The role of $CD4^+$ helper T cells in immunity to acute Friend retroviral infection

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Dedicated to my Parents

The chess board is the world,

the pieces are the phenomena of the universe,

the rules of the game are what we call the laws of Nature.

A Liberal Education
Thomas Henry Huxley, 1868

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1. INTRODUCTION

"Retroviruses are unique among infectious agents, both in the way they interact with the host cells and organism and in the consequence of this interaction - not only to the life of the infected host but also in some cases to the host's descendants. No other infectious agent of higher eukaryotes regularly integrates its genetic information into the host genome; no other regularly acquires host genes into its genome, no other can infect the germ line of its host; no other has played such an important part in so many aspects of modern biology." (Coffin, 1997)

1.1 History of retroviral research (Cann, 2001; Coffin, 1997)

- 1908: Vilhelm Ellerman and Oluf Bang, were studying leukemia in chickens and succeeded in transferring the disease from one to another by cell-free tissue filtrates.
- 1911: Peyton Rous reported cell free transmission of sarcoma in chicken and isolated the infectious agent *Rous sarcoma Virus* (RSV).
- 1951: Gross observed vertical (germ line) transmission of cancers
- 1957: The Friend murine leukemia virus discovered by Charlotte Friend provided an animal model system for the study of erythropoiesis and the multistep nature of cancer.
- 1960's: Howard Temin knew that retrovirus genomes were composed of RNA and observed that replication was inhibited by actinomycin D (that inhibits DNA synthesis), this drug doesn't inhibit the replication of other RNA viruses. Temin and Baltimore simultaneously demonstrated that retrovirus particles contain an RNA-dependent DNA polymerase-reverse transcriptase (Nobel prize awarded to Baltimore and Temin, 1975).
- 1969: Huebner and Todaro proposed the viral oncogene hypothesis - the transmission of viral and oncogenic information as genetic elements. This explained the vertical (germ line) transmission of 'cancers', first observed by Gross, 1951.
- 1981: Gallo and co-workers discovered Human T-cell leukaemia virus, which was the first pathogenic human retrovirus to be identified.
- 1983: Barre-Sinoussi *et al* (1983) discovered Human immunodeficiency virus - the causative agent of AIDS.

1.2 Retroviruses

1.2.1 Structure and genome of Retroviruses (Voisset, 2000)

Retroviruses are enveloped viruses with a core containing a single-stranded RNA genome of positive polarity. The virions are 80–100 nm in diameter. The envelope is composed of a lipid bilayer containing single glycopeptides. The outer envelope glycoprotein (SU) is responsible for receptor binding and is linked by disulphide bonds to the transmembrane glycoprotein (TM) which holds the SU protein in the envelope and is responsible for membrane fusion. Inside the envelope membrane is the rough Matrix protein (MA) that obscures the icosahedral capsid (CA). Inside the capsid is the core including the RNA genome, nucleocapsid (NC), Reverse transcriptase (RT) and, Integrase (IN) (Fig 1.1).

The retrovirus genome consist of two molecules of RNA, which are single stranded, (+) sense and have a 5′cap and 3′poly-tail (equivalent to messenger RNA or mRNA) (Tang, Kuhen, and Wong-Staal, 1999). These vary in size from ~ 8-11 kb depending upon the retroviral species. The two RNA molecules are physically linked by hydrogen bonds. In addition, there is a specific type of transfer RNA (tRNA) presented in all particles that is required for replication. Gene order in all retroviruses is invariant: 5′-gag-pol-env-3′. A simple retrovirus contains three principal genetic domains.

1. The *env* gene encodes the two envelope proteins
2. The *gag* (group specific antigen) gene encodes four proteins that compose the core of the virus.
3. The *pol* (polymerase) gene encodes three enzymes in mature retroviral particles:
    - **Protease** that cleave the gag polyprotein into its constituent four peptides
    - **Reverse transcriptase** that transcribes the RNA genome into its DNA component
    - **Integrase** that catalyzes the integration of the double-stranded DNA copy into the host genome

In addition, there are specific cis-acting sites that are important for replication such as a primer binding site to initiate reverse transcription, a packaging site for incorporation of the genome into virions, and a site for 3′polyadenylation of nascent mRNA molecules.

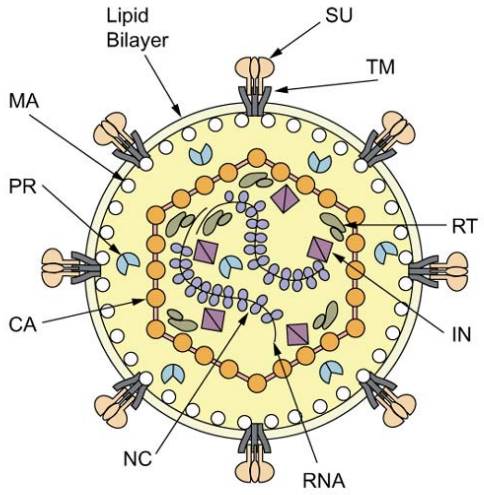


Figure 1.1: Schematic structure of a retroviral particle (Voisset, 2000)
TM, transmembrane components; SU, surface components; IN, integrase; CA, capsid protein; MA, matrix protein; NC, nucleocapsid protein; RT, reverse transcriptase; PR, retroviral protease.

1.2.2 Taxonomy of retroviruses (http://www.ncbi.nlm.nih.gov/retroviruses)

Retroviruses are part of a large and diverse family of enveloped RNA viruses defined by common taxonomic denominators that include structure, composition, and replicative properties. According to the Baltimore Classification retroviruses belong to the group VI of viruses: *Viruses with single-stramded (+) sense RNA with DNA intermediate in life cycle*. The family Retroviridae is further subdivided into eight genera (Table 1.1).

Table 1.1: Taxonomy- Group VI: RNA Reverse Transcribing Viruses; Family Retroviridae (http://www.ncbi.nlm.nih.gov/retroviruses)

Genus	Type Species	Genome	Virion Morphology	Hosts
Alpharetrovirus	*Avian leucosis virus* *Avian carcinoma virus* *Avian myelocytomatosis virus* *Fujinami sarcoma virus* *Rous sarcoma virus* *UR2 sarcoma virus* *Y73 sarcoma virus*	Simple	central, spherical core "C particles"	Vertebrates
Betaretrovirus	*Enzootic nasal tumour virus of goats* *Jaagseikte sheep retrovirus* *Mason-Pfizer monkey virus* *Mouse mammary tumour virus* *Ovine enzootic nasal tumour virus* *Squirrel monkey retrovirus*	Simple	Central, spherical core "B particles"	Vertebrates
Deltaretrovirus	*Bovine leukemia virus* *Human T-lymphotropic virus 1* *Human T-lymphotropic virus 2* *Simian T-lymphotropic virus 1* *Simian T-lymphotropic virus 2* *Simian T-lymphotropic virus 3*	Complex	central, spherical core	Vertebrates
Epsilonretrovirus	*Walleye dermal sarcoma virus* *Snakehead retrovirus*	Simple		Vertebrates
Gammaretrovirus	*Abelson murine leukemia virus* *Feline leukemia virus* *Friend murine leukernia virus* *Gibbon ape leukemia virus* *Moloney murine leukemia virus* *Moloney murine sarcoma virus*	Simple	central, spherical core "C particles"	Vertebrates

	Murine type C retrovirus *Porcine endogenous retrovirus* *RD114 retrovirus* *Rauscher murine leukemia virus* *Reticuloendotheliosis virus* *Spleen focus-forming virus* *Woolly monkey sarcoma virus* *Xenotropic MuLV-related virus VP62*			
Lentivirus	*Bovine immunodeficiency virus* *Caprine arthritis-encephalitis virus* *Equine infectious anemia virus* *Feline immunodeficiency virus* *Human immunodeficiency virus 1* *Human immunodeficiency virus 2* *Jembrana disease virus* *Ovine lentivirus* *Simian immunodeficiency virus* *Simian immunodeficiency virus SIV-mnd 2* *Simian - Human immunodeficiency virus* *Visna/Maedi virus*	Complex	cone-shaped core	Vertebrates
Spumaretrovirus	*African green monkey simian foamy virus* *Bovine foamy virus* *Equine foamy virus* *Feline foamy virus* *Human foamy virus* *Human spumaretrovirus* *Simian foamy virus* *Macaque simian foamy virus*	Complex	central, spherical core	Vertebrates

1.2.3 Life cycle of retroviruses

The life cycle of a retrovirus begins with specific **"attachment"** of retroviral particles to a host cell membrane, via cellular receptor and viral surface protein interactions. Some retroviruses also employ a secondary receptor, referred to as the co-receptor. Some retroviral receptors and co-receptors have been identified. For example, CD4 and various members of the chemokine receptor family on human T cells serve as HIV receptors and co-receptors. The second and third steps are **"penetration"** and **"uncoating"**, respectively. Retroviruses penetrate the viral envelope and fuses with the cellular plasma membrane to permit the viral core consisting mostly of gag-derived proteins, full-length genomic RNA, and viral enzymes to enter into the infected cell. The specificity of the virus-cell interaction is determined largely by the envelope protein of the retrovirus. The fourth step in retrovirus life-cycle is **"replication"**. Reverse transcriptase transcribes the genomic RNA into a double-stranded DNA molecule. The reverse transcriptase has three enzymatic activities: RNA-directed DNA polymerase synthesises one DNA strand, DNA-directed DNA polymerase synthesises the complementary strand, and RNAse H degrades the viral RNA strand. Reverse transcription is primed by a cellular tRNA that is packaged into retrovirus virions. After nuclear translocation, the genomic viral DNA is integrated in the cellular genomic DNA by the integrase and is then called provirus. The proviral DNA structure is composed of long terminal repeat (LTR) sequences located at each end of the integrated genome, surrounding *gag*, *pol* and *env* coding genes. The LTR sequences provide the transcriptional control elements for provirus expression. The LTR is divided into three domains: U3 (unique 3´), R (repeat), and U5 (unique 5´). Since, an integrated provirus resembles other multiexon cellular genes it utilizes the host cell machinery for replication, expression, and protein production (Voisset, 2000).

Following integration, the DNA genome is transcribed back into mRNA with the help of promoters. Transcription begins at the upstream end of the R domain and proceeds through the genome, terminating in a polyadenylation signal at the downstream end of the 3´ LTR. The U3 domain that contains basal promoters (PRO, such as a TATA box) and upstream enhancers (ENH) are important for retroviral transcription. The enhancers are regulatory elements in DNA that bind cellular proteins that act to "open" condensed DNA so that it is accessible to polymerase complex (Nathanson N et al., 1997). Retroviruses make use of splicing and ribosomal frameshifting to compress maximal information into a small genome.

Splicing is regulated by the cellular apparatus which interacts with *cis*-acting sequences present in the mRNA. The proteins encoded by *gag*, *pol*, and *pro* genes are expressed from a full length genomic RNA (vRNA). In *Murine Leukemia virus*, the *pro* gene is separated from the gag gene by terminal suppression. The *env* protein is expressed from a spliced mRNA. In more complex retrovirus (e.g., *Lentivirus*), several mRNAs are produced and especially the pattern of splicing in HIV is very complex (Tang, Kuhen, and Wong-Staal, 1999).

Step five is termed "**assembly"** in which retrovirus capsids are assembled in an immature form at various locations in the host cell. The virion capsid contains two (+) RNA strands. A small region called the packaging signal (φ), spanning the 3´end of the LTR and the 5´end of the *gag* gene, mediates specific binding to the nucleocapsid protein. This is followed by an "**egress"** stage, in which after assembly of RNA and viral proteins, the virion core acquires the envelope protein by budding from the plasma membrane of the host. Finally, step seven is **"maturation".** In this step, viral protease cleaves the gag-pol precursor in the core to carry out the maturation of virions. This step can occur along with the budding process or immediately after the external budding process. The progeny virions produced can initiate new infection cycles by binding to the receptor (s) of an appropriate target cell, entry by membrane fusion, and uncoating of the virion capsid virions (Voisset, 2000). Life-cycle of HIV is illustrated in Fig 1.2.

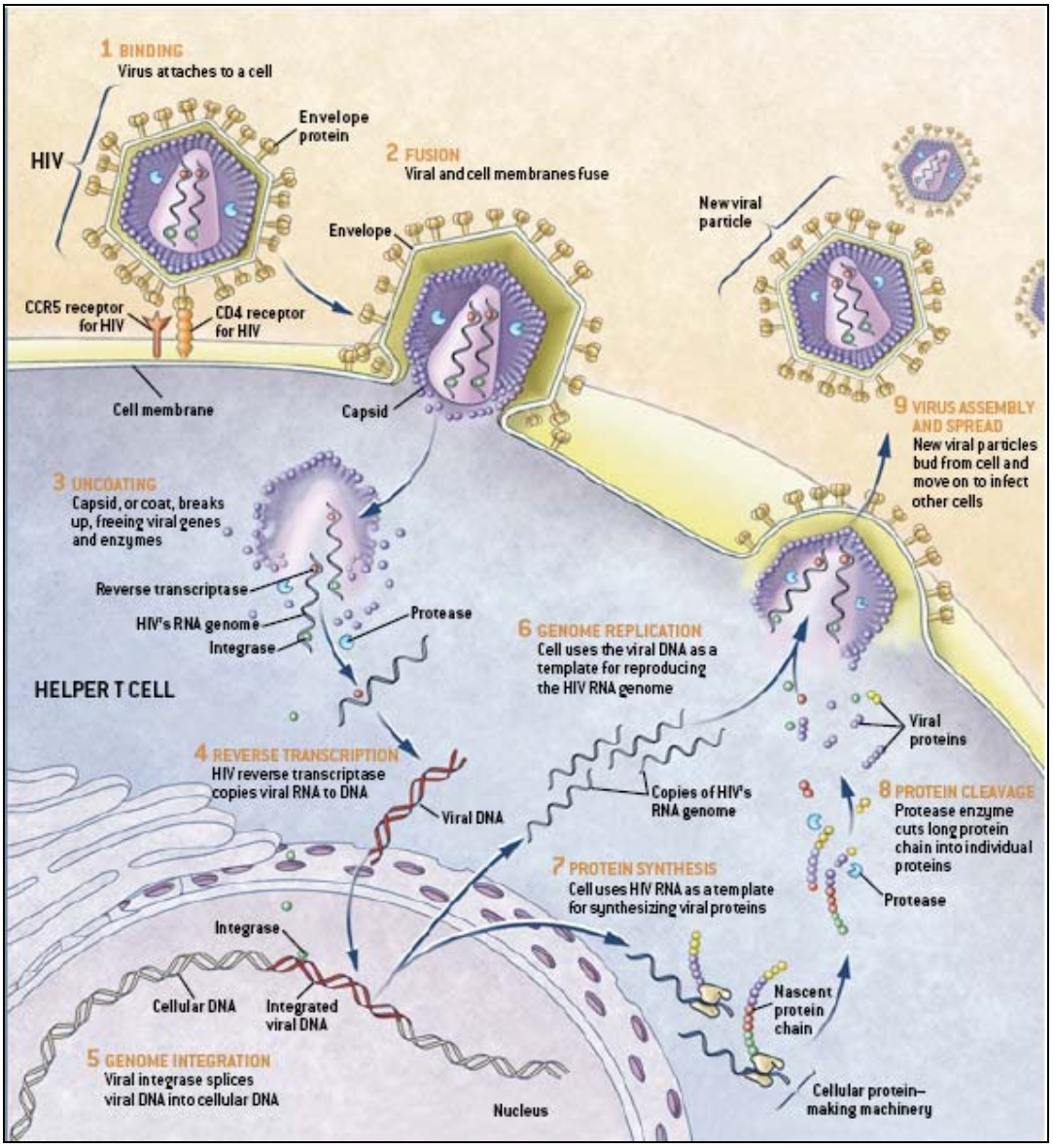


Figure 1.2: The retroviral life cycle
(http://www.sif.it/SIF/en/portal/journals/highlight_broglia/highlight_broglia_figs)

The life cycle of retroviruses can be broadly divided into six essential steps, which are shown schematically in the figure: The HIV enters a host cell (bind to it and inject their genes into the interior), copy their genes and proteins (by using the host cell's machinery and raw material), and pack the fresh copies into new viral particles able to spread to and infect other cells.

1.2.4 Genomic variation between complex *versus* simple retroviruses

Simple retroviruses usually carry only the elementary information such as information for reverse transcriptase, RNase H, integrase, and protease whereas complex retroviruses code for additional regulatory proteins derived from multiply spliced messages. *Murine leukaemia viruses* (MuLVs) are prototype simple retroviruses, while the *Human immunodeficiency virus* (HIV-1) of the *Lentivirus* genus is an example for a complex retrovirus. Lentiviruses contain additional genes that are essential for or contributory to efficient virus replication and persistence. HIV-1, encodes two regulatory genes (*tat, rev*) and four accessory genes (*vpr, vpu, nef, vif*) that contribute to viral replication and host pathogenesis (Fig 1.3). The *tat* and *rav* genes are responsible for transcriptional and posttranscriptional activation of viral gene expression respectively (Tang, Kuhen, and Wong-Staal, 1999).

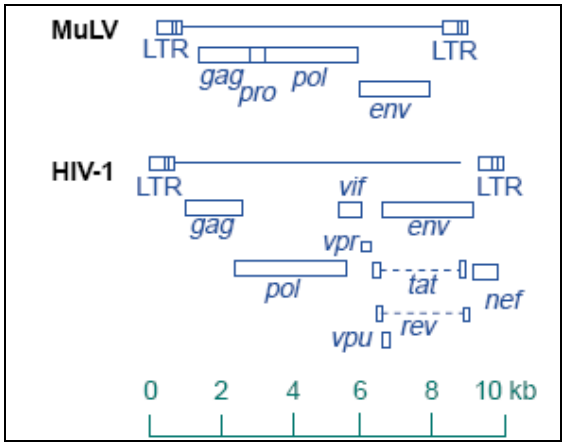


Figure 1.3: Representative retroviral genomes of Type C retroviruses (MuLV) and lentiviruses (HIV-1) (Power, 2001)

The figure shows the major structural genes of retroviruses (*gag, pol, env*), and showing the greater complexity of the lentiviral genomes because of the accessory genes, including *rev, tat, vif, vpu, nef, vpr*.

1.3 Retrovirus and host interactions

Retroviruses give rise to a broad spectrum of diseases, including neurological disorders, malignant transformation, and immunodeficiencies such as AIDS.

1.3.1 Retrovirus induced oncogenesis

Most oncogenic RNA viruses belong to the family Retroviridae and the three major classes of oncogenic retroviruses are the nonacute transforming viruses, the acute transforming viruses, and the trans-acting viruses. The characteristic differences between these three classes are mentioned in Table 1.2.

Table 1.2: Major categories of oncogenic retroviruses (Nathanson, 2001)

Category	Mechanism of transformation (clonality)	Replication competence	Examples
Nonacute transforming	Insertional up-regulation of cellular proto-oncogenes (clonal)	Competent	F-MuLV, ALV, FeLV
Acute transforming	Action of viral oncogenes (polyclonal)	Defective, requires helper virus	ASV, MSV, FeSV, SFFV
Trans-acting transforming	Action of viral accessory genes (oligoclonal)	Competent	HTLV-1, BLV

ALV - *Avian leukemia virus*; ASV - *avian sarcoma virus*; BLV- *bovine leukemia virus*; FeLV - *Feline leukemia virus*; FeSV - *Feline sarcoma virus*; HTLV - *Human T-cell leukemia virus*; F-MuLV – *Friend murine leukemia virus*; SFFV – *Spleen focus forming virus*; MSV - *Murine sarcoma virus.*

1.3.2 Mechanism of Oncogenesis

Tumor formation is a multistep process that in some cases could be virus-mediated and sometimes not. Tumor induction often requires activation of a cellular oncogene and down-regulation of tumour suppressor genes. Each oncogenic virus tends to produce a characteristic range of tumors, related to the cells that it infects and the cell-specific activity of the enhancers in its LTR (Nathanson, 2001).

1.3.2.1 Nonacute transforming retroviruses: Insertional mutagenesis

These retroviruses have the organization of simple retroviruses, are replication competent and transform by promoter or enhancer insertion. Murine Leukemia viruses (MuLV), which were subject of research in this study, have been studied in great detail as representatives of nonacute transforming retroviruses. MuLV are replication-competent simple retroviruses which are host-specific. The DNA provirus integrates at different sites in the genome of the infected cell and persists for the lifetime of the specific cell type. Transformation is due to the effect of the LTR of the provirus upon expression of host genes. The effect can be exerted in two ways - promoter insertion and enhancer activation. In promoter insertion, the DNA provirus is integrated upstream and in the same orientation to a proto-oncogene (a normal cellular gene that can influence cell growth). Transcription is initiated in a LTR of the provirus and reads through the downstream cellular gene, increasing its rate of transcription. If the cellular gene that is up-regulated has an influence on cellular growth, then transformation may result. The resulting tumors are usually clonal but the transformed target cells will depend upon the genomic site of insertion (Fig 1.4a). Alternatively, for enhancer activation, the provirus DNA is located near a cellular gene but is oriented to read away from the cellular gene, the enhancer sequences in the provirus may bind cellular factors that “open” condensed DNA and enhance transcription of neighbouring cellular genes regardless of their orientation (Fig 1.4b). Again, if the gene influences cellular growth, it results in transformation. Hence, enhancer activation is a more common phenomenon than promoter insertion, as it can occur at more sites in the cellular genome (Nathanson, 2001).

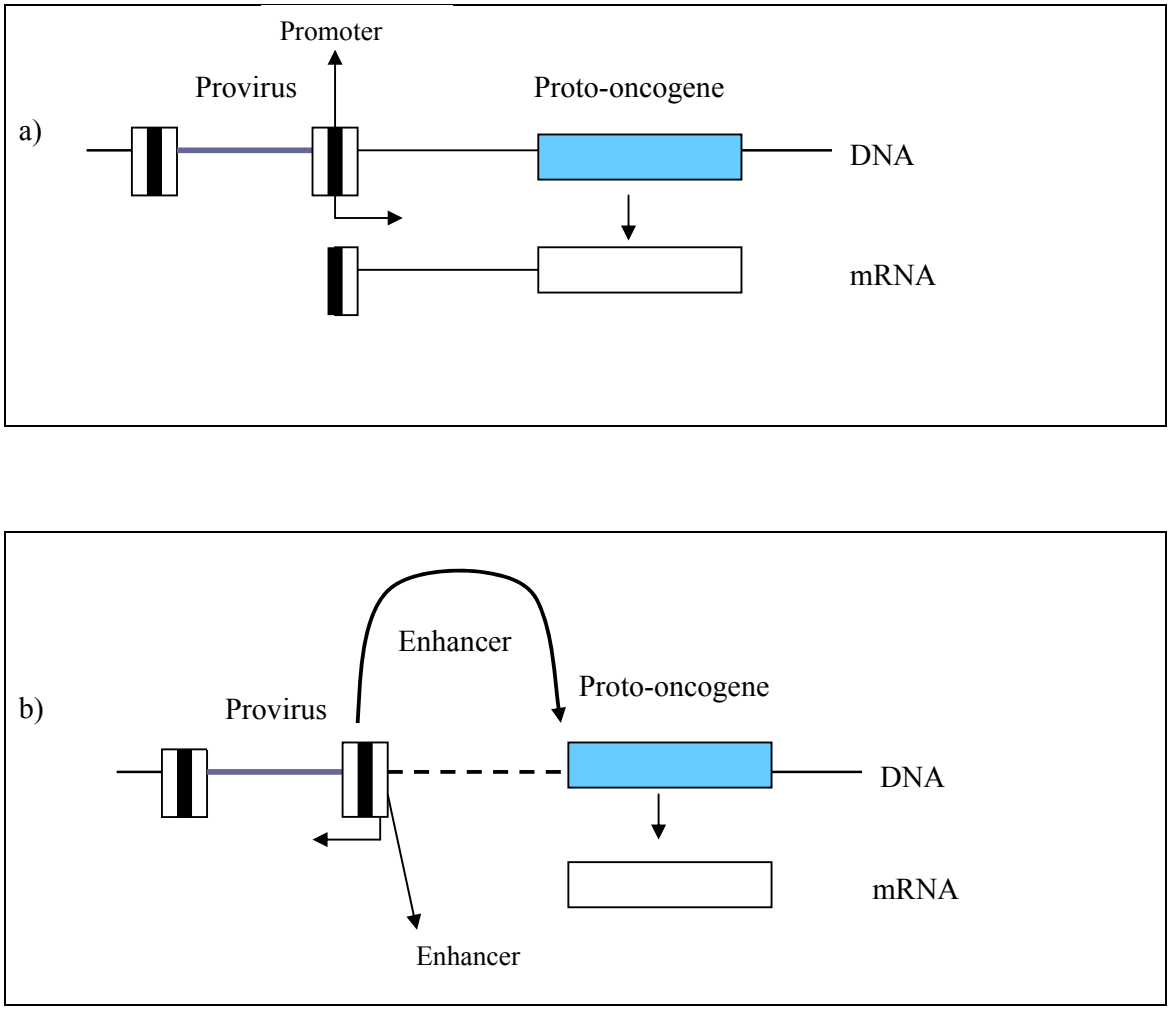


Figure 1.4: Mechanisms of transformation (Nathanson, 2001)
a) Promoter insertion b) Enhancer activation

1.3.2.2 Acute transforming Retroviruses: Viral oncogenes

In these viruses, the viral genome contains a viral oncogene (*v-onc*) that possesses a specific transforming activity at a high level. Most v-onc genes were derived from proto-oncogenes (*c-onc*). Typically, acute transforming viruses are defective for replication as they have lost their genes such as *gag* and *env* during ancestral recombination event. Hence, they can grow only in the presence of a replication-competent nontransforming retrovirus. Unlike nonacute transforming viruses, acute transforming viruses have a shorter incubation period and

transformed cells are polyclonal in nature in addition to specific tumour induction (Nathanson, 2001).

1.3.2.3 Trans-acting retroviruses: Viral accessory genes

These retroviruses encode several accessory genes which play a dual important role in viral replication and transformation capacity. However, the mechanism of trans-activation mediated oncogenesis is complex and there is also the requirement of subsequent nonviral genetic events for tumour induction. HTLV-1 is the best studied example in this group of viruses (Nathanson, 2001).

1.4 Infection and Immunity

Protection against infection is fundamental to the survival of all animals and is mediated by the immune system, comprising of both innate and adaptive mechanisms to deal with invading pathogens (Fig 1.5).

1.4.1 Innate immunity

The first line of defence against invading microbes is offered by the innate immune system comprising of various innate immune effector cells, including macrophages, dendritic cells (DCs), neutrophils and natural killer (NK) cells and various protein components of the complement system. Pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), on the cell surface of macrophages and DCs recognize the conserved pathogen-derived molecules or the pathogen associated molecular patterns (PAMPs). Binding of PAMPs to TLRs leads to production of pro-inflammatory cytokines and chemokines. The initial interaction of T cells with APCs is mediated by cell-adhesion molecules such as selectins, integrins, members of the Ig superfamily and some mucinlike molecules which help to attract other effector cells to the site of infection. L-selectin expressed on naïve T cells guides the exit of naïve T cells from the blood into peripheral lymphoid tissues. Tissue DCs ingest antigens at sites of infection and are activated as part of the innate immune response (C.A.Janeway; Travers, 2001).

1.4.2 Adaptive immunity

Immature dendritic cells following interaction with the antigen during innate immune response get activated to become mature DCs. Mature DCs have increased expression of co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) on their surface. The receptor for B7 molecules on the T cells is CD28, a member of immunoglobulin (Ig) superfamily like B7 molecules. Macrophages and B cells also get activated to express B7 molecules on their surface. DCs, macrophages and B cells are often termed as professional antigen presenting cells (APCs) although DCs are much stronger APCs than macrophages and B cells. DCs migrate rapidly from the periphery via afferent lymphatic channels to draining lymph nodes. Pathogen activated DCs present these pathogen-derived antigens to T cells and promote the differentiation of naive T cells to various subtypes of effector $CD4^+$ and $CD8^+$ T cells to commence the adaptive immune cascade. Peptides from intracellular pathogens that multiply in the cytoplasm are carried to the cell surface by MHC class I molecules and presented to $CD8^+$ T cells which clonally expand in the presence of B7 molecules expressed on APCs to produce cytotoxic T cells (CTLs) that can kill infected target cells via production of cytotoxic molecules such as granzymes A and B and perforin. Peptide antigens processed from pathogens multiplying in intracellular vesicles and those derived from ingestion of extracellular bacteria and toxins are carried to the cell surface by MHC class II molecules and presented to $CD4^+$ T cells which then receive a second signal from DCs provided by molecules of the B7 family to differentiate into helper T cells (Th), type Th1 and Th2. Interactions between CD28/B7.1 drive immune responses towards Th1 type while CD28/B7.2 favour Th2 type immune responses. $CD4^+$ Th1 cells secrete interferon-γ (IFN-γ), Tumor Necrosis factor- (TNF) - α and β, which promote the anti-microbial activity of macrophages and helps B-cell production of IgG2a antibodies, whereas Th2 cells provide help for B-cell production of IgG1, IgA and IgE, which form the effector molecules of the humoral immune response (Janeway, 2001).

Activated T cells expresses a number of accessory proteins required for sustaining co-stimulatory signals and one such protein is CD40 ligand (CD40L), which binds to CD40 on APCs. Binding of CD40-CD40L triggers further activation of APCs to express more B7 molecules, thus stimulating further T-cell proliferation. Interaction of Th cells with B cells

and $CD8^+$ T cells requires ligation of CD40 (expressed on B cells and $CD8^+$ T cells) by CD40L (expressed on $CD4^+$ T cells). Activated T cells under the influence of co-stimulatory

signal synthesize interleukin-2 cytokine and its receptor. IL-2 production determines whether a T cell will proliferate and become an armed effector cell. Antigen recognition in the absence of co-stimulation inactivates naïve T cells, inducing a state of anergy and the anergic T cells are unable to proliferate further due to the absence of IL-2. This leads to T-cell tolerance to the antigen and this mechanism is mostly used when self-antigens are presented by APCs. Some CD28-related proteins which share structural homology with CD28 such as CTLA-4 (CD152) when bind to B7 molecules deliver an inhibitory signal to the activated T cells while inducible co-stimulator (ICOS) induced on activated T cells binds to the ligand of ICOS (LICOS), distinct from B7 molecules and delivers an activating signal. Resolution of an infection is accompanied by the death of most of the effector cells to restore tissue integrity and the generation of long-lived memory cells. Immunological memory is the hallmark of adaptive immunity and is the property of remembering specific adaptive immune responses so as to develop a more rapid and greater response in the future to the same pathogen (Janeway, 2001).

