPs a limited model for the investigation of HIV-1/AIDS.

A small animal model for the investigation of HIV-1 virology, pathogenesis, and antiviral immune responses, as well as for the development and testing of vaccine candidates, and the screening of novel anti-viral strategies represents a desirable goal. The use of domestic cats has emerged as an alternative to accomplish this task. Cats are a natural host of the lentivirus feline immunodeficiency virus (FIV). Different studies have observed that this model is capable of sustaining a productive infection, and that FIV can be detected in different feline organs (e.g., thymus, bone marrow, female reproductive tract), and body fluids. The production of virus specific antibodies (i.e., IgG, and IgA) in infected cats has also been reported (Pedersen, Yamamoto et al. 1989; English, Nelson et al. 1994). Interestingly, after the viral incubation period, infected cats develop a progressive immune deterioration accompanied by $CD4^+$ T cell depletion, thus, mimicking the disease observed in HIV-1 infected humans (Willett, Flynn et al. 1997; Burkhard and Dean 2003).

Finally, the similarities found between FIV and HIV-1 RTs have permitted the use of cats to evaluate different RT inhibitors and the emergence of resistance to such antiretrovirals. FIV has shown to be sensitive to nucleoside RT inhibitors such as zidovudine and lamivudine. In contrast, nonnucleoside RT inhibitors and protease inhibitors cannot inhibit FIV (Van Rompay 2005). This last observation hampers the practical application of the FIV model for the screening of antiretroviral combinations in the FIV model. Despite the valuable body of information generated from studies using cats, limitations exist in these animals. The extended viral

incubation period seen in infected cats (7-9 years), has made difficult the proper evaluation of the efficacy of antiretroviral drugs. Unlike HIV-1, FIV does not have tropism for the $CD4^+$ receptor and, additionally, it targets $CD8^+$ T lymphocytes and B cells.

In the quest to further refine the search for an in vivo model that most accurately resemble the biological properties of HIV-1 and its associated pathology, new animal models have been generated. Mice are attractive candidates for HIV-1 research because they are inexpensive, easy to manipulate, can be housed in small facilities and in large numbers, and they reproduce quickly. However, normal laboratory mice are not susceptible to HIV-1 infection because the virus is species-specific and only infects and causes disease in humans. Fortunately, the ever increasing knowledge about murine genetics has facilitated the establishment of approaches to make these animals permissive to HIV-1 infection and to reproduce the events occurring during human infection at the molecular and clinical levels. Thus, transgenic mice were developed as a means to overcome the replication barriers faced by HIV-1 in wild-type laboratory mice. These replication barriers include the following: *1)* The inability of HIV-1 to interact with the murine CD4 and CXCR4 or CCR5 cell receptors, which in humans are the principal receptor and co-receptors for HIV-1 entry into target cells, respectively (Landau, Warton et al. 1988); *2)* Inefficient viral transcription due to the lack of association between HIV-1 Tat and the murine cyclin T1 (a complex required for viral transcription and elongation) (Kwak, Ivanov et al. 1999); *3)* Defects in virus assembly, leading to low levels of infectious virus and poor dissemination (Bieniasz and Cullen 2000). To circumvent these limitations, transgenic mice expressing the human genes necessary for HIV-1 replication have been generated (van Maanen and Sutton 2003). Although transgenic mice have been useful to monitor early events in virus infection, the limited replication supported by these animals has hampered

the study of pathogenesis and the efficacy of antiretroviral drugs and vaccine candidates (Boberg, Brave et al. 2008).

