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Studies of early retrovirus-host interactions

Viral determinants for pathogenesis and the influence of sex on the susceptibility to Friend murine leukaemia virus infection.

Dr. philos. thesis 2003
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ACKNOWLEDGMENTS

The present work has been carried out at UNIGEN Center for Molecular Biology and at the Department of Laboratory Medicine, Children's and Women's Health, Faculty of Medicine, The Norwegian University of Science and Technology, Trondheim, Norway.
Financial support given by the Norwegian Cancer Society is gratefully acknowledged.

Especially, I would like to express my great gratitude to

- Are Dalen, my supervisor, for recruiting me, and for believing in me: Your innovative and enthusiastic mind has been a huge inspiration.

- Hong Yan Dai for her supervising during the first years: The "FIS-2 project" would not been possible without you.

- my co-authors Hong Yan Dai, Gunn Irene Troseth, Merete Gunleksrud, Liss Anne Solberg Lavik, Lena Irene Kristiansen, and Harald Aarset for excellent work. I would like to give a special thank to Liss Anne. S. Lavik: Without you, this thesis would never been finished.

-Sidsel Krokstad for her friendship and encouragement through all these years.

Further, I would like to thank all the people who have been connected to the *Virus research group* for interesting years, and the staff at the animal facility in St Olavs Hospital HF for help and skilful animal care.

I also thank everybody at UNIGEN and later at the Department of Cancer research and Molecular medicine for creating such a nice working environment, helping out with problems, and for including me. Personal thanks to Svanhild Schønberg for listening to my frustrations and sharing my joys at the final stages of my thesis; and to Marit Otterlei, Liv Ryan, Lilliand Leistad, Nina Beate Liabakk, Kristind Lund, Heidi Sterten, and, again, Sidsel Krokstad and Liss Anne S. Lavik for sense and nonsense over bottles of vine.

I am grateful to friends and family for their love and support. An immense thank to my mother and father for their never-ending interest and concern. And most importantly, thanks to my dear husband Atle and our two sons, Viktor and Ravn Thor, for being. You have been incredibly patient!

Trondheim, Mai 2003

Torunn Bruland

LIST OF PAPERS

I. **Dai, H.Y., Troseth, G. I., Gunleksrud, M., Bruland, T., Solberg, L. A., Aarseth, H., Kristiansen, L. I. & Dalen, A. (1998).** Identification of genetic determinants responsible for the rapid immunosuppressive activity and the low leukemogenic potential of a variant of Friend leukemia virus, FIS-2. *Journal of Virology* **72**, 1244-1251.

II. **Bruland, T., Dai, H. Y., Lavik, L. A. S. & Dalen, A. (2002).** Early dissemination rates of Friend murine leukaemia virus variants correlate with late pathogenesis. *APMIS* **110,** 899-912.

III. **Bruland, T., Dai, H. Y., Lavik, L. A. S., Kristiansen, L. I. & Dalen, A. (2001).** Gender-related differences in susceptibility, early virus dissemination and immunosuppression in mice infected with Friend murine leukaemia virus variant FIS-2. *Journal of General Virology*. **82**, 1821-1827.

IV. **Bruland, T., Lavik, L. A. S., Dai, H. Y. & Dalen, A. (2003).** A glucocorticoid response element in the LTR U3 region of Friend murine leukaemia virus variant FIS-2 enhances virus production *in vitro* and is a major determinant for sex differences in susceptibility to FIS-2 infection *in vivo*. *Journal of General Virology* **84,** 917-923.

V. **Bruland, T., Lavik, L A. S., Dai, H. Y. & Dalen, A.** Identification of Friend murine retrovirus infected immune cells and studies of the effects of sex and steroid hormones in the early phase of infection. *Submitted to APMIS*

ABBRIVATIONS

Glossary of virology & immunology is enclosed in the appendix section.

AIDS	Acquired immunodeficiency syndrome
AR	androgen receptor
BSA	bovine serum albumin
CAT	chloramphenicol acetyl transferase
ConA	concavalin A
DR	direct repeat
d.p.i	days post inoculation
FCS	foetal calf serum
FIS	Friend ImmunoSuppressive virus (Friend murine leukaemia virus variant)
FIV	Feline immunodeficiency virus
F-MuLV	Friend murine leukaemia virus
FV	Friend leukaemia virus complex
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HIV	Human immunodeficiency virus
IHC	immunohistochemistry
ISH	*in situ* hybridisation
IU	infectious unit
i.p.	intraperitoneally
LP-BM5-MuLV	The MAIDS virus complex
MAIDS	Murine acquired immunodeficiency syndrom
MMTV	Mouse mammary tumour virus
Mo-MuLV	Moloney murine leukaemia virus
MCF	Mink cell focus-inducing virus
NCS	new-born calf serum
NF1	Nuclear factor 1
PBS	phosphate-buffered salin
PFC	plaque-forming cell
p.i.	post infection/inoculation
PR	progesterone receptor
RT	reverse transcriptase
s.c.	subcutanous
SRBC	sheep red blood cell
SIV	Simian immunodeficiency virus
SU	surface glycoprotein
TGF-β	transforming growth factor-β

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."*
JM Coffin, SH Huges, and HE Varmus in The Interaction of Retroviruses and Their Host (1997).

I: Brief introduction to retroviruses.

Taxonomy

The replication strategy of any virus depends on the nature of its genetic material. In this respect, all viruses can be divided into seven groups ("Baltimore Classification"). Retroviruses, which represent a large group of viruses found in many species, are classified in the group VI: *Viruses with single-stranded (+) sense RNA with DNA intermediate in life cycle*. The family *Retroviridae* in this group is currently furthered subdivided into seven genera defined by evolutionary relatedness (Table 1). The first five of these genera represent viruses with oncogenic potential, and were therefore previously classified into the subfamily *Oncovirinae*. According to the International Committee of Taxonomy of Viruses (ICTV) this classification is no longer appropriate, since these viruses are no more closely related (or similar) to one another than they are to members of the other previously designed subfamilies (*Lentivirinae* and *Spumavirinae*).

Historically**,** *oncovirinae* were divided into groups based on their morphology in negative stained EM pictures: **A-types** are non-enveloped immature particles only seen inside cells and believed to result from endogenous retrovirus-like genetic elements. **B-types** are enveloped extracellular particles with a condensed, acentric core and prominent envelope spikes (virus encoded glycocprotein). **C-types** are as B-types, but with a central core and barely visible spikes, while **D-types** are usually slightly larger than the others (to 129nm). Currently, most virus comparisons are based on sequence conservation (Table 1).