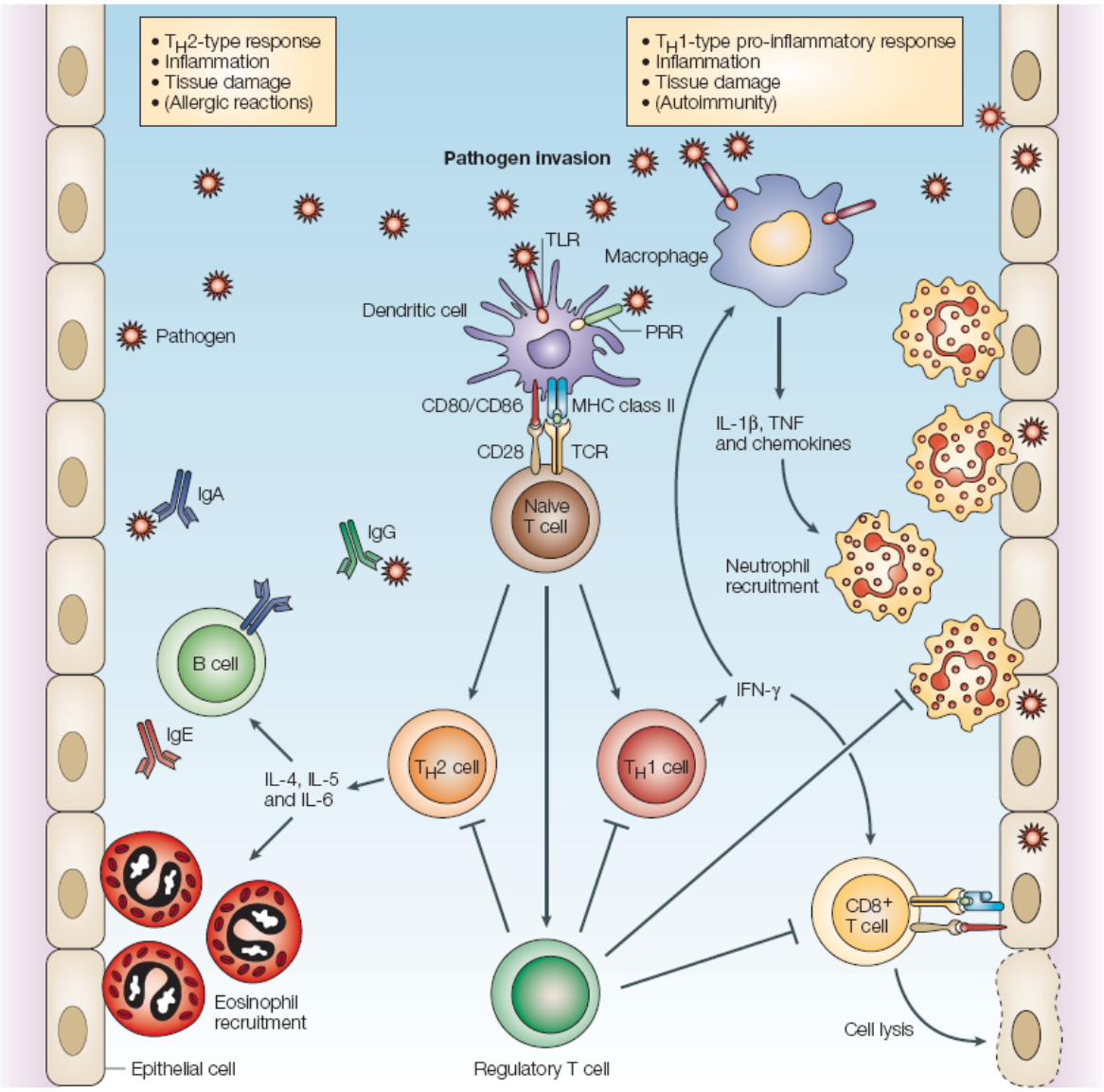


Figure 1.5: Immunity to infection (Mills, 2004)

1.4.3 Helper T cells

T helper (Th) cells are a subpopulation of $CD4^+$ T cells which are specialized in providing supporting signals to other cell types such as macrophages, B cells and $CD8^+$ T cells either through secretion of cytokines or through direct interactions to induce immunity to pathogens (Th cells or $CD4^+$ T cells are used as synonym terms in this thesis unless otherwise mentioned).

1.4.3.1 Helper T cells in virus infections

$CD4^+$ T cells provide "help" to a number of key immune responses involved in controlling viral infections. Unlike $CD8^+$ T cells, which are essential during the acute stages of infection, Th cells are found essential in controlling viral replication towards the chronic stages of infection. Lymphocytic choriomeningitis virus (LCMV) is a noncytopathic virus that is cleared primarily by $CD8^+$CTLs. However, Th cells are required for the control of pathogenic strains of LCMV that can establish chronic infection in mice. Also, in the absence of $CD4^+$ T cells, vaccination of mice with LCMV peptides is less effective in eliciting CTL responses and neutralizing antibody responses that protect against pathogenic viral challenge. When CD4 - deficient mice were challenged with LCMV, virus-specific CTL responses were able to initially control viremia, however, with time, the $CD8^+$ CTLs lost its cyotoxic activity which resulted in rise in viremia. In murine influenza virus infection, viral clearance is antibody mediated and lack of CTLs has no effect on the control of viremia. However, transfer of influenza-virus specific $CD4^+$ T cells to nude mice lacking MHC class II expression can induce clearance of the virus. This implies that $CD4^+$ T cell help for B cell-mediated production of antibodies rather than direct $CD4^+$ T cell effector function induce control of influenza virus in the mouse model (Norris and Rosenberg, 2002). Vesicular stomatitis virus (VSV) infection of immunocompetent mice induces a rapid neutralizing IgM response that occurs independently of T cell help, followed by production of neutralizing IgG antibodies that are strictly dependent on $CD4^+$ T cell help. The neutralizing IgG response seems to be crucial for recovery from primary infections and for protection against reinfection. Furthermore, priming of immunocompetent mice that have been primed with VSV rapidly eliminates a recombinant vaccinia virus expressing the VSV-G (a peptide derived from the glycoprotein (G) of VSV) in a $CD4^+$ T cell-dependent manner (Maloy et al., 1999).

Viral infection is known to stimulate the production of IL-12 by DCs and macrophages, and is a potent inducer of Th1 responses. The successful control of vaccinia infection depends on the production of T cell–derived cytokines, most notably IFN-γ and TNF-α by $CD4^+$ T cells. In VSV infection, Th1 and Th2 cells provide different levels of anti-viral protection. Effector Th1 cells induced against VSV or vaccinia virus expressing the VSV-G possess much greater antiviral protective capacity than their Th2 counterparts. Th1 cells were able to mediate protection against viruses controlled by induction of IgG2a antibodies (VSV) or cytokines

(vaccinia). In contrast, although Th2 cells protected against systemic infection with VSV by inducing neutralizing IgG1 antibodies, they were unable to eradicate vaccinia virus (Maloy et al., 2000). Similarly, during polio virus infection in mice, Thl cells can mediate a protective immune response through helper activity for humoral immunity (Mahon et al., 1995). In the mouse zosteriform model that mimics several aspects of reactivated HSV infection of humans, protective immunity could be induced routinely against zosteriform lesions caused by HSV. The protection is mediated by $CD4^+$ T cells obtained from mice immunized with plasmid DNA-encoding glycoprotein B (gB) of HSV-1 and displayed a Th1 cytokine profile (Manickan et al., 1995). Acute central nervous system disease caused by Measles virus in Lewis rats is attenuated by transfer of individual MV protein-specific $CD4^+$ T cells, without the participation of $CD8^+$ T cells or neutralizing antibodies (Reich et al., 1992). In humans too, Th cells are requisite for viral clearance and resolution of disease in Hepatitis C virus infection. Importantly, HCV-specific Th cell responses are essential for eliminating virus during the acute phase of disease and these responses are also required for permanent maintenance of viral control. Hence, Th cells play a key role in inducing effective immune responses during acute viral infection and orchestrating effective immune function in chronic human viral infections (Altfeld and Rosenberg, 2000).

1.4.3.2 Helper T cells in HIV infections

The crucial importance of Th cells in immunity is dramatically demonstrated by the epidemic of AIDS. Activated Th cells are the main target of HIV-1 and are killed by infection and HIV-1 induced apoptosis. HIV-1 infection is characterized by both the absence of HIV-1 specific Th cells and eventual decline in virus-specific CTLs, inevitably resulting in AIDS. Qualitative impairment of Th cell function occurs very early in the course of infection. There is weak proliferation of HIV-1 specific Th cells especially to the HIV whole protein in comparison to HIV gag protein p24, suggesting that the virus was not able to induce an overall strong virus-specific Th cell responses (Wahren et al., 1987). Certain other HIV patients displayed lower or below normal levels of lymphokine production by macrophages, although the antimicrobial activity of these cells were intact and when the patient's monocytes were subsequently treated with exogenous IFN-γ, there was enhanced and effective intracellular antimicrobial activity (Murray et al., 1984). Furthermore, virus-specific $CD8^+$ T cells can be maintained in the peripheral circulation at high frequency in the absence of circulating peripheral $CD4^+$ T cells. However, their ability to secrete IFN-γ in response to

virus-specific stimulation was compromised possibly due to lack of help from $CD4^+$ T cells (Spiegel et al., 2000).

The ability of HIV long-term nonprogressors to control viremia is dependant on both virus-specific Th responses and CTL responses. In a small cohort of HIV-1-infected long-term nonprogressors, robust Th proliferative responses exclusively to HIV gag protein p24 contribute to immunological control of virus replication. Anti-viral effector responses in these patients are mediated by increased levels of IFN-γ, the chemokine RANTES and the macrophage inflammatory proteins MIP-1α and MIP-1β, and no IL-4 indicating that the response is Th1–like (Rosenberg et al., 1997). Only HIV p24 - specific Th responses correlated with *gag*-specific CTL responses likely reflecting the immunodominance of *gag* for inducing CTL responses in chronically HIV infected patients who can control viremia. In the absence of Th responses, the presence of HIV-1-specific CTL was not associated with decreased viral replication (Kalams et al., 1999). Apart from HIV *gag* p24, other *gag* proteins, gp17 and gp55 also induce potent HIV-1 specific Th cell effector responses.

1.4.4 Regulatory T cells

Regulatory T cells (Treg cells) are a specialized subpopulation of T cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens. There are essentially two major populations of Treg cells - natural (or constitutive) and inducible (or adaptive) Treg cells. Natural $CD4^+CD25^+$Treg cells constitute 5 -10% of peripheral T cells in normal mice. Natural regulatory T cells express the cell-surface marker CD4, the IL-2Rα chain (CD25) and the transcriptional repressor Foxp3 (forkhead box p3). These cells mature and migrate from the thymus and then enter peripheral tissues, where they suppress the activation of self-reactive T cells. On the contrary, the inducible regulatory T cell populations are IL-10 secreting Treg cells called T_R1 and TGF-β secreting Treg called T_H3. They are generated from naïve $CD25^+$ or $CD25^-$ T cells in the periphery when they encounter antigen presented by semi-mature dendritic cells and under the influence of IL-10, TGF-β and possibly IFN-α. T_R1 cells secrete high levels of IL-10, no IL-4 and very low levels of IFN-γ while T_H3 cells secrete very high levels of TGF-β. In addition to the $CD4^+$ Treg cells, there exists also a population of $CD8^+$ Treg cells that secrete either IL-10 or TGF-β (Fig 1.6). Other rare immunosuppressors are the $CD8^+$γδ T cells and the IL-10 and TGF-β-producing γδ T cells that can prevent insulin-dependent diabetes in

mice and suppress the anti-tumor activity of CTLs and NK cells. Furthermore, natural killer T cells (NKT) cells also secrete IL-10 (Mills, 2004). Natural $CD4^{+}CD25^{+}$ Treg cells were first defined in 1995 by Sakaguchi and colleagues. CD25, although a marker for natural regulatory T cells is also expressed on activated Th cells and some cells which are $CD25^{-}$ also have suppressive function. Alternative markers for Treg cells include cell-surface expression of CD38, CD62L, CD103 or glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR), $CD45RB^{low}$ or intracellular expression of the transcriptional repressor Foxp3. Foxp3 is the most unique marker of natural Treg cells. Inducible regulatory T cells are characterized by the cytokine production profile (IL-10 and TGF-β) rather than the expression of cell-surface markers (Mills, 2004).

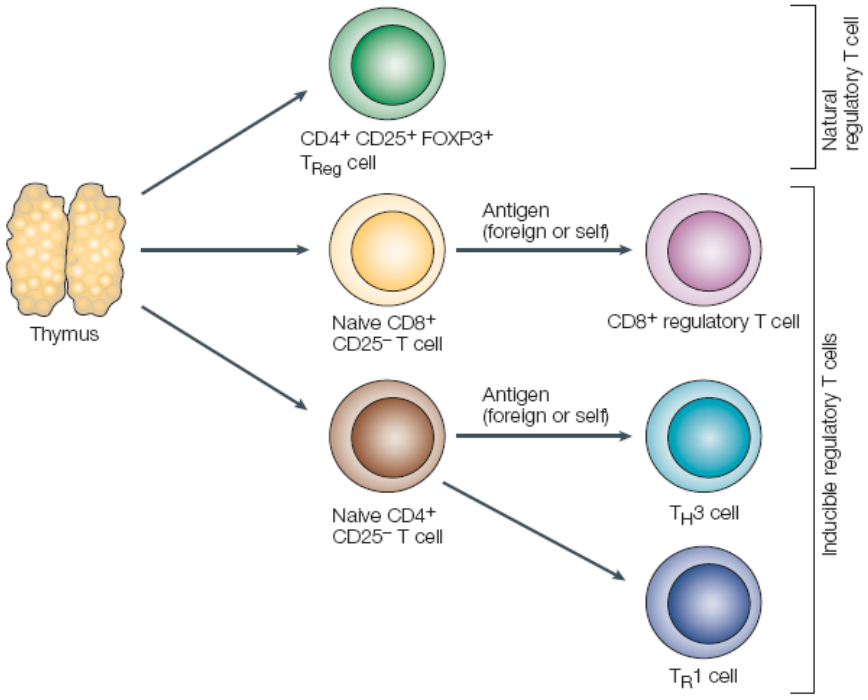


Figure 1.6: Natural and Inducible regulatory T cells (Mills, 2004)

1.4.4.1 Regulatory T cells in virus infections

Treg cells have a key role in protection in autoimmune diseases, allograft rejection and allergy by suppressing potentially pathogenic immune responses mediated by effector Th1 cells, Th2 cells or CTLs. However, during an infection, down-regulation of these very immune responses can have two-sided consequences –

a. Protective effect on the host tissues as accelerated pro-inflammatory responses could cause collateral tissue damage and immunoregulatory mechanisms induced by Treg are essential to control this immunopathology (Fig 1.7). In HCV infection, there is inhibition of HCV-specific T cells in chronically infected individuals by IL-10 producing $CD4^+$ and $CD8^+$ Treg cells in the liver which helps in reduce liver inflammation. HCV-infected patients with low numbers of $CD4^+CD25^+$ T cells often develop an autoimmune syndrome, known as Mixed Cryoglobulinemia, which is characterized by B-cell proliferation and autoantibody production. Infection of mice with Theiler's virus induced a Demyelinating disease mediated by $CD4^+$ T cells, and the transfer of $CD8^+$ Treg cells prevented inflammation and the pathogenic effects of the $CD4^+$ T cells (Mills, 2004). Furthermore, mice deprived of Treg cells in genital HSV-2 infection resulted in enhancement of Th1 cell responses in draining lymph nodes at the site of infection, but delayed the entry of the immune cells into the HSV-infected tissue. Treg-deficient mice succumbed more rapidly, developing severe lesions and hind limb paralysis 4 to 5 days earlier than Treg-sufficient mice. Thus, the authors postulated an immune response promoting role for Tregs in acute infections in which the site of pathogen replication is in non-lymphoid tissues (Lund et al., 2008).

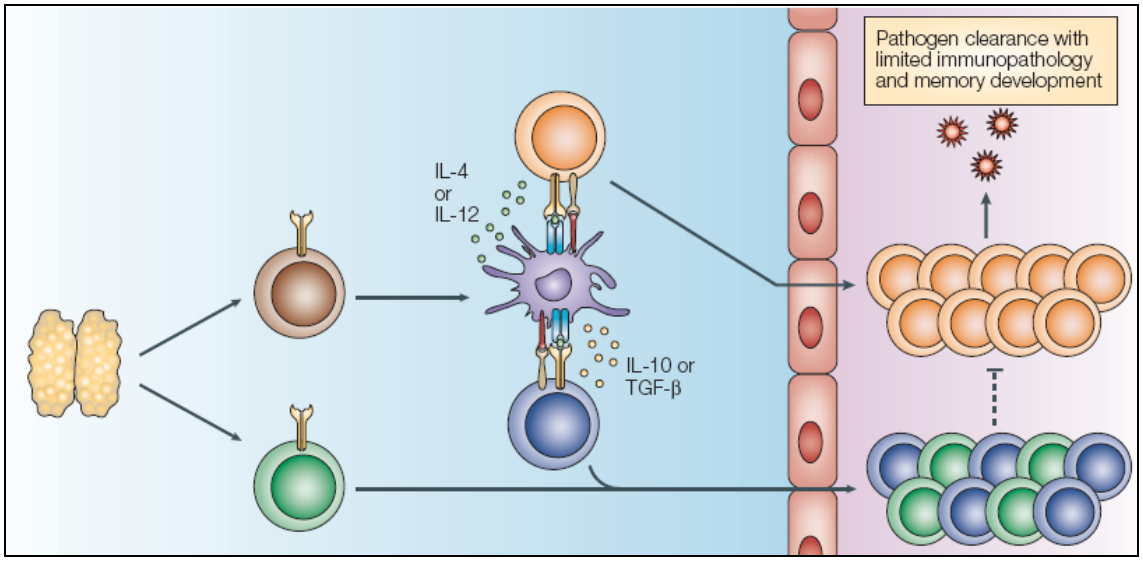


Figure 1.7: Protective immunity by Tregs (Mills, 2004)

b. Immunosuppressive effect on effector T cells can also be beneficial to the virus, by prolonging recovery of the infected host leading to persistent infections. Many viruses stimulate the production of immunosuppressive cytokines for instance, IL-10 or TGF-β that can cause dysfunction of effector T cells (Fig 1.8). Patients with chronic HCV infection have circulating HCV-specific $CD4^{+}T_R1$ cells and $CD8^{+}$ Treg cells. Blocking the anti-inflammatory activity of Treg cells by adding neutralizing IL-10 specific antibody significantly increased HCV-specific IFN-γ production by effector T cells *in vitro*. Furthermore, depletion of $CD4^{+}CD25^{+}$ Treg cells from PBMCs increase the frequency of IFN-γ producing $CD8^{+}$ and $CD4^{+}$ T cells in response to CMV antigens. Removal of $CD4^{+}CD25^{+}$ Treg cells in HSV infected mice also increased the virus specific $CD8^{+}$ T cell response and improved viral clearance. In EBV infected humans, induction of TR1 cells which are specific for LMP1 (latent membrane protein 1) of EBV inhibit TH1 cell responses to other EBV proteins facilitating viral persistence and promoting the induction of EBV-associated tumors (Mills, 2004).

c.

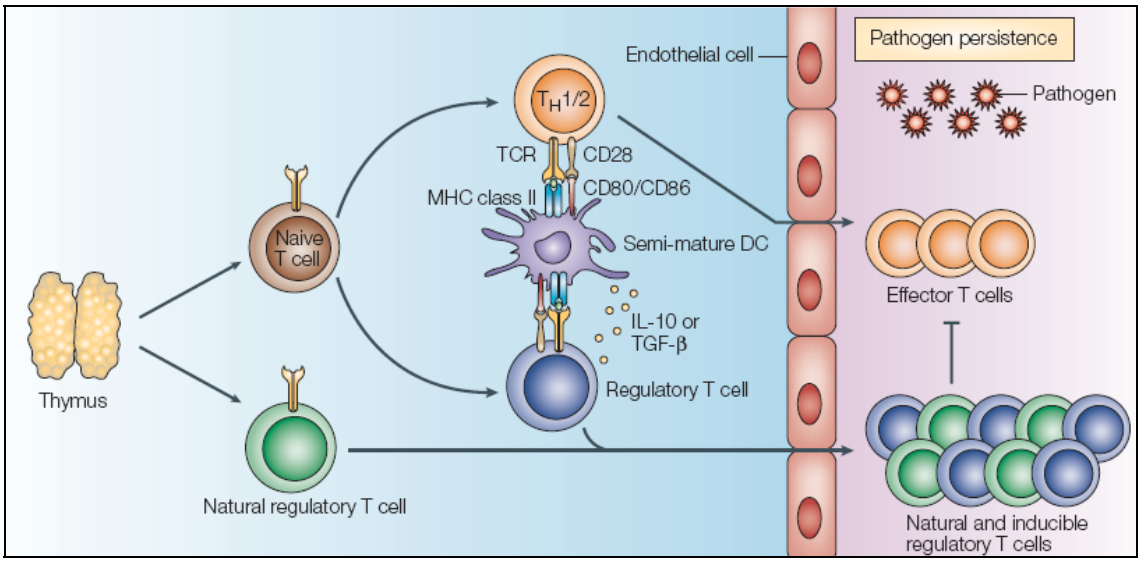


Figure 1.8: Immunosuppressive activity of Tregs (Mills, 2004)

1.4.4.2 Regulatory T cells in retrovirus infections

Retroviruses, such as HIV, which usually persist in the host for life, are potentially dangerous due to reactivation of the viruses in immunocompromised situations. In HIV infection, even before the decline in the number of $CD4^+$ T cells begins, immune responses to HIV are suppressed. T cell dysfunction plays an important role in AIDS disease progression. One explanation for impaired T cell function arises from recent studies on Treg cells in HIV-infected patients. Immunosuppressive $CD4^+$ T cells, which express the common Treg marker CD25, and secrete immunosuppressive cytokines IL-10 and TGF-β have been shown to inhibit both $CD4^+$ and $CD8^+$ HIV-specific T cell responses. In general, IFNγ production and T cell proliferation were affected by Treg cells in HIV-infected patients. In addition, the cytotoxic activity of virus-specific $CD8^+$ T cells was shown to be suppressed by Treg cells (Kinter et al., 2004). Recent results in HIV-positive patients and SIV-infected macaques showed that the accumulation of Treg in lymphoid tissues was associated with high HIV virus loads and reduced CTL activity (Andersson et al., 2005; Estes et al., 2006). It is assumed that viral replication and effector T cell responses may promote Treg compartmentalization into lymphoid organs. The question remains whether natural Treg cells or a population of induced Treg cells, play the major role in HIV pathogenesis and chronic infection. The accumulation of Treg cells in HIV-infected lymph nodes argues for an induction, or at least expansion, of these cells during infection. Furthermore, immunomodulatory proteins, such as gp120, *pol*, and *gag* from HIV stimulate IL-10 or TGF-β production from macrophages and DC, which in addition to directly suppressing Th1 cells also provide the appropriate cytokine milieu for the differentiation of T_R1 or T_H3 cells (Mills and McGuirk, 2004). During FIV infection in cats, there is increased activation of $CD4^+CD25^+CTLA4^+$ Treg cells that inhibit IL-2 production by $CD4^+CD25^-$T cells from normal cats. Furthermore, ablation of IL-10 secreting Treg cells in mice prevented the progression of murine AIDS (Nathanson, 2001). The phenotype, the mechanism of induction or expansion, and the interaction of Treg cells with effector T cells during retroviral infection need to be determined to develop therapeutic means for intervention. Demonstration of induced Treg cells that respond to the pathogen or pathogen-derived antigens is summarized in Table 1.3 and response of Natural Treg cells to pathogen is summarized in Table 1.4.

Table 1.3: Pathogen-induced regulatory T cells and their role in virus infections (Mills, 2004)

Virus	Cell type	Antigen specific	Cytokine secreted	Responses suppressed	Manipulation of Tregs	Effect on immune response, immunopathology, and pathogen load
Friend virus	Mouse T_R1 cell	ND	IL-10	IFN-γ production by $CD8^+$T cells	Depletion with GITR-specific antibody	Increases IFN-γ secreting $CD8^+$T cells and reduces viral load (Dittmer et al., 2004)
Friend virus	Mouse T_R1 cell	ND	ND	granzyme A &B, perforin production & expression of CD107a by $CD8^+$T cells	Adoptive transfer of Treg cells	Decreases production of granzymes and reduce expression of CD107a by effector $CD8^+$T cells and increases viral load (Zelinskyy et al., 2006)
Murine leukemia virus	Mouse T_R1 $CD4^+CD25^+$ T cell	ND	IL-10	ND	$CD25^+$ T cell depletion	Prevents spleen pathology and disease progression but has no effect on viral load (Beilharz et al., 2004)
HCV	Human T_R1 cell	Yes	IL-10	PBMC IFN-γ production	ND	ND (Brady et al., 2003; MacDonald et al., 2002)
HCV	Human $CD8^+$ T cell	Yes	IL-10	Antigen-specific PBMC proliferation	ND	ND (Accapezzato et al., 2004)
EBV	Human T_R1 cell	Yes	IL-10	T-cell proliferation and IFN-γ production to recall antigen	ND	ND (Marshall, Vickers, and Barker, 2003)
HIV	Human $CD8^+$ T cell	Yes	TGF-β	Vaccinia virus specific $CD8^+$T cell IFN-γ production	ND	ND (Garba et al., 2002)

CD107a – degranulation marker for cytotoxic $CD8^+$T cells; EBV - Epstein–Barr virus; GITR - glucocorticoid-induced tumour-necrosis factor receptor-related protein; HCV - hepatitis C virus; IFN-γ - interferon-γ; IL-10 - interleukin-10; ND, not determined; PBMC - peripheral-blood mononuclear cell; TGF-β - transforming growth factor-β; Th, - T helper; TLR4 - Toll-like receptor 4; T_R1 - T regulatory 1.

Table 1.4: Natural $CD4^+CD25^+$ Treg cells and their role in virus infections (Mills, 2004)

Virus	Species	Antigen specific	Cytokine secreted	Responses suppressed	Manipulation of Tregs	Effect on immune response, immunopathology, and pathogen load
HSV	Mouse	ND	IL-10	Antigen-specific $CD4^+$ T cell IFN-γ production	*In vivo* depletion	Increases Th1 cell responses, $CD4^+$ T cell infiltration and stromal keratitis (Suvas et al., 2004)
HIV	Human	ND	ND	Antigen-specific $CD4^+$ T and $CD8^+$ T cell proliferation and cytokine production	*In vitro* depletion	Increases HIV-specific $CD8^+$T cell IFN-γ production (Aandahl et al., 2004; Kinter et al., 2004)

1.5 Friend virus complex as a model to study immunity to Retroviruses

One drawback in the ongoing research for HIV vaccines is the unavailability of good animal models. The only species that can reproducibly be infected with HIV-1 apart from humans are chimpanzees. However, the replication rate is really slow in these animals and it usually takes almost a decade to cause disease at least in some monkeys. Add to that, chimpanzee models have been very expensive to work with and many ethical concerns hamper large scale HIV vaccine experiments. An alternative to this model are macaques, which could be infected with *Simian Immunodeficiency virus* (SIV) or chimeric SIV/HIV viruses known as SHIVs. Since, both HIV and SIV belong to the same family of primate Lentivirus, using macaques have been the most crucial and good model for HIV vaccine research. However, there are a wide range of limitations in the approaches towards understanding the protective immunological mechanisms in retroviral infections. There are limited number of monkeys, limited knowledge about molecular immunology of monkeys, and a lack of certain immunological tools for studying and manipulating the macaque immune system. Mouse models have always been advantage over humans or other animal species in terms of elucidating fundamental concepts in immunology. This is because mouse models are easier to

handle, different immunological manipulations can be tried and mice with different genetic backgrounds including congenic, transgenic and knockout mice can be studied. The critical drawback is that both HIV and SIV cannot infect mice and hence other oncogenic murine retroviruses have to be considered. Again, a large number of the known oncogenic murine retroviruses only induce disease in newborn and only very few of them can induce a lethal or semi-lethal disease in adult immunocompetent mice. Friend retrovirus is one such murine retrovirus and has been a useful model since past 25 years for studying the basic mechanisms of immunological control and escape in both acute and persistent retroviral infections (Dittmer and Hasenkrug, 2001).

1.5.1 Friend virus – induced disease

Friend viral erythroleukemia was discovered in 1956 by Charlotte Friend and has since become the model for understanding host genetic barriers to retroviral diseases. It has helped in identifying mouse genes that control susceptibility to virus-induced cancer and also been a good model for testing anti-retroviral drugs. Unlike other retroviruses that cause rapid-onset neoplasms Friend virus lack oncogenes and contains only retroviral-specific nucleic acid sequences. Friend Virus is a complex of two viruses: Friend murine leukemia virus (F-MuLV), a replication competent helper virus that is nonpathogenic in adult mice; and spleen focus-forming virus (SFFV), a replication-defective virus that is the pathogenic component (Kabat, 1989). SFFV cannot produce its own particles so it spreads by being packaged in F-MuLV-encoded particles produced in cells co-infected by both viruses. SFFV unlike other replication defective retroviruses does not contain a viral oncogene but encodes a truncated form of the viral envelope glycoprotein, gp55 which associates specifically with the erythropoietin receptors (EpoR) at the cell surface of erythroid precursor cells (Li et al., 1990). Binding of gp55 to EpoR leads to an increased replication of the erythroid precursors namely Erythropoietic Burst Formation Unit (BFU-E) and Colony forming unit-Erythroid (CFU-E). Erythroblastosis occurs within 48 hrs after infecting susceptible strains of mice with FV and the erythroblasts migrate from the bone marrow to the spleen. Following this is the site-specific integration of SFFV DNA near the Spi1 gene locus, which is an ets family transcriptional activator. The integration leads to activation of Spi1 gene and loss of tumor suppressor p53 (Kabat, 1989) (Fig 1.9). By 9 days post infection, erythropoietic foci cover the spleen which enlarges from 0.09 g to 2 - 4 g and transplantable erythroleukemia cells are produced as early as 15-20 days post infection (Ney and D'Andrea, 2000). Resistant strains of

mice are those that mount immune responses with sufficient speed and potency to prevent FV-induced leukemia (Hasenkrug and Dittmer, 2000). However, even the most resistant strains of mice are never able to completely clear virus-infected cells and develop a life-long chronic infection. All experiments described in this study will be performed with resistant mice, which do not develop acute leukemia but become chronically infected for life.