The recent emergence of immunodeficient mouse strains that can be transplanted with human cells and tissues (see overview to humanized mice) has greatly contributed to the field of HIV/AIDS research. Unlike transgenic mice, which are genetically manipulated to express human genes (e.g., critical for HIV-1 replication), humanized mice are capable of developing a human hemato-lymphoid system containing a variety of immune cell types. Therefore, these models have become useful in vivo systems for the study of pathogens (e.g., HIV-1) with tropism for human immune cells. The first two human-mouse chimeric models utilized in various HIV-1-related studies were SCID-hu-PBL and SCID-hu mice (Jamieson, Aldrovandi et al. 1996; Mosier 1996). Because both models sustain virus infection and replication, they have contributed to studies of HIV-1 pathogenesis, the in vivo efficacy of different antiretroviral drugs, and gene therapy (Goldstein, Pettoello-Mantovani et al. 1996; Poignard, Sabbe et al. 1999; Bai, Banda et al. 2002; Lapenta, Santini et al. 2003; Stoddart, Bales et al. 2007). Despite the many advances made in the understanding of HIV-1 modus operandi, these conventional models present some challenges. For instance, in the SCID-hu-PBL model, viral infection is restricted to a relative short period of time (8-12 weeks, approximately) due to the absence of continual multilineage human hematopoiesis (Tary-Lehmann, Saxon et al. 1995). Unlike SCID-hu-PBL mice, SCID-hu mice support viral infection for an extended period of time; however, this is restricted to the implanted organs. Furthermore, human T cells are mainly present in the thymic organ and very low levels of these lymphocytes are observed in peripheral lymphoid organs and blood. Finally, B cells, monocytes/macrophages, and dendritic cells are absent in these mice (Roncarolo, Carballido et al. 1996). In general, the absence of a functional peripheral human-lymphoid tissue,

the minimal development of human primary immune responses, as well as the lack of the chronic phase and the persistent decrease of T cells observed in HIV-1 infected humans, have restricted the pathogenesis studies related to HIV-1 using the SCID-hu-PBL and SCID-hu models.

Attempts to overcome the different challenges present in the previous generations of humanized mice have encouraged the production of new and improved immunodeficient mice strains. One such example is the human-bone marrow-liver-thymus (hu-BLT) mouse (Ito et al., 2002; Melkus et al., 2006). In this model, the transplantation of human thymus and liver is followed by reconstitution with autologous human fetal liver $CD34^+$ cells. hu-BLT- SCID mice display a complete, self-renewing reconstitution of human T and B lymphocytes, macrophages, and dendritic cells. Such a repertoire of immune cells facilitate the generation of primary human immune responses and HIV-1 replication and transmission. Additionally, it has been documented that BLT mice are susceptible to mucosal HIV-infection due to the presence of HIV-1-susceptible $CD4^+$ T lymphocytes in the reproductive tract of females (Sun et al., 2007; Denton et al., 2008; Brainard et al., 2009). Because of these features, BLT mice have been successfully used to demonstrate the prophylactic efficacy of different antiretrovirals against vaginal HIV infection (Denton et al., 2008). Despite the contributions of this model to the study of HIV-1 pathogenesis and prevention of viral infection; the restricted access to human fetal tissues, and the surgical procedure involved in the generation of BLT mice make this model technically challenging and time consuming.

Alternatively, mice with a more immune-suppressed phenotype and showing a functional human immune system have been reported, among them NOD/SCID/$\gamma c^{-/-}$,

$Rag2^{-/-}\ \gamma c^{-/-}$, and $Rag1^{-/-}\ \gamma c^{-/-}$ mice which are engrafted with human $CD34^+$ hematopoietic stem cells (HSCs) (Berges and Rowan 2011). The weaker mouse immunity results in lower

resistance for engrafted cells, while the use of HSCs in the graft results in self-renewal of the graft and long term engraftment. As mentioned earlier, the $Rag2^{-/-}$ $\gamma c^{-/-}$ mouse model is a double knockout mouse with mutations in Rag2 and the γc genes. These mutations prevent the development of functional murine T, B, and NK cells (Traggiai et al., 2004; Baenziger et al., 2006). Transplantation of human HSCs to produce humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mice leads to the novo development of the major functional components of the human adaptive immune system and the production of a variety of human cell types, including the major cellular targets of HIV in both central and peripheral lymphoid organs (Traggiai et al., 2004).

Currently, there is extensive evidence showing that humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mice are an excellent model system by which to study different aspects of HIV biology, its related immunodeficiency, and therapeutic interventions. Several studies have demonstrated that these mice are susceptible to HIV infection and that following infection the human $CD4^+$ T cells are gradually depleted, thus resembling what is observed in humans infected with HIV (Baenziger et al., 2006; Berges et al., 2006, 2010, 2011; Zhang et al., 2007; Choudhary et al., 2009).