Table 1. Taxonomy: Group VI: RNA Reverse Transcribing Viruses

Family	Genus	Previous genus for type species	Type Species	genome	Hosts
Retroviridae	*Alpharetrovirus*	Avian type C retrovirus	*Avian leukosis virus* (ALV)	simple	Vertebrates
	Betaretrovirus	Mammalian type B retrovirus	*Mouse mammary tumour virus* (MMTV)	simple	Vertebrates
	Gammaretrovirus	Mammalian type C retrovirus	*Murine leukaemia virus* (MLV)	simple	Vertebrates
	Deltaretrovirus	BLV-HTLV retroviruses	*Bovine leukaemia virus* (BLV)	complex	Vertebrates
	Epsilonretrovirus	Type D retrovirus group	*Wally dermal sarcoma virus*	simple	Vertebrates
	Lentivirus		*Human immunodeficiency virus-1* (HIV-1)	complex	Vertebrates
	Spumaretrovirus		*Human spumavirus* (HSRV)	complex	Vertebrates
Metaviridae	*Metavirus*		*Saccharomyces cerevisiae Ty3 virus*		Fungi
	Errantivirus		*Drosophila melanogaster gypsy virus*		Invertebrates
Pseudoviridae	*Pseudovirus*		*Saccharomyces cerevisiae Ty1 virus*		Invertebrates
	Hemivirus		*Drosophila melanogaster copia virus*		Invertebrates

Generated from Cann (2001) and the ICTV (International Committee of Taxonomy of Viruses) database
http://www.ncbi.nlm.nih.gov/ICTVdb/canintro1.htm

Unless specified otherwise, the following section is based on Coffin *et al.* (1997) and Cann (2001).

Retroviruses structure and genome

All retroviruses contain minimally three genes, *gag*, *pol*, and *env*, which encode the structural proteins as well as the enzymes required for virus integration. There is a universal nomenclature for retroviral proteins:

Table 2: Retroviral proteins essential for replication (illustrated in Fig. 4A).

gene	**Name**	**Protein**	**Function:**
gag	**MA**	Matrix	a matrix protein that lines envelope
gag	**CA**	Capsid	a capsid protein that protects the core
gag	**NC**	Nucleocapsid	a capsid protein; protects genome and forms the core
pro	**PR**	Protease	essential for gag protein cleavage during maturation
pol	**RT**	Reverse transcriptase	reverse transcribes the RNA genome; has also RNAseH activity
pol	**IN**	Integrase	needed for integration of the provirus
env	**SU**	Surface glycoprotein	outer envelope glycoprotein and major virus antigen
env	**TM**	Transmembrane protein	inner component of the mature envelope glycoprotein

The outer envelope glycocprotein (SU) is responsible for receptor binding and is linked by disulphide bonds to the transmembrane glycocprotein (TM) which holds the SU protein in the envelope and is responsible for membrane fusion. Inside the envelope membrane is the rather amorphous matrix (MA) protein that obscures the capsid (CA), which is believed to be icoasahedral. Inside the capsid is the core including the RNA genome, nuclocapsid (NC), Reverse transcriptase (RT) and, integrase (IN).

All retrovirus genome consist of two molecules of RNA, which are s/s, (+) sense and have 5'cap and 3' poly- (A) (equivalent to mRNA). These vary in size from ~8-11 kb. The two RNA molecules are physically linked by hydrogen bonds. In addition, there is a specific type of tRNA (usually trp, pro, or lys) presented in all particles (required for replication). Gene order in all retrovirus is invariant: **5'-gag-pol-env-3'**. The *gag* directs the synthesis of internal virion proteins that form the matrix, the capsid, and the nucleoprotein structures.

The mature product of the *pol* gene is a complex of polypeptides, which includes three distinct enzymatic activities (reverse transcriptase, RNAse H, and integrase). The *env* codes the surface and transmembrane components of the viral envelope protein (summerized in Table 2). An additional, smaller, coding domain present in all retroviruses is *pro*, which encodes the virion protease. Complex retroviruses have additional genes (illustrated in Fig. 1c and Fig. 5; and described below).

Viral life cycle

The steps involved in a generalized retrovirus life cycle are shown in Fig. 1b. The HIV-1 life cycle is shown in more detail in Fig. 4. To initiate the infection, the SU envelope glycoprotein binds to a specific receptor on the surface of the host target cell. The specificity of this interaction does much to determine the cell-tropism and pathogenesis of different retroviruses, or different variants of the same virus. Murine retroviruses are sub-divided on the basis of receptor-determined host species specificity: **Ecotropic** viruses infect only mouse cells, **xenotropic** viruses infect only non-mouse cells (e.g., rat), and **amphotropic** viruses infect both mouse and non-mouse cells (Battini *et al.,* 1992). In recent years, a number of different retrovirus receptor molecules have been identified (reviewed in Sommerfelt, 1999). The cell-surface receptor for ecotropic murine leukaemia viruses (MuLVs) has been shown to be a cationic amino acid transporter (Kim *et al.,* 1991; Wang *et al.,* 1991; see Fig. 10).

Penetration and **uncoating** are poorly understood, but it is clear that uncoating is only partial, resulting in a core (nucleocapsid) particle within the cytoplasm. **Reverse transcription** occurs inside the ordered structure of this core particle. The mechanism of reverse transcription of retrovirus RNA genomes, in which two molecules of RNA are converted into a single double-stranded DNA **provirus**, is illustrated in Fig. 2.

