Pathogenesis of HIV-1 Nef in adult mice

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Preface

This thesis is submitted to the McGill University Faculty of Graduate Studies and Research in a manuscript-based format. This thesis consists of five chapters and describes a novel animal model of AIDS and pathogenesis of HIV-1 Nef in adult mice. Chapter 1 is general introduction and review of literature relevant to this research project. The experimental results are presented in chapters 2, 3 and 4. Chapter 2 and 4 are manuscripts in preparation. Chapter 3 is a manuscript based on preliminary data. Each of these chapters has its own abstract, introduction, materials and methods, results, discussion and bibliography sections. Connecting texts are included in the preface of each chapter. Chapter 5 is general discussion and conclusions. Other experimental results and claims for original research have been included in the appendix section.

Abstract

Development of a suitable animal model of AIDS is much needed in AIDS research to study infection and pathogenesis as well as to evaluate methods of prevention and treatment of HIV infection. Small animals such as rodents are attractive candidates for AIDS research due to the availability of various inbred and genetically engineered strains, extensive knowledge or their immune system, especially in mice, and the relative ease of breeding and maintaining animal colonies. Transgenic small animal models carrying entire HIV genome or selected genes have been instrumental to understand functions of HIV genes *in vivo* and their role in HIV pathogenesis. The type of cells in which HIV genes are expressed seems to be an import prerequisite for the study of HIV gene functions in transgenic mice. Mice constitutively expressing the entire HIV-1 genome or HIV-1 *nef* gene in $CD4^+$ T cells and in the cells of macrophage/dendritic lineage develop an AIDS-like disease very similar to AIDS disease in humans. Similarly, expression of Nef in adult mice, using inducible system, results in the AIDS-like disease. This disease is characterized by thymic atrophy, impaired thymocyte maturation, loss of $CD4^+$ T cells, increased activation and turnover of T cells, which can occur in the absence of lymphypenia, and non-lymphoid organ disease involving the lungs and kidneys. Susceptibility of adult mice to the pathological effects of Nef suggests that the AIDS-like disease in the constitutively expressing Nef Tg mice is not due to developmental defects caused by early expression of Nef. This model highlights the important role of Nef in HIV-1 pathogenesis. The high similarity in the disease in these Tg mice with human AIDS strongly suggest that these mice are a relevant model to study

AIDS. This study further evidence that mouse cells can support functions of Nef and these Tg mice represent a unique model to study Nef functions *in vivo* in the context of the primary immune system. Moreover, the inducible Nef Tg model has given us the ability to control the level and time of expression of Nef which was impossible to do in the previously reported constitutive Nef Tg mouse models. These mice will be useful to study immune reconstitution since Nef expression can be turned off after withdrawal from dox.

Résumé

Le développement d'un modèle animal adéquat du SIDA est une nécessité pour la recherche sur le SIDA, afin d'étudier l'infection et la pathogenèse du VIH ainsi qu'évaluer les méthodes de prévention et de traitement de l'infection par le VIH. De petits animaux, tels les rongeurs, sont d'excellents candidats pour la recherche sur le SIDA en raison de la disponibilité de multiples lignées congéniques et génétiquement modifiées, de notre connaissance approfondie de leur système immunitaire, particulièrement celui des souris, et de la facilité relative de produire des croisements et de maintenir des colonies. Des modèles de petits animaux transgéniques porteurs du génome entier du VIH, ou de gènes sélectionnés, ont servi à comprendre les fonctions des gènes du VIH *in vivo* et leur rôle dans la pathogenèse du VIH. Le type de cellules dans lesquelles les gènes du VIH sont exprimés semble être un prérequis important pour l'étude des fonctions des gènes du VIH dans les souris transgéniques. Les souris exprimant, de façon constitutive, le génome entier du VIH-1 ou le gène VIH-1 Nef dans les cellules T $CD4^+$ et dans les cellules de la lignée macrophage/dendritique développent une maladie très semblable au SIDA humain. Cette maladie est caractérisée par l'atrophie thymique, la perturbation de la maturation des thymocytes, la disparition des cellules T $CD4^+$, l'activation et le renouvellement accrus des cellules T, qui peuvent survenir en l'absence de lymphopénie, et par une maladie des organes non-lymphoïdes impliquant les poumons et les reins. La susceptibilité des souris adultes aux effets pathologiques de Nef suggère que la maladie semblable au SIDA humain qui se développe dans les souris dans lesquelles Nef est exprimé de façon constitutive n'est pas

due à des problèmes de développement résultant de l'expression de Nef prématurément. Ce modèle souligne donc l'importance du rôle de Nef dans la pathogenèse du VIH-1. Les similarités flagrantes entre la maladie dans ces souris Tg au SIDA humain suggèrent fortement que ces souris sont un modèle pertinent pour l'étude du SIDA. Cette étude démontre aussi que les cellules murines sont capables de supporter les fonctions de Nef, et que les souris Tg représentent un modèle unique pour l'étude des fonctions de Nef *in vivo* dans le contexte du système immunitaire primaire. D'autant plus, le modèle Tg inductible de Nef nous a permis de contrôler le niveau et le timing d'expression de Nef, ce qui n'était pas possible dans les modèles murins Tg préalablement documentés dans lesquels l'expression de Nef était constitutif. Ces souris pourront servir à des études sur la reconstitution du système immunitaire, vu que l'expression de Nef peut être cessée suite à l'arrêt de la doxycycline.

Acknowledgements

I express my deepest gratitude to my supervisor, Dr. Paul Jolicoeur for his excellent mentorship and guidance throughout the course of this investigation. I thank my committee members, Dr. Nicole Bernard and Dr. Mattias Götte for their constructive criticism of my work during the committee meetings, and Dr. Claude Lazure for his timely advice. I also thank Dr. Zaher Hanna for being there whenever I needed any information, Dr. Pavel Chrobak for all the discussions and his valuable suggestions regarding my experiments, Dr. Denis Kay and Dr. Chunyan Hu for reading the histology slides. I extend my thanks to all my present and past lab-mates, specially Elena Priceputu, Ginette Masse, Soheila Afkhami, Stephanie Lemay, Isabelle Corbin, Eve-Lyne Thivierge, Marie-Eve Higgins, Clara Forestier, Audrey-Ann Kustec, Katarzyna Jesien, Alice Hobeika, Benoit Laganiere and Jonathan Kergoat for their help and good spirit. A word of thanks goes to Eric Massicote and Matin Dupuis from flow cytometry core facility, Annie Vallee from histology unit and Dominic Filion from microscopy unit of IRCM for their excellent technical support. My special thanks go to Catherine, my family and my friends for their love and support all these years.

Contributions of authors

Chapter 2: A novel inducible HIV-1 Nef trangenice mouse model

Pavel Chrobak helped with the generation of bone marrow chimeras and data analysis. Zaher Hanna helped with design of transgene constructs. He has generated the HIV^{Nef} construct. Paul Jolicoeur provided funding and mentorship for the project. Mir Munir Rahim generated the transactivator Tg mice, performed the experiments and analysis.

Chapter 3: HIV-1 Nef induced T cell activation in the absence of lymphopenia

Pavel Chrobak helped with the generation of bone marrow chimeras and FACS analysis of thymus. Zaher Hanna helped with design of transgene constructs particularly the HIV^{Nef} construct. Paul Jolicoeur provided funding and mentorship for the project. Mir Munir Rahim generated bone marrow chimeras and performed FACS analysis of the lymphoid organs.

Chapter 4: Normal development and function but impaired memory phenotype of $CD8^+$ T cells in Transgenic Mice Expressing HIV-1 Nef.

Elena Priceputu performed the FACS analysis of activation markers and BrdU incorporation by $CD8^+$ T cells. She also helped with T cell proliferation assays. Pavel Chrobak helped with the design of experiments for analysis of $CD8^+$ T cell development. Zaher Hanna has constructed the $CD4C/HIV^{Nef}$ Tg mice. Paul Jolicoeur provided funding and mentorship for the project. Mir Munir Rahim performed FACS analysis of lymphoid organs, T cell proliferation assays and the experiments with LCMV.

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Abbreviations

7-AAD: 7-aminoactinomycin D

AIDS: Acquired immunodeficiency syndrome

APC: Antigen presenting cells

BM: Bone marrow

BrdU: Bromodeoxyuridine

CAF: $CD8^+$ T-lymphocyte antiviral factor

CTL: Cytotoxic T lymphocyte

CA: Capsid

CypA: Cyclophilin A

CEF: Chicken embryonic fibroblasts

CNS: Central nervous system

Dox: Doxycycline

DTg: Double-transgenic

DP: Double positive

DN: Double negative

DC: Dendritic cell

DNA: Deoxyribonucleic acid

DTH: Delayed type hypersensitivity

Env: Envelop

ELISA: Enzyme linked immunosorbent assay

FSGSC: Focal segmental glomerulosclerosis

FACS: Fluorescence activated cell sorting

FITC: Flrorescein isothiocyanate

GFP: Green fluorescent protein

GALT: Gut associated lymphoid tissue

GEF: Guanine exchange factor

hCMV: Human cytomegalovirus

HIV: Human immunodeficiency virus

HTLV: Human T lymphotropic virus

HIVAN: HIV-associated nephropathy

IP: Immunoprecipitation

IB: Immunoblot

ICTV: International Committee on Taxonomy of Viruses

IN: Integrase

IP3R: Inositol triphosphate receptor

KHL: Keyhole limpet hemocyanin

KS: Kaposi's sarcoma

kDa: Kilodalton

LC: Langerhans cell

LTR: Long terminal repeat

LCMV: Lymphocytic choriomeningitis virus

MA: Matrix

MBP: Myelin basic protein

mAb: Monoclonal antibody

MOI: Multiplicity of infection

MHC: Major histocompatibility complex

MAPK: Mitogen-activated protein kinase

MMTV: Mouse mammary tumor virus

Nef: Negative effector

NOD: Nonobese diabetic

NC: Nucleocapsid

NES: Nuclear export signal

NFAT: Nuclear factor of activated T cells

ORF: Open reading frame

PIC: Preintegration complex

PR: Protease

Pol: Polymerase

PHA: Phytohemagglutinin A

PBMC: Peripheral blood mononuclear cell

PI3K: Phosphatidylinositol 3 kinase

PAK: p21-activated kinase

PBL: Peripheral blood lymphocytes

PE: Phycoerythrin

pLN: Peripheral lymph node

rtTA: Reverse tetracycline-dependent transactivator

RNA: Ribonucleic acid

RT: Reverse transcriptase

RNAPII: RNA polymerase II

RRE: Rev response element

RBD: RNA-binding domain

STg: Single-transgenic

SPF: Specific pathogen free

SP: Single positive

SIV: Simian Immunodeficiency virus

SCID: Severe combined immunodeficiency

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TM: Transmembrane

TGN: Transgolgi network

TCR: T cell receptor

TNF-β: Tumor necrosis factor β

TGF-β: Transforming growth factor β

Tet: Tetracycline

TRE: Tetracycline responsive elements

tTA: Tetracycline-dependent transactivator

Thy/liv: Thymus/liver

Tg: Transgene

Vif: Viral infectivity factor

Vpr: Viral protein R

Vpu: Viral protein U

WT: Wild type

Chapter 1: Introduction and review of literature

1.1. Acquired immunodeficiency syndrome (AIDS)

AIDS is a multiorgan disease with pathologies in both lymphoid and non-lymphoid organs. It is caused by infection of humans with human immunodeficiency virus (HIV-1 and HIV-2). HIV uses the CD4 receptor along with the chemokine receptors CXCR4 or CCR5 to infect its target cells [1-4]. HIV-1 strains that preferentially use the CXCR4 co-receptor are termed T-tropic (X4) and primarily infect $CD4^+$ T cells, while those that use the CCR5 co-receptor are termed M-tropic (R5) strains and infect macrophages, dendritic and $CD4^+$ T cells. The major hallmark of AIDS is destruction of $CD4^+$ T cells leading to loss of immune competence. The loss of $CD4^+$ T cells begins with the primary infection and continues throughout the course of infection [5,6]. The disease course can be divided into 3 stages, namely, primary infection followed by a long latency period that will ultimately lead to the late stage infection and AIDS. A diagramatic representation of the natural history of HIV-1 infection is shown in **Fig.1**.

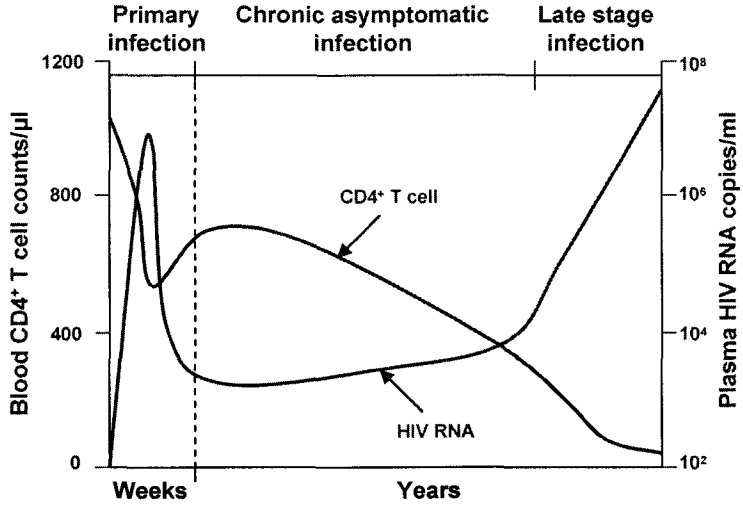

Figure 1.1. Diagramtic representation of natural history of HIV-1 infection. Adapted from Pantaleo *et al.*, 1993 [7].

1.1.1. Primary infection

Mucosal surfaces are the major sites of primary HIV infection. HIV-1 has been shown to cross epithelial cell line barrier using transcytosis and productively infect mononuclear cells located at the basolateral side of the epithelial barrier [8]. Macrophages and dendritic cells (DCs) located in the submucosal tissues, and Langerhans cells (LCs) in mucosal epithelium are initial targets for HIV-1 infection. Both LCs and macrophages express CCR5 on their surface and fuse with R5 HIV-1 envelopes [9]. $CD4^+$ T cells can be infected directly by HIV-1 or through the infectious synapse formed at the site of contact between DCs and $CD4^+$ T cells [10]. The infected $CD4^+$ T cells migrate and spread the infection to regional lymph nodes. Within 2-3 weeks after transmission, the virus becomes well established in the lymphoid tissue reservoir and the threshold of replication is reached within 2-6 week resulting in plasma viraemia [6,11]. Primary infection and seroconversion may be associated with symptoms including fever, skin rash, oral ulcers and lymphadenopathy [11]. The peak viraemia is associated with a transient reduction in $CD4^+$ T cells in peripheral blood. Severe depletion of $CD4^+$ T cells occur in the gut-associated lymphoid tissue (GALT) which harbours the majority of T lymphocytes in the body [12-14]. Although suppression of HIV-1 by highly active antiretroviral therapy (HAART) results in near-complete restoration of blood $CD4^+$ T cells, their restoration in the gut is delayed and incomplete [12,13]. Similar massive depletion of $CD4^+$ T cells in GALT has been observed in SIV-infected rhesus macaques [15,16]. Memory $CD4^+$ T cells were the major cell population infected with SIV. In one study 30-60% of memory $CD4^+$ T cells throughout the body were infected with SIV at the peak of infection and about half of all memory $CD4^+$ T cells were destroyed directly by virus infection during the

acute phase [16]. The peak viraemia in HIV-infected individuals coincides with a vigorous HIV-specific cell mediated immune response involving both $CD4^+$ and $CD8^+$ T cells [17-19]. $CD8^+$ cytotoxic T lymphocyte (CTL) response is the first virus-specific immune response detected in acute HIV-1 infection and is the major component of the host immune response leading to the control of virus replication following primary HIV-1 infection [18,19]. Once the viraemia peak has been resolved, it reaches a set steady state level and the $CD4^+$ T cell levels return towards normal within several weeks or months of infection. Despite the significant rebound of $CD4^+$ T cell levels, it does not regain pre-infection levels [6]. Plasma virus load is a strong predictor of the rate of $CD4^+$ T cell loss and progression to AIDS [20,21]. The lower the viral set steady state level, the better the long-term prognosis for the infected individual.

1.1.2. Latency or chronic infection

The primary infection by HIV-1 is followed by a long asymptomatic chronic infection which ranges over 10-12 years. The disease is still active and progressive with virus replication in the lymphoid tissues despite absence or very low viraemia and very small number of infected cells in the peripheral blood [5,22]. The frequence of HIV-1-expressing cells was found to be 1/1000 $CD4^+$ T cells in peripheral blood [22]. During this period, there is active virus replication, clearance and continuous rounds of *de nova* infection, as well as rapid turn over of $CD4^+$ T cells [23-25]. The increased T cell turnover was proposed to be related to the strong and persistent immune activation in HIV-1 infected individuals [25]. The composite life span of plasma virus and virus producing cells is remarkably short with the half life in the order of 2±0.9 days. Approximately 30%

of plasma virus is replenished daily, which means a production rate in the range of 10^7-10^9 virions/day [23]. In one study the half life of T lymphocytes in HIV-infected individuals was less than 1/3 as long as those of HIV-seronegative individuals [26]. The same study reported no compensation by increased production of $CD4^+$ T cells [26]. Hence, the high level of HIV replication, short T cell survival time and failure to increase T cell production results in a continuous decline in $CD4^+$ T cell numbers, ultimately culminating in a state of immune deficiency most likely due, atleast in part, to impaired helper T cell functions.

1.1.3. Late stage infection or clinical AIDS

In the late stage disease, when $CD4^+$ T cell counts in blood is below 200/μl, there is a rapid increase in the rate of $CD4^+$ T cell decline. The phenotype of the virus isolated at late stage infection is different from those isolated during the chronic infection phase. Progression to AIDS is associated with emergence of HIV-1 variants that are more cytopathic *in vitro* and that replicate more efficiently in a wide variety of different human cells [27,28]. The virus isolates from AIDS patients productively infect several $CD4^+$ tumor cell lines while those isolated from asymptomatic individuals show no or only transient virus production from $CD4^+$ tumor cell lines [27]. The frequency of productively infected cells at late stage infection is over 100 fold greater than the chronic infection phase. HIV-1 clones isolated from asymptomatic individuals are non-syncytuim-inducing and mainly R5-tropic, while the isolates from patients progressing to AIDS are highly syncytium-inducing [29]. Hence, progression of HIV-1 infection is associated with a selective increase of X4-tropic HIV-1 variants in peripheral blood. This switch from

CCR5 to CXCR4 co-receptor usage results in increased infection and destruction of T lymphocytes. The severe loss of $CD4^+$ T cells results in the inversion of $CD4^+$:$CD8^+$ T cell ratio in AIDS patients [30]. Such immunocompromised individuals are highly susceptible to opportunistic infections by *Pneumocystis carinii*, *Cryptococcus neoformans*, *Candida albicans*, herpes simplex virus and cytomegalovirus among others [30,31].

1.2. Immune activation and AIDS pathogenesis

HIV infection results in a state of chronic immune activation, which is characterized by elevated markers of activation on T cells, B cells, NK cells and monocytes, as well as increased levels of proinflammatory cytokines and chemokines in plasma and lymph nodes [32]. This results in increased T cell turnover in HIV-infected individuals [25,33,34]. A direct correlation has been observed between the immune activation and disease progression in HIV infected patients [35-37]. Direct link between $CD4^+$ T cell changes and immune activation has also been reported in both acute and chronic phases of HIV infection [38,39]. The role of immune activation in AIDS pathogenesis is highlighted by studies of a cohort of sex workers in Kenya, who are exposed to repeated sexually transmitted infections resulting in immune activation, increased viremia, decline in $CD4^+$ T cell levels and rapid progression to AIDS [40]. Similarly, when SIV_{mac251}-infected macaques were repeatedly immunized with SIV-independent antigens to mimic state of chronic immune activation, the animals had significantly reduced survival compared to non-immune-stimulated control animals [41]. Further evidence come from

studies of SIV-infected sooty mangabey monkeys, natural hosts of SIV, which do not develop aberrant immune activation and immunopathologies in contrast to pathogenic SIV and HIV infections [42].

The cause of HIV-induced immune activation is multifactorial. Various *in vitro* studies have implicated HIV-1 gene products such as Env gp120 and Nef to cause immune activation [43-49]. The envelope protein gp120 can induce immune activation through binding to CD4 and correceptors, CCR5 and CXCR4 [43,44]. Nef has been shown to recruit and increase association of TCR-signaling molecules to membrane rafts and potentiate T cell activation [46,48,49]. Soluble Nef is present in the serum of HIV-1 infected individuals and is cytotoxic to human $CD4^+$ T cells [50]. Soluble Nef has been shown to induce T cell activation either through a direct affect or indirectly by inducing monocytes/macrophages to produce inflammatory factors that can in turn activate T cells [51-53]. T cells in transgenic mice expressing HIV-1 Nef in T cell populations show activated/memory phenotype [54-57]. All these experiments indicate to important role of Nef in HIV-inducted T cell activation.

HIV-1 infection is associated with increased levels of plasma lipopolysaccharides (LPS) which positively correlate with immune activation [58]. Increased level of LPS in blood is indicative of microbial translocation which could be due to massive depletion of T cells and possibly macrophages and dendritic cells from the gut resulting in disruption of gut mucosal barrier [58]. Another potential factor that could contribute to T cell activation in HIV-infected individuals is dysfunction of $CD4^+$ regulatory T cells (T_{regs}). T_{regs} were found to be susceptible to infection by HIV-1 [59]. The same study also report

profound defects in T_{regs} from HIV-infected individuals with low $CD4^+$ T cell numbers and high percentage of activated T cells [59].

HIV infection is also associated with B cell dysfunctions [60]. B cell activation in HIV infection is manifested as hypergammaglobulinemia, expansion of B cell areas of lymphoid tissues and increased expression of activation, proliferation and terminal differentiation markers on circulating B cells [61]. A role of HIV-1-infectected macrophages has been described in B cell activation [62]. HIV-1 Nef gene was shown to induce secretion of ferritin through activation of NF-κB in macrophages which was necessary and sufficient to induce B cell activation [62].

1.3. Human immunodeficiency virus-1 (HIV-1)

HIV-1 belongs to the family of retroviruses and is the etiologic agent of AIDS. According to the International Committee on Taxonomy of Viruses (ICTV), Retroviridae family is divided into Orthoretrovirinae and Spumaretrovirinae subfamilies. The six genus classified under the Orthoretrovirinae subfamily are Alpha-, Beta-, Gamma-, Delta-, Epsilon- and Lenti-viruses. HIV-1 belongs to the genus Lentiviruses. Two unique features of lentiviruses that distinguish them from other retroviruses are that they encode a number of regulatory and accessory genes not encoded by the genomes of simple retroviruses and their ability to productively infect some terminally differentiated non-dividing cells. The HIV genome is composed of 2 copies of single-stranded RNA of about 10 kb long [6]. HIV-1 genome encodes several structural, regulatory and accessory proteins. The structural proteins, Gag, Pol, and Env, are the major components of the

viral nucleocapsid and envelope. The two regulatory proteins, Tat (transcriptional transactivator) and Rev (regulator of virion gene expression) are crucial to activate transcription from the HIV-1 long terminal repeat (LTR) and transport of viral mRNAs from the nucleus to the cytoplasm, respectively. While the four accessory proteins, Nef (negative effector), Vif (viral infectivity factor), Vpr (viral protein R), and Vpu (viral protein U) play important roles in pathogenesis and immune evasion. Organization of HIV-1 genome and virion is shown in **Figure 1.2**.

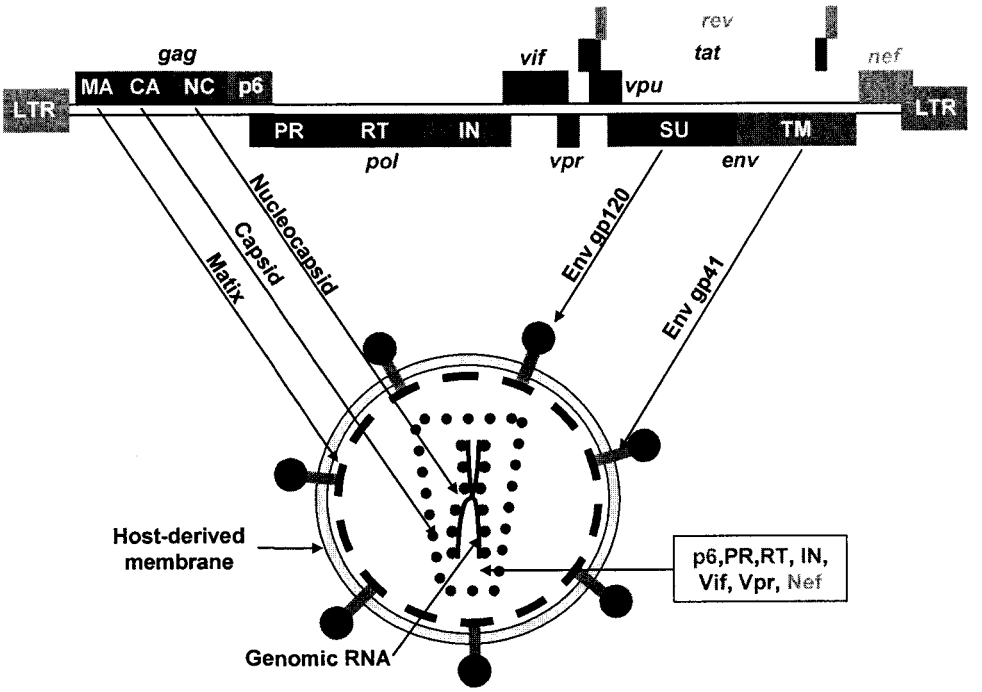

Figure 1.2. Diagramatic representation of HIV-1 genome and virion organization. Adapted from Frankel and Young, 1998 [63].

The replication cycle of HIV-1 begins with binding of virus envelope to its receptor and coreceptor on target cells. CD4 molecules on the surface of T lymphocytes, macrophages and dendritic cells serve as receptor for HIV-1 [1,4]. The coreceptors for HIV-1 include the chemokine receptors CCR5 and CXCR4 [2,3]. CD4 binding induces conformational changes in Env protein which are important for membrane fusion and entry of the virus [64]. Once inside the cell, viral RNA is reverse transcribed into proviral DNA in the cytoplasm by viral RT [63]. At this point the proviral DNA is present in a pre-integration complex (PIC). The viral proteins Gag and Vpr, present in PIC, aid in transport of this complex to the nucleus where the proviral DNA gets integrated into the cellular genome [65]. HIV-1 LTR contains variety of elements that direct binding of RNA polymerase II (RNAPII) as well as several cellular transcription factors [66]. Viral protein Tat modulates the activity of transcription factors and RNAPII at the 5' LTR of HIV-1 [67,68]. HIV-1 transcription yields multiply spliced ~2 kb, single spliced ~4 kb, and unspliced ~9 kb RNAs. The viral protein Rev mediates export of the ~9 kb unspliced and ~4 kb single spliced viral mRNAs from the nucleus to the cytoplasm [69]. Viral transcripts are translated on cellular ribosomes in the cytoplasm. Once the viral structural protein Gag and Gap-Pol are synthesized they encapsidate viral genomic RNA and assemble into core particles that are targeted to the membrane. The viral protein Env is also targeted to the membrane where interactions between Env and Gag mediate incorporation of Env in the budding virions [70,71]. Once budding is complete, the virions undergo morphological changes known as maturation which involves cleavage of Gag and Gag-Pol polyproteins by viral protease (PR) enzyme [72,73]. The mature virus is ready to start another round of infection.

HIV-1 manipulates fundamental host cell processes in complex ways to achieve optimal replicative efficiency. HIV-1 genes play important roles in viral life cycle as well as AIDS pathogenesis. Virus infectivity depends on complex interplay between viral genes and various host factors. A brief description of viral genes and their functions is presented below.

1.3.1. HIV-1 Gag

HIV-1 Gag is a 55 KDa polyprotein (Pr55Gag) which is cleaved by the viral protease shortly after budding from the host cell into mature Gag proteins p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC), p6 and two small spacer peptides, p1 and p2 [72,73]. Pr55Gag plays important role in assembly of virus and is sufficient for production of virus particles in the absence of other viral proteins [74].

MA is the N-terminal component of Pr55Gag and is important for targeting Gag and Gag-Pol precursor polyproteins to the plasma membrane. The two features of MA involved in membrane targeting are the N-terminal myristate group and a highly basic region that binds phospholipids [75]. MA is also involved in the incorporation of viral Env protein into mature virions. Mutations in the MA domain of Gag were shown to abolish incorporation of Env and the resulting mutant virions were non-infectious [71]. Subsequently a direct interaction between the MA domain and the transmembrane (TM) region of Env was demonstrated [70].

The CA domain of Gag forms the shell surrounding the viral RNA genome and core-associated proteins [73]. The CA protein is composed of an N-terminal core domain and a C-terminal dimerization domain. The core domain is involved in maturation and

incorporation of cyclophilin A (CypA) in the virion [76]. CypA loosens the CA-CA interactions, thereby facilitating dissembly after virus entry into the host cell [76]. The dimerization domain of CA is involved in Gag-Gag interactions [77]. C-terminal domain of CA has also been shown to be involved in incorporation of Gag-Pol precursor protein into the mature viral particle [78].

The NC domain of Gag is involved in encapsidation of viral genomic RNA. The RNA packaging signal (ψ site) in HIV-1 is composed of 4 stem-loops (SL1-SL4) and interacts with the NC domain [79]. In particular, the SL3 has been shown to interact with NC with high affinity [80]. By interacting with the viral genomic RNA during assembly, NC acts as an RNA chaperone and mediates RNA stability and maturation [78].

Finally, the p6 domain has been shown to be involved in the release of virus after budding from the surface of the cells. Mutations within this domain prevent release of budded virions [81]. Mutations in p6 domain also abolish incorporation of Vpr into the virions indicating the important role of p6, particularly the amino acids near the C-terminus, in this process [82].

1.3.2. HIV-1 Pol

HIV-1 pol gene product is a polyprotein which is cleaved to give rise to 3 proteins with distinct enzymatic activites: reverse transcriptase (RT), integrase (IN) and protease (PR). RT is a hetermodimer of subunits p66 and p51. Each subunit contains a polymerase domain composed of four subunits termed the fingers, palm, thumb and connection, and the p66 contains an additional RNase H domain [83-85]. RT catalyses both RNA-dependent and DNA-dependent DNA polymerization reactions, and converts viral

genomic RNA to proviral DNA that will be integrated into the host genome [63]. Reverse transcription is initiated from a $tRNA_{Lys}$ which acts as the primer and also induces conformation changes in p66 and stimulation of enzymatic activity [86]. The RNaseH domain functions to cleave the RNA portion of RNA-DNA hybrid generated during the reverse transcription [63]. Mistakes during DNA polymerization and the strand transfers in reverse transcription reactions are partly responsible for the generation of quasi-species and retroviral variations [87]. HIV-1 RT fidelity was found to be higher in RNA-dependent compared to DNA-dependent DNA synthesis reactions [88]. An *in vivo* mutation rate of $3.4X10^{-5}$ mutations/bp/cycle has been reported for HIV-1 [89]. This is about 20 fold lower than the error rate of purified HIV-1 RT calculated *in vitro*, suggesting that HIV-1 reverse transcription is not as error prone as predicted from in vitro fidelity studies of purified RT [89].

IN is active as an oligomer and catalyses the integration of proviral DNA into the host geneome [63]. IN monomers are composed of an N-terminal domain that forms dimers, the catalytic domain and the C-terminal domain with nonspecific DNA-binding activity. Mutations within the C-terminal domain abolish both integration and processing activity but not the disintegration activity, a property of the isolated catalytic domain [90]. This suggests that the C-terminal domain does not have catalytic activity and is not the only DNA-binding domain in the protein and supports a model involving separate viral and target DNA-binding sites on IN [90]. From the structural studies, the C-terminal was proposed to bind, bend and orient viral DNA during integration [91]. Mutations within the central catalytic domain can have pleiotropic effects on viral replication [92].

PR is an aspartyl protease responsible for processing of Gag and Pol polyproteins, a step crucial for maturation of budding virions [63]. Mutation of the aspartic acid residue in the catalytic site of PR abolishes enzymatic activity and mutant virions are non-infectious [93]. Cleavage of viral Gag and Gag-Pol polyproteins by viral PR initiates at the membrane of the infected cell during virus budding and release. Besides helping with maturation of virus, PR activity is also required for efficient release of budding virions [94].

1.3.3. HIV-1 Env

The Env glycoprotein, also termed gp160, is synthesized as a polyprotein precursor molecule which is cleaved into a noncovalently associated gp120 surface (SU) and gp41 transmembrane (TM) subunits [95-97]. The Env protein contains domains which mediate subunit-subunit recognition, interaction and assembly [98]. Mature oligomeric Env protein is transported to the membrane where they are incorporated into the budding virions. The TM subunit of Env interacts with the MA domain of Gag and this is involved in the incorporation of Env in the budding virions [70,71]. The gp120 contain domains which are highly conserved (C) between virus isolates, as well as hypervariable (V) domains [95]. These domains binds to CD4 on the surface of the target cells and the CD4-binding determinants of Env have been mapped to the C2, C3 and C4 conserved regions of gp120 [99]. CD4 binding induces conformational changes in gp120 which are important for membrane fusion and entry of the virus [64]. Env protein is also involved in determining the tissue tropism of the virus. Overlapping regions of gp120 was shown to determine T cell- or macrophage tropism [100]. This involves interaction with the

chemokine receptors CXCR4 and CCR5, which is determined by the hypervariable V1/V2 and V3 regions of gp120, respectively [101].

1.3.4. HIV-1 Tat

HIV-1 Tat is a 14-16 kD protein that is required for virus replication. Tat binds to specific sequences of TAR (Transactivation response) elements located in the R region of the 5' LTR of viral RNA [102]. Tat protein consists of five different domains: the N-terminal domain, the cysteine-rich domain, the core, the basic domain and the C-terminal domain. The cysteine-rich domain is responstible for intra-molecular disulfinde bridge formation, whereas the basic region contains signals for nuclear localization and binding to TAR-RNA [67]. Tat regulates viral gene expression by modulating the activity and association of cellular transcription factors with RNA polymerase II (RNAPII) at the 5' LTR [67,68]. Tat associates with RNAPII complexes during early transcription initiation and elongation after promoter clearance and before synthesis of the full-length TAR RNA transcript [103]. Two Tat molecules appear to be involved in performing various functions during single round of HIV-1 mRNA synthesis [103]. Tat also regulates the expression of a number of cellular genes and plays a key role in AIDS progression and the development of AIDS-associated malignancies, such as Kaposi's sarcoma [104,105] and the colorectal cancers in long-term HIV/AIDS survivors [106], by interfering with cellular processes such as proliferation, differentiation, and apoptosis [107,108]. Tat has been shown to inhibit transcription of p53 [109]. In hibition of p53 activity in presence of Tat is associated with loss of p21/waf1 resulting in loss of G_1/S checkpoint and inappropriate entry in S phase leading to apoptosis [110]. Besides these functions in the infected cells, Tat can also affect

uninfected cells away from the site of infection. Tat is released from infected cells and interacts with different cell membrane-associated receptors such as the chemokine receptors [111,112]. Extracellular Tat is internalized through cell membrane lipid rafts mediated caveolar endocytosis [113]. The protein also has been shown to modulate reverse transcription (RT) reaction by two different mechanisms. First, it can promote placement of tRNA on the viral RNA and secondly, it can suppresses RT activity at late stages of the viral life cycle. The cysteine-rich and core domains of Tat are responsible for suppression of RT reaction to prevent premature reverse transcription of viral RNA in the cytoplasm and facilitate packaging of intact genome [114].

1.3.5. HIV-1 Rev

Rev is an 18 kD protein involved in transport of viral RNAs from the nucleus to the cytoplam. HIV-1 transcription yields multiply spliced ~2 kb, single spliced ~4 kb, and unspliced ~9 kb RNAs [63]. While the ~2 kb transcripts are able to pass through the nuclear membrane and enter the cytoplasm, the ~4 kb and ~9 kb RNAs need to be actively transported through the nuclear membrane. Rev binds to the Rev response element (RRE) in multimeric form and promotes the nuclear export of the ~9 kb unspliced and ~4 kb single spliced viral mRNAs [69]. Rev contains RNA-binding domain (RBD) and an arginine-rich nuclear localization signal (NLS) sequence in its N-terminal domain, and a nuclear export signal (NES) in the C-terminal [115,116]. The NLS is required for transport of Rev from cytoplasm to the nucleus for its mRNA export function. The RBD of Rev binds to the RRE sequence, which is a 351 nucleotide RNA structure present within the *env* intron of unspliced and singly spliced viral mRNAs [117]. Phosphorylation

of Rev at Ser5 and Ser8 sites by casein kinase II has been shown to enhance Rev-RRE interaction [118,119]. Rev has been shown to bind to a number of cellular proteins known to be involved in the nuclear export of viral mRNA species [120]. Rev-mediated nuclear export of inton-containing RNA species is different from nuclear export of cellular mRNAs because most cellular mRNAs exit the nucleus after being fully spliced. However, the mechanism seems to be similar and linked to the Ran-GTPase cycle [121]. The interaction between Rev NES with CRM/exportin 1 transport receptor is essential to Rev-mediated export of viral RNAs. Following Rev-RRE-CRM association, this complex migrates to the nuclear pore complex (NPC), where the various nucleoporins bind Rev and allow translocation into the cytoplasm [122].

1.3.6. HIV-1 Vif

Vif is a 23 kD basic protein that is expressed late during infection. It contributes to viral infectivity and is essential for virus replication in major target cells, namely $CD4^+$ T cells and macrophages, and various T cell lines, which are non-permissive for Vif-deleted HIV-1 [123]. Vif is largely localized within the cytoplasm in soluble form or associated with the cytoplasmic side of the cell membrane [124]. The C-terminus of Vif is essential for its association with the membrane and its functions. Soluble cytosolic form of Vif is closely associated with the cytoskeletal component, intermediate filament vimentin [125]. Vif is also incorporated in the virus particles and is associated with the inner core of the virus [125]. HIV-1 Vif is phosphorylated at Thr96, Ser144, Thr155, Ser165 and Thr188 sites [126,126]. Phosphorylation at Thr96 and Ser165 are by the ERK1 and ERK2 mitogen-activated protein kinases [126]. Phosphorylation at Ser144 is important

as mutation of this residue to alanine resulted in a loss of activity and an almost complete inhibition of viral replication [127].

Vif has been reported to bind to a number of cellular proteins, including tyrosine kinase (Hck), a component of the HIV-1 PIC, Ku70 and Sp140 nuclear protein [128,129]. Hck has been shown to inhibit HIV-1 production and infectivity in Vif-defective virus, while having no effect on the wild-type virus in single-cycle replication assays [128]. Similarly, Sp140, an IFN-gamma-inducible protein, has been shown to be present in all non-permissive cells and Vif partially localizes Sp140 to the cytoplasm from the nucleus [129].

A cellular protein termed CEM15, identified as apolipoprotein B mRNA editing enzyme APOBEC-3G, was shown to impart resistance to Vif-deficient HIV-1 infection in the non-permissive cells. Introduction of this protein in the permissive cells rendered them non-permissive to infection by Vif-deficient HIV-1 and this antiviral action is overcome in the presence of Vif [130]. APOBEC-3G is a cytidine deaminase and induces guanine to adenine hypermutation in the plus-strand of newly synthesized viral DNA [131,132]. These hypermutations adversely affect the expression and function of viral gene products. Vif prevents hypermutation of virus DNA by APOBEC-3G. Vif has been shown to form a complex with human CEM15 and prevent its encapsidation into newly synthesized virions [133]. Vif prevents APOBEC-3G encapsidation by inducing its post-translational degradation through ubiquitination and targeting to the 26S proteasome via its SLQ(Y/F)LA motif [134,135]. Subsequently, Vif was shown to interact with cellular protein Cul5, elongins B and C, and Rbx1 to form an Skp1-cullin-F-box (SCF)-like complex capable of inducing ubiquitination and degradation of APOBEC-3G. The

ability of Vif to suppress antiviral activity of APOBEC-3G was specifically dependent on Cul5-SCF function [136].

1.3.7. HIV-1 Vpr

Vpr is a 15 kD regulatory protein which is predominantly present in the nuclear matrix of HIV-1 infected PBMCs [137]. Vpr is also packaged in the HIV-1 virion which requires the p6 and NC sequences of Gag protein [137]. Once viral RNA is reverse transcribed after infection, the viral DNA and associated viral proteins, termed the pre-integration complex (PIC), must enter the nucleus for subsequent steps in viral replication. The two major functions of Vpr are transport of the PIC into the nucleus and induction of G2 arrest [138]. Both the Gag MA and Vpr impart nucleophilic properties on the PIC and allow transport of this complex to the nucleus of non-dividing cells such as macrophages [65]. While the nuclear localization property of Vpr correlates with its ability to target PIC to the nucleus early in the infection, the G2-arrest phenotype correlates with its ability to activate viral transcription after the infection is established [139]. The nuclear localization and cell cycle arrest functions of Vpr are not interrelated and are mediated by separate functional domains. The amino acid terminal alpha-helical region of Vpr is required for its nuclear localization properties and the C-terminal basic domain controls the cell cycle arrest by Vpr [140,141]. Vpr has been shown to associate with DDB1- and Cullin4A-containing ubiquitin-ligase complex through VprBP/DCAF-1 [142-146]. This association is important for Vpr mediated G2 arrest of cell cycle. Vpr can also retard cell proliferation independent of G2 arrest of the cell cycle. While C-terminally truncated

Vpr fails to induce G2 arrest, it can still cause G1 arrest and retain the ability to prevent cell proliferation [147].

In addition to nuclear localization and cell cycle arrest, Vpr is also involved in transactivation of the HIV-1 LTR and other cellular promoters, increased viral infectivity and apoptosis [120]. Extracellular Vpr is present in the sera and cerebral splinal fluid (CSF) of HIV-1 infected individuals and is effectively transduced across the cell membrane leading to G2 arrest and apoptosis [148]. Additionally, Vpr inhibits IL-12 production by enhancing glucocorticoid activity, thereby suppressing innate and cellular immunities of infected individuals [149].

1.3.8. HIV-1 Vpu

Vpu is a 17 kD oligomeric integral membrane phosphoprotein [63]. Vpu distinguishes HIV-1 isolates from HIV-2 and SIV since only HIV-1 genome encodes for this protein [150]. The two major functions of Vpu in the viral life cycle are degradation of CD4 receptors and enhancement of virion release from infected cells [120]. These two activities of Vpu are mediated by separate functional domains [151]. Vpu with deletion of the cytoplasmic domain is unable to induce CD4 degradation but has residual biological activity for virus release. The N-terminus of Vpu, encoding the transmembrane (TM) anchor, represents the active domain important for regulation of virus release [151]. Vpu can induce degradation of both glycosylated as well as non-glycosylated membrane-associated CD4 molecules by specifically targeting sequences in the cytoplamic domain of CD4 [152]. To enhance virus release, Vpu counteracts an assembly restriction that is

present in human cells [153]. This assembly restriction has been identified to be mediated by host protein CD317 also known as BST2 and termed as tetherins [154].

1.3.9. HIV-1 Nef

The 27-35 kDa regulatory Nef protein is conserved in HIV-1, HIV-2 and SIV. Myristoylation of Nef protein at its 5' end leads to its localization at the plasma membrane [155]. Messenger RNA (mRNA) coding for Nef protein is detected early after infection of lymphocytes and monocytes. Nef-coding mRNA is detected as early as 4 hours after infection and significantly increased by 12 hours post-infection [156]. Also Nef expression predominates over other regulator genes such as *tat* and *rev*. Various functions of Nef include downregulation of surface molecules such as CD4, CD28, MHC-I and MHC-II, modulation of TCR-mediated signaling, modulation of cell survival and enhancement of virus infectivity [157]. Role of Nef in AIDS pathogenesis and its functions are described in more detail in the following sections.