It has been also documented that humanized $Rag2^{-/-}$ $\gamma c^{-/-}$ mice contain HIV-susceptible human cells in the rectal and vaginal mucosa, and that these animals are vulnerable to HIV-1 infection following rectal or vaginal exposure to cell-free virus. Furthermore, mucosal HIV infection resulted in viremia and associated $CD4^+$ T cell depletion in peripheral blood. Infected cells were detected in different organs, including the intestinal mucosa, giving evidence of a systemic viral spread (Berges et al., 2008). It has been demonstrated that after administration of antiretrovirals, HIV-1-infected $Rag2^{-/-}$ $\gamma c^{-/-}$ mice show suppression of viremia below the limits of detection and $CD4^+$ T cell recovery. On the other hand, interruption of drug therapy resulted in viral rebound and renewed loss of $CD4^+$ T cells. These studies provide evidence that HIV-1

infected Rag2$^{-/-}$ γc$^{-/-}$ mice are capable of recapitulating many aspects of antiretroviral therapies in humans (Choudhary et al., 2009; Sango et al., 2010).

In a different study using the same mouse model, Neff and co-workers documented that oral administration of raltegravir (an integrase inhibitor) and maraviroc (an FDA-approved CCR5 inhibitor) protects humanized Rag2$^{-/-}$ γc$^{-/-}$ mice from HIV-1 infection via the vaginal route (Neff et al., 2010). Taken together, these findings demonstrate that the humanized Rag2$^{-/-}$ γc$^{-/-}$ mouse is an ideal in vivo model to study HIV-1 pathobiology as well as the effects of anti-HIV therapies. Therefore, in this study we proposed to use humanized Rag2$^{-/-}$ γc$^{-/-}$ mice as a model to evaluate the in vivo antiviral efficacy of the D-peptide inhibitor of HIV-1 entry, namely, chol-PIE12-trimer.

RESEARCH OBJECTIVES

The purpose of this study was to use the humanized Rag2$^{-/-}$ γc$^{-/-}$ mouse model to determine the *in vivo* antiviral efficacy of the HIV-1 entry inhibitor chol-PIE12-trimer.

Specific Aims:

1. Assess the stability of chol-PIE12-trimer in Rag2$^{-/-}$ γc$^{-/-}$ mice.
2. Evaluate the antiviral efficacy of chol-PIE12-trimer in HIV-1 infected Rag2$^{-/-}$ γc$^{-/-}$ mice.

MATERIALS AND METHODS

Generation of Rag2$^{-/-}$ γc$^{-/-}$ mice

Neonatal Rag2$^{-/-}$γc$^{-/-}$ mice were conditioned by irradiation at 350 rads, and later transplanted with human cord blood-derived CD34^{+} cells as previously described (see production of immune deficient Rag2$^{-/-}$ γc$^{-/-}$ mice). Approximately 8-10 weeks post-reconstitution, mice

were screened for human cell engraftment. Blood was collected by tail bleed and red blood cells were lysed. The white cell fraction was stained with antibodies and FACS analysis was performed as previously mentioned.

chol-PIE12-trimer IC_{50} calculation

In this study we used HIV-1_{Ba-L} (R5 tropic virus) for in vitro and in vivo experiments. HIV-1_{Ba-L} (4.0 x 10^6 $TCID_{50}$/ml) was obtained from Suzanne Gartner, Mikulas Popovic, and Robert Gallo, through the AIDS Research and Reference Reagent Program Division of AIDS, NIAID, NIH (Gartner, Markovits et al. 1986). The inhibitory concentration (IC_{50}) of chol-PIE12-trimer against HIV-1_{Ba-L} was assessed by a luciferase infectivity assay using the TZM-bl cell line (NIH AIDS Research and Reference Reagent Program catalogue no. 8129). TZM-bl cells were derived from HeLa cells and they constitutively express human CD4, CCR5, and CXCR4. This cell line also carries integrated reporter genes for luciferase and beta-galactosidase under the control of the HIV-1 LTR promoter. TZM-bl cells were seeded in a 96 well plate and cultured overnight in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. The next day, 70-90% confluent cells were infected with HIV-1_{Ba-L} (1.0x 10^3 $TCID_{50}$) in the presence of different concentrations of chol-PIE12-trimer (serial dilutions from 1250 pM to 0.4 pM). 42 hours post infection, the cells were lysed, and luciferase activity (expressed as relative luminescence) was measured. The IC_{50} of chol-PIE12-trimer was calculated by fitting the data to the equation y = 1-X/ (m2+x), where y = luciferase activity and m2 is the IC_{50} value (Fig. 16).

chol-PIE12-trimer dosing and pharmacokinetic analysis in mice

Three non-humanized $Rag2^{-/-}\gamma c^{-/-}$ mice of approximately 30g each received a single subcutaneous dose of 4.0 µM of inhibitor in 100 ul of HEPES buffer. Blood was collected by tail bleeding at 0 min, 30 min, 1h, 2h, 4h, 8h, 12h, and 24h post drug administration. Serum was collected from whole blood, and analyzed by viral infectivity assay as described previously (Welch, VanDemark et al. 2007) to determine the half-life of chol-PIE12-trimer.