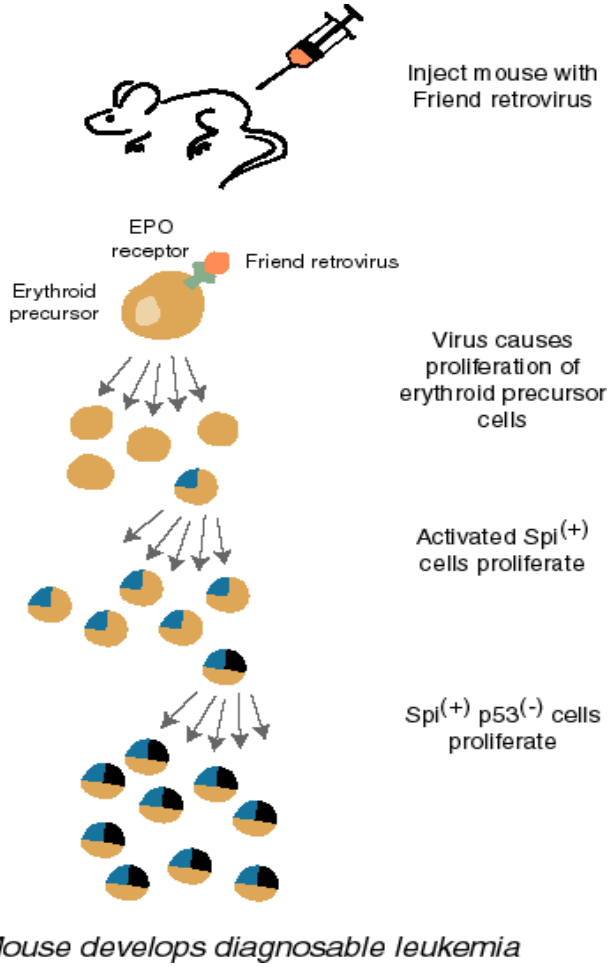


Figure 1.9: Friend Virus-Induced Erythroleukemia in a Model Organism
(http://www.fhcrc.org)

1.5.1.1 Factors influencing susceptibility to FV-induced disease

Susceptibility to FV-induced splenomegaly is genetically determined and controlled by various host genes that are important in disease induction and progression. Host genes such as Fv-2, Fv-5, Kit, Mgf, affect the potential of infected target cells to proliferate and differentiate. Fv5 determines whether FV will cause anemia or polycythemia and mutations in certain strains of mice (Shibuya and Mak, 1982). Mutations in Kit, Mgf genes can affect

normal erythropoiesis of cells (Bennett et al., 1968; Steeves et al., 1968). Fv-2 is an important gene locus that determines the proliferation of SFFV-infected erythroblasts since it influences the ability of gp55 to interact with and stimulate the erythropoietin receptor (Lilly, 1970). Fv-2 does not interfere with retroviral entry into cells or the retroviral life cycle (Behringer and Dewey, 1985). Susceptibility at the Fv-2 locus (Fv-$2^{s/s}$) develops lethal erythroleukemia in most cases. In contrast, Fv-2-resistant (Fv-$2^{r/r}$) mice such as C57BL/6 and other strains are protected from the FV-induced disease (Ney and D'Andrea, 2000).

Fv-2 encodes a truncated form of Stk (SF-Stk), a member of the Met subfamily of receptor tyrosine kinases (Persons et al., 1999). Stk is closely related to RON (its human homologue), avian v-sea and mouse Met (Smith, Vogt, and Hayman, 1989). The susceptible phenotype, Fv-$2^{s/s}$ have increased expression of SF-Stk. Stk is the receptor for macrophage stimulating factor (MSP), which can induce migration of peritoneal macrophages and other cell types. EpoR is a member of a large family of cytokine receptors while Stk is a member of a large family of tyrosine kinase which may have general role as modifiers of cytokine receptor signaling. SF-stk is speculated to increase the affinity of EpoR-gp55 interaction or increase the expression of cell surface EpoR, or acts as a co-receptor that activates additional downstream signaling events that are required for erythroblast transformation. In addition, activation of Spi1 can in turn activate SF-Stk through Ets sites in the SF-Stk promoter, resulting in progression of FV-susceptible mice towards erythroleukemia as reviewed in (Ney and D'Andrea, 2000).

1.5.1.2 Factors influencing recovery from FV-induced splenomegaly

Recovery from Friend virus disease depends upon an array of genes mapped to the major histocompatibility complex (MHC) H-2 region of the mice. The gene locus, which influences the resistance of adult mice to FV-induced disease, corresponds to an allele of the H-2D structural gene which encodes a class I product and hence can influence the effectiveness of the CTL response against FV (Chesebro, Miyazawa, and Britt, 1990). H-2D region displays a gene-dose effect whereby H-$2D^{b/b}$ mice show the highest recovery incidence, H-$2D^{b/d}$ mice are intermediate, and H-$2D^{d/d}$ mice are lowest. Such a gene-dose effect is probably dependent upon the expression levels of D^b class I molecules used to present viral peptides to CTLs. H-2A is a class II MHC gene that also help in better recovery from FV-induced disease. For H-2A, high recovery is associated with the H-2^b haplotype with a dominant effect (Chesebro,

Miyazawa, and Britt, 1990). The H-2A$^{b/b}$ genotype or the H-2A$^{b/k}$ genotype promotes CD4^{+} T-cell mediated isotype switching of FV-specific antibodies and improves responsiveness to vaccinia/FV envelope vaccination. While the low recovery genotypes, such as H-2A$^{k/k}$ fail to mount T cell proliferative responses after FV infection and hence have low incidence of recovery (Ney and D'Andrea, 2000). H-2E, another class II MHC gene's effect on immunity to FV is more complex as it has both positive and negative effects on protection from FV induced disease. H-2E molecules are not expressed on the cell surface in H-2^{b} mice because of lack of a functional E α gene. However, a functional α gene can be introduced by breeding with mice carrying another haplotype such as H-2^{a}. H-2$^{a/b}$ heterozygous mice have H-2E α chains from the H-2^{k} haplotype which associates with β chains from the H-2^{b} haplotype to form functional cell surface heterodimers that present F-MuLV envelope peptides to CD4^{+} T cells.

In addition to the four MHC genes there is also one non MHC gene, recovery from Friend virus 3 (Rfv-3) gene locus. Rfv-3 is a single autosomal gene encoding a resistance trait that influences retroviral neutralizing antibody responses and viremia (Chesebro and Wehrly, 1976; Chesebro and Wehrly, 1979; Doig and Chesebro, 1979). Rfv-3 is encoded by Apobec3, which maps to the same chromosome region as Rfv-3 and has broad inhibitory activity via induction of neutralizing antibodies against retroviruses, including HIV (Santiago et al., 2008). Furthermore, there are also certain other host genes such as Fv-1, Fv-4 that interfere with the infection of target cells by the retrovirus. Fv-1 encodes a *gag*-related protein and interferes with the retroviral life cycle (Best et al., 1996), while, Fv-4 encodes a protein related to the MuLV *env* protein, which blocks ecotropic retroviral receptors (Ikeda et al., 1985). Hence, to sum up, recovery from FV-induced leukemia is associated with an H-2^{b} haplotype, while mice of an H-2^{a} or H-2^{d} haplotype, such as BALB/c, respectively readily succumb to the disease.

1.5.2 Immunity to FV infection

1.5.2.1 Role of cytotoxic CD8^{+} T cells in FV immunity

Recovery from FV infection requires immunological help from all the effector arms of the immune system, including antibodies, CD4^{+} T cells and CD8^{+} T cells. Similar to HIV infections, virus titer peak quite rapidly around 7 days post infection in the acute phase then

drop off rapidly as immune responses develop and gain control over the infection. $CD8^+$ T cells are critical for recovery from the acute phase of FV infection (Hasenkrug, Brooks, and Chesebro, 1995; Robertson et al., 1992) and even highly resistant mouse strains such as C57BL/6 fail to control acute infections in the absence of a $CD8^+$ T cell response (Hasenkrug, 1999). The major viral protein recognized by CTLs *in vivo* is the F-MuLV *env* protein (Collins, Britt, and Chesebro, 1980). In addition, secondary CTL generated *in vitro* indicate that many FV-specific CTL were directed against the *gag* polyprotein of F-MuLV (Holt, Osorio, and Lilly, 1986; Klarnet et al., 1989). $CD8^+$ CTL responses correlate with reduction of splenomegaly in FV-infected mice and the protection is mediated by IFN-γ, Fas-FasL and cytotoxic molecules - perforin, granzymes A and B which eliminate virus-infected cells by apoptosis of target cells. In FV infection, the requirement for one or more of these molecules is dependent upon the time point of infection. During acute phase of infection, viral replication is controlled by the granule exocytose pathway mediated by a concerted action of perforin and granzyme A and B but all these three molecules can independently reduce viral loads. However, when all three molecules are completely absent, the mice have high viral loads and develop FV-induced erythroleukemia. On the contrary, Fas/FasL's role is dispensable for the control of early FV replication, however, during persistent infections, Fas/FasL is crucial even for the high recovery mice (Zelinskyy et al., 2004). Despite recovery from acute viremia due to CTL responses, virus is never completely cleared in high recovery mice and they remain persistently infected at low levels for life. Quite in contrast to acute FV infection, $CD8^+$ T cells do not seem to have an impact on protection from chronic infection as depletion of $CD8^+$ T cells in persistently infected mice has no effect on virus levels (Hasenkrug, Brooks, and Dittmer, 1998). Although, FV-specific $CD8^+$ T cells expressing activation markers are detected in persistently infected mice, they are functionally impaired in their ability to produce lytic granules- perforin, granzymes A and B and also have reduced secretion of IFN-γ. The down-regulation of granzymes B is at the transcriptional level while the regulation of both perforin and granzymes A are at posttranscriptional levels indicating that complex regulatory mechanisms are involved in down-regulation of these effector molecules (Zelinskyy et al., 2005).

1.5.2.2 Role of immunosuppressive Regulatory T cells in FV immunity

The $CD8^+$ T cells critically important during acute FV infection are rendered functionless during chronic FV infection. One explanation of this transition is that some form of

immunological escape has occurred. It is known from other infection models that escape from $CD8^+$ T cells maybe mediated by poor recognition of infected cells. However, virus-specific $CD8^+$ T cells from FV-specific CD8 TCR transgenic mice when transferred into persistently infected mice can efficiently recognise and get activated to expand clonally in response to the FV antigen. However, these transferred FV-specific $CD8^+$ T cells were only able to reduce viral loads in acutely infected mice and not persistently infected mice even though they are activated and proliferative. These $CD8^+$ T cells were functionally impaired as their ability to produce IFN-γ is markedly decreased in chronically infected mice. This clearly indicates suppression of $CD8^+$ T cell immunity. The immunosuppressive action is brought about by Treg cells as when mice receive both FV-specific donor $CD8^+$ T cells and $CD4^+$ T cells from persistently infected mice, they have diminished population of IFN-γ-producing $CD8^+$ T cells and increased population of host $CD4^+$ T cells producing IL-10 (Dittmer et al., 2004). The glucocorticoid-induced tumour necrosis factor receptor (GITR) is associated with Treg cell functions (Shimizu et al., 2002). Immunosuppressive action by IL-10 secreting $CD4^+$ T cells is reversed when host mice receive anti-GITR antibody along with FV-specific donor $CD8^+$ T cells, and this helps to significantly decrease spleen virus levels and enhance production of IFN-γ and TNF-α by $CD8^+$ T cells. Hence, in the FV model, the virus utilizes Treg cell system to escape $CD8^+$ T cell responses to cause persistent infections in the host (Dittmer et al., 2004). The deterioration in the function of effector $CD8^+$ T cells begins early during acute infection. The activation of effector $CD8^+$ T cells is highest during the first two weeks of FV infection with increased production of granzymes and CD107a, a surrogate marker for degranulating $CD8^+$ T cells. Subsequently, the percentage of activated $CD8^+$ T cells starts to decline and also its ability to produce granzymes and CD107a. This $CD8^+$ T cell dysfunction begins at the same time point as Treg cells (described by the increased expression of CD25, GITR, CD103, Foxp3) expanded, suggesting that early development of $CD8^+$ T cells dysfunction correlates with peak expansion of Treg cells that likely contributes towards viral persistence (Zelinskyy et al., 2006).

1.5.2.3 Role of B cells in FV immunity

Virus-neutralizing antibodies are essential for recovery from FV infection and their production is influenced by a non-MHC gene, Rfv-3. Low recovery mice ($H\text{-}2^{a/b}$ or $H\text{-}2^{a/a}$) with $Rfv\text{-}3^{r/s}$ genotype recovers from viremia via production of virus-specific antibodies that neutralise virus and lyse virus-infected cells in the presence of complement (Chesebro and

Wehrly, 1976; Chesebro and Wehrly, 1979; Doig and Chesebro, 1979). Additionally, low-recovery mice recessive in Rfv-3 gene (Rfv-$3^{s/s}$) have suppressed FV-specific antibody responses such as BALB/c, A/WySn and A.BY mice, fail to produce anti-FV antibodies, and remain viremic until death (Chesebro and Wehrly, 1976; Doig and Chesebro, 1979). However, in another study, near normal responses were observed in mice having the H-$2^{a/b}$ or H-$2^{b/b}$ genotype, whereas mice having the H-$2^{a/a}$ genotype were suppressed. This H-2 effect was observed not only in mice having heterozygous C57BL/10 × A background genes, including Rfv-$3^{r/s}$, but also was apparent in mice having homozygous A-strain background genes, including Rfv-$3^{s/s}$. The suppression in susceptible H-$2^{a/a}$ mice was characterized by a partial suppression of the IgM response and a profound suppression of both the primary and secondary IgG responses. Since, H-$2^{a/a}$ mice are unable to develop potent effector T cell responses and therefore, suppression in antibody production was probably due to the lack of additional T-cell help for the class switch from IgM to IgG antibodies (Morrison, Nishio, and Chesebro, 1986).

1.5.2.4 Vaccine - induced protection against FV

Protection from FV infection can be elicited by several different types of vaccines including killed and attenuated viruses, viral proteins, peptides and recombinant vaccinia vectors expressing FV genes. Recombinant vaccinia viruses using different combinations of FV epitopes, show that protective epitopes are localized to F-MuLV *gag* and *env* proteins, although F-MuLV *env* protects against infection better than *gag* proteins (Earl et al., 1986; Miyazawa, Nishio, and Chesebro, 1992). The gp70 *env* protein contains at least one CTL epitope, three Th epitopes, and two neutralizing antibody epitopes (Hasenkrug and Chesebro, 1997). In mice immunized with the envelope vaccine, recovery from challenge with FV is due to induction of neutralizing antibody (IgG) and virus-specific T cell responses, thereby defining a protective vaccine to stimulate both $CD4^+$ and $CD8^+$ T cell subsets and B cells (Earl et al., 1986; Morrison et al., 1987). MHC background of the mice used for immunization plays an important role to determine the efficacy of a vaccine. Only mice that express MHC class-II alleles such as H-$2A^b$ can be protected when immunized with vaccinia virus recombinants expressing F-MuLV *env* protein. MHC class-II molecules are involved in antigen presentation to $CD4^+$ T cells, thereby establishing importance of $CD4^+$ T cells in protective immunization (Miyazawa, Nishio, and Chesebro, 1988; Morrison et al., 1987). Immunization with chimeric envelope vaccines (vvCh1 vaccine) containing only part of *env*

protein gp70 with Th cell and B cell epitopes was enough to mediate protection via $CD8^+$ T cells and $CD4^+$ T cells. However, when the full-length Friend envelope protein (vvFr57) was used, protection was independent of $CD8^+$ T cells but required $CD4^+$ T cells for rapid production of virus-neutralizing antibodies which limited the viral spread in the immunized mice (Hasenkrug et al., 1996). Recombinant vaccinia viruses protect mice against acute FV disease but not against establishment of persistent infection. Live-attenuated retroviruses containing attenuated N-tropic F-MuLV vaccine virus can elicit broad lymphocyte responses and efficient B and T cell memory and can induce complete protection against FV-induced splenomegaly, plasma viremia and persistent spleen infection (Dittmer, Brooks, and Hasenkrug, 1999a; Dittmer, Race, and Hasenkrug, 1999; Earl et al., 1986). Moreover, spleen cells from immunized mice when adoptively transferred into naive mice demonstrate that B cells and both $CD4^+$ T cells and $CD8^+$ T cell subsets are required to transfer protection against acute viremia, splenomegaly and persistent infection. Immune $CD8^+$ T cells alone were not protective but could reduce viremia and facilitate recovery from FV-induced splenomegaly in the recipient mice. Again, CD4-depletion in recipient mice prior to $CD8^+$ T cell transfer abolished the protection and this suggests the requirement of endogenous $CD4^+$ T cells for $CD8^+$ T cell – mediated protection even in the presence of transferred $CD8^+$ T cells and neutralising antibodies (Dittmer, Brooks, and Hasenkrug, 1999b; Dittmer and Hasenkrug, 2000). In summary, from vaccination studies it is obvious that Th priming is as critical for retrovirus immunity as CTL priming.

1.5.2.5 Role of Helper T cells in FV immunity

The $CD4^+$ T cell responses is specific for determinants in the F-MuLV *env* protein and two helper epitopes from the gp70 protein of envelope are targeted by Th cells. One peptide binds to $H\text{-}2A^b$ molecules and the other to H-2E molecules, thus providing ligands for recognition by $CD4^+$ T cells (Iwashiro et al., 1993; Miyazawa, Nishio, and Chesebro, 1988; Shimizu et al., 1994). In addition, a Th epitope has also been identified in the p15 (MA) region of F-MuLV *gag*. *Gag*-specific $CD4^+$ T cells are effective in controlling retrovirus infections and hence are potential targets for the development of effective anti-retrovirus vaccines (Sugahara, Tsuji-Kawahara, and Miyazawa, 2004). $CD4^+$ T cells play very critical role in controlling FV replication and spread in persistently infected mice as depletion of $CD4^+$ T cell subsets in these mice show a marked increase in splenomegaly. Viral loads in spleens and blood was much higher in CD4-depleted relapsed mice compared to non-depleted mice chronically infected with FV. The $CD4^+$ T cells control viral replication during persistent

infections independently of $CD8^+$ T cells and virus-neutralising antibody response (Hasenkrug, Brooks, and Dittmer, 1998). The mechanism of $CD4^+$ T-cell mediated anti-viral effect during persistent FV infection is via production of IFN-γ. Friend virus-specific $CD4^+$ T-cell clone revealed that these cells produce IFN-γ *in vitro*, which acts with two distinct mechanisms of antiviral activity. $CD4^+$ T cells can lyse infected target cells, and they can also suppress virus replication by production of IFN-γ. *In vivo*, neutralization of IFN-γ using monoclonal antibodies increased the levels of virus in persistently infected mice and a significant percentage of IFN-gamma-deficient mice were unable to maintain long-term control over Friend virus infections. But, in contrast to $CD4^+$ T-cell depletions, this was not sufficient to induce a relapse of splenomegaly in a significant proportion of the mice (Iwashiro et al., 2001). In addition, knockout mice deficient in $CD4^+$ T cells have an 80% incidence of late-onset splenomegaly (Hasenkrug, 1999) while IFN-g-deficient mice have only a 32% incidence (Iwashiro et al., 2001). Thus, it appears that $CD4^+$ T-cell mediated mechanisms in addition to IFN-γ help to control persistent FV infection.

In summary, FV infection model is a comprehensive model to study very early events in retrovirus infections and also aid to understand the differences in immune responses in genetically diverse animals. A more defined role for $CD8^+$ T cells in recovery from acute FV infection and suppression of its effector functions under the influence of Treg cells have been characterised. Furthermore, $CD4^+$ T cells are known to be important in recovery from persistent FV infections. Additionally, $CD8^+$ T cell, $CD4^+$ T cell and B cell - mediated immune responses are critical for vaccine induced protection against persistent FV infection. Treg cells are responsible for dysfunction of $CD8^+$ T cells in persistent FV infection. It will be important to answer the question of how important the $CD4^+$ T cell response is for the control of acute virus replication and for the resistance of certain mouse strains to virus–induced disease, which will be unravelled in the current study.

2. AIM & SCOPE OF WORK

The ability to evade the host's immune system and establish chronic infection is a feature of many human viruses such as retroviruses, hepatitis viruses and herpes viruses. Chronic viral infections lead to diseases such as AIDS, liver cancer, and severe complications in immunocompromised transplantation and cancer patients. Unfortunately, there is no mouse model available to study immune responses against HIV infections. Hence, we have used a murine retrovirus, namely Friend virus (FV) as a model to study basic mechanisms of immunological control and escape during acute retroviral infection. The role of virus-specific cytotoxic T cells (CTLs) is very well-characterised in the immune control of viral infections. However, the role of helper T cells (Th) in retroviral immunity is not well understood. The FV mouse model was used in the current study to characterise the anti-retroviral effector functions of Th cells. FV is a retroviral complex that induces lethal erythroleukemia in most strains of mice. Recovery from acute infection with FV is dependent upon perforin and granzyme-mediated cytotoxic killing of FV-infected cells by CD8-positive Cytotoxic T lymphocytes ($CD8^+$CTLs). On the other hand, Th cells are known to be critical in controlling FV replication and spread in persistently infected mice. However, prior to this study there was no concrete information about the requirement of Th cells during recovery from acute retroviral infections.

(C57BL/10 x A.BY) F1 mice are high recovery mice as they are able to develop potent immune responses against acute FV infection and do not develop lethal leukemia. However, they are never able to completely clear the virus and remain persistently infected for life. High recovery mice will be depleted of $CD4^+$ T cells and infected with FV to study the effect of CD4 T cell depletion on viral replication and disease progression during an acute infection. $CD4^+$ T cells are known to be important for mediating help for CD8 and B cell effector responses in various infectious diseases but significance of CD4 T cell help in acute retroviral infections remain elusive. For this purpose, FV-infected mice depleted of $CD4^+$ T cells will be investigated to determine whether $CD4^+$T cells can influence memory CD8 T cell responses and neutralising antibody production during acute FV infection. In humans, variations at immune response loci (HLA class I and II gene) determine the clinical course and outcome of HIV-1, HCV and HBV infections. Recovery from FV infections is also known to be dependent on the MHC background of mice. However, the reason for its differential induction of immune responses in these mice has not been investigated as yet.

MHC class-II tetramer technology will be used to characterize and compare the kinetics of $CD4^+$ T cell responses against acute FV infection in high and intermediate recovery mice. Importantly, adoptive transfer experiments showed that FV-specific $CD8^+$ T cells with an effector phenotype lose their protective function during chronic FV infection under the suppressive effect of Regulatory T (Treg) cells. We want to utilize the CD4 T-cell receptor (TCR) transgenic (tg) mouse model in which the CD4 TCR is specific for FV-envelope gene to elucidate the role of virus-specific $CD4^+$ T cell populations in antiviral immunity *in vivo*. Since, Treg cells induce CD8 dysfunction during acute FV infection; we envisage a possible immunosuppressive effect of Treg cells on $CD4^+$ T cells. For this purpose, we want to use a transgenic mouse model (DEREG) in which Treg cells can be specifically depleted and in this manner, we can analyse the effect of Treg cells on effector responses of FV-specific Th cells.

Thus, our studies would help in widening our horizons on the existing knowledge on FV immunity by exploring the importance of Th cells during an acute retroviral infection and also looking at the influence of Treg cells on suppressing effector function of Th cells *in vivo*.

3. MATERIALS

3.1. Laboratory animals

3.1.1 Wild-type mice

C57BL/6 (B6) : Resistance genotype (H-$2^{b/b}$, $Fv1^{b/b}$, $Fv2^{r/r}$, $Rfv3^{r/r}$)
Harlan Winkelmann GmbH, Borchen, Germany

(C57BL/10 x A.BY)F1 (Y10) : Resistance genotype (H-$2^{b/b}$, $Fv1^{b/b}$, $Fv2^{r/s}$, $Rfv3^{r/s}$), Inbred at Central Animal Labor, University Hospital Essen, Germany

(C57BL/10.A x A.BY)F1 (Y10.A) : Resistance genotype (H-$2^{b/b}$, $Fv1^{b/b}$, $Fv2^{r/s}$, $Rfv3^{r/s}$), Inbred at Central Animal Labor, University Hospital Essen, Germany

3.1.2. Congenic mice

CD45.1-congenic B6 : Genotype (B6.SJL-$Ptprc^{a}$ $Pep3^{b}$/BoyJ)
Inbred at Central Animal Labor, University Hospital Essen, Germany

3.1.3. Transgenic mice

CD4 TCRβ-transgenic (tg) mice: Created by Dr. George Kassiotis's group (Division of Immunoregulation, National Institute for Medical Research, London, United Kingdom) and were subsequently maintained at animal facilities of University Hospital Essen.

CD4 TCRβ-tg mice (Antunes et al., 2008) transgenically express the TCRβ chain of a TCR specific to an MHC class II-presented epitope of the surface (SU) product of the F-MuLV envelope (env) gene (Iwashiro et al., 1993). The transgenic TCRβ chain is able to associate with endogenous TCRα chains creating a polyclonal repertoire. 4% of peripheral $CD4^{+}$ T

cells in CD4 TCRβ-tg mice are reactive to F-MuLV env protein. CD4 TCRβ-tg mice were crossed with CD45.1-congenic B6 mice for adoptive transfer experiments.

DEREG mice: Created by Dr. Tim Sparwasser's group (Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Technische Universität München, Munich, Germany) and maintained at animal facilities of University Hospital Essen.
DEREG (depletion of regulatory T cell) mice were generated from bacterial artificial chromosome (BAC) technology. These mice express a diptheria toxin receptor (DTR) enhanced green fluorescent protein (eGFP) fusion protein under the control of the *foxp3* locus. Usage of DEREG mice allows both detection and inducible depletion of $Foxp3^{+}$ Treg cells. DEREG.Y10 mice were generated from crossing of DEREG mice with ($H\text{-}2^{b/b}$, $Fv1^{b/b}$ und $Fv2^{s/s}$ $2^{s/bs}$) (A.BY) F1 mice.

All mice used were sex-matched and were 8-16 weeks of age at the beginning of experiments. The central animal laboratory kept the mice under special pathogen-free (SPF) conditions which were maintained for the entire experimental phase. The mice had free access to drinking water and standard food. The litters of the (CD45.1-congenic B6 x CD4 TCRβ-tg mice) and DEREG.Y10 mice were genotyped for the expression of transgenes in 6-weeks old mice. DEREG.Y10 mice used for the experiments were essentially males.

3.2. Virus

The FV stock used for the experiments was a FV complex containing B-tropic Friend Murine Leukemia Helper Virus (F-MuLV) and polycythemia-inducing spleen focus-forming virus (SFFV) (Chesebro, Miyazawa, and Britt, 1990).

3.3. Cell lines

Mus dunni cell line is susceptible to infection with FV and was used to determine the productivity of virus infected cells *in vitro*.

3.4. Equipment and materials

The equipment and materials used in this study are listed in Tables 3.1 and 3.2 below.

Table 3.1 Equipment

Item	***Manufacturer***
Reflected-light microscope CK 2	*Hund, Wetzlar*
Biofuge fresco	*Heraeus, München*
Centrifuge 5415 C	*Eppendorf, Hamburg*
CO_2 incubator	*Thermo, Dreieich*
Single channel pipettes (10, 20, 100, 200, 1000 µl)	*Eppendorf, Hamburg*
FACS calibur flow cytometer	*Becton Dickinson, Heidelberg*
Freezer	*LIEBHERR, Ochsenhausen*
Heating block	*Grant, QBC*
Refrigerator	*LIEBHERR, Ochsenhausen*
LSRII flow cytometer	*Becton Dickinson, Heidelberg*
Megafuge 1.0R	*Heraeus, München*
Sorvall centrifuge fresco	*Thermo, Dreieich*
Laminar flow	*KOJAIR®, Meckenheim*
Infrared lamps	*Phillips, Amsterdam*
Neubauer cell counting chamber	*Becton Dickinson, Heidelberg*

Table 3.2 Materials

Material	***Manufacturer***
Beakers	*Schott, Mainz*
Disposable syringes (5 ml; 10 ml)	*B. Braun, Melsungen*
Erlenmeyer flasks	*Schott, Mainz*
FACS tubes	*Becton Dickinson, Heidelberg*
Cannulae (G23; G25; G27)	*Becton Dickinson, Heidelberg*
Microtest™ cell culture plates, 96 wells	*Falcon BD, Heidelberg*

Material	***Manufacturer***
U-shaped microplates (96 wells)	*Greiner bio-one, Frickenhausen*
Parafilm	*American National Can, Chicago*
Forceps, pointed and curved	*Oehmen, Essen*
Plastic pipettes (sterile; 1 ml; 5 ml; 10 ml; 25 ml)	*Greiner bio-one, Frickenhausen*
PP screw-cap tubes (15 ml; 50 ml)	*Greiner bio-one, Frickenhausen*
Reaction tubes (1,5 ml; 2 ml)	*Eppendorf, Hamburg*
Scissors, large and small	*Oehmen, Essen*
Cell culture flasks (T25; T75; T175)	*Greiner bio-one, Frickenhausen*
Cell culture plates, sterile (6; 24 and 96 well)	*Greiner bio-one, Frickenhausen*
Cell microstrainer (70 µm)	*Falcon BD, Heidelberg*

3.5. Chemicals and media

Unless not otherwise annotated, the following chemicals listed were procured from the companies Applichem, Merck, Roth and Sigma.