1.4. Nef: Structure and Function

The Nef proteins of HIV-2 and SIV are slightly larger, containing approximately 250 amino acids, than the 206 amino acid HIV-1 Nef protein. When *nef* sequences of 186 different strains of HIV-1 from five different subtypes were compared, the sequence conservation was high with an average sequence identity of 84% and an average similarity of 89% [158]. The full length HIV-1 Nef structure consists of six α-helices (α 1- α 6) and a β-pleated sheet of five antiparallel β-strands (β 1- β 5) [158]. The structure of

HIV-1 Nef and domains involved in the interaction with various host proteins is shown in **Figure 1.3** [158]. The Nef structure is partitioned in 4 units: a flexible myristoylated membrane anchor region of variable length (1-56), followed by the PxxP loop (57-80), the core domain (81-206) and a C-terminal flexible loop (148-180). Nef protein has a considerably large solvent-exposed surface area due to its high degree of less folded, flexible regions [158]. The large accessible surface in combination with the high portion of flexible regions may account for the numerous protein interactions that have been reported for Nef. The cellular interacting partners of Nef and the Nef domains involved in these interactions are listed in **Table 1.1** [157,159].

Initially Nef was thought to be a negative regulator of HIV replication. Nef protein suppressed replication of wild-type and *nef* mutant proviruses, and the expression of reporter genes linked to HIV-1 long terminal repeat (LTR) [160]. In lymphoid cell lines, the presence of Nef was shown to suppress replication of some strains of both HIV-1 and HIV-2 [161]. This was thought to be important for establishment of HIV latency. It was also shown that myristoylation of Nef is required for efficient suppression of transcription for HIV-1 LTR [155]. However, these early findings were not supported by further studies that showed the important role of Nef in AIDS pathogenesis [157].

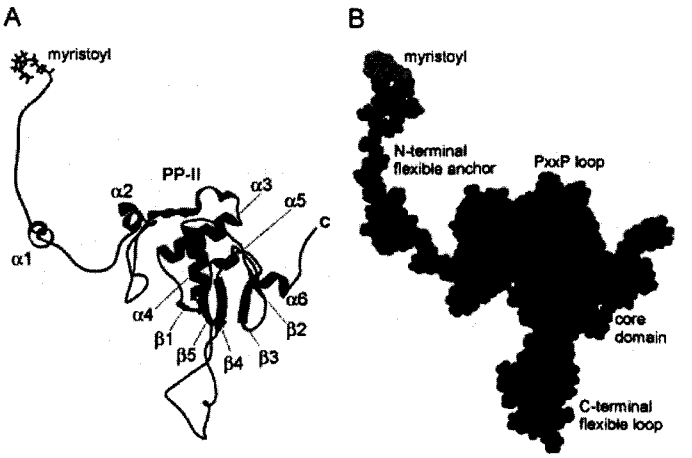

Figure 1.3. Structure of HIV-1 Nef. (A) Ribbon presentation. (B) Molecular surface presentation. Used with permission [158].

Table 1.1. Cellular proteins known to interact with Nef

Cellular protein	Critical domain(s) of Nef	Associated protein domain(s)	References
CD4	$MGxxxS_1$, WL_{57}, L_{110}, FPD_{121}, $D/ExxxLL_{165}$, EE_{154}, DD_{174}	Cytoplasmic tail	162-164
CD28	$MGxxxS_1$	Unidentified	165
MHC-I	$MGxxxS_1$, M_{20}, $EEEE_{62}$, PxxP, FPD_{121}, EE_{154}	Cytoplasmic tail	166-169
MHC-II	$MGxxxS_1$, $D/ExxxLL_{165}$, $EEEE_{62}$, PxxP	Unidentified	170-172
Hck	PxxP	SH3 domain	173
Lck	PxxP	SH3 and SH2 domains	174,175
Fyn	PxxP	SH3 domain	176,177
Lyn	PxxP	SH3 domain	176,177
Src	PxxP	SH3 domain	176,177
PI3K	C- and N-termini	p85 subunit	178
PAK 1/2	PxxP Leu_{112}, RR_{105}, FPD_{121}	Unidentified	179,180
Vav	PxxP	SH3 domain	181
MAPK	PxxP	SH3 domain	182
PKCθ isoform	Unidentified	Unidentified	183
Raf1 kinase	$DDPxxE_{174}$	Unidentified	184
TCRζ chain	PxxP	Unidentified	185,186
ASK1	Unidentified	Unidentified	187
p53	N-terminus (1-57)	Unidentified	188
PACS1	$EEEE_{62}$	Unidentified	189
PACS2	$EEEE_{62}$	Unidentified	190
Rack1	N-terminus and central core	C-terminus	191
IP3R	Unidentified	Unidentified	192
β-COP	EE_{154}	Unidentified	193,194
AP-1/2/3	D/ExxxLL	μ and β1 subunits	195
Thioesterase	FPD_{121}	Unidentified	196
V1H	DD_{174}	Unidentified	197

1.4.1. Nef as a determinant of AIDS Pathogenesis

Infection of rhesus monkeys with SIV_{mac239} having a premature stop signal at the 93rd codon of *nef* results in low viral load during the course of persistent infection and reduced pathogenesis. Revertants with a coding codon at this position came to predominate in infected animals suggesting a strong selective pressure for the functional form of Nef *in vivo* [198]. Nef deletions and mutations have been reported in long-term survivors with nonprogressive HIV-1 infection [199-204]. Kirchhoff and coworkers amplified HIV-1 *nef* sequences from peripheral blood mononuclear cells (PBMC) of five patients with long-term nonprogressive HIV-1 infection and found defective forms of *nef* in one patient [202]. Among various deletions in different mutant, the 118 bp deletion removing a highly conserved acidic domain and a highly conserved (Pxx) motif, and placing downstream sequences out of frame was the predominant deletion observed. Deacon and coworkers detected deletions in the *nef* gene and the region of overlap between *nef* and the U3 region of the LTR in blood donor infected with HIV-1 and a cohort of six blood recipient infected from this donor with stable and normal CD4 lymphocyte counts 10 to 14 years after infection [200]. Deletions located in *nef* and the *nef*/U3 overlap region has also been reported in a long-term nonprogressor who acquired HIV-1 infection through sexual route [204]. Mariani and coworkers characterized *nef* alleles obtained from PBMCs of four long-term nonprogresser hemophiliacs that had acquired HIV-1 from contaminated factor VIII concentrates [203]. Majority of *nef* sequences from three of the four patients were functional for CD4 downregulation. In one of the patients, the Nef protein recovered early in the infection was able to downregulate CD4 but Nef proteins recovered at various time points over 12 year period were non-

functional. Truncated Nef proteins were also detected in asymptomatic patients of HIV-2 infection [199]. Out of the 60 infected individuals 6 had truncated Nef proteins, which is at a higher prevalence than the HIV-1 infected cohorts of long-term nonprogressors. Although these studies demonstrate the association of a particular HIV gene defect and absence of disease progression, defects in Nef are not responsible for most of the cases of nonprogression.

The importance of Nef in AIDS pathogenesis has also been demonstrated in animal models of AIDS. Expression of HIV-1 Nef in T cells of mice leads to CD4 cell surface downregulation and altered T cell activation [56,57]. Expression of selected HIV-1 genes in $CD4^+$ T cells and the cells of monocyte/macrophage lineage revealed the pivotal role of Nef in pathogenesis of AIDS-like disease induced by HIV-1 in transgenic mice [54]. In this study the AIDS-like disease characterized by weight loss, diarrhea, wasting, premature death, thymus atrophy, loss of $CD4^+$ T cells, interstitial pneumonitis and tubulo-interstitial nephritis closely resembling human AIDS, was induced in transgenic mice by HIV-1 *nef* gene alone. Similarly, expression of SIV Nef in $CD4^+$ T cells and the cells of monocyte/macrophage lineage also results in severe AIDS-like disease in transgenic mice [205]. HIV–1 Nef was also shown to affect replication and pathogenicity of HIV-1 in fetal thymus and liver implants in severe combined immunodeficient (SCID-hu) mice [206,207]. Nef inactivation resulted in decreased replicative potential and severely attenuated pathogenic potential in SCID-hu mice. Duus and coworkers showed in SCID-hu mice that the replication activity of HIV-1 clone derived from an accidentally infected laboratory worker isolate (HXB/LW) was separable from its pathogenicity [181]. HXB/LW encodes defective *nef* and *vpr* genes, replicates to high level in SCID-hu mice but has

significantly reduced pathogenic activity. Restoration of *nef* ORF in HXB-LW resulted in enhanced pathogenic potential at levels observed in NL4-3 infection of SCID-hu mice with no significant effect on its replication. This suggests that replication of HXB/LW is genetically separable from its pathogenic activity [181].

1.4.2. Role of Nef in Viral Infectivity

The mechanisms by which Nef influences viral replication and infectivity is a subject of intense research. Positive influence of HIV-1 Nef on viral infection and replication in primary and established cell lines has been demonstrated by various studies. When mitogen-activated PBMCs are infected with HIV-1, the structural viral protein Gag p24 is detected earlier than the infection with Nef mutant HIV-1 [208]. A positive affect of Nef on viral replication was also observed in primary macrophages infected with R5-tropic clone of HIV-1. Dependence on intact Nef function is more pronounced at low multiplicity of infection (MOI) [208]. Nef also confers a positive growth advantage to HIV-1 in $CD4^+$ lymphocytes [209]. Transfection of T-lymphoblastoid cell line CEM with infectious clones of HIV-1 with Nef mutation results in 100 fold less p24 production compared to wild-type virus [210]. In the same study, mutation in Nef resulted in 3 to 5 fold decrease in p24 production in single-cycle infection but equal amount of p24 in single-cycle transfections suggesting Nef-independence after provirus is established. The myristoylation signal is important for this function and the positive influence of Nef on viral growth rate was due to the infectivity advantage conferred to the virus by an intact *nef* gene [210]. Using truncated or deleted versions of Nef, Aiken and Trono showed that Nef protein increased viral infectivity by 4 to 40 fold [211]. The

infectivity of Nef-deleted virus was enhanced by providing the protein in virus producer cells but not target cells indicating Nef effect at the stage of viral particle formation. In trans-complementation assays, Nef proteins from a number of primary HIV-1 isolates and to a lesser extend from HIV-2 ST and SIV_{mac239} enhanced infectivity of Nef deleted HIV-1. Nef was also shown to exert a positive influence on HIV-1 replication by stimulating proviral DNA synthesis. The synthesis of both minus-strand strong stop DNA and full-length double-stranded DNA was impaired in the absence of Nef [211]. Since both early and late steps of proviral DNA synthesis are affected, Nef does not seem to affect stand transfer, but instead augments the efficiency of reverse transcription [211]. Similar to HIV-1 Nef, SIV Nef also accelerates SIV replication in human and rhesus PBMCs, which correlates with greater infectivity of Nef producing virus [212]. HIV-1 and SIV Nef are functionally interchangeable. Substitution of HIV-1 Nef with SIV Nef and *vice versa*, enhances viral infectivity and replication of the recombinant virus compared to its Nef–defective counterpart [212]. Nef has been shown to physically interact with Tat and enhance Tat-induced HIV-1 LTR-mediated gene expression [213]. The enhancement of Tat-induced HIV transcription by Nef was found to depend on association of Nef with Lck, serine kinase and a polycomb group protein Eed in Nef-associated kinase complex (NAKC) [214,215]. Subsequently, a host facter, hnRNP-k or K protein, was shown to interact with Nef in the context of NAKC and activate Lck and Erk1/2, resulting in enhancement of Tat-mediated HIV transcription [216]. $CD4^+$ T cells of HIV-sera negative Ethiopian individuals (ETH) exhibit elevated levels of surface activation markers CD45RO and HLA-DR as well as the chemokine receptors CCR5 and CXCR4 making them highly susceptible to HIV-1 infection *in vitro* without the need for stimulation by

mitogen [217]. Peripheral blood lymphocytes from ETH individuals support significant level of Nef-defective HIV-1. This supports the notion that Nef enhances the basal level of T cell activation and consequently, viral replication [217].

1.4.3. Nef-mediated CD4 downmodulation

CD4 is a 55 kDa cell surface glycoprotein and primary cellular receptor for HIV and SIV. Downmodulation of cell surface CD4 is well studied function of Nef and was demonstrated for the first time in $CD4^+$ T cell line (CEM T4) infected with HIV-1 Nef [218]. Downregulation of CD4 may be important for HIV replication and pathogenesis in many ways. Firstly, CD4 downmodulation may enhance HIV replication by preventing detrimental superinfection events [219]. Secondly, high levels of CD4 interfere with HIV budding [219]. Finally, this function of Nef may represent a strategy to control signaling events in the infected cells [219]. Nef downregulates cell surface CD4 expression without affecting steady-state levels of CD4 mRNA and CD4 translation [220]. Mutations of CD4 intracellular serine residues at positions 408, 415 and 431, prevent phorbol ester-induced downregulation of CD4. However, this mutated CD4 is downmodulated by Nef indicating a different mechanism of CD4 cell surface downmodulation by HIV Nef [220]. Nef is also involved in cell surface CD4 downregulation in the context of viral infection. CD4 downregulation occurs considerably faster in CEM T lymphocytes infected with the wild-type HIV-1 strain, compared to *nef*-mutated virus or HIV-1 mutant producing a nonmyristoylated form of Nef [221]. SIV Nef has also been shown to downregulate cell surface expression of CD4 [222].

Both SIV and HIV-1 Nef alter the stability of CD4 protein, which can be prevented by treatment of cells with chloroquine, primaquine, NH_4Cl and monensin known to neutralize and elevate the pH of acidic compartment such as lysosomes [221-223]. Nef induces CD4 degradation late in the biosynthetic pathway without affecting its transport and glycosylation through the endoplamic reticulum and *cis*-Golgi [222]. Downmodulation of surface CD4 depends on signals present within 20 amino acid long membrane-proximal cytoplasmic domain of CD4 molecule [221]. Surface expression of a chimeric molecule with the extracellular and transmembrane regions of CD8 linked to the 20 amino acid long membrane-proximal residues of the CD4 cytoplasmic domain ($CD884_{STOP418}$) is efficiently downregulated by HIV-1 Nef. A dileucine motif at position 413 and 414 within this domain is critical for CD4 downregulation by Nef. Mutation of the dileucine motif completely abolishes downregulation of CD4 and $CD884_{STOP418}$ chimeric molecules [221]. Using heteronuclear NMR spectroscopy, Grzesiek and coworkers showed that this cytoplasmic domain of CD4 binds directly to Nef without any accessory proteins. This binding is specific as it delineates a well-defined area on the surface of Nef and is abrogated when the central dileucine motif is mutated [162]. The regions of Nef important for CD4 downmodulation are restricted to the C- and N-terminal parts of the molecule in both HIV-1 and SIV. Mutations located between amino acid residues 36 and 56 in the N-terminal part and between residues 174 and 179 in the C-terminal part of Nef abrogate its ability to downregulate surface expression of CD4 [163].

Nef increases the rate of internalization of CD4 from cell surface [164,221,223,224]. The increased endocytosis of CD4 in Nef producing cells is abrogated by mutation of dileucine in CD4 cytoplasmic tail [224]. Substituting HIV-1 Nef for the cytoplasmic

domain of an integral membrane protein with intact extracellular and transmembrane domains, such as CD4 (44Nef) and CD8 (88Nef), is sufficient to direct the resulting molecule to the endocytic pathway [224]. Pulse-chase experiments showed that shortly after synthesis, CD4 molecules were delivered to cell surfaces and accumulated there with similar kinetics in the presence and absence of Nef. Following delivery on cell surface, CD4 molecules are rapidly internalized in presence of Nef [223]. However, Mangasarian and coworkers showed that Nef can induce intracellular retention of CD4 molecules to some degree besides accelerated endocytosis from cell surface and this was more pronounced for 44Nef chimeric molecules [224]. When CEM cells expressing HIV-1 Nef were labeled with CD4 specific monoclonal antibody, the surface staining was abrogated and multiple cytoplasmic fluorescent dots were observed. In Nef expressing cells, CD4 accumulated in acidic intracellular vesicles that stained with transferring-rhodamine, a specific marker of early endosomes, indicating that CD4 molecules accumulated in early endosomes in presence of Nef [164]. Immunogold electron microscopic studies of Namalwa B lymphoid cells stably producing CD4 and Nef or 44Nef chimera showed a significant increase in the recruitment of CD4 and 44Nef molecules into the clathrin-coated pits. 44Nef chimeric molecule also accumulated in crescent-shaped intracellular compartment, which partly overlapped with Golgi complexes stained with giantin-specific antibody, consistent with increased intracellular retention of this molecule [224]. Nef interacts with the μ chains of adaptor complexes, key components of clathrin-coated pits [195]. The adaptor complex μ2 chain interacts strongly with HIV-2 and SIV Nef but weakly with HIV-1 Nef in a yeast two hybrid assay. The 59 N-terminal residues of SIV Nef, containing a double tyrosine motif at positions 28 and 39, are sufficient for this

interaction. Despite the lack of interaction with adaptor complexes and inhibiting increased CD4 endocytosis, the double tyrosine mutation does not completely abrogate CD4 downregulation by SIV Nef. This mutant Nef was shown to block recycling of internalized CD4 to the cell surface. Recycling of CD4 is more efficient by full-length SIV Nef suggesting that the signals responsible for the lysosomal targeting activity of SIV Nef must be downstream of amino acid 59 and genetically separable from its endocytosis activity [195]. Although μ2 chain interacts weakly with HIV-1 Nef, a GST-HIV-1 Nef fusion protein could capture *in vitro* translated μ2 as well as adaptor complexes from the cytoplasm of human lymphoid T cells suggesting that μ2 may also be a downstream mediator of HIV-1 Nef action [195]. Another factor demonstrated to be involved in endocytosis of CD4 molecules by HIV-1 Nef was the catalytic subunit of Vacuolar ATPase (V-ATPase), which is required for acidification of endosomes and lysosomes [197]. The internalization of Nef and CD4 correlates with the ability of Nef to bind V-ATPase, and required the ED residues near the C-terminus of Nef. This binding may depend on a larger part of the C-terminal flexible loop rather than a specific single short motif as was shown for SIV Nef [225]. Antisense V-ATPase and dominant negative clathrin blocked internalization and drastically reduced the rate of endocytosis of CD8Nef chimera. These experiments showed that the catalytic subunit of V-ATPase capable of binding to Nef participates in internalization and endocytosis of CD4 by Nef via clathrin-coated pits [197]. Once CD4 is internalized it must be targeted to lysosomal degradation or recycled back to the cell surface. Nef targets CD4 to lysosomal degradation by acting as a connector between the receptor and the endosomal compartment [193]. This is achieved through interaction of Nef with β-COP, an essential

component of molecular machinery of membrane trafficking [193,194]. Mutation of a highly conserved acidic dipeptide (EE^{115}) in a C-terminal disordered loop of Nef abrogates this interaction and the ability of Nef to target CD4 from the early to the late endosomal compartments [193]. Nef has also been shown to bind to human thioesterase II protein and this interaction is important for CD4 downregulation, but the exact role of thiosterase II in this process is not known [196].

1.4.4. Nef-mediated downregulation of CD28 cell surface expression

Nef downregulates cell surface expression of CD28, a major co-stimulatory receptor required for maximal T cell activation [226,227]. Multiple variants of SIV Nef are capable of downregulating CD28 cell surface expression, which is dependent on the C-terminal dileucine motif of Nef [227]. Mutations that abolish the interaction of Nef with the AP-2 clathrin adaptor disrupts CD28 downregulation and also CD28 co-localization with AP-2 and Nef suggesting that Nef induces CD28 endocytosis through an AP-2 clathrin adaptor-dependent pathway [165]. Both SIV and HIV-1 Nef induce endocytosis of CD28 and utilize partially overlapping but distinct sets of amino acid residues in the membrane-proximal region of CD28 cytoplasmic domain. The ability of Nef to downregulate CD28 and CD4 cell surface expression are genetically separable [165]. It is hypothesized that by reducing availability of CD28 at the cell surface, Nef impairs the ability of $CD4^+$ T cells to respond to antigenic stimulation and return to a resting state which is still able to support viral replication [227]. Alternatively, the downregulation of surface CD28 may limit the adhesion of infected $CD4^+$ T cells to the APC following a productive antigen presentation event. This promotes disengagement of the activated T cell from the APC

and its subsequent movement into circulation or to other APCs thus facilitating the spread of the virus [165].

1.4.5. Downregulation of Major Histocompatibility Complex-I (MHC-I) surface expersion by Nef

Nef, from various strains of HIV-1, HIV-2 and SIV, downregulates surface expression of MHC-I [166,228]. Whereas MHC-I complex synthesis and transport through the endoplasmic reticulum and *cis*-Golgi apparatus occur normally in Nef expressing cells, surface MHC-I molecules are rapidly internalized and accumulate in endosomal vesicles where they are degraded. By reducing surface levels of MHC-I, HIV can evade immune responses and promotes survival of HIV infected cells [228]. Nef promotes accumulation of MHC-I molecules in γ-adaptin positive trans-Golgi compartment and in transport vesicles containing AP-1 adaptor complexes [166,167]. Since Nef mutants defective in CD4 downregulation retain the ability to downregulate MHC-I, Nef employs different mechanisms to induce the endocytosis of MHC-I complexes and CD4 [167]. Mutations that disrupt the SH3 domain-binding surface of Nef also disrupt MHC-I downregulation. This indicates the involvement of SH3 domain-containing proteins in this process. Besides the SH3 domain-binding surface of Nef a cluster of acidic amino acid residues at positions 62-65 of Nef ($EEEE_{65}$) are also required for the internalization and accumulation of MHC-I molecules in the *trans*-Golgi [167]. Downregulation of MHC-I complexes also requires a tyrosine residue (Y_{320}) in the cytoplasmic domain of MHC-I heavy chain [166,167]. Unlike HLA-A and –B, HLA-C molecules carry a cysteine at position 320 instead of tyrosine, hence, its surface expression is not modulated by Nef [166]. HIV-1

Nef binds to the cytoplasmic tail of MHC-I molecules that are downregulated by Nef (HLA-A) in Y_{320}-dependent manner [168]. Although HIV-1 and SIV Nef seem to use similar mechanisms to downregulate MHC-I expression, they use different surfaces for the molecular interactions that are required for this function [169]. The ability of SIV_{239} Nef to downregulate MHC-I requires a unique C-terminal region in SIV Nef which is not found in HIV-1 Nef and the PxxP helix in SIV Nef is dispensable for this function. This C-terminal region of SIV Nef is also dispensable for the interaction with clathrin adapters and is not required to enhance viral replication and infectivity [169]. The exact mechanism of MHC-I downregulation by Nef is not completely known and is still a matter of controversy. Nef seems to relocalize surface MHC-I to TGN through the PACS-1-dependent protein-sorting pathway [189]. Nef interacts with PACS-1 and this interaction requires the cluster of acidic amino acid residues $EEEE_{65}$ towards the N-terminal of Nef protein. A chimeric intergral membrane protein, containing Nef as its cytoplasmic domain, localizes to the TGN after internalization in an acidic-cluster-dependent and PACS-1-dependent manner. Mutations in other determinants of Nef shown to be essential for MHC-I downregulation, such as the N-terminal α-helix and the proline-rich repeat [167], does not disrupt targeting of the chimeric intergral membrane protein containing Nef as its cytoplasmic domain to the TGN indicating that they are probably involved in some other steps necessary for MHC-I downregulation [189]. A model of HIV-1 Nef mediated MHC-I downregulation was proposed by Blagoveshchenskaya and coworkers [229]. The model suggested a hierarchy of the Nef sorting motifs in MHC-I downregulation that is initiated with binding of the $EEEE_{65}$ motif to PACS-1 and targeting of Nef to the TGN The TGN targeting is required for the PxxP-mediated

PI3K/ARNO-catalyzed activation of ARF6 and subsequent MHC-I endocytosis. Finally, M_{20} is required to sequester internalized MHC-I from the ARF6 endosomes to the TGN, thus preventing efficient recycling of MHC-I to the cell surface [229]. However, this model was challenged by Larsen and coworkers, who showed that ARF6 is not directly involved in Nef-mediated MHC-I downregulation. Also the inhibition of PI3K, the upstream activator of ARF6, has no effect of the internalization step but its activity is required to direct MHC-I complexes to the TGN [230]. Recently Nef has been shown to interact with the sorting protein PACS-2, which is required for targeting Nef to the late Golgi/TGN region to assemble the multikinase complex and downmodulate MHC-I [190].

1.4.6. Downregulation of Major Histocompatibility Complex-II (MHC-II) surface expression by Nef

HIV-1 Nef downregulates surface expression of mature peptide-loaded MHC-II molecules while it upregulates the invariant chain (Ii) surface expression associated with immature MHC-II molecules in the infected cells [170]. In Nef transfected cells, the inceased Ii chain is associated with MHC-II α and β chains as immature MHC-II molecules. A 35-50% decrease in mature peptide loaded MHC-II complexes was detected in these cells [170]. Reduced surface levels of mature MHC-II leaves the infected cells incompetent to present antigens and stimulate specific T cells in MHC-II dependent manner [170]. In the context of HIV-1 infection, only Nef has the capacity to enhance Ii and reduce mature MHC-II expression at the cell surface. This function of Nef is conserved in many HIV-1 Nef alleles as well as SIV Nef [171]. Downregulation of MHC-II and upregulation of Ii by Nef are genetically separable from each other and from other

Nef functions such as CD4 and MHC-I downregulation [170,171]. Nef does not affect the synthesis of MHC-II and Ii but reduces the rate of degradation of Ii associated to MHC-II [172]. Decreased surface levels of MHC-II could be the result of intracellular accumulation of mature MHC-II complexes. Nef induces a strong increase in the number of multivascular bodies (MVB), which contain high amounts of Ii and MHC-II. These may represent the location where immature and mature MHC-II complexes accumulate in the presence of Nef. Nef also specifically inhibits the transport of Ii chain-containing complexes to the lysosomal compartments hence reducing their rate of degradation [172].

1.4.7. Modulation of T cell receptor (TCR) and cellular signalling by Nef

Thymocytes of transgenic mice expressing HIV-1 Nef show hyperactivity to anti-CD3 stimulation [54]. Microarray analysis of gene expression profile has shown that the transcriptional program induced by expression of Nef is 97% identical to that induced by stimulation through TCR [45]. Among the genes induced by Nef were 15 transcriptional factors capable of transactivating viral long terminal repeat (LTR). The transcriptional program induced by Nef depends on the presence of functional TCRζ chain and ZAP70 proteins [45]. This indicates that Nef functions at the same level or upstream of these proteins in the TCR signaling pathway [45]. HIV-1 Nef is myristoylated and targeted to the cell membrane where it associates with membrane microdomains known as rafts [49]. Nef has been shown to increase the association of TCR-signaling molecules such as TCRζ and Lck to the raft fraction of T cell membrane prior to activation [46]. By doing so Nef decreases the activation threshold of the T cells making them hyperactive to TCR stimulation. Nef also has the capacity to facilitate raft fusion resulting in increased size

of the rafts following TCR stimulation [46]. Such an event is associated with polarization of the rafts, TCR and its associated signaling molecules to the site of immunological synapse. Although other studies have argued the capacity of Nef to modulate lipid rafts, Nef has been shown to be recruited to immunological synapses within minutes of TCR stimulation [48]. Other studies have shown HIV-1 infection to inhibit formation of immunological synapse and clustering of TCR and Lck at the site of contact between the lymphocytes and antigen presenting cells (APC). These defects were in large part caused by Nef [231]. In the infected cells Lck was retained in the endosomal compartment. Moreover, tyrosine phosphorylation of proteins at the synapse was reduced and the pattern of tyrosine phosphorylated proteins was altered in HIV-infected lymphocytes [231]. Nef-mediated inhibition of actin polymerization and synapse recruitment of Lck was found to be associated with PAK2 kinase activity, where as endosomal retention of Lck ocurred independent of PAK2 activity and required integrity of the microtubule rather than the actin filament system [232]. Modulation of actin organizer N-Wasp activity by Nef has been implicated to cause defects in maturation of the immunological synapse [233].

HIV and SIV Nef interact with various components of the TCR signaling pathway and modify their functions. The interaction of Nef with Src family of tyrosine kinase is through its hightly conserved PxxP motif. Nef binding to the SH3 domain of Hck is the highest affinity reported for an SH3-mediated interaction [173]. Nef can interact with Lck through the SH3 domain as well as the SH2 domain in phosphytyrosine independent manner [174,175]. In the same assay Nef was shown to interact with mitogen-activated protein kinase (MAPK). Interaction of Nef with Lck and MAPK leads to reduced *in vitro* kinase activity of both proteins [174]. This is relevant to Nef-induced modulation of TCR

signaling because Lck is intricately involved in mediating signals from coreceptors CD4 and CD8, IL-2R and TCR. In a separate study, TCR-dependant activation of MAP kinase pathway was increased in $CD4^+$ T cells expressing HIV-1 Nef [234]. Direct interaction of Nef with c-Raf-1 kinase, an important downstream transducer of cell signaling through the c-Raf1-MAP kinase pathway, has been demonstrated [184]. Nef has also been shown to interact and modulate the activity of theta isoform of protein kinase C (θPKC), an important kinase involved in antigen mediated T cell activation [183]. Interaction of Nef with receptor of activated C-kinase 1 (Rack1) has been shown to enhance its *in vitro* phosphorylation by PKC [191].

Nef enhances T cell activation and IL-2 production by Jurkat cell line and primary human $CD4^+$ T cells when stimulated though T cell receptor (TCR) and costimulatory CD28 receptor [47]. Increased production of IL-2 by Nef-expressing Jurkat cells was due to increased phosphatidylinositol 3 kinase (PI3K) activity induced by Nef [235]. Nef interacts directly with the p85 subunit of PI3K [178]. This interaction involves the C-terminus of p85 and several residues in the C-terminus of Nef. This association was also shown to be required for p21-activated kinase (PAK) activation as well as increased viral replication [178]. PAK is another interacting partner of Nef also called Nef-associated kinase or NAK [179,180,236]. This is a highly conserved property of both HIV and SIV Nef alleles. Nef-induced cytoskeletal rearrangement, activation of JNK-SAPK cascade and enhanced viral replication depend on PAK activity [237]. Similarly Vav, a guanine exchange factor (GEF) has been shown to be necessary for these functions of Nef as well as activation of PAK [181]. The current model for PAK activation is that the interaction of PxxP motif of Nef with C-terminal SH-3 domain of Vav activates its GEF activity

leading to the formation of Cdc42-GTP and Rac1-GTP proteins. These GTPases in turn activate PAK which triggers downstream signaling events such as cytoskeletal rearrangement and JNK activation [181]. Nef has also been shown to activate Rac GTPase activity through interaction with a complex containing dedicator of cytokinesis 2 (DOCK2) and engulfment and cell motility 1 (ELMO1) factors [238]. This interaction was also shown to be involved in inhibition of T cell migration in response to the chemokine stromal cell-derived factor 1α (SDF-1α) [238,239].

Interaction of Nef with cellular factors such as inositol triphosphate receptor (IP3R), has been described to induce T cell activation independent of TCR engagement [192]. HIV-1 Nef induces activation of nuclear factor of activated T cells (NFAT), a transcription factor that plays an important role in coordination of T cell activation, via TCR-independent modulation of calcium metabolism and calcineurin activation [240]. Direct interaction of Nef with IP3R has been show in both Nef-transfected Jurkat cells and HIV-infected human PBMCs. This interaction leads to activation of calcium signaling in a TCR-independent manner and hence activation of NFAT [192]. Induction of T cell activation independent of TCR maybe important in the context of reduced surface levels of TCR on cells expressing SIV Nef [226]. Both SIV and HIV-2 Nef have been shown to directly interact with TCRζ chain and down-regulate surface expression of TCR [185,186,226,241]. Under such circumstances, activation of cells independent of TCR would create conditions conducive for viral replication.

In addition of T cell activation, HIV-1 Nef has also been implicated in B cell activation in HIV/AIDS [62]. Swingler and coworkers found increased levels of plasma ferritin in HVI-1-infected individuals which correlated with the ezxtent of

hypergammaglobulinemia [62]. They showed that HIV-1 Nef induces the secretion of ferritin from infected macrophages through activation of NFκB that causes B cell activation and differentiation to immunoglobulin-secreting cells [62].

1.4.8. Nef-mediated modulation of cell survival

HIV infection causes widespread apoptosis of both infected and uninfected cells [242]. A comparison of apoptotic cell death in HIV-1 infected individuals with different primate models showed that abnormal levels of $CD8^+$ T cell apoptosis was present in both pathogenic and non-pathogenic models while abnormal levels of $CD4^+$ T cell apoptosis was only present in the pathogenic models [243]. This study demonstrated the relevance of altered $CD4^+$ T cell apoptosis to AIDS pathogenesis. By inducing apoptosis of virus-specific cytotoxic T lymphocytes (CTL), the virus can prevent development of an effective CTL response and evade the immune responses. SIV Nef has been shown to increase surface expression of FasL (CD95L) on both $CD4^+$ and $CD8^+$ T cells [244]. Induction of FasL on Nef expressing cells is dependent on interaction of Nef with TCRζ chain [245]. Thus Nef can form signaling complexes with TCR to bypass the requirement of antigen to mediate T cell activation and subsequent upregulation of FasL expression. Expression of FasL on infected cells can trigger apoptosis of virus-specific CTL, which themselves express Fas (CD95). Nef can also sensitize $CD4^+$ T cell to apoptosis because it also augments surface expression of Fas on the infected cells [246].

Many viruses including HIV are able to protect infected cells from apoptotic cell death to suit their replication cycle. Early in the viral life cycle, HIV must avoid cell death until viral particles are made and leave the cell. Nef has been implicated to

suppress apoptosis of infected host cells by various mechanisms [187,188,247]. Nef has been shown to interact with apoptosis signal-regulated kinase 1 (ASK1), a member of MAPKKK family involved in Fas and TNFα-induced apoptosis [187]. Nef inhibits ASK1 activation by interfering with the dissociation of its negative regulator, thioredoxin. This inhibitory affect of Nef on ASK1 was shown to suppress TNFα-induced apoptosis and protect the infected cells despite increased Fas and FasL expression [187]. In a separate study Nef was shown to phosphorylate pro-apoptotic factor Bad in serine residues 112 and 136, which inactivates Bad and promotes cell survival [247]. Phosphorylation of Bad in presence of Nef was dependant on its association with PI3K and PAK activation but independent of protein kinase B (PKB)/Akt activation. Furthermore, Nef-mediated increased survival of HIV infected cells was associated with increase viral particle release [247]. Finally Nef has been shown to interact with tumor suppressor protein p53 and decrease its half life [188]. This interaction was direct, involving the N-terminal amino acid residues 1-57 of Nef, and decreased the proapoptic, transcriptional and DNA binding activities of p53. Levels of p53 were reduced in HIV-1 infected cells but not by *nef*-deleted virus and the infected cells were protected against UV-induced apoptosis [188].

1.5. Small animal models of AIDS

Development of a suitable animal model of AIDS is much needed in AIDS research to study infection and pathogenesis as well as to evaluate methods of prevention and treatment of HIV infection. Small animals such as rodents are attractive candidates for AIDS research due to the availability of various inbred and genetically engineered

strains, extensive knowledge or their immune system, especially in mice, and the relative ease of breeding and maintaining animal colonies. A small animal model of AIDS would be instrumental for evaluation of therapeutic agents and could aid studies of host-virus interactions since both the virus and the host are feasible for genetically manipulations. Unfortunately, rodents are not susceptible to HIV infection and multiple blocks to HIV-1 replication exist in rodent cells [248-250]. Genetically engineered mouse (NIH 3T3), rat (Rat2) and hamster (CHO) cell lines, which stably express permissive cyclin T1 protein are able to support reverse transcription, integration and early gene expression of HIV-1, if viral entry restrictions are bypassed. However, viral replication was blocked in mouse and rat cell lines while hamster cell line could support some level of HIV-1 replication [249]. Multiple blocks encountered by HIV-1 during replication in rodent cells are: (i) host cell-specific reduction in CD4- and coreceptor-dependent virus entry, (ii) reduced levels of full-length unspliced HIV-1 transcripts in rodent cell lines, (iii) reduced expression of structural genes and inefficient processing of Gag precursor resulting in formation of largely non-infectious viral particles. Koito and coworkers found that majority of Gag in rodent cells was largely present in cytosolic complexes and remained unprocessed. They also showed that avian chicken embryonic fibroblasts (CEF), quail QT6, and mink lung Mv.1.Lu cells supported Gag processing, assembly and release more efficiently than rodent cells [250]. Using vesicular stomatitis virus G envelope glycoprotein (VSV-G)-pseudotyped HIV-1, Nitkiewicz and coworkers showed that primary murine astrocytes, lymphocytes and macrophages are susceptible to productive infection [251]. While NIH 3T3 cells presented blocks to HIV-1 replication, no such block to HIV-1 replication existed in primary murine cells once the block to virus entry is circumvented and virus

replication was completed through the production of infectious progeny virus. However, there was a relative defect in export of viral particles in these cells [251]. Also, rabbit T-cell and macrophage cell-lines can be productively infected with high-titered HIV-1 stock. These cells were shown to exhibit properties similar to HIV-1 infected human cells [252]. Moreover, rabbit cells expressing human CD4 and CCR5 are highly permissive to M-tropic strains of HIV-1 infection and replication at levels similar to those in human cells [253]. Importantly, rodent cells can support the key functions of HIV-1 pathogenicity factor Nef. Downregulation of surface CD4 and MHC-I molecules by Nef, association with activity of cellular signaling effector PAK and enhancement of virus infectivity by Nef are conserved in rodent cells. This is importance since the validity of small animal model of AIDS depends on its ability to support the functions of viral genes.

In an attempt to study susceptibility of small animal to HIV-1 infection, Morrow and coworkers inoculated small animals such as rats, hamsters, guinea-pigs, rabbits, musk shrews and mice, either at birth or as adult, with blood components from HIV infected individuals or cell-free virus. None of the inoculated animals presented any unexplained pathologies or mortalities. Moreover, these animals did not seroconvert nor had any virus in their peripheral blood mononuclear cells (PBMC) and spleen cells [248]. However, several groups have shown low but persistent infection of rabbits, mice and cotton rats with HIV-1 [254-258]. High titers of virus were needed to infect these animals and none of the animals developed AIDS-associated pathologies. Filice and coworkers showed that intraperitoneal inoculation of rabbits with HIV-1 or chronically infected H9 cells consistently induced persistent infection and seroconversion 2 weeks after inoculation [254]. Budding particles and extracellular virions with morphology typical of

HIV-1 were visualized in the mononuclear cells of infected rabbits by electron microscopy. Infection of rabbits in this study required prior treatment of the animals with thioglycollate to activate cells of macrophage/monocyte lineage followed by intraperitoneal inoculation with HIV-1. Susceptibility of New Zealand white rabbits to HIV-1 infection has been reported by 2 independent groups [255,257]. Kulaga and coworkers showed infection of rabbits inoculated with HIV-1-infected cells alone and those that were first infected with HTLV-1 and subsequently with HIV-1. Infected rabbits seroconverted within 6 weeks of inoculation. Only the HTLV-1/HIV-1 coinfected rabbits showed signs of illness which included diarrhea, weight loss and transient neurologic impairment. In one animal, a rapidly progressing mammary adenocarcinoma was detected [255]. Reina and coworkers have shown infection of New Zealand white rabbits by intraperitoneal inoculation with cell-free HIV-1 [257]. Infected rabbits developed humoral immune response to HIV-1 antigen, which persisted for 3 years. They were able to recover HIV-1 particles from infected rabbits, which contained many defective particles and showed poor infectivity. Only one animal developed changes in lymph node structure reminiscent of those observed in HIV-1 infected humans [257]. Persistent infection of Swiss mice with HIV-1 has been demonstrated by Locardi and coworkers [256]. In this experiment, the mice were treated with thioglycolate prior to intraperitoneal inoculation with HIV-1-infected U937 cell clone producing high titers of the virus. Anti-HIV antibodies in blood and virus-specific sequences in DNA samples from PBMCs were detected more then 500 days after original injection. None of the mice showed any pathological changes during this time period [256]. Langley and coworkers demonstrated a very low level of infection of cotton rat with HIV-1 [258]. This infection stimulated a

strong, specific and long-lasting immune response and was maintained up to 1 year post-infection with viral neutralizing antibodies present in some animals. Passage of the virus *in vivo* was possible, however, passaged infections were consistently at low levels and no overt disease was produced [258]. To circumvent the unavailability of suitable HIV-1 infection receptors and coreceptors in mice, Potash and coworkers replaced the coding region of gp120 in HIV-1 with that of gp80 from ecotropic murine leukemia virus and used the resulting chimeric virus, EcoHIV, to infect mice [259]. A single inoculation with EcoHIV established infection in >75% of the inoculated mice. Viral DNA was detected in $CD4^+$ but not $CD4^-$ lymphocytes. All the mice that carried viral DNA in spleen also produced virus-specific antibodies. A second chimeric virus based on clade D HIV-1/NDK (EcoNDK) was also highly infectious in mice. The virus was detected in spleen and brain 3 weeks p.i., and it induced expression of infection response genes such as MCP-1, STAT-1, IL-1β and complement component C3 in the brain tissues [259].

Despite the extensive efforts to infect rodents with HIV-1, the infection level has remained low. Alterative methods have been used to modify these animals to generate models of AIDS. These modifications can be categorized into 3 groups: (i) transgenic animals expressing human CD4, human chemokine receptors CXCR4 or CCR5 and other permissive factor for HIV infection, (ii) xenotrasplantation of immunodeficient mice to generate human immune cells susceptible to HIV infection, and (iii) transgenic mice expressing whole or part of HIV-1 genome. Although these models have contributed enormously to the study of HIV pathogenesis, they have their own disadvantages. Some of the disadvantages associated with small animal models of AIDS are: (i) the relatively short life-span of these animals limiting the study duration, (ii) loss of genetric markers

upon breeding that may influence the disease phenotype, (iii) due to differences from humans the results cannot always be extrapolated, (iv) differences in the composition and the duration of maintenance of human immune cells in human-hemato-lymphoid-system mice, (v) expression of the transgene in much larger proportion of cells in transgenic mice compared to the proportion of cells infected in humans, and (vi) absence of virus replication in relevant rodent cells. Various small animal models of AIDS are described below.

1.5.1. Transgenic animals expressing human CD4 and human chemokine receptors CCR5 or CXCR4

Susceptibility of rodent cells to productive infection by HIV-1, when the entry block is overcome suggests that these animals could be engineered to make them susceptible to HIV infection by providing the suitable receptor and coreceptors [251,253]. Rabbits, mice and rats have been used to generate infectable small animal model of AIDS but with limited success [260-263]. Using CD2 enhancer elements, Dunn and coworkers expressed human CD4 in T lymphocytes of huCD4 transgenic rabbits [260]. Blood lymphocytes from these mice were more susceptible to HIV-1 infection *in vitro* as compared to the lymphocytes from nontransgenic rabbits and they produced higher levels of viral proteins. When huCD4 transgenic rabbits were inoculated with HIV-1 producing cells, viral DNA was detected at low copies in PBMC DNA and virus could be recovered from these animals at 2 weeks p.i. but the number of cell infected was still relatively low. Antibodies to HIV-1 were first detected in the infected animals by ELISA between 3 and 8 weeks p.i. and by 12 weeks p.i. antibodies against several viral proteins were present.