Antiviral efficacy of chol-PIE12-trimer in $Rag2^{-/-}$ $\gamma c^{-/-}$ mice

8-10 week post-reconstituted $Rag2^{-/-}\gamma c^{-/-}$ mice (n= 10) were infected intraperitoneally with 1 x 10^4 $TCID_{50}$ of $HIV\text{-}1_{Ba\text{-}L}$. Beginning at one week post-infection, blood samples were collected weekly from infected and control mice. Plasma viral load and human $CD4^+$ T cell levels were monitored for 11 weeks (Fig. 17). For detection of cell-free HIV-1, RNA was extracted from 25-60 µl of EDTA-treated plasma and then subjected to Q-RT-PCR using a primer set specific for the HIV-1 LTR sequence and a corresponding LTR specific probe as described previously (Berges, Akkina et al. 2008). Human $CD4^+$ T cell levels were assessed by FACS analysis. Whole blood was collected and red blood cells lysed as previously described. Peripheral blood cells were stained for hCD3-PE and hCD4-PECy5 markers and analyzed on a BD FACS Canto (Becton, Dickinson, CA) analyzer. T cell levels were calculated as a ratio of the entire CD3 population ($CD4^+$ $CD3^+$: $CD4^-$ $CD3^+$). To establish a baseline of CD4+ T cell levels, all mice were monitored one week prior to infection (mean 60% CD4: CD3 ratio) (Fig.17).

Five weeks post-infection, infected mice (n=5) were administered daily (for 4 weeks) with 700 nM of chol-PIE12-trimer inhibitor by the subcutaneous route. The antiviral protection of chol-PIE12-trimer to HIV-1 challenged animals was tested by weekly monitoring the levels of plasma viral load (Q-RT-PCR) and $CD4^+$ T cell counts (FACS) as described above. These values

were compared with reading obtained from HIV-1 challenged but chol-PIE12-trimer untreated mice (control mice). Drug treatment was stopped after week 9 post-infection, but animals continued to be monitored for two more weeks (Fig. 17). Two mice from each group were sacrificed immediately after the last drug treatment. Lymphoid organs were collected from sacrificed mice for histology analysis as well as to evaluate the concentrations of inhibitor on each organ.