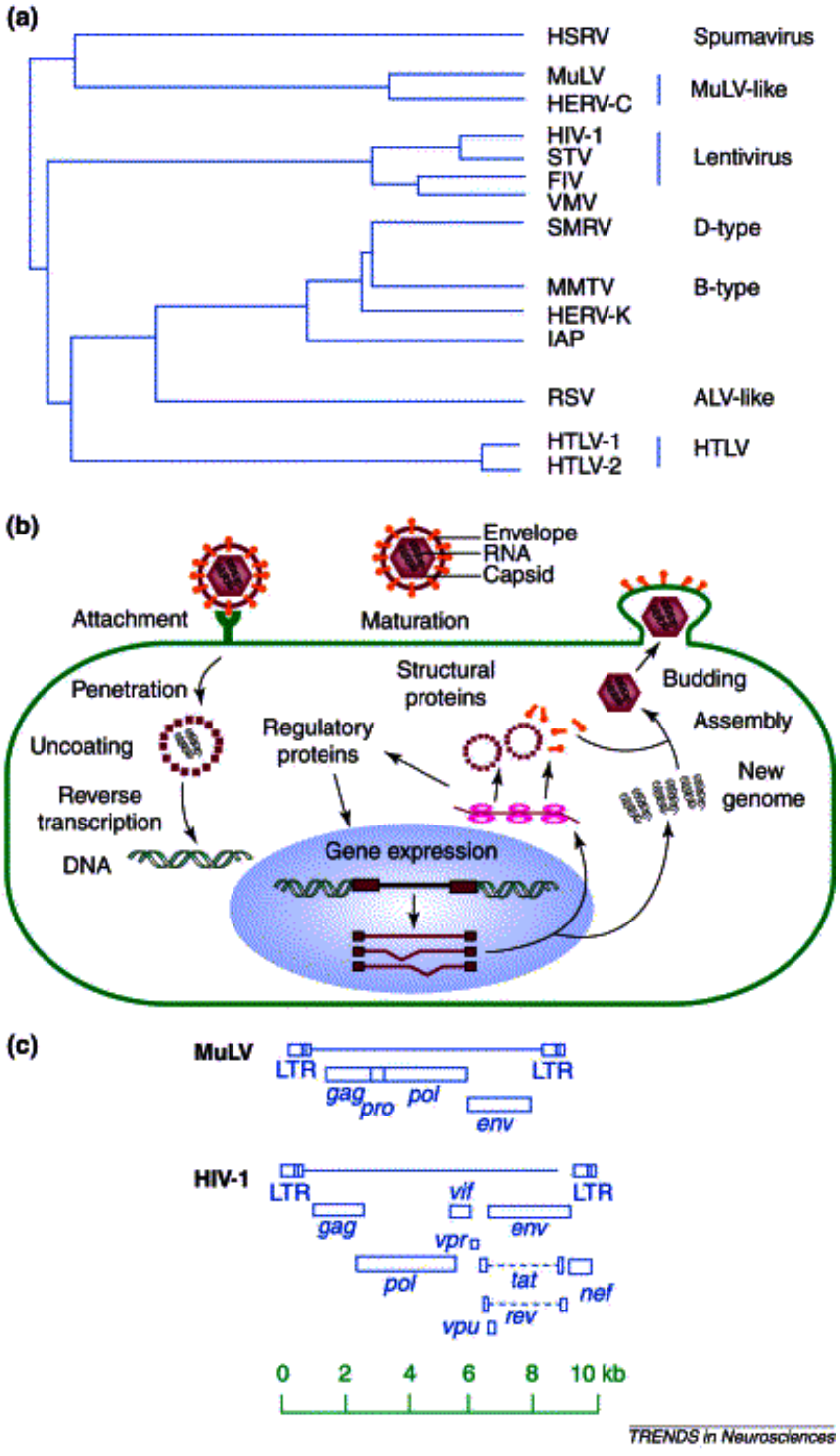

Figure 1. (a) Retroviral phylogenetic tree, (b) the life cycle of retroviruses, and (c) representative genomic structures of simple (F-MuLV) and complex (HIV-1) retroviruses. See text for details. The figure is from Power (2001).

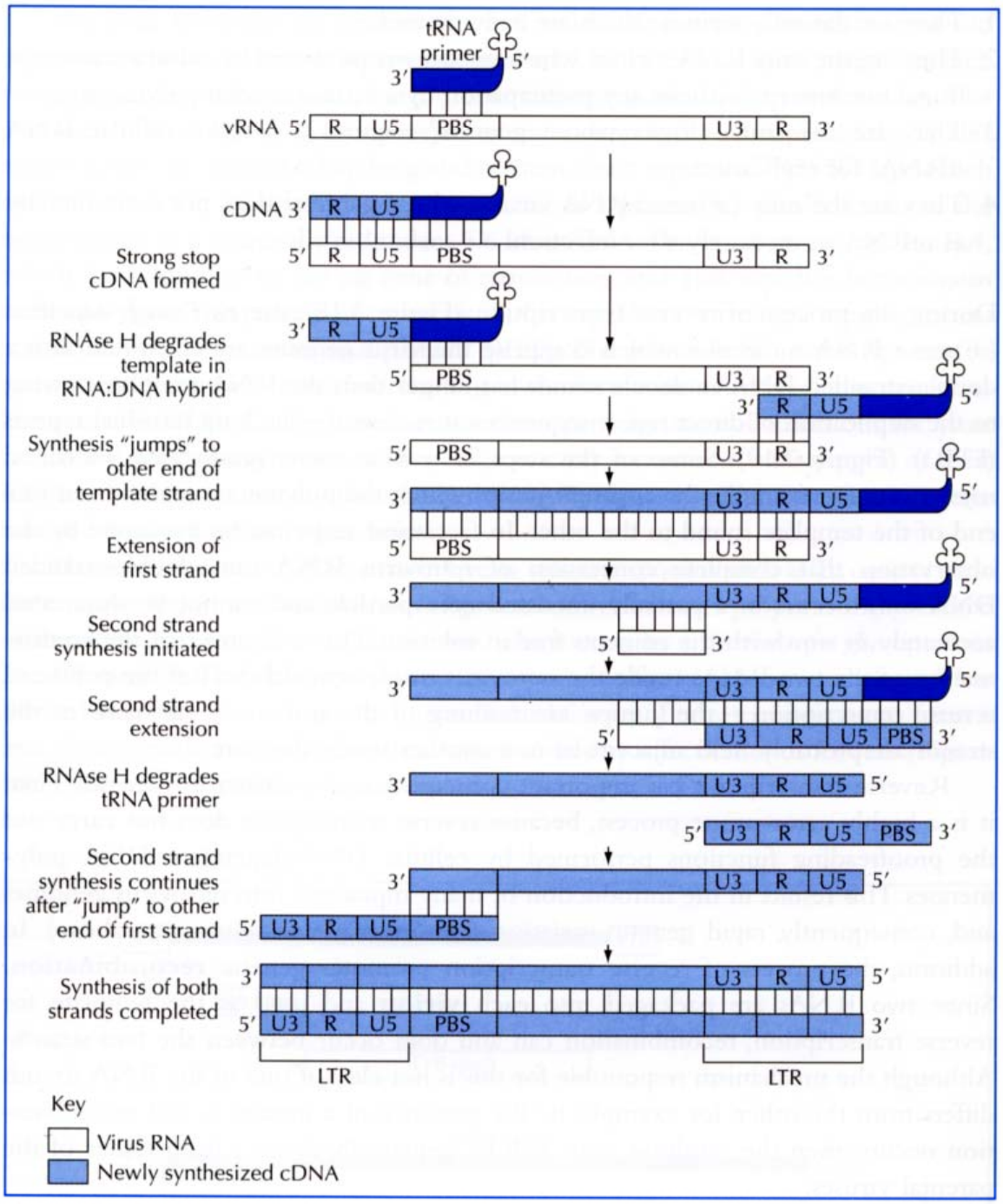

Figure 2. Reverse transcription of retrovirus RNA genomes. The figure is from Cann (2001).

The RNA genome of retroviruses is flanked by two short, redundant R sequences at both termini. These are adjacent to unique sequences, U5 and U3, found at the 5' and 3' ends, respectively. The provirus differ from the vRNA in being longer by one U3, R, U5 sequence. As a result there is a direct repeat of this sequence presented at each end of the provirus genome, known as the long terminal repeat (**LTR**). Three forms of double-stranded DNA are found in retrovirus-infected cells following reverse transcription: linear DNA and two circular forms, which contain either one or two LTRs. The linear form **integrates** into the host's genome. Genomic integration is thought to be semirandom, occurring preferentially at transcriptional active sites (Scherdin *et al.,* 1990). Retroviral infection of a cell is permanent, as proviruses are almost never lost from the chromosome.

Once integrated into the host cell genome, the DNA provirus is dependent on the host-cell transcription machinery for **gene expression.** Viral transcription is carried out by cellular RNA polymerase II, which initiates in the upstream LTR at the U3-R border and terminates in the downstream LTR at the R-U5 border (yielding RNA identical to genomic RNA). For polymerase II promoters, sequences upstream from initiation start site control transcription (Dynan & Tjian, 1985). Thus, the U3 region sequences of the LTR are particular important for retroviral transcription. This region contains proximal and distal promoter elements, as well as enhancer sequences (see Fig. 3). The sequences that govern viral transcription and the repertoire of transcription factors used by a particular retrovirus reflect the unique characteristics of its own replication mechanism (e.g., simple retroviruses that infect lymphoid cells depend on the activity of lymphoid-specific transcription factors). Thus, the ability of a retrovirus to propagate in a given cell type is mainly determined by the tissue specificity of the enhancer sequence in the LTR and the viral envelope (see above).