None of the animals showed signs of disease associated with HIV-1 infection [260]. Browning and coworkers expressed human CD4 and CCR5 in transgenic mice using T-cell specific *lck* proximal promoter [261]. Similar to huCD4 transgenic rabbits, human CD4 and CCR5 expression on mouse T- cells permitted these cells to be infected by M-tropic HIV-1 isolates. Although HIV-1 *gag* DNA and *tat/rev* RNA sequences were detected in the *in vitro* infected transgenic mouse splenocytes, the amount of p24 antigen production was 1-2 logs less than that observed after infection of human leukocytes and infectious virus was not isolated from the culture supernatant by secondary coculture with human PBMCs. When transgenic mice were inoculated with HIV-1 isolates, infected cells could be detected in the spleen and lymph nodes but virus could not be recovered from these cells. This indicates that although expression of human CD4 and CCR5 permitted entry of HIV into the mouse cells, significant HIV infection was prevented by other blocks to HIV replication present in mouse cells [261]. In another study, Sawada and coworkers expressed human CD4 and CXCR4 specifically in mouse $CD4^+$ T cells using the CD4 promoter elements [262]. Transgenic mouse thymocytes were susceptible to infection by T-tropic strain of HIV-1 *in vitro*. Both early- and late-stage viral DNA was detected in the DNA isolated from transgenic thymocytes exposed to HIV. HIV Gag p24 was detected in the culture supernatant but was significantly lower than that produced by human PBMCs [262]. They also observed that expression of human CXCR4 on mouse $CD4^+$ T cells resulted in their migration and accumulation in the bone marrow with concomitant disappearance from the blood. This led them to the hypothesis that CXCR4 expression on $CD4^+$ T cells of HIV-1 infected individuals may contribute to AIDS pathogenesis by recruiting susceptible $CXCR4^{high}$ $CD4^+$ T cells to the bone marrow which contains active

HIV replication loci [262]. Finally rats have been engineered to express human CD4 and CCR5 in their $CD4^+$ T cells, macrophages and microglia [263]. Both primary macrophages and microglia from transgenic rats could be productively infected with R5-tropic strains of HIV-1. They produce and secrete p24 in the culture supernatant and could be inhibited by reverse transcriptase inhibitor AZT. Moreover, culture supernatant from infected transgenic macrophage could productively infect a second culture from the same animals indicating to the presence of infectious virus. Although human CD4 and CCR5 expression permitted infection of transgenic rat lymphocytes, they were not productively infected and did not secrete significant levels of p24. Clearly viral replication is restricted in the rat lymphocytes but not in macrophages and microglia [263]. Further analysis showed that virus entry, reverse transcription, nuclear import of *de novo* synthesized viral DNA genome and its integration into the genome of rat cells is as efficient in transgenic rat lymphocytes as human T cells [264]. In contrast, early viral gene expression was impaired in rat T cells and could be enhanced by transient expression of human Cyclin T1 in rat T cells [264]. When huCD4/CCR5 trangenic rats were inoculated with HIV-1, 2-LTR circles were detected in the spleen on day 3 and 16 p.i. Integrated proviral DNA could be amplified from genomic DNA from spleen cells and viral RNA could be detected in plasma samples 7 days p.i. [263]. As a proof of principle that HIV-infectable transgenic model can be used for rapid evaluation of therapeutic agents, the huCD4/CCR5 transgenic rats were used to evaluate inhibitors targeting virus entry (enfuvirtide) and reverse transcription (efavirenz) [265]. Prophylactic treatment of rats with enfuvirtide and efavirenz resulted in 92.5% and 98.8% reduction, respectively, of HIV-1 cDNA load in spleen 4 days p.i. [265]. This study demonstrated the potential application of

a small animal model of AIDS for preclinical evaluation of inhibitory potency and pharmacokinetic properties of antiviral compounds.

1.5.2. Human-hemato-lymphoid-system mice

Immunodeficient mice can receive human hematopoietic xenotransplants and support their development into mature human cells. These, so called humanized mice are susceptible to infection by human viruses targeting human lymphoid cells and tissues [266]. A number of different immunodeficient mice including severe combined immunodeficiency (SCID), nonobese diabetic (NOD)-SCID or $Rag2^{-/-}$ mice with deficiency for the common cytokine-receptor γ-chain ($Il2rg^{-/-}$), NOD/SCID/$Il2rg^{-/-}$ and $Rag2^{-/-}Il2rg^{-/-}$ respectively, have been used as host for human hemato-lymphoid cell and tissue transplantation [266-271]. Using SCID mice as the host, McCune and coworkers transferred human fetal liver hematopoietic cells, human fetal thymus and lymph nodes to generate SCID-hu mice, while Mosier and coworkers transferred human peripheral blood leukocytes (PBL) to generate hu-PBL-SCID mice [267,268]. In SCID-hu mice, human fetal tissues are implanted under the kidney capsule. The engrafted human fetal thymus undergoes considerable growth and resembles human thymus in microscopic anatomy and the representative thymocyte populations. Human hematopoietic precursors home to and differentiate in the engrafted human fetal thymus. However, appearance of human lymphocytes in the periphery is partial and transient [267]. When intact fragments of human fetal thymus and liver were co-implanted in SCID mice, structures similar to human bone marrow associated with multilineage human hematopoietic differentiation could be observed in the conjoint human thy/liv implant. Human T cells were detected in the

peripheral circulation of these mice in small numbers which persisted up to 15 months in some animals [272]. On the other hand, in hu-PBL-SCID mice, no hematopoiesis takes place but the transferred human PBL expands and reconstitutes a functional immune system in these mice. All the major cell populations present in human PBL are found in the lymphoid tissue and blood of hu-PBL-SCID mice, but their relative proportions differ from that of human PBL [268].

Both SCID-hu and hu-PBL-SCID mice have been used as model to study HIV pathogenesis [273,274]. Direct inoculation of HIV-1 in the conjoint human thy/liv organ of SCID-hu mice results in multiple rounds of infection. Viral RNA is detected in most of the infected cells and some cells produce detectable levels of viral proteins. Infected cells were detected in both the medulla and cortex of the thymus but majority were present in the medulla [273]. Infection of conjoint organ results in depletion of thymocytes [275]. The first thymocytes to be deleted are $CD4^+8^+$ double positive (DP) and $CD4^+8^-$ single positive (SP) thymocytes and at later stages the $CD8^+4^-$ SP thymocytes. The level of viral DNA rises during the first 2-3 weeks and then deceases which coincide with the loss of $CD4^+$ cells [275]. Intravenous (i.v.) inoculation of SCID-hu mice with HIV-1 leads to infection of implanted human lymph nodes (LN) 10-14 days after inoculation. Both T cells and macrophages in the LNs were infected and the mice were viremic 2 weeks after infection [276]. Direct injection of a molecular clone of HIV-1 isolate, JR-CSF, in the thy/liv implant of SCID-hu mice leads to infection of $CD4^+$ SP and $CD8^+$ SP thymocytes and to a lesser degree infection of $CD4^+8^+$ DP thymocytes. On the other hand, more pathogenic isolate of HIV-1 SM leads to infection of mainly the $CD4^+8^+$ DP thymocytes [219]. Viral RNA was also detected in the thymic epithelial (TE) cells in the infected thy/liv

implants. This infection of the thymus resulted in marked distruption of thymic microenvironment leading to depletion of thymocyte and destruction of TE cells [219]. Depletion of thymocytes occurs in two phases in the infected thymus of SCID-hu mice [277]. An initial rapid depletion without any increase in apoptosis is followed by a more gradual $CD4^+$ SP thymocyte depletion and increased apoptosis. The peak proviral load coincides with rapid depletion of $CD4^+$ SP thymocyte. $CD4^+$ SP thymocyte depletion appears to be through a direct non-apoptotic killing mechanism [277]. Although the chemokine receptor CXCR4-utilizing isolates of HIV-1 (X4) are more pathogenic in SCID-hu mice, CCR5-utilizing isolates of HIV-1 (R5) have also been shown to infect these mice [278]. Only the R5-tropic HIV-1 clones isolated after AIDS diagnosis caused depletion of $CD4^+$ SP thymocytes in SCID-hu mice. These isolates also replicated to higher levels than the early R5 isolates and viral replication was highly correlated with $CD4^+$ SP thymocyte depletion [278]. In all the studies with SCID-hu mice cited above, the virus was directly inoculated in the thy/liv implants due to absence of significant numbers of circulating human T cells in these mice, which could be target for HIV infection. Kollmann and coworkers were able to increase the number of human T cells in the peripheral lymphoid tissues of SCID-hu mice by increasing the quantity of human fetal thymus and liver tissue implanted under the renal capsule of both kidneys [279]. In these mice, inoculation of HIV-1 in thy/liv implant in one kidney capsule could systematically disseminate and infect the other thy/liv implant as well as human T cells in the peripheral blood. In addition, infection of thy/liv implant occurred when HIV-1 was inoculated intraperitoneally in these mice. Both HIV-1 *gag* DNA and RNA was detected in the

thy/liv implant, PBMC, spleen and LN of SCID-hu mice infected either by intraimplant injection or by intraperitoneal inoculation [279].

SCID-hu mice have been used to study the importance of HIV-1 accessory genes, such as *nef*, *vpr*, *vpu* and *vif*, in viral infectivity and pathogenesis [206,207,280]. While *vpr* mutant strain of HIV-1 is as pathogenic as the wild type virus in SCID-hu mice, *vpu* and *vif* mutant viruses show moderately less pathogenicity and the *nef* mutant virus is significantly attenuated [207]. Mutation of *vpu* and *vif* had significant effect on the infectivity of HIV-1 in these mice. Deletion of *nef* significantly attenuated infectivity as well as pathogenisity of both X4 and R5 strains of HIV-1 in SCID-hu mice [206,207]. Similarly, a laboratory adapted HIV-1 clone, HXB/LW, which has premature termination of *vpr*, *vpu* and *nef* ORFs, does not deplete thymocytes despite high levels of replication in thy/liv implants of SCID-hu mice [280]. An intact *nef* ORF restored the pathogenicity of HXB/LW strain in these mice. This study demonstrated that HIV-1 replication and pathogenicity are genetically separable and *nef* can function as a pathogenic factor in HXB/LW clone [280].

SCID-hu mice have been used to study the efficacy of antiviral compounds against HIV [276,281,282]. Administration of 3'-azido-3'-deoxythymidine (AZT) to SCID-hu mice 24 hours before intrathymic inoculation and continued treatment for another 2 weeks completely suppressed viremia. Withdrawal from AZT treatment or lowering the dose of AZT resulted in increased number of infected cells and increased percentage of infected animals [276,281]. Similarly, 2'-3'-didoxyinosine (ddIno) protected animals against HIV-1 infection in a dose dependent manner [276]. These studies showed that AZT and ddIno are efficacious in SCID-hu mice in doses that are equivalent to those used

clinically. Recently, SCID-hu mice were used to test the efficacy of bevirimal, a new class of maturation inhibitor which inhibits cleavage of spacer peptide 1 (SP1) from the C-terminal of capsid resulting in defective core condensation [282]. Bevirimal treatment of SCID-hu mice one day prior to infection and 3 weeks after infection inhibited viral replication in dose-dependent manner, reducing viral RNA by >2 $\log_{10}$ and p24 concentration by >90%. This treatment also protected the thy/liv implant from virus-mediated thymocyte depletion. The plasma concentration at which bevirimat showed efficient anti-viral activity is achievable in humans by oral dosing. Consistent with its mode of action, bevirimal inhibited cleavage of p25 (CA-SP) to p24 in dose-dependent manner [282].

Unlike SCID-hu mice, the hu-PBL-SCID mice have mature human T and B cells in their periphery that can be targets for HIV infection [268,274]. Intraperitoneal injection of cell-free or virus-infected autologous T lymphoblasts leads to infection of hu-PBL-SCID mice 3-4 weeks after virus inoculation [274]. Virus was detected in peritoneal lavage (PL) cells, spleen, blood and lymph nodes, and could be isolated on human phytohemagglutinin A (PHA)-stimulated lymphoblasts. This infection resulted in loss of human $CD4^+$ T cells and impaired human immunoglobulin concentrations. Virus recovery decreased between 6-8 weeks after infection probably due to loss of human $CD4^+$ T cells, but a substantial fraction of animals (33-50%) established persistant infection for upto 16 weeks [274]. Rizza and coworkers observed that at 2-24 hours after transfer to SCID mice, human PBLs are highly activated and express early activation marker CD69. At 1-2 weeks post-reconstitution, the percentage of $CD69^+$ cells progressively decline and at 2 weeks they are replaced my $CD45RO^+$ memory T cells [283].

Intraperitoneal injection of HIV-1 two hours after human PBL injection in SCID mice resulted in a generalized and productive infection with a dramatic loss of human $CD4^+$ T cells. In addition, serum levels of human IgM, IgA and sIL-2R were significantly reduced in the infected animals. The majority of immune dysfunctions induced in the 2 hour infection were not induced when hu-PBL-SCID mice were infected 4 weeks after reconstitution. This study provided evidence that the state of activation of human T cells at the time of infection with HIV-1 is a crucial factor in determining the immune impairment observed in AIDS patients [283]. Fais and coworkers showed up-regulation of CCR5 on human $CD4^+$ T cells after injection of human PBLs in SCID mice [284]. Infection of hu-PBL-SCID mice with X4 or R5 strains of HIV-1 led to distinct pattern of $CD4^+$ T cell depletion and immune dysfunction depending on the state of activation/maturation of human T cells at the time of *in vivo* infection. While the R5 strains always caused $CD4^+$ T cells depletion and immune dysfunction independent of time of virus inoculation, the X4 strains caused $CD4^+$ T cell depletion and immune dysfunction only when inoculated early after reconstitution when human T cells were highly activated [284]. Hence, the possibility of X4 strains of HIV-1 to infect, spread and induce $CD4^+$ T cell depletion depends on the state of activation of T cells and the expression of correceptor CXCR4 at the time of infection.

Variations of hu-PBL-SCID model have been generated to study different aspects of HIV pathogenesis and therapy [285-287]. Koyanagi and coworkers studied the effects of mouse genetic background on human T cell reconstitution and HIV infectivity [285]. Inhibition of natural killer (NK) functions of SCID mouse by treatment with mAb TMβ1 was found to dramatically improve reconstitution efficiency of human lymphocytes.

Higher levels of HIV-1 replication were observed in these mice compared to the conventional hu-PBL-SCID mice. In the hu-PBL-SCID mice on a non-obese diabetic background (hu-PBL-NOD-SCID), higher levels of HIV-1 viremia, reaching upto 100-1000 times higher than conventional hu-PBL-SCID mice, was observed [285]. This high level of viremia resulted in systemic infection involving the lungs, liver and brain of these mice. This study demonstrated that the genetic background and innate immunity are critical factors in the development of primary HIV-1 viremia and subsequent invasion of central nervous system [285]. In another study, Boyle and coworkers engrafted SCID mice with PBL from HIV-infected patients to generate hu-HIV/PBL-SCID mice [286]. The percentage of $CD4^+$ T cell recovery in these mice varied significantly as a function of both HIV infection of the donor and treatment. Viral RNA and severe $CD4^+$ T cell depletion was observed 7 days and 18-25 days after engraftment, respectively. Since in these mice the virus comes from the same source as the lymphocytes, it is possible to preserve the intricate relationship between virus and host factor. These mice were used to test 3 different modes of intervention [286]. Treatment with 2'-β-fluoro-2',3'-dideoxyadenosine, a nucleoside analogue, significantly reduced $CD4^+$ T cell depletion and frequency of virus isolation. Administration of mAb against TNF had minimal effects while adoptive transfer of donor Ig with neutralizing activity against HIV-1 did not affect the frequency of $CD4^+$ T cell depletion and virus isolation [286]. Mosier and coworkers used PBLs from healthy individuals at different intervals after immunization with vaccinia gp160 HIV-1 envelope glycoprotein and booster injections of recombinant gp160 to generate hu-PBL-SCID mice [287]. These mice were resistant to infection by homologous virus, demonstrating the potential of these mice to be used for evaluating

efficacy of candidate vaccines. In this study, a positive correlation was observed between donor T cell proliferative response to gp160 and the level of protection. No such correlation was observed for the presence of neutralizing antibody level in donors, suggesting that cellular immune responses to HIV-1 should be given more emphasis in HIV vaccine development [287].

Besides the SCID mice, other immunodeficient mice with improved reconstitution of human immune system have also been used as model to study AIDS pathogenesis. NOD/SCID/IL2Rγc^{null} and Rag2$^{-/-}$γc$^{-/-}$ mice support multilineage human hematopoiesis when engrafted with human hematopoietic stem cells (HSC) and progenitor cells [266,269]. The humanized NOD/SCID/IL2Rγc^{null} mice (hNOG) are susceptible to infection with both the R5 and X4 strains of HIV-1 [288]. They maintain high levels of viremia for over 40 days. R5 virus-infected mice showed high levels of HIV DNA copies in spleen and bone marrow, and X4 virus-infected mice showed high levels of HIV DNA copies in the thymus and spleen. This correlates with the expression of CCR5, which is expressed on CD4$^+$ T cells in blood, spleen and bone marrow, while CXCR4 is expressed mainly on CD4$^+$ thymocytes. Human antibodies against both HIV-1 Env gp120 and Gag p24 antigens were detected in mice after exposure to high titers of HIV-1 [288]. When hNOG mice were generated without the myeloablation procedures, which include whole body irradiation, the mice lived longer and showed viremia for over 3 months examined [289]. Loss of CD4$^+$ T cells was observed in the infected mice, which was more predominant in R5 virus-infected mice. In X4 virus-infected mice loss of CD4$^+$8$^+$ DP thymocytes was observed consistent with the expression of CXCR4 on thymocytes [289].

The humanized $Rag2^{-/-}\gamma c^{-/-}$ mice (RAG-hu or DKO-hu), generated by two independent groups, are also susceptible to infection by both R5 and X4 strains of HIV-1 [270,271]. The infected mice maintained viremia for upto 30 weeks post-infection [270]. HIV-1 infection of these mice resulted in depletion of $CD4^+$ T cells [270,271]. Moreover, the RAG-hu mice have been shown to support mucosal transmission of HIV-1 via vaginal and rectal routes [290]. Although mice exposed to X4 virus were infected, their transmission through intact mucosa was not as efficient as the R5 virus. Mucosally transmitted virus spread systematically to lymphoid tissues and resulted in $CD4^+$ T cell depletion, which was delayed and less severe compared to intraperitoneally infected RAG-hu mice [290]. Similarly, NOD-SCID mice engrafted with human fetal thymic and liver tissues followed by reconstitution with autologous human $CD34^+$ HSCs, referred to as bone marrow/thymus/liver (BLT) humanized mice, are susceptible to infection by mucosally transmitted HIV-1. However, in these mice, intentional abrasion of the rectal epithelium prior to viral exposure was required for infection to occur [182].

With the recent advances and improvements in human-hemato-lymphoid-system mice, these mice will prove to be instrumental in studies of HIV pathogenesis and evaluation of therapeutic and preventive measures.

1.5.3. Transgenic small animals expressing whole or part of HIV genome

One of the major advantages of small animal models is the feasibility to manipulate their genome. Small animals can be engineered to express heterologous gene(s) in specific cells or tissues to study their functions throughout the developmental and adult life of the animal. Transgenesis bypasses the species-specific infectability

barriers and can reveal the effects of individual genes. Transgenic small animal models carrying entire HIV genome or selected genes have been generated for *in vivo* analysis of HIV genes [291]. These are described below.

1.5.3.1. HIV proviral genome trangenics

Leonard and coworkers generated transgenic mice containing intact copies of HIV proviral DNA [292]. In these mice, HIV transgene expression was under the control of sequences of HIV long terminal repeats (LTR). The F1 progeny of one of the founder lines developed disease characterized by epidermal hyperplasia, lymphadenopathy, spleneomegaly, pulmonary lymphoid infiltration, growth retardation and early death. Infectious virus particles could be isolated from the affected mice [292]. This study demonstrated that primary mouse cells support gene expression from the virus LTR and subsequence assembly of infections virus particles. Dickie and coworkers generated transgenic mice expressing the genes of a defective provirus, in which sequences within *gag* and *pol* genes were deleted [293]. Expression of HIV RNA was detected in skin and skeletal muscles of these mice. HIV RNA was also detected in kidney and other tissues at lower levels compared to the skin. These mice developed kidney disease resembling HIV-associated nephropathy (HIVAN) in HIV-infected humans. HIV gene expression was detected in the affected but not in the unaffected transgenic kidneys. Also HIV-related proteins were present in the glomeruli of affected transgenic kidneys [293]. Further analysis of the kidney disease in these mice revealed progressive glomerular and tubular abnormalities, such as focal segmental glomerulosclerosis (FSGSC) and microcystic tubular dilations, accompanied by increased accumulation of the basement membrane

components laminin, collagen type IV and heparine sulfate proteoglycan [294]. The development of similar nephropathy in transgenic mice implicated HIV-1 gene products in the pathogenesis of HIVAN [293,294]. Transgenic mice homozygous for this transgene manifest growth failure syndrome similar to that observed in children with AIDS [295]. In addition to this, homozygous transgenic mice exhibit lymphoproliferation, reduced percentage of $CD4^+$ T cells but an increased absolute number of splenic $CD4^+$ and $CD8^+$ T cells and thymic hypoplasia [295]. In the homozygous transgenic mice, HIV gp120 was detected in the suprabasal keratinocytes and the mice developed proliferative epidermal lesions [296]. Cutaneous injury in these mice increased the viral gene expression, which resulted in development of papillomas, implicating the role of HIV gene products in the pathogenesis of proliferative epidermal disorders associated with HIV-1 infection [296]. When transgenic mice were generated with HIV-1 proviral DNA containing deletions of sequences of *gag*, *pol* and *nef* genes, the transgenic mice did not develop cataracts, papillomatous skin lesions and growth failure [297]. Nevertheless, these mice developed kidney disease similar to but less severe than transgenic mice with only *gag* and *pol* deletions [297,298]. When *nef* gene was bred back onto the Δ*gag-pol-nef* background, glomerulosclerosis was more severe compared to the Δ*gag-pol-nef* mice [298]. These studies showed that *nef* gene is an essential cofactor in the development of papillomatosis, but is not required for development of HIVAN, although it does potentiate glomerular injury induced by other HIV-1 gene products.

Generally the level of expression of HIV genes is low in HIV LTR-regulated proviral transgenic mice. Heterologous promoter sequences can drive high levels of transgene expression and target gene expression to specific tissues in transgenic mice.

Transgenic mice containing complete HIV provirus DNA fused to mouse mammary tumor virus (MMTV) LTR express high levels of viral RNA and proteins in tissues, such as mammary, hardenain, and salivary glands, epididymis, thymus and spleen, known to support transcription from MMTV LTR [299]. However, no abnormal phenotype was observed in these mice. Expression of HIV-1 gene products in the relevant cells targeted by HIV-1 infection in humans is necessary for development of an AIDS-like disease in the transgenic mice [300]. Using human CD4 gene promoter sequences flanked by the enhancer of the mouse CD4 gene, Hanna and coworkers expressed HIV-1 gene products in the $CD4^+$ T cells and the cells of dendritic/macrophage lineage of transgenic mice [300]. These mice develop a severe AIDS-like disease which was characterized by wasting, atrophy, loss of normal architecture and depletion of thymocytes and peripheral T cells in the lymphoid organs, kidney disease with tubulointerstitial nephritis as most frequent histological change and lung lesions. Transgene expression was detected in the glomeruli of transgenic kidneys, lending more support to the causative role of HIV gene products in pathogenesis of HIVAN. The severity of the disease was dependent on the level of transgene expression. This study showed that expression of HIV gene products at sufficient levels in relevant cell types of transgenic mice results in a disease with characteristics very similar to human AIDS and particularly pediatric AIDS [300].

Besides the transgenic mice, rats have also been used to study the functions of HIV gene products through transgenesis. Transgenic rats containing a *gag-pol* deleted HIV-1 provirus regulated by HIV-1 LTR express viral RNA in the lymph nodes, thymus, spleen, liver and kidneys [301]. These rats develop clinical manifestations such as cataracts, weight loss, skin lesions, neurological abnormalities, respiratory difficulty, interstitial

fibrosis and mononuclear cell infiltration in lungs, lymphoid depletion and fibrosis in mesenteric lymph nodes, loss of splenocytes and kidney disease similar to HIVAN [301]. Immunologically these mice have impaired T helper-1 (Th-1) immunity, as evidenced by a reduced delayed type hypersensitivity (DTH) reaction to keyhole limpet hemocyanin (KHL) antigen, and reduced levels of interferon-γ (IFN- γ) production by PBMCs, both in terms of number of IFN- γ producing T cells and the expression level of IFN- γ in the positive cells, after stimulation *in vitro* [301,302]. The number of $CD4^+$ and $CD8^+$ T cells with effector/memory surface phenotype is reduced while there is a reciprocal increase in the number of naïve T cells in these rats. Peripheral T cells from transgenic rats also have an increased susceptibility to activation-induced apoptosis *in vitro* [302]. The $CD4^+$ T cells from these rats express reduced levels of CD28, IL-2 and anti-apoptotic factor Bcl-xL following activation *in vitro* [303]. In addition, transgenic rat $CD4^+$ T cells show reduced tyrosine dephosphorylation of Lck at Y505 site (required for activation) following activation *in vitro*. Thus, transgenic rat T cells show qualitative and quantitative defects in T cell receptor (TCR) signaling that may contribute to the compromised immune system of these rats [303].

1.5.3.2. HIV structural gene transgenics

Trangenic mice containing HIV-1 *env* sequence encoding gp120 placed under the control of promoter sequences of murine glial fibrillary acidic protein (GFAP) gene express *gp120* mRNA in the astrocytes that comprise a substantial proportion of the central nervous system (CNS) [304]. Although *gp120* mRNA was transcribed, gp120 protein was undetectable in the brain of the transgenic mice. Nevertheless, transgenic

mice developed extensive CNS damage indicating that gp120 was expressed in the brain of these mice. Neurological changes in transgenic mice included vacuolization of dendrites, decreased synapto-dendritic complexity and loss of large pyramidal neurons. The brains of transgenic mice also had widespread reactive astrocytosis, one of the earliest and most consistent changes found in the HIV-infected human CNS, and were most pronounced with higher levels of gp120 expression [304]. The close resemblance of these neurological changes to those found in brains of AIDS patients provides support for the causative role of gp120 in HIV-1 associated CNS damage.

1.5.3.3. HIV regulatory/accessory gene transgenics

Mice transgenic for HIV-1 *tat* gene under the control of HIV-1 LTR sequences express detectable levels of the transgene in the skin [305]. Transgenic male mice develop skin lesions that resemble Kaposi's sarcoma (KS) seen in AIDS patients and show high incidence of hepatocellular carcinoma after long latency [305,306]. The skin lesions of transgenic mice were of progressive nature. At 12 to 18 months of age, ~15% of male transgenic mice developed skin tumors, some of which were erythematous. The multifocal areas of dermal hypercellularity and their subsequent development to malignant tumors were correlated with the expression of Tat in the skin of the male mice. Because the dermal lesions closely resembled KS, a direct role of HIV and particularly the *tat* gene was suggested in development of KS [305]. A significant number of male transgenic mice (42.2%) over the age of 18 months developed liver lesions that could be classified into 3 different, but overlapping, groups based on their histology: liver cell dysplasia, hepatic adenoma (HA) and hepatocellular carcinoma (HCC) [306]. The most

common histological finding in these mice was HCC. Expression of HIV-1 *tat* mRNA was detected at a very low level in the liver of only one transgenic founder line. Specific expression of Tat in the liver may not be required for the development of liver lesion, rather extrahepatic growth signals from the Tat expressing cells in these mice may initiate the changes in the liver [306].

Targeted expression of *tat* gene in the T cell compartment of transgenic mice can be obtained by placing *tat* under the control of human CD2 regulatory elements [307]. Expression of Tat was confined to the lymphoid tissues, such as thymus, spleen, lymph nodes and peripheral blood. No defects in the development and distribution of T cells were apparent in the CD2-*tat* transgenic mice. However, when activated *in vitro*, T cells from transgenic mice expressed higher levels of tumor necrosis factor β (TNF-β), transforming growth factor β (TGF-β) and interleukin-4 receptor (IL-4R) mRNAs compared to the non-transgenic T cells. The increased cytokine levels in transgenic mice did not alter mitogen- or antigen-stimulated T cell responses. Unlike the HIV-1 LTR-*tat* transgenic mice the CD2-*tat* transgenic mice did not develop any skin lesions [307].

Pathological functions of Nef have been evaluated in transgenic mice expressing Nef in various tissues and cell populations [54,56,57,91,308,309]. Dickie and coworkers expressed HIV-1 Nef in transgenic mice under the control of sequences of HIV-1 LTR and MMTV LTR [308]. Expression of Nef was detected in the basal cell layer of epidermis in the HIV-1 LTR-Nef transgenic mice. These mice developed proliferative skin lesions. The MMTV LTR-Nef transgenic mice did not develop any pathologic signs despite expression of Nef in tissues known to support MMTV LTR-driven transcription [308]. Using the transcriptional control elements from the gene encoding the δ subunit of CD3-

TCR complex, Skrownski and coworkers expressed HIV-1 Nef in the T cells of transgenic mice [57]. Nef expression was detected in thymus of these animals but not in the periphery, possibly due to elimination of Nef expressing T cells in the periphery. Significant decrease of $CD4^+$ T cells in the PBL and surface expression of CD4 was detected in the transgenic mice. Expression of Nef in the thymus altered development of $CD4^+$ thymocytes and their activation responses. Thymocytes from transgenic mice showed elevated responses to *in vitro* stimulation compared with those of the non-transgenic controls [57]. In a separate study, HIV-1 Nef was expressed in the T cells of transgenic mice using the human CD2 regulatory elements [56]. The CD2-Nef transgenic mice expressed Nef in the thymocytes as well as peripheral T cells. This resulted in downregulation of CD4 on the surface of thymocytes, decrease in percentage of $CD4^+$ SP thymocytes and decrease in the percentage of $CD4^+$ T cells in the periphery [56]. In contrast to the CD3-Nef transgenic mice, a decreased response to *in vitro* activation was observed in thymocytes of CD2-Nef transgenic mice compared to the non-transgenic mice [56,57].

The important role of HIV-1 Nef as the pathogenic factor of AIDS disease was demonstrated in transgenic mice ($CD4C/HIV^{Nef}$) when Nef was expressed in the relevant cells targeted by HIV in humans [54]. The expression of Nef was under the control of human CD4 promoter sequences previously used to generate the $CD4C/HIV^{WT}$ transgenic mice [300]. Expression of Nef gene alone induced an AIDS-like disease very similar to the disease in $CD4C/HIV^{WT}$ transgenic mice and to human AIDS [54,300]. The severity of disease was correlated to the level of Nef expression in these mice. In addition to the loss of thymic and peripheral $CD4^+$ T cells, the thymocytes of these mice

were in a state of activation and hyperresponsiveness with respect to tyrosine phosphorylation of several substrates of TCR signaling, including LAT and MAP kinase [54]. Similarly, expression of SIV Nef in transgenic mice under the control of the same promoter sequences (CD4C) resulted in an AIDS-like disease [205]. The AIDS-like disease in the HIV-1 Nef transgenic mice was associated with accumulation of immature dentritic cells with impaired capacity to present antigens *in vitro*, activated peripheral $CD4^+$ T cells having impaired proliferation *in vitro*, increased expression of Fas/FasL and T cell death [55,115,310]. These mice were used to study kidney and wasting disease. A small molecule inhibitor of NF-κB pathway was shown to increase the life span, kidney and lean body preservation, demonstrating the therapeutic relevance of NF-κB pathway inhibitors to the treatment of HIV-associated disorders [311]. In addition, these mice have shed light on the critical domains of Nef and their involvement in the pathogenesis of AIDS-like disease *in vivo* [312,313].

Specific expression of Nef in the oligodendrocytes of transgenic mice using the myelin basic protein (MBP) promoter resulted in vacuolar myelopathy which is a frequent CNS complication of HIV-1 infected patients [309]. Similarly, podocyte-specific expression of Nef using the sequences of mouse nephrin gene (Nphs1) promoter resulted in full spectrum of HIVAN [91,314]. All these studies together have demonstrated that HIV-transgenic small animals are valuable resources to study the relevance of particular HIV genes in AIDS pathogenesis and decipher molecular mechanisms which could be targeted for therapeutic purposes.

1.6. Research Objectives

HIV transgenic rodents have proved to be useful model to study the role of HIV genes in AIDS pathogenesis. In particular, the CD4C/HIV^{Nef} transgenic mice have been instrumental in highlighting the important role of Nef in AIDS pathogenesis. The AIDS-like disease of these mice closely resembles human AIDS and particularly neonatal AIDS, making it a unique model to study the functions of Nef *in vivo* [54]. However, in this model, as well as other HIV transgenic mice, transgene expression is constitutive and begins early in life. Expression of Nef may interfere with the normal developmental processes early in life and give rise to phenotypes, which may not reflect the true role of Nef in the pathogenesis of disease observed in these mice. Not all organs are fully developed at birth. For example, kidney development continues after birth and in rats the kidneys undergo functional and morphological maturation for several weeks post-natal [315,316]. Early expression of Nef, which has been detected in the kidneys of CD4C/HIV^{Nef} transgenic mice (unpublished data), may interfere with the function and maturation of kidneys in neonate mice. This may result in phenotypes due to developmental defects that may be different from the effects of Nef expression in adult kidneys. To overcome this problem one could induce the expression of transgene in the fully developed adult animal, just as humans are infected with HIV in adulthood. This would require the use of inducible systems that efficiently turn on trangene expression in presence of an inducer.

In the present study we have used a tetracycline-inducible system to generate HIV-1 Nef transgenic mice, in which Nef expression can be turned on in adult mice upon treatment with doxycycline (dox), a derivative of tetracycline. The tetracycline-inducible

system can function in either ON or OFF modes as show in **Figure 4.1** [317,318]. In the tet-Off system, the transactivator (tTA) which is a fusion protein containing the repressor of tetracycline-resistance operon from *Escherichia coli* transposon Tn10 (tetR) and activating domain of herpes simplex virus VP16 protein, induces transcription from a minimal promoter of human cytomegalovirus (hCMV) fused to seven tet operator sequences, also called the tetracycline responsive elements (TRE), in the absence of tetracycline but not in its presence [317]. In the tet-On system, the transactivator (rtTA) has reverse DNA binding properties as compared to tTA and was generated by random mutagenesis of the tetR [318]. In this system presence of tetracycline induces transcription from the TRE promoter sequences. Further mutagenesis of the tTA resulted in generation of a new transactivator, termed rtTA2^S-M2, with enhanced sensitivity to dox, improved stability in eukaryotic cells and lower background expression in the absence of dox [319]. Both the tet-Off and tet-On systems have been shown to regulate transgene expression *in vivo*, in dox-dependent and tissue-specific manner [320,321].

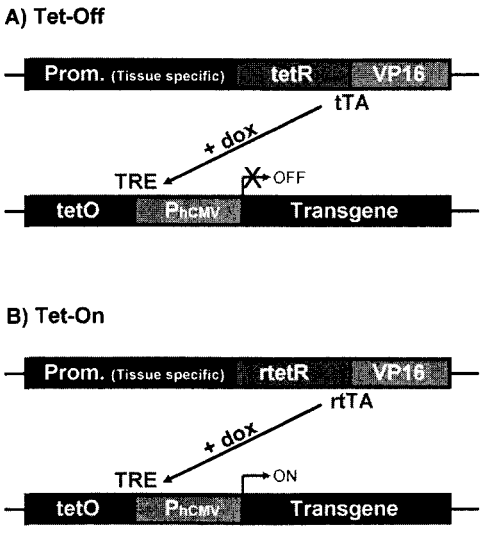

Figure 4.1. Diagramatic representation of tetracycline inducible system. The Tet-Off (A) and Tet-On (B) versions are shown. Adapted from Kistner et al., 1996 [321].

In a recent study, Balasubramanyam and workers have used tet-Off (tTA) system with phosphoenolpyruvate carboxykinase promoter to express HIV-1 Vpr in the liver and in white and brown adipose tissues of adult mice in an attempt to study the affects of Vpr on lipid and energy metabolism [322]. We have use the tet-On (rtTA and $rtTA2^S$-M2) system to induce expression of HIV-1 Nef in the $CD4^+$ T cells and cells of macrophage/dendritic lineage of mice using the tissue-specific promoter CD4C, used previously to generate the constitutively Nef expressing $CD4C/HIV^{Nef}$ Tg mice [54]. Expression of Nef can be induced in adult animals when treated with dox and repressed in the absence of dox treatment. To our knowledge, this is the first such model generated to express Nef in tissue-specific and inducible manner. We show that adult mice are succeptible to the effects of Nef. These mice are used to test the hypothesis that HIV-1 Nef is the major pathogenic factor in the AIDS-like disease of the constitutively Nef expressing $CD4C/HIV^{Nef}$ transgenic mice by avoiding any developmental defects that may have been cause by early expression of Nef. In addition, these mice will be used to test the hypothesis that Nef causes T cell activation in Nef Tg mice by studying T cell activation in the context of a complete T cell compartment in bone marrow chimeric mice. This model will provide further evidence that functions of HIV gene products such as Nef is conserved in murine cells, thereby providing an *in vivo* model to study molecular mechanisms of Nef function. Since transgene expression can be turned off by withdrawal from dox, these animals can also be used to study the immune reconstitution seen in HIV-infected patients after anti-retroviral therapy.

1.7. Bibliography

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Chapter 2: A novel inducible HIV-1 Nef trangenice mouse model

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2.1. Preface

Nef is a 27-35 kDa HIV and SIV regulatory protein which is myristoylated at its 5' end and localized at the plasma membrane [1]. Various functions of Nef include downregulation of surface molecules such as CD4, CD28, MHC-I and MHC-II, modulation of TCR-mediated signaling, modulation of cell survival and enhancement of virus infectivity [2]. Pathological functions of Nef have been evaluated in transgenic mice expressing Nef in various tissues and cell populations [3]. Our lab previously generated CD4C/HIV^{Nef} Tg mice which express Nef in the CD4^{+} T cells and the cells of macrophage/dendritic lineage under the control of sequences of human CD4 promoter and mouse CD4 enhancer elements [4]. These mice express Nef early in life and develop pathologies very similar to those seen in humans infected with HIV-1. Expression of Nef in the relevant cells of HIV infection seems to be very important for the development of the AIDS-like disease in these mice. To show that the disease in these mice is not due to developmental defects caused by early expression of Nef, we generated inducible HIV-1 Nef Tg mice using the tetracycline inducible system and the same CD4C promoter elements for tissue-specific expression of Nef. In this study we show that the AIDS-like disease in constitutively expressing CD4C/HIV^{Nef} Tg mice is not due to developmental defects caused by early expression of Nef and adult mice are also susceptible to pathological effects of Nef. Moreover we have generated a novel inducible mouse model to study the functions of Nef *in vivo*, as function of time and level of expression in the context of a primary immune system.

2.2. Abstract

The CD4C/HIV^{Nef} Tg mice express Nef in CD4^{+} T cells and in the cells of macrophage/monocyte/dendritic lineage, and develop an AIDS-like disease similar to human AIDS. In these mice, Nef expression begins at birth and is constitutive throughout the life of the animal. To rule out the contribution of any developmental defects caused by early expression of Nef, we generated inducible HIV-1 Nef Tg mice using the tetracycline-inducible system. Faithful expression of Nef transgene is induced in the (CD4C/rtTA X TRE/HIV^{Nef}) or (CD4C/rtTA2S-M2 X TRE/HIV^{Nef}) double-Tg mice upon 1 week of doxycycline (dox) treatment in drinking water. Down-regulation of CD4 surface expression is observed as early as 2 weeks after Tg induction. Long-term treatment of these mice with dox also leads to loss, apoptosis and activation of CD4^{+} T cells. In addition, these phenotypes can be transferred by bone marrow transplant, which indicates to a hematopoietic cell autonomous effect. Finally, the dox-induced double Tg mice develop non-lymphoid organ diseases similar to that of CD4C/HIV^{Nef} Tg mice and of human infected with HIV-1. This study rules out any developmental defects as a result of early Nef expression and demonstrates that adult mice are susceptible to the action of Nef. These Tg mice represent a unique model which is likely to be instrumental to understand the cellular and molecular pathways of Nef action as well as the main characteristics of immune reconstitution following dox withdrawal.

2.3. Introduction

Transgenic small animal models carrying entire HIV genome or selected genes have provided useful information on the role of HIV genes in AIDS pathogenesis. Transgenic mice containing intact copies of HIV proviral DNA, defective provirus with deletions of sequences within *gag* and *pol* genes or individual HIV-1 genes (*env* gp120, *tat* and *nef*) develop various pathologies, some of which resemble pathologies found in human AIDS while others do not [4-17]. The cell type context in which the HIV-1 transgene is expressed plays an important role in determining the type of pathological lesions. Mice expressing the entire coding sequence of HIV-1 (CD4C/HIV^{WT}) or HIV-1 Nef (CD4C/HIV^{Nef}) alone in the relevant target cells of HIV-1, namely CD4$^+$ T cells, macrophages and dendritic cells, develop pathologies very similar to those in human AIDS [4,8]. The CD4C-HIV^{Nef} Tg mice develop an AIDS-like disease characterized by immunodeficiency, loss of CD4$^+$ T cells, thymic atrophy, activation of T and B cells, loss of germinal centre formation and pathologies in heart, lungs and kidney similar to those in human AIDS [4]. Similarly, expression of SIV Nef in transgenic mice under the control of the same promoter sequences (CD4C) result in an AIDS-like disease [18]. These studies demonstrated that Nef plays an important role in the pathogenicity of the AIDS-like disease induced by HIV-1 in transgenic mice.

In CD4C/HIV^{Nef} Tg mice, Nef expression begins early in life and is constitutively expressed throughout the life of the animal. This early expression of Nef may interfere with the normal developmental processes early in life and give rise to phenotypes, which may not reflect the true role of Nef in the pathogenesis of disease observed in these mice.

Temporal regulation of Nef expression in adult mice using inducible system would allow assessment of affects of Nef in fully developed adult animals. A commonly used inducible system for *in vivo* studies is the tetracycline inducible system [19,20]. This system can function in either ON or OFF modes [21,22]. The tet-On system makes use of a transactivator (rtTA) which is a fusion protein containing the modified repressor of tetracycline-resistance operon from *Escherichia coli* transposon Tn10 (tetR) and activating domain of herpes simplex virus VP16 protein. Tetracycline and its derivatives (doxycycline) promote binding of rtTA and induces transcription from a minimal promoter of human cytomegalovirus (hCMV) fused to seven tet operator sequences (tetO), also called the tetracycline responsive elements (TRE) [22]. Expression of rtTA under the control of sequences of a tissue-specific promoter will allow tissue specific induction of transgene from TRE promoter elements [20].

In the present study was used the tet-On (rtTA and rtTA2^{S}-M2) system to induce expression of HIV-1 Nef in the CD4^{+} T cells and cells of macrophage/dendritic lineage of mice using the tissue-specific promoter CD4C, used previously to generate the constitutively Nef expressing CD4C/HIV^{Nef} Tg mice [4]. To our knowledge, this is the first such model generated to express Nef in a tissue-specific and inducible manner. These mice robustly express Nef when treated with doxycycline (dox), and develop a disease very similar to that seen in constitutively Nef expressing CD4C/HIV^{Nef} Tg mice. This model rules out any developmental defects that may have been caused by early expression of Nef in CD4C/HIV^{Nef} Tg mice, and shows that adult mice are also susceptible to the effects of Nef.

2.4. Materials and Methods

2.4.1 Transgene construction and generation of Tg mice. The CD4C promoter and HIV^{Nef} Tg has been described before [4]. An *Aat*II and *BamH*I fragment of pRetro-Off plasmid containing TRE sequences were fused to HIV^{Nef}-SV40 polyadenylation sequences in Litmus29 vector. The TRE-HIV^{Nef}-SV40 fragment was excised from this vector with *Aat*II and cloned in *Not*I site of HPRT targeting vector/pBR322. This construct was linearized with *Puv*I and used to transfect mouse ES cells. ES cell clones containing single copy of Tg inserted at the HPRT locus by homologous recombination were selected and injected into mouse blastocysts to generate TRE/HIV^{Nef} Tg mice. To obtain the rtTA transgene, the DNA fragment containing human CD2 promoter sequences fused to rtTA was PCR amplified from hCD2/rtTA Tg mouse genomic DNA and cloned in pBluescript KS vector. An *EcoR*I fragment containing only rtTA sequences was excised from this construct, blunt ended with Klenow and fused to CD4C promoter and SV40 polyadenylation sequences in pBR322 vector. The $rtTA2^{S}$-M2 sequences were excised from pUHrT62-1 vector (gift of Dr. Berens, Erlangen-Nuernberg University, Germany) with *EcoR*I and *BamH*I, blunt ended with Klenow and fused to CD4C promoter and SV40 polyadenylation sequences in pBR322 vector. The CD4C/rtTA and CD4C/$rtTA2^{S}$-M2 Tg were excised with *EcoR*I and microinjected into fertilized (C37BL/6 X C3H) F2 oocytes to generate Tg mice, as described before [4]. Mice carrying the Tg were bred as heterozygotes on C3H/HeNHsd background. To generate double Tg (DTg) mice, TRE/HIV^{Nef} Tg mice were bred with CD4C/rtTA or CD4C/$rtTA2^{S}$-M2 Tg mice. The GFP reporter mouse (tetO/H2B-GFP) was purchased

from Jackson labs and bred with TRE/HIV^{Nef} Tg mice to generate DTg mice. To induce Tg expression, DTg progeny were treated with dox in drinking water. All the mice were housed in specific pathogen free (SPF) environment and all experiments were approved by the Institutional Animal Ethics Committee.