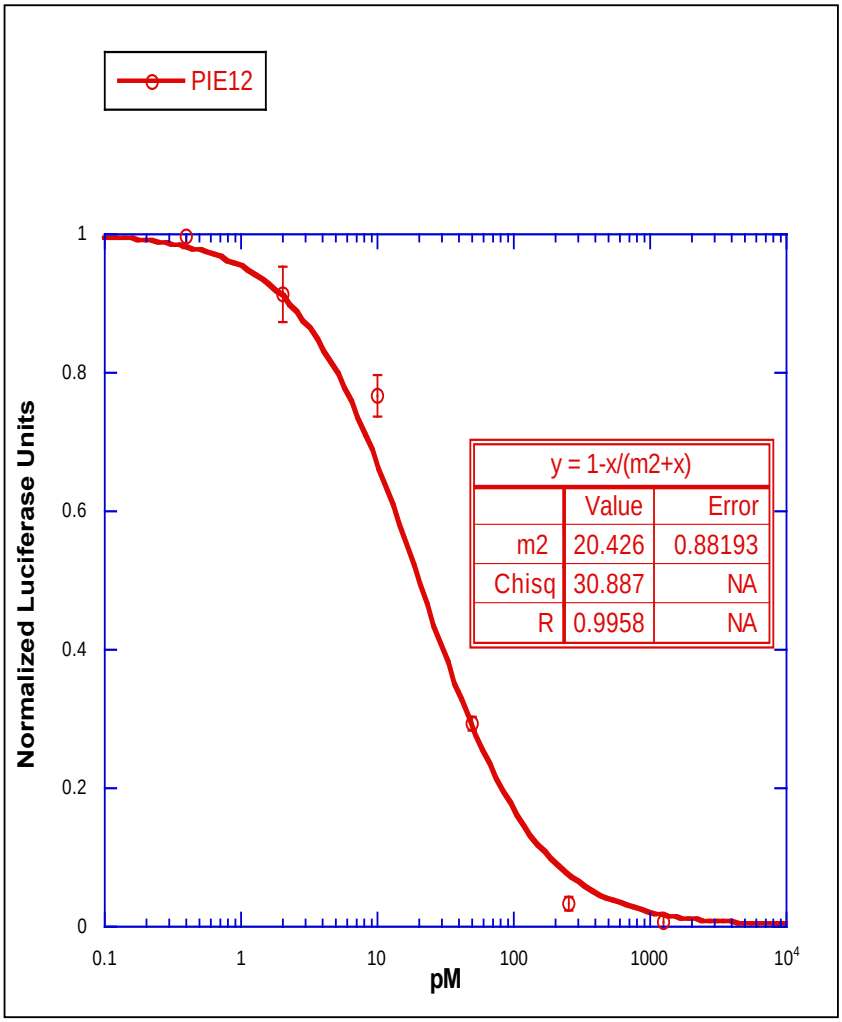

Figure 16. IC_{50} curve for chol-PIE12-trimer against Hiv-$1_{Ba\text{-}L}$.

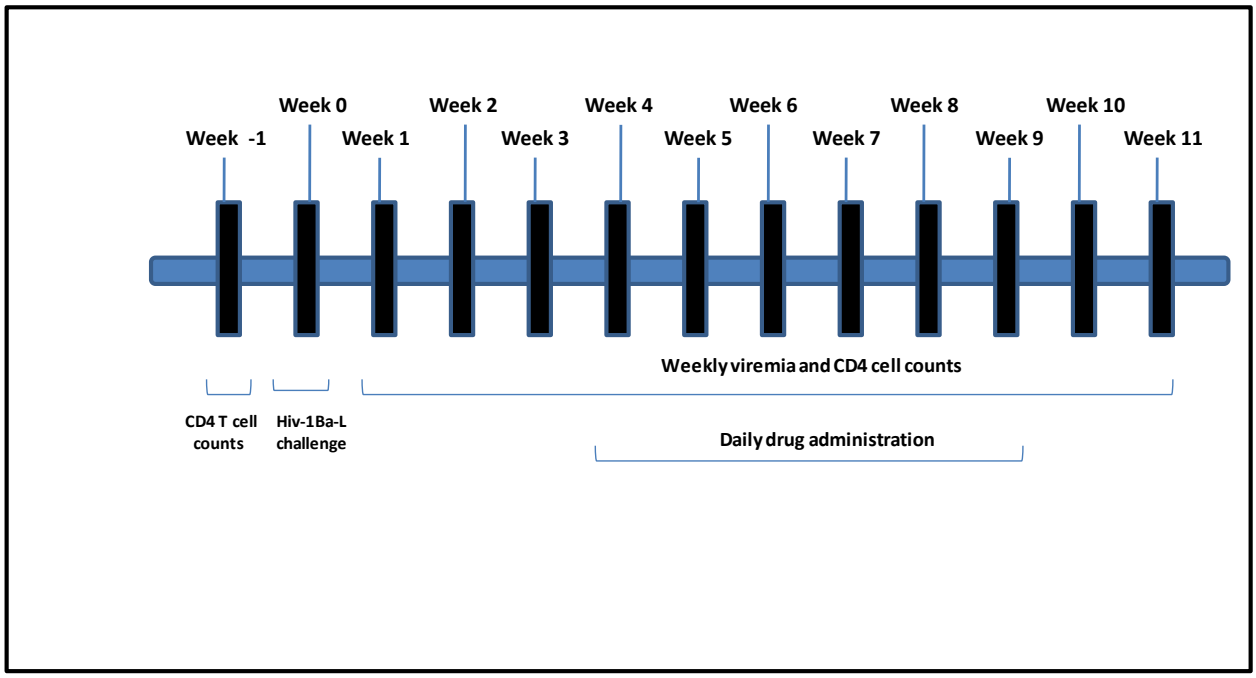

Figure 17. Experimental design for the evaluation of the antiviral efficacy of chol-PIE12-trimer D-peptide in Rag2-/- γc -/- mice.

RESULTS AND DISCUSSION

Determination of IC50 of PIE12-trimer against HIV-1_{Ba-L}

The IC_{50} of chol-PIE12-trimer has been already determined for many other HIV-1 isolates including HIV-1_{JRFL} (Welch, Francis et al. 2010), but never against HIV-1_{Ba-L} (the target virus for these studies). As determined by the luciferase assay described in methods, the IC_{50} value of chol-PIE12-trimer was 0.02 nM (Fig. 16).