To compress maximal information into a small genome, retroviruses make use of splicing and ribosomal frameshifting. 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 leukaemia 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 (reviewed in Tang *et al.,* 1999).

The final steps in the retrovirus life cycle are viral **assembly** and **release** from the cell. For MuLVs and HIV, the assembly occur at the cell surface. Thickened patches begin to form in the membrane (*env* proteins on outer surface, and *gag* proteins at the inside). The genome is packaged as the particle buds out through the membrane. **Maturation -** the phase of infection during which newly formed virus particles become infectious **-** involves cleavage events catalyzed by the protease and condensation of the core.

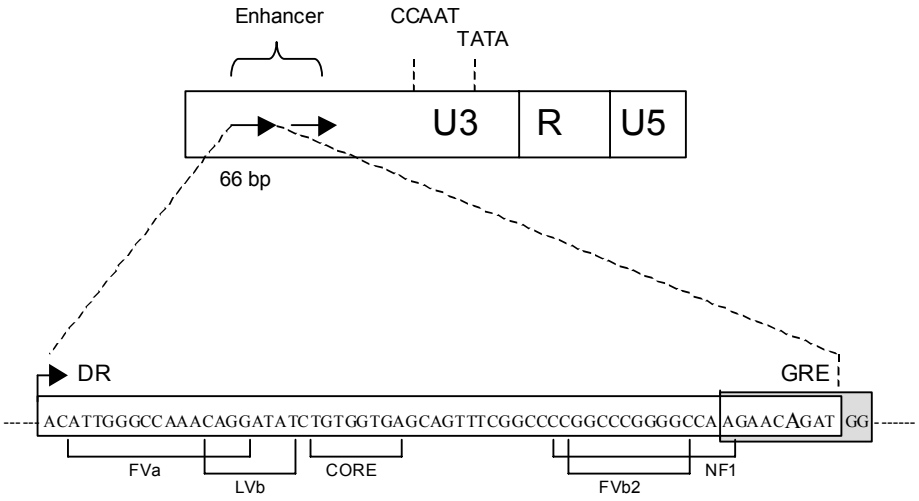

Figure 3. Proviral LTR.
The organization of a prototypical *Gammaretrovirus* proviral LTR with its three regions U3, R, and U5 is illustrated at the top. The arrowheads indicate two direct repeat (DR) sequences, understood to be the viral enhancer (Golemis *et al.,* 1990). The direct repeats (DRs), the promoter (CCAAT), and the site of transcriptional initiation (TATA box) are all located in the U3 region. As in many viral and cellular enhancer elements, each DR contains binding sites for multiple nuclear proteins.

The Friend murine leukaemia virus variant FIS-2 LTR contains only one copy of a 66-bp repeat (Dai *et al.,* 1994). Bindings site for identified viral core element (core) and nuclear factors (Speck & Baltimore, 1987; Manely *et al.,* 1989) sequence are underlined: Friend virus factor a and b2 (FVa, Fvb2), Leukaemia virus factor b (LVb) and Nuclear factor one (NF1). The gray box frames a glucocorticoid response element (GRE) presented in the FIS-2 LTR U3 region (Dai *et al.,* 1994).

Complex *versus* simple retroviruses

Retroviruses are broadly divided in two categories, simple and complex, distinguishable by the organization of their genomes (Table 1 and Fig. 1c). Simple retroviruses usually carry only the elementary information, whereas complex retroviruses code for additional regulatory nonvirion proteins derived from multiply spliced messages. Murine leukeamia viruses (MuLVs) are prototype simple retroviruses, while the Human immunodeficiency virus (HIV-1) of the *Lentivirus* genus is among the most complex retroviruses known. The lentiviruses are exogenous, nononcogenic (currently thought to induce cancer indirectly) retroviruses, causing persistent infections. These viruses usually infect cells of the immune system (T-cells, macrophages), and lentivirus infections are not cleared by the immune system, leading to accumulated damage over a period of many years (*lenti* for slow). An important characteristic not found in other retroviruses is their ability to infect nondividing cells (Lewis & Emerman, 1994). HIV infection of non-dividing cellular targets like macrophages requires successful passage of the viral preintegrated complex (PIC) across an intact nuclear envelope (see Fig. 4).

Compared with other retroviruses, lentiviruses have a larger genome (see Fig. 1c). Lentiviruses most distinguished property is that they encode essential regulatory and accessory genes that allow regulation of their own expression in the cell (reviewed in Tang *et al.,* 1999). Besides the common *gag*, *pol*, and *env* encoded proteins, HIV-1 has six additional genes encoding the regulatory proteins Tat and Rev, together with the accessory proteins Vif, Vpr, Vpu, and Nef (Fig. 5).

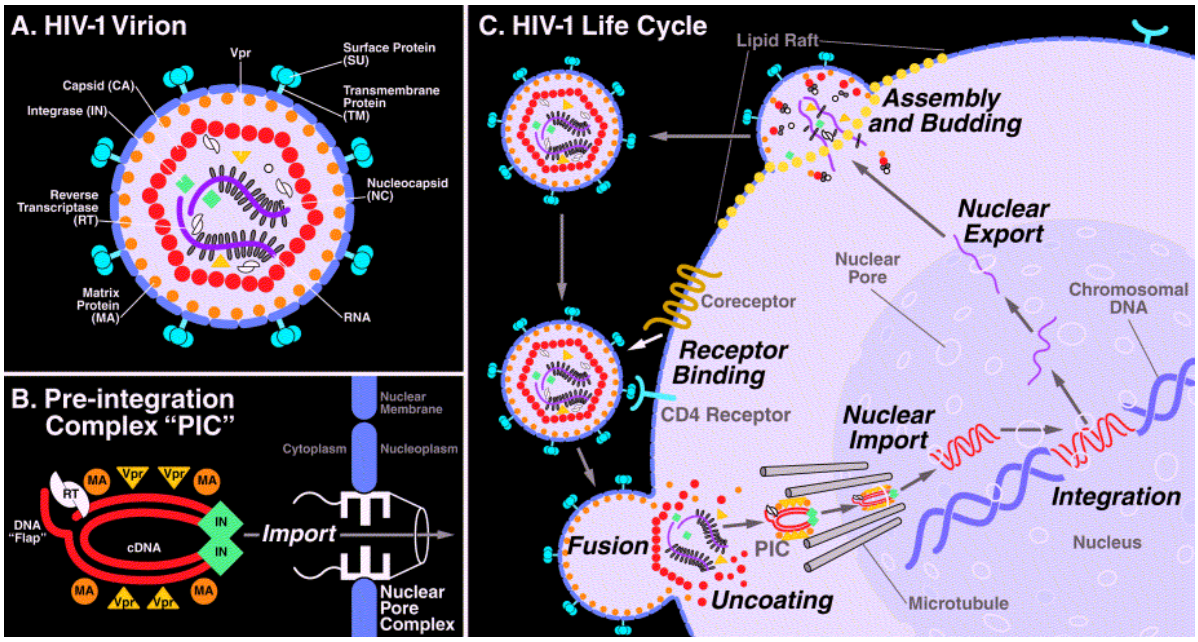

Figure 4. HIV life cycle.