2.4.2. Northern blot analysis. RNA was isolated from thymus using the TRIzol reagent (Invitrogen) following manufacturer's instructions. 15μg of RNA was resolved on formaldehyde agarose gel, transferred to nylon membrane and hybridized with ^{32}P-labelled HIV-1 probe, as described before [4]. The membranes were washed and rehybridized to actin probe.

2.4.3. Western blot analysis. Protein extracts were prepared from different tissues using RIPA buffer in presence of protease inhibitors. The amount of protein in the lysates was determined using micro-BCA assay (Sigma-Adrich). 100μg protein was resolved on SDS-PAGE, transferred to membrane and immunoblotted with polyclonal rabbit antibodies against Nef and actin and goat anti-rabbit Alexa 680-conjugated secondary antibody. Detection was done with Odyssey infrared detector.

2.4.4. Preparation of peritoneal macrophages. Peritoneal cells were harvested by washing peritoneal cavity twice with 5ml RPMI medium containing 10% FBSI. Cells were collected in Petri dishes and macrophages were allowed to attach to the bottom of plates overnight. Macrophages were washed and collected from protein extraction.

2.4.5. Antiphophotyrosine immunoblot. The hybridoma producing hamster anti-mouse CD3ε (145-2C11) was purchased from American Type Culture Collection (ATCC; Rockville, Md.). The anti-CD3 mAb was purified with a protein G affinity column. Thymocytes were stimulated with anti-CD3 antibody and protein extracts were prepared in presence of protease and phosphatase inhibitors. Protein quantification was done using micro-BCA assay (Sigma). 100μg of protein was resolved on SDS-PAGE and immunobloted with anti-phosphotyrosine antibody (4G10) (UBI), followed by anti-actin and anti-Nef antibodies. Detection was done with Odyssey infrared detector after immunoblotting with Alexa 680-conjugated secondary antibodies.

2.4.6. Cryosections and Green fluorescent protein detection. Mice were perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). Tissues were dissected out and further fixed in 4% paraformaldehyde for 1 hour at 4°C. Fixed tissues were kept in 10% sucrose solution (prepared in PBS) overnight and then transferred to 15% sucrose solution of few hours before freezing in O.C.T. (Tissue-Tek). 5μm sections were immunostained with Texas red-conjugated anti-mouse IgM antibodies and mounted with Vectashield containing DAPI. Fluorescent signals were detected with Axiovert S100TV fluorescent microscope using appropriate filters.

2.4.7. Flow cytometry. Cell suspensions were prepared from lymphoid organs and stained with antibodies, as described previously [4]. Fluorescein isothiocyanate (FITC), phycoerythrin (PE) and allopycocyanin (APC)-labeled mAb, including anti-CD4, CD8, TcRαβ, CD69, CD25, CD44, CD45RB, CD62L, CD45.2 were purchased from

Cederlane. Irrelevant rat IgG1, rat IgG2a, rat IgG2b and Armenian hamster IgG1 were used as isotype controls. Annexin V-FITC and 7-aminoactinomycin D (7-AAD) were purchased from Cederlane, and propidium iodide (PI) was from Sigma. Staining with Annexin V, PI and 7-AAD is as described before [23]. Flow cytometric analysis was done with FACS Calibur and Cell Quest software (Becton Dickinson).

2.4.8. Microscopic analysis. Organs to be assessed were fixed in 3.7% formaldehyde and embedded in paraffin. 5μm sections were stained with hematoxylin and eosin. All sections were assessed blindly using bright field microscopy.

2.5. Results

2.5.1. Construction of Tg mice.

To construct the inducible TRE/HIV^{Nef} Tg line, the NL4-3 HIV-1^{MutG} genome [4] (designated here HIV^{Nef}) was used. This construct harbors mutations of each of the HIV-1 ORF (*gag, pol, env, vif, vpu, rev, tat and vpr*), except *nef*, thus coding only for the Nef protein. The HIV^{Nef} DNA fragment with its downstream SV40 polyadenylation signal, were ligated downstream of tetracycline responsive regulatory element (TRE) in an HPRT targeting vector **(Fig. 2.1A).** This construct was used to transfect embryonic stem (ES) cells and to generate Tg mice with a single copy of transgene integrated at the HPRT locus in the X chromosome. This founder (F) TRE/HIV^{Nef} Tg line was designated F130391. The CD4C/rtTA and CD4C/rtTA2^{S}-M2 transactivator lines were generated by ligating the transactivator rtTA and rtTA2^{S}-M2 fragments, respectively, downstream of

CD4C regulatory sequences, as previously described **(Fig. 2.1B).** The rtTA2^{S}-M2 gene is a mutated version of the rtTA gene found to exhibit lower basal activity and higher sensitivity to dox [24]. Southern blot analysis of all the Tg mice founders showed a grossly intact transgene structure (data not shown). One founder (F148571) CD4C/rtTA and two founders (F176042 and F176043) CD4C/rtTA2^{S}-M2 Tg lines were used in the experiments. All Tg lines were maintained on C3H/HeNHsd background and progeny was routinely genotyped. Double Tg (DTg) mice were generated by breeding the TRE/HIV^{Nef} Tg mice with either of the transactivator Tg lines.

2.5.2. Dox-dependent expression of HIV-1 in the double Tg (DTg) mice

The levels of HIV-1 transgene expression was first determined by Northern **(Fig. 2.2A)** and Western **(Fig. 2.2B, C)** blot analysis of RNA and proteins, respectively, from different organs of (TRE/HIV^{Nef} X CD4C/rtTA) and (TRE/HIV^{Nef} X CD4C/rtTA2^{S}-M2) DTg mice fed with dox in drinking water for one week. All untreated DTg mice showed no or very low levels of HIV expression, indicating low leakiness. Transgene induction was dox-and dose-dependent, with each transactivator Tg line. The three main transcripts of HIV-1 (8.8 kb, 4.3 kb and 2 kb) could be detected by Northern blot analysis **(Fig. 2.2A)**. Western blot analysis with anti-Nef antibody showed the expression of Nef in lymphoid tissues and peritoneal macrophages **(Fig. 2.2B, C)**. The expression levels vary between different founder lines, most likely reflecting the positional effect at the site of CD4C/rtTA or CD4C/rtTA2^{S}-M2 transgene integration. We also observed that, in all founder lines, Tg expression was lower in female than in male DTg mice. This could be due to the X chromosome inactivation phenomenon in female cells which could affect

expression of Nef Tg integrated in the X chromosome of TRE/HIV^{Nef} Tg line. The molecular basis of this phenomenon has not been investigated. At the highest dose of dox used (2 mg/ml) for one week, Nef expression was comparable to that of CD4C/HIV^{Nef} (F27367) Tg mice previously shown to develop an AIDS-like disease [4]. Therefore, a dox concentration of 2mg/ml in drinking water was used for all other subsequent experiments, unless otherwise stated. Thus, HIV-1 Nef is highly expressed in the inducible Tg mice after treatment with dox in drinking water.

To further determine the specificity of trangene expression and to monitor the identity of the cells expressing HIV-1 in DTg mice, we utilized a green fluorescent protein (GFP) reporter mouse, tetO/H2B-GFP, which expresses histone H2B-GFP fusion protein under the control of TRE promoter sequences [25], inducible by rtTA or rtTA2^{S}-M2 transactivators in presence of dox. GPF expression was analyzed in lymphoid tissues by FACS **(Fig. 2.2D)** and in peripheral lymphoid and non-lymphoid tissue cryosections by fluorescent microscopy **(Fig. 2.2E)** following treatment of (CD4C/rtTA X tetO/H2B-GFP) and (CD4C/rtTA2^{S}-M2 X tetO/H2B-GFP) DTg mice with dox (2mg/ml) for one week. Double Tg mice not receiving dox and single Tg tetO/H2B-GFP mice were used as controls to monitor leaky expression. In all populations of T and B cells, a low GFP expressing population was detected in all DTg animals, including in those not treated with dox and in single Tg tetO/H2B-GFP Tg mice **(Fig. 2.2D and Table 2.1)**. In the thymus, only low level of leakiness was observed. After dox treatment, most CD4^{+}8^{+} double positive (DP) and CD4^{+}8^{-} single-positive (SP) thymocytes of the three founder transactivator Tg lines express high levels of GFP, as expected. Most CD8^{+}4^{-} SP thymocytes of the three founders did not express high levels of GFP, but a significant

proportion expressed moderate to high levels. Interestingly, a significant percentage of $CD4^-8^-$ double negative (DN) thymocytes of two (F176042, F176043) of the 3 founder lines were GFP-positive. In the peripheral lymph nodes (pLN), both $CD4^+$ T cells and a smaller proportion of $CD8^+$ T cells expressed GFP. The proportion of GFP^{Hi} $CD4^+$ T cells was higher in DTg mice from founder line F148571 than from the other two founder lines (F176042 and F176043). $CD4^+$ T cells from F176042 DTg mice were mainly GFP^{med}, while those of F176043 DTg mice showed high levels of GFP^{med} leakiness. A small proportion of $CD8^+$ T cells and B cells expressed GFP at high or moderate levels, considering the significant leakiness of GFP expression in $CD8^+$ T cells of the parental tetO/H2B-GFP mice **(Fig. 2.2D and Table 2.1)**.

Analysis of cryosections of LNs and spleen from dox-treated mice, by fluorescent microscopy, showed the presence of GFP expressing cells in these organs. When immunostained with Texas Red-labeled anti-IgM antibody, most of the GFP expressing cells were localized to the T cell zone in these organs **(Fig. 2.2E)**. Only small numbers of GFP expressing cells were present in the control mice due to the leaky expression of GFP in tetO/H2B-GFP Tg line. Besides the lymphoid organs, GFP expressing cells were also detected in the lungs and liver: these could represent infiltrating T cells and macrophages in the lungs and Kupffer cells in the liver, as judged by their distinct nuclear morphology **(Fig. 2.2E)**. Hence, transgene appears to be efficiently and faithfully induced in organs of DTg mice from the three transactivator founder lines when treated with dox in drinking water. These results are consistent with our previous work on the expression of various surrogate genes with these CD4C regulatory elements [4,8,26].

2.5.3. Survival of Dox-treated Tg mice

The (TRE/HIV^{Nef} X CD4C/rtTA) and (TRE/HIV^{Nef} X CD4C/rtTA2^{S}-M2) DTg mice were started on dox treatment (2mg/ml) at the age of 1-1.5 month and groups of mice (n = 8-10) were kept on this regimen for ~6 months, in order to study the long term effects of the induced HIV-1 Nef. Controls represented dox-treated TRE/HIV^{Nef}, CD4C/rtTA, CD4C/rtTA2^{S}-M2 STg and non-Tg mice, as well as untreated DTg mice. Dox treatment had no apparent detrimental effects on non-Tg and STg mice which remained in apparent good health throughout the experiment, up to 6 month. All dox-treated DTg mice from the F148571 (males and females) and female mice from F176042 and F176043 (since males died at 1 month, see below) transactivator founder lines also remained healthy. Only dox-treated male DTg mice from F176042 and F176043 had a much shorter survival than its untreated control DTg littermates. This shorter survival is likely to reflect the very severe renal disease developing in these mice, as documented by the severe proteinuria already present after 10 days following initiation of dox treatment (**Fig. 2.3A**).

At autopsy, gross examination confirmed the presence of severe kidney (smaller size, irregular surface, pale), lung (consolidation) pathologies as well as severe atrophy of the thymus and LN in dox-treated DTg male mice of the F176042 and F176043 founder lines. In the other DTg mice from F148571, F176042 (female mice) and F176043 (female mice) founder lines, the same lesions were present but they were much less severe. Histologic examination revealed in each organ, the same typical AIDS-like pathological changes that we previously described in CD4C/HIV Tg mice: loss of LN

and spleen architecture, lymphocytic interstitial pneumonitis and nephropathy (to be reported separately) **(Fig. 2.3B)**.

2.5.4. Thymocyte depletion in adult Tg mice expressing Nef

Expression of Nef in the thymus of CD4C/HIV^{Nef} Tg mice leads to depletion of thymocytes [4]. To determine whether thymocyte depletion can be induced by Nef expression in adult mice, 4-6 weeks old (CD4C/rtTA X TRE/HIV^{Nef}) and (CD4C/rtTA2^{S}-M2 X TRE/HIV^{Nef}) DTg mice were treated with dox for 6-7 weeks and thymus cellularity as well as thymocyte population profiles were analyzed by FACS. Nef expression resulted in significant depletion of thymocytes in mice from all of the transactivator founder lines **(Fig. 2.4)**. In male mice from F176042 and F176043 transactivator founder lines, severe thymocyte depletion occurred after 1 week dox treatment at low dose of dox (0.5mg/ml). In these mice thymus cellularity was over 10 fold lower then the untreated control DTg mice. This thymocyte loss was reflected in all thymocyte populations **(Fig. 2.4A and Table 2.2)**. Besides thymocyte loss, surface CD4 expression was reduced on CD4^{+}8^{-} SP and CD4^{+}8^{+} DP thymocytes of Nef expressing mice **(Fig. 2.4A and Table 2.2)**. No significant loss of peripheral lymph node (pLN) T lymphocytes was observed after 1 week dox treatment, however, CD4 surface expression was downmodulated on CD4^{+} T cells **(Fig. 2.4A and Supplementary Table 2.1)**.

In the DTg male mice from F148571 and DTg female mice from F176042 and F176043 transactivator founder lines, treated with dox, significant loss of thymocytes was observed after 6 weeks of dox treatment at 2mg/ml **(Fig. 2.4B and Table 2.2)**. Thymocyte loss in these mice was reflected in CD4^{+}8^{+} DP as well as mature

$TCR\alpha\beta^{hi}CD4^{+}8^{-}$ SP and $TCR\alpha\beta^{hi}CD8^{+}4^{-}$ SP thymocytes **(Table 2.2)**. The CD4/CD8 ratio was affected in mice from F148571 indicating to faster depletion of $CD4^{+}8^{-}$ SP compared to $CD8^{+}4^{-}$ SP thymocytes. In DTg female mice from F176043 transactivator founder line, significant loss of $TCR\alpha\beta^{hi}CD4^{+}8^{-}$ SP and $TCR\alpha\beta^{hi}CD8^{+}$4- SP was observed despite normal thymic cellularity. No thymocyte depletion was observed in DTg female mice from F148571 transactivator founder line most likely due to very low transgene expression (data not shown). In all DTg mice treated with dox, surface expression of CD4 was downmodulated compared to the untreated control mice **(Table 2.2)**. Hence, expression of Nef in thymus of adult mice results in thymocyte loss and downmodulation of surface CD4 expression.

2.5.5. Impaired $CD4^{+}8^{-}$ SP thymocyte maturation in Nef expressing adult mice

Thymocytes undergo selection and maturation events in the thymus to form immunocompetent $CD4^{+}$ and $CD8^{+}$ T cells. We studied maturation of thymocytes in dox-treated DTg mice (F148571 male, F176042 and F176043 female) by FACS analysis of markers such as TCRαβ, CD69, CD2, CD5 and IL-7R. FACS analysis showed significantly lower percent of $TCR\alpha\beta^{hi}$ thymocytes and appearance of $TCR\alpha\beta^{low}$ thymocyte population in dox-treated DTg mice from all the founder lines **(Fig. 2.5A)**. In addition, dox-treated DTg mice also showed significantly lower percentage of $TCR\alpha\beta^{hi}CD69^{+}$ thymocytes, which indicates that a lower proportion of thymocytes are selected for further maturation **(Fig. 2.5B)**. The developmental markers CD2 and CD5 were significantly downmodulated on higher proportion of $TCR\alpha\beta^{hi}CD4^{+}8^{-}$ SP thymocytes from F148571 and F176042 transactivator founder lines **(Fig. 2.5C)**. In

contrast, TCRαβ^{hi}CD8^{+}4^{-} SP thymocytes showed surface downmodulation of CD2 alone. Surface expression of IL-7R was also downmodulated on TCRαβ^{hi}CD4^{+}8^{-} SP thymocytes from F176042 transactivator founder line **(Fig. 2.5C)**. Expression of these developmental markers was not downmodulated in mice from F176043 transactivator founder line, which maintained thymus cellularity after dox treatment **(Table 2.2)**. Together these results demonstrate that Nef expression alters maturation of CD4^{+} T cells in adult mice.

2.5.6. Down-regulation of surface CD4 and CD4^{+} T cell loss in peripheral lymphoid tissues of inducible Tg mice

Constitutive expression of Nef starting early in life of Tg mice (CD4C/HIV^{Nef} Tg mice) results in down-regulation of CD4 cell surface expression and depletion of CD4^{+} T cells [4]. We monitored these two parameters in blood and lymphoid tissues of adult DTg mice treated or not treated with dox. CD4 surface expression, monitored by FACS, was found to be downregulated in blood as early as 2 weeks post induction **(Fig. 2.6A)**. In the F148571 founder line, a rapid fall in the percentage of CD4^{+} T cells in blood was observed within 2 weeks after induction **(Fig. 2.6A),** followed by a gradual loss monitored over a period of 5 months **(Fig. 2.6B).** By 3-4 months after induction, CD4^{+} T cell loss was ~50% compared to the uninduced control DTg mice.

FACS analysis was then performed on peripheral lymphoid tissues of adult mice treated with dox for 6 weeks and 6 months. At the time of sacrifice, mice were ~3 months old for the 6 week treatment group and 7-8 months old for the 6 months treatment group, except for DTg male mice of the F176042 and F176043 founder lines which had a much

shorter survival (see above). Peripheral LN showed significant depletion of $CD4^+$ T cells in all dox-treated DTg mice from the three transactivator founder lines. This was reflected in a lower percentage of cells, lower absolute cell numbers and decrease CD4/CD8 ratios at both 6 weeks and 6 months of dox treatment **(Fig. 2.6C, Table 2.3 and Supplementary Table 2.2)**. Similar loss of $CD4^+$ T cells was observed in spleen and mesenteric lymph nodes of dox-treated mice (date not shown). Downmodulation of surface CD4 expression was observed in pLN of mice from F148571 founder line (**Table 2.3**). There was also some loss of $CD8^+$ T cells in the pLN nodes of mice from founder lines F176042 and F176043 **(Table 2.3)**, which is consistent with the Tg expression in small proportion of $CD8^+$ T cells of these 2 founder lines **(Fig. 2.2D)**. We also observed a decrease in B cell numbers in the peripheral lymphoid tissues **(Table 2.3)**. None of the above mentioned phenotypes were observed in single-Tg or non-Tg mice treated with the same dose of dox, ruling out the possible detrimental effects of dox treatment on these cell populations (data not shown). These results show that Nef expression causes depletion of T lymphocytes and downmodulation of surface CD4 expression on peripheral T cells in adult mice.

2.5.7. Peripheral T cells exhibit an activated/memory-like phenotype in dox-treated Tg mice

We previously reported that $CD4^+$ T cells from $CD4C/HIV^{Nef}$ Tg mice exhibit an activated/memory-like phenotype being $CD25^+$ $CD44^+$ $CD45RB^{low}$ $CD62L^{Low}$ and $CD69^+$ [27]. To determine whether Nef-expressing adult $CD4^+$ T cells would show the same phenotype, the surface phenotype of peripheral LN T cells from the same 6 weeks

and 6 month dox-treated DTg mice studied above, were analyzed by FACS after labeling with different antibodies against activation and developmental markers. Results were slightly different for each founder line. In the F148571 founder dox-treated DTg mice, a larger proportion of $CD4^+$ T cells expressed CD69 and CD25 cell surface markers and were $CD44^{high}$, $CD45RB^{low}$ and $CD62^{low}$ cells compared to the untreated control male mice **(Fig. 2.7A)**. Similar results were obtained for DTg female mice of the F176042 founder line, although the activation phenotype was less pronounced **(Fig. 2.7A)**. In contrast, no increase in the proportion of cells with an activated/memory-like phenotype was observed in the dox-treated DTg mice of the F176043 founder line, compared to the untreated control mice, despite depletion of $CD4^+$ T cells as severe as in F148571 founder DTg mice **(Fig. 2.7)**. Most likely, the difference can be attributed to the level of Tg expression, being lower in the F176043 DTg mice, compared to those in F148571 DTg mice, as judged by the GFP^{Hi} fluorescence intensity **(Fig. 2.2D)** and by the absence of CD4 cell surface downregulation in F176043 and its presence in F148571 DTg mice **(Table 2.3)**. A similar FACS analysis of $CD8^+$ T cells from the same mice showed signs of activation in the DTg mice from F148571 and F176042 transactivator founder lines **(Supplementary Fig. 2.1)**. The activation status of T cells in founder lines F176042 and F176043 males could not be determined due to the massive atrophy of the pLNs and the severe depletion of T cells in these dox-treated DTg mice.

In addition to having activated peripheral T cells, thymocytes from dox-treated DTg mice are hyperresponsive to TCR stimulation. Western blot analysis revealed higher steady state levels of phosphotyrosine (pY) proteins in thymocytes from dox-treated male DTg mice from F148571 founder line compared to the untreated control

mice **(Fig 2.7B)**. Stimulation of thymocytes with anti-CD3 antibody alone resulted in further increase in the level of pY proteins in dox-treated mice but not in untreated control mice **(Fig 2.7B)**. These results together suggest that Nef induces a state of activation and hyperresponsiveness to TCR stimulation in Tg thymocytes.

2.5.8. Peripheral CD4$^+$ T cells undergo increased apoptosis/cell death in dox-treated Tg mice

We have previously found that the CD4$^+$ and CD8$^+$ T cells from CD4/HIV^{Nef} Tg mice show a higher level of apoptosis/cell death than their non-Tg littermates [23]. To determine whether T cell depletion in dox-treated mice was associated with apoptotic cell death, we measured apoptosis/cell death in cells of lymphoid organs by annexinV/PI or 7AAD staining and FACS analysis. Increased proportions of CD4$^+$ T cells were positive for annexinV/PI or 7AAD in the pLNs of all DTg mice treated with dox compared to the untreated control mice, but reached statistical significance only in mice from the F148571 transactivator founder line **(Fig. 2.8A and B)**. A similar trend towards an increased apoptotic/dead cell phenotype was observed in the CD8$^+$ T cell compartment but was not statistically significant **(Fig. 2.8C)**. Similar increase in apoptotic/dead cells in dox-treated mice were observed in other lymphoid tissues as well (data not shown).

2.5.9. T cell phenotype is more severe when HIV-1 Nef is expressed early in life

The pathological changes described above, which result from the expression of Nef during adulthood, are remarkably similar to those previously observed in CD4C/HIV^{Nef} Tg mice expressing Nef early in life. However, the severity of the various

phenotypes could not be compared stringently, because they arise in two different Tg lines. This issue is of clinical relevance, because AIDS has been reported to have a shorter incubation period in pediatric cohorts [28,29]. These children have usually been infected early in life, i.e. in uterus, during delivery or through breast feeding. The availability of inducible HIV-1^{Nef} Tg mice offers the opportunity to compare the severity of the various phenotypes in function of the time of Nef expression (early vs. adult) in the same Tg line.

For this experiment, HIV-1 Nef expression was induced by treating pregnant females with dox in drinking water during the 2^{nd} week of pregnancy, and maintained throughout the weaning period and in adulthood. Nef protein was detected in the thymi of 2 week-old pups born to dox-treated mothers (F148571) **(Fig. 2.9A)**. The pups were then maintained on dox for up to 6 months of age and FACS analysis was performed on their lymphoid tissues to determine the extent of T cell loss. Down- regulation of surface CD4 and $CD4^+$ T cell loss are observed in lymphoid tissues of all (TRE/HIV X CD4C/rtTA) DTg mice compared to their CD4C/rtTA or TRE/HIV single Tg and non-Tg littermates on dox treatment **(Fig. 2.9B and C)**. As expected, peripheral $CD4^+$ T cells of the DTg mice with early Tg induction show activated/memory like phenotype **(Fig. 2.9E)** and undergo increased cell death compared to their single Tg and non-Tg littermates on dox **(Fig. 2.9D)**. Both phenotypes were more severe than when Tg expression was induced at adult age. We also observed an increase of B cells in dox-treated mice compared to the control mice **(Fig. 2.9C)**. Together, these results suggest that expression of Nef early in the life of Tg mice may be more detrimental for the immune system than

when expressed later. These results are reminiscent of the faster progression to AIDS in HIV-infected children than in adult humans.

2.5.10. T cell phenotype of inducible Nef Tg mice is cell autonomous

We performed bone marrow transplantation to determine if the T cell phenotypes induced by expression of Nef in adult Nef Tg mice was transferable. Bone marrow cells from DTg male of F148571 transactivator founder line ($CD45.2^+$) was depleted of mature lymphocytes and transferred to lethally irradiated non-Tg B6.SJL-Ptprca Pepcb/BoyJ mice ($CD45.1^+$) by intravenous (i.v.) injection. Donor cells are $CD45.2^+$ and can be distinguished from residual host cells ($CD45.1^+$) by FACS analysis. Mice were rested for 2 months to allow reconstitution of their hemato-lymphoid system and then treated with dox for 2 months. BM transplanted mice not treated with dox were used as controls. Nef expression in the host derived T cells resulted in downregulation of surface CD4 in the thymus as well as loss of $TCR\alpha\beta^{hi}CD4^+8^-$ SP and $CD4^+8^+$ DP thymocytes **(Fig. 2.10A, B and C)**. T cells loss was also observed in the pLN of dox-treated but not in untreated mice **(Fig. 2.10D and E)**. In addition, higher percentage of donor BM-derived $CD4^+$ T cells showed activated/memory phenotype in dox-treated compared to untreated mice **(Fig. 2.10F)**. These results suggest that the Nef-induced T cell phenotypes in Nef Tg mice are cell autonomous.

2.6. Discussion

Our lab previously reported development of an AIDS-like disease in Tg mice (CD4C/HIV^{Nef}) expressing HIV-1 Nef in CD4$^+$ T cells and cells of dendritic/macrophage lineage [4]. Nef expression begins early in life of these mice and may interfere with normal developmental processes. To rule out contribution of any developmental defects to the AIDS-like disease in these mice, we generated inducible Nef Tg mice. We report here development of an AIDS-like disease in mice expressing Nef in adult life, which is very similar to the disease reported in constitutively expressing CD4C/HIV^{Nef} Tg mice.

2.6.1. Adult mice are susceptible to pathogenic effects of Nef

Nef can be expressed faithfully in tissue specific manner using tetracycline inducible system. In (CD4C/rtTA x TRE/HIV^{Nef}) and (CD4C/rtTA2^S-M2 x TRE/HIV^{Nef}) DTg mice, where rtTA and rtTA2^S-M2 are tetracycline-dependent transactivators [22,24], Nef expression is induced from TRE promoter mainly in the CD4$^+$8$^-$ SP and CD4$^+$8$^+$ DP thymocytes, as well as in the peripheral CD4$^+$ T cells, when treated with dox. Nef is also expressed at lower levels in small proportion of other thymocyte populations and peripheral T and B lymphocytes in these mice. Expression of Nef in adult mice results in downmodulation of surface CD4 as well as loss of thymocytes, particularly CD4$^+$8$^-$ SP and CD4$^+$8$^+$ DP thymocytes, as well as in loss of peripheral CD4$^+$ T cells. Both HIV and SIV Nef have been shown to downregulated CD4 surface expression by increasing the rate of internalization of CD4 from cell surface and directing them to the endocytic pathway [30-35]. HIV-1 infection has been shown to result in depletion of thymocytes in the

thy/liv conjoint organ of SCID-hu mice [36]. The first thymocytes to be deleted are $CD4^+8^+$ double positive (DP) and $CD4^+8^-$ single positive (SP) thymocytes and at later stages the $CD8^+4^-$ SP thymocytes. Infection of $CD4^+8^-$ SP, $CD8^+4^-$ SP and $CD4^+8^+$ DP thymocytes have been reported with different HIV-1 isolates in these mice [37]. Deletion of *nef* significantly attenuated infectivity as well as pathogenicity of both X4 and R5 strains of HIV-1 in SCID-hu mice, demonstrating the importance of Nef in pathogenesis of HIV-1 infection in this mouse model of AIDS [38-40]. Viral RNA was also detected in the thymic epithelial (TE) cells in the infected thy/liv implants, which resulted in marked disruption of thymic microenvironment leading to depletion of thymocyte and destruction of TE cells [37]. However, our bone marrow transplantation study rules out the involvement of TE cells in thymocyte depletion observed in Nef Tg mice. Increased thymocytes apoptosis has been demonstrated in constitutively expressing CD4C/HIV^{Nef} Tg mice [23]. Expression of Nef in the thymus of adult mice also results in impaired maturation of $CD4^+8^-$ SP thymocytes. This was revealed by downmodulation of surface markers, such as TCRαβ, CD69, CD2, CD5 and IL-7R, on $CD4^+8^-$ SP but not $CD8^+4^-$ SP thymocytes. A similar impaired maturation of $CD4^+8^-$ SP thymocytes has been observed in CD4C/HIV^{Nef} Tg mice (Chrobak *et al.*, manuscript in preparation). Impairment of thymopoiesis may contribute to reduced peripheral $CD4^+$ T cells in addition to Nef-mediated T cell depletion by increased cell death.

In addition to T cell depletion, Nef expressing thymocytes and peripheral $CD4^+$ T cells show an activated phenotype and are in a state of hyperresponsiveness to TCR stimulation, judged by tyrosine phosphorylation of proteins in thymocytes and analysis of activation markers on peripheral $CD4^+$ T cells. A similar phenotype was earlier reported

for the constitutively expressing Nef Tg mice [4,27]. Nef has been shown to increase the association of TCR signaling molecules such as TCRζ and lck to the raft fraction of T cell membrane prior to activation [41]. By doing so Nef decreases the activation threshold of the T cells making them hyperactive to TCR stimulation. Microarray analysis of gene expression profile has shown that the transcriptional program induced by expression of Nef is 97% identical to that induced by stimulation through TCR [42].

Besides the immunological disease, expression of Nef in adult mice also results in pathologies in non-lymphoid organs such as kidney and lungs. These phenotypes are very similar to those reported in CD4C/HIV^{Nef} Tg mice [4]. Expression of Nef in the relevant cells for HIV infection seems to be crucial for development of the non-lymphoid organ disease in mice. Tg mice expressing Nef under the control of promoter elements different from CD4C promoter elements do not develop the full spectrum of pathologies observed in our model [13-15]. The close resemblance of the disease in our inducible Nef Tg model and the CD4C/HIV^{Nef} mice demonstrates that the AIDS-like disease in these mice is not caused by any developmental defects as a result of early Nef expression and that adult mice are also susceptible to pathogenic effects of Nef. This study further confirms that murine cells can support Nef functions.

2.6.2. Severity of T cell phenotypes depends on time and level of Nef expression

The level of expression of Nef varies between the 3 transactivator founder lines used in this study. This may be due to positional affect as a result of random integration of the transgene in the mouse genome. The physical location of the transgene with respect to other genes and their regulatory elements including enhancer elements can

influence the level of expression of the transgene. Random integration may affect the level of expression of the transactivator (rtTA or $rtTA2^{S}$-M^{2}) and subsequently the level of dox-dependent Nef expression. We have observed lower Nef expression in the DTg female compared to the DTg male mice from the 3 transactivator founder line. This is due to the insertion of TRE/HIV^{Nef} Tg in the X chromosome of this founder line. Since X chromosome inactivation is a random process in female mouse cells [43], this may result in inactivation of Tg-bearing X chromosome in a subset of female cells, thereby, decreasing the amount of Nef protein detected by western blot analysis of whole organ. Consequently, the disease in the female mice is less severe than the male mice from their corresponding transactivator founder lines. In the mice from F176042 and F176043 transactivator founder lines, severe loss of thymocytes is detected in the male mice treated with dox for one week. However, thymocyte loss is apparent in the female mice, from the same transactivator founder lines, after 2 months of dox treatment. By one month of dox treatment, the male mice develop a fatal disease characterized by wasting, lymphoid organ atrophy, proteinurea and kidney disease, while the female mice are in apparent good health upto 6 months of dox-treatment despite T cell loss and appearance of proteinurea and kidney disease. The female mice from F148571 do not develop any aberrant pathology due to very low level of Nef expression.

The difference in the level and cellular specificity of dox-dependent Tg expression, between different transactivator founder lines, is apparent from studies of inducible GFP reporter mouse. While in the thymus from F148571 and F176042 transactivator founder lines, majority of $CD4^{+}8^{+}$ DP and $CD4^{+}8^{-}$ SP thymocytes express high levels of GFP, in the thymus from F176043 transactivator founder line, medium

GFP expressing subset is apparent in both the thymocyte populations. Consequently the impairment of thymocyte maturation is more apparent in mice from F148571 and F176042 transactivator founder lines. Amongst the peripheral $CD4^+$ T cells, GFP expression is highest in mice from F148571 transactivator founder line followed by mice from F176042 and F176043 transactivator founder lines. This correlates with the reduced CD4 surface expression on peripheral $CD4^+$ T cells from F148571 only, and the presence of T cells with activated/memory phenotype in the mice from F148571 and F176042 but not from F176043 transactivator founder line. This could also explain the significant increase in T cell death seen in mice from F148571 but not in mice from F176042 and F176043 transactivator founder lines. A positive correlation has been established between plasma viral load and disease progression in HIV infected patients [44-46]. It is not surprising to expect higher levels of Nef in patients with high plasma viral load. Hence, it seems probable that Nef, among other factors, will contribute to rapid progression of disease in these patients.

Integration site may also affect the cell type and tissue specificity of Tg expression. Cells expressing GFP in dox-dependent manner are present in non-lymphoid organs, such as lungs, liver and kidney, of mice from F176042 and F176043 but not in mice from F148571 transactivator founder line. While mice from F176042 and F176043 transactivator founder lines develop pathologies in lungs and kidneys, the mice from F148571 do not develop non-lymphoid organ disease. The correlation between the non-lymphoid organ disease and Tg expression implicates Nef expression in these organs as the principle factor responsible for development of these pathologies. The absence of non-lymphoid organ disease in the mice from F148571, which express high levels of Tg

in the thymus and peripheral lymphoid organs, also rules out the involvement of lymphoid cells in development of pathologies in the non-lymphoid organs. The identity of the cells expressing Tg in non-lymphoid organs and their contribution to pathogenesis of Nef in these organs needs further investigation.

The inducible Nef Tg mice has enabled us to study effects of Nef *in vivo* as function of the time of Nef expression. Transgene expression can be induced *in utero* and neonate mice by treatment of pregnant female mice with dox [47-49]. We have detected Nef expression in 2 week old pups born to female mice treated with dox during pregnancy. We have also observed differences in the severity of T cell phenotypes in the inducible Nef Tg mice as a function of the time of Nef expression. T cell phenotypes such as activation and cell death were more pronounced in mice in which Nef expression was induced early in life as compared to the mice in which Nef was induced at adult stage. This issue is of clinical relevance, because AIDS has been reported to have a shorter incubation period in pediatric cohorts [28,29]. Approximately 20% of HIV-infected infants develop AIDS in the first year of life and the remainder develop AIDS as a nearly constant rate of 8% per year, reaching the median at 4.8 years [29]. In HIV infected infants the plasma virus level remains high for many months after appearance of viremia, which is thought to be as a consequence of viral replication within an expanding lymphoid mass in young infants [50]. Similar to HIV infected adult individual, rapid progression of disease in infants is also associated with high plasma viral loads [51].

In summary, expression of Nef in adult mice results in an AIDS-like disease similar to the disease reported previously in Tg mice expressing Nef early in life. The severity of the disease depends on the level and time of Nef expression. The inducible

Nef Tg mice described here is a unique model to study Nef functions *in vivo* in relation to the level and time of expression of Nef, and *in vitro* in primary immune cells. Moreover, these mice will be useful to study immune reconstitution since Nef expression can be turned off after withdrawal from dox.

2.7. Acknowledgments

We thank Eric Massicotte and Martine Dupuis from flow cytometry core facility for their help with flow cytometry, Annie Vallee for preparation of histology slides and Dominic Filion for the help with microscopy. We also thank Isabelle Corbin, Stephanie Lemay, Audey-Ann Kustec, Benoit Laganiere and Jonathan Kergoat for their excellent technical assistance.

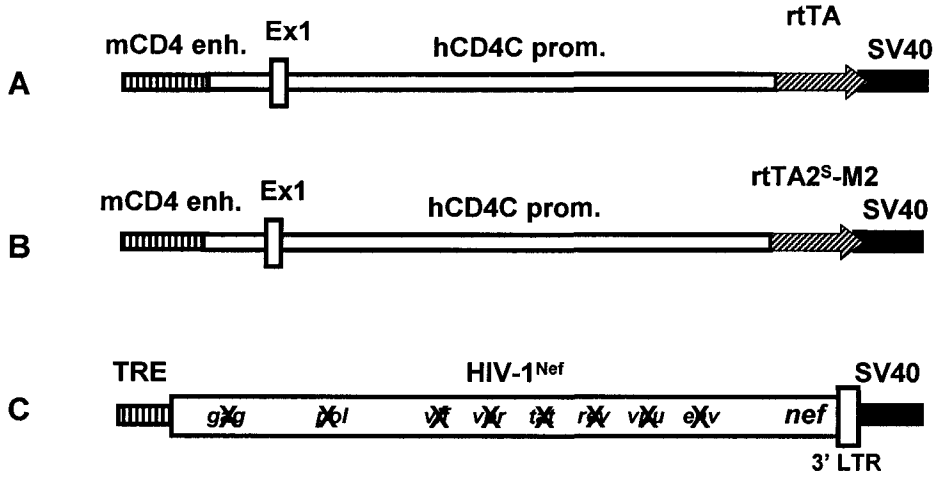


Figure 2.1. Diagrammatic representation of transgene structure. **(A)** CD4C/rtTA, Human CD4 promoter (thin bar) fused to mouse enhancer (striped bar); rtTA (striped arrow); SV40 poly(A) sequences (dark bar). **(B)** CD4C/rtTA2^{S}-M2, Human CD4 promoter (thin bar) fused to mouse enhancer (striped bar); rtTA2^{S}-M2 (striped arrow); SV40 poly(A) sequences (dark bar). **(C)** TRE/HIV^{Nef}, TRE promoter sequences (striped bar); HIV-1^{Nef} sequences (thick bar) in which all HIV-1 genes except *nef* is disrupted (X); SV40 poly(A) sequences (dark bar).

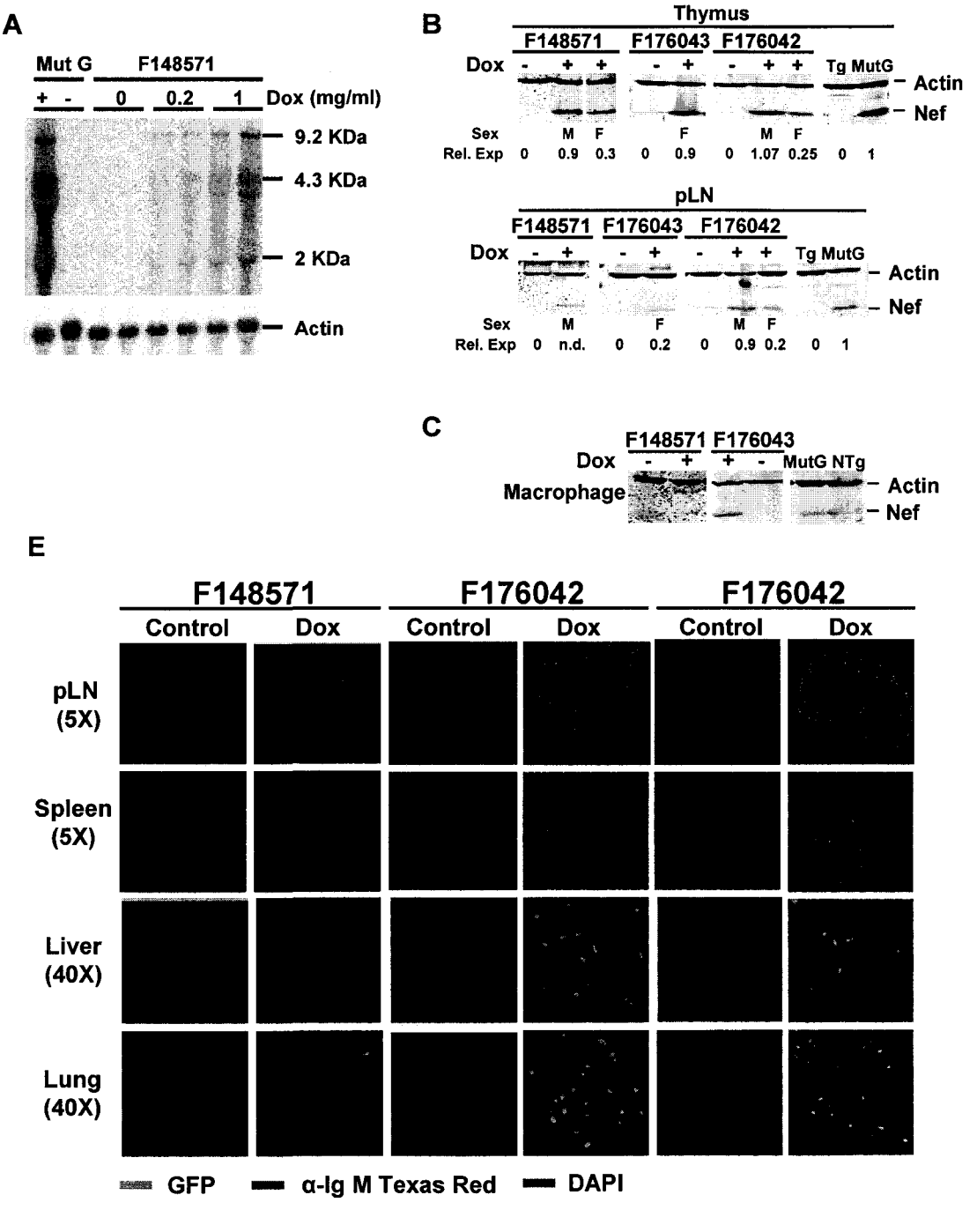

A
Mut G
F148571
+ -
0
0.2
1
Dox (mg/ml)
9.2 KDa
4.3 KDa
2 KDa
Actin
B
Thymus
F148571
F176043
F176042
Dox
Tg MutG
Actin
Nef
Sex
Rel. Exp
pLN
C
F148571
F176043
MutG
NTg
Macrophage
E
F148571
F176042
F176042
Control
Dox
pLN (5X)
Spleen (5X)
Liver (40X)
Lung (40X)
GFP
α-Ig M Texas Red
DAPI

Figure 2.2.