7AAD (7-Aminoactinomycin) [Molecular Probes], AEC (3-Amino 4-Ethylencarbazole), Acetic acid, Ammonium chloride, Avidin-FITC [Pharmingen], Bouin´s solution, BSA (Bovine serum albumin), Brefeldin A, Chloroform, Cytofix/Cytoperm [BD Pharmingen], N-N-dimethylformamide, DMSO (Dimethyl sulfoxide) DEPC, EDTA, Ethanol, Ether, Ethidium bromide, Formalin (40%), FCS (Fetal calf serum) [Biochrom], Foxp3 staining kit [eBioscience], Glucose, Guinea pig complement serum, Heparin, Hexadimethrine bromide (Polybrene A), Hydrochloric acid, Hydrogen peroxide, Ionomycin [CalBiochem], Isopropanol, LB broth base [USB], Ketamine, β-mercaptoethanol, Methyl-^{3}H thymidine, OPD (o-Phenylenediamin), phenol, Picric acid, PBS [Invitrogen], PMA (Phorbol-12-Myristat 13-Acetate), RPMI 1640 Medium [Gibco], Sodium acetate, Sodium oxide, Sodium Azide, Streptavidin-APC [Pharmingen], Streptavidin-FITC [Pharmingen], Streptavidin-PE [Pharmingen], Streptavidin-PerCP [Pharmingen], sulfuric acid, Tris-(hydroxymethyl)-aminomethane, Trypan blue, Urea.

3.6. Antibiotics

Ampicillin (Sigma)

Penicillin / Streptomycin (Gibco)

3.7. Buffers and Media

All solutions and buffers (unless otherwise noted) were prepared using double distilled water (see Table 3.3).

Table 3.3 Buffers and Media

Description	Composition
AEC working solution	AEC solution was diluted 1:20 with 0.05 M sodium acetate and the reaction was started with 0.5 µl 30 % H_2O_2 / ml solution.
AEC solution (4 mg/ml)	AEC tablets were dissolved in N-N-dimethylformamide
Culture medium	500 ml RPMI 1640 10% FCS 0.5% Penicillin/Streptomycin mixture
Freezing mediums	40% FCS 10% DMSO 50% RPMI medium
PBBS	1 l Phosphate buffered saline (PBS) 1.0 g glucose
MACS buffer	1 l PBS 2mM EDTA 0.5% BSA
FACS buffer	1 l PBS 0.02% Na-azide 0.5% BSA

3.8. Antibodies

Unless otherwise noted, the antibodies (AB) were procured from Becton Dickinson Pharmingen, Heidelberg (see Table 3.4).

Table 3.4 Antibodies for flow cytometry

Antibodies	**Clone**
AK 720; monoclonal mouse anti-MuLV env antibody (isotype IgG1)	
Horse radish peroxidase-coupled goat anti mouse immunoglobulin Dako]	
CD3e Purified NA/LE Hamster anti-mouse antibody	145-2C11
CD4-FITC; monoclonal rat anti-mouse antibody	GK 1.5
CD4-PE; monoclonal rat anti-mouse antibody	H129.19
CD4-PerCP; monoclonal rat anti-mouse antibody	RM4-5
CD4-APC; monoclonal rat anti-mouse antibody	RM4-5
CD4-Biotin; monoclonal rat anti-mouse antibody [eBioscience]	GK 1.5
CD4-AF700; monoclonal rat anti-mouse antibody [eBioscience]	RM4-5
CD8a-FITC; monoclonal hamster anti-mouse antibody [Caltag]	5H10
CD8a-PerCP; monoclonal rat anti-mouse antibody [eBioscience]	53-6.7
CD25-perCP-Cy5.5; monoclonal rat anti-mouse antibody	PC61
CD25-APC; monoclonal rat anti-mouse antibody	PC61
CD28 Purified anti-mouse antibody	37.51
CD43-FITC monoclonal rat anti-mouse antibody	1B11
CD43-PE ; monoclonal rat anti-mouse antibody [eBioscience]	1B11
CD45.1-FITC, monoclonal rat anti-mouse antibody [eBioscience]	A20
CD45.1-APC, monoclonal rat anti-mouse antibody [eBioscience]	A20
CD62L-FITC; monoclonal rat anti-mouse antibody [eBioscience]	MEL-14
CD62L-APC; monoclonal rat anti-mouse antibody [eBioscience]	MEL-14
CD62L-PECy7, monoclonal rat anti-mouse antibody [eBioscience]	MEL-14
CD69-PE; monoclonal hamster anti-mouse antibody [Caltag]	H1.2F3
CD127-FITC; monoclonal rat anti-mouse antibody [eBioscience]	A7R34
CD127-PE; monoclonal rat anti-mouse antibody	SB/199

Antibodies	Clone
Fc block; Affinity purified anti-mouse CD16/CD32	93
Foxp3- FITC; monoclonal rat anti-mouse antibody [eBioscience]	FJK-16S
Foxp3- PE; monoclonal rat anti-mouse antibody [eBioscience]	NRRF30
Foxp3- APC; monoclonal rat anti-mouse antibody [eBioscience]	FJK-16S
IFN-γ Biotin rat anti-mouse antibody [eBioscience]	XMG1.2
Mac-1-FITC; monoclonal rat anti-mouse antibody	WT.5
Mac-1-APC; monoclonal rat anti-mouse antibody	WT.5
Valpha2 TCR-FITC; monoclonal rat anti-mouse antibody [eBioscience]	B20.1
Valpha2 TCR-PE; monoclonal rat anti-mouse antibody [eBioscience]	B20.1

3.9. Fluorochromes

The antibody-coupled fluorochromes and their absorption- and emission maxima are listed in Table 3.5.

Table 3.5 Characteristics of fluorochromes

Flurophore	Abbreviation	Absorption (nm)	Emission (nm)
Fluorescein isothiocyanate	FITC	488	518
Phycoerythrin	PE	488	575
Peridinin-chlorophyll-protein complex	PerCP	488	675
Phycoerythrin – Cy7	PE Cy7	488	785
Allophycocyanin	APC	633	660
Alexa Fluor 700	AF 700	633	723

3.10. Tetramers

PE-labelled MHC class I H2-D^b tetramers were custom-made from Beckman Coulters, INC, U.S.A. The variant peptide (ABU-ABU-L-ABU-LTVFL) loaded on the tetramers is recognised by D^bGagL-specific $CD8^+$ T cells (Stromnes et al., 2002).

PE-labelled (I-Ab) MHC class II tetramers loaded with I-A^b-restricted MoMSV-envelope epitope (H19-Env) peptide-encoding (EPLTSLTPRCNTAWNRLKL) sequence. MHC class-II tetramers were constructed by the Schumacher Laboratory (The Netherlands Cancer Institute, Amsterdam) and were used for the detection of I-A^b FV envelope specific $CD4^+$ T cells (Schepers et al., 2002).

4. METHODS

4.1. Animal trials

The animal experiments were conducted according to the guidelines of the Federation of European Laboratory Animal Science Association.

4.2. Replication of the Friend Virus *in vivo*

The FV stock was prepared as a 10% spleen cell homogenate from BALB/c mice infected 12 days previously with 1500 spleen focus-forming units (SFFU) of uncloned virus stock. The spleen cell homogenate was diluted with PBBS with 2 mM EDTA and stored at -80°C. For the investigation of the viral concentration, Y10.A mice were infected with different dilutions of the spleen homogenates, and after 9 days the spleen was removed. Bouin's solution was used to stain the malignant cell foci on the spleen resulting from the FV infection. The number of these foci yielded the viral concentration in SFFU. The stock was free of contamination from lactate dehydrogenase virus (LDV). For the infection of the mice with FV the viral stock was diluted with sterile PBS. 500 µl with 6000 - 20,000 SFFU were injected intravenously (i.v) using a G25 cannula in a lateral tail vein of the C57BL/6 and DEREG mice.

4.3. *In vivo* depletion of lymphocyte subsets

For depletion of $CD4^+$ T cells, Y10 mice were intraperitoneally injected with 0.5 ml of supernatant fluid obtained from hybridoma cell culture for CD4-specific monoclonal antibody (MAb) YTS 191.1. CD4 depletion was carried out using this antibody at days -6, -4, -2 and 0, 2 and 4 days post FV infection. Depletion of regulatory T cells was carried out in naïve or 3 weeks FV-infected DEREG.Y10 mice by injecting the mice with 1 µg Diphtheria toxin per mouse intraperitoneally on alternate days for 1 week.

The MAb YTS191.1 was of immunoglobulin G2b isotype and was produced and used as ascites fluid or culture supernatant fluid. The hybridoma cell line was a kind gift from Dr. Kim Hasenkrug (Laboratory of Persistent Viral Diseases, Rocky Mountain laboratories, NIAID, Montana, USA), and was stored in liquid Nitrogen chamber for later use.

4.3.1. Culturing of YTS191.1

Principle

CELLMAX® Artificial capillary system (Spectrum Laboratories INC. USA) was used to simulate an *in vivo* microenvironment for the cells to grow rapidly and produce a high amount of the desired monoclonal antibody (mAb) into a small volume of medium. In the CELLMAX, cells were inoculated into the extra capillary space (ECS) of the artificial capillary cartridge and settled onto the outer surface of the capillaries. The cells were subsequently nourished within this network of artificial capillaries, where nutrients and oxygen in the perfusing medium readily diffused through the capillary walls to nourish the cells. Artificial capillary cell culture, therefore, provides a three-dimensional growth environment in which there is cell-cell contact, rapid influx of oxygen and nutrients into the ECS with concomitant diffusion of metabolic waste across the capillary walls where it is diluted into the reservoir bottle. Secreted antibodies and other growth factors are preserved within the ECS thereby maintaining a stable environment similar to *in vivo* physiological conditions.

Protocol

The frozen anti-CD4 MAb stock was thawed and first cultured in Tissue culture (TC) flasks with supplemented RPMI medium. The artificial capillary module was sterilized before inoculating cells. The sterile reservoir cap/bottle assembly was the connected to the artificial capillary module. Subsequently, the cartridge was then connected to the flow path. The capillary system was precultured with complete growth medium (RPMI-1640 + 10% FCS + Pen/Strep). Only rapidly dividing cells with at least 90% viability was used. Cells to be inoculated were split not more than 2 days prior to inoculation. Approximately 5 x 10^7 cells were resuspended in a volume of complete RPMI slightly larger than the ECS volume of the cartridge. The ECS volume in the cartridge was approximately 13 ml while the flow path contained about 35 ml of the medium. The reservoir volume was 500 ml during initial days and later changed to 1000 ml. The total system volume was noted to determine the rates of glucose consumption as a measure of the rate of growth and medium consumption.

Glucose consumption rate calculations

Quantity of glucose consumed in grams = $(V_f) \times (G_f) + (V_r) \times (G_r) - (V_t) \times (G_c)$

Where,

V_f = liters of fresh media at the time of previous glucose reading

V_r = liters of unreplaced media at the time of previous glucose reading

V_t = liters of total amount of media in the reservoir, tubing and the cartridge at the time of the current glucose reading

G_f = glucose concentration (g/l) of fresh media

G_r = glucose concentration (g/l) of media at the time of previous glucose measurement

G_r = glucose concentration (g/l) of fresh media at the present time

The medium was replaced when the glucose concentration reached approximately 50% of the glucose concentration of fresh medium. Hence, fresh RPMI-1640 with a glucose concentration of 2.0 g/l was usually replaced in 24 hrs when its glucose concentration reached 1.0 to 1.5 g/l. The serum concentration in the medium was changed in a step-wise manner at each subsequent replacement of the medium; i.e., 10% to 7.5%, 7.5% to 5%, 5% to 2.5%, until the lower limit of serum reduction being 1% to 2% level. If the daily glucose consumption rate decreased significantly subsequent to a reduction in serum, the serum concentration was increased to the previous level. Antibodies were harvested from the ECS that contained 13 ml of the medium. The cell culturing using the artificial capillary system was stopped when enough volume of the antibody batches were produced.

4.4. Measurement of Friend virus induced splenomegaly

Palpation for splenomegaly has been the standard protocol applied to measure the extent of Friend disease (Chesebro *et al.,* 1974; Earl *et al.,* 1986; Polsky and Lilly, 1991). Each infected mouse was palpated at weekly intervals under general anaesthesia and rated on a scale of 1^+ to 4^+ according to its spleen size (Fig 4.1). Normal (1^+) spleen weights range from 0.1 to 0.25 g. Spleens greater than twice normal size (more than 0.4 g) but not large enough to reach the ventral midline were rated as 2^+. Experienced personnel can easily distinguish such spleens from normal spleens. Spleens large enough to reach the ventral midline were rated as 3^+ (weight between 0.8 and 1.6 g). Spleens that extended across the abdominal

midline, and caused protrusion of the abdominal wall were rated as 4^+ (weight greater than 1.6 g) (Hasenkrug, K.J et al; 1998).

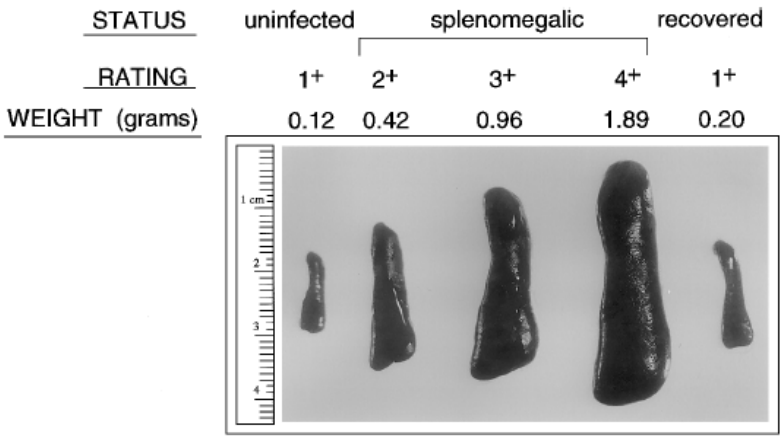


Figure 4.1: FV-induced splenomegaly in (C57BL/10.A x A.BY) F1 adult mice (Hasenkrug, K.J et al; 1998).

4.5. Preparation of single cell suspensions of spleen

For the removal of spleens, required number of mice were anaesthetised with Isofluran® and killed by cervical dislocation. Spleens were collected in 6-well plates kept on ice bath. Each spleen was weighed and the spleen was homogenised using a sterile sieve to make single cell suspension of spleen cells using a sterile plunger of a 5 ml syringe. The coarser components were removed and the single cell suspension was transferred into a 15 ml tube. The spleen suspension was filled up to 10 ml with PBBS due to the high cell count. An aliquot was taken from each suspension to count viable cells by Trypan blue exclusion assay. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable (Fig 4.2). Aliquot was diluted with 0.4% Trypan blue stain and 10 µL of the diluted aliquot solution is transferred onto the Neubauer cell counting chamber and cover-slipped to be examined under microscope (at 10x) for cell counting.

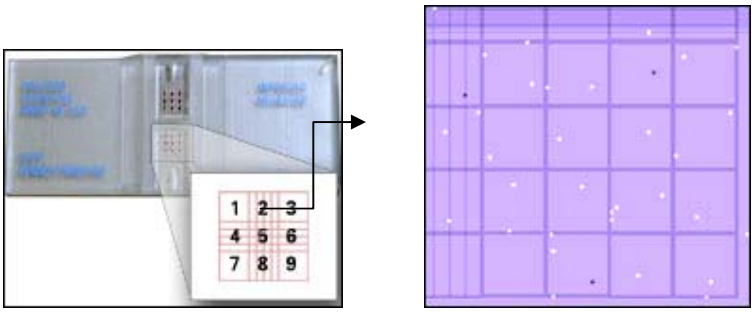


Figure 4.2: Illustration of viable cell counting using Trypan Blue exclusion assay.
Grid with viable cells appear white and dead cells appear blue from the Trypan Blue dye.
(http://www.bme.gatech.edu/vcl/Tissue_Engineering/Background/6_cell_passaging.htm)

Formula for counting number of viable cells for the total cell concentration:

Number of cells/ml = number of cells over a large square x dilution factor x 10^4

Meanwhile, the suspensions in the tubes were centrifuged at 300 x g at 10°C for 10 minutes. The cell pellet was resuspended with either PBBS or RPMI medium to get a desired final concentration of spleen cells to be 1 x 10^8 cells/ml.

4.6. MACS technology for enrichment of CD4 T-cell subset

The Midi MACS Separation system was used for the purification of $CD4^+$ T cells from CD4 TCRβ-tg mice. Using the magnetic activated cell sorting (MACS) technology, spleen cells were labelled with super paramagnetic Micro Beads (Miltenyi Biotec) of CD4 and purified by immunomagentic separation on LS columns.

Principle

MACS® Technology is based on MACS Micro Beads, MACS Separators, and MACS Columns. MACS Micro Beads are super paramagnetic particles of approximately 50 nanometers in diameter. They are composed of a biodegradable matrix, and it is therefore not necessary to remove them from cells after the separation process. Usually, MACS Micro

Beads do not alter structure, function, or activity status of labelled cells and are not known to interfere with subsequent experiments. Separation of cells using MACS technology takes place inside the columns. When a MACS Column is placed in a MACS Separator, a strong permanent magnet, a high-gradient magnetic field is induced on the column matrix which is enough to retain cells labeled with minimal amounts of MACS Micro Beads. Unlabelled cells (negative fraction) are eluted out while labeled cells (positive fraction) bound to the column are released after removal of the column from the magnet. Thus, with MACS Technology both labelled and unlabelled cell fractions can easily be isolated with high purity. The entire procedure of positive selection or depletion takes less than 30 minutes, and cells can immediately be used for further experiments (http://www.miltenyibiotec.com) (Fig 4.3).

Single cell spleen suspensions were prepared and 1 x 10^8 cells/ml were stained with MACS CD4 microbeads and incubated for 15 minutes. The cells were washed with 10 ml of MACS buffer and centrifuged. During this period, LS columns were equilibrated in the magnetic field of the MACS separator. LS columns were washed with MACS buffer and each sample to be enriched for $CD4^+$ T cell population was passed through the magnetic columns. Unspecific cell populations were collected as the negative fractions and $CD4^+$ cell population bound to the column was then taken out of the separator to be collected separately. Positive fraction collected in the tubes is centrifuged and the pellets are resuspended with 1 ml PBS. Fraction of the samples was taken to analyse purity of the cell fractions by flow cytometry.

Step 1

Step 2

Step 3

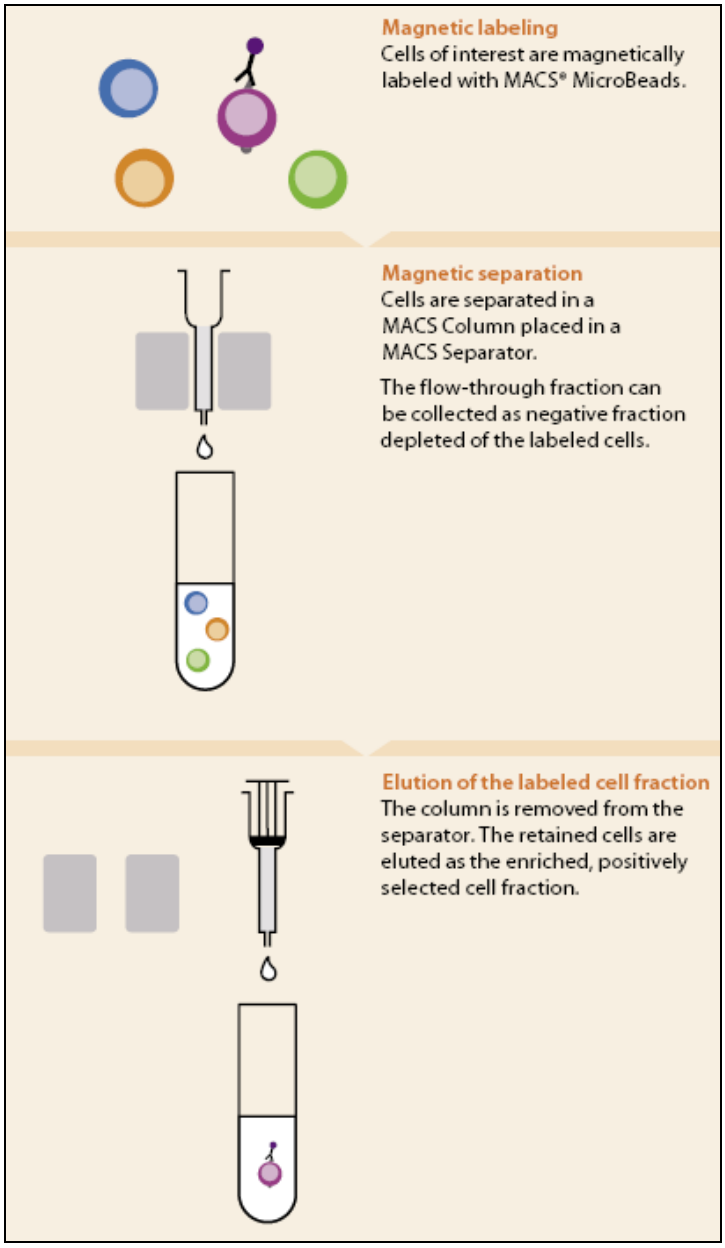


Figure 4.3: Purification of positive fraction of cells by direct magnetic labelling using MACS technology
(http://www.miltenyibiotec.com)

4.7. Cultivation and storage of *Mus dunni* cells

The adherent *Mus dunni* cells were cultivated at 37°C in a steam-saturated 5% CO_2 atmosphere and, according to the number of cells required were passaged in different cell culture flasks containing complete RPMI. The cells were passaged at 95% confluence. After decanting the medium, the cells were washed briefly with 2 ml of 5% Trypsin-EDTA and the nonconfluent cells were decanted. Another 2 ml of 5% Trypsin-EDTA was added to the flask and incubated at 37°C for 5 min until the cells separated from the bottom of the culture flask. A fraction of the cells was drawn out to count the number of cells using the Trypan blue exclusion assay. The process was completed with a cold full medium. $1x10^5$ cells were transferred from the cell suspension to a new culture flask with 10 ml fresh medium. For longer storage of *Mus dunni* cells, $1x10^6$cells/ml were resuspended in a freezing medium. Aliquots of 2 ml were transferred into freeze tubes and frozen at -20°C for 2-3 hrs, stored overnight at -80°C and transferred into liquid nitrogen. To thaw the cells, they were heated in a water bath of 37°C, transferred into 10 ml of heated medium (37°C) and centrifuged for 10 mins at 300 x g and 4°C. The pellet was rinsed again to remove the DMSO from the medium completely. Respectively 400 µl and 1600 µl of the cell suspension were transferred into T25 cell culture flasks and filled up to a final volume of 10 ml.

4.8. Estimation of viral loads by infectious center assay

Infectious center assay (Robertson et al., 1991) was used to determine the number of infectious centers in the spleen which corresponded to the number of spleen focus forming virus per ml. Serial ten-fold dilutions of single-cell spleen suspensions (1×10^8 cells/ml) from FV infected mice were titrated and plated onto 6-well plates containing 2×10^4 cells of susceptible *Mus Dunni cells* in 3 ml of complete RPMI medium for each well. The plates were cocultivated for 3 days in the 5% CO_2 incubator. 3 wells with F-MuLV and 3 wells without viruses or infectious cells served as control tests. In the control with F-MuLV, polybrene A (4 µg/ml) had to be added so that the free virus could bind better to the cell surfaces. Virus from the infected cells was able to infect the *Mus Dunni* cells via cell-cell contact. These replicated viruses were passed on to the daughter cells. A virus focus was created and each focus corresponded with one infected cell. At the end of the incubation period, plates were taken out from the incubator and were fixed for 5 mins with 95% ethanol

and subsequently rinsed with PBS and then with PBS containing 0.1% BSA. Later, plates were incubated sequentially with FMuLV-envelope specific monoclonal antibody (mAb) 720 (Robertson, M.N et al; 1991) at R.T for 1 hr. The plates were rinsed twice with PBS containing 0.1% BSA. The second antibody coupled with horse-radish peroxidase (goat anti mouse IgG1-HRP; 0.05 mol/l) was added to the plates and incubated at R.T for 1 hr during which the secondary antibody bound to the Fc region of the first antibody. The plates were washed again with PBS containing 0.1% BSA and freshly-made AEC substrate solution was added to each plate and incubated in darkness for 20 min at RT. The soluble substrate AEC converted into an insoluble red product by the HRP. The red spots marked the virus foci on the cell layer (Fig 4.4). Plates were then washed with running water and dried overnight to count the foci. The number of infectious spleen cells per mouse was calculated using the mean number of foci per dilution and the total count of spleen cells per mouse (Table 4.1).

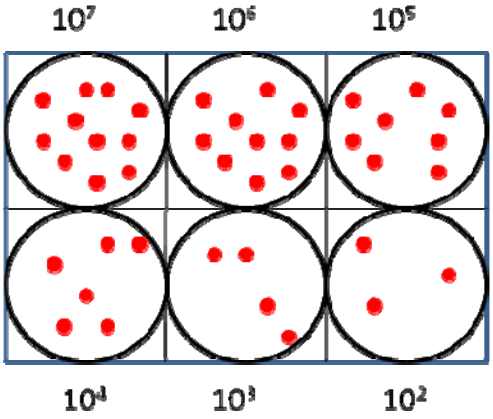


Figure 4.4: Viral foci in serially-diluted splenocytes co-cultivated with FV susceptible *Mus dunni* cells.

Table 4.1: Calculation of Infectious centers per spleen

Sample	Cells (dilution)	Foci (n)	Foci/10^8 cells (a)	Total cells per spleen x 10^8 (b)	ICs per spleen (Foci/10^8 cells x Total spleen cells x 10^8) (a x b)
Spleen 1	10^6	60	6000/10^8	2	12 x 10^3 foci in spleen 1
	10^5	4 (n<10, don't choose)			

4.9. Virus-neutralizing antibody assay

Mus dunni cells were plated onto 24 well plates one day prior the assay. Each row of wells corresponded to one sample and each well contained 7.5 x 10^3 cells in 2ml complete RPMI. The following day, polybrene was added to the *Mus dunni* cells. The plasma samples from mice were heat-inactivated at 56^0C water bath for 30 mins to deactivate the virus. In a 96-well plate, an aliquot of F-MuLV stock whose concentration was previously titrated was added to each well in addition to guinea pig complement and serially-diluted plasma samples (Morrison et al., 1987). As positive controls, in 3 wells, plasma samples were replaced by mAb 48 with known neutralizing effect. While for negative controls, PBS instead of plasma sample was added to 9 wells. The plate was incubated for 37^0C for exactly 1 hr. Cold PBS was added to all the wells and a fraction of each diluted sample was added to the corresponding 24-well plates passaged with *Mus dunni* cells. All the plates were kept in the 37^0 C incubator for 3 days. Cells were then fixed with ethanol and incubated sequentially with FMuLV-envelope specific monoclonal antibody (mAb) 720 and developed with secondary antibody IgG1-HRP. Viral foci were identified by developing it with AEC substrate. The titer was defined as the plasma dilution at which greater than 75% of the input virus was neutralized.

4.10. Tetramers and Tetramer staining

Tetramer staining has become the gold-standard method for T cell analyses and isolation of a very small number of antigen-specific precursor cells. A relatively new method established in the year 1996 by a group of eminent scientists Davis, McHeyzer-Williams and Altman was basically designed to tag T cells in an antigen-specific manner. Schumacher´s laboratory in Holland developed mouse (I-A^b) MHC class II tetramers in which the MHC heterodimers are expressed in conjunction with a genetically fused peptide in insect cells. In brief, heterodimers of the extracellular domains of the MHC class II alpha and beta chain are produced in insect cells with the T cell epitope covalently attached to the beta chain. Velcro leucine zippers are included to promote heterodimerization and a His-tag and biotinylation signal (bio-tag) are attached to the MHC class II alpha and beta chain respectively for subsequent purification and tetramer formation. Four identical biotin-containing pMHC

complexes are attached to fluorescently labelled streptavidin for binding to specific T cell receptors (TCRs) (Fig 4.5). Tetramers bind to T cells that express T cell receptors specific for the cognate peptide – MHC complex and can therefore be used to track Ag-specific T cells by flow cytometry. Although the yield of MHC class II tetramers is low as compared to MHC class I complexes, the resulting MHC class II tetramers stain antigen-specific $CD4^+$ T cells in specific fashion. Needless to say, MHC –II tetramer positive cells are effector $CD4^+$ T cell populations.

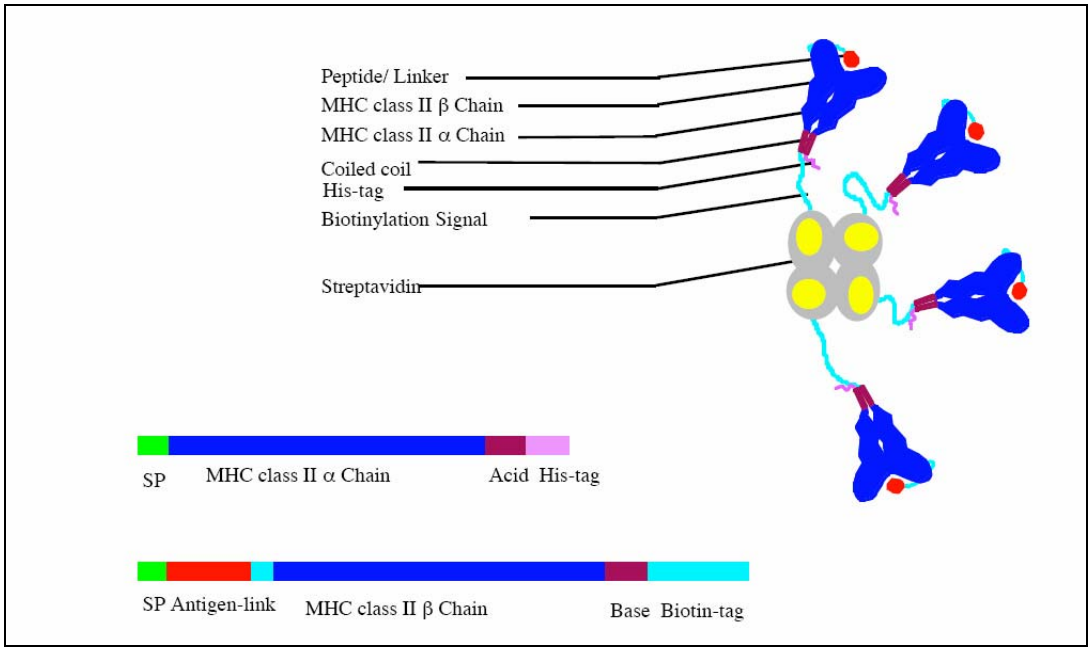


Figure 4.5: Schematic representation of MHC class-II tetramer components (http://research.nki.nl/schumacherlab/MHC%20tetramers.htm)

The ($I\text{-}A^b$) class-II tetramers were used for detection of $I\text{-}A^b$ FV envelope specific $CD4^+$ T cells. 1 x 10^6 nucleated spleen cells were incubated with PE-labelled $I\text{-}A^b$ tetramers for 2 hrs at 37^0C and later stained with surface molecules to quantify the population of FV-specific activated $CD4^+$ T cells by flow cytometry. After washing, cells were stained for APC-labelled anti-CD4, and FITC-labelled anti-monocytes Mac-1 for 15 mins at 4^0C. Cells were washed twice, resuspended in buffer containing 7AAD and analysed by Flow cytometry and 150,000 to 400,000 lymphocyte gated events per sample and analyses were done using BD Cell Quest software.