Pharmacokinetics of chol-PIE12-trimer in humanized Rag2$^{-/-}\gamma c^{-/-}$ mice

Prior to evaluating the efficacy of chol-PIE12-trimer in HIV-1 infected Rag2$^{-/-}\gamma c^{-/-}$ mice, we determined the half-life of the drug in mouse plasma by measuring the levels of the inhibitor in plasma following subcutaneous administration of chol-PIE12-trimer. The results obtained from the inhibitory assay (inhibition of luciferase production in infected cells) indicated that the concentration of the D-peptide dropped approximately 50-fold over 24 hours (from 4,000 nM to ~ 80 nM). Thus, the half-life of chol-PIE12-trimer was calculated to be 5 hours. Based on this value, we established a daily dose of drug (starting concentration of 700 nM) for the efficacy study in vivo. We projected that with this initial dose, the trough concentration of drug 24 hours post-administration would be 15 nM, which is approximately 800-fold above our measured tissue culture IC_{50} for HIV-1_{Ba-L}.

Antiviral efficacy of chol-PIE12-trimer in humanized Rag2$^{-/-}\gamma c^{-/-}$ mice

Two well-accepted and characterized parameters used to monitor disease progression in HIV-1 infected humans are the levels of plasma viral load and $CD4^+$ T cell counts. Thus, before assessing the in vivo antiviral potency of chol-PIE12-trimer, we first evaluated the state of infection and the $CD4^+$ T cell levels in humanized Rag2$^{-/-}\gamma c^{-/-}$ mice reconstituted with human

HSCs and infected with HIV-1. Blood samples were collected at weekly intervals (Fig.17) and the plasma fraction was subjected to Q-RT-PCR analysis to monitor levels of plasma viremia. The mean HIV-1 viral load recorded one week prior to drug administration was 1.0×10^6 genome copies /ml plasma in infected mice (Fig. 18). CD4+ T cell levels started to decline at 3 weeks post- infection in all infected mice (Fig. 19).

At 5 weeks post-infection, five infected mice were treated with chol-PIE12-trimer (experimental group) on a daily basis for 4 weeks (Fig.17) and 5 mice were left untreated. Viral load and $CD4^+$ T cell levels were monitored weekly in the presence of the inhibitor (Fig.18, 19). One week post-drug administration, a decline in plasma viremia of approximately 1.5 log was observed in the treated group, while the viral load in mice with no chol-PIE12-trimer treatment (control group) was constant ($\sim 1.0 \times 10^6$ copies /ml) (Fig.18). Interestingly, two weeks post-drug administration there was a rebound of approximately 1 log in viral load in the experimental mice. Untreated mice maintained steady levels of viral load (Fig.18). No statistical difference between treated and untreated animals was observed in levels of viral load at any time point in treated versus control animals. However, there was as significant difference ($p = 0.02$) in viral load when comparing the last time point of drug administration with the point after in treated mice. But a similar increase was seen in untreated mice (Fig. 18).

Even when viremia was partially suppressed in the presence of chol-PIE12-trimer, we observed a gradual recovery in the levels of $CD4^+$ T cell counts in the blood of animals treated with the inhibitor (Fig. 19). Unlike the treated mice, the untreated animals experienced a drastic drop in the CD4 T cell counts in peripheral blood (~30% decrease at week 6 post-infection) (Fig. 19). Discontinuation of chol-PIE12-trimer resulted in a new decline of CD4 T cells (~ 20% at 1 week after drug interruption). But a similar decline was seen in untreated animals (Fig. 19).

Since a control group of uninfected mice treated with chol-PIE12-trimer was not included in this study, it is not possible to draw a strong conclusion that the recovery of $CD4^+$ T cells seen during drug administration is the result of chol-PIE12-trimer preventing HIV-1 infection. Thus, the increasing levels of $CD4^+$ T cell counts observed in the presence of the inhibitor could be due to cell stimulation by the drug, or by preventing T cells from exiting the blood.