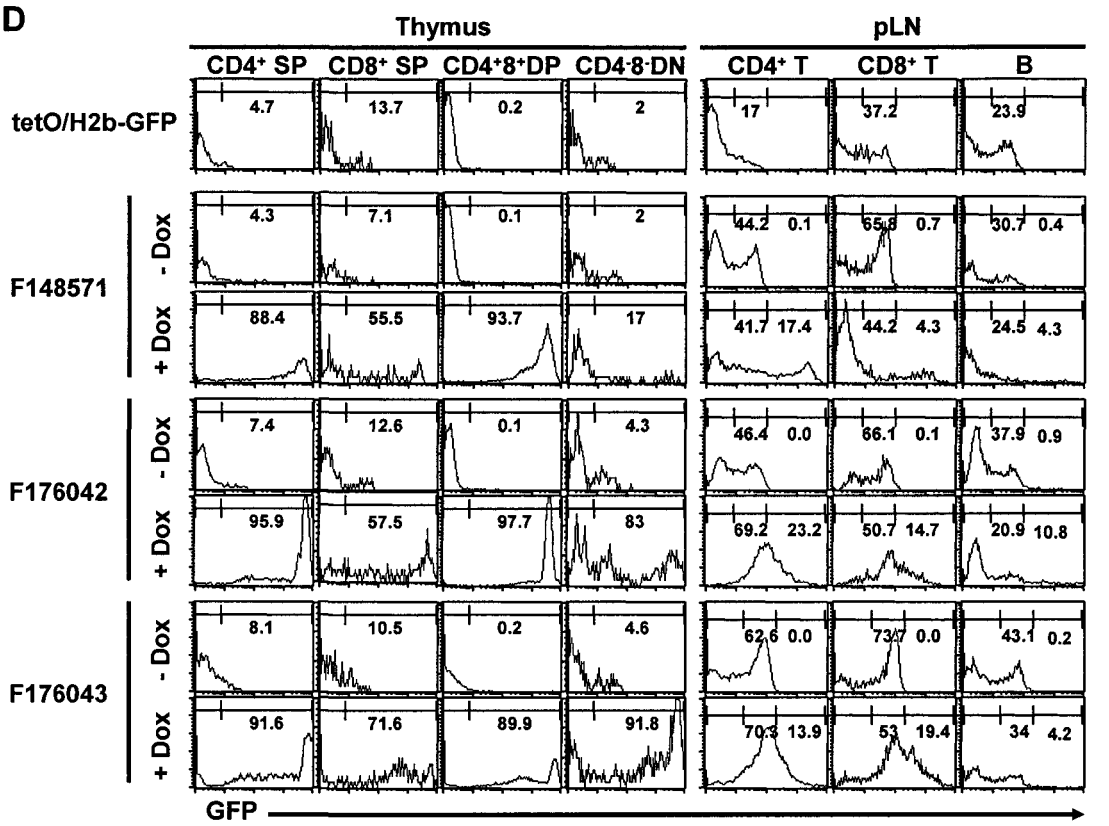


Figure 2.2. Doxycycline-dependent transgene expression. **(A)** Northern blot analysis of HIV-1 RNA in thymus of mice treated with different concentrations of dox in drinking water for 1 week. Total RNA (10μg) extracted from thymus was hybridized with HIV-1 specific probe. RNA from CD4C/HIV^{Nef} was used as positive control. To control sample loading, blots were washed and rehybridized with actin specific probe. **(B)** Western blot analysis of Nef protein in thymus and peripheral lymph nodes, and **(C)** western blot analysis of Nef protein in peritoneal macrophages of different transactivator founder lines treated with dox (2mg/ml) in drinking water for 1 week. Protein extracts (100μg) from different organs were blotted with anti-Nef serum and anti-actin antibody. Protein samples from CD4C/HIV^{Nef} were used as positive controls. **(D)** Histogram plots of GFP expression detected by FACS analysis in different immune cell populations of mice treated with dox (2mg/ml) for one week and untreated controls. The percentage of GFP$^+$ cells is indicated. CD4, CD4$^+$ SP thymocytes; CD8, CD8$^+$ SP thymocytes; DP, CD4$^+$8$^+$ DP thymocytes; DN, CD4$^-$CD8$^-$ DN thymocytes; CD4 T, CD4$^+$ T cells; CD8 T, CD8$^+$ T cells; B, B cells. **(E)** Fluorescent microscopic detection of GFP expression in cryosections of peripheral lymph nodes, spleen, liver and lungs of different mouse founder lines treated with dox (2mg/ml) for one week and untreated control animals. pLN and spleen sections were immuno-stained with anti-IgM Texas red to visualize the B cell zones. All sections were stained with DAPI to visualize the nucleus.

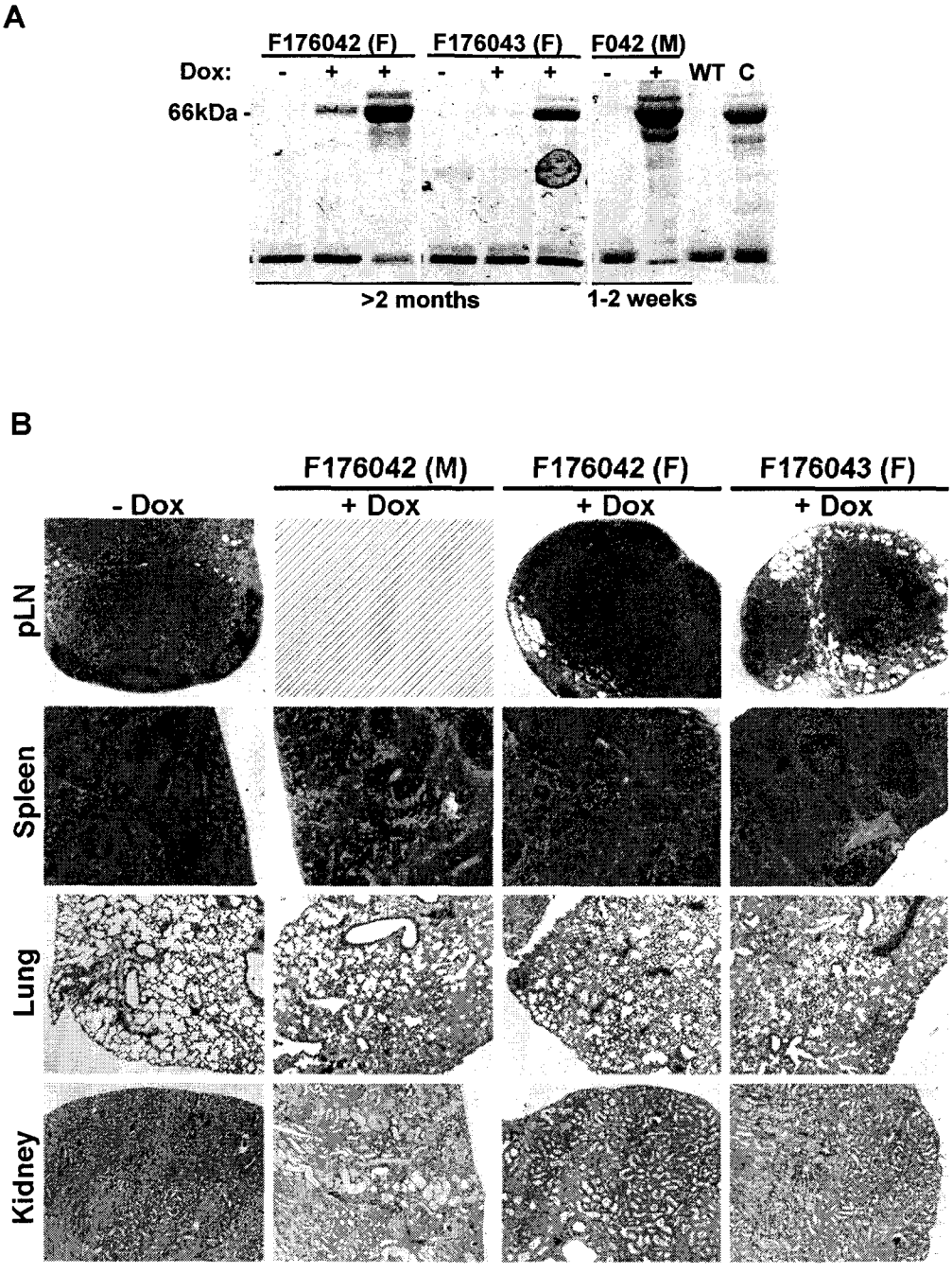


Figure 2.3. Pathology of Nef expression in adult mice. **(A)** Commassie blue stained SDS-PAGE showing proteinuria, apparent as 66kDa serum albumin in urine from dox-treated mice. F, female; M, male; WT, wild-type; C, CD4C/HIV^{Nef}. **(B)** Histological sections of pLN, spleen, lungs and kidney in dox-treated and untreated mice from F176042 and F176043 transactivator founder lines, shown at low magnification. Note the loss of T cell zone and hypocellularity of B cell zone in pLN, particularly in F176042 female mice, and loss of architecture of spleen in dox-treated mice. pLN from F176042 male mice was not available due to severe lymphoid atrophy (upper panel, 2nd from left). Also note the tubular atrophy and dilation in kidney of dox-treated mice.

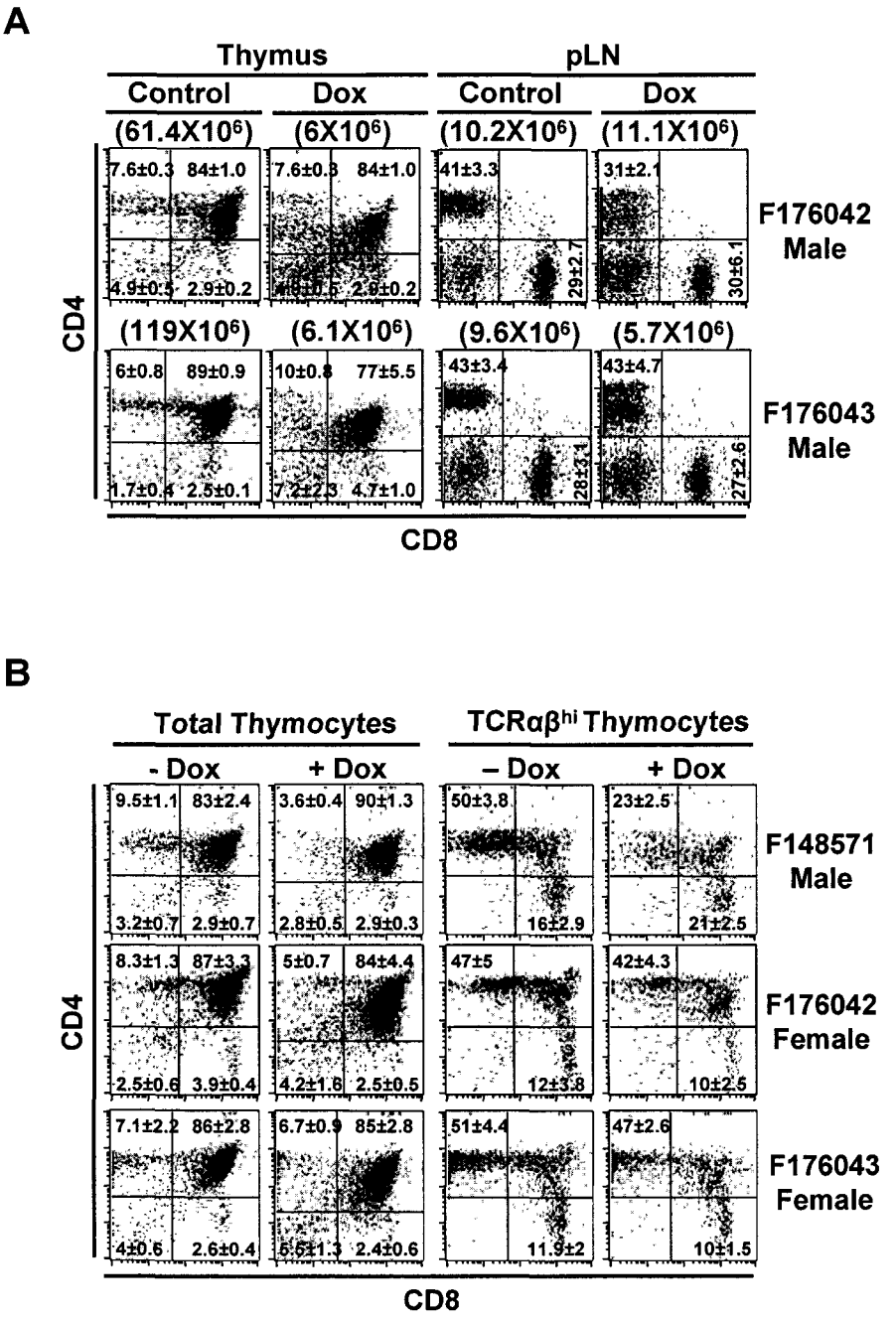


Figure 2.4. Flow cytometric analysis of thymocyte populations in dox-treated mice. **(A)** FACS analysis of cell populations in thymus and pLN of male mice from F176042 and F176043 transactivator founder lines treated with 0.5mg/ml dox in drinking water for 1 week and untreated control mice. The percentage of cells and standard deviations in each quadrant is indicated. Total cell numbers for each organ is indicated in parentheses. **(B)** FACS analysis of cell populations in thymus of male mice from F148571 and female mice from F176042 and F176043 transactivator founder lines treated with 2mg/ml dox in drinking water for 6 weeks and untreated control mice. The percentage of cells and standard deviations in each quadrant is indicated.

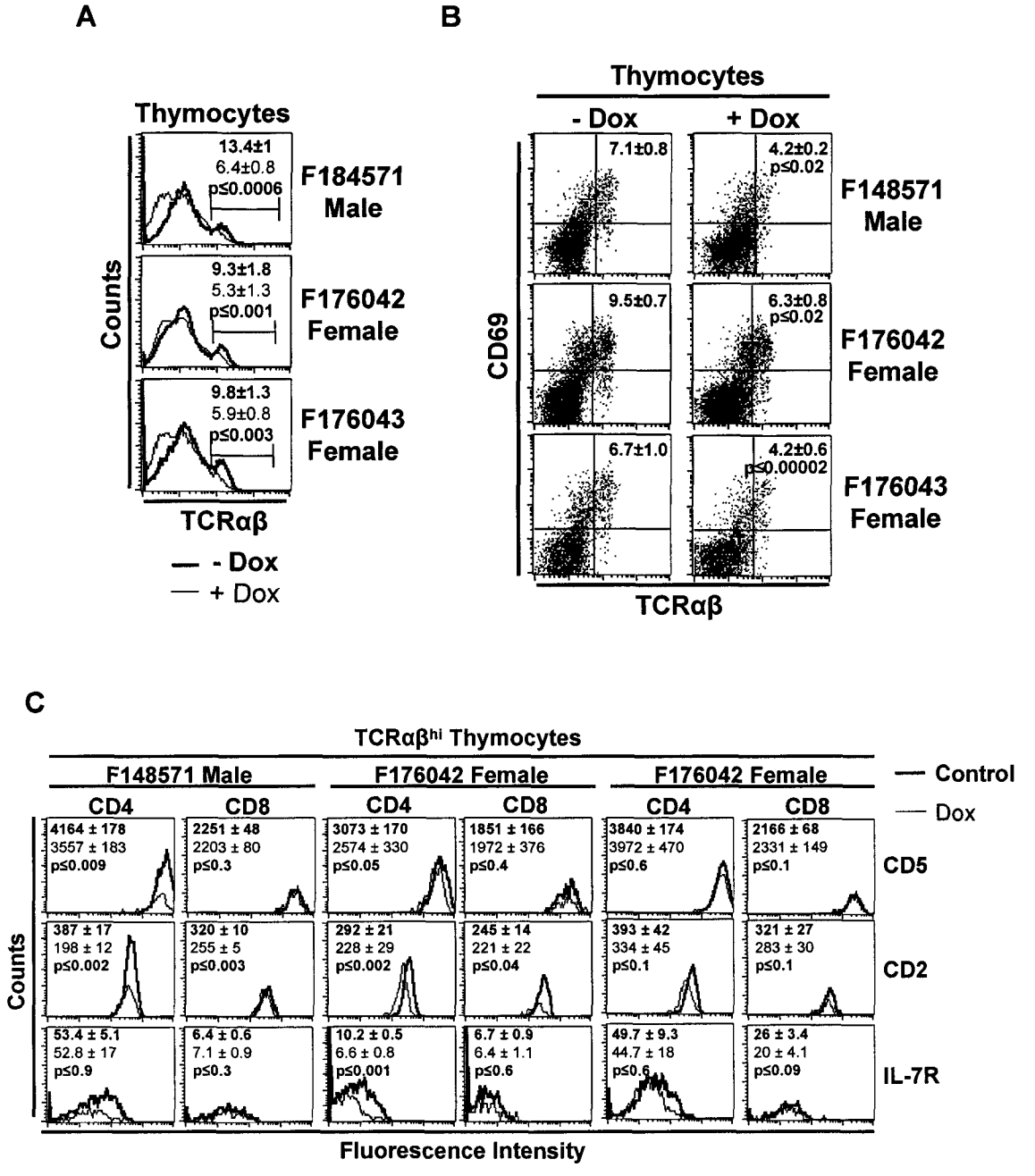


Figure 2.5. Analysis of thymocyte maturation in dox-treated mice. **(A)** Expression of TCRαβ on thymocytes from 3 transactivator founder lines treated with 2mg/ml dox in drinking water for 2 months. Percentage of TCRαβ^{hi} cells is shown in bold font for untreated control mice and in regular font for dox-treated mice. **(B)** Expression of TCRαβ and CD69 in thymus of dox-treated and untreated control mice. Percentages and standard deviations for TCRαβ^{hi}CD69^{hi} cells are indicated. **(C)** Analysis of maturation markers (CD2, CD5 and IL-7R) on TCRαβhi thymocytes. MFI of each marker is indicated in bold font for the untreated control mice and in regular font for dox-treated mice. Statistical analysis was performed by Student's *t*-test and *p*-values are shown.

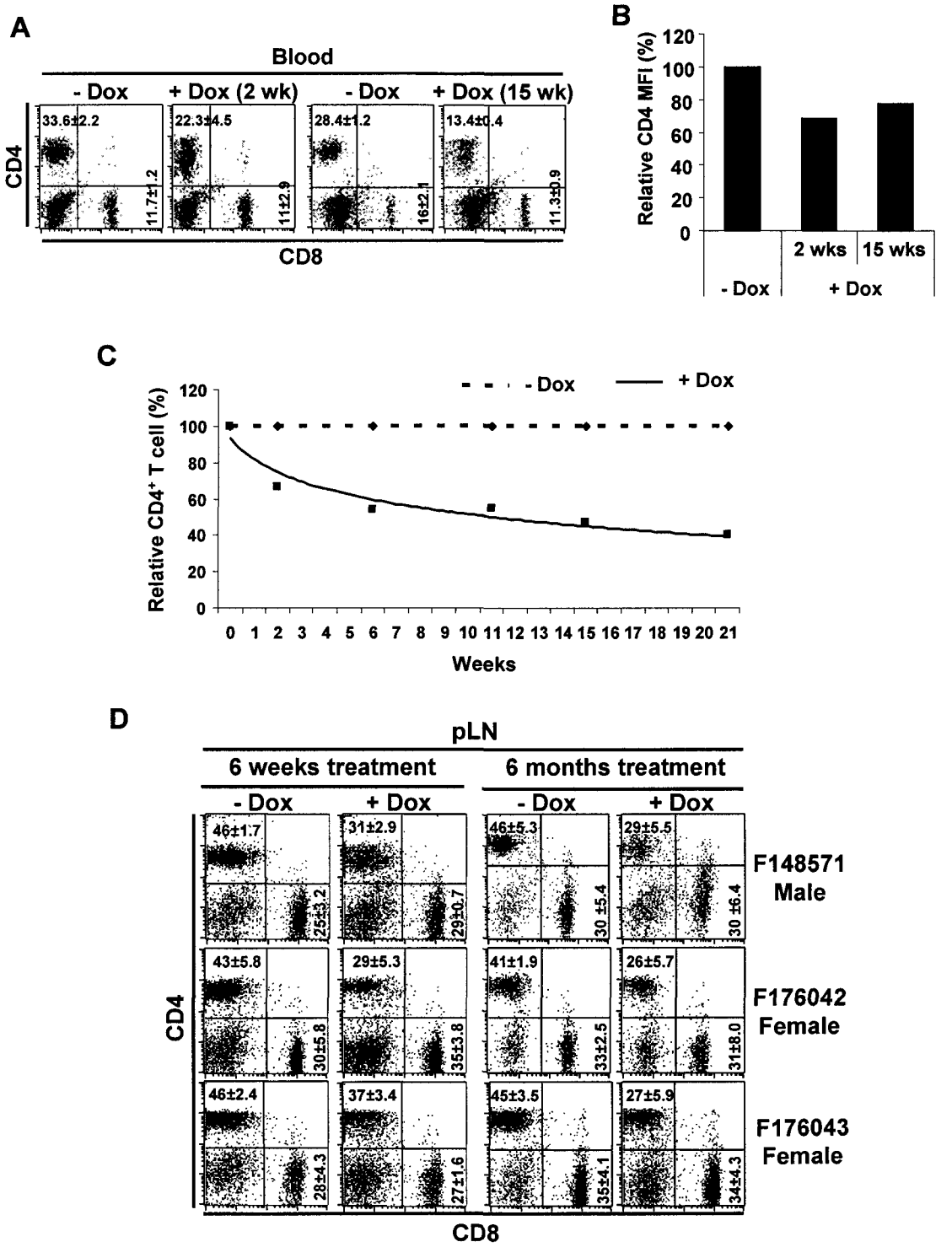

A
Blood
- Dox
+ Dox (2 wk)
- Dox
+ Dox (15 wk)
33.6±2.2
11.7±1.2
22.3±4.5
11±2.9
28.4±1.2
16±2.1
13.4±0.4
11.3±0.9
CD4
CD8
B
Relative CD4 MFI (%)
120
100
80
60
40
20
0
2 wks
15 wks
- Dox
+ Dox
C
- Dox
+ Dox
Relative CD4+ T cell (%)
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Weeks
D
pLN
6 weeks treatment
6 months treatment
- Dox
+ Dox
- Dox
+ Dox
46±1.7
25±3.2
31±2.9
29±0.7
46±5.3
30 ±5.4
29±5.5
30 ±6.4
F148571
Male
43±5.8
30±5.8
29±5.3
35±3.8
41±1.9
33±2.5
26±5.7
31±8.0
F176042
Female
46±2.4
28±4.3
37±3.4
27±1.6
45±3.5
35±4.1
27±5.9
34±4.3
F176043
Female
CD4
CD8

Figure 2.6.

Figure 2.6. Flow cytometric analysis of peripheral T cells in dox-treated mice. (A) FACS analysis of T cell population in blood of male mice from F148571 founder line treated with dox (2mg/ml). Duration of treatment is shown in parentheses. Percentages and standard deviations of cells in each quadrant is indicated. **(B)** Bar graph showing relative CD4 MFI in blood of dox-treated and untreated mice. **(C)** Graph showing relative loss of $CD4^+$ T cells in blood of the founder line F148571 over time after transgene induction. Dashed line, untreated control mice; solid line, dox-treated mice. **(D)** FACS analysis of cell populations in pLN of mice treated with 2mg/ml dox for 6 weeks and untreated control mice of the 3 founder lines. The percentage and standard deviation for cells in each quadrant is indicated.

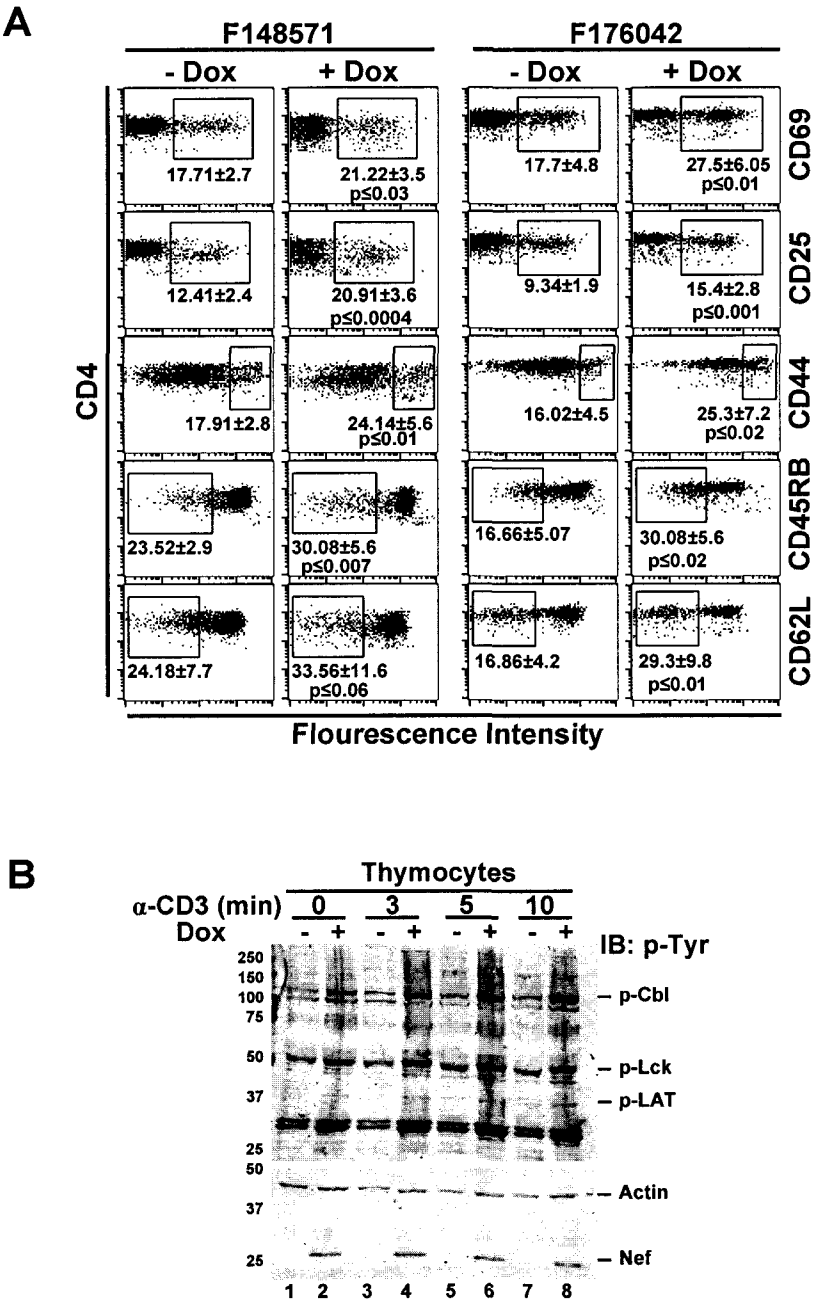


Figure 2.7. Immunophenotype of pLN CD4$^+$ T cells from dox-treated mice. (A) FACS analysis of activation/developmental markers (CD69, CD25, CD44, CD45RB, CD62L) on CD4$^+$ T cells from male mice of F148571 and female mice of F176042 founder lines treated with dox (2mg/ml). Percentage and standard deviation of cells enclosed in box is indicated. Statistical analysis was performed by Student's *t*-test and *p*-values is shown. **(B)** Phosphotyrosine (pY) proteins immunoblot of thymocyte lysate from dox-treated and untreated DTg male mice from F148571 founder line after stimulation with anti-CD3 antibody. The membrane was washed and reblotted with anti-actin and anti-Nef antibody to control loading and detect Tg expression.

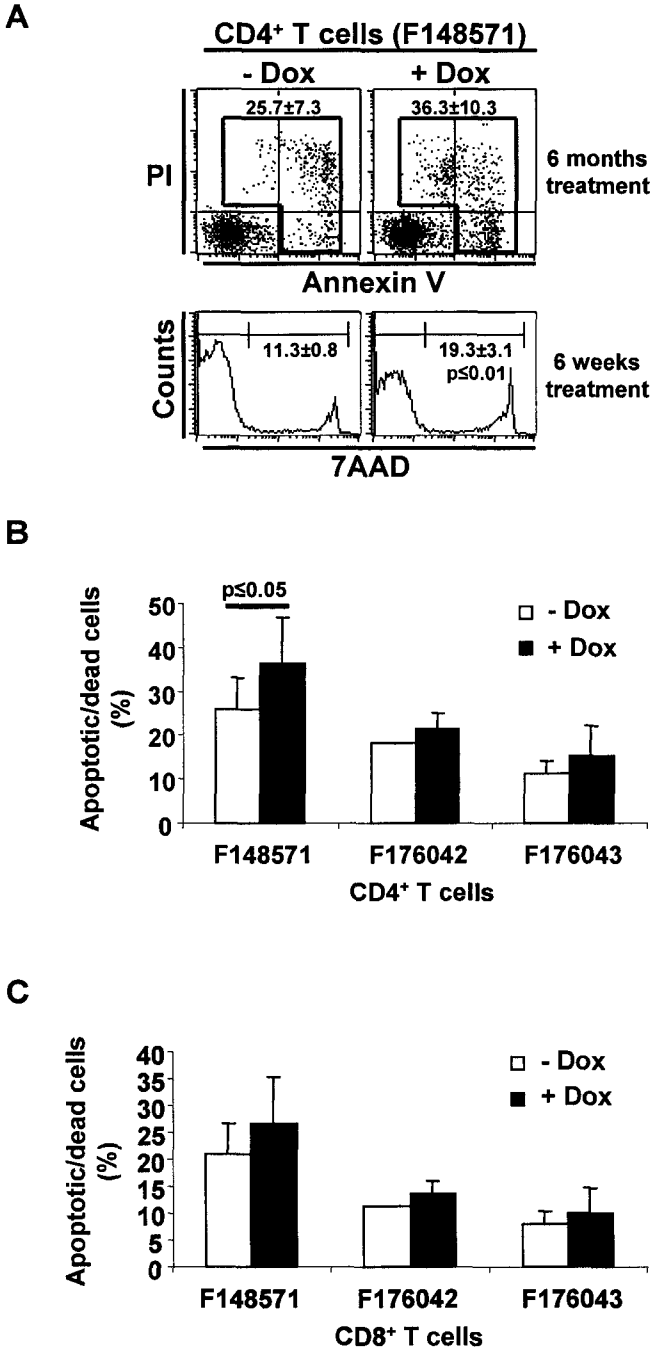


Figure 2.8. CD4$^+$ T cell death in dox-treated mice. **(A)** FACS analysis of apoptotic and dead cell among the CD4$^+$ T cells of male mice from F148571 founder line treated with dox (2mg/ml). Apoptotic/dead cells were analyzed by FACS analysis after staining cells with anti-CD4 and anti-CD8 MAbs and 7AAD or annexin V/PI. The percentage and standard deviation of apoptotic/dead cells is indicated. Bar graph showing percent of apoptotic/dead cells among CD4$^+$ T cells **(B)** and CD8$^+$ T cells **(C)** in the pLN. Statistical analysis was performed by Student's *t*-test and *p*-values are shown.

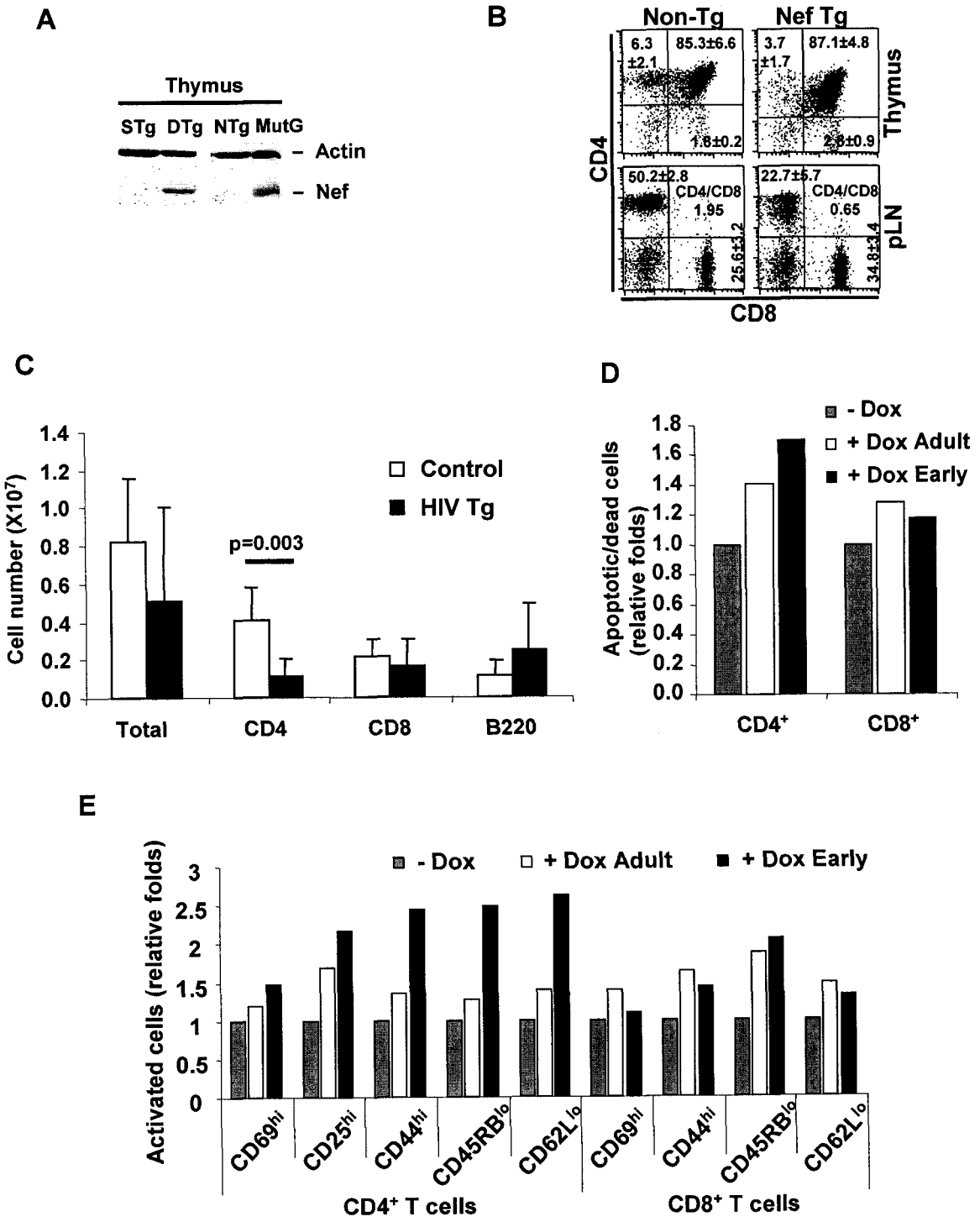

A
Thymus
STg DTg NTg MutG
Actin
Nef
B
Non-Tg
Nef Tg
6.3 ±2.1
85.3±6.6
1.6±0.2
3.7 ±1.7
87.1±4.8
2.3±0.9
Thymus
50.2±2.8
CD4/CD8 1.95
22.7±5.7
CD4/CD8 0.65
pLN
CD4
CD8
C
Cell number (X10^7)
Control
HIV Tg
p=0.003
Total
CD4
CD8
B220
D
Apoptotic/dead cells (relative folds)
- Dox
+ Dox Adult
+ Dox Early
CD4+
CD8+
E
Activated cells (relative folds)
- Dox
+ Dox Adult
+ Dox Early
CD69hi
CD25hi
CD44hi
CD45RBlo
CD62Llo
CD69hi
CD44hi
CD45RBlo
CD62Llo
CD4+ T cells
CD8+ T cells

Figure 2.9.

Figure 2.9. Early induction of Tg expression. Pregnant mice were fed with dox (2mg/ml) in drinking water and the pups were kept on dox upto the age of 6 months. **(A)** Western blot analysis of Nef protein in thymus of 2 weeks old pup born to female mice on dox treatment. Total thymus protein extract (100μg) was blotted with anti-Nef serum and anti-actin antibody. Protein sample from CD4C/HIV^{Nef} was used as positive control. STg, mice single Tg for either TRE/HIV or CD4C/rtTA; DTg, mice double Tg for TRE/HIV and CD4C/rtTA; NTg, non-Tg mice. **(B)** FACS analysis of cell populations in thymus and pLN of male mice from F148571 transactivator founder line with early Nef expression. The percentage of cells and standard deviations in each quadrant is indicated. The CD4/CD8 ration in pLN is also indicated. **(C)** Bar graph showing pLN cell numbers of DTg mice (HIV Tg) and littermate STg (Control) treated with dox (2mg/ml). **(D)** Relative increase in proportions of apoptotic/dead cells among the CD4$^+$ T cells of mice treated with dox at early or adult life compared to the control mice. **(E)** Relative increase in proportion of CD4$^+$ T cells showing activated/memory phenotype from mice treated with dox at early and adult life. Statistical analysis was performed by Student's *t*-test and *p*-values are shown.

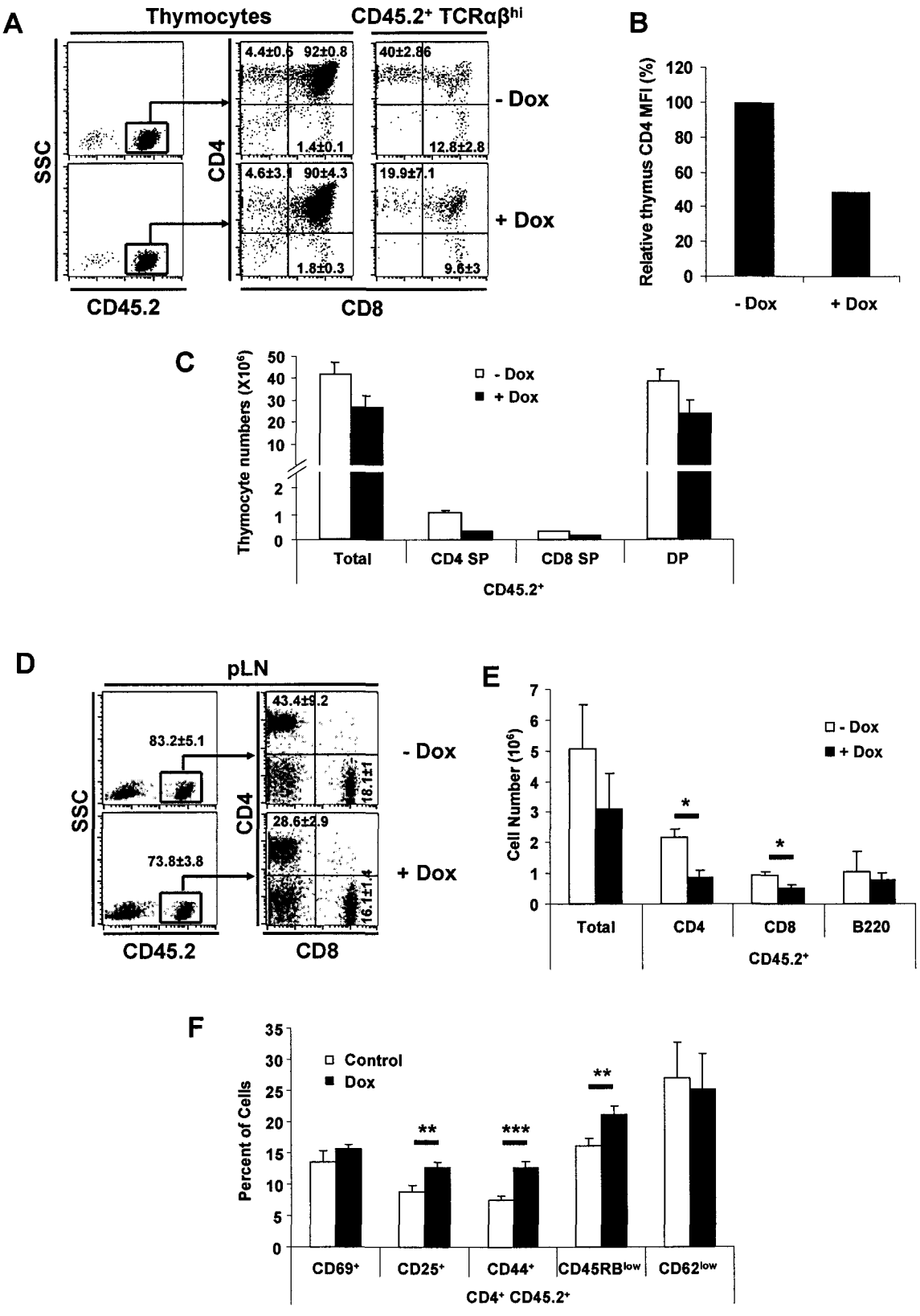
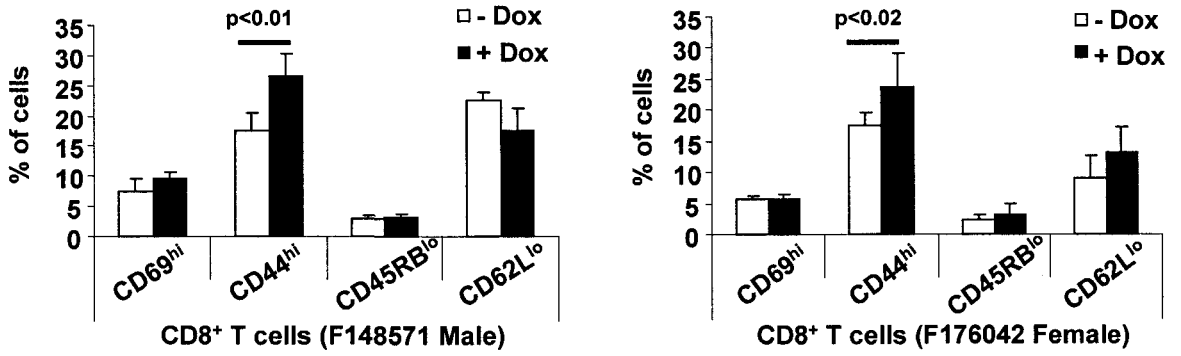

A
Thymocytes
CD45.2+ TCRαβhi
SSC
CD4
CD45.2
CD8
4.4±0.6
92±0.8
1.4±0.1
40±2.86
12.8±2.8
- Dox
4.6±3.1
90±4.3
1.8±0.3
19.9±7.1
9.6±3
+ Dox
B
Relative thymus CD4 MFI (%)
120
100
80
60
40
20
0
- Dox
+ Dox
C
Thymocyte numbers (X10⁶)
50
40
30
20
10
2
1
0
- Dox
+ Dox
Total
CD4 SP
CD8 SP
DP
CD45.2+
D
pLN
SSC
CD4
CD45.2
CD8
83.2±5.1
43.4±9.2
18.1±1
- Dox
73.8±3.8
28.6±2.9
16.1±1.4
+ Dox
E
Cell Number (10⁶)
7
6
5
4
3
2
1
0
- Dox
+ Dox
*
*
Total
CD4
CD8
B220
CD45.2+
F
Percent of Cells
35
30
25
20
15
10
5
0
Control
Dox
**
***
**
CD69+
CD25+
CD44+
CD45RBlow
CD62low
CD4+ CD45.2+

Figure 2.10.

Figure 2.10. T cell phenotypes in bone marrow transplanted chimeric mice. **(A)** FACS analysis of thymocyte populations in dox-treated and untreated control bone marrow transplanted chimeric mice. Percentage and standard deviation of cells in each quadrant is indicated. **(B)** Bar graph showing thymocyte cell numbers. **(C)** Bar graph showing relative decrease in CD4 MFI in thymus of dox-treated Vs untreated control chimeric mice. **(D)** FACS analysis of T cell populations in pLN of dox-treated and untreated control chimeric mice. Percentage and standard deviation of cells in each quadrant is indicated. **(E)** Bar graph showing pLN cell numbers. **(F)** Bar graph showing percentage of $CD4^+$ T cells showing activated/memory phenotype based on analysis of markers such as CD69, CD25, CD44, CD45RB and CD62L. Statistical analysis was performed by Student's *t*-test and *p*-values are shown. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0005$.



Supplementary Figure 2.1. Immunophenotype of pLN $CD8^+$ T cells from dox-treated mice. Percentage of $CD8^+$ T cells showing activated/memory phenotype based on expression of activation/developmental markers (CD69, CD25, CD44, CD45RB, CD62L) in dox-treated mice from F148571 and F176042 transactivator founder lines.

Table 2.1. Percent of cells expressing high levels of GFP in thymus and pLN of (CD4C/rtTA X tetO/H2B-GFP) and (CD4C/rtTA2^S-M2 X tetO/H2B-GFP) DTg mice treated with dox (2mg/ml) for 1 week.

Mouse line	Dox Treatment	GFP^{high} in thymus (%)				GFP^{high} in pLN (%)		
		CD4$^+$SP	CD8$^+$ SP	CD4$^+$8$^+$ DP	CD4$^-$8$^-$ DN	CD4$^+$ T	CD8$^+$ T	B
F148571	No	4.3±2.1	7.1±2.1	0.14±0.14	2±3.2	0.14±1.04	0.7±0.9	0.4±0.1
	Yes	88.3±5.5	55.5±8.3	93.7±8.4	17.8±12.1	17.3±11.5	4.2±5.4	4.3±1.4
F176042	No	7.4±1.1	12.6±3.9	0.15±0.07	4.3±5.7	0±0	0.1±1.14	0.9±0.07
	Yes	95.9±1.6	57.5±7.9	97.7±1.9	83±23	23.2±12.3	14.7±5.1	10.7±2.7
F176043	No	8.1±3.9	10.5±0.9	0.2±0.02	4.6±2.9	0	0	0.25±0.2
	Yes	91.6±7.1	71.6±8.3	89.8±11.3	91.7±5.6	13.9±3.7	19.3±10.1	4.2±1.0

Table 2.2. Cell numbers of thymocyte populations from mice of different transctivator founder lines after dox treatment to induce HIV-1 Nef expression.