(A) The mature virion is fully assembled after budding.

(B) Completion of reverse transcription gives rise to the HIV preintegration complex (PIC). Unique but redundant nuclear import signals resides within the HIV integrase, matrix, Vpr proteins, and the "DNA flap" (corresponds to a triple-stranded intermediate created during reverse transcription). These signals appear to facilitate PIC transport through the limiting nuclear pores. It should be noted, that integration and infection are reduced in non-dividing host cells, were deoxynucleotide concentrations are below Km value of retroviral RTs (Coffin *et al.,* 1997).

(C) The HIV life cycle. HIV fuses with the CD4 and cognate coreseptor, initiating fusion and uncoating. The subsequent complex facilitates conversion of viral RNA into cDNA and sheds several proteins while it traverses the cytoplasm along microtubule towards the nucleus. The PIC somehow enters through the limiting nuclear pore complex to gain access to host chromosomal DNA. After integration, genomic viral RNA is exported along with the immature viral particle components, which assemble and bud together out of the cytoplasm through lipid rafts.

Reviewed in Greene & Peterlin (2002). The figure is from Sherman & Greene (2002).

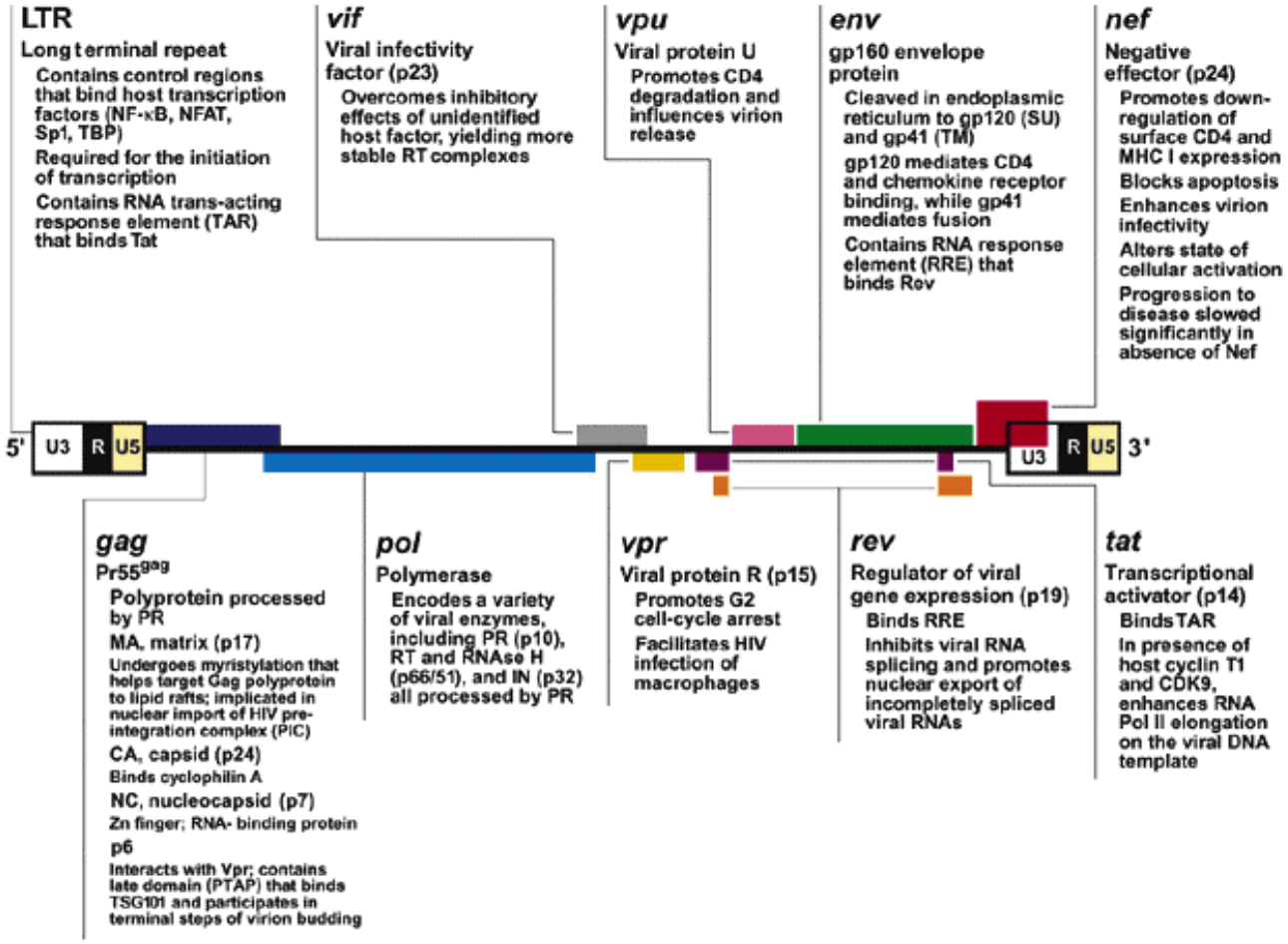

Figure 5. Genome of the HIV provirus.
An overview of the organization of the ~9-kilobase genome of the HIV provirus and a summary of the functions of its 9 gene encoding 15 proteins. The figure is from Greene & Peterlin (2002)

II: Retroviruses and pathogenesis

Retroviruses give rise to a broad spectrum of diseases, including neurological disorders, malignant transformation, and immunodeficiencies. The AIDS epidemic associated with HIV infections is currently the single greatest threat to public health worldwide (Piot *et al.,* 2001). It should be emphasized, though, that most retroviruses do not give rise to any disease symptoms. Further, except from HIV and HTLV-1, the majority of disease-causing retroviruses are found in non-humans (Coffin *et al.,* 1997). Nevertheless, studies of retrovirus induced diseases have made huge contributions to e.g., cancer research, with subsequent revolutionary impact on fundamental concepts in modern biology (see Table 3). Only a brief introduction to retrovirus induced malignant transformations and immunosuppression, including HIV-1/AIDS, will be presented here. A glossary of frequently used terms in medical virology & immunology is enclosed in the appendix section.

Retrovirus induced oncogenesis.

It is now well accepted that evolution of cancer is a multistep process associated with the activation of oncogenes and inactivation of tumour suppressor genes (reviewed in Yokoto, 2000). Studies of retroviral-induced oncogenesis in animal systems led to the initial discovery of viral oncogenes (*v-onc*) and their cellular homologues (*c-onc*), and provided critical insights into their role in the neoplastic process (Coffin *et al.,* 1997; Jonkers & Berns, 1996; Butel, 2000). The retroviruses which can transform cells fall into three groups: (i) Transducing, acutely transforming viruses, which carry oncogenes (*v-onc*); (ii) *cis*-activating, chronic transforming viruses, which do not have an oncogene present but activate *c-onc* in cell genome by proviral insertion (see Table 4); and (iii) *trans*-activating viruses, which activate cellular proteins by trans-activating virus protein (e.g., HTLV-1 encoded Tax).