For the MHC-I staining the cells were incubated with H-2D^b class-I tetramers together with surface markers CD8 and CD43 for 15 min at 4^0C. Cells were washed twice, resuspended in buffer containing 7AAD and analysed by Flow cytometry and 150,000 to 300,000 lymphocyte gated events per sample and analyses were done using BD Cell Quest software.

4.11. Flow cytometry

Flow cytometry is a method to differentiate and count cells and microparticles that are tagged with fluorescent antibodies. Until a decade ago, the flow cytometer was seen only in research laboratories. Recently, it has evolved from its highly specialized research tool status to a commonplace clinical assay. The impetus for this change has been that flow cytometry is the only technique capable of quantitative measurements of multiple features of individual cells in a rapid manner (J. Philip McCoy, 1994). Flow cytometry is a generic term, while FACS (Fluorescence activated cell sorter) is a trademark of the Becton-Dickinson Corporation.

4.11.1. Methodology of Flow cytometry

A flow cytometer is made up of three main systems: fluidics, optics, and electronics (http://www.bdbiosciences.com) (Fig 4.6).

• The fluidics system hydrodynamically focuses the cell stream to the laser beam for interrogation.

• The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors.

• The electronics system converts the detected light signals into electronic signals that can be processed by the computer

For some instruments equipped with a sorting feature, the electronics system is also capable of initiating sorting decisions to charge and deflect particles. Any suspended particle or cell from 0.2–150 micrometers in size is suitable for analysis. Cells from solid tissue must be disaggregated before analysis.

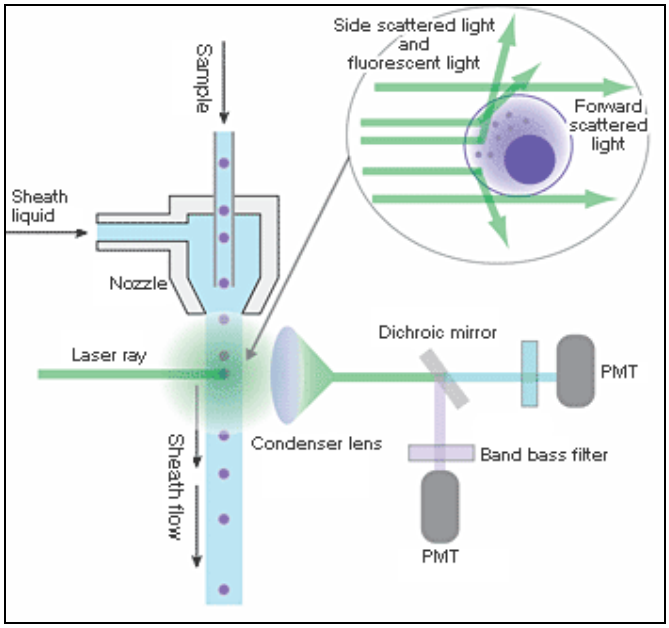


Figure 4.6: Principle of Flow cytometry

(http://www.bdbiosciences.com)

Light scattering

A flow cytometer operates by causing a fluid stream to pass single file through a beam of light generated by a laser. The photons of light, which are disrupted and scattered and emitted by the cells following their interaction with the laser beam, are separated into constituent wavelengths by a series of filters and mirrors (J. Philip McCoy, 1994). The deflection of the beams depends upon impact of the laser beams on the cells and is divided into two categories. The light which detects the relative size of the single cells is called forward scatter (FSC) light, and the light which is deflected by 90° and thus reveals data about the inner granularity of the cells is called sideward scatter (SSC) light (http://www.bdbiosciences.com) (Fig 4.7).

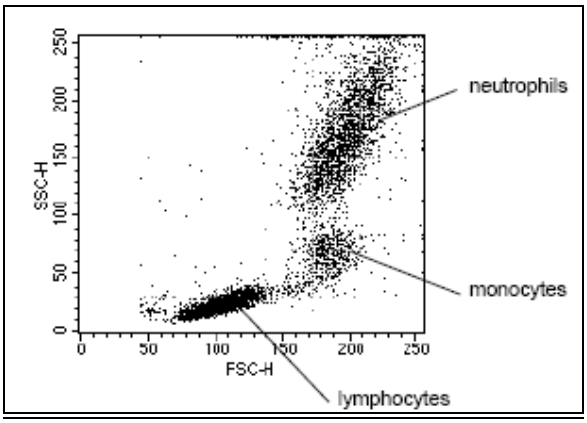


Figure 4.7: Preliminary identification of cells based on FSC v/s SSC
(http://www.bdbiosciences.com)

Fluorochromes

A fluorescent compound (fluorochrome) absorbs light energy over a range of wavelengths that is characteristic for that compound. This absorption of light causes an electron in the fluorescent compound to be raised to a higher energy level. The excited electron quickly decays to its ground state, emitting the excess energy as a photon of light. This transition of energy is called fluorescence (http://www.bdbiosciences.com). These methods enable, amongst other things, a quantitative investigation of the surface molecules. The basis for this is an antigen antibody reaction conducted with fluorescently-marked antibodies which are aimed at particular surface molecules. The single cell suspensions are analysed by flow cytometry (Fig 4.8).

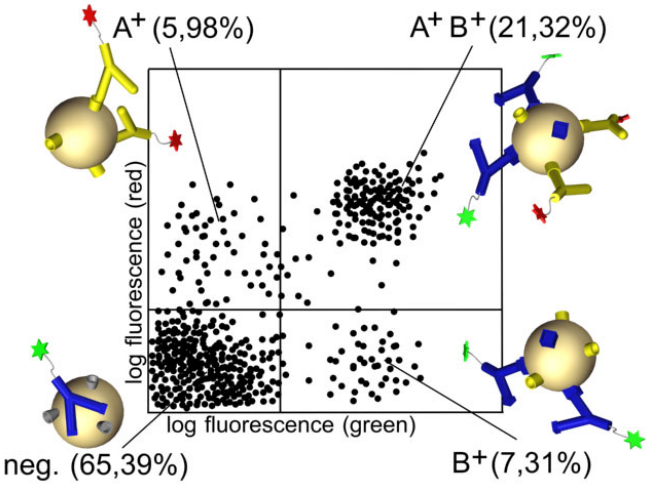


Figure 4.8: Specific binding of fluorochrome-labelled antibodies to cell surface antigens
(http://www.bdbiosciences.com)

Signal detection

Light signals are generated as particles pass through the laser beam in a fluid stream. These light signals are converted to electronic signals (voltages) by photodetectors and then assigned a channel number on a data plot. A voltage pulse is created when a particle enters the laser beam and starts to scatter light or fluoresce. Once the light signals, or photons, strike one side of the PMT or the photodiode, they are converted into a proportional number of electrons that are multiplied, creating a greater electrical current. The electrical current travels to the amplifier and is converted to a voltage pulse. The highest point of the pulse occurs when the particle is in the center of the beam and the maximum amount of scatter or fluorescence is achieved. As the particle leaves the beam, the pulse comes back down to the baseline (http://www.bdbiosciences.com) (Fig 4.9).

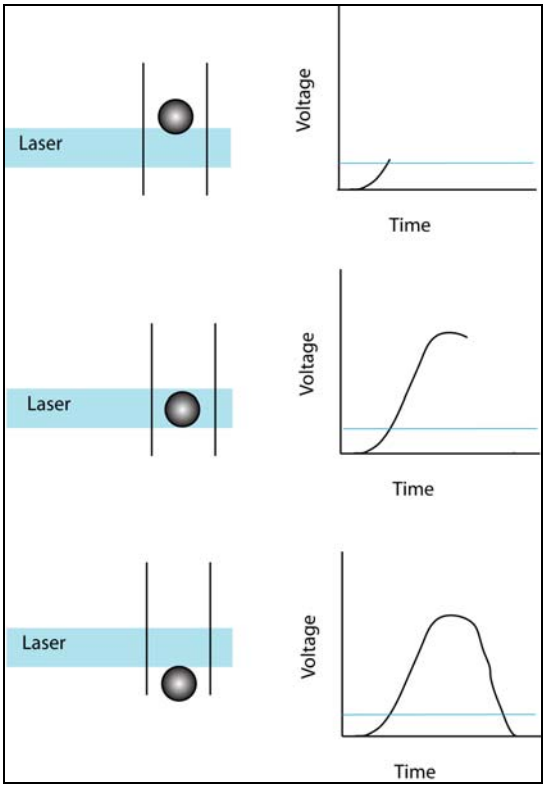


Figure 4.9: Formation of a voltage pulse
(http://www.bdbiosciences.com)

4.11.2. Staining of cells and FACS analyses

$1x10^6$ cells from each tissue were suspended in the FACS buffer, centrifuged for 5 min with 300 x g at RT and the supernatant was removed. The cell pellet were resuspended in 100 µl FACS buffer containing 0.5 - 2 µl conjugated antibodies of various surface bodies (CD4, CD8, CD43, CD69, CD62L, CD127, CD25, CD45.1) and incubated in the dark for 20 min at 4°C. After a double rinse in the FACS buffer, the cells were resuspended with 400 µl FACS buffer. Data were acquired from 100,000 to 250,000 lymphocyte-gated events per sample.

For the estimation of intracellular production of IFN-γ, spleen cells were stimulated with either plate-bound CD3 antibody in the presence of 2 µg/ml of CD28 antibody and 2 µg/ml of brefeldin A for 5 h at 37^0 C. The cells were then stained for surface expression of CD4, CD45.1 and CD43, fixed and permeabilised with Cytofix/Cytoperm solution (BD) and incubated with anti-mouse IFN-γ-Biotin for 30 mins at RT. Cells were washed once with Cytofix /Cytoperm- wash buffer and then incubated with FITC labelled Streptavidin for 30 mins at RT. After a double rinse in the FACS buffer, the cells were resuspended in a volume of 400 µl with FACS buffer and analysed in detail. Data were acquired from 100,000 to 250,000 lymphocyte-gated events per sample. Foxp3 expression was detected by intracellular staining using the anti-Foxp3 antibody and the Foxp3 staining set. The fluorescently-stained characteristics of the cells were measured on FACS Calibur and LSRII flow cytometer and evaluated either with Cell quest software or FlowJo software.

4.11.3. Exclusion of dead cells in flow cytometry

The exclusion of dead cells and cellular debris in flow cytometry was carried out using the dye 7-Amino-Actinomycin (7AAD). 7AAD has a high DNA binding constant and is efficiently excluded by intact cells. It is useful for DNA analysis and dead cell discrimination during flow cytometric analysis. When excited by 488 laser light, 7-AAD fluorescence is detected in the far red range of the spectrum (650 nm long-pass filter). In healthy cells, the intact cell membrane prevents the fast permeation of 7AAD and thus it is only slowly absorbed by the healthy cells. However, should a cell be apoptotic or is damaged or killed in the measuring process, then the cell membrane loses its protective function and 7AAD rapidly diffuses into the cell. There, it is taken up in the cell nucleus and DNA with which it intercalates. The cell is thus marked as “dead” and can be detected by the flow cytometer.

7AAD with the ratio of 1:250 was added to the stained cells in 400 µl FACS buffer and the sample was immediately analysed.

4.12 Statistical analyses

Statistical analyses and graphical presentations were computed with Graph Pad Prism version 5. Statistical difference (p-value) between the different parameters was analyzed by Mann-Whitney U test. The p-value is a probability with a value ranging from zero to one. In this work, all p-values ≤ 0.05 were determined significant.

5. RESULTS

5.1. Helper T cells are important in controlling viral replication and preventing erythroleukemia during acute FV infections

Helper T (Th) cells are important in vaccine-induced protection against FV infections (Dittmer and Hasenkrug, 2000) and in restricting FV replication and spread in persistently infected mice (Hasenkrug, Brooks, and Dittmer, 1998). What remains to be known is their role during acute FV infection of mice that recover from FV-induced disease. To better understand the importance of $CD4^+$ T cells in recovery from acute FV infections, mice were injected with anti-CD4 MAbs to deplete $CD4^+$ T cells while the control animals received PBS solution as mentioned in Materials and Methods (Fig 5.1). The *in vivo* effectiveness of the CD4 depletion protocol was tested. After 1 week of CD4 antibody injection, greater than 98% depletion of $CD4^+$ T cells was achieved (Fig 5.2A). One week later, CD4-depleted mice and non-depleted control mice were infected with a similar dose of FV. Spleens from these mice were palpated at weekly intervals to monitor splenomegaly (Fig 5.3A). CD4-depleted mice had much enlarged spleens than the non-depleted mice at 4 weeks post infection (wpi). This time point was therefore chosen to determine how CD4 T-cell depletions affected FV-induced erythroleukemia and the degree of viral replication in the spleen. At 4 wpi, the levels of $CD4^+$ T cells in mice depleted for $CD4^+$ T cells was still significantly lower than in non-depleted mice (Fig 5.2B) indicating that the effect of the CD4 depletion lasts for at least for 4 weeks.

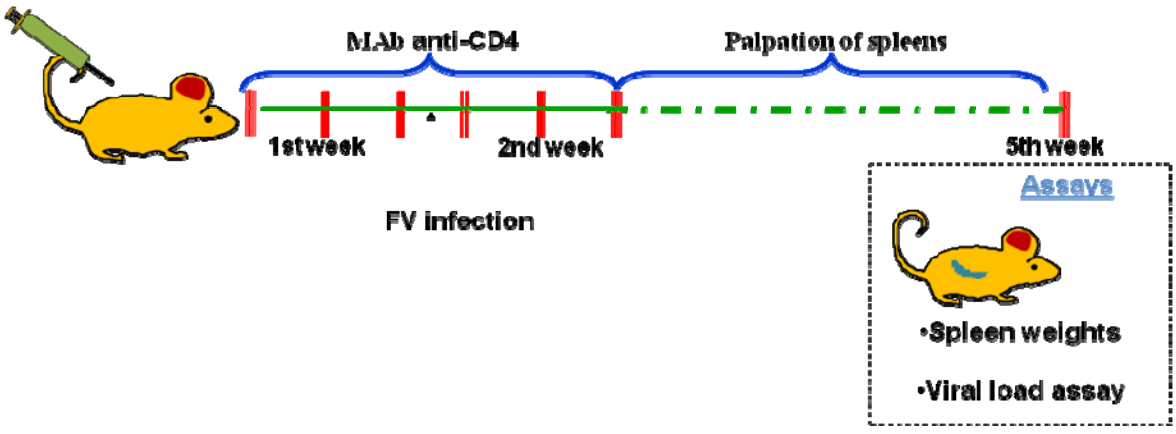
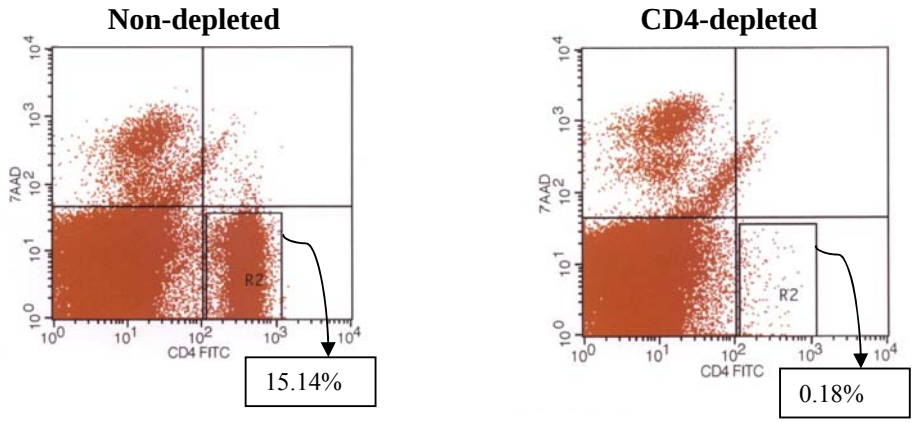


Fig 5.1: CD4 depletion protocol

The figure demonstrates protocol for the CD4 depletion and acute FV infection established in (C57BL/10 x A.BY) F1 mice. Mice were intraperitoneally injected with 0.5 ml of supernatant fluid obtained from hybridoma cell culture for CD4-specific mAb YTS 191.1. CD4 depletion was carried out at days -6, -4, -2 and 0, 2 and 4 days post FV infection.

A



B

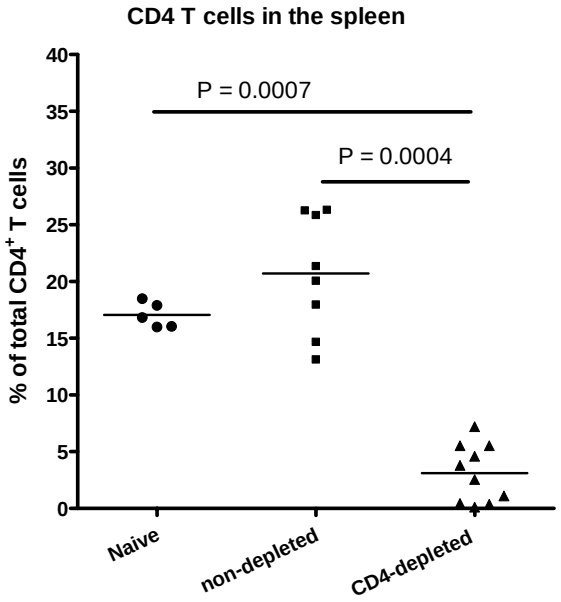
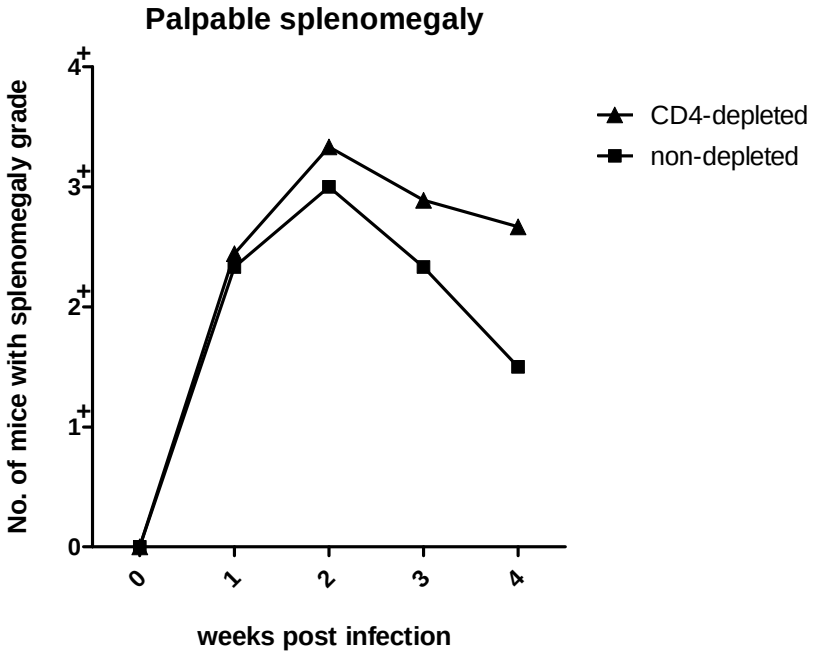


Fig 5.2: Efficiency of CD4 depletion

A) CD4 T cell depletion was performed for 1 week in (C57BL/10 x A.BY) F1 mice as described in Materials & Methods. Live spleen cells were isolated from a representative CD4-depleted mouse and a non-depleted mouse to compare percentages of total $CD4^+$ T cells in whole lymphocyte population. Efficiency of CD4 depletion at 1 week post anti-CD4 treatment is demonstrated in the representative data from a single mouse. Percentages of positive (lower right quadrant) are given on the dot blots. B) Acute FV infection was established in (C57BL/10 x A.BY) F1 mice and depletion of $CD4^+$ T cells was performed as described in Materials & Methods. Percentage of total $CD4^+$ T cells in naïve (●), non-depleted, FV-infected (■) and CD4-depleted, FV-infected (▲) mice was determined at 4wpi. Each dot represents an individual mouse. Mean percentages are indicated by a line. P values were determined by the Mann-Whitney U test for statistical analysis and are shown in the figure. The experiment was repeated three times with comparable results.

At 4wpi, spleen weights of CD4-depleted mice ranged from 0.54 g to 4.07 g with mean spleen weight of 2.021 g (Fig 5.3C). This was in sharp contrast to the spleen weights of the non-depleted mice which had mean spleen weight of 0.21 g indicating that FV-induced disease had resolved by that time, almost comparable to the non-infected mice which had a mean spleen weight of 0.12 g (Fig 5.3C). The representative photographs of spleens from each group of mice clearly indicate the differences in disease progression (Fig 5.3B). These results suggest that the absence of $CD4^+$ T cells affects the normal recovery from FV-induced disease and results in gross splenomegaly in CD4-depleted mice.

A



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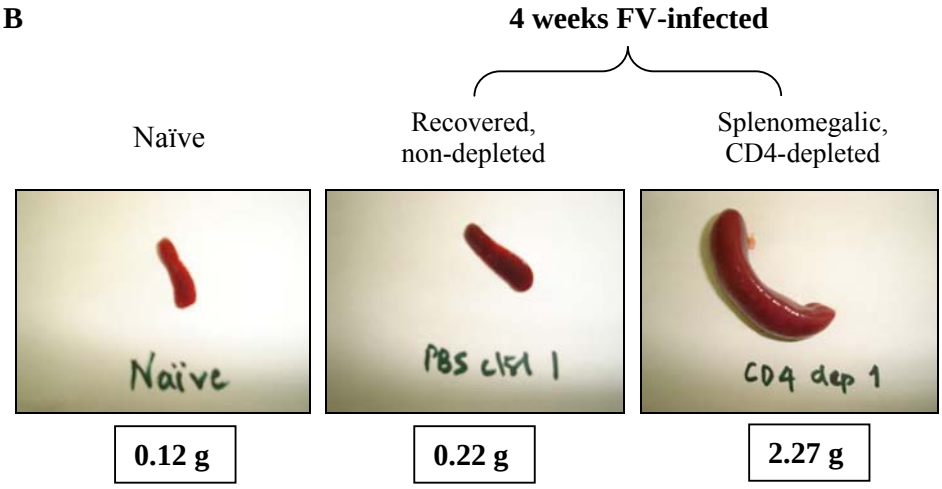

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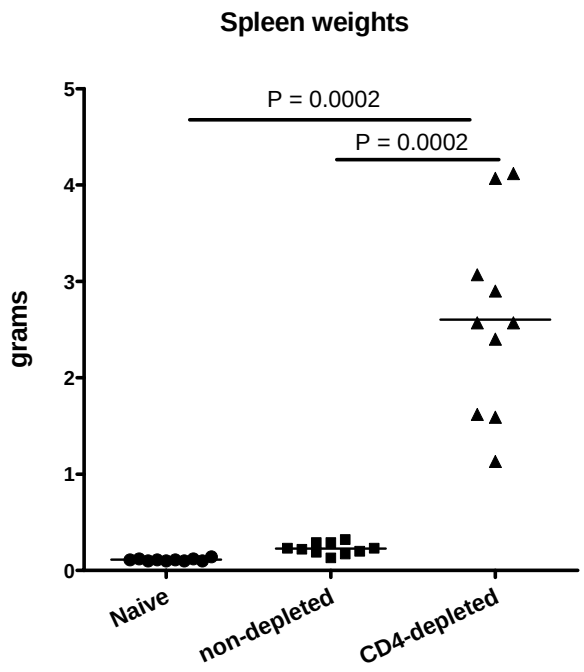


Fig 5.3: FV-induced splenomegaly in CD4-depleted mice

Acute FV infection was established in (C57BL/10 x A.BY) F1 mice and depletion of $CD4^+$ T cells was performed as described in Materials & Methods. A) CD4-depleted and non-depleted mice were palpated at weekly intervals to monitor splenomegaly and were rated on a scale of 1^+ to 4^+ as mentioned in Materials & Methods. At 4wpi, CD4-depleted mice had palpable splenomegaly in comparison to non-depleted mice. B) Representative photographs of spleens at 4wpi display an enlarged spleen from a CD4-depleted, FV-infected mouse when compared to spleens from a naïve mouse and non-depleted, FV-infected mouse. The spleen weights are shown below. C) Spleen weights of naïve (●), non-depleted, FV-infected (■) and CD4-depleted, FV-infected (▲) mice were determined at 4wpi. Each dot represents an individual mouse. Mean spleen weights are indicated by a line. P values were determined by the Mann-Whitney U test for statistical analysis and are shown in the figure. The experiment was repeated three times with comparable results.

Infectious center assays were performed to determine whether the absence of $CD4^+$ T cells affected levels of viral replication in the spleen. Spleen cell suspensions from CD4-depleted and non-depleted mice were plated onto indicator cells to produce infectious centers as described in Materials and Methods. Infectious center assay results revealed that there were significantly more infectious centers in CD4-depleted mice (mean = 1.375×10^8) than in nondepleted mice (mean = 2.376×10^3) analysed by the Mann-Whitney U test (Fig 5.4). Thus, depleting the CD4 T cell population resulted in increased FV replication during acute

FV infection and progression of FV-induced disease from erythroproliferation to erythroleukemia in the resistant mouse strain (C57BL/10 x A.BY) F1.

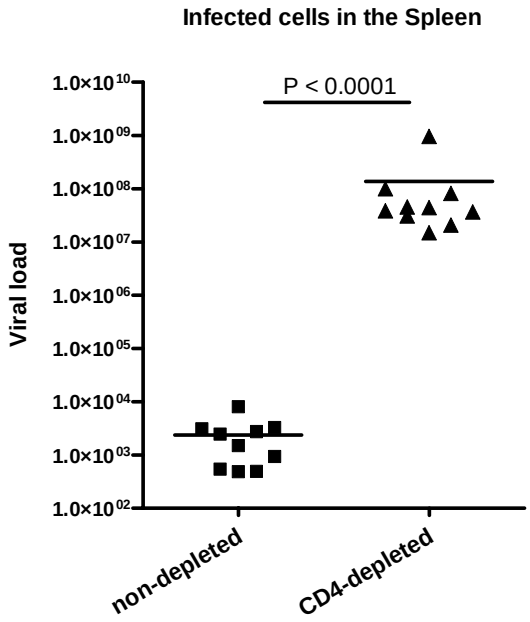


Fig 5.4: CD4 depletion hampers control over virus replication

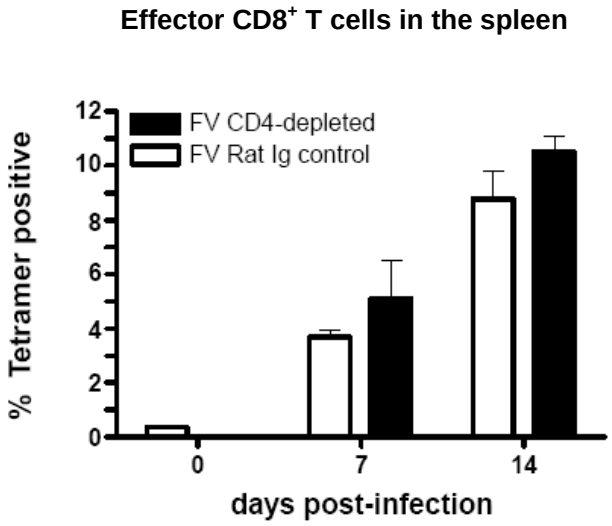
Acute FV infection was established in [(C57BL/10 x A.BY) F1 mice and depletion of CD4 T cell subset was performed as described in Materials & Methods. Spleen cell suspensions were plated onto indicator cells to produce infectious centers as described in Materials & Methods. Viral load in spleens of naïve (●), non-depleted, FV-infected (■) and CD4-depleted, FV-infected (▲) mice was determined at 4wpi. Each dot represents an individual mouse. Mean viral loads are indicated by a line. P values were determined by the Mann-Whitney U test for statistical analysis and are shown in the figure. The experiment was repeated three times with comparable results.

5.2 CD4 T cell help is needed for maintenance of FV- specific effector and memory $CD8^+$ T cell responses and for the induction of neutralizing antibodies by B cells during acute FV infection

We have established in our current study that CD4 depletions resulted in loss of immunity to FV infection in resistant mice. The mechanisms by which $CD4^+$ T cells mediated protection against FV needed to be investigated next. $CD4^+$ T cells are also called helper T cells (Th) as they coordinate various immune responses with B cells and cytotoxic $CD8^+$ T cells. There has been contrasting evidence related to the requirement of help from $CD4^+$ T cells in the activation, growth and survival signals in the $CD8^+$ T cell response. Overall, primary CTL

responses seems to be $CD4^+$ T-cell help independent during some infections (LCMV, vaccinia virus, *L. monocytogenes*) but help is required for the generation of long-lived memory $CD8^+$ T cells to mount a stronger immune response against these pathogens during rechallenge. On the other hand, in other infections (adenovirus, influenza virus, HSV-1), primary CD8 T cell responses are dependent on $CD4^+$ T-cell help. Since, nothing is known about the significance of $CD4^+$ T cells in mediating help to $CD8^+$ T cells in retroviral infections; it was of keen interest to investigate the mode of CD4 T-cell mediated protection in the Friend retroviral infection model. At 7dpi and 14dpi, FV specific effector $CD8^+$ T cell responses in the spleen characterized by ($CD8^+CD43^+Tet^+$ T cells) were not affected by $CD4^+$ T cell depletions (Fig 5.5A). However, at 4wpi, there was a remarkable decrease in the population of FV specific effector $CD8^+$ T cells in CD4-depleted mice when compared to non-depleted mice (Fig 5.5B). Furthermore, absolute cell numbers of FV specific effector memory cells characterized by ($CD8^+Tet^+CD127^+$ T cells) was also significantly lower in CD4-depleted mice as compared to non-depleted mice (Fig 5.5C). Thus, early recovery from FV infections mediated by $CD8^+$ T cell responses were CD4 T-cell help independent but long-term maintenance of $CD8^+$ T cell responses required help from $CD4^+$ T cells.