Mouse line	Dox Treatment	Cell numbers (X10^6)				CD4/CD8 ratio	CD4 MFI (%)
		Total	CD4$^+$CD8$^-$	CD8$^+$CD4$^-$	CD4$^+$CD8$^+$		
CD4C/rtTA;TRE/HIV							
F148571 Male	Nil	70.66±10	4.74±0.73	1.61±0.62	58.8±6.87	2.94	100
	6 weeks	48±3.26*	0.73±0.18**	0.66±0.08*	44.8±2.58*	1.1	67.4
CD4C/rtTA2^S-M2;TRE/HIV							
F176042 Male	Nil	61.4±17.4	2.72±0.78	0.92±0.31	51.8±14.8	2.94	100
	1 weeks	6±2.2**	0.59±0.21**	0.18±0.08*	2.8±1.08**	3.22	52.4
F176042 Female	Nil	109±13.8	4.99±1.8	1.25±0.53	92.6±13.2	3.98	100
	6 weeks	80.5±25.6*	1.72±0.29**	0.42±0.14*	68.5±23.6*	4.02	66.1
F176043 Male	Nil	119±31	5.37±2.03	2.07±0.64	106±27.9	2.58	100
	1 weeks	6.75±3.22**	0.27±0.09*	0.08±0.04**	5.35±2.73**	3.15	42.1
F176043 Female	Nil	210±11.5	10.7±2.68	2.4±0.15	180±21.7	4.46	100
	6 weeks	202±26.2	5.82±1.74*	1.21±0.18**	172±21.7	4.79	67.7

*, p<0.05; **, p<.005 by Student's T-test

Table 2.3. Cell numbers of lymphocyte populations from mice of different transctivator founder lines after dox treatment to induce HIV-1 Nef expression.

Mouse line	Dox Treatment	Cell number (X10^6)				CD4/CD8 ratio	CD4 MFI (%)
		Total	CD4$^+$ T	CD8$^+$T	B		
CD4C/rtTA;TRE/HIV							
F148571 Male	Nil	17.8±2.02	8.16±0.71	4.521±0.32	4.55±1.15	1.8	100
	6 weeks	5.05±1.1**	1.57±0.46***	1.48±0.31***	1.65±0.33*	1.06	87.8
CD4C/rtTA2^S-M2;TRE/HIV							
F176042 Female	Nil	18.2±3.7	7.38±1.03	5.49±1.16	2.77±1.22	1.34	100
	6 weeks	13.1±1.66*	4.02±0.97***	4.61±0.74	3.56±0.91	0.87	106
F176043 Female	Nil	19.7±2.06	9.16±1.38	5.48±0.82	4.69±0.97	1.67	100
	6 weeks	8.1±2.99**	3.51±0.8**	2.22±0.77**	2.27±1.16*	1.58	103

*, p<0.05; **, p<.005; ***, p<0.0005 by Student's T-test

Supplementary Table 2.1. Cell numbers of lymphocyte populations in male mice from F176042 transctivator founder lines treated with 0.5mg/ml dox for 1 week.

Mouse line	Dox Treatment	Cell number ($X10^6$)				CD4/CD8 ratio	CD4 MFI (%)
		Total	$CD4^+$ T	$CD8^+$ T	B		
CD4C/rtTA2^S-M2;TRE/HIV							
F176042 Male	Nil	10.2±2.93	4.18±0.92	2.96±0.77	2.78±1.29	1.41	100
	1 weeks	11.1±3	3.45±0.9	3.32±0.28	3.84±1.69	1.03	73.7
F176043 Male	Nil	9.6±2.18	4.16±0.84	2.7±0.83	1.67±0.55	1.5	100
	1 weeks	5.7±3.71	2.61±1.94	1.47±0.98	1.12±0.77	1.77	73.6

Supplementary Table 2.2. Cell numbers of thymocyte populations in mice from different transctivator founder lines treated with 2mg/ml dox for 6 months.

Mouse line	Dox Treatment	Cell number ($X10^6$)				CD4/CD8 ratio	CD4 MFI (%)
		Total	$CD4^+$ T	$CD8^+$ T	B		
CD4C/rtTA;TRE/HIV							
F148571 Male	Nil	6.14±1.72	2.89±1.04	1.81±0.41	1.04±0.42	1.59	100
	6 months	5.97±2.06	1.77±0.6 *	1.95±0.89	1.66±0.90	0.91	88.2
CD4C/rtTA2^S-M2;TRE/HIV							
F176042 Female	Nil	12.6±4.92	5.33±2.16	4.19±1.54	2.19±1.00	1.27	100
	6 months	5.25±2.36 *	1.34±0.52 **	1.65±0.88 *	1.39±0.87	0.81	104
F176043 Female	Nil	6.85±1.3	2.99±0.53	2.59±0.69	0.80±0.31	1.15	100
	6 months	5.16±1.08	1.43±0.45 **	1.87±0.33	1.25±0.38	0.76	99.2

*, p<0.05; **, p<.005 by Student's T-test

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Chapter 3: HIV-1 Nef-induced T cell activation in the absence of lymphopenia

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(Manuscript based on preliminary results)

3.1. Preface

HIV-1 Nef has been shown to modulate TCR signaling and cause immune activation *in vitro* [1-3]. We and others have shown activation of T cells in transgenic mice expressing HIV-1 Nef in T cell populations [4-7]. Expression of Nef in Tg mice results in loss of T cells which creates lymphopenic conditions conducive for T cell activation. To demonstrate direct involvement of Nef in causing T cell activation, one must demonstrate activation of Nef expressing T cells in the context of an intact T cell compartment. This can be done in chimeric mice generated with a mixture of Nef Tg and non-Tg bone marrow or fetal liver cells. Unfortunately, Nef expressing T cells are unable to compete with non-Tg T cells to reconstitute the peripheral T cell compartment and constitute only a very small proportion of peripheral T cells in the mixed chimeras. The inducible Nef Tg mouse model has offered us the opportunity to generate mixed chimeras containing a significant proportion of Nef Tg T cells since in this system Nef is not expressed unless induced. Using chimeric mice containing inducible Nef Tg and non-Tg T cells we have studied the role of Nef in T cell activation and any indirect affect on non-Tg T cells *in vivo*.

3.2. Abstract

Chronic immune activation is thought to play an important role in HIV-1 pathogenesis. The cause of this chronic immune activation is not completely understood. Expression of HIV-1 Nef in T cells of Tg mice results in T cell loss as well as T cell

activation and increased turnover. Loss of peripheral T cells results in lymphopenic conditions in these mice which can induce T cell activation. We used inducible Nef Tg mice to study the involvement of Nef in T cell activation. Nef expression is induced in these mice upon treatment with doxycyline. Bone marrow chimeras were generated from a mixture of non-Tg and inducible Nef Tg bone marrows. The chimeras contained a significant proportion of Nef Tg cells in their lymphoid tissues. Treatment of these bone marrow chimeras with dox resulted in loss of Nef Tg peripheral $CD4^+$ T cells while the non-Tg peripheral T cells were unaffected, unlike the thymus where both the Tg and, to a lesser extent, the non-Tg thymocytes were affected. Moreover, Nef Tg peripheral $CD4^+$ T cells showed an activated phenotype in the absence of lymphopenia. Our results show that in bone marrow chimeras, T cell activation can occur in the absence of lymphopenia and is most likely driven by expression of Nef.

3.3. Introduction

HIV infection results in a state of chronic immune activation, which is characterized by elevated markers of activation on T cells, B cells, NK cells and monocytes, as well as increased levels of proinflammatory cytokines and chemokines in plasma and lymph nodes [8]. This results in increased T cell turnover in HIV-infected individuals [9-11]. A direct correlation has been observed between the immune activation and disease progression in HIV infected patients [12-14]. A direct link between $CD4^+$ T cell changes and immune activation has also been reported in both acute and chronic phases of HIV infection [15,16]. The role of immune activation in AIDS pathogenesis is

highlighted by studies of a cohort of sex workers in Kenya, who are exposed to repeated sexually transmitted infections resulting in immune activation, increased viremia, decline in $CD4^+$ T cell levels and rapid progression to AIDS [17]. Similarly, when SIV_{mac251}-infected macaques were repeatedly immunized with SIV-independent antigens to mimic state of chronic immune activation, the animals had significantly reduced survival compared to non-immune-stimulated control animals [18]. Further evidence come from studies of SIV-infected sooty mangabey monkeys, natural hosts of SIV, which do not develop aberrant immune activation and immunopathologies in contrast to pathogenic SIV and HIV infections [19]. Various factors that may contribute to this immune activation include direct or indirect involvement of of HIV-1 gene products such as Env gp120 [20,21] and Nef [1-3], increased plasma LPS levels due to microbial translocation from the gut [22] and impaired regulatory T cell (T_{reg}) function [23] in HIV-infected individuals.

HIV-1 Nef is a 27-35 kDa protein shown to downmodulate T cell surface markers (CD4, CD28, etc), modulate T cell receptor (TCR) signaling and cell survival, and play a role in viral infectivity and pathogenesis [24]. We and others have shown activation of T cells in transgenic mice expressing HIV-1 Nef in T cell populations [4-7]. The observation that the level of T cell activation depends on the level of Nef expression in the T cells of $CD4C/HIV^{Nef}$ Tg mice is suggestive of a direct involvement of Nef [5]. However, Koenen and coworkers have reported that T cell activation in Nef Tg mice is induced by lymphopenia [25]. In their study, chimeric mice were generated from a mixture of non-Tg and Nef Tg bone marrows, with a full T cell compartment, and Nef Tg T cells did not show activated phenotype. However, Nef Tg T cells constituted only 1% of peripheral T cells in these chimeras and the level of Nef expression in these cells is questionable [25].

The low reconstitution level by Nef Tg cells can be attributed to the competition by the non-Tg cells. To overcome the competitive disadvantage imparted by expression of Nef, we have used bone marrow from inducible Nef Tg mice to generate chimeras. The inducible Nef Tg mice express Nef only when treated with doxycycline and develop pathologies similar to CD4C/HIV^{Nef} Tg mice (Rahim *et al.*, unpublished).

In this study we report that bone marrow chimeras, generated from a mixture of non-Tg and inducible Nef Tg bone marrows, contain a significant proportion of Nef Tg cells in their lymphoid tissues. Treatment of these bone marrow chimeras with dox results in loss of Nef Tg peripheral $CD4^+$ T cells while the non-Tg peripheral T cells are unaffected. In the thymus, both the Tg and, to less extend, the non-Tg thymocytes were affected. Moreover, Nef Tg peripheral $CD4^+$ T cells show an activated phenotype in the absence of lymphopenia. Our results show that in bone marrow chimeras, T cell activation can occur in the absence of lymphopenia and is most likely driven by expression of Nef.

3.4. Materials and Methods

3.4.1 Mice. The (CD4C/rtTA X TRE/HIV^{Nef}) double-Tg mice are described elsewhere (Rahim et al., unpublished). The B6.SJL-Ptprca Pepcb/BoyJ ($CD45.1^+$) mice were obtained from Jackson Laboratory (Maine, USA) and breeding colony was established in our animal facility. The C3H/HeNHsd ($CD45.2^+$) mice were obtained from Harlan (Indiana, USA). To generate $CD45.1^+.2^+$ mice, B6.SJL-Ptprca Pepcb/BoyJ ($CD45.1^+$) were bred with C3H/HeNHsd ($CD45.2^+$) mice. To induce Nef expression,

mice were treated with dox (2mg/ml) in drinking water. Mice that were not treated with dox were used as control. All mice were housed in specific pathogen free (SPF) facility and all experiments were approved by the Institutional Animal Ethics Committee.

3.4.2. Antibodies and reagents. Fluorescein isothiocyanate (FITC), phycoerythrin (PE) and allopycocyanin (APC)-labeled mAb, including anti-CD45.1, CD45.2, CD4, CD8, TcRαβ, CD69, CD44, CD62L were purchased from Cederlane. The hybridomas producing rat anti-mouse CD4 (GK1.5), CD8 (53-6.72) and Thy1.2 (30H12) were purchased from American Type Culture Collection (ATCC; Rockville, Md.). Sheep anti-rat IgG magnetic dynabeads were purchased from Invitrogen. Doxycycline was purchased from Sigma-Aldrich.

3.4.3. Bone marrow transplantation. Tibia and femur were flushed with Iscove's medium to obtain the bone marrow. T cells were depleted using rat monoclonal antibodies against mouse CD4, CD8 and Thy1.2, followed by magnetic separation with sheep anti-rat IgG dynabeads (Invitrogen). The B6.SJL-Ptprca Pepcb/BoyJ mice were irradiated with a sublethal dose of 950 rads. A total of 5 $X10^6$ bone marrow cells were transferred to the recipient mice through i.v. injection. Immune reconstitution and chimerism in the blood were monitored by FACS analysis.

3.4.4. Flow cytometric analysis. Cell suspensions were prepared from lymphoid organs and stained with antibodies, as described previously [4]. Data acquisition was done

with FACS Calibur or LSR (Becton Dickinson) and Cell Quest software (Becton Dickinson) was used for data analysis.

3.4.5. Statistical analysis. Statistical comparison between groups was performed using the standard two-tailed Student's *t* test. The difference between groups was considered significant at $p<0.05$.

3.5. Results

3.5.1. Generation of mixed bone marrow chimeric mice

We have generated the inducible (CD4C/rtTA X TRE/HIV^{Nef}) HIV-1 Nef Tg mice which express Nef in CD4$^+$ T cells and cell of dendritic and monocyte/macrophage lineage when treated with doxycyline (dox) (Rahim *et al.*, unpublished). These mice develop an AIDS-like disease similar to the constitutively Nef expressing CD4C/HIV^{Nef} Tg mice generated previously in our lab [4]. To generate mixed bone marrow chimeras, bone marrow cells from inducible Nef Tg mice and non-Tg mice were cotransfered to sub-lethally irradiated B6.SJL-Ptprca Pepcb/BoyJ host mice. The donor and host immune cells could be distinguished by the CD45 allele expression. Nef Tg donor cells were CD45.1$^-$.2$^+$, non-Tg donor cells were CD45.1$^+$.2$^+$ and host was CD45.1$^+$.2$^-$. Two months after bone marrow transfer, reconstitution of host with donor-derived cells was confirmed in blood by FACS analysis **(Fig. 3.1A)**. Despite the 50:50 ratio of Tg to non-Tg bone marrow at the time of transfer, reconstitution was always greater with non-Tg cells. This is most likely due to very low level of leaky Nef expression in the inducible

system which can be disadvantageous to the Nef Tg cells. Approximately 38% of cells in the peripheral blood were of Nef Tg origin **(Fig. 3.1A)**. To induce Nef expression, the mice were treated with dox (2mg/ml) in drinking water for a period of 2 months, following which the mice were sacrificed and lymphoid organs were analyzed by flow cytometry. Chimeric mice which were not treated with dox were used as controls. Staining with antibody against CD45.1 and CD45.2 revealed Tg and non-Tg chimerism in all T cell populations in the thymus and pLN. Nef Tg cells constituted approximately 31% and 25% of cells in the thymus and pLN, respectively **(Fig. 3.1B and C)**. As expected, the proportion of Tg cells decreased when the mice were treated with dox, due to Nef-induced loss of T cells.

3.5.2. Depletion of both Nef Tg and non-Tg thymocytes in dox-treated mixed chimeric mice

FACS analysis was performed on thymus of dox-treated and untreated mixed chimeras after staining with antibodies against CD45.1, CD45.2, CD4 and CD8 **(Fig. 3.2A)**. We observed lower levels of surface CD4 expression on Tg as compared to non-Tg thymocytes as a result of the low level of leaky Nef expression in uninduced state **(Fig. 3.2B)**. Expression of Nef in Tg thymocytes after treatment with dox resulted in further downmodulation of surface CD4 expression on Tg thymocytes but not on non-Tg thymocytes **(Fig. 3.2B)**. Expression of Nef in the thymus also resulted in loss of thymocytes, which was apparent in Tg as well as the non-Tg thymocytes **(Fig 3.2C)**. However, depletion of non-Tg thymocytes was less severe than depletion of Tg thymocytes. Depletion was mainly confined to the $CD4^+8^+$ DP populations in non-Tg

thymocytes, while all Tg thymocytes populations were significantly depleted upon Nef expression **(Fig. 3.2D)**. Since the thymic stromal cells are host-derived and do not express Nef, the effect of Nef on Tg thymocytes seems to be cell autonomous. Whether depletion of non-Tg $CD4^+8^+$ DP thymocytes is a bystander effect of Nef expressing thymocytes needs further investigation.

3.5.3. Depletion of peripheral $CD4^+$ T cells caused by Nef expression

FACS analysis was performed on pLN of dox-treated and untreated mixed chimeras after staining with antibodies against CD45.1, CD45.2, CD4 and TCRαβ **(Fig. 3.3A)**. Similar to our observations in the thymus, lower levels of surface CD4 expression was recorded on Tg as compared to non-Tg $CD4^+$ T cells **(Fig. 3.3B)**. Expression of Nef after treatment with dox further downmodulated surface CD4 expression on Tg $CD4^+$ T cells but not on non-Tg $CD4^+$ T cells **(Fig. 3.3B)**. Furthermore, Tg $CD4^+$ T cell numbers were reduced in the pLN from dox-treated mixed chimeras as compared to the untreated control chimeras **(Fig. 3.3C)**. Although Tg $CD8^+$ T cell numbers were lower in dox-treated mixed chimeras as compared to the untreated control chimeras, it did not reach statistical significance. The non-Tg $CD4^+$ T and $CD8^+$ T cell numbers were unaffected in dox-treated mixed chimeras **(Fig. 3.3C)**. The total cellularity of pLN was maintained in dox-treated chimeras. These results demonstrate that the loss of Tg $CD4^+$ T cells is caused by Nef in a cell autonomous basis and no bystander effect is seen on non-Tg T cells.

3.5.4. Activated/memory phenotype of Tg CD4$^+$ T cells in the absence of lymphopenia

Despite some loss of Tg CD4$^+$ T cells upon induction of Nef expression by dox treatment, the total pLN cellularity was unaffected. Equal cell numbers were present in the pLN of dox-treated and untreated mixed chimeras **(Fig. 3.4A)**. To determine the activation status of T cells in the pLN, expression of activation/developmental markers such as CD69, CD44 and CD62L were assessed by FACS analysis. A higher proportion of Tg CD4$^+$ T cells were CD69$^+$ and CD44$^+$CD62L$^-$ in the dox-treated compared to the untreated control mixed chimeras **(Fig. 3.4B)**. However, the proportion of non-Tg CD4$^+$ T cells showing CD69$^+$ and CD44$^+$CD62L$^-$ phenotype was equal in both dox-treated and untreated mixed chimera groups **(Fig. 3.4B)**. These results show that T cells can attain activated phenotype in the absence of lymphopenia and since this phenotype is restricted to the Tg T cell population only, it is most likely driven by Nef expression in these cells.

3.6. Discussion

In this study, we report that in bone marrow chimeric mice, with a full T cell compartment, CD4$^+$ T cells expressing HIV-1 Nef attain an activate/memory phenotype. Since this T cell phenotype, in the absence of lymphopenia, is restricted to Nef-expressing cell only, it is most likely a direct affect of Nef. This is in contrast to the study by Koenen and coworkers, who reported that T cell activation is induced by lymphopenia in their HIV-1 Nef Tg mice [25]. Their argument is based on the observations that CD4$^+$ and CD8$^+$ T cells undergo proliferative responses when transferred to

lymphopenic hosts such as mice exposed to sublethal doses of irradiation and genetically modified $Rag2^{-/-}$ or $CD3\varepsilon^{-/-}$ mice [26-29]. This is thought to be an active homeostatic mechanism to fill the peripheral pool of naïve T cells. Loss of T cells in their Nef Tg mice creates lymphopenic conditions which may be conducive to such a homoeostatic mechanism, inducing proliferation of remaining T cells. Their observation that naïve wild type T cells, when transferred to lymphopenic Nef Tg mice, acquire effector/memory phenotype is an expected phenomenon seen under any lymphopenic condition [26]. This does not rule out the involvement of Nef in the activation of Tg T cells. There is also the possibility of presence of soluble Nef in the serum of these mice which may affect the non-Tg T cells besides the affect of the lymphopenic microenvironment. Soluble Nef is present in the serum of HIV-1 infected individuals and is cytotoxic to human $CD4^+$ T cells [30]. Soluble Nef has been shown to induce T cell activation either through a direct affect or indirectly by inducing monocytes/macrophages to produce inflammatory factors that can in turn activate T cells [31-33]. Koenen and coworkers also showed that in bone marrow chimeras from wild type and Nef Tg bone marrow, with full T cell compartment, Nef Tg T cells retain their naïve phenotype. In their chimeras the Nef Tg T cells constituted only 1% of total T cell compartment. Nef expressing cells are at disadvantage and are out competed by the wild type cells to reconstitute the immune compartments. Moreover, not all T cells in Nef Tg mice express high levels of Nef and the 1% Nef Tg T cells that do make it to the periphery maybe because these cells inherently express low levels of Nef compared to the cells which do not make it. Although, these cells have lower surface CD4 expression compared to the wild type T cells, which can be considered as the surrogate marker for Nef expression,

the level of Nef in these cells may not be sufficient to induce the activated phenotype. We have previously reported in CD4C/HIV^{Nef} Tg mice that the severity of disease as well as T cell activation levels depend on the level of Nef expression [4,5]. Also in the inducible Nef Tg mice, T cell activation is seen only in the mice from founder lines expressing high levels of Tg in the peripheral T cells (Rahim et al., unpublished). We have overcome the problem of low number of Nef Tg T cell reconstitution in the mixed bone marrow chimeras by using the inducible Nef Tg mice, since these mice do not express Nef in the absence of dox. In the bone marrow chimeras generated from mixture of non-Tg and inducible Nef Tg bone marrow, Tg cells constituted approximately 31% and 25% of cells in the thymus and pLN, respectively, before treatment with dox. In the pLN, expression of Nef, upon dox treatment, resulted in significant loss of Nef Tg $CD4^+$ T cells without affecting non-Tg T cells and total cellularity. Nef Tg $CD4^+$ T cells also showed activated/memory phenotype when treated with dox. Since these phenotypes are restricted to Tg T cells only and occur in a full T cell compartment, Nef expression seems to be the most likely mediator of these effects.

An unexpected but interesting phenotype in the mixed bone marrow chimeric mice treated with dox was the significant loss of non-Tg $CD4^+8^+$ DP thymocytes, although less severe than the loss of Nef Tg thymocyte. At the moment we cannot comment whether this is a true bystander effect caused by the Nef expressing thymocytes. We can rule out the involvement of thymic stromal cells since these cells come from the non-Tg host and do not express Nef. Most of the thymocytes in the Tg mice express high levels of the transgene (Rahim et al., unpublished). A large number of thymocytes are destroyed during thymic development [34]. This may release soluble Nef

that will affect other cells within the thymus. As discussed earlier, soluble Nef is present in the serum of HIV-1 infected individuals and can affect lymphocytes and macrophages *in vitro* [30,31,33,35]. Further studies are needed to address the nature and mechanism of this bystander effects observed in the thymus of mixed bone marrow chimeras.

In summary, this study shows that expression of Nef in the T cell populations of HIV-1 Nef Tg mice is the most likely cause of the activated/memory phenotype of $CD4^+$ T cells.

3.7. Acknowledgments

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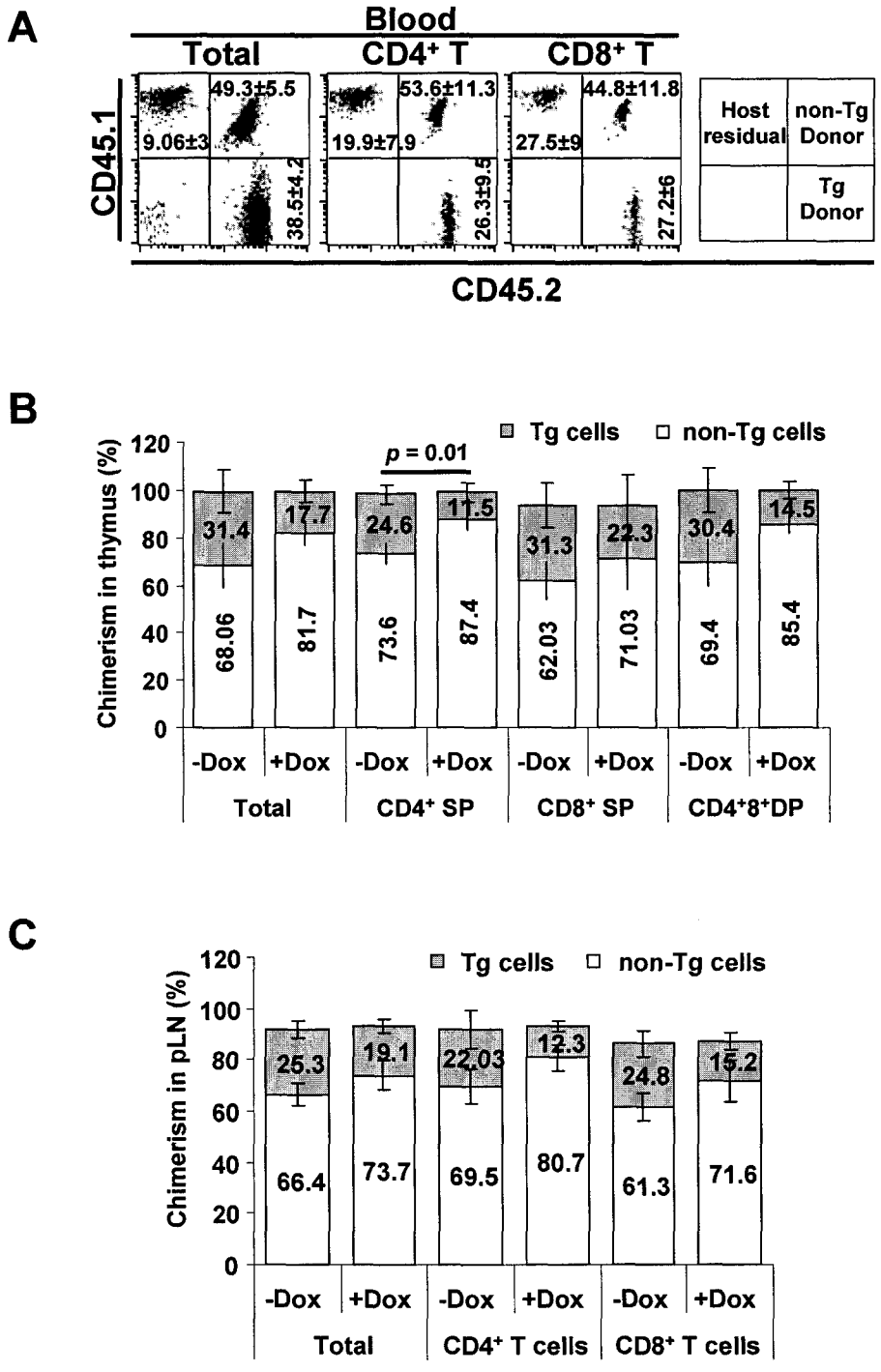
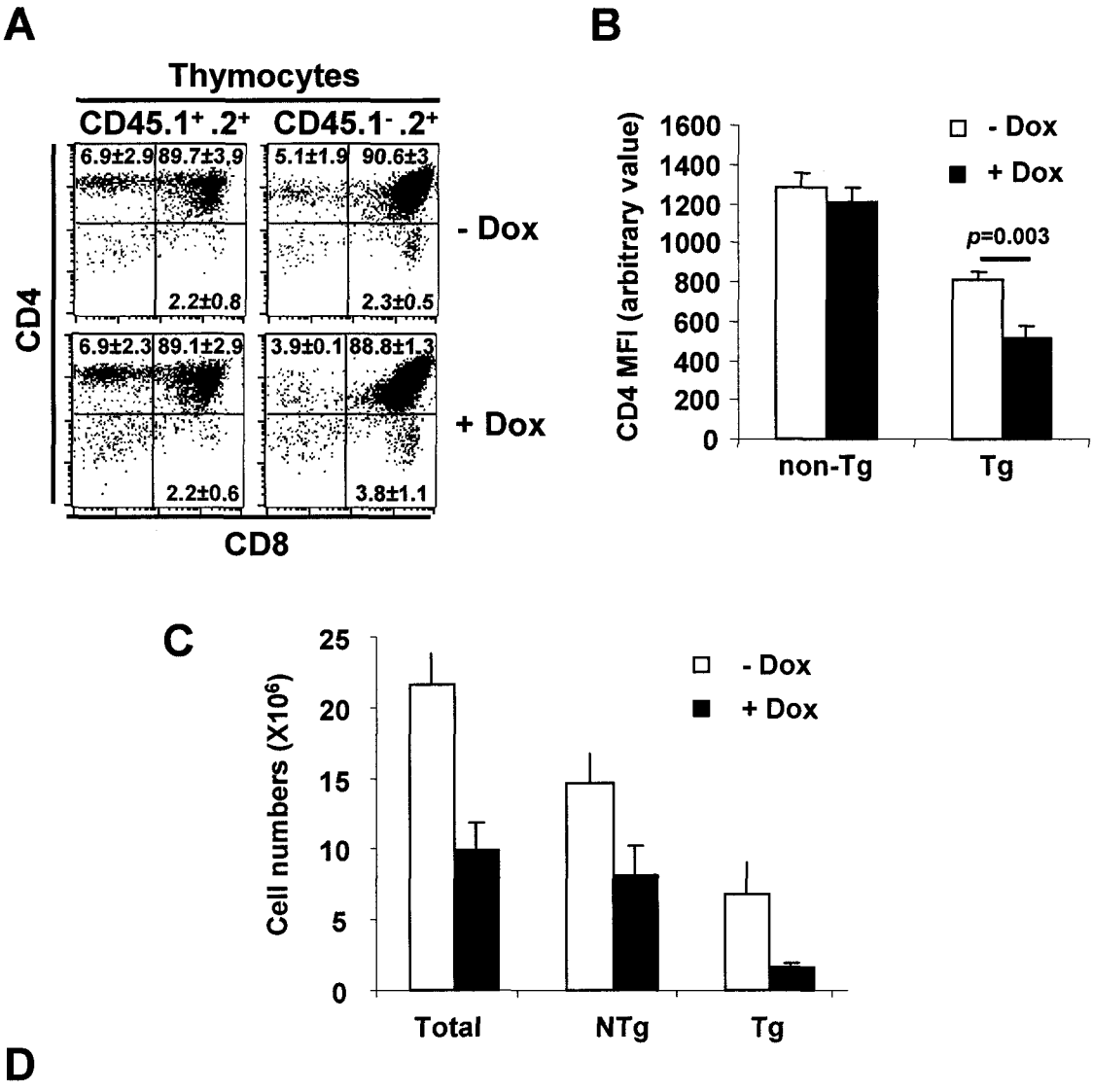


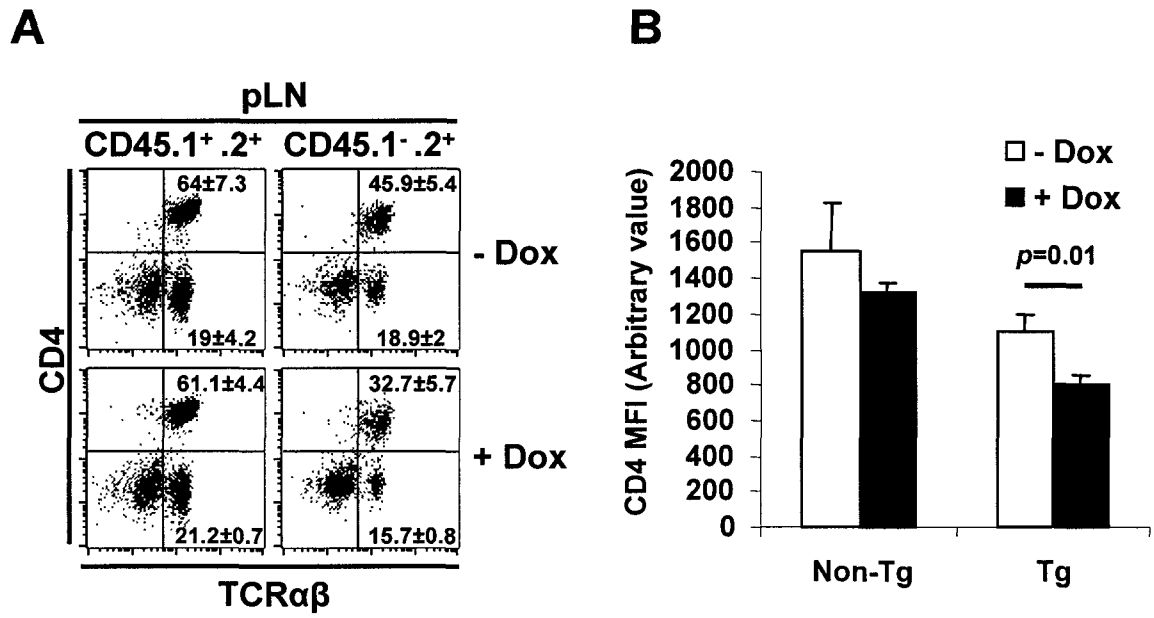
Figure 3.1. Chimerism in central and peripheral lymphoid compartments. (A) FACS analysis of chimerism in T cells from blood before commencement of dox treatment. Percentage and standard deviation of cells in each quadrant is indicated. Bar graphs showing chimerism in thymus (B) and pLN (C) from chimeras treated with dox and untreated controls. Average percentages are indicated.



D

Donor cells	Tg Induction	Cell numbers ($X10^6$)				CD4/CD8 ratio
		Total	$CD4^+8^-$ SP	$CD8^+4^-$ SP	$CD4^+8^+$ DP	
$CD45.1^+ .2^+$	No Dox	14.6±2	0.97±0.2	0.32±0.08	13.2±2.3	3.07
	Dox	8.1±2.1*	0.56±0.2	0.18±0.06	7.24±1.9*	3.08
$CD45.1^- .2^+$	No Dox	6.82±2.2	0.32±0.01	0.15±0.02	6.22±2.1	2.07
	Dox	1.7±0.26*	0.07±0.01***	0.07±0.03*	1.5±0.21*	0.98

Figure 3.2. Affect of Nef expression in thymus of mixed bone marrow chimeras. (A) FACS analysis of non-Tg ($CD45.1^+.2^+$) and Nef Tg ($CD45.1^-.2^+$) thymocytes in dox-treated and untreated chimeras. Percentage and standard deviation of cells in each quadrant is indicated. (B) Bar graph showing mean fluorescence intensity of surface CD4 staining on non-Tg and Tg thymocytes from dox-treated and untreated chimeras. (C) Bar graph showing cell numbers in the thymus of dox-treated and untreated chimeras. (D) Table showing cell numbers and CD4/CD8 ratio in the thymus from dox-treated and untreated chimeras.



C

Donor cells	Tg Induction	Cell numbers ($X10^6$) Total	$CD4^+$ T	$CD8^+$ T	CD4/CD8 ratio
$CD45.1^+ .2^+$	No Dox	4.19±0.37	2.66±0.1	0.81±0.23	3.3
	Dox	5.09±1.9	3.07±1.1	1.1±0.45	2.82
$CD45.1^- .2^+$	No Dox	1.61±0.36	0.74±0.19	0.31±0.1	2.38
	Dox	1.27±0.25	0.41±0.03*	0.2±0.03	2.05

Figure 3.3. Effect of Nef expression in pLN of mixed bone marrow chimeras. (A) FACS analysis of non-Tg ($CD45.1^+.2^+$) and Nef Tg ($CD45.1^-.2^+$) lymphocyte in dox-treated and untreated chimeras. Percentage and standard deviation of cells in each quadrant is indicated. (B) Bar graph showing mean fluorescence intensity of surface CD4 staining on non-Tg and Tg $CD4^+$ T cells from dox-treated and untreated chimeras. (C) Table showing cell numbers and CD4/CD8 ration in the pLN from dox-treated and untreated chimeras.

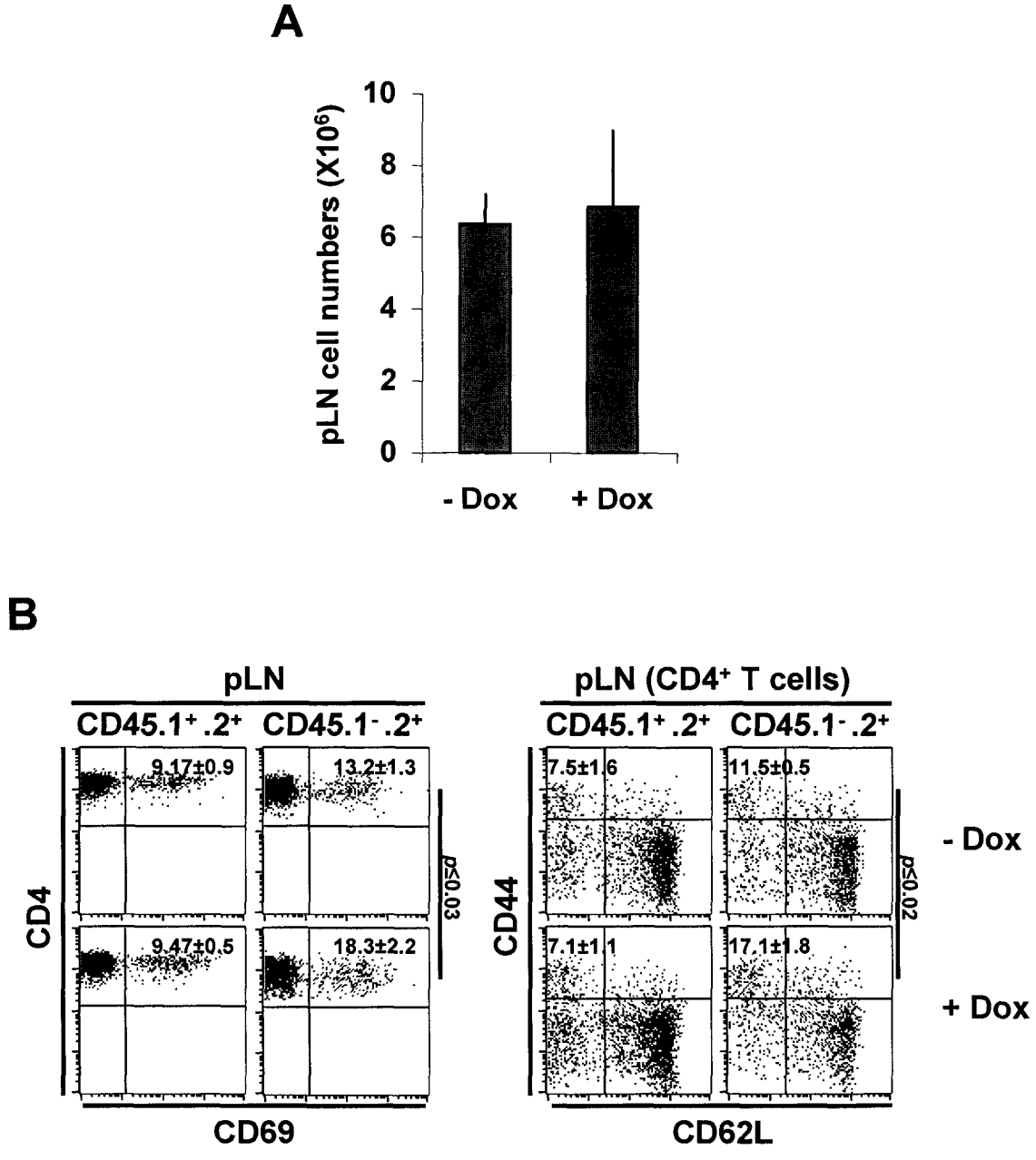


Figure 3.4. Activated/memory phenotype of Nef Tg CD4$^+$ T cells in pLN. (A) Bar graph showing cell numbers in pLN from dox-treated and untreated chimeras. (B) FACS analysis of activation markers (CD69, CD44 and CD62L) on non-Tg (CD45.1$^+$.2$^+$) and Nef Tg (CD45.1$^-$.2$^+$) peripheral CD4$^+$ T cells. Percentage and standard deviation of cells with activated/memory phenotype (CD69$^+$ and CD44$^+$CD62L$^-$) are indicated. Student's *t* test was performed and *p* values are shown.

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Chapter 4: Normal development and function but impaired memory phenotype of CD8$^+$ T cells in transgenic mice expressing HIV-1 Nef.

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(Manuscript in preparation)

4.1. Preface

$CD8^+$ T cells play an important role in the control of virus replication following primary HIV-1 infection. Since we have shown that expression of Nef in T cell populations of transgenic mice impairs thymopoiesis, we decided to study development of this T cell population in Nef Tg mice. In this study the effects of Nef expression on $CD8^+$ T cell development and function in the CD4C/HIV^{Nef} Tg mice was studied. The CD4C/HIV^{nef} Tg mice express Nef in $CD4^+$ T cells and in the cells of macrophage/monocyte/dendritic lineage, and develop an AIDS-like disease. HIV-1 infection has been shown to impair thymopoiesis in SCID-hu mouse model and HIV-1 infected infants with thymus dysfunction [1,2]. CD4C/HIV^{Nef} Tg mice express Nef at high levels in the thymus, particularly in the $CD4^+8^+$ DP thymocyte, and show impaired development of $CD4^+$ SP thymocytes (Chrobak et al., unpublished data). Since $CD8^+$ T cells also develop from the same $CD4^+8^+$ DP thymocyte, we decided to assess the effects of Nef on $CD8^+$ T cell development in these mice. We also studied $CD8^+$ T cell functions *in vitro* and *in vivo*, as well as generation and maintenance of memory response following virus infection in these mice.

4.2. Abstract

The CD4C/HIV^{nef} Tg mice express Nef in $CD4^+$ T cells and in the cells of macrophage/monocyte/dendritic lineage, and develop an AIDS-like disease. In the present study, we characterized the $CD8^+$ T cells compartment of these mice. Our results

show that both negative and positive selections of $CD8^+$ T cells proceed normally in the thymus of Tg mice. However, an increased number of immature $CD8^+CD69^+$ thymocytes accumulate in the thymus of Tg mice, indicating that their further development to mature cells may be impaired. Peripheral $CD8^+$ T cells of HIV Tg mice have an activated phenotype and enhanced cell division *in vivo*. These cells proliferate as efficiently as the wild type cells when stimulated *in vitro* with antigenic peptide. When challenged with $LCMV^{Armstrong}$, Nef Tg mice show a strong $CD8^+$ T cell response and clear the virus as efficiently as the wild type mice. However, the generation and maintenance of LCMV-specific $CD8^+$ memory T cells is impaired in the Tg mice which was partially rescued by adoptive transfer of non-Tg naïve $CD4^+$ T cells. Our results show that thymic selection of $CD8^+$ T cells is not impaired in HIV-1 Nef Tg mice. HIV-1 Nef Tg mice have competent peripheral $CD8^+$ T cells and develop an efficient primary $CD8^+$ T cells response to $LCMV^{Armstrong}$ infection but are not capable of maintaining memory phenotype due to lack of $CD4^+$ T helper activity.

4.3. Introduction

$CD8^+$ cytotoxic T lymphocyte (CTL) response is the first virus-specific immune response detected in acute HIV-1 infection and is the major component of the host immune response leading to the control of virus replication following primary HIV-1 infection [3,4]. HIV-specific CTLs are present in HIV-infected individuals and are directed against both the structural as well as regulatory genes of HIV-1 [5-8]. In SIV-infected macaque, deletion of $CD8^+$ T cells results in a dramatic rise in the plasma viremia

showing the strong dependence on $CD8^+$ T cells for viral control [9]. Also a significant inverse correlation has been observed between the frequencies of HIV-specific CTLs, recognized by tetramer staining, and plasma RNA viral load [10]. These CTLs may control HIV-1 replication by two mechanisms. First, virus-specific CTLs can recognize virus-infected cells and lyse them in an HLA class I restricted manner [11,12]. Second, these cells produce β-chemokines, which may bind HIV-1 coreceptor on the surface of $CD4^+$ T cells and block viral entry, and as yet unidentified $CD8^+$ T-lymphocyte antiviral factor (CAF) capable of inhibiting HIV-1 replication postentry in a STAT-1 dependant manner [12-14].