Table 3: Selected major breakthroughs in the science of Retrovirology up to the discovery of human immunodeficiency virus.

	Major findings	References
1908	Vilhelm Ellermann and Oluf Bang searched for an infectious cause (bacterium) for leukaemia in chicken. They succeeded in transferring the disease from one chicken to another by cell-free tissue filtrates, and thereby showed that virus caused the chicken leukosis.	Ellermann & Bang, (1908)
1911	Peyton Rous reported cell free transmission of sarcoma in chicken and isolated the infectious agent, *Rous Sarcoma Virus* (RSV). This discovery was followed by many other examples of acutely transforming retroviruses. Several of the retroviruses isolated became important model systems, actively studied at the cellular and molecular levels to this day.	Rous (1911) Received the Nobel prize in 1966
1951	Gross observed vertical (germ line) transmission of cancers	Gross (1951)
1957	The Friend murine leukaemia virus provided an animal model system for the study of erythropoiesis and the multistep nature of cancer.	Friend (1957)
1958	Focus assay for RSV introduced the "one cell, one virus paradigm"	Temin & Rubin (1958)
1960's	Howard Temin knew that retrovirus genome were composed of RNA and observed that replication was inhibited by actinomycin D (that inhibits DNA synthesis). This drug does not inhibit the replication of other RNA viruses. Temin and Baltimore simultaneously published the observations that retrovirus particles contain an RNA-dependent DNA polymerase - reverse transcriptase.	Temin & Mizutani (1970) Baltimore (1970) Received the Nobel prize in 1975.
1969	Huebner and Todaro proposed the viral oncogene hypothesis (the transmission of viral and oncogen information as genetic elements). Explained the vertical transmission of cancers, first observed by Gross (1951)	Huebner & Todaro (1969)
1981	Human T-cell leukaemia virus discovered, the first pathogenic human retroviruses	Gallo *et al.,* (1981)
1983	Human immunodeficiency virus (HIV) discovered - the causative agent of AIDS.	Barre-Sinoussi *et al.,* (1983) Gallo *et al.,* (1984)

Adapted from Cann (2001) and Coffin *et al.* (1997).

Retroviruses that lack *v-onc* genes are usually replication competent, do not transform cells in culture, and induce tumours with long latent period *in vivo*. Most retroviruses that lack oncogenes cause hematopoietic malignancies, although a few of these viruses induce carcinomas. In the next section, some murine retroviruses that lack *v-onc* are described as examples.

Table 4: Cellular oncogenes activated by insertion of retroviruses lacking oncogenes.

General class	Oncogene	Virus	Protein product
Non-receptor protein TK	*LcK*	Mo-MuLV	Tyrosine kinase
	c-fms	**F-MuLV**	CSF receptor
Serine/threonine TK	*Piml*	Mo-MuLV	Serine/threonine kinase
Growth factor	*Fgf3/Int2*	MMTV	Fibroblast growth factor
	Wnt1/Int1	MMTV	Secreted glycocprotein
	Wnt3/Int4	MMTV	Secreted glycoprotein
G-protein	*c-Ki-ras*	**F-MuLV**	GDP/GTP binding
Transcription factor	*Ets1*	Mo-MuLV	Transcription factor
	c-myb	Mo-MuLV	Transcription factor
	c-myc	Mo-MuLV	Transcription factor
Cyclin	*Fis1/Cyclin D1*	**F-MuLV**	G_1 cyclin
	Vin1/ Cyclin D2	Mo-MuLV	G_1 cyclin

CSF: Colony stimulating factor; TK: tyrosine kinase; Mo-MuLV: Moloney murine leukaemia virus; **F-MuLV: Friend murine leukaemia virus**. Adapted from Coffin *et al.* (1997) and Butel (2000). The list is representative, not exhaustive. In humans, no mechanism such as oncogene activation by proviral insertion has been reported, but genes identified in animal models are also found mutated or overexpressed in human tumours.

Mouse mammary tumour virus (MMTV) of the genus *betarerovirus* can induce mammary carcinomas in mice late in life by activation of proto-oncogenes after integration in their vicinity. Common integration site for MMTV is close to *int* genes that codes for extracellular growth factors (reviewed in Jonkers & Berns, 1996). Exogenous MMTV is spread via the milk of infected females and is acquired by suckling pups. On rare occasions, an exogenous MMTV provirus is inserted into germ or early embryonic cells, thereby becoming a stable inherited endogenous provirus. MMTV, like most retroviruses, is dependent on cell division to complete its replication cycle. Interesting, it requires a

functional immune system to achieve efficient infection of the mammary gland, and MMTV has developed at least two strategies to exploit the immune response. During primary infection, MMTV is transmitted in the milk from the mother to the newborn and is taken up in the intestine, where it infects local lymphocytes (reviewed in Ross, 2000). Infected B-cells express a 3'-LTR-encoded superantigen (*Sag*) on their surfaces that interacts with the Vβ chains of the T-cell receptor on specific T-cell subsets (reviewed in Acha-Orbea & MacDonald, 1995). These immune reaction results in a preferential clonal expansion of infected B-cells, thus facilitating the persistence of the virus in the organism until the target mammary tissue develops. Further, MMTV also activates B-cells via interaction with toll-like receptor 4 (TLR4) at an early stage of infection, and this activation is independent of viral gene expression (Rassa *et al.,* 2002). Thus, the use of a receptor involved in innate immunity (Takeda & Akira, 2001) represent a second way this virus has evolved to take advantage of the immune system.

Since MMTV does not encode an oncogene and cause tumours by integrating near cellular oncogenes and activating or altering their transcription, the more cells that become infected, the greater the likelihood that an oncogenic integration will occur. The mammary gland specificity of the oncogenic property of MMTV depends on the high viral replication rate and consequent high reinfection rate in the mammary epithelial cells, which are stimulated by pregnancy-related hormones (Coffin *et al.,* 1997; Ross, 2000).

The Friend murine leukaemia virus complex (FV) was first characterized by Charlotte Friend (Friend, 1957) and has become one of the best animal tumour models to study the multistep nature of cancer (Ben-David & Bernstein, 1991; Ney & D'Andrea, 2000). The FV complex consists of two components: the spleen focus-forming virus (**SFFV**), a replication defective virus which is responsible for an acute pathogenicity, and Friend murine leukaemia virus (**F-MuLV**), which acts as a helper for the replication of the defective SFFV. Although SFFV lacks a classical oncogene and consists entirely of retroviral-specific sequences, it encodes a unique envelope glycoprotein (gp55), which