We were encouraged by the significant protection of helper T cell levels in humanized mice treated with chol-PIE12-trimer, which indicated that the drug was effective at preventing an AIDS phenotype. However, there was no significant difference in viral load in treated animals. The possible reasons for the low impact of chol-PIE12-trimer on plasma viral load might be 1) the presence in mice of chronically infected reservoirs that constantly supply peripheral blood and lymphoid tissues with newly produced virus. We speculate that one such viral reservoir could be human macrophages, which are targeted by HIV-1 early during infection. It is well known that because of their extended life span and resistance to the cytopathic effects of the virus, these cells contribute to the dissemination and persistence of HIV-1 in infected humans, even in those under antiretroviral therapies (Crowe, Zhu et al. 2003; Alexaki, Liu et al. 2008). 2) Because chol-PIE12-trimer is an entry inhibitor of HIV-1, its mechanism of action has no effect in cells that are already infected. Thus, the late drug administration (5 weeks post-infection) can be another factor contributing to no change in viremia. 3) The inability to generate an effective human adaptive immune response against HIV-1 may prevent humanized $Rag2^{-/-}\gamma c^{-/-}$ mice from being able to eliminate chronically infected cells (Baenziger, Tussiwand et al. 2006; Gorantla, Sneller et al. 2007). Thus, it is possible that chol-PIE12-trimer may be more effective at reducing viral load in humans than in humanized mice.

One approach to test the hypothesis of chronically infected macrophages could be the use of dual immuno-staining experiments using antibodies specific for HIV-1 p24 and the macrophage marker CD68. Cells positively stained for both p24 and CD68 will confirm the hypothesis that chronically infected macrophages contribute to the high levels of viremia seen in infected mice even in the presence of chol-PIE12-trimer. In addition, antibody-mediated complement lysis of chronically infected macrophages (using a mouse anti-human antibody specific for CD14) is another strategy that may contribute to the reduction of viral load in the context of macrophages. Alternatively, complement-mediated responses with mouse anti-human antibody against CD3, could indicate if chronically infected T cells are contributing to viremia. If targeting of a specific cell type by this method resulted in a rapid reduction in viral load, we would conclude that that cell type is a reservoir of HIV-1 replication and is contributing to the persistently high viral load in the face of drug treatment.

CONCLUSIONS

In summary, we have determined the pharmacokinetics of chol-PIE12-trimer in mice. We have also demonstrated that in humanized $Rag2^{-/-}\gamma c^{-/-}$ mice, chol-PIE12-trimer prevents $CD4^{+}$ T cells depletion in infected mice, even in the presence of invariable levels of viremia.