Despite the strong CTL response and initial control of viremia in primary infection, most individuals ultimately lose control of viral replication and progress to AIDS. The lack of $CD4^+$ helper T cells may render virus-specific CTLs non-functional. This is supported by the lack of inverse correlation between viral load and CTL frequency in patients with $CD4^+$ T cell counts below 400 cells/μl of blood [15]. HIV-specific CTLs also express lower levels of perforin and have diminished cytolytic activity [16]. In HIV-1 seropositive individuals the mean fluorescence (MF) of anti-CD8αβ staining in peripheral blood lymphocytes (PBL) is lower than the seronegative individuals and is positively correlated to the $CD4^+$ T cell counts [17]. Similar observation has been made in HIV-1 infected children with evidence of thymic defects and these cells were found to have weaker responses against antigens from CMV [18]. In both children and adults, there is a gradual loss of naïve $CD8^+$ T cells during the asymptomatic stage of HIV infection with a reciprocal increase in memory cells [19,20]. However, the maturation of memory HIV-specific $CD8^+$ T cells is altered and is predominantly composed of pre-terminally differentiated cells [21]. This could be the result of lack of $CD4^+$ helper activity which has

been shown to be crucial for maintenance of memory $CD8^+$ T cells in mice [22]. The central memory (T_{CM}) $CD8^+$ T cells have shorter half-life in HIV-infected individuals and their numbers are decreased as compared to healthy uninfected individuals [23]. Also expression of IL-7Rα on $CD8^+$ memory T cells is reduced in HIV-infected individuals which may impeded their function [23,24].

Taking into account the importance of $CD8^+$ T cells in suppression of viral replication in primary HIV-1 infection, we decided to study the $CD8^+$ T cell compartment of transgenic (Tg) mouse model of HIV-1 pathogenesis (CD4C/ HIV^{nef}) developed in our laboratory [25]. The CD4C-HIV^{nef} Tg mice express Nef in $CD4^+$ T cells and in the cells of macrophage/monocyte/dendritic lineage, and develop an AIDS-like disease characterized by immunodeficiency, loss of $CD4^+$ T cells, thymic atrophy, activation of T and B cells, loss of germinal centre formation and pathologies in heart, lungs and kidney similar to those in human AIDS. $CD8^+$ T cell development was studied in the thymus to assess the effect of HIV-1 Nef expression on positive and negative selection of $CD4^+8^+$ double positive (DP) thymocytes, which are the precursor cells for $CD8^+$ single positive (SP) thymocytes [26]. $CD8^+$ T cell functions were studied in *in vitro* stimulations and in *in vivo* infection with lymphocytic choriomeningitic virus (LCMV). Furthermore, generation of LCMV-specific memory $CD8^+$ T cells was assessed in these mice after infection with LCMV. Our results show that $CD8^+$ T cell development proceeds largely unaffected and are functionally competent in the Nef Tg mice. We observed a skewed maturation of LCMV-specific memory $CD8^+$ T cells, which was due to lack of $CD4^+$ helper activity in these mice.

4.4. Materials and Methods

4.4.1. Mice. CD4C/HIV^{Nef} (previously designated CD4C/HIV^{MutG}) Tg mice have been described earlier [25]. HY TCR and OT-1 TCR Tg mice were provided by Dr. Vibhuti Dave (IRCM, Montreal) and were crossed with CD4C/HIV^{Nef} Tg mice to obtain double Tg mice. All mice were maintained on a C57BL/6 background and littermates between the ages of 6-8 weeks for older mice and 1-2 weeks for younger mice were used in the experiments. All mice for this work were kept under specific pathogen-free conditions and all experiments were approved by the Institutional Animal Ethics Committee.

4.4.2. Antibodies and reagents. Fluorescein isothiocyanate (FITC), phycoerythrin (PE) and allopycocyanin (APC)-labeled mAb, including anti-CD4, CD8, TcRαβ, TCRγδ, CD69, CD25, CD44, CD45RB, CD62L were purchased from Cederlane. PE-labeled anti-HY TCR (T3.70) was from eBioscience and FITC-labeled anti-OT-1 (Vα2) TCR was from Cederlane. The hybridoma producing hamster anti-mouse CD3ε (145-2C11) was purchased from American Type Culture Collection (ATCC; Rockville, Md.). The anti-CD3 mAb was purified with a protein G affinity column. Mouse IL-2 was purchased from Roche Diagnostics GmbH. Irrelevant rat IgG1, rat IgG2a, rat IgG2b and Armenian hamster IgG1 were used as isotype controls. Bromodeoxyuridine (BrdU)-FITC flow kit was purchased from BD Pharmingen. OVAp peptide was a gift from Dr. V. Dave (IRCM, Montreal). LCMV^{Armstrong}, VL5 hybridoma cells, BHK21 and MC57G cells were gifts from Dr. C. Perreault (University of Montreal, Montreal) and Dr. W-K. Suh (IRCM,

Montreal). LCMV gp33-specific tetramer was purchased from Immunomics division, Beckman Coulter (San Diego, CA).

4.4.3. Flow cytometric analysis. Immunostaining was performed as described previously [25]. FACS Calibur and Cellquest software (Becton Dickinson) were used for flow cytometric analysis.

4.4.4. Purification of $CD8^+$ T cells. $CD8^+$ T cells were purified by cell sorting with MoFlo cell sorter (Cytomation Inc.). Peripheral LN cells were stained with PE-labeled MHC-II, anti-CD4, anti-B220 and anti-TCR$\gamma\delta$ as described previously [27]. The $CD8^+$ T cells were sorted by gating on PE-negative population.

4.4.5. CFSC fluorescent-dye cell labeling and division assay. CFSE labeling was performed as described previously [27]. CFSE-labeled cells were plated at 10^5 cells/well in a 96-well microtiter plates (NUNC^{TM}) and stimulated with plate bound anti-CD3 (5μg/ml) and recombinant mouse IL-2, ConA (5μg/ml) or OVAp (1μg/ml) for 3 days. For stimulation of purified $CD8^+$ T cells with OVAp, splenocytes from non-Tg mice irradiated at 2500 rads were used as antigen presenting cells. Unstimulated cells were used as CFSE baseline control. CFSE-labeled cells were analyzed by flow cytometry.

4.4.6. LCMV infections. LCMV^{Armstrong} was produced on BHK21 cells and titrated on MC57G cells by immunological focus assay [28,29]. Mice were infected with 10^6

pfu of LCMV intraperitonially (ip.). All the experiments were carried out in containment level 2 (CL2) facility. LCMV titer in spleen of infected mice was determined by immunological focus assay on MC57G cells [29]. LCMV gp33-specific tetramer staining was performed following manufacturer's instructions.

4.4.7. Quantitative RT-PCR. To detect transgene expression, Q-RT-PCR was performed on RNA isolated from sorted $CD4^+$ and $CD8^+$ T cells using QuantiTect SYBR Green PCR Kit (Qiagen) following manufacturer's instructions. The sequence of primers used for detecting HIV transcripts were CCCCACTGGGCTCCTGGTTGCAGC located in exon 1 of the human CD4 gene and CAGTCGCCGCCCCTCGCCTCTT specific for HIV genome.

4.4.8. Statistics. Statistical analysis by Student's *t*-test was performed as described previously [27].

4.5. Results

4.5.1. Expression of HIV Nef in $CD8^+$ T cells from Tg mice

We previously reported that the CD4C regulatory elements can derive expression of surrogate genes in a small percentage of $CD8^+$ T cells [30]. To confirm these results, we used alternative experimental approaches. First, we used the inducible green fluorescent protein (GFP) reporter mouse, tetO/H2B-GPP, expressing histone H2B-GFP fusion protein under the control of the tetracycline-responsive regulatory elements (TRE) [31].

GFP expression can be induced in these mice by the transactivator rtTA or its mutant derivatives rtTA2^{S}-M2 in the presence of doxycycline (dox), a derivative of tetracycline. Tg mice express each of these two transactivator genes under the control of the CD4C sequences (CD4C/rtTA, CD4C/rtTA2^{S}-M2) (Rahim *et al.*, unpublished). For the present experiment, Tg mice were bred with the tetO/H2B-GFP mice and double Tg (DTg) mice were induced with dox (2mg/ml) in drinking water for 1 week. FACS analysis of GFP expression showed a high expression of GFP in peripheral LN CD4^{+} T cells with the three transactivator founder lines studied, as expected (Rahim *et al.*, unpublished). Only a small proportion of CD8^{+} T cells expressed GFP at high or moderate levels, especially if the leaky expression of GFP in this cell population in the tetO/H2B-GFP single Tg mice is taken into account (**Fig. 4.1A**). In the thymus, a larger proportion of CD8^{+} single positive (SP) thymocytes and most of the CD4^{+} SP and CD4^{+}8^{+} DP thymocytes expressed GFP (**Fig. 4.1A**).

We then carried out a second experiment in which peripheral CD8^{+} T cells were purified from CD4C/HIV^{Nef} Tg mice by cell sorting and HIV expression was directly measured by quantitative real-time RT-PCR in the RNA extracted from them. For comparison, control CD4^{+} T cells were processed in parallel. Results of this experiment revealed that the levels of expression of the HIV transgene in CD8^{+} T cells was low, at about less than 2% of that measured in actual CD4^{+} T cells (**Fig. 4.1B**). Together, these experiments indicated that only a small percentage of CD8^{+} T cells in CD4C/HIV^{Nef} Tg mice express HIV-1 Nef at low levels. This is consistent with the reported low CD4-dependent infection of CD8^{+} T cells in patients infected with HIV-1 [32-34].

4.5.2. Negative selection of class-I restricted CD8$^+$ T cells is not affected by HIV-1 Nef in Tg mice

CD8$^+$ T cells develop from the CD4$^+$8$^+$ double positive (DP) thymocytes which have undergone negative and positive selection in the thymus. In CD4C/HIV^{Nef} Tg mice the transgene is expressed in DP thymocytes. To determine the effect of HIV-1 Nef expression on negative selection of class-1 restricted thymocytes, CD4C/HIV^{Nef} Tg mice were bred with HY TCR Tg mice. The HY TCR Tg mice express a T-cell receptor (TCR) transgene encoding reactivity to male antigen HY presented by class-I MHC [35]. In male HY TCR Tg mice, the thymus is normally small and thymocyte numbers are low due to depletion of self-reactive T-cells. Since adult CD4C/HIV^{Nef} Tg mice show pathologies in non-lymphoid organs, which could adversely affect the lymphoid tissues, we decided to analyse adult as well as very young mice (<2 week-old) before the appearance of any organ disease. Young (<2 week-old) and older (4-6 week-old) (CD4C/HIV^{Nef} X HY TCR) double Tg male mice showed lower CD8$^+$ SP and CD4$^+$8$^+$ DP thymocytes numbers compared to female double Tg mice and even to male HY TCR Tg mice **(Fig. 4.2A and B)**. CD8$^+$ T cell numbers were similar in the peripheral lymphoid organs (data not shown). In addition, older (4-6 week-old) double Tg male mice had severe thymic atrophy and splenomegaly compared to HY TCR Tg mice, as well as downregulation of cell surface CD4, depletion of thymic CD4$^+$ SP and peripheral CD4$^+$ T cells similar to the phenotype observed in CD4C/HIV^{Nef} Tg mice [25].

Therefore, the self-reactive HY TCR Tg CD4$^+$8$^+$ DP thymocytes are depleted efficiently in the thymus of DTg male mice. These results indicate that negative selection

of class-I restricted $CD8^+$ T cells still proceeds in the presence of HIV-1 Nef and may even be more efficient than in non-Tg mice.

4.5.3. Positive selection of class-I restricted $CD8^+$ T cells proceeds in the presence of HIV-1 Nef with the accumulation of immature $CD8^+$ SP thymocytes

Positive selection of $CD8^+$ T cells was first studied in female HY TCR Tg mice, in which HY TCR expressing thymocytes are not negatively selected. In both young (<2 weeks old) and older (4-6 weeks old) female ($CD4C/HIV^{Nef}$ X HY TCR) double Tg mice, $CD4^+8^+$ DP thymocytes numbers were lower than in HY TCR single Tg females, while there was an increase in DN thymocyte numbers and some increase in $CD8^+$ SP thymocyte numbers **(Fig. 4.2B)**. Surprisingly, $CD8^+$ T cell numbers were much lower in the peripheral lymphoid organs of younger female mice, although they were similar in older female mice compared to HY TCR Tg female mice **(Fig. 4.2C)**.

OT-1 TCR Tg mice were also used to study positive selection. These Tg mice express a TCR (Vα2Vß5) specific for the OVA-derived peptide SIINFEKL (OVAp) presented in the context of $H\text{-}2K^b$ and are a useful model to study thymic positive selection [36]. In the ($CD4C/HIV^{Nef}$ X OT-1 TCR) double Tg mice, the increase in $CD8^+$ SP thymocyte numbers was more prominent than in ($CD4C/HIV^{Nef}$ X HY TCR) double Tg mice **(Fig. 4.3A)**. Since upregulation of CD69 in $CD8^+$ SP thymocytes indicate that these cells have undergone positive selection, we used this marker to follow the fate of $CD8^+$ SP thymocytes in the presence of Nef. In ($CD4C/HIV^{Nef}$ X OT-1 TCR) double Tg mice, a larger proportion (~64%) of the $CD8^+$ SP thymocytes were $CD69^{hi}$ than in OT-1 TCR single Tg mice (~45%), suggesting an accumulation of cells with immature phenotype

(Fig. 4.3B, upper panel). The percentage of OT-1 $TCR^{hi}CD69^{lo}$ thymocytes, which represent the mature thymocytes, was similar in both the double and single Tg mice **(Fig. 4.3B, lower panel)**. Also, the $CD8^+$ T cell numbers in the peripheral lymphoid organs was similar in the double and single Tg mice (data not shown).

Together, these results indicate that more DP cells receive a positive selection signal in the presence of HIV-1 Nef to form $CD8^+$ SP thymocytes. However, it appears that not all of them develop to mature $CD8^+$ T cells. Rather, they seem to accumulate in the thymus as immature $CD69^{hi}$ $CD8^+$ SP cells. Our data also suggest an additional effect of HIV-1 Nef on thymocyte development, i.e., a block in the transition from DN to DP thymocytes, as indicated by increased numbers of DN and reduced numbers of DP thymocytes in (CD4C/HIV^{Nef} X HY TCR) Tg mice.

4.5.4. Peripheral $CD8^+$ T cells from Tg mice exhibit an activated phenotype and increased proliferation in vivo

The development of $CD8^+$ T cells does not seem to be grossly affected in HIV-1 Nef Tg mice and equal numbers of these cells are present in the peripheral lymphoid organs of Tg mice compared to non-Tg control mice. Cell surface phenotype of peripheral LN $CD8^+$ T cells from CD4C/HIV^{Nef} Tg mice was determined by labeling cells with antibodies against activation and development markers and analysis by FACS. A larger proportion of $CD8^+$ T cells from Tg mice were $CD44^{hi}$ associated with activated T cells, as compared to non-Tg control mice **(Fig. 4.4A and B)**. The difference in cell surface expression of CD69, CD25, CD45RB and CD62L was less pronounced.

To determine whether the activated phenotype of $CD8^+$ T cells in Tg mice was associated with increased proliferation, we assessed cell proliferation *in vivo* following labeling with BrdU provided in drinking water for a period of 2 weeks. Incorporation of BrdU in newly synthesized DNA was revealed by FACS analysis of $CD8^+$ T cells in spleen and pLN. Increased proportion of Tg $CD8^+$ T cells was found to be labeled with BrdU compared to the non-Tg $CD8^+$ T cells **(Fig. 4.4C and D)**. This indicates to an enhanced cell proliferation of peripheral $CD8^+$ T cells in HIV-1 Nef Tg mice. Despite the increased cell divisions, the numbers of peripheral $CD8^+$ T cells is not increased in Tg mice. This could be due to increased cell death observed among $CD8^+$ T cells from Tg mice compared the non-Tg mice [37].

4.5.5. Peripheral $CD8^+$ T cells from Tg mice are able to proliferate when stimulated in vitro but this function is impaired in the context of other cells of the Tg mice

To determine the functional competence of peripheral $CD8^+$ T cells of CD4C/HIV^{Nef} Tg mice, (CD4C/HIV^{Nef} X OT-1 TCR) double Tg total peripheral LN cells were stimulated *in vitro* with anti-CD3 antibody and mouse IL-2, ConA or OVAp (SIINFEKL) peptide for 3 days and cell division was monitored by CFSE dilution of pre-loaded cells, by gating on $CD8^+$ T cells. When stimulated with anti-CD3 antibodies or ConA, (CD4C/HIV^{Nef} X OT-1 TCR) double Tg $CD8^+$ T cells proliferated as efficiently as the control OT-1 TCR Tg $CD8^+$ T cells stimulated under similar conditions **(Fig. 4.5A and B)**. This suggests that the signaling cascade activated by direct engagement of T cell receptor (TCR) with anti-CD3 antibody functions normally in the cells from Nef Tg

mice. In contrast, $CD8^+$ T cells from (CD4C/HIV^{Nef} X OT-1 TCR) double Tg mice were found to proliferate at a significantly slower rate than control OT-1 TCR Tg $CD8^+$ T cells when stimulated with OVAp (**Fig. 4.5C**). While ~42.8% of OT-TCR $CD8^+$ T cells had divided 4 times and ~11.4% had divided 5 times, only ~11.5% and ~1.8% of the double Tg $CD8^+$ T cells had divided 4 and 5 times, respectively. Conversely, ~10.1% and ~31.5% of double Tg of $CD8^+$ T cells were in division 1 and 2, respectively, as compared to ~2.2% and ~10.6% of OT-TCR $CD8^+$ T cells **(Fig. 4.5D)**. Since LN contain many populations of immune cells, including $CD4^+$ T cells and dendritic cells whose function is impaired in the CD4C/HIV^{Nef} Tg mice [37,38], the proliferation defect of $CD8^+$ T cells from Nef-expressing mice could result from this altered microenvironment of the peripheral LN. To study this question, we sorted $CD8^+$ T cells from (CD4C/HIV^{Nef} X OT-1 TCR) double and OT-1 TCR single Tg peripheral LN by negative staining and stimulated them *in vitro* with OVAp in the presence of irradiated non-Tg splenocytes (as antigen presenting cells) and mouse IL-2 for 3 days. Both single Tg and double Tg $CD8^+$ T cells proliferated with similar efficiency in this context **(Fig. 4.5E)**. These results indicate that $CD8^+$ T cells from CD4C/HIV^{Nef} Nef Tg mice have the ability to proliferate efficiently when stimulated *in vitro,* but this function is compromised when these cells are stimulated in the presence of other immune cells, some which express HIV-1 Nef.

4.5.6. Tg mice are able to mount an efficient primary $CD8^+$ T cell response against LCMV

To assess $CD8^+$ T cell function *in vivo*, CD4C/HIV^{Nef} Tg and non-Tg control mice were infected intraperitoneally (i.p.) with 10^6 pfu of the *Armstrong* strain LCMV

known to cause an acute infection and induce a strong $CD8^+$ T cell response in mice. Virus titers were determined in the spleen of infected mice on day 3 and 8 post-infection (p.i.). On day 3 p.i., over 10^6 pfu LCMV per gram of spleen was detected in both Tg and non-Tg mice. Virus titres in spleen were down by ~1000 fold on day 8 p.i. in Tg as well as non-Tg mice **(Fig. 4.6A)**. LCMV gp33 tetramer staining and FACS analysis of spleen $CD8^+$ T cells on day 7 p.i. showed no significant difference in the numbers of gp33-specific $CD8^+$ T cells in Tg Vs non-Tg mice **(Fig. 4.6B and C).** These LCMV gp33-specific $CD8^+$ T cells showed an activated $CD62L^{lo}$ phenotype **(Fig. 4.6B)**, consistent with their being the effector cells. These results show that, despite the lack of $CD4^+$ helper T cells and defective dendritic cell functions in CD4C/HIV^{Nef} Tg mice, these mice have conserved the ability to mount a robust $CD8^+$ T cell response against $LCMV^{Armstrong}$ and to clear the virus.

4.5.7. Skewed phenotype of LCMV-specific $CD8^+$ memory T cells in HIV-1 Nef Tg mice partially restored by adoptive transfer of naïve non-Tg $CD4^+$ T cells

Since the primary response against LCMV appears normal in CD4C/HIV^{Nef} Tg mice, we then investigated whether these mice are able to generate and maintain long term LCMV-specific $CD8^+$ memory T cells. Tg and non-Tg control mice were infected with LCMV as before and were analyzed by LCMV gp33 tetramer staining and FACS analysis for the presence of LCMV gp33-specific $CD8^+$ memory T cell more than 2 months after infection. Representative FACS profiles are show in **Figure 4.7 (panels A and B)**. Significantly reduced numbers of LCMV gp33-specific $CD8^+$ memory T cells were present in the spleen and peripheral LN of Tg compared to non-Tg mice **(Fig. 4.7C**

and D, upper panels). Also, the phenotype of these Tg memory cells was skewed towards low surface expression of CD62L, which may correspond to the effector memory subset. Increased proportion of LCMV gp33-specific $CD8^+$ memory T cells were $CD62L^-$ in both the spleen and pLN while the $CD62L^+$ subset was reduced in Tg mice compared to the non-Tg mice **(Fig. 4.7C and D, lower panels)**. It is known that $CD4^+$ helper T cell functions are necessary for the maintenance of $CD8^+$ memory T cell in mice [22]. Therefore, we transferred naïve non-Tg $CD4^+$ T cell in Tg mice prior to infection with $LCMV^{Armstrong}$. This resulted in an increase in the numbers as well as partial recovery of the $CD62L^+$ subset of $CD8^+$ memory T cells in spleen of Tg mice **(Fig. 4.7C, lower panel)**. No increase in the numbers of $CD8^+$ memory T cells was observed in the pLN but a similar partial recovery of the $CD62L^+$ subset of $CD8^+$ memory T cells was evident in Tg mice that received $CD4^+$ T cells **(Fig. 4.7D, lower panel)**. Together these results suggest that HIV-1 Nef Tg mice have impaired $CD8^+$ memory phenotype due to lack of peripheral $CD4^+$ T cells.

4.6. Discussions

We studied the $CD8^+$ T cell compartment of $CD4C/HIV^{Nef}$ Tg mice, which express HIV-1 Nef under the control of sequences of a fused human CD4 promoter and mouse CD4 enhancer elements (CD4C), previously shown to drive expression of the Tg in $CD4^+$ T cells and the cells of macrophage/monocytes/dendritic lineage [25,30]. Using tetracycline-inducible GFP Tg mice we also detected Tg expression driven by the CD4C promoter in a significant proportion of $CD8^+$ SP thymocytes among other thymocytes. In

contrast, only a very small proportion of peripheral $CD8^+$ T cells expressed the Tg. This is consistent with the low level of infection of $CD8^+$ T cells in HIV-1 infected individuals. Activation of human $CD8^+$ T cells through their TCR turns on CD4 gene expression making them targets for HIV infection *in vitro* [39,40]. These CD4 expressing $CD8^+$ T cells represent <3% of the peripheral blood mononuclear cells (PBMC) in HIV-1 infected individuals and are productively infected with HIV-1 at levels approaching that of $CD4^+$ T cells [32-34]. Peripheral $CD8^+$ T cells of HIV-1 Nef Tg mice show activated phenotype based on the analysis of surface expression of CD44 and may promote some activity of the CD4C promoter and Nef expression.

4.6.1. Thymic selection of MHC-I restricted $CD8^+$ thymocytes is not impaired in $CD4C/HIV^{Nef}$ Tg mice

Human thymus is a site of infection by HIV-1, which impairs thymopoiesis as demonstrated in SCID-hu mouse model and HIV-1 infected infants with thymus dysfunction [1,2]. $CD4C/HIV^{Nef}$ Tg mice express Nef at high levels in the thymus, particularly in the $CD4^+8^+$ DP thymocyte, and show impaired development of $CD4^+$ SP thymocytes (Chrobak et al., unpublished data). We studied $CD8^+$ SP thymocytes development in these mice with the use of TCR Tg (HY TCR Tg and OT-1 TCR Tg) mice commonly used as models to study thymocyte selection processes. The HY TCR Tg mice express a T-cell receptor (TCR) transgene encoding reactivity to male antigen HY presented by class-I MHC [35]. In male HY TCR Tg mice, the thymus is normally small and thymocyte numbers are low due to depletion of self-reactive T-cells while in female mice these cells are not depleted and develop to $CD8^+$ T cells. OT-1 TCR Tg mice

express a TCR (Vα2Vß5) specific for the OVA-derived peptide SIINFEKL (OVAp) presented in the context of H-2K^b and are positively selected to form CD8$^+$ T cells [35]. Both negative and positive selections of CD8$^+$ T cells proceed in the presence Nef in Tg mice. The expression of Nef in male HY TCR Tg mice did not rescue the elimination of HY-specific DP thymocytes. In the female HY TCR Tg and OT-1 TCR Tg mice, expression of Nef did not impair positive selection of CD8$^+$ T cells, rather an increase in CD8$^+$ SP thymocyte numbers was observed. Surprisingly, appearance of CD8$^+$ T cells in the periphery of female HY TCR Tg mice was delayed in young mice suggestive of defect in the final maturation of these cells in the thymus. Larger proportions of CD8$^+$ SP thymocytes remain immature and accumulate in the thymus of OT-1 TCR Tg mice expressing Nef. However, the proportion of mature CD8$^+$ SP thymocytes with TCRαβ^{hi}CD69^{lo} phenotype is not affected. Hence, unlike the CD4$^+$ T cells, expression of Nef in the thymus of mice does not grossly impair CD8$^+$ T cell development and mature CD8$^+$ T cells are recruited to the periphery. Neither does the lower surface expression of CD4 in the DP thymocytes expressing Nef impair their selection and development to CD8$^+$ SP thymocytes similar to the CD4 knock-out mice which do not show any defects in CD8$^+$ T cell development [41].

4.6.2. Peripheral CD8$^+$ T cells from CD4C/HIV^{Nef} Tg mice respond well to antigenic stimulation in vitro and in vivo

The peripheral CD8$^+$ T cells of Nef expressing mice show activated phenotype and enhanced division *in vivo*. This is similar to what was reported for CD4$^+$ T cells of these mice [27]. The increased *in vivo* cell division in these mice is counter balanced by

increased apoptotic cell death; hence no net increase in $CD8^+$ T cells numbers in observed [37]. The proliferation capacity of $CD8^+$ T cells in response to *in vitro* stimuli is maintained in the Tg mice. However, when peripheral LN cells are stimulated with antigenic peptide, such as OVAp (OT-1 TCR-specific antigen), $CD8^+$ T cells form Tg mice proliferate slower than the non-Tg mice. Dendritic cells (DC), which are the professional antigen presenting cells, have a lower capacity to stimulate a mixed lymphocyte reaction and present antigens in CD4C/HIV^{Nef} Tg mice [38]. HIV-1 Nef has been shown to downregulate MHC-I surface expression on DCs and impairs presentation of antigens to HIV-specific $CD8^+$ T cells [42]. HIV-1 Nef has also been show to induce $CD8^+$ T cell apoptosis by exploiting DC death receptors [43]. Providing non-Tg antigen presenting cells in the form of irradiated splenocytes restored proliferation of $CD8^+$ T cells from Tg mice in response to OVAp stimulation. Hence, the signaling pathway down-stream of TCR is unaffected in $CD8^+$ T cells of Tg mice consistent with the fact that only a small proportion of these cells are expressing Nef at low levels.

Infection of mice with lymphocytic choriomeningitis virus (LCMV) leads to generation of protective cell-mediated immune response and clearance of the virus [44]. HIV-1 Nef Tg mice generate a strong $CD8^+$ T cell response against $LCMV^{Armstrong}$ and clear the virus as efficiently as the non-Tg mice. LCMV gp33-specific $CD8^+$ T cells are detected in the chronically infected Tg mice and show activated phenotype consistent with them being effector cells in the absence of $CD4^+$ T helper activity. It has been shown that $CD4^+$ T cells are not essential for the control of acute infection by $LCMV^{Armstrong}$ but are required for the resolution of chronic LCMV infection [45,46].

4.6.3. Generation of CD8$^+$ memory T cells is impaired in CD4C/HIV^{Nef} mice due to lack of CD4$^+$ T helper activity

Following activation and expansion of $CD8^+$ T cells in response to antigenic stimulation *in vivo*, majority of effector $CD8^+$ T cells die by apoptosis, while a small population (~5-10%) will differentiate into a small but long-lasting population of memory $CD8^+$ T cells [47]. LCMV-specific memory $CD8^+$ T cells are detected in mice several weeks after viral clearance and are distinguished into three major subsets based on the surface coexpression of CD62L and CCR7: $CD62L^+CCR7^+$ central memory (T_{CM}), $CD62L^-CCR7^+$ effector memory (T_{EM}) and $CD62L^-CCR7^-$ intermediate memory (T_{IM}) T cells [48,49]. Lower numbers of LCMV-specific memory CD8 T cells are generated in Nef Tg mice compared to the non-Tg mice. Moreover, the maturation of memory cells in the Tg mice is skewed towards the $CD62L^-$ subpopulation resulting in a significantly smaller population of $CD62L^+$ memory subset. Skewed maturation of HIV-specific memory $CD8^+$ T cells have been shown in HIV-1 infected individuals [21]. In their study, Champagne and coworkers identified four subsets of HIV- and CMV-specific memory $CD8^+$ T cells based on surface expression of CD8, CD45RA and CCR7. While CMV-specific $CD8^+$ memory pool was consisted mainly (~50%) of terminally differentiated $CD45RA^+CCR7^-$ cells, HIV-specific pool was predominantly (~70%) consisted of pre-terminally differentiated $CD45RA^-CCR7^-$ cells and less than ~5% terminally differentiated memory cells [21]. Amongst the HIV-specific $CD8^+$ memory T cells, the T_{CM} population was found to have a shorter half-life and is preferentially lost while the T_{EM} population accumulates [23]. In addition the expression of IL-7Rα on $CD8^+$ memory T cells is reduced which may impede their function [23,24]. Besides the memory $CD8^+$ T

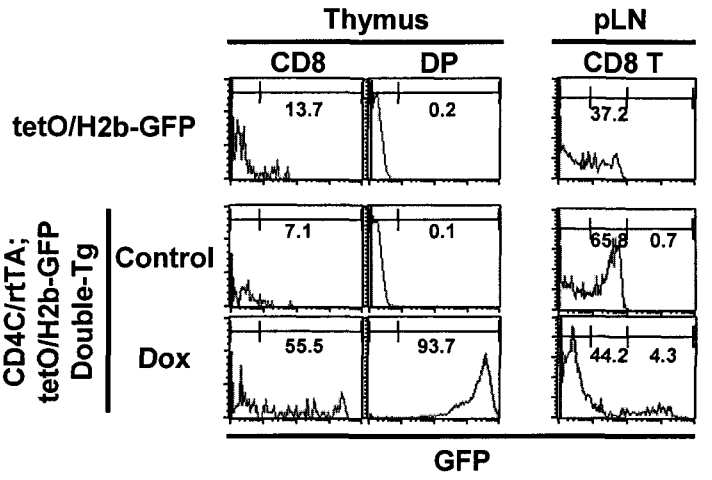
cells, defects in memory $CD4^+$ T cells and memory B cells, specific for many bacterial and viral antigens including *Sterptococcus pneumoniae*, diphtheria, tetnus, pertussis, measles and HIV-1, have been shown in HIV-1 infected individuals [50-56]. The skewed maturation of memory $CD8^+$ T cells in HIV-infected individuals and in the Nef Tg mice could be due to the lack of $CD4^+$ T helper activity which has been shown to be essential for the generation of memory $CD8^+$ T cells in mice [57-60]. Indeed we were able to partially rescue the memory $CD8^+$ T cell maturation defects in Nef Tg mice by adoptive transfer of non-Tg naïve $CD4^+$ T cells. The reason why we could not obtain complete rescue could be due to insufficient number of $CD4^+$ T cells transferred, the timing of $CD4^+$ T cell transfer or involvement of other factors in the generation of memory $CD8^+$ T cells in these mice.

In summary, the development and function of $CD8^+$ T cells is normal in CD4C/HIV^{Nef} Tg mice while the generation of memory $CD8^+$ T cells is impaired due to the lack of $CD4^+$ T helper cell activity in these mice.

2.7. Acknowledgments

We thank Eric Massicotte and Martine Dupuis from flow cytometry core facility for their help with flow cytometry. We are grateful to Dr. Claude Perreault (University of Montreal, Montreal) and Dr. Woong-Kyung Suh (IRCM, Montreal) for providing reagents for LCMV experiments and Dr. Vibuthi Dave (IRCM, Montreal) for providing OVAp peptide. We also thank Stephanie Lemay, Ginette Masse and Benoit Laganiere for their excellent technical assistance.

A



B

	Relative Tg Expression (%)	
	$CD4^+$ T cells	$CD8^+$ T cells
non-Tg	0	0
Nef Tg	100	1.6

Figure 4.1. Transgene expression in $CD8^+$ thymocytes and $CD8^+$ T cells. (A) GFP expression in (CD4C/rtTA X tetO/H2b-GFP) double Tg mice with and without doxycycline (dox) in drinking water (2mg/ml). Baseline expression of GFP in tetO/H2b-GFP single Tg mice is shown. Average percent of GFP expressing cells is indicated for each population of cells. (B) Table showing relative expression of Tg in sorted peripheral $CD8^+$ T cells as compared to $CD4^+$ T cells of CD4C/HIV^{Nef} Tg mice measure by quantitative RT-PCR.

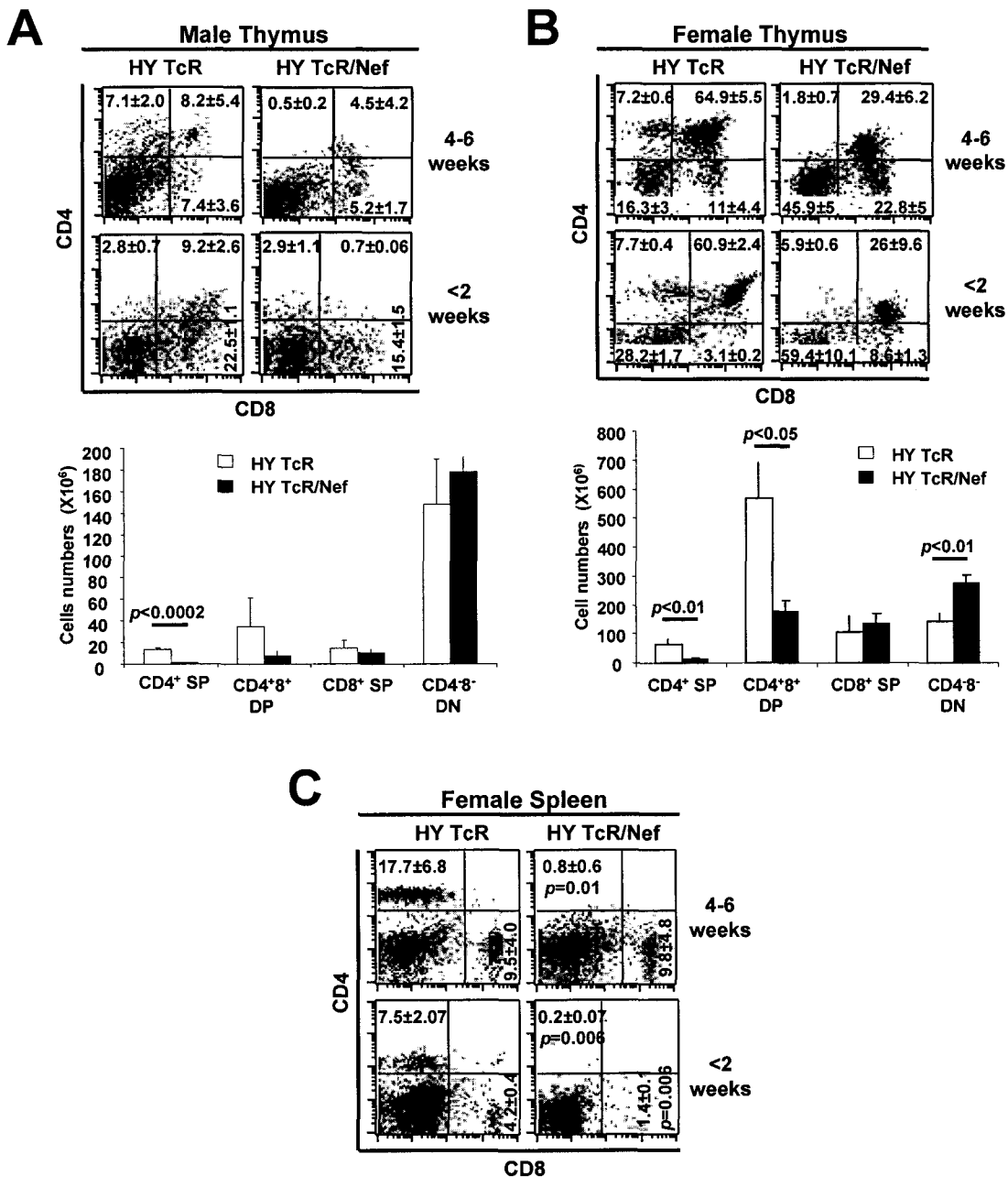


Figure 4.2. Thymic selection of MHC class-I restricted CD8$^+$ thymocytes. (A) Thymic FACS profiles of male HY TcR Tg and male (HY TcR X CD4C/HIV^{Nef}) double Tg mice at <2 weeks and 4-6 weeks of age. Absolute cell numbers of different thymocyte populations in 4-6 weeks old mice are shown in lower panel. (B) Thymic profiles of female HY TcR Tg and female (HY TcR X CD4C/HIV^{Nef}) double Tg mice at <2 weeks and 4-6 weeks of age. Absolute cell numbers of different thymocyte populations in 4-6 weeks old mice are shown in lower panel. (C) FACS profile of spleen from female HY TcR Tg and female (HY TcR X CD4C/HIV^{Nef}) double Tg mice at <2 weeks and 4-6 weeks of age. Statistical analysis was performed by Student's *t*-test and *p*-values are shown.

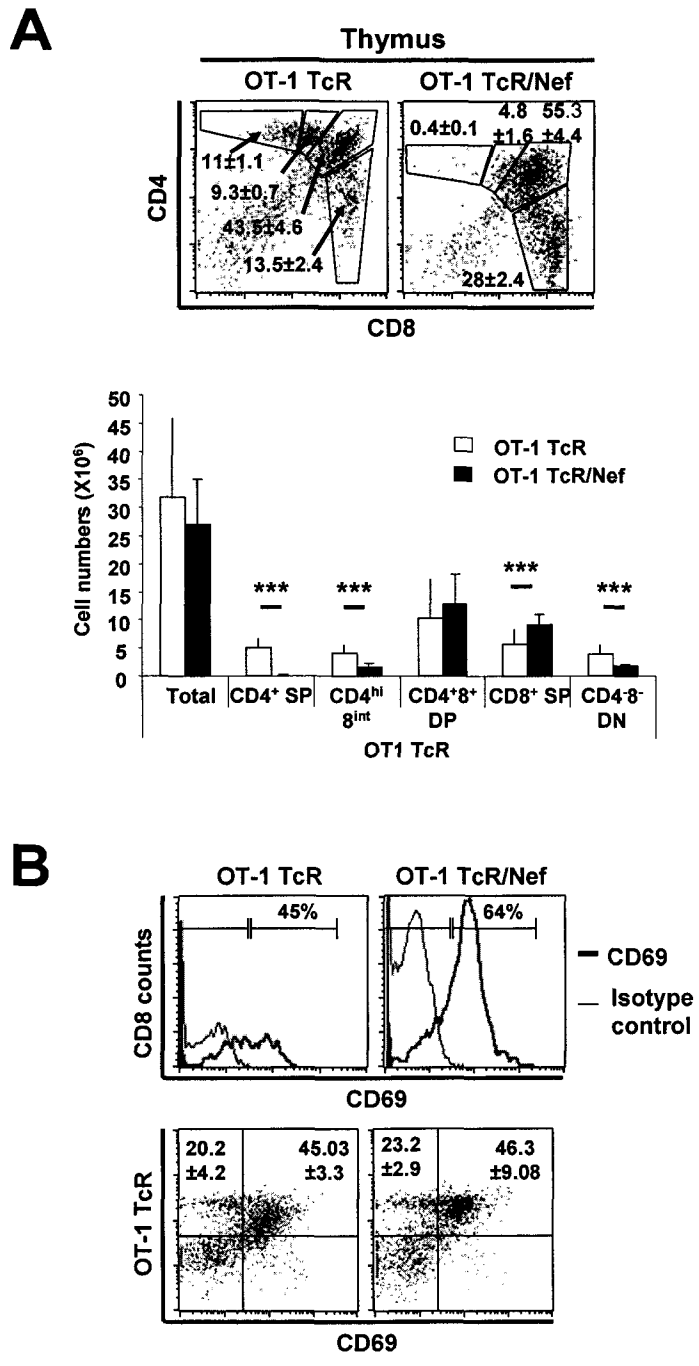


Figure 4.3. Positive selection of MHC class-I restricted CD8$^+$ thymocytes. (A) Thymic FACS profiles of OT-1 TcR Tg and (OT-1 TcR X CD4C/HIV^{Nef}) double Tg mice. Absolute cell numbers of different thymocyte populations expressing OT-1 TcR are shown in lower panel. (B) CD69 surface expression on thymocytes of OT-1 TcR Tg and (OT-1 TcR X CD4C/HIV^{Nef}) double Tg mice. Statistical analysis was performed by Student's *t*-test. ***: $p \leq 0.0005$

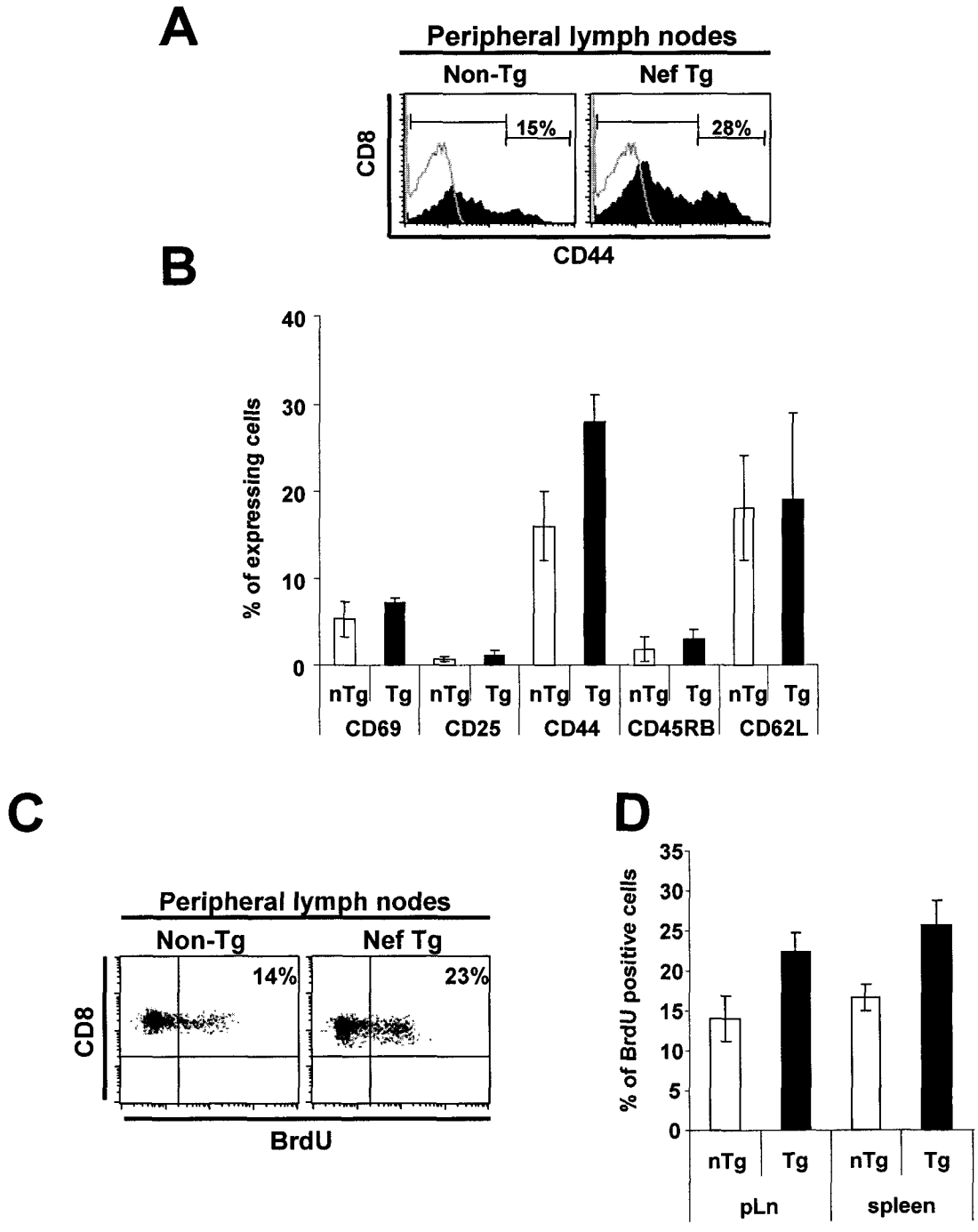


Figure 4.4. Activated phenotype of peripheral CD8$^+$ T cells. (A) Histogram plot, representative of one experiment, showing CD44 surface expression in shaded area. Isotype control is shown by grey line. (B) Bar graph representation of expression of activation markers on CD8$^+$ T cells in non-Tg Vs HIV-1 Nef Tg mice. (C) Incorporation of BrdU in CD8$^+$ T cells of peripheral LN analyzed by FACS after staining with anti-BrdU antibody. Dot plot of one representative experiment is shown. (D) Bar graph showing increased incorporation of BrdU in CD8$^+$ T cells of pLN and spleen from non-Tg Vs HIV-1 Nef Tg mice.