interacts specifically with the EpoR at the cell surface, resulting in activation of the receptor and subsequent activation of erythroid signal transduction pathways (Li *et al.,* 1990). However, the interaction of the SFFV envelope glycoprotein (gp55) with the EpoR is not sufficient to transform cells. The emergence of clonal tumorogenic cells during later stages of the disease is dependent upon proviral insertional mutagenesis (Jonkers & Berns, 1996). Critical elements of the disease involve the interaction of host gene products Sfpi/PU.1 and SF-Stk, as well as EpoR. SFFV proviral integration at *Sfpi-1* (spleen focus forming virus proviral integration site 1) leads to rearrangement and transcriptional activation of the PU.1 gene, which encodes an Ets-related transcriptional factor that is normally expressed in machrophages and B-cells (reviwed in Ruscetti *et al.,* 1999). The Stk is a member of a large family of surface tyrosine kinase receptor, while EpoR is a member of a large family of cytokine receptors. The binding of gp55 to EpoR stimulates uncontrolled erythroblast proliferation and increases the migration of erythroid precursors from the bone marrow to the spleen. Such expansion of mitotically activated target cells is thought to be essential for FV-induced malignant transformation because of the increased probability of proviral integration. Thus, when adult mice of susceptible strains (described below) are infected with FV, their spleens rapidly enlarge because of virus induced polyclonal proliferation of erythroid precursors cells. Subsequent proviral integration at the *Sfpi-1* (*ets*) oncogene locus combined with inactivation or mutation of the p53 tumour suppressor gene produces fully malignant erythroleukaemia. This process results in gross splenomegaly at 8-9 days post infection and transplantable erythroleukaemia cells as early as 15-20 days post infection (for review on Friend erytroleukaemia, see Ruscetti, 1999; Ney & D' Andrea, 2000).

Two different Friend virus strains have been isolated, FVa and FVp, which, due to different SFFV virus components induce different subsets of early erythroid cells to expand polyclonally: SFFVa (anaemia) causes expansion of non-haemoglobin synthesizing cells which are dependent on erythropoietin, whereas SFFVp (polycythemia) induces erythropoietin-independent cells actively synthesizing haemoglobin. The helper virus F-

MuLV is capable of inducing erythroleukaemia independently of SFFV when injected into newborn mice of susceptible strains. The F-MuLV-induced erythroleukaemia has a disease pattern very similar to that induced by SFFVa, and also results in early rapid expansion of epo-dependent erythroid precursors, followed by clonal outgrowth of malignant erythroleukaemia cells (reviewed in van Lohuizen & Berns; 1990).

Murine leukaemia viruses (MuLVs) of the genus *gammaretrovirus* can induce a large spectrum of pathologic responses in mice, with a predominance of hematopoietic tumours. Transformation is usually achieved by retroviral integration at the vicinity of a cellular proto-oncogene. Several insertion sites have been identified, and among them are many loci that contain genes that become deregulated due to proviral integration (see Table 4). Although MuLVs can infect many tissues and cell types, each virus will induce a specific type of tumour (e.g., T or B lymphomas, myeloid leukaemia, or erythroleukaemia). Several studies have shown that the strength or tissue specificity of the LTR enhancers may affect the pathogenic behaviour of nonacute retroviruses (selected references: Evans & Morrey, 1987; Ishimoto *et al.,* 1987; Thiesen *et al.,* 1988; reviewed in Athas *et al.,* 1994, and Fan, 1990). Fan (1990) suggested that a retrovirus with a strong enhancer might more efficiently activate a proto-oncogene and more rapidly induce tumours than one with a weaker enhancer. Further, the tissue-specific replication of different MuLVs is conferred by their LTRs (Evans & Morrey, 1987).

Experiments with different MuLVs such as **Mo-MuLV** and **F-MuLV**, which induces T-cell lymphoma and erythroleukaemia in neonatal mice, respectively, have shown that both the type of leukaemia induced and the incubation period for leukaemia induction are largely determined by the U3 region of the respective LTR: (i) The distinct disease specificity of Mo-MuLV and F-MuLV may be entirely switched by exchanging a segment of the U3 region of the LTR (Chatis *et al.,* 1984; Golemis *et al.,* 1989). (ii) A two-nucleotide mutation in the enhancer core of Mo-MuLV (TGTGGTAA to TGCCGTAA) altered the disease specificity from 100% T-cell lymphoma to 65% erythroleukaemia (Speck *et al.,*

1990). (iii) A deletion in the tandem repeat of the F-MuLV LTR (see Fig. 3) attenuated the leukemogenesis of F-MuLV (Li *et al.,* 1987; Sitbon *et al.,* 1991). Overall, these studies showed that subtle alterations in the highly conserved structure of the mouse type C retrovirus U3 region (Golemis *et al.,* 1990) could be sufficient to confer distinct biological properties to the virus.

Retrovirus induced immunosuppression

Most, if not all, pathogenic retroviruses induce a generalized immunosuppression in the infected host. Human and simian immunodeficiency viruses (HIV and SIV) induce a pronounced immunosuppression, ultimately leading to AIDS (described below). Marked impairment of the immune system has also been described in cats infected with the feline immunodeficiency virus (FIV) (Pedersen *et al.,* 1989), and in mice infected with several murine leukaemia viruses (MuLVs) as well as the Friend leukamia virus complex (Bendinelli *et al.,* 1985; Friedman *et al.,* 1983; Soldaini et al., 1989). Furthermore, murine AIDS (MAIDS), characterized by a lymphoproliferative disease and a profound anergy which involves mostly $CD4^+$ cells, develops following infection with the MAIDS (LP-BM5 MuLV) virus complex (reviwed in Mosier, 1996).

The prototype immunosuppressive virus, **HIV-1,** causes a spectrum of clinical problems beginning at the time of seroconversion, and terminating with **AIDS** (acquired immunodeficiency syndrome) and death. During primary infection with HIV, high levels of viremia develop within days to weeks (Daar *et al.,* 1991). Viremia reaches a peak and the $CD4^+$ cell count temporary decreases. Subsequently, as the host mounts a vigorous immune response that partially controls viral replication, a (hypothesized) viral set point is reach, reflecting a tenuous balance between production and destruction of virions. The host enters a largely asymptomatic phase during which viremia persists, and billions of virions and $CD4^+$ cells are produced and destroyed daily. Eventually, progression to disease occurs, characterized by steadily increasing viremia, decreasing $CD4^+$ cell counts, and, finally,

profound immunosuppression, clinically recognized as AIDS. Various complications characterize AIDS, including wasting, neurological impairment, opportunistic infections and malignancies (reviewed in Levy, 1998; Mindel & Tenant-Flowers, 2001).

Diverse clinical courses can occur after HIV infection (reviewed in Haynes *et al.,* 1996; Hogan & Hammer, 2001, a and b): (i) **Rapid progressors** are HIV-infected subjects which progress to AIDS within the first 2 to 3 years of HIV infection. (ii) **Long-term nonprogressors** are clinical asymptomatic after 7 to 10 years and have stable $CD4^+$ T cell level. Some of these HIV-infected persons will be AIDS free for decades. (iii) **Typical progressors** are projected to develop AIDS within median time of approximately 10 years from initial infection (Fig. 6).