A



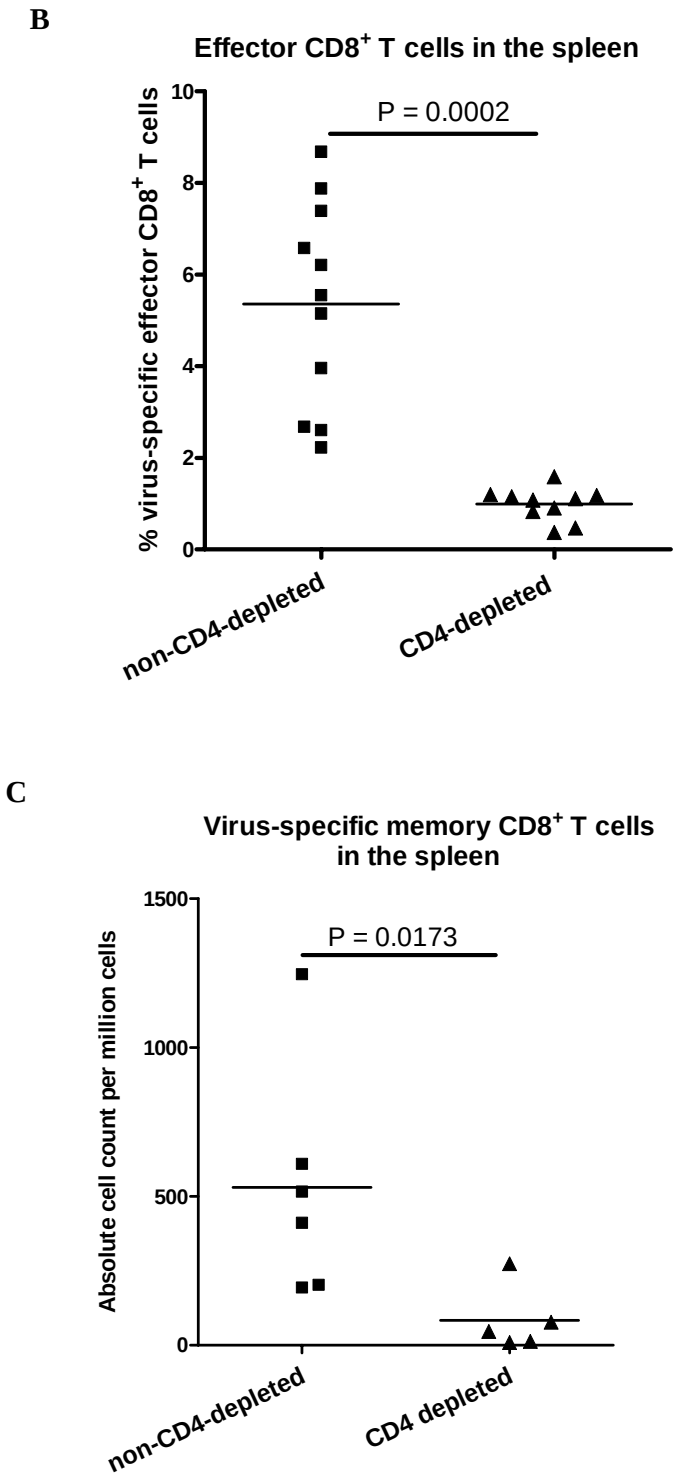


Fig 5.5: CD4 T-cell help is required for maintenance of FV- specific effector and memory CD8$^+$ T cells during acute FV infection.

Acute FV infection was established in (C57BL/10 x A.BY) F1 mice and depletion of CD4 T cells was performed as described in Materials & Methods. A) Spleen cells from CD4-depleted and non-depleted mice were isolated at 7dpi and 14dpi and stained for cell-surface CD8 and FV-specific D^{b}gagL MHC

class I tetramer, and analysed by Flow cytometry. The graph shows the mean percentages ± standard errors of the means (SEM) of $CD8^+$ T cells that are tetramer positive (n = 7 to 10 mice per group). There were no significant differences in percentage of FV-specific tetramer positive $CD8^+$ T cell responses between rat Ig-injected control mice and CD4-depleted mice at both time points. (B) & (C) Spleen cells from CD4-depleted and non-depleted mice were isolated at 4wpi. B) Gated live $CD8^+$ T cells were co-stained for the activation-associated glycoform of CD43 and FV-specific D^bgagL MHC class I tetramer. Percentage of FV-specific effector $CD8^+$ T cells was determined in non-depleted, FV-infected (■) and CD4-depleted, FV-infected (▲) mice. C) Gated live $CD8^+$ T cells were co-stained for FV-specific D^bgagL MHC class I tetramer and CD127 to detect virus-specific memory $CD8^+$ T cells. Absolute cell numbers of memory $CD8^+$ T cells were determined in non-depleted, FV-infected (■) and CD4-depleted, FV-infected (▲) mice. Mean percentages for (B-C) are indicated by a line. P values for (A-C) were determined by the Mann-Whitney U test for statistical analysis and are shown in the figures. Experiments were repeated two times with comparable results.

It is of common knowledge that CD4 T cell help is indispensable for production of neutralising antibodies in various infections such as HIV, Measles virus, VSV, Influenza and LCMV. Furthermore, virus-neutralizing antibodies are essential for recovery from FV infection as concluded from vaccine studies. Hence, to determine if B cells require help from CD4 T cells in induction of neutralising antibodies, frozen plasma samples from CD4-depleted and non-depleted mice were tested for FV neutralising antibody levels as described in Materials and Methods. The mean neutralising titer for FV-infected non-depleted mice was 1:254 while in the FV-infected mice, the mean titer was 1:44 and it was statistically different (Fig 5.6). Thus, helper T cells are critical for aiding B cells for induction of neutralising antibodies in addition to maintenance of effector memory $CD8^+$ T cells during acute FV infection.

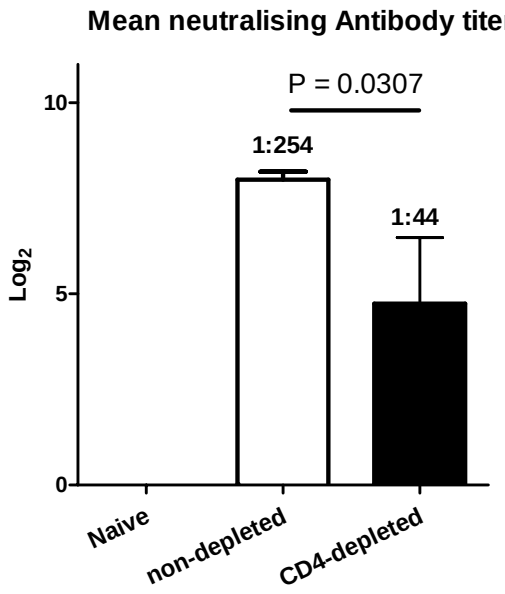


Fig 5.6: CD4 T cell help is required for efficient induction of neutralizing antibodies by B cells during acute FV infection.

Acute FV infection was established in [(C57BL/10 x A.BY) F1 mice and depletion for CD4 T cell subset was performed as described in Materials & Methods. Frozen plasma samples from naive, CD4-depleted and non-depleted mice were tested for neutralising antibody levels. Neutralising antibody titers are log_2 geometric means determined by the last dilution that produced 75% F-MuLV neutralization (n = 6 mice per group). P values were determined by the Mann-Whitney U test for statistical analysis and are shown in the figure. The experiment was repeated two times with comparable results.

Thus, *in vivo* depletion of Th cells in acute FV infection demonstrated that Th cells were not only vital in controlling viral spread and onset of erythroleukemia but also in the maintenance of CD8 and neutralizing antibody responses.

5.3 Resistance to FV-induced disease correlated with the magnitude of H-2A-restricted $CD4^+$ T cell responses

From previous work, it is known that recovery from FV-induced disease depends upon an array of genes mapped to the major histocompatibility complex (MHC) H-2 region of the mice, of which H-2A region influences effectiveness of Th cell responses. The $H\text{-}2A^{b/b}$ genotype is associated with high recovery while the $H\text{-}2A^{b/k}$ genotype is associated with intermediate recovery from FV infection. The H-2A genotype influences FV-specific $CD4^+$ T

cell responsiveness, isotype switching of FV-specific antibodies and vaccination against FV (Ney and D'Andrea, 2000). Hence, we investigated how the genetic background of a mouse controls the protective Th cell response during acute FV infection. Therefore, we quantified the population of FV-specific effector $CD4^+$ T cells taken directly *ex vivo* from high recovery ($H\text{-}2A^{b/b}$) and intermediate recovery ($H\text{-}2A^{b/k}$) mice at various time points of FV infection. We used tetramer technology to stain spleen cells with MHC class-II tetramers loaded with $I\text{-}A^b$-restricted MoMSV-envelope epitope (H19-Env) peptide-encoding (EPLTSLTPRCNTAWNRLKL) sequence.

Fig 5.7 demonstrates kinetics of FV-specific $CD4^+$ T cell responses characterised by $Tetramer^+CD4^+$ T cells ($CD4^+Tet^+$ T cells) in the two different mouse strains. This kinetic study showed that the peak of FV-specific effector Th cell response was at 10 dpi in both high recovery and intermediate recovery mice which corresponded well with peak viral loads at 7 dpi (unpublished data). However, there was a significant difference in peak $CD4^+Tet^+$ T cell responses with high recovery mice having 58% more effector $CD4^+$ T cells as compared to intermediate recovery mice. To corroborate if the population of $CD4^+Tet^+$ T cells had an effector phenotype, spleen cells were also stained for the activation markers CD43 and CD69 at 10 dpi. Quite expectedly, $CD4^+$ T cells from high recovery mice had significantly higher expression ($p < 0.05$) of the activation markers CD69 and CD43 in comparison to intermediate recovery mice (Fig 5.8). Thus, the resistance of mice to FV-induced disease correlated with the magnitude of the H-2A-restricted $CD4^+$ T cell response.

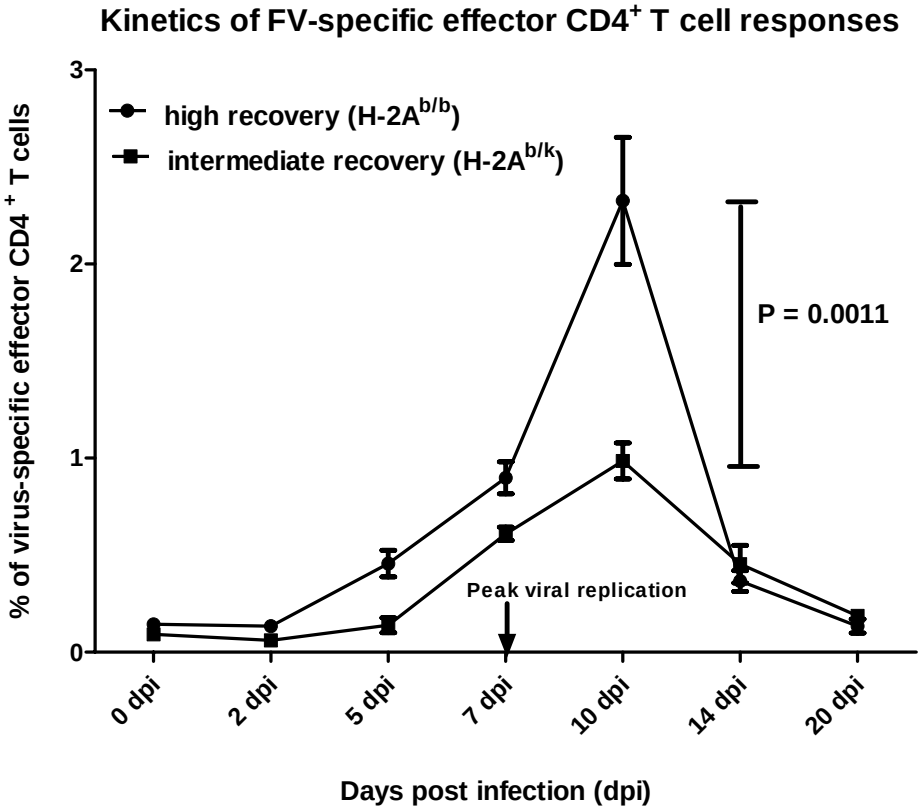


Fig 5.7: Kinetics of FV- specific effector CD4$^+$ T cell responses

High recovery (H-2A$^{b/b}$) (C57BL/10 x A.BY) F1 mice and intermediate recovery (H-2A$^{b/k}$) (C57BL/10.A x A.BY) F1 mice were acutely infected with FV and percentages of virus-specific CD4$^+$T cells reactive with I-A^b MHC class-II tetramers specific for FV-envelope epitope (EPLTSLTPRCNTAWNRLKL) were compared at various time points of infection. Spleen cells were analyzed by flow cytometry for the expression of cell surface CD4 and FV-specific MHC class-II tetramer. The figure shows a kinetic plot of mean percentages ± SEM of CD4$^+$Tet$^+$ T cells (n = 7 to 10 mice per group). P values were determined by the Mann-Whitney U test for statistical analysis. The experiment was repeated three times with comparable results.

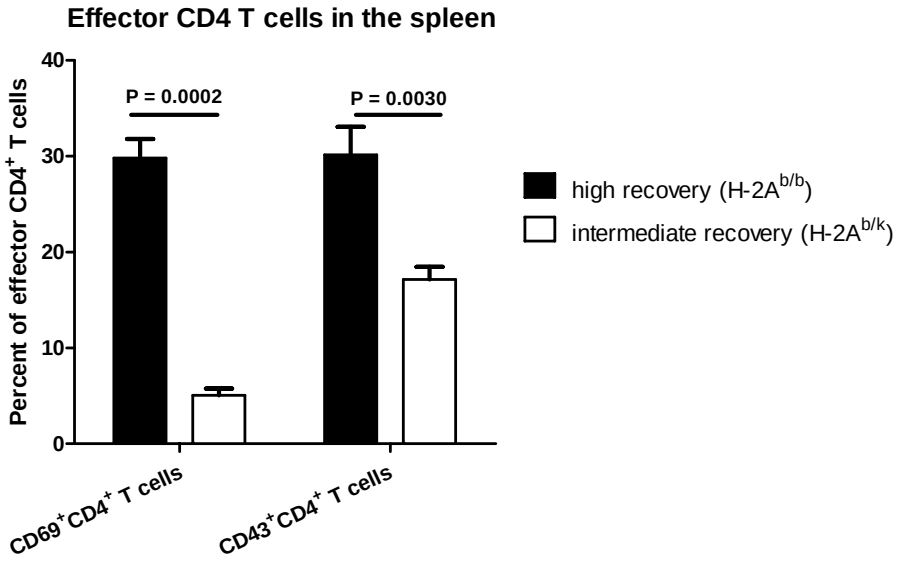


Fig 5.8: Phenotypic analysis of $CD4^+$ T cells in high and intermediate recovery mice

High recovery ($H\text{-}2A^{b/b}$) (C57BL/10 x A.BY) F1 mice and intermediate recovery ($H\text{-}2A^{b/k}$) (C57BL/10.A x A.BY) F1 mice were acutely infected with FV for 10 days. General levels of CD4 T-cell activation were assessed by measuring the expression of activation markers CD69 and CD43 gated on live $CD4^+$ T cells. The graph shows mean percentages ± SEM of $CD4^+$ T cells expressing cell surface CD69 and CD43 (n = 6 to 10 mice per group). P values were determined by the Mann-Whitney U test for statistical analysis. The experiment was repeated three times with comparable results.

These data support the findings from vaccination experiments which established the impact of the genetic background of mice on FV-induced recovery. Due to the genotype $H\text{-}2A^{b/b}$, mice were able to develop potent immune responses against FV and recover better as compared to $H\text{-}2A^{b/k}$ mice, which have only one $H\text{-}2^b$ allele and hence were not able to present antigens as effectively as high recovery mice resulting in an intermediate recovery from acute FV infection.

5.4 FV- specific CD4$^+$ T cells produced IFN-γ production and reduced viral loads in FV-infected mice

Although, we were able to provide sufficient evidence to state the immunoprotection by Th cells during acute FV infection, it would be of interest to investigate the function of adoptively transferred FV-specific CD4$^+$ T cells in acute FV infection *in vivo*. Using adoptively transferred FV-specific CD8$^+$ T cells from CD8 TCR transgenic mice, it was shown that such cells dramatically reduce viral loads during acute FV infection, however, fail to reduce viral loads in persistently infected mice (Dittmer et al., 2004). Hence, we made use of mice transgenically expressing the TCRβ chain of a TCR specific to an MHC class II-presented epitope of the surface (SU) product of the F-MuLV envelope (env) gene (Antunes et al., 2008) to perform similar studies.

Naive CD4$^+$ T cells from CD4 TCRβ-tg mice that are specific for FV envelope were enriched by MidiMACS separation system and were transferred into recipient mice infected with FV for different time periods (Fig 5.9). Donor CD4$^+$ T cells were differentiated from the endogenous host CD4$^+$ T cells since donor and recipient mice were from a different genetic background. In this study, T cells from donor mice express CD45.1 while T cells from the recipient mice express CD45.2. Before transfer, it was verified that the phenotype of the isolated CD4$^+$ T cells from CD4 TCRβ-tg mice was naïve. Donor CD4$^+$ T cells were stained for activation marker CD43 and lymphocyte homing receptor CD62L for which the expression is high on naive cells while it is down-regulated on activated T cells. Therefore, the naïve phenotype was represented as CD43^{low} and CD62L^{high} (Fig 5.10).

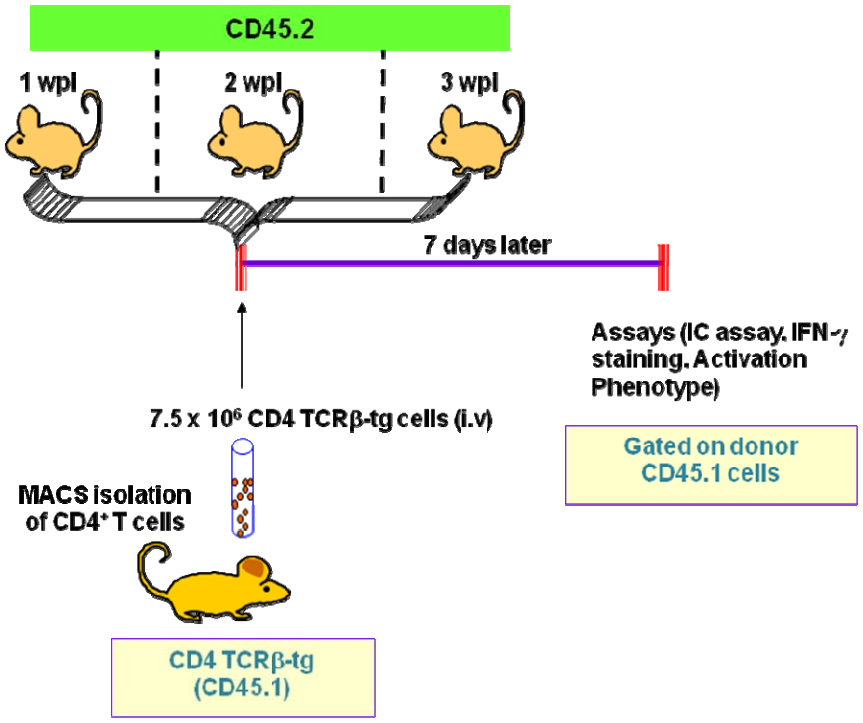


Fig 5.9: Adoptive transfer protocol

High recovery (H-2A$^{b/b}$) (C57BL/10 x A.BY) F1 mice expressing CD45.2 were acutely infected with FV either for 1 week, 2 weeks or 3 weeks. 7.5 x 10^6 CD4$^+$ TCRβ-tg cells from CD4 TCRβ-tg mice expressing CD45.1 were transferred into FV-infected recipient mice. One week after transfer, CD4$^+$ T cells from recipient mice were re-isolated for *ex vivo* phenotype and functional analysis in the spleen.

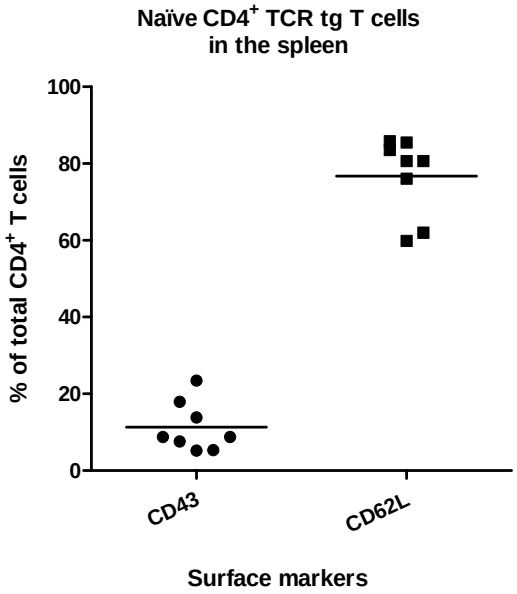


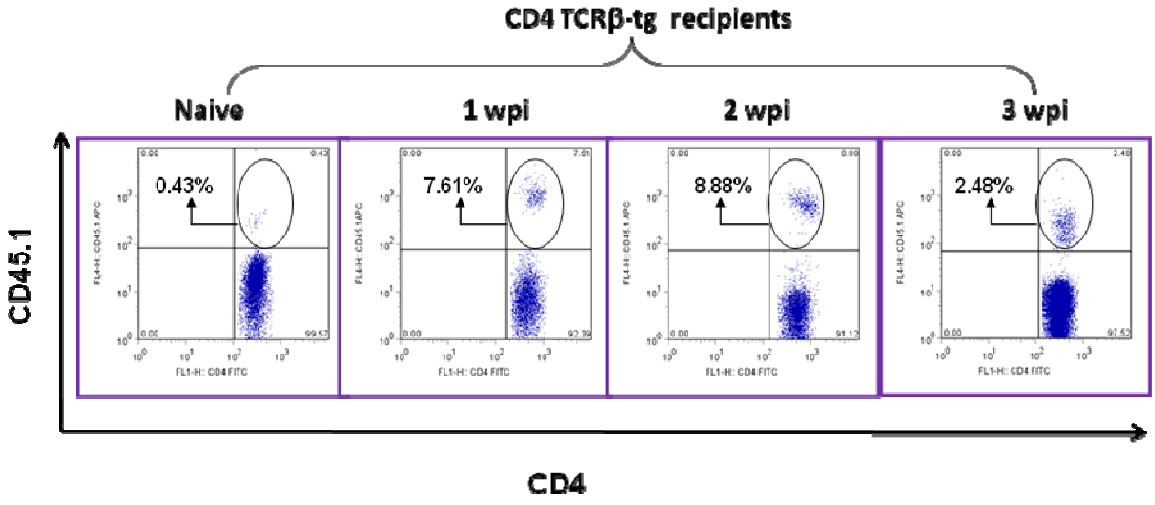
Fig 5.10: Phenotype of CD4$^+$ TCRβ-tg T cells

Spleen cells from naïve FV-specific CD4 TCRβ-tg mice were isolated and phenotyped for surface expression of CD43 and CD62L by flow cytometry before being transferred into FV-infected

recipients. The graph shows percentage of total $CD4^+$ TCRβ-tg T cells expressing CD43 and CD62L surface molecules. Each dot represents an individual mouse. Mean percentages are indicated by a line. The experiment was repeated three times with comparable results.

One week later after adoptive transfer of $CD4^+$ TCRβ-tg T cells into FV-infected recipient mice, $CD4^+$ T cells were re-isolated and analysed by flow cytometry. First, using this CD4 TCRβ-tg model, phenotypic analysis of donor $CD4^+$ T cells responding to FV were studied in host mice, which were infected with FV for 1, 2 or 3 weeks. Adoptive transfer of FV-specific TCRβ-tg $CD4^+$ T cells into naïve control mice indicated that only poor expansion of the donor cells occurs when no specific antigen is present. However, a strong expansion of FV-specific TCRβ-tg $CD4^+$ T cells was observed after transfer into mice infected for 1, 2 or 3 weeks (Fig 5.11A). Expansion of the donor cells was paralleled by an activation of the cells after recognizing their cognate antigen. Whereas very few cells that were transferred into naïve mice displayed an effector phenotype ($CD43^{high}$ and $CD62L^{low}$), the numbers of effector $CD4^+$ T cells was strongly increased after transfer into infected mice. Highest percentages of $CD4^+$ TCRβ tg T cells with effector phenotype were reisolated from animals infected with FV for 1 week, which correlated nicely with the viral loads in the recipient mice (Fig 5.11B).

A



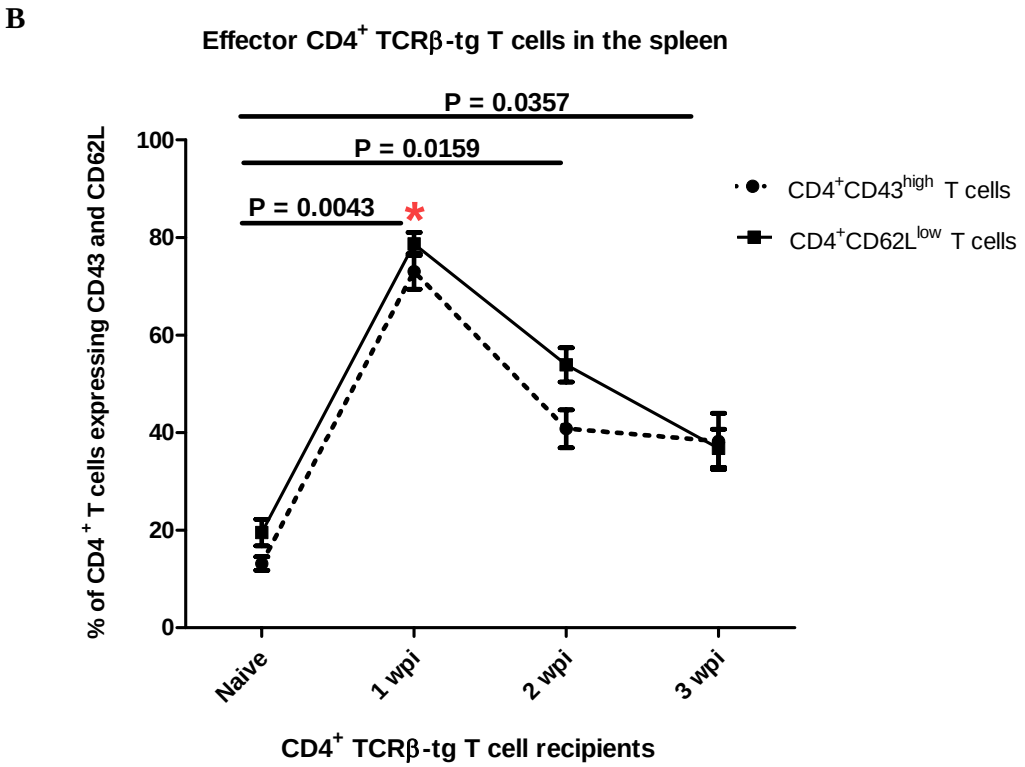


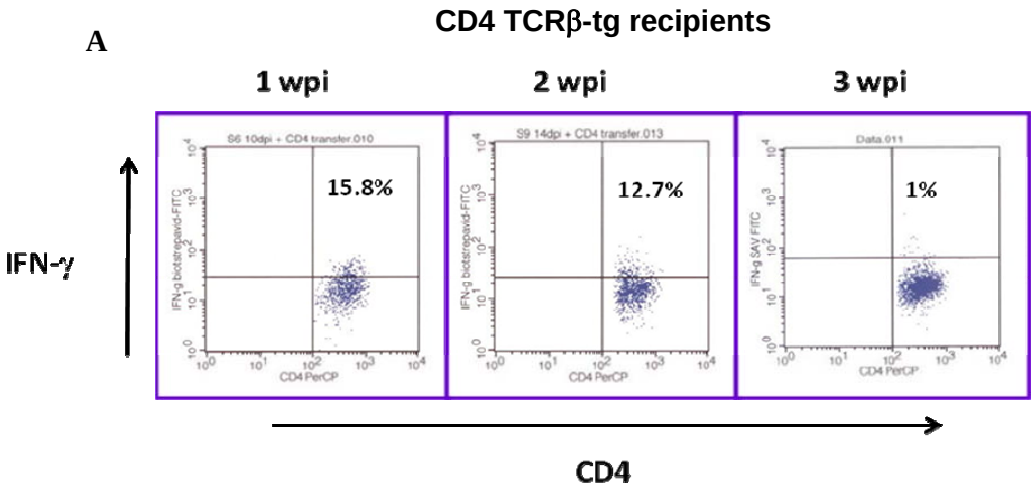
Fig 5.11: Phenotype of adoptively transferred CD4$^+$ TCRβ-tg T cells

High recovery (H-2A$^{b/b}$) (C57BL/10 x A.BY) F1 mice expressing CD45.2 were acutely infected with FV either for 1 week, 2 weeks or 3 weeks. 7.5 x 10^6 CD4$^+$ TCRβ-tg cells from CD4 TCRβ-tg mice expressing CD45.1 were transferred into FV-infected recipient mice. One week after transfer, CD4$^+$ T cells from spleens of FV-infected recipient mice were re-isolated and investigated. A) Representative data of expansion of donor CD4$^+$ T cells (CD45.1$^+$ T cells) re-isolated from an individual naïve, 1, 2, or 3 weeks FV-infected recipient mouse. B) Mean percentages ± SEM of CD4$^+$ TCRβ-tg T cells expressing CD43 and CD62L surface molecules (n = 7 mice per group). Asterisk (*) indicates that mean percentages ± SEM of CD4$^+$CD43^{high} T cells and CD4$^+$CD62L^{low} T cells in 1 week FV-infected recipient mice was significantly higher ($P < 0.05$) than 2 or 3 weeks FV-infected or naïve recipient mice. P values were determined by the Mann-Whitney U test for statistical analysis. The experiment was repeated two times with comparable results.