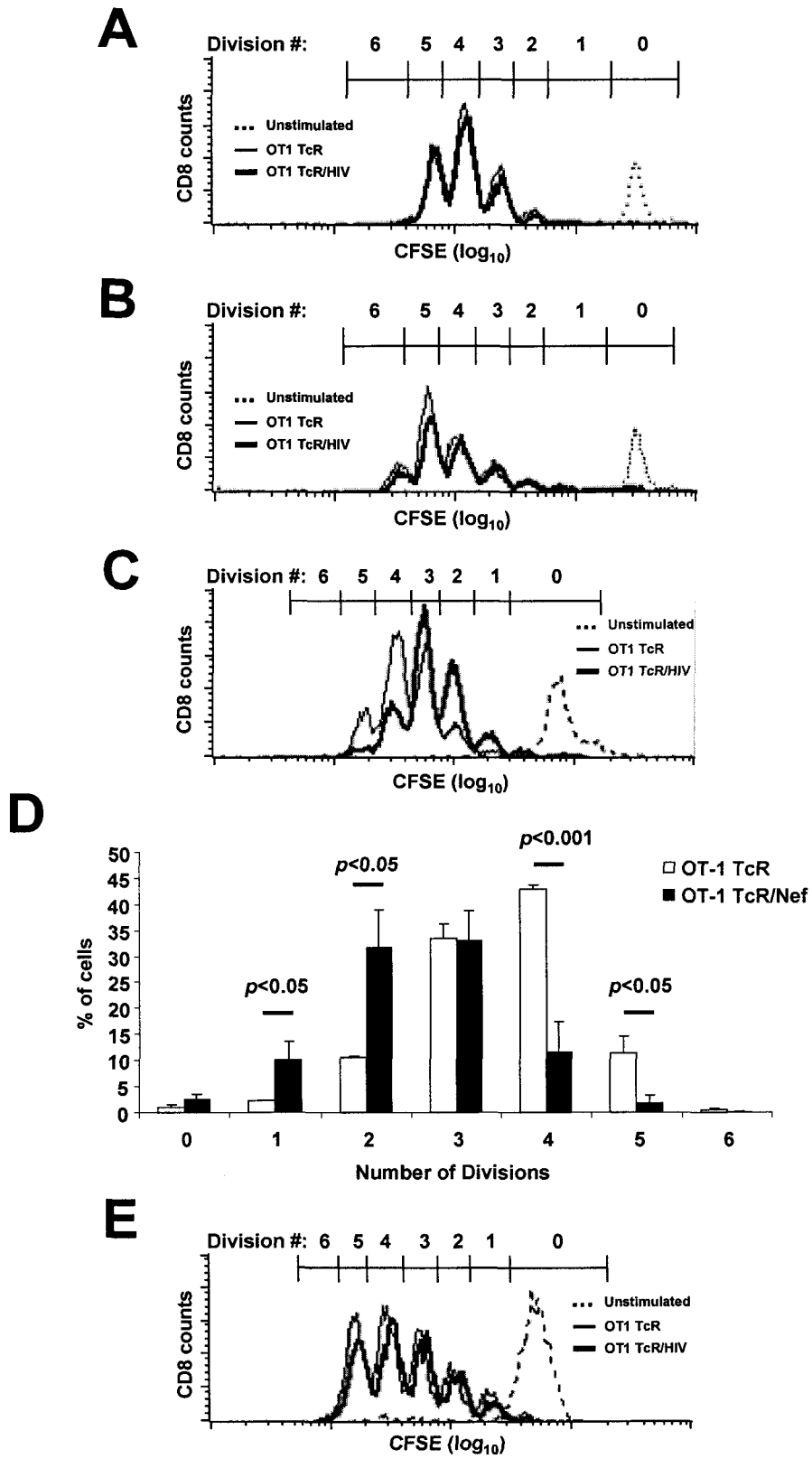

A
Division #: 6 5 4 3 2 1 0
CD8 counts
Unstimulated
OT1 TcR
OT1 TcR/HIV
CFSE (log10)
B
Division #: 6 5 4 3 2 1 0
CD8 counts
Unstimulated
OT1 TcR
OT1 TcR/HIV
CFSE (log10)
C
Division #: 6 5 4 3 2 1 0
CD8 counts
Unstimulated
OT1 TcR
OT1 TcR/HIV
CFSE (log10)
D
p<0.001
p<0.05
p<0.05
OT-1 TcR
OT-1 TcR/Nef
% of cells
50
45
40
35
30
25
20
15
10
5
0
0 1 2 3 4 5 6
Number of Divisions
E
Division #: 6 5 4 3 2 1 0
CD8 counts
Unstimulated
OT1 TcR
OT1 TcR/HIV
CFSE (log10)

Figure 4.5.

Figure 4.5. *In vitro* stimulation and $CD8^+$ T cell proliferation. CFSE labeled OT-1 TcR Tg and (OT-1 TcR X CD4C/HIV^{Nef}) double Tg total peripheral LN cells stimulated with plate-bound anti-CD3 and 100U/ml mouse IL-2 (A), 5μg/ml ConA (B) or 1μg/ml OVAp (C) *in vitro* for 3 days. Number of divisions is indicated on the top. (D) Bar graph representation of percent of peripheral LN $CD8^+$ T cells at different divisions after *in vitro* stimulation with OVAp. (E) CFSE labeled flow cytometry sorted $CD8^+$ T cells from OT-1 TcR Tg and (OT-1 TcR X CD4C/HIV^{Nef}) double Tg peripheral LN stimulated with 1μg/ml OVAp *in vitro* for 3 days in presence of 30000 irradiated non-Tg splenocytes and 100U/ml mouse IL-2. Number of divisions is indicated on the top. Statistical analysis was performed by Student's *t*-test and *p*-values are shown.

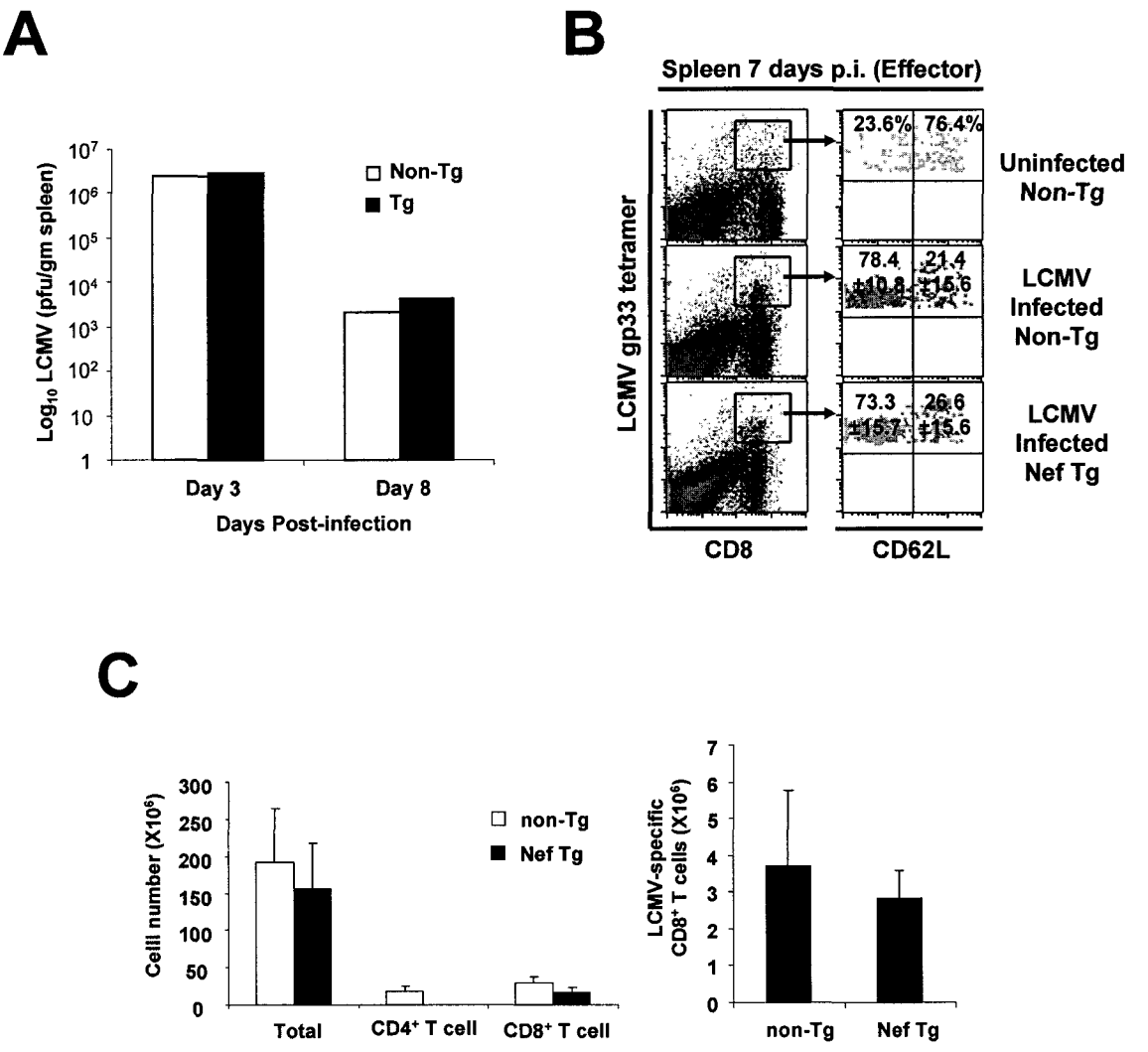


Figure 4.6. CD8$^+$ T cell response and virus clearance in LCMV^{Armstrong} infected mice. (A) LCMV titres in the spleen of non-Tg and HIV-1 Nef Tg mice on day 3 and day 8 p.i. Mice were infected with 10^6 pfu LCMV^{Armstrong} by intraparitoneal route. Virus titer per gram of spleen is represented as $\log_{10}$ scale. (B) LCMV gp33-specific effector CD8$^+$ T cells in spleen of uninfected non-Tg, LCMV^{Armstrong} infected non-Tg and Tg mice on day 7 p.i. (C) Spleen cellularity (left panel) and LCMV gp33-specific effector CD8$^+$ T cells numbers (right panel) in spleen of LCMV^{Armstrong} infected non-Tg and Tg mice on day 7 p.i.

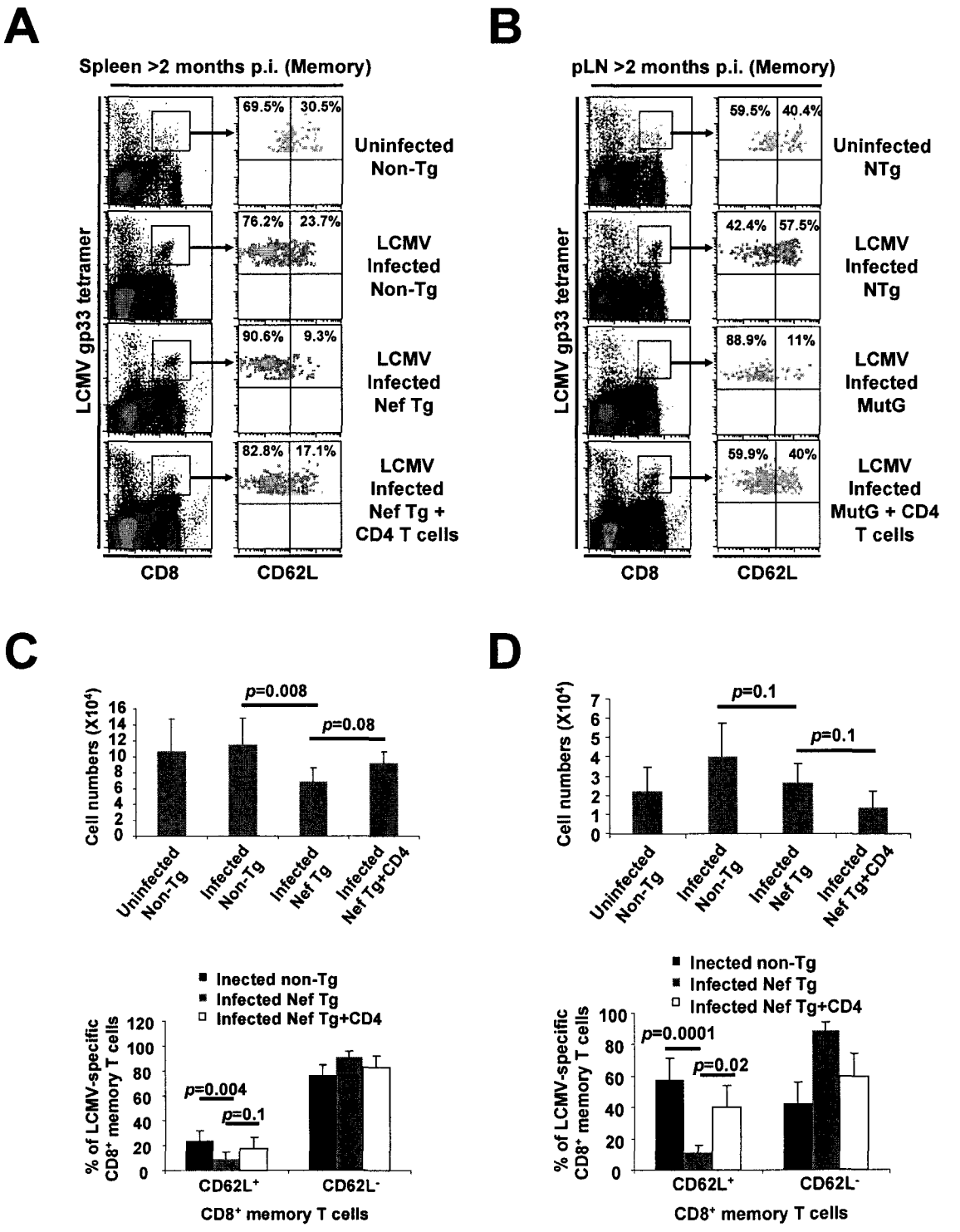

A
Spleen >2 months p.i. (Memory)
LCMV gp33 tetramer
CD8
CD62L
69.5%
30.5%
Uninfected Non-Tg
76.2%
23.7%
LCMV Infected Non-Tg
90.6%
9.3%
LCMV Infected Nef Tg
82.8%
17.1%
LCMV Infected Nef Tg + CD4 T cells
B
pLN >2 months p.i. (Memory)
59.5%
40.4%
Uninfected NTg
42.4%
57.5%
LCMV Infected NTg
88.9%
11%
LCMV Infected MutG
59.9%
40%
LCMV Infected MutG + CD4 T cells
C
Cell numbers (X10⁴)
p=0.008
p=0.08
Uninfected Non-Tg
Infected Non-Tg
Infected Nef Tg
Infected Nef Tg+CD4
Inected non-Tg
Infected Nef Tg
Infected Nef Tg+CD4
% of LCMV-specific CD8⁺ memory T cells
p=0.004
p=0.1
CD62L⁺
CD62L⁻
CD8⁺ memory T cells
D
Cell numbers (X10⁴)
p=0.1
p=0.1
Uninfected Non-Tg
Infected Non-Tg
Infected Nef Tg
Infected Nef Tg+CD4
Inected non-Tg
Infected Nef Tg
Infected Nef Tg+CD4
% of LCMV-specific CD8⁺ memory T cells
p=0.0001
p=0.02
CD62L⁺
CD62L⁻
CD8⁺ memory T cells

Figure 4.7.

Figure 4.7. LCMV-specific $CD8^+$ memory T cells in $LCMV^{Armstrong}$ infected mice. LCMV gp33-specific memory $CD8^+$ T cells in spleen (A) and pLN (B) of uninfected non-Tg, $LCMV^{Armstrong}$ infected non-Tg, HIV-1 Nef Tg and HIV-1 Nef Tg mice to which naïve non-Tg $CD4^+$ T cells were adoptively transferred prior to infection. Mice were infected with 10^6 pfu $LCMV^{Armstrong}$ by intraparitoneal route. FACS analysis was performed >2 months p.i. on spleen and pLN. Bar graphs showing LCMV gp33-specific memory $CD8^+$ T cell numbers in spleen (C, upper panel) and pLN (D, upper panel) of uninfected and $LCMV^{Armstrong}$ infected mice more >2 months after primary infection. Bar graphs representing skewed LCMV gp33-specific memory $CD8^+$ T cell phenotype in spleen (C, lower panel) and pLN (D, lower panel) of LCMV infected HIV-1 Nef Tg Vs non-Tg mice and its partial restoration by adoptive transfer of naïve non-Tg $CD4^+$ T cells to the HIV-1 Nef Tg mice. Statistical analysis was performed by Student's *t*-test and *p*-values are shown.

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Chapter 5: General discussion and conclusions

Small animals such as rodents are attractive candidates for AIDS research due to the availability of various inbred and genetically engineered strains, extensive knowledge or their immune system, especially in mice, and the relative ease of breeding and maintaining animal colonies. A small animal model of AIDS would be instrumental for evaluation of therapeutic agents and could aid studies of host-virus interactions since both the virus and the host are amenable for genetically manipulations. Unfortunately, rodents are not susceptible to infection by HIV. HIV encounters multiple blocks during replication in rodent cells [1-3]. To circumvent these blocks, various approaches have been used to modify small animals such as rabbits, rats and mice so that they could be used as models for AIDS. These approaches can be categorized into 3 groups: (i) transgenic animals expressing human CD4, human chemokine receptors CXCR4 or CCR5 and other permissive factor for HIV infection, (ii) xenotrasplantation of immunodeficient mice to generate human immune cells susceptible to HIV infection, and (iii) transgenic mice expressing whole or part of HIV-1 genome. The small animal models of AIDS generated by these approaches have provided information on different aspects of HIV replication and pathogenesis.

Transgenic small animal models carrying entire HIV genome or selected genes have been generated for *in vivo* analysis of HIV genes [4]. Transgenesis bypasses the species-specific infectability barriers and can reveal the effects of individual genes. Small animals can be engineered to express individual HIV genes in cell- or tissue-specific manner and study their functions throughout the developmental and adult life of the animal. Transgenic mice have been used to study pathological functions of Nef in various tissues and cell populations [5-10]. HIV-1 Nef is a 27-35 kDa protein shown in *in*

vitro experiments to downmodulate T cell surface markers (CD4, CD28, etc), modulate T cell receptor (TCR) signaling and cell survival, and play a role in viral infectivity and pathogenesis [11]. Some of these functions of Nef, including CD4 downmodulation, modulation of TCR signaling, increased T cell activation and turnover, are conserved in mouse cells as demonstrated in HIV-1 Nef Tg mice [6-8,12]. In addition to the immunological phenotypes, Nef has also been implicated in the non-lymphoid organ pathologies in heart, lungs and kidneys in Nef Tg mice, particularly the CD4C/HIV^{Nef} Tg mice reported earlier by Hanna and coworkers [8]. The expression of Nef in the cell populations relevant for HIV infection, such as CD4$^+$ T cells, macrophages and dendritic cells, using the CD4C promoter sequences, seems to be important to obtain the lymphoid and non-lymphoid pathologies characteristic of HIV-infected patients. This model more appropriately resemble neonate AIDS since Nef expression begins early in life and is maintained throughout the life of the animal. However, early expression of Nef may interfere with normal developmental processes and may result in phenotypes that do not reflect the true functions of Nef.

To prevent any developmental defect associated with early Nef expression and to show that adult mice are susceptible to effects of Nef, we generated inducible Nef Tg mice. Using the tetracycline inducible system [13,14] along with the CD4C promoter sequences, used earlier in our lab to generated CD4C/HIV^{Nef} Tg mice [8], we have been able to express Nef in tissue-specific manner when the mice are treated with dox. In this thesis we report that adult mice are susceptible to effects of Nef and develop disease similar to constitutively Nef expressing CD4C/HIV^{Nef} Tg mice. Our results show that the

AIDS-like disease in Nef Tg mice is not as a result of developmental defects caused by early expression of Nef.

5.1. Altered thymopoiesis in adult mice expressing Nef

Tetracycline-dependent transactivators (rtTA and rtTA2^{S}-M2) expressed under the control of CD4C promoter sequences, are able to induce expression of Tg (GFP or Nef) from the TRE promoter sequences mainly in the lymphoid tissues of Tg mice. Tg expression is highly dependent on the presence of dox. From studies with GFP reporter tetO/H2B-GFP Tg mice, Tg expression was detected mainly in the $CD4^{+}8^{-}$ SP and $CD4^{+}8^{+}$ DP thymocytes, as well as in the peripheral $CD4^{+}$ T cells, when treated with dox. Tg is also expressed at lower levels in small proportion of other thymocyte populations and peripheral T and B lymphocytes in these mice. Expression of Nef in adult mice results in downmodulation of surface CD4 as well as loss of thymocytes, particularly $CD4^{+}8^{-}$ SP and $CD4^{+}8^{+}$ DP thymocytes. This is similar to reported downregulation of surface CD4 by HIV and SIV Nef *in vitro* [15-17] and *in vivo* in Nef Tg mice [6-8,18]. HIV-1 infection has been shown to result in depletion of thymocytes in the thy/liv conjoint organ of SCID-hu mice [19]. The first thymocytes to be deleted are $CD4^{+}8^{+}$ double positive (DP) and $CD4^{+}8^{-}$ single positive (SP) thymocytes and at later stages the $CD8^{+}4^{-}$ SP thymocytes. Deletion of *nef* significantly attenuated infectivity as well as pathogenicity of both X4 and R5 strains of HIV-1 in SCID-hu mice, demonstrating the importance of Nef in pathogenesis of HIV infection in this mouse model of AIDS [20-22]. Infection of $CD4^{+}8^{-}$ SP, $CD8^{+}4^{-}$ SP and $CD4^{+}8^{+}$ DP thymocytes have been reported with different HIV-1 isolates in these mice. In addition, viral RNA has also detected in the thymic

epithelial (TE) cells in the infected thy/liv implants, which resulted in marked disruption of thymic microenvironment leading to depletion of thymocyte and destruction of TE cells [23]. However, our observation of thymocyte loss in bone marrow chimeras, in which TE cells are of wild type host origin and do not express Nef, rules out the involvement of TE cells in thymocyte depletion observed in Nef Tg mice.

Since the Nef Tg is integrated in the X chromosome of TRE/HIV^{Nef} Tg mice, Nef expression is silenced by X chromosome inactivation process in a subset of female cells. As a result of this, Nef expression is higher in male mice compared to the female mice. Consequently, in the male mice from 2 transactivator founder lines (F176042 and F176043), Nef expression leads to severe thymocyte loss and thymic atrophy within one week of dox treatment, while in female mice from the same founder lines, thymocyte depletion occurs after 2 months of dox treatment. Whether such a massive depletion of thymocytes in male mice is due of high levels of Nef expression in these cell or caused by other mechanisms, such as disruption of thymic microenvironment and cytokine imbalance, is currently under investigation with the use of a series of bone marrow and fetal liver transplantation experiments.

The male mice from founder line F148571 and female mice from founder lines F176041 and F176042 were used for long term studies of the thymus. In these mice, expression of Nef resulted in impaired maturation of $CD4^+8^-$ SP thymocytes after 2 months of dox treatment. This was revealed by downmodulation of surface markers, such as TCR$\alpha\beta$, CD69, CD2, CD5 and IL-7R, on $CD4^+8^-$ SP but not $CD8^+4^-$ SP thymocytes. A similar impaired maturation of $CD4^+8^-$ SP thymocytes has been observed in CD4C/HIV^{Nef} Tg mice (Chrobak *et al.*, manuscript in preperation). This phenotype

was only observed in mice from founder lines expressing Tg at high levels (F146571 and F176042), as revealed from the experiments with the inducible GFP reporter mouse. We also observed that thymocytes from Nef Tg mice, after Tg induction by dox treatment, are in a state of hyperresponsiveness to TCR stimulation, as judged by the increased levels of tyrosine phosphorylation of proteins before and after TCR stimulation. Similar phenotype was earlier reported for the constitutively expressing Nef Tg mice [8]. Nef has been shown to increase the association of TCR-signaling molecules such as TCRζ and lck to the raft fraction of T cell membrane prior to activation [24]. By doing so Nef decreases the activation threshold of the T cells making them hyperactive to TCR stimulation. Impairment of thymopoiesis results in lower thymic output and may contribute to reduced peripheral $CD4^+$ T cells in addition to Nef-mediated T cell depletion by increased cell death. Decreased thymic output has been reported in HIV-1 infected adults and children, which is restored after HAART initiation [25-27]. A combination of decreased thymic output and increased T cell turnover has been proposed to contribute to the perturbation of T cell homeostasis in HIV-1 infected individuals [26].

An interesting phenomenon, we observed in mixed bone marrow chimeras generated with a mixture of inducible Nef Tg and non-Tg bone marrow cells, was the depletion of non-Tg $CD4^+8^+$ DP thymocytes when treated with dox. At the moment we cannot comment whether this is a true bystander effect caused by the Nef expressing thymocytes in the same thymus. Most of the thymocytes in the Tg mice express high levels of the transgene (Rahim et al., unpublished). A large number of thymocytes are destroyed during thymic development [28]. This may release soluble Nef that will affect other cells within the thymus. Soluble Nef is present in the serum of HIV-1 infected

individuals and can affect lymphocytes and macrophages *in vitro* [29-32]. Further studies will be performed to address the nature and mechanism of this bystander effects observed in the thymus of mixed bone marrow chimeras.

5.2. Peripheral T cell loss and activation in adult mice expressing Nef

Studies with the GFP reporter mouse (tetO/H2B-GFP) showed that the major T cell population expressing the Tg at high level is the $CD4^+$ T cells. Nef expression in these cells leads to downmodulation of surface CD4 expression as evidenced in the blood of these mice within 2 weeks of dox treatment. In the male mice from F176042 and F176043 founder lines, massive atrophy of peripheral lymph nodes was observed within 1 month of dox treatment in contrast to the thymic atrophy which was observed within 1 week of dox treatment. This is in agreement with report by Bourgeois and coworkers who showed that decay of peripheral T cells is delayed by 2-3 weeks from cessation of thymocyte production by ablation of Rag [33]. This is further evidence for the important role of thymic function in T cell homeostasis. In the mice from founder lines with slow depletion of thymocytes, loss of $CD4^+$ T cells occurs after 2 months dox treatment. In addition, $CD4^+$ T cells from Nef expressing mice show activated/memory phenotype and increase cell death. These phenotypes have been previously reported in the constitutively expressing CD4C/HIV^{Nef} Tg mice and other mice expressing Nef in different T cell populations [6,7,12,34]. Various i*n vitro* studies have implicated HIV-1 Nef to cause immune activation [24,35,36]. HIV-1 pathogenesis in humans is associated with chronic immune activation, which results in increased T cell turnover [37-40]. A direct correlation has been observed between the immune activation and disease progression in HIV infected

patients [41-43]. The exact cause of the chronic immune activation is not completely understood. The results from Nef Tg mouse models suggest that Nef may contribute to T cell activation besides other factor in HIV-1 infected individuals. However, Koenen and coworkers have reported that T cell activation in their Nef Tg mice is induced by lymphopenia [44]. In chimeric mice generated from a mixture of non-Tg and Nef Tg bone marrows, with a full T cell compartment, Nef Tg T cells did not show activated phenotype. However, Nef Tg T cells constituted only 1% of peripheral T cells in their chimeras and the level of Nef expression in these cells is questionable [44]. Here, we revisited this question, using bone marrow from inducible Nef Tg and non-Tg mice. We have been able to generate bone marrow chimeras in which Nef Tg cells constituted approximately 31% and 25% of cells in the thymus and pLN, respectively, before treatment with dox. In the pLN, expression of Nef, upon dox treatment, resulted in significant loss of Nef Tg $CD4^+$ T cells without affecting non-Tg T cells and total cellularity. Nef Tg $CD4^+$ T cells also showed activated/memory phenotype when treated with dox. Since these phenotypes are restricted to Tg T cells only and occur in a full T cell compartment, Nef expression seems the most likely mediator of these effects. We have observed in the inducible Nef Tg mice, T cell activation in mice from founder lines shown, by GFP reporter assays, to express high level of Tg (F148571 and F176042). Similarly, in the constitutively expressing CD4C/HIV^{Nef} Tg mice, T cell activation was more severe in $CD4^+$ T cells expressing high levels of Nef compared to the $CD4^+$ T cells expressing low levels of Nef in the same animal [12]. These results together support the direct involvement of Nef in causing T cell activation in Nef Tg mice.

The inducible Nef Tg mice have enabled us to study effects of Nef *in vivo* as function of the time of Nef expression. Transgene expression can be induced *in utero* and in neonate mice by treatment of pregnant female mice with dox [45-47]. We have detected Nef expression in 2 weeks old pups born to female mice treated with dox during pregnancy. T cell phenotypes such as activation and cell death were found to be more pronounced in mice in which Nef expression was induced early in life as compared to the mice in which Nef was induced at adult stage. This issue is of clinical relevance, because AIDS has been reported to be more severe in pediatric cohorts [48,49]. The incubation period of pediatric AIDS patients is short and approximately 20% develop AIDS in the first year of life [50].

5.3. Non-lymphoid organ disease in adult mice expressing Nef

Expression of Nef in adult mice results in pathologies in non-lymphoid organs such as kidney and lungs. These phenotypes are very similar to those reported in CD4C/HIV^{Nef} Tg mice [8]. These pathologies include tubular atrophy, tubular dilation and focal glomerular sclerosis in kidney, and foci of lymphocyte infiltration resembling lymphocytic interstitial pneumonitis in the lungs of dox-treated mice. Expression of Nef in the relevant cells for HIV infection seems to be important for development of the non-lymphoid organ disease in mice. Tg mice expressing Nef under the control of promoter elements different from CD4C promoter elements do not develop the full spectrum of pathologies observed in our model [5-7]. Moreover, expression of Nef in the cells of these organs seems to be crucial for the development of such pathologies. Cells expressing GFP in dox-dependent manner are present in non-lymphoid organs, such as lungs, liver

and kidney **(Fig. 6.1 in Appendix)**, of mice from F176042 and F176043 but not in mice from F148571 transactivator founder line. While mice from F176042 and F176043 transactivator founder lines develop pathologies in lungs and kidneys, the mice from F148571 do not develop non-lymphoid organ disease. The absence of non-lymphoid organ disease in the mice from F148571, which express high levels of Tg in the thymus and peripheral lymphoid organs, also rules out the involvement of lymphoid cells in development of pathologies in the non-lymphoid organs. The identity of the cells expressing Tg in non-lymphoid organs and their contribution to pathogenesis of Nef in these organs needs further investigation. The close resemblance of the disease in our inducible Nef Tg model and the CD4C/HIV^{Nef} mice demonstrates that the non-lymphoid organ disease in these mice is not caused by any developmental defects as a result of early Nef expression.

5.4. Normal development and function of CD8$^+$ T cells in CD4C/HIV^{Nef} Tg mice

The impaired thymopoiesis in Nef Tg mice led us to study T cell development in more details. We used the constitutively expression CD4C/HIV^{Nef} Tg mice for these studies. Development and maturation of CD4$^+$ T cells as well as transition of cells from CD4$^-$8$^-$ DN to CD4$^+$8$^+$ DP thymocyte populations are altered in these mice (Chrobak et al., manuscript in preparation). Since CD4$^+$ and CD8$^+$ T cells develop from the same CD4$^+$8$^+$ DP progenitor cells in the thymus [28], we reasoned that development of CD8$^+$ T cells might also be affected by expression of Nef in thymocytes of these mice. When CD4C/HIV^{Nef} Tg mice were bred with class-I MHC-restricted TCR Tg mice (HY TCR Tg and OT-1 TCR Tg mice), CD8$^+$ T cell developement was not grossly impaired and

mature $CD8^+$ T cells were recruited to the periphery. Both negative and positive selections of $CD8^+$ T cells proceed in the presence Nef in these mice. However, increased numbers of $CD8^+$ SP thymocytes were formed which retained immature $CD8^+CD69^{hi}$ phenotype and accumulated in the thymus of Nef expressing mice. The proportion of mature $CD8^+$ SP thymocytes with $TCR\alpha\beta^{hi}CD69^{lo}$ phenotype did not increased because of increased numbers of immature $CD8^+$ SP thymocytes. A larger proportion of $CD4^+8^+$ DP thymocytes seem to be positively selected to form $CD8^+$ SP then $CD4^+$ SP thymocytes in presence of Nef. Similar phenotype has been observed when $CD4C/HIV^{Nef}$ Tg mice are bred with class-II MHC restricted AND TCR Tg mice (Chrobak et al., submitted for publication). Further studies will be conducted to understand the molecular mechanism of this phenotype.

Equal numbers of $CD8^+$ T cells are present in the peripheral lymphoid tissues of Nef Tg mice and non-Tg mice. However, peripheral $CD8^+$ T cells of Nef expressing mice show activated phenotype and enhanced division *in vivo*. This is similar to what was reported for $CD4^+$ T cells from these mice [12]. The increased *in vivo* cell division in these mice is counter balanced by increased apoptotic cell death, hence no net increase in $CD8^+$ T cells numbers is observed [34]. The proliferation capacity of $CD8^+$ T cells in response to *in vitro* stimuli is maintained in the Nef Tg mice. The signaling pathway down-stream of TCR is unaffected in $CD8^+$ T cells of Tg mice consistent with the fact that only a small proportion of these cells are expressing Nef. Infection of Nef Tg mice with $LCMV^{Armstrong}$ induces a strong $CD8^+$ T cell response that clears the virus as efficiently as the non-Tg mice. LCMV gp33-specific $CD8^+$ T cells are detected in the chronically infected Tg mice and show activated phenotype consistent with them being

effector cells in the absence of $CD4^+$ T helper activity. Hence the $CD8^+$ T cell functions *in vivo* are maintained in CD4C/HIV^{Nef} Tg mice.

5.5. Impaired $CD8^+$ memory T cells in CD4C/HIV^{Nef} mice due to lack of $CD4^+$ T helper activity

LCMV-specific memory $CD8^+$ T cells are detected in wild type mice several weeks after viral clearance and are distinguished into three major subsets based on the surface coexpression of CD62L and CCR7: $CD62L^+CCR7^+$ central memory (T_{CM}), $CD62L^-CCR7^+$ effector memory (T_{EM}) and $CD62L^-CCR7^-$ intermediate memory (T_{IM}) T cells [51,52]. Lower numbers of LCMV-specific memory $CD8^+$ T cells are generated in Nef Tg mice compared to the non-Tg mice. Moreover, the maturation of memory cells in the Tg mice is skewed towards the $CD62L^-$ subpopulation resulting in a significantly smaller population of $CD62L^+$ memory subset. Skewed maturation of HIV-specific memory $CD8^+$ T cells have been shown in HIV-1 infected individuals [53]. $CD4^+$ T helper activity has been shown to be essential for the generation of memory $CD8^+$ T cells in mice [54-57]. Indeed we were able to partially rescue the memory $CD8^+$ T cell maturation defects in Nef Tg mice by adoptive transfer of non-Tg naïve $CD4^+$ T cells. Hence, $CD8^+$ T cells in Nef Tg mice are functional during the acute phase of virus infection but due to lack of $CD4^+$ T helper activity in these mice, they cannot generate memory T cells.

In conclusion, expression of HIV-1 Nef in mice (adult and neonate) leads to a constellation of pathologies involving both the lymphoid and non-lymphoid organs closely resembling pathologies in HIV-infected individuals. This model highlights the

important role of Nef in HIV-1 pathogenesis. The high similarity in the disease in these Tg mice with human AIDS strongly suggest that these mice are a relevant model to study AIDS. This study also shows that mouse cells can support functions of Nef and these Tg mice represent a unique model to study Nef functions *in vivo* in the context of the primary immune system. The ability to generate chimeric mice containing a significant proportion of Nef Tg T cells, using bone marrow or fetal liver from inducible Nef Tg mice, will allow us to answer questions regarding role of Nef in T cell activation, bystander affect, the role of lympoid tissue microenvironment and the involvement of immune cells in nonlymphoid organ diseases. The inducible Nef Tg model has also given us the ability to control the level and time of expression of Nef which was impossible to do in the previously reported constitutive Nef Tg mouse models. Moreover, these mice will be useful to study immune reconstitution since Nef expression can be turned off after withdrawal from dox.

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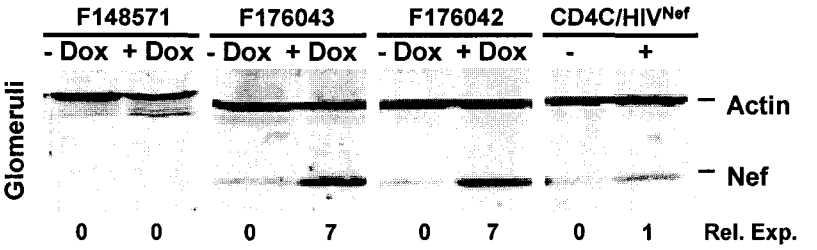
Appendix

6.1. Transgene expression in the kidney of inducible Nef Tg mice

Expression of Nef was detected in the glomeruli from dox-treated (CD4C/rtTA2^{S}-M2 X TRE/HIV^{Nef}) double-Tg mice (F176042 and F176043) by western blot analysis. Nef was not expressed in the kidneys from (CD4C/rtTA X TRE/HIV^{Nef}) double-Tg mice (F148571) treated with dox **(Fig. 6.1A)**. Similarly, using the GFP reporter mouse strain (tetO/H2B-GFP), GFP expressing cells were detected in the glomeruli of mice from F176042 and F176043 but not from F148571 transactivator founder lines **(Fig. 6.1B)**. Transgene was not expressed in the kidneys of mice not treated with dox. Only the mice from F176042 and F176043 transactivator founder lines developed kidney disease. Expression of Nef in the kidneys of these mice seems to be crucial for the development of kidney disease.

A



B

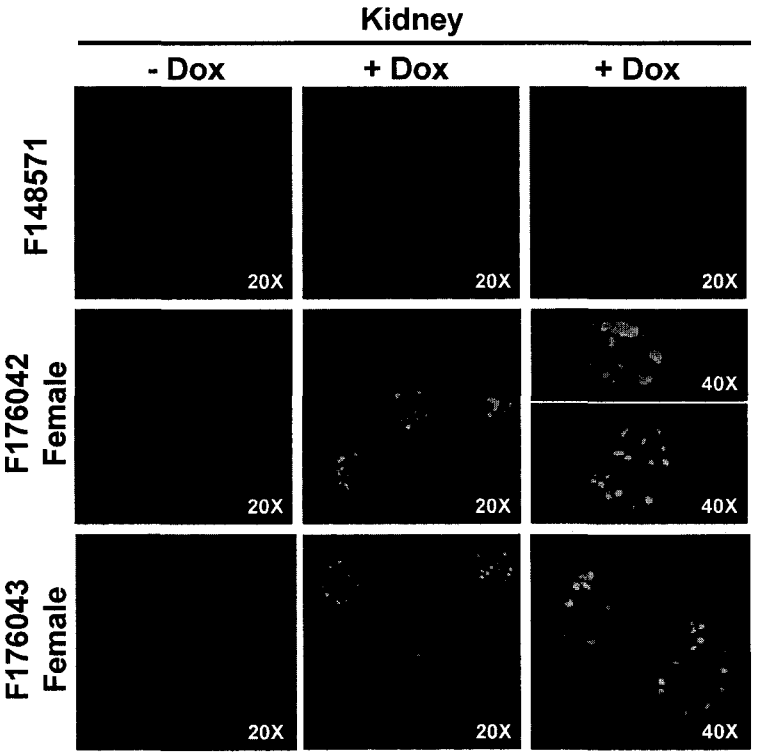


Figure 6.1. Detection of Tg expression in the glomeruli of dox-treated mice. (A) Protein extracts were prepared from glomeruli of dox-treated and untreated inducible Nef Tg mice. 100μg protein was electrophoresed on SDS-PAGE and transferred to membrane. The membrane was probed with antibodys against Nef and Actin followed by Alexa680-conjugated secondary antibody. (B) Frozen kidney sections from dox-treated and untreated inducible GFP reporter mice were stained with DAPI to visualize nuclei. GFP expressing cells in the glumeruli were detected by fluorescent microscopy.

6.2. Activation of p21 associated kinase 2 (PAK2) in inducible Nef Tg mice

To determine activation of PAK2 in inducible Nef Tg mice, *in vitro* kinase assay (IVKA) was performed on thymocyte lysates from dox-treated mice after immunoprecipitation with antibody against PAK2. Thymocytes from untreated mice were used as control. Three major phosphorylated proteins of approximately 62, 85 and 95 kDa were detected in thymocyte lysate from dox-treated but not from untreated mice **(Fig. 6.2)**. The 62 kDa protein is most likely a PAK member. This result shows that expression of Nef in mouse thymocytes results in phosphorylation of PAK2 and other proteins associated with it.

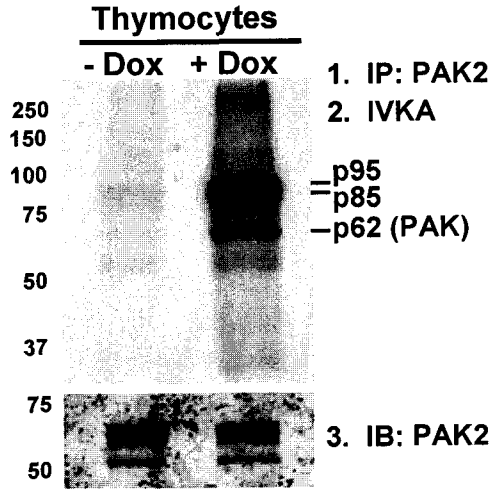


Figure 6.2. Activation of PAK2 in Nef expressing thymocytes. Thymocyte lysate from dox-treated and untreated mice were immunoprecipitated with antibody against PAK2 followed by IVKA. Proteins were resolved on SDS-PAGE and visualized by autoradiography after transfer to membrane. Membrane was immunobloted (IB) with antibody against PAK2 to determine protein loading.

6.3. Claims to original research

This thesis describes the pathogenic affects of HIV-1 Nef in adult mice. To our knowledge this is first such mouse model which expresses HIV-1 Nef in tissue-specific and inducible manner. For the first time we have been able to control the time and level of Nef expression and study the effects of Nef expression as function of these two parameters in the same Tg mouse line. The phenotypes described in **chapter 2**, such as thymocyte depletion, altered thymocyte maturation, peripheral T cell depletion, T cell activation and apoptosis, and pathologies in the lung and kidney, occur in the context of a fully developed organ system. This study rules out any developmental defects that may have been caused by early expression of Nef in the constitutively Nef expressing Tg mice. In **chapter 3** we have revisited the question about the role of Nef in causing T cell activation *in vivo*. Using of bone marrow chimeric mice, with a full T cell compartment, we clearly demonstrate that T cell activation in these chimeras is a direct consequence of expression of Nef in these cells. Finally, in **chapter 4** we describe characterization of $CD8^+$ T cell compartment in CD4C/HIV^{Nef} Tg mice. This is the first time $CD8^+$ T cell development and functions have been addressed in these mice. We report that CD4C/HIV^{Nef} Tg mice are unable to generated $CD8^+$ T memory response to viral infections due to lack of $CD4^+$ helper functions.

The inducible Nef Tg mouse model described in this thesis well offer us the opportunity to study functions of Nef *in vivo* and *in vitro* in primary immune cells. Moreover, these mice will be useful to study immune reconstitution since Nef expression can be turned off after withdrawal from dox treatment.