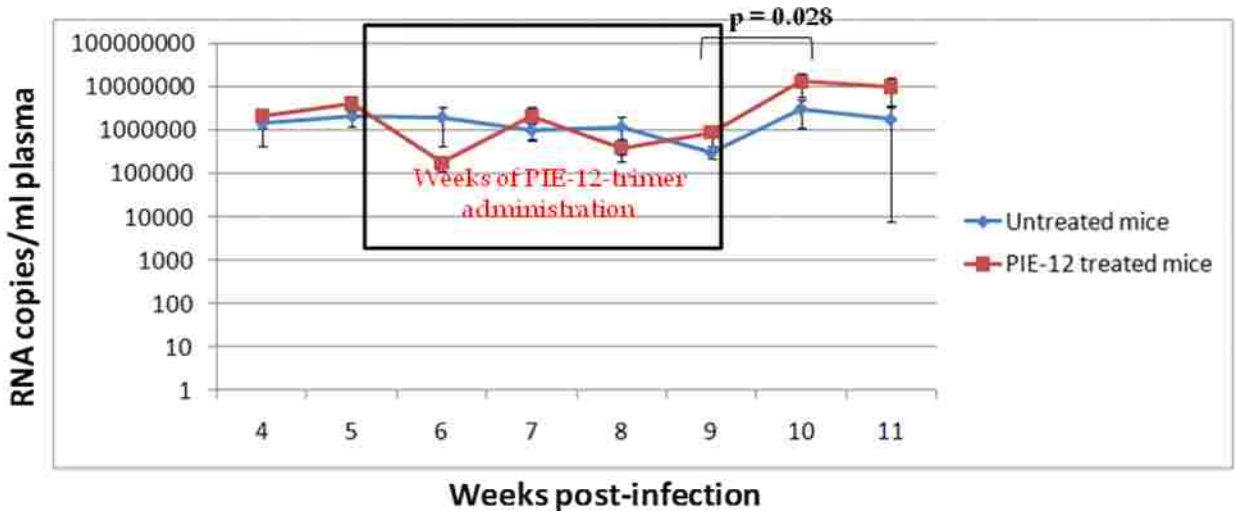

Figure 18. Effect of chol-PIE12-trimer on plasma viral load of infected humanized Rag2$^{-/-}\gamma c^{-/-}$ mice. Mice were infected with 1 x 10^4 $TCID_{50}$ of HIV-1_{Ba-L}. Five weeks post-infection, mice began to be treated daily with 700nM of chol-PIE12-trimer inhibitor by the subcutaneous route. Blood was collected weekly and viral RNA was extracted from the plasma fraction. Viral load was assessed by Q-RT-PCR. The square indicates the weeks of chol-PIE12-trimer administration to infected mice (week 5 to 9). n= 5 for each group, with n = 3 after week 9. Differences were considered statistically significant for p values < 0.05.

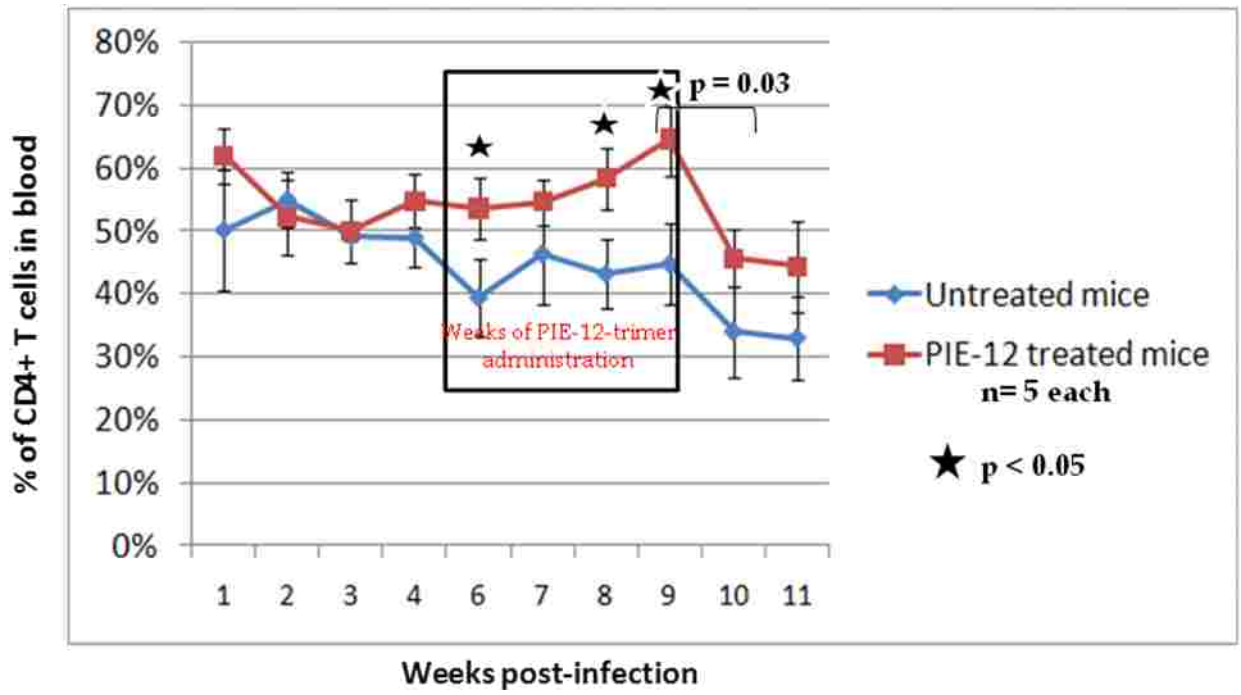

Figure 19. Effect of chol-PIE12-trimer on CD4+ T cell levels of infected humanized Rag2-/-γc-/- mice. Humanized mice were infected with 1 x 104 TCID50 of HIV-1Ba-L and at 5 weeks post infection, they began to be treated with 700nM of chol-PIE12-trimer inhibitor by the subcutaneous route. Peripheral blood was collected once a week (postinfection). Cells were stained with the T cell markers CD3 and CD4, and analyzed by FACS to determine the levels of CD4+ T cells. CD4+ T cell levels were calculated as a percent of individual mouse baseline levels. The square indicates the weeks of chol-PIE12-trimer administration to infected mice (week 5 to 9). n= 5 for each group; Differences were considered statistically significant for p values < 0.05.

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