To examine the functional role of the adoptively transferred CD4$^+$ TCRβ-tg T cells during acute FV infection, intracellular cytokine staining for IFN-γ was determined one week after transfer of CD4$^+$ TCRβ-tg T cells transferred into mice infected for 1, 2 or 3 weeks. The percentage of IFN-γ producing donor CD4$^+$ T cells in mice infected with FV for 1 or 2 weeks was significantly elevated in comparison to donor CD4$^+$ T cells transferred into naïve control mice (Fig 5.12A and 5.12B). In contrast, transfer of FV-specific CD4$^+$ TCRβ-tg T cells into mice infected with FV for 3 weeks resulted in reduction in IFN-γ producing cells (1%) in

comparison to mice infected with FV for 1 week (15%) or 2 weeks (12.7%). Representative flow cytometry plots are shown in Fig 5.12A. The IFN-γ production by donor $CD4^+$ T cells in 3 weeks FV-infected recipient mice had declined to levels similar to that observed by donor $CD4^+$ T cells transferred to naïve mice (Fig 5.12B).

Fig 5.11 shows that FV-specific $CD4^+$ TCRβ-tg T cells after transfer into mice infected with FV for 1, 2, or 3 weeks were substantially activated and acquired an effector phenotype in response to FV antigen. However, IFN-γ production was diminished in $CD4^+$ TCRβ-tg T cells transferred to mice infected with FV for 3 weeks. Our group's previous work demonstrate a similar dysfunction of FV specific $CD8^+$ T cells, which although have an effector phenotype are unable to produce IFNγ and the cytolytic molecules perforin, granzyme A, and granzyme B following adoptive transfer into persistently infected mice (Dittmer et al., 2004; Zelinskyy et al., 2005). Hence, to quantify IFN-γ producing effector FV-specific $CD4^+$ TCRβ-tg T cells in infected mice, we performed a co-staining experiment for IFN-γ and CD43 activation marker. A significant number of donor $CD4^+$ T cells re-isolated from recipient mice infected for 1 or 2 weeks had an effector phenotype and produced IFN-γ. Surprisingly, in spite of sufficient activation of donor $CD4^+$ T cells re-isolated from mice infected with FV for 3 weeks, their ability to produce IFN-γ was strongly reduced (Fig 5.12C). Thus, although FV-specific $CD4^+$ T cells expanded and up-regulated activation marker following adoptive transfer into acutely infected mice, the cells were functionally impaired at 3 weeks post infection.



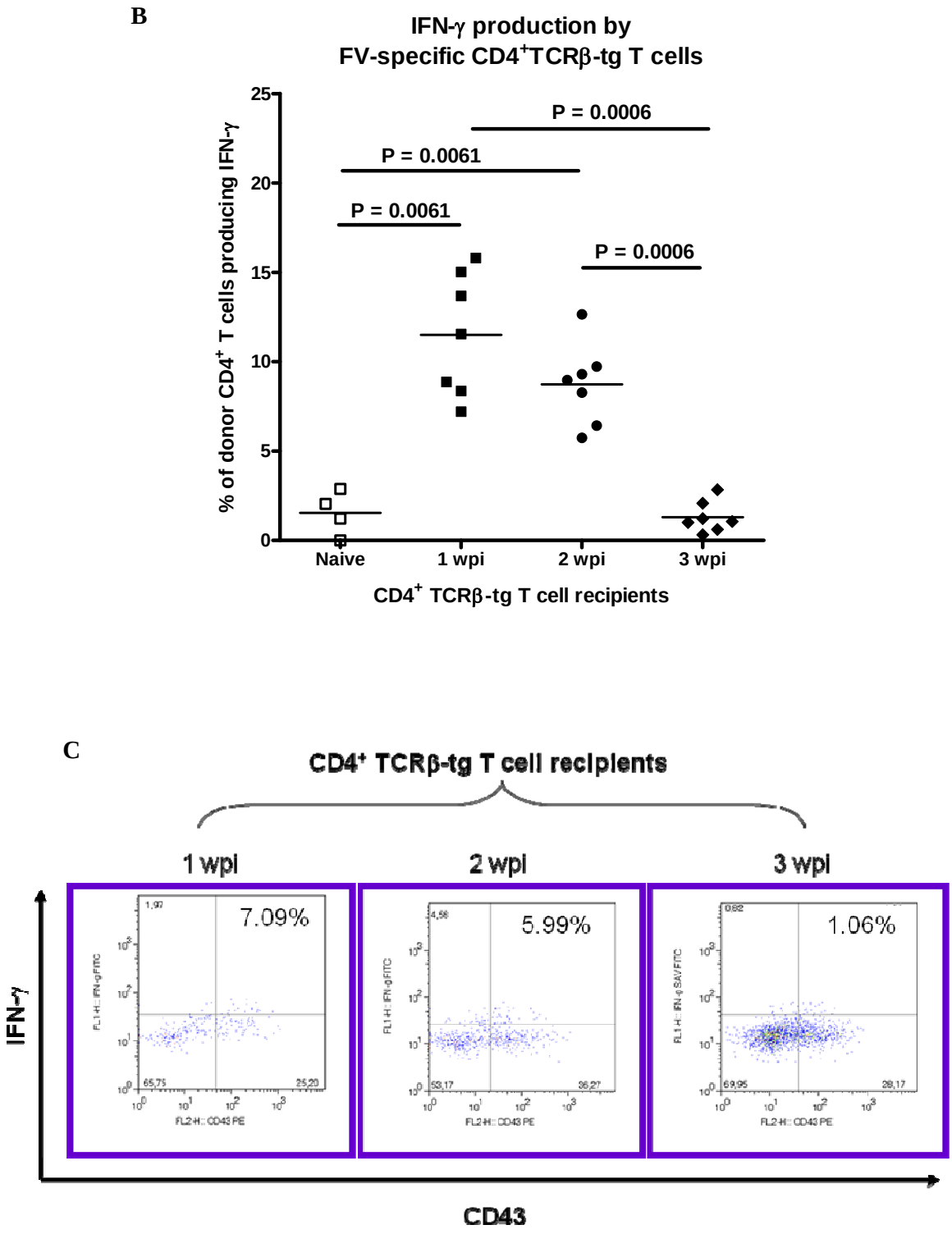


Fig 5.12: Functional analysis of adoptively transferred CD4$^+$ TCRβ-tg T cells

High recovery (H-2A$^{b/b}$) (C57BL/10 x A.BY) F1 mice expressing CD45.2 were acutely infected with FV either for 1 week (■), 2 weeks (●) or 3 weeks (♦). 7.5 x 10^6 CD4$^+$ TCRβ-tg cells from CD4 TCRβ-tg mice expressing CD45.1 were transferred into FV-infected recipient mice and naïve mice (□). One week after transfer, CD4$^+$ T cells from spleens of FV-infected recipient mice were re-isolated and intracellular IFN-γ was measured on gated donor (CD45.1) CD4$^+$ T cells. A) IFN-γ production by donor FV-specific CD4$^+$ TCRβ-tg T cells in a representative mouse. B) The graph

shows percentage of donor $CD4^+$ T cells producing IFN-γ after transfer into FV-infected recipient mice. The mean percentages are indicated by a line. P values were determined by the Mann-Whitney U test for statistical analysis and are shown in the figures. Each dot represents an individual mouse. C) Donor $CD4^+$ T cells co-expressing IFNγ and CD43 in a representative mouse. (A & C) Percentages of positive cells (upper right quadrant) are given on the dot blots. (A-C) Experiments were repeated three times with comparable results.

Given the rapid expansion and activation of the adoptively transferred $CD4^+$ T cells in response to their cognate viral antigen, it was of interest to determine whether the active transferred cells had any effect on viral loads during acute FV infection. Furthermore, due to the suppression in IFN-γ production of $CD4^+$ TCRβ-tg T cells transferred into mice infected with FV for 3 weeks, it was crucial to know their impact on viral replication. At 1 week post-transfer, the average infectious center levels in mice infected with FV for 1 week were reduced from 3 x 10^7 cells per spleen to 4 x 10^5 cells per spleen, a reduction of 100-fold (Fig 5.13). Similarly, approximately 75-fold reduction in viral loads was observed in 2 weeks FV-infected mice that received $CD4^+$ TCRβ-tg T cells (Fig 5.13). On the contrary, $CD4^+$ TCRβ-tg T cells transferred into mice infected with FV for 3 weeks did not have the capacity to reduce viral loads *in vivo* (Fig 5.13).

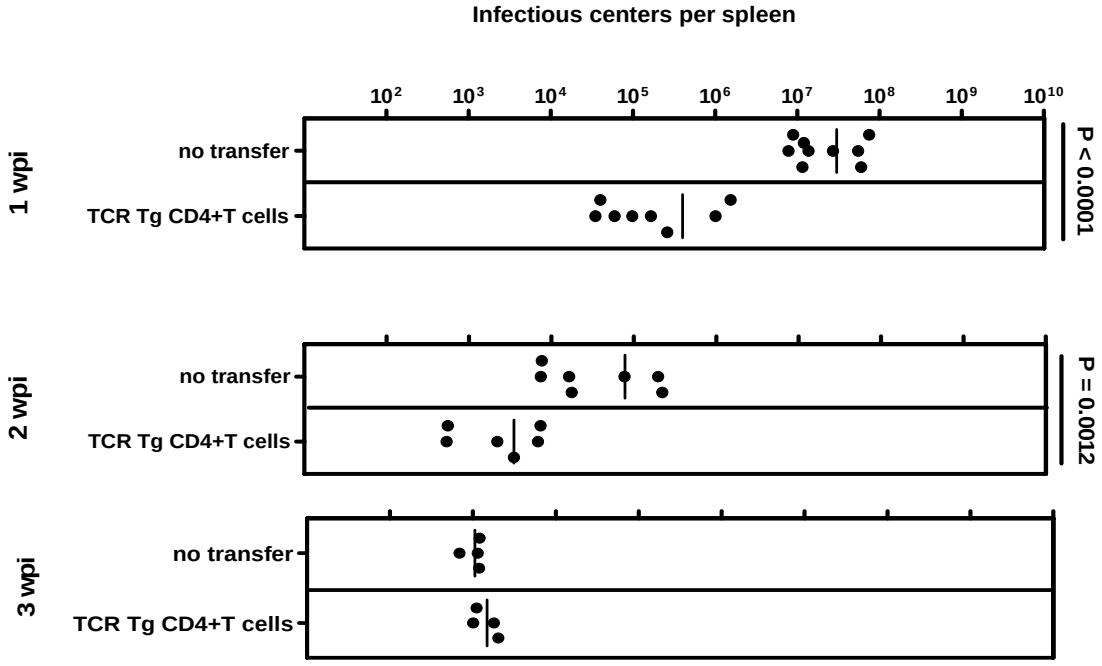


Fig 5.13: Infectious center levels after adoptive transfer of FV-specific $CD4^+$ TCRβ-tg cells

High recovery ($H\text{-}2A^{b/b}$) (C57BL/10 x A.BY) F1 mice expressing CD45.2 were acutely infected with FV either for 1 week, 2 weeks or 3 weeks. 7.5 x 10^6 $CD4^+$ TCRβ-tg cells from CD4 TCRβ-tg mice expressing CD45.1 were transferred into FV-infected recipient mice. Spleen cell suspensions were

plated onto indicator cells to produce infectious centers as described in Materials & Methods. Each dot represents an individual mouse. The mean viral loads are indicated by a line. P values for the difference in means (log10) were determined by the Mann-Whitney U test for statistical analysis and are shown in the figures. The experiment was repeated two times with comparable results.

These results indicated that the $CD4^+$ TCRβ-tg T cells were effective at reducing viral loads in mice infected with FV for 1 or 2 weeks but ineffective at reducing viral loads in 3 weeks FV-infected mice. Thus, although the FV-specific $CD4^+$ T cells expanded and upregulated the activation marker CD43 following adoptive transfer into acutely infected mice, the cells were functionally impaired in mice infected with FV for 3 weeks resulting in loss of their anti-viral activity.

5.5 Suppression of FV-specific $CD4^+$ T cell responses by Regulatory T cells

The functional impairment in FV-specific $CD8^+$ T cells during chronic infection is mediated by $CD4^+$ $Foxp3^+$ regulatory T (Treg) cells, which already expand during the late phase of acute FV infection (2 - 3wpi) (Dittmer et al., 2004; Zelinskyy et al., 2006). In the current study, we speculated that the low frequency of IFNγ-producing FV-specific $CD4^+$ T cells at 3wpi could also be a result of CD4 T cell dysfunction by Treg cells. To determine whether Treg cells were inhibiting the anti-viral functions of virus-specific $CD4^+$ T cells, we used a mouse model in which Foxp3 expressing Treg cells can be experimentally depleted. Injection of diphtheria toxin (DT) into transgenic DEREG mice (Lahl et al., 2007) that express the DT receptor under the control of the Foxp3 promoter can selectively deplete $Foxp3^+$ Treg cells *in vivo*. High recovery DEREG mice expressing CD45.2 were crossed with A.BY mice and these F1 generation mice were termed as DEREG.Y10.

Down-regulation of IFNγ responses in donor $CD4^+$ T cells was observed in mice infected with FV for 3 weeks. Therefore, for the current study, naïve FV-specific $CD4^+$TCRβ-tg $CD4^+$ T cells were transferred into DEREG.Y10 mice infected with FV for 3 weeks. At the same time, FV-infected recipient mice also received Diphtheria toxin (DT) intraperitoneal injections. After 1 week posttransfer, $CD4^+$TCRβ-tg T cells were re-isolated from FV-infected DEREG.Y10 mice to examine the impact of Treg depletion on IFN-γ production and viral loads in spleen (Fig 5.14). Effectiveness of Treg depletion was assessed to verify

whether injection of DT at regular intervals for one week leads to selective depletion of $Foxp3^+$ Treg cells *in vivo*. On day 7, a complete obliteration in the frequency of $CD4^+Foxp3^+GFP^+$ Treg cells in DT-treated DEREG.Y10 mice was observed (Fig 5.15). Thus, Treg cells could be depleted very efficiently in DEREG.Y10 mice. However, a resident population of $CD4^+Foxp3^+GFP^-$ Treg cells was always present in all the DT-treated DEREG.Y10 mice in spite of different alterations in the DT treatment protocol. This was because not all of the Foxp3 expressing Treg cells in DEREG.Y10 mice are transgenic and therefore these non-transgenic $CD4^+Foxp3^+GFP^-$ Treg cells cannot be depleted by DT injection.

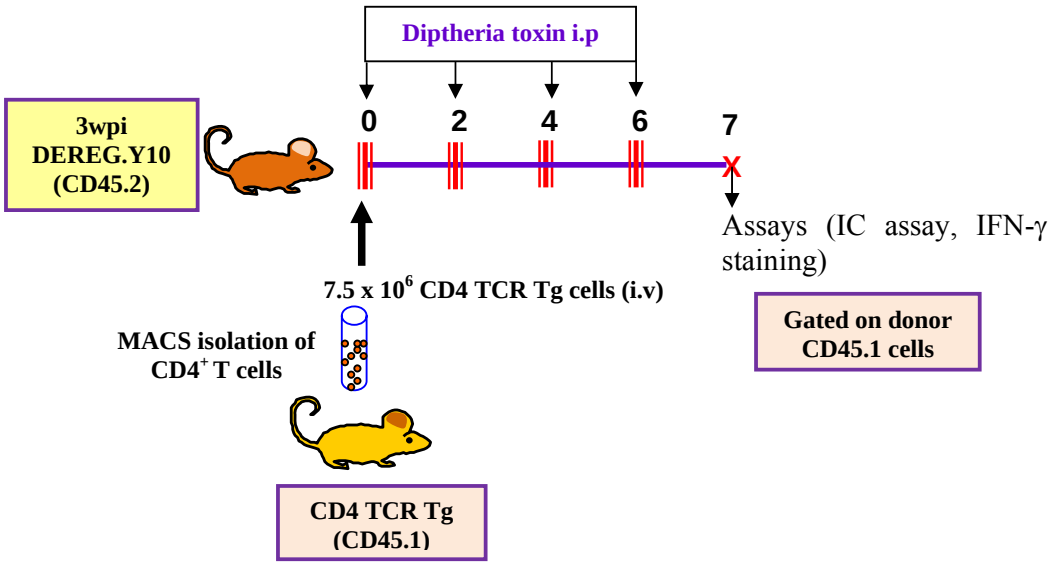


Fig 5.14: Protocol for adoptive transfer and Treg depletion with DT

High recovery ($H\text{-}2A^{b/b}$) DEREG.Y10 mice expressing CD45.2 were acutely infected with FV for 3 weeks. 7.5 x 10^6 $CD4^+$ TCRβ-tg T cells from CD4 TCRβ-tg mice expressing CD45.1 were transferred into FV-infected recipient mice. Simultaneously, FV-infected DEREG.Y10 mice also received DT on alternate days for 1 week. 7 days posttransfer, $CD4^+$ TCRβ-tg T cells from FV-infected mice were re-isolated and assayed for IFN-γ production. In addition, infectious centers in the spleen were determined.

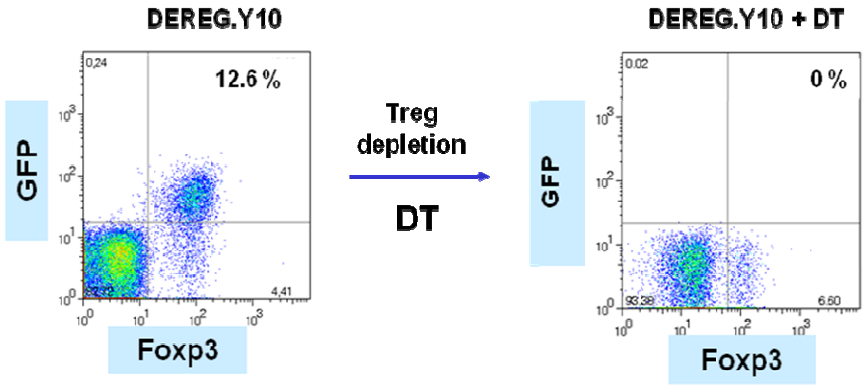
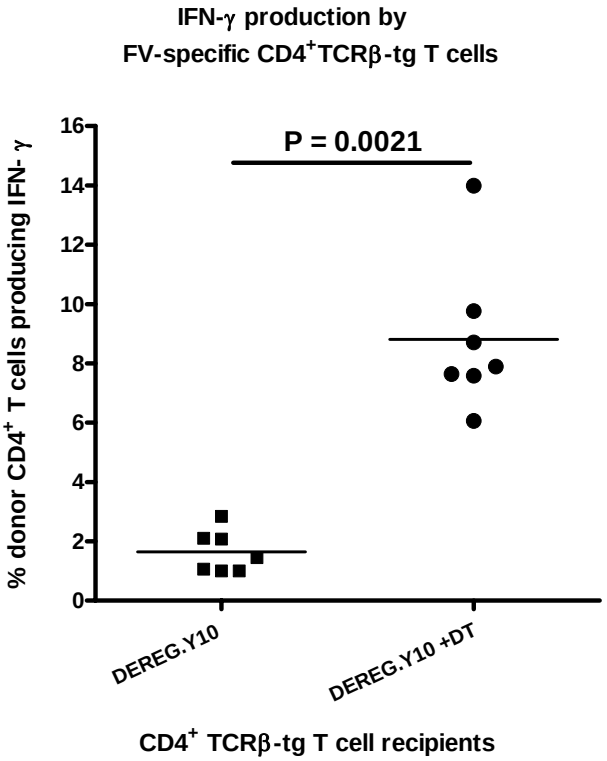


Fig 5.15: Efficiency of Treg depletion in DEREG.Y10

High recovery (H-2A$^{b/b}$) DEREG.Y10 mice were injected with DT on alternate days for 1 week. Spleen cells were stained for intracellular Foxp3 production and gated on CD4$^+$ T cells. Loss of Treg cells (CD4$^+$Foxp3$^+$GFP$^+$ T cells) on day 7 in a representative DEREG.Y10 mouse treated with DT is shown. Percentages of CD4$^+$Foxp3$^+$GFP$^+$ T cells (upper right quadrant) are given on the dot blots.

CD4$^+$ TCRβ-tg T cells transferred into DEREG.Y10 mice infected with FV for 3 weeks and treated with DT was associated with a significant rise in the percentage of IFN-γ producing CD4$^+$ T cells of both host and donor origin compared to non-depleted mice (Fig 5.16A and 5.16B). Thus, removal of Treg cells in DEREG.Y10 mice infected with FV for 3 weeks restored the loss in IFN-γ production by CD4$^+$ T cells. Hence, it is clearly evident that Treg cells play a significant role in suppressing immune functions of Th cells in the late phase of acute FV infection.

A


IFN-γ production by
FV-specific CD4+TCRβ-tg T cells
P = 0.0021
% donor CD4+ T cells producing IFN- γ
16
14
12
10
8
6
4
2
0
DEREG.Y10
DEREG.Y10 +DT
CD4+ TCRβ-tg T cell recipients

B

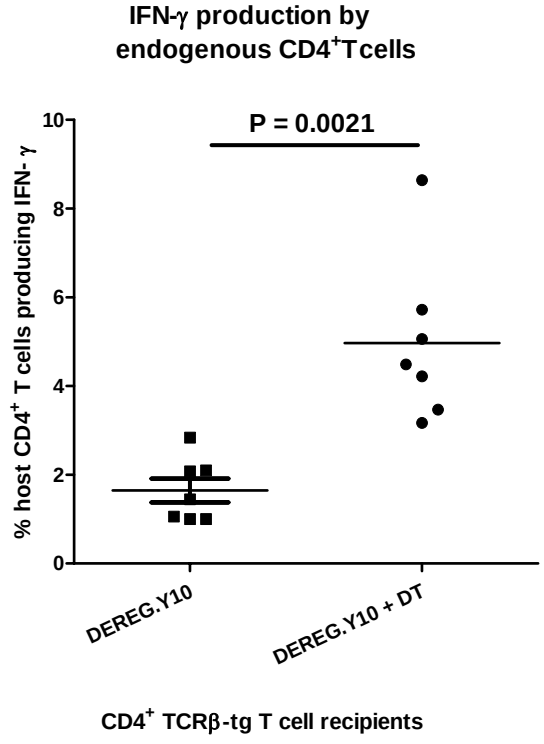

IFN-γ production by
endogenous CD4+Tcells
P = 0.0021
% host CD4+ T cells producing IFN- γ
10
8
6
4
2
0
DEREG.Y10
DEREG.Y10 + DT
CD4+ TCRβ-tg T cell recipients

Fig 5.16 IFN-γ production by CD4$^+$ T cells in DT treated FV-infected DEREG.Y10 mice
High recovery (H-2A$^{b/b}$) DEREG.Y10 mice expressing CD45.2 were acutely infected with FV for 3 weeks. 7.5 x 10^6 CD4$^+$ TCRβ-tg T cells from CD4 TCRβ-tg mice expressing CD45.1 were transferred into FV-infected recipient mice. Simultaneously, mice also received DT on alternate days for 1 week. 7 days posttransfer, (A) CD4$^+$ TCRβ-tg T cells from FV-infected mice were re-isolated from the spleen and assayed for IFN-γ production. The graph shows percentage of donor CD4$^+$ T cells producing IFN-γ. B) The graph shows percentage of endogenous (CD45.2$^+$) IFN-γ producing CD4$^+$ T cells of the host. Each dot represents an individual mouse. The mean percentages are indicated by a line. P values were determined by the Mann-Whitney U test for statistical analysis and are shown in the figures. The experiment was repeated two times with comparable results.

We observed that adoptively transferred CD4$^+$ TCRβ-tg T cells into (C57BL/10 x A.BY) F1 mice infected with FV for 3 weeks had no influence on viral loads (Fig 5.13). Since IFN-γ production by CD4$^+$ T cells was augmented following depletion of Treg cells, it was of interest to determine the outcome of Treg depletion on viral loads after transfer of FV-specific CD4$^+$ TCRβ-tg T cells. At 1 week post-transfer and DT treatment, the average infectious center levels in DEREG.Y10 mice infected with FV for 3 weeks were reduced from 1.479 x 10^7 per spleen to 1.194 x 10^4 per spleen, a reduction of approximately 1000-fold (Fig 5.17). Thus, Treg cells expanding during late phase of acute FV infection, down-regulate anti-viral functions of Th cells aiding viral replication.

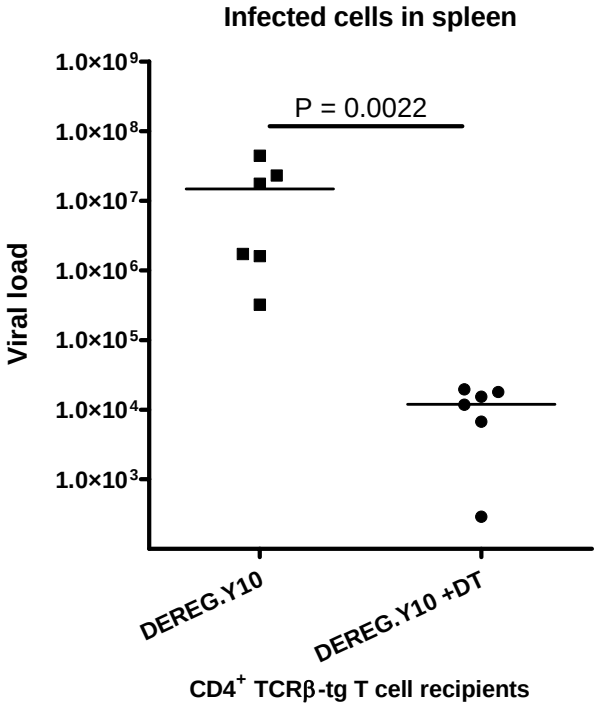


Fig 5.17: Effect of Treg depletion on viral loads in spleen

High recovery (H-2A$^{b/b}$) DEREG.Y10 mice expressing CD45.2 were acutely infected with FV for 3 weeks. 7.5 x 10^6 CD4$^+$ TCRβ-tg T cells from CD4 TCRβ-tg mice expressing CD45.1 were transferred into FV-infected recipient mice. Simultaneously, DEREG.Y10 mice also received DT on alternate days for 1 week. 7 days posttransfer, spleen cells from FV-infected mice were re-isolated from the spleen and assayed for viral loads. Spleen cell suspensions were plated onto indicator cells to detect infectious centers as described in Materials & Methods. Each dot represents an individual mouse. The mean viral loads are indicated by a line. P values were determined by the Mann-Whitney U test for statistical analysis and are shown in the figure. Experiment was repeated two times with comparable results.

In our current study, we established that Th cells were critical for recovery from acute Friend retroviral infections and CD4 T cell help was important in maintaining CD8$^+$ T cell and neutralizing antibody responses. Furthermore, we also demonstrated that the magnitude of Th cell responses in FV resistant mice was much higher than in FV susceptible mice owing to the different genetic background. Adoptively transferred FV-specific Th cells were able to produce anti-viral IFN-γ and reduce viral loads in recipient mice. However, at 3 wpi, Th cells with effector phenotype lost their ability to produce IFN-γ and to control viral replication. Suppression in IFN-γ production by CD4$^+$ T cells was due to the contemporaneous presence of Tregs which when experimentally eliminated can lead to increased antiviral activity of CD4$^+$ T cells. In conclusion, we could corroborate that regulatory T cells can cause immunosuppression of helper T cell effector functions and contribute towards retroviral persistence.

6. DISCUSSION

Helper T-cells (Th) represent a critical and integral part of a functional immune system and is well characterised in many viral infections but its role in retroviral immunity is poorly understood. The focus of this study was to elucidate the contribution of Th cells during acute FV infections in terms of recovery from FV induced disease. *In vivo* depletion of $CD4^+$ T cells underscored the vital role of Th cells in controlling viral spread and onset of FV-induced erythroleukemia. High recovery (C57BL/10 x A.BY) F1 mice depleted of $CD4^+$ T cells during acute FV infection were unable to recover from FV-induced splenomegaly and lethal leukemia. However, with increasing duration of infection, non-depleted control mice were able to resolve the infection and had reduced spleens unlike CD4-depleted mice, which had grossly enlarged spleens at that time point. This correlated with very high viral loads in CD4-depleted mice when compared to non-depleted mice. It has been previously shown for persistent FV infections that depletion of $CD4^+$ T cells resulted in a marked increase in FV-induced disease. The viral titers from CD4-depleted persistently infected mice has been shown to be higher than titers from non-depleted mice. However, CD8 depletions doesn't generate any relapse of FV-induced disease (Hasenkrug, Brooks, and Dittmer, 1998). Hence, control of persistent FV infection is CD8-independent and solely dependent upon CD4-mediated effector responses. On the other hand, recovery from acute FV infection requires strong effector responses elicited by CD8-positive cytotoxic T lymphocytes ($CD8^+$ CTLs) that control viral replication (Zelinskyy et al., 2004). Our results reveal that in spite of intact $CD8^+$ T cell responses in acute FV infections, removal of $CD4^+$ T cell compartment in high recovery mice renders them susceptible to FV-induced disease. Thus, $CD4^+$ T cells are crucial for controlling FV replication during acute infections and persistent infections.

In the current study, $CD4^+$ T cells were also shown to be critical in maintaining $CD8^+$ T cell and neutralizing antibody responses. $CD4^+$ T cells provide "help" to a number of key immune responses of CD8 T cells and B cells involved in antiviral immunity. The role of $CD4^+$ T cells in aiding CD8 T cell responses remain a controversial field. $CD4^+$ T cell mediate help for efficient CD8 T cell responses including clonal expansion, development of effector function and the generation of long-term memory. However, in our current work, the absence of CD4 T cells during the early phase (upto 2 weeks) of acute FV infection did not influence the FV-specific $CD8^+$ T cell response in FV-infected mice. Along these lines, it has been well documented for other murine infections of *Lymphocytic choriomeningitis virus* (LCMV),

Ectromelia virus, *Vaccinia virus* (VV), *Listeria monocytogenes* (*L. monocytogenes*) that CD4 T-cell help is not needed during the priming phase of $CD8^+$ T cell responses (Buller et al., 1987; Janssen et al., 2003; Novy et al., 2007; Shedlock and Shen, 2003; Sun and Bevan, 2003). This lack of requirement for $CD4^+$ T cells during the programming phase of acute infection is probably because most pathogens elicit strong inflammatory stimuli to directly activate APCs and induce the production of inflammatory cytokines (Buller et al., 1987; Wu and Liu, 1994). In contrast, primary CD8 T cell responses to some pathogens such as *Adenovirus*, *Influenza virus*, *Herpes simplex virus* (HSV-1), *L. monocytogenes*, are dependent on help from $CD4^+$ T cells (Marzo et al., 2004; Riberdy et al., 2000; Smith et al., 2004; Yang et al., 1995). Interestingly, we have demonstrated here that during the late phase of acute infection, deficiency of $CD4^+$ T cells in FV-infected mice affected the maintenance of FV-specific effector $CD8^+$ T cells. A recent work implicated that the diminished clonal expansion of $CD8^+$ T cells in response to primary vaccinia virus infection in the absence of $CD4^+$ T cells is caused by poor survival of activated CD8 T cells *in vivo*.

Th cells are also required during the maintenance phase of the long-lived memory $CD8^+$ T cell pool (Buller et al., 1987; Janssen et al., 2003; 2007; Shedlock and Shen, 2003; Sun and Bevan, 2003). In line with this theory, we provided evidence that during the late acute phase of FV infection, mice lacking $CD4^+$ T cells had poor expansion of FV-specific memory $CD8^+$ T cells expressing the memory cell marker CD127. CD4 T-cell help provided during the primary response to infections like LCMV, VV, *L. Monocytogenes*, delivers the necessary "instructive signals" for the generation of fully functional memory CD8 T cells that respond rapidly to secondary challenge (Janssen et al., 2003; Shedlock and Shen, 2003). Furthermore, CD4 T-cell help is critical to sustain $CD8^+$ CTL responses during chronic viral infection. Helper T-cell deficient hosts infected by persistent LCMV undergo functional deterioration and deletion of antiviral $CD8^+$ T cells (Matloubian, Concepcion, and Ahmed, 1994). Additionally, $CD8^+$ T cell-mediated control of chronic murine gammaherpesvirus 68 (MHV-68) infection is eventually lost in the absence of $CD4^+$ T cells (Cardin et al., 1996). In HIV-1 infection, virus-specific $CD8^+$ T cells can be maintained in the peripheral circulation at high frequency in the absence of circulating $CD4^+$ T cells. However, their ability to secrete IFN-γ in response to virus-specific stimulation is compromised due to lack of help from $CD4^+$ T cells (Spiegel et al., 2000). It needs to be further investigated in our retrovirus infection model whether diminished expansion of FV-specific effector $CD8^+$ T cells was due to decreased survival of activated $CD8^+$ T cells in the absence of $CD4^+$ T cells. In addition,

future studies are required to elucidate the importance of CD4 T-cell help in the generation of functional FV-specific memory CD8 T cells during both primary and secondary FV infection.

Induction of anti-viral neutralizing antibodies are an important mode of defense against most viruses in conjunction with cellular immune responses. Lack of HIV-1 specific Th cells in established HIV-1 infection hampers the host's body's ability to generate effective antibody responses to the virus as reviewed in (Pantaleo, 2001). Lack of CD4 T-cell help resulted in significant loss of FV-specific neutralizing antibodies during acute FV infections. CD4 T-cell dependent humoral immune response is also essential for clearance of *Influenza virus*, *mouse polyoma virus* (PyV), *Vesicular stomatitis virus* (VSV), HSV and VV infections (Kemball et al., 2007; Maloy et al., 1999; Maloy et al., 2000; Manickan et al., 1995; Spriggs et al., 1992). Priming of multiple effector arms of the immune system including CD8, CD4 and virus-neutralizing antibodies are vital for effective sterilizing immunity against FV (Dittmer, Brooks, and Hasenkrug, 1999). In this context, the requirement of all three effectors for FV immunity is put into perspective by our current work. It is apparent that B cell and CD8 T cell responses were influenced by CD4 T cell help for recovery from FV induced disease during acute infections, thereby strengthening the requirement of CD4 T cells in FV immunity.

Recovery from FV-induced disease depends upon H-2A genotype, which influences FV-specific $CD4^+$ T cell responsiveness against FV (Chesebro, Miyazawa, and Britt, 1990). The exact mechanism of H-2A genotype associated recovery from FV is not precisely understood. Here we used MHC class-II tetramers to characterize and compare the kinetics of $CD4^+$ T cell responses against FV in high and intermediate recovery mice. It is for the first time that FV-specific effector Th cell responses are quantified using MHC class-II tetramer technology. Our study extends earlier results to show that resistance to FV-induced disease correlated with the magnitude of H-2A-restricted $CD4^+$ T cell responses (Chesebro, Miyazawa, and Britt, 1990). Frequency of tetramer-positive CD4 T cells in high and intermediate recovery mice followed similar kinetics during acute FV infection. However, the magnitude of FV-specific $CD4^+Tet^+$ T cell responses in high recovery mice was much stronger than in intermediate recovery mice. This is because high recovery mice with two $H\text{-}2^b$ alleles are able to present more CD4 epitopes than intermediate recovery mice, which have only one $H\text{-}2^b$ allele. In persistent PyV infection, $CD4^+$ T-cell mediated immune responses are variable among allogeneic strains of inbred mice (Kemball, Szomolanyi-Tsuda, and Lukacher, 2007).

In other infectious diseases, like *Porphyromonas gingivalis* (Gemmell et al., 2002) and *Toxoplasma gondii* (Liesenfeld et al., 1996) infections, CD4 T-cell mediated immune responses are also shown to be dependent on MHC genes. For humans, variation at immune gene loci (HLA class I and II genes) determine the clinical course and outcome of HIV-1, HCV and HBV infections. For instance, HLA-B*27 and HLA-B*57 patients are associated with clearance of HCV infection and delaying AIDS while HLA-B*35 accelerates AIDS and HLA-DRB1*0701 is associated with HCV persistence. Additionally, HLA-A*0301 is associated with HBV clearance unlike HLA-B*08, which is associated with virus persistence as reviewed in (Martin and Carrington, 2005). Thus, strategies for vaccine development need to consider genetic diversity of immune response genes that can influence resistance or susceptibility to several different diseases.

Previous studies have investigated the kinetics of FV-specific effector $CD8^+$ T cells expansion in high recovery mice. Interestingly, peak tetramer-positive CD8 T cell responses is far greater in magnitude (Robertson et al., 2007; Zelinskyy et al., 2006) than peak tetramer-positive CD4 T cell responses observed in our studies. Using class I and class II tetramers, it has been shown for LCMV (Homann, Teyton, and Oldstone, 2001) and *Moloney murine sarcoma and leukemia virus complex* (MoMSV) (Schepers et al., 2002) infections that the burst size of the virus-specific $CD8^+$ T cell response is considerably larger (~ 20 - 40) times than that of virus-specific $CD4^+$ T cells. Clonal expansion of $CD8^+$ T cells is more profound than $CD4^+$ T cells as seen in infections like HIV, *Epstein-Barr virus* (EBV), LCMV, *influenza virus*, *Tetanus toxoid* (TT). This is mainly because better expansion of $CD8^+$ clones are being required to achieve efficient direct CTL killing, whereas the effects of $CD4^+$ T cells are potentiated via cytokine production by these cells (Maini et al., 1999). Moreover, clonal expansion of $CD4^+$ T cells is more tightly regulated by homeostatic control molecules such as Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) (Chambers, Sullivan, and Allison, 1997). Thus, it appears that induction of $CD4^+$ and $CD8^+$ T-cell responses differ in both activation requirements and in magnitude due to their differential clonal expansion rate.

Apart from being the “Helper” arm of the immune system, $CD4^+$ T cells have a direct anti-viral role in secreting cytokines such as IFN-γ and TNF-α with effector functions. IFN-γ production by $CD4^+$ T cells is a key component in the control of persistent FV infection in mice. One additional mechanism for control of persistent FV by $CD4^+$ T cells is possibly by cytolytic activity as demonstrated *in vitro* (Iwashiro et al., 2001b). In the current study, we

investigated the anti-viral effect of FV-specific Th cell response using CD4 TCRβ transgenic mice (CD4 TCRβ-tg) specific for the F-MuLV envelope epitope. Quantification of adoptively transferred FV-specific CD4 TCRβ-tg T cells into acutely infected mice display rapid expansion and activation of donor T cells in response to FV antigen. Donor FV-specific effector $CD4^+$ T cells displayed a rapid rise in IFN-γ levels until 2 weeks post infection. This correlated well with a significant reduction in viral loads in mice that received FV-specific CD4 TCRβ-tg T cells. Effector function of $CD4^+$ T cells via IFN-γ production is also observed in Hepatitis B virus (HBV) transgenic mouse model (Franco et al., 1997). IFN-γ production by HIV-1 specific $CD4^+$ T cells is associated with control of viremia in HIV-infected individuals that did not progress to AIDS (Rosenberg et al., 1997). However, the effector functions of FV-specific $CD4^+$ T cells was lost 3 weeks post FV infection with a dramatic drop in the frequency of IFN-γ producing FV-specific $CD4^+$ T cells. As a result of which, the adoptively transferred FV-specific $CD4^+$ T cells were incompetent to reduce viral loads. Thus, although FV-specific $CD4^+$ T cells expanded and up-regulated activation marker following adoptive transfer into acutely infected mice, the cells were functionally impaired at 3 weeks post infection. A similar down-regulation in effector functions has been already shown for FV-specific $CD8^+$ T cells with effector phenotype that are functionally suppressed during chronic FV infection. These cells fail to produce IFNγ and the cytolytic molecules perforin, granzyme A, and granzyme B (Dittmer et al., 2004; Zelinskyy et al., 2005), which are critical for the control of FV infection in resistant mice (Zelinskyy et al., 2004). Consequently, $CD8^+$ T cells from chronically infected mice are unable to degranulate and kill FV-labelled target cells *in vivo* (Zelinskyy et al., 2005).

It is interesting that although FV-specific CD8 and CD4 T cells have anti-viral effects during FV infection, mice are never able to completely clear the virus. During an ongoing acute infection, viruses adopt several strategies to evade immunesurveillance to survive in their host for life. Suppression of immune responses is one of the most common tactics efficiently used by viruses for its benefit. Measles virus infection displays inhibition of activation and proliferation of T cells or directly affecting their viability by means of viral proteins as reviewed in (Schneider-Schaulies and Dittmer, 2006). Primary SIV infection in rhesus monkeys also induces loss of IFN-γ and IL-2 production in $CD4^+$ T cells, which is associated with both a selective depletion of memory $CD4^+$ T cells and a loss of the functional capacity of the memory $CD4^+$ T cells (Sun et al., 2007). “CD8 T cell exhaustion” is postulated as the

reason for loss of effector function in persistent LCMV infection (Onami et al., 2002). Our current work on CD4 dysfunction in acute FV infection and previous studies on CD8 dysfunction in persistent FV infection (Dittmer et al., 2004), show that T cell dysfunction seem to be a common feature in many chronic viral infections.

Immunosuppressive $CD4^+$ Treg cells, are known as the primary mediators of peripheral tolerance in autoimmune diseases. However, they also limit beneficial responses by suppressing immune responses and limiting antitumor immunity as reviewed in (Vignali, Collison, and Workman, 2008). Treg cells, which express the common marker CD25 inhibit both HIV- specific $CD4^+$ and $CD8^+$ T cell responses. In general, IFNγ production and T cell proliferation are affected by Treg cells cells in HIV-infected patients (Aandahl et al., 2004; Kinter et al., 2004; Weiss et al., 2004). Evidence from studies on *Hepatitis C virus* (HCV) indicates that the expansion of $CD4^+CD25^+$ Treg cells may account for the suppression of effector $CD8^+$ T cell immunity in patients with persistent viremia (Sugimoto et al., 2003). Other studies have also reasoned expansion of Treg cells as a strategy to suppress anti-retroviral CTL responses (Iwashiro et al., 2001; Vahlenkamp et al., 2004; Dittmer et al., 2004, Estes et al., 2006, Krathwohl et al. 2006). This is true for FV infections where $CD4^+$ $Foxp3^+$ Treg cells expanding during the late phase (2-3weeks) of acute FV infection (Zelinskyy et al., 2006) and are responsible for the functional suppression of $CD8^+$ T cells observed during persistent FV infections (Dittmer et al., 2004). Interestingly, FV-infected dendritic cells (DC) can mediate expansion of Treg cells with immunosppressive potential. FV infection of DCs led to a defect in DC maturation as infected cells expressed very little co-stimulatory molecules. Although, naïve T cells were still activated by FV-infected DC, this activation did not result in antigen-specific T cell proliferation thereby inducing a state of tolerance rather than immunity (Balkow et al., 2007).

In our study, the down-regulation in IFN-γ production by FV-specific $CD4^+$ T cells was observed at 3 weeks post infection, around the same time of peak expansion of Treg cells. In order to elucidate the role of Tregs in inhibiting $CD4^+$ T cell responses, we used a transgenic mouse model, in which Treg cells can be specifically depleted (DEREG). In our current study, we clearly demonstrated that DEREG mice infected with FV for 3 weeks had a sharp augmentation in IFN-γ production by FV-specific $CD4^+$ T cells after DT-induced depletion of $Foxp3^+$ Treg cells. This correlated well with a reduction in infectious titers in the spleens of DT-treated DEREG mice. In contrast, a very recent paper reported that ablation of Treg cells

in HSV-2-infected mice resulted in an accelerated fatal infection with increased viral loads instead of enhanced immunity to the virus (Lund et al., 2008). The authors also used a mouse model in which they could deplete Treg cells by DT injection. Depletion of Treg cells amplified the immune responses in draining lymph nodes at the site of infection, but delayed the entry of the immune cells into the HSV-infected tissue. Thus, the authors postulated an immune response promoting role for Treg cells in acute infections in which the site of pathogen replication is in non-lymphoid tissues. In contrast to these findings, our study using FV-infected DEREG mice provide first experimental evidence that Treg cells were directly involved in suppression of anti-viral $CD4^+$ T cell responses during an acute retroviral infection. The suppressive activity of Treg cells on antiviral immunity is the predominant effect in viral infections in which the primary targets of the virus are cells of the lymphoid organs. This suggests that the predominant biological effect of Treg cells in different viral infections can be quite diverse ranging from boosting to suppressing anti-viral immune responses and it depends on the site of viral replication.

Although, the concept of immunosuppressive Treg cells came into being three decades ago, the precise mechanisms underlying Treg-mediated suppression remain elusive. Several mechanisms of suppression have been proposed and one of the mechanism is via production of immunosuppressive cytokines such as Interleukin-10 (IL-10) and Transforming growth factor (TGF-β). In autoimmune diseases, effector functions of T cells are inhibited by IL-10 and TGF-β by blocking the maturation and activation of dendritic cells (DCs) or other antigen presenting cells (APCs) as reviewed in (Vignali, Collison, and Workman, 2008). IL-10 and TGF-β secreted by $CD25^+CD4^+$ Treg cells suppresses IFN-γ production by effector T cells in HIV-infected individuals (Kinter et al., 2004; Weiss et al., 2004). In chronic FV infection, the generation of class I restricted CTL activity was suppressed *in vitro* by additive actions of TGF-β and CTLA-4 expressed on Treg cells (Iwashiro et al., 2001a). A second mechanism used by Treg cells is by inducing cytolysis of effector T cells. For instance, naïve $CD4^+$ $CD45RA^+$ cells of human origin when stimulated *in vitro* with specific antibodies can also develop into Treg cells with cytolytic potential. These cells can kill B cells, NK cells and CTLs in a granzyme-B-dependent and perforin-dependent manner resulting in the suppression of crucial immune responses (Grossman et al., 2004). Additionally, activated Treg cells can induce apoptosis of effector T cells *in vitro* and *in vivo* through a TRAIL-DR5 (tumor-necrosis-factor-related apoptosis-inducing ligand-death receptor 5) pathway (Ren et

al., 2007) and also via up-regulation of galectin-1 (Garin et al., 2007). Another mechanism of apoptosis-mediated suppression is by metabolic disruption of effector T cells induced by Treg cells via IL-2 deprivation mediated apoptosis. Treg cells compete with T cells for consumption of growth and/or survival factors such as IL-2. Treg cells deprives effector T cells of IL-2 leading to the death of effector cells and this mode of suppression can be observed *in vitro* (Pandiyan et al., 2007). However, *in vivo* work using human Treg cells suggest that IL-2 depletion alone cannot render apoptosis of effector T cells since they are not exclusively dependent on IL-2 for its proliferation (Oberle et al., 2007). A third mechanism for Treg-mediated suppression is through expression of the ectoenzymes CD39 and CD73 by Tregs. Concordant expression of CD39 and CD73 catalyzes the production of pericellular adenosine, which resulted in suppression of effector T cell function through activation of the adenosine receptor 2A (Borsellino et al., 2007; Deaglio et al., 2007; Kobie et al., 2006).

A fourth mechanism of suppression is through direct interaction of Treg cells with effector T cells via gap junctions. Natural Treg cells harbor high levels of cyclic adenosine monophosphate (cAMP), a potent inhibitor of T cell proliferation and IL-2 synthesis. Treg cells suppress effector T cell function directly by transferring the potent cAMP into responder T cells through membrane gap junctions (Bopp et al., 2007). A cell-to-cell contact dependent Treg-mediated suppression *in vitro* was also demonstrated for FV infection. $CD4^+CD25^+$ T cells isolated from mice chronically infected with FV can suppress IFN-γ production by activated $CD8^+$ T cells in a direct cell-to-cell contact dependent manner in the absence of APCs (Robertson et al., 2006). Furthermore, FV induced Treg cells express Connexins, which are gap-junction proteins and help in the formation of gap junctions (unpublished data). Thus, direct cell-cell contact dependent mechanism is a novel strategy used by Treg cells to use a classical second messenger to induce suppression of neighbouring T cells through a ubiquitous system of intercellular communication such as gap junction. A fifth mechanism known is that Treg cells can also directly target DCs to modulate the maturation and/or function of DCs that are necessary for the activation of T cells. $CD4^+CD25^+$ Treg cells express CTLA-4 molecules which interacts with CD80 and/or CD86 on the surface of DCs to induce production of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs and is a negative signal for T-cell activation (Fallarino et al., 2003; Mellor and Munn, 2004). Furthermore, Lymphocyte-activation gene 3 (LAG3), a CD4 homologue selectively expressed on activated T cells bind to MHC Class II molecules

expressed by immature DCs inducing suppression of DC maturation and their immunostimulatory capacity (Huang et al., 2004; Workman and Vignali, 2005).

In the context of retroviral infections, it was postulated that infection of DCs leading to defect in maturation is an escape mechanism used by FV to specifically expand $Foxp3^+$Treg cells (Balkow et al., 2007). *In vitro* suppression of CD8 T cells by Treg cells was not mediated by the immunosuppressive cytokines IL-10 (Dittmer et al., 2004; Iwashiro et al., 2001a), but required coordinated effect of TGF-ß and the molecule CTLA-4 (Iwashiro et al., 2001a). Furthermore, FV induced Treg cells didn't express granzymes, thus ruling out granzyme-dependent Treg mediated apoptosis of effector T cells (unpublished data). There is plethora of mechanisms possibly used by Treg cells to render immunosuppression of effector T cells, however, which mechanism is precisely used is still unknown. Investigation of mechanisms responsible for immunosuppression of effector T cells can be simplified by using molecular tools such as gene array technology for screening genes expressed by virus-induced Treg cells. siRNA technology is also a helpful tool in knocking down the genes of interest in these assays to determine the molecular mechanisms underlying Treg-mediated T-cell dysfunction.

Our studies have established a critical role for $CD4^+$ T cells in controlling viral spread and onset of erythroleukemia in an indirect manner through the maintenance of CD8 and neutralizing antibody responses during acute FV infection. A direct anti-viral role for $CD4^+$ T cells using FV-specific transgenic mouse model has been shown during the early phase of FV infection. Loss of anti-viral effects of $CD4^+$ T cells occurring in the later phase was directly associated with the contemporaneous presence of Treg cells thereby contributing towards viral persistence. Depletion of Treg cells during FV infection resulted in an augmented anti-viral T cell response and superior control of virus replication. Hence, down-regulation of Treg responses during infections with lymphotropic viruses such as FV might still be an interesting therapeutic approach. However, prolonged deletion of Treg cells has been reported to result in autoimmune diseases in newborn and adult mice (Lahl et al., 2007; Lund et al., 2008) questioning whether a therapy based on suppression of Treg cells or their function would be beneficial. However, the short-term depletion of Treg cells that we applied in the current study did not result in severe immunopathology even during an ongoing anti-viral immune response. The findings presented here have implications for vaccination against viruses that escape immunesurveillance to establish persistent infections. Our results should encourage researchers to develop new reagents to manipulate Treg and Th cells *in vivo* that

may then be used for the therapy of acute or chronic infections with lymphotropic viruses. Additionally, our results also highlight the importance of genetic background in induction of potent CD4 T cell immunity. Therefore, apart from targeting effectors of cellular and humoral immunity, influence of genetic heterogeneity on T and B cell immune responses is central for an effective vaccine in order to confer total protection against infectious pathogens.

SUMMARY

Specific immune T lymphocytes play an important role in the resistance to a variety of viral disease. In the past few years, the Friend retroviral (FV) model has been extensively used to study the mechanisms of immunity against retroviruses. These studies have provided insights into fundamental roles of $CD8^+$ T cells in spontaneous recovery from acute FV infections and Helper T (Th) cells in the control of chronic FV infection. However, prominence of Th cell immunity in acute FV infection was not well-defined. The primary aim of our study was therefore to investigate the role of Th cells in recovery from acute FV infection. *In vivo* depletion of CD4 T cells largely affected the normal recovery process from FV-induced disease in high recovery mice with depleted animals progressing to fatal eryhroleukemia. Absence of CD4 T cells also accelerated viral replication and spread in spite of an intact CD8 T cell compartment. This suggested that recovery from acute FV infections required coordinated CD8 and CD4 T-cell mediated immune responses. In many viral infections, CD4 T cell help is required for the maintenance of CD8 effector responses and expansion of memory CD8 T cells. Thus, FV-infected mice depleted of CD4 T cells were investigated for the influence of Th cells on the levels of effector and memory CD8 T cells. Early recovery from FV infection mediated by CD8 T cells didn't require any help from CD4 T cells but long-term maintenance of effector and memory CD8 T cells was dependent on CD4 T cells. CD4 T cells were also critical for aiding B cells for induction of neutralizing antibodies in response to FV. Genetic heterogeneity of mice influences susceptibility to FV-induced disease. An MHC class-II tetramer based analysis of FV-specific Th cell responses during acute FV infection demonstrated peak tetramer-positive CD4 T cell responses far greater in magnitude in high recovery mice than intermediate recovery mice. Owing to the presence of two $H\text{-}2A^b$ alleles in high recovery mice, they were able to present more CD4 epitopes and therefore induced potent immune responses against FV unlike intermediate recovery mice with only one $H\text{-}2A^b$ allele. FV-specific T-cell receptor (TCR) transgenic CD4 T cells were transferred into FV infected mice to perform a detailed kinetic analysis of anti-retroviral CD4 T cell responses during acute FV infection. Adoptively transferred FV-specific Th cells were able to produce anti-viral IFN-γ and reduced viral loads in recipient mice. However, at 3 wpi, Th cells with effector phenotype lost their ability to produce IFN-γ resulting in loss of their anti-viral activity. The functional impairment of FV-specific CD8 T cells in persistent FV infections was shown to be mediated by Regulatory T (Treg) cells. Treg-mediated immunosuppresion is one of the strategies used by viruses to evade immunesurveillance in

their hosts. In order to dissect the role of Tregs in mediating immunosuppression of FV-specific Th cells, we used DEREG mice in which Treg cells can be experimentally depleted. Absence of Treg cells led to a significant increase in the frequency of IFN-γ producing FV-specific Th cells. Enhancement in IFN-γ production increased the antiviral activity of $CD4^+$ T cells. Thus, Treg cells can cause immunosuppression of Th cell immunity and contribute towards viral persistence. Considered together, our findings imply that therapeutic manipulation of Treg / Th – cell number and/or function could improve immune control of retroviral infections. Strategies for vaccine development also need to take into account the genetic diversity of MHC genes that can influence resistance or susceptibility to several different infectious diseases.

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9. Appendix

9.1. Abbreviations

Abbreviations	Full name
7AAD	7-Amino-Actinomycin D
AEC	3-Amino-9-Ethyl-Carbazol
AIDS	Acquired Immune Deficiency Syndrome
Ab	Antibody
APC	Antigen presenting cells
APC	Allophycocyanin
BSA	Bovine Serum Albumin
°C	Degree Celsius
CD	Cluster of differentiation
CTL	Cytotoxic T cells
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DC	Dendritic T cells
DEREG	Depletion of regulatory T cells
DMEM	Dulbecco's Modified Eagles's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
d.p.i	Days post infection

DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
dsRNA	Double-stranded RNA
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraaceticacid
Env	Envelope protein
FACS	Fluorescence Activated Cells Scanner (Flow cytometer)
FCS	Fetal Calf Serum
FFU	Focus Forming Units
FITC	Fluorescein isothiocyanate
Fig	Figure
F-MuLV	*Friend Murine Leukemia Virus*
Foxp3	Forkhead box P3
FV	*Friend Virus*
g	Gram
Gag	Group specific antigen
GFP	Green fluorescent protein
GM-CSF	Granulocyte-Macrophage colony-stimulating factor
GTP	Guanosine triphosphate
h	Hours

HBV	*Hepatitis B Virus*
HCV	*Hepatitis C Virus*
HSV	*Herpes simplex virus*
HAART	Highly Active Antiretroviral Therapy
H-2	Histocompatibility-2
HIV-1	*Human Immunodeficiency Virus Type 1*
HIV-2	*Human Immunodeficiency Virus Type 2*
HRP	Horse Radish Peroxidase
HLA	Human leukocyte antigen
HTLV-1	*Human T-Cell Leukemia Virus-1*
IAV	*Influenza A Virus*
IFN-γ	Interferon gamma
IFNAR	Interferon-α Receptor
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
JAK	Januskinase
kDa	Kilodalton
l	Liter

LCMV	*Lymphocytic choriomeningitis virus*
L. monocytogenes	*Listeria monocytogenes*
LTR	Long Terminal Repeat
M	Molar
MCMV	*Mouse Cytomegalovirus*
mDCs	Myeloid Dendritic cells
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
ml	Millilitre
µl	Microlitre
mM	Millimolar
MAb	Monoclonal antibody
MMTV	*Mouse mammary tumour virus*
Mo-MuLV	*Moloney-Murine leukemia virus*
PyV	*Polyoma virus*
mRNA	messenger RNA
NFκB	Nuclear Factor κB
NK	Natural Killer cells
ODN	Oligodeoxynucleotide

PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid Dendritic cells
PE	Phycoerythrin
pH	-log $[H^+]$
PKR	Protein kinase R
Pol	Polymerase
PRP	Pathogen Recognition Receptors
qPCR	quantitative PCR
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute Medium 1640
RSV	*Respiratory Syncytial virus*
R.T	Room temperature
SARS	Severe Acute Respiratory Syndrome
SFFU	Spleen Focus Forming Units
SFV	*Simian Foamy Virus*
SHFV	*Simian Haemorrhagic Fever Virus*

SIV	Simian Immunodeficiency Virus
ssRNA	Single stranded-RNA
STAT	Signal Transducer and Activator of Transcription
SV5	*Simian Parainfluenza Virus*
Tet	Tetramer
TCR	T-cell receptor
TGF-β	Transforming Growth Factor-beta
Th	T helper cells
TLR	Toll-Like Receptor
TRAIL-DR5	Tumor-necrosis-factor-related apoptosis-inducing ligand-death receptor 5
TNF-α	Tumor necrosis factor-alpha
tg	Transgenic
Treg	Regulatory T cells
U	Units
VV	*Vaccinia virus*
Vol.	Volume
VSV	*Vesicular Stomatitis Virus*
Y10.A	(B.10A x A.BY)F1-generation
Y10	(B10 x A.BY)F1-generation

9.2. List of figures

9.3. List of tables

PUBLICATIONS

1. Nicole Gerlach, Kathrin Gibbert, Christina Alter, **Savita Nair**, Gennadiy Zelinskyy, Cassandra M. James and Ulf Dittmer. (2009). **Anti-retroviral effects of type I interferon subtypes in vivo.** *Eur J Immunol*. **39**(1):136-46.

2. Shelly J. Robertson, Christoph G. Ammann, Ronald J. Messer, Aaron B. Carmody, Lara Myers, Ulf Dittmer, **Savita Nair**, Nicole Gerlach, Leonard H. Evans, William A. Cafruny, and Kim J. Hasenkrug. **Suppression of acute anti-Friend virus CD8+T cell responses by co-infection with lactate dehydrogenase-elevating virus**. (2008). *J Virol* **82** (1), 408-418.

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2. **Talk and Poster** presentation on "Protective role of $CD4^+$ T cells in acute Friend retroviral infections", 4th Spring school on Immunology in Ettal - Bavaria, Germany (Mar 2nd- Mar 5th 2008).
3. **Talk and Poster** presentation on "Role of $CD4^+$ T cells in acute and persistent Friend retroviral infections", 6^{th} GFV (Gesellschaft für Virologie) workshop on Immunobiology of Viral infections in Deidesheim, Germany (Oct 10^{th} – Oct 12^{th} 2007).
4. **Talk and Poster** presentation on "Role of $CD4^+$ T cells in acute and persistent Friend retroviral infections", 37^{th} Annual Meeting of the German Society for Immunology" in Heidelberg, Germany (Sep 5^{th} - Sep 8^{th} 2007).

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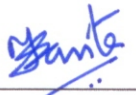

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Erklärungen

Erklärung:

Hiermit erkläre ich, gem. § 6 Ab. 2, Nr. 7 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „The role of $CD4^+$ helper T cells in immunity to acute Friend retroviral infection" zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Savita Nair befürworte.

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Hiermit erkläre ich, gem. § 6 Ab. 2, Nr. 8 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst habe und mich keiner anderer als der angegeben Hilfsmittel bedient habe.

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Erklärung:

Hiermit erkläre ich, gem. § 6 Ab. 2, Nr. 6 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

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