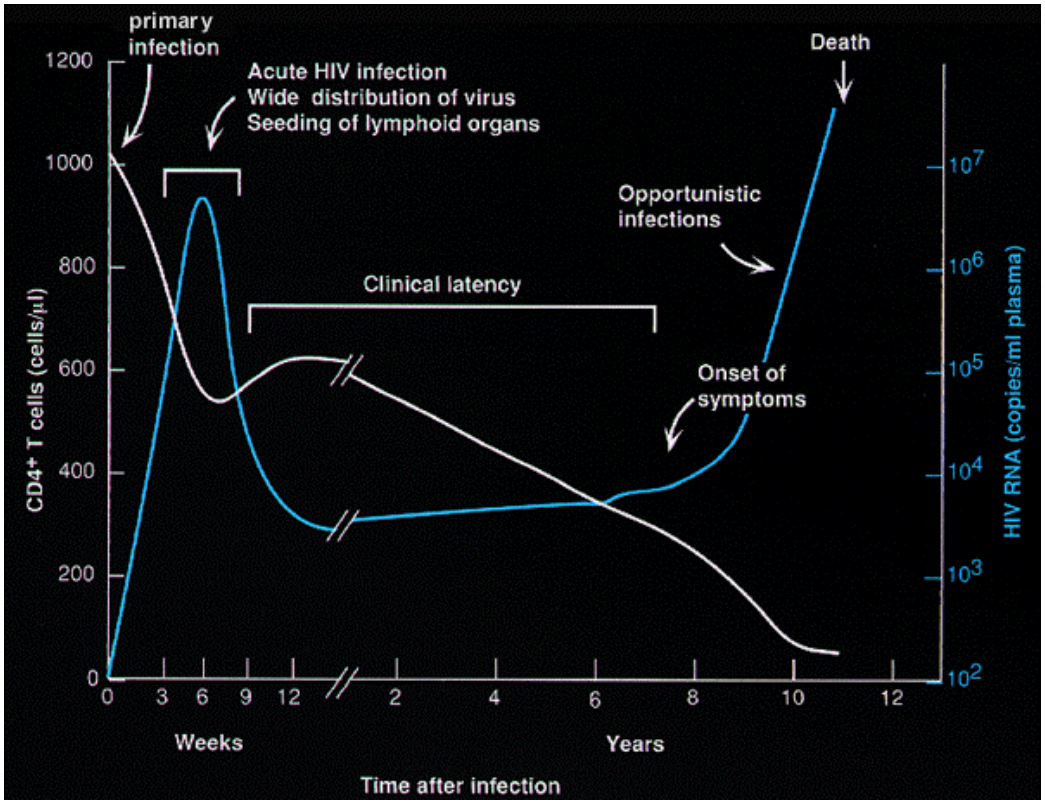

Figure. 6. A schematic diagram of events occurring after HIV infection. Patterns of $CD4^+$ T-cell decline and virus load increase vary greatly from one patient to another, as do the actual values of viral RNA load. Further, the course of HIV infection varies widely among individuals (se text). The figure is generated from Coffin *et al.* (1997), and is representative for a typical progressor.

Immunosuppression by retroviruses is a complex phenomenon involving many different cell types of the immune system, and several mechanisms for immunosuppression by retroviruses have been suggested, including (i) killing of infected target lymphocyte; and (ii) modulation of cytokine production and/or induction of a T_H1/T_H2 imbalance (see Fig. 7) (reviewed in Bendinelli *et al.,* 1985; Cann, 2001; Coffin *et al.,* 1997; Denner, 1998).

Retroviruses that cause immune deficiency often do so by infecting activated lymphocytes, including those involved in the immune response to the retroviruses. Thus, the most likely explanation is that immunological dysfunction are produced directly by viral replication in the affected cells. For example, HIV-1 preferentially infects HIV-specific $CD4^+$ T-cells (Douk *et al.,* 2002). The HIV infection of $CD4^+$ T-cells leads to the loss of critical regulatory cells in the immune system, with $CD4^+$ T-cell number and viral load being the most sensitive predictors of disease progression (Fig. 6). Recent observations support a close linkage between immune activation and $CD4^+$ cell depletion in HIV infection and only an indirect relationship of these parameters to the virus rate of replication (Sousa *et al.,* 2002; reviewed in Grossman *et al.,* 2002).

One of the most thoroughly studied viral candidates for the immunosuppressive actions of retroviruses is the transmembrane envelope protein (TM). A hydrophilic 26-amino acid sequence of the otherwise hydrophobic TM protein, p15E, of feline and murine leukaemia virus is conserved among TM-proteins of murine, feline, simian, human-T-cell leukaemia retroviruses, and partly HIV (reviewed in Denner, 1998). Cianocilo *et al*., (1985) synthesized a 17 amino acid peptide, CKS-17, representing the conserved domain within this region. Several studies have shown that this prototypic immunosuppressive domain elevate intracellular cAMP (Haraguchi *et al.,* 1995a), activates MAP-kinases (Takahashi *et al.,* 2001), and suppress numerous immune functions (Table 5). It has been reported that activation of the MAP kinase pathway plays a role in enhancing HIV infection and replication (Yang & Gabuzda, 1999), as well as suppressing T_H1-related cytokine production (Feng *et al.,* 1999). Haraguchi *et al.,* (1995b) have shown that CKS-17 can act

as an immunomodulatory epitope causing imbalance of the T_H1- and T_H2- related cytokine production and suppression of cell mediated immunity (discussed below; illustrated in Fig. 7). CKS-17 may excert its immunosuppressive activity via activation of the cAMP/protein kinase A (PKA) patway and inhibition of the protein kinase C (PKC) pathway (reviwed in Haraguchi *et al.,* 1995c). Recently, Andahl *et al*., (2002) showed that cAMP through activation of PKA type 1 is a universal inhibitor of T-cell function. Further, hyperactivation in the cAMP/PKA system has been implicated in the T-cell dysfunctions associated with HIV (Aandahl *et al*., 1998; reviwed in Torgersen *et al.,* 2002) and MAIDS-associated T- and B-cell anergy (Rahmouni *et al.,* 2001).

Table 5: Immunosuppressive activity of the retroviral TM protein

In vitro	*In vivo*
• mitogen induced proliferation of T and B lymphocytes • mixed lymphocyte reaction • IL-2-induced proliferation of T cell lines • respiratory burs of monocytes • chemotaxis of monocytes • natural killer cell (NK-cells) • erythroid colony-forming units (CFU)	• machrophage accumulation • antibody response to cell surface antigens • enhancement of tumour progression

From Denner (1998)

However, it is not known how much of the pathology (e.g., AIDS) is related to individual genetic traits of the immune system and how much is caused by the virus. Thus, although immunosuppression by retroviruses was first described over 40 years ago (Old, 1959), and although enormous efforts have been undertaken to study its mechanisms because of the AIDS epidemic, the answer to how it works is still enigmatic.

The next paragraph gives a brief description of animal models currently used to study the basic mechanisms whereby a retrovirus persists and immunosuppress the host.

III: Animal models for retrovirus-induced immunosuppression

For the first decade of the AIDS epidemic, researcher conducted experiments in chimpanzees held in primate colonies (Fultz *et al.,* 1989). Because these animals are endangered and expensive, researchers have more recently used rhesus monkeys, originally from India. These monkeys develop an AIDS-like disease when infected with either simian immunodeficiency virus, SIV, or a laboratory-made SIV/HIV hybrid called SHIV (reviewed in Joag, 2000). Other currently available animal models of AIDS includes feline immunodeficiency virus infection (FIV) of cats (Pedersen *et al.,* 1989), HIV-1 infections of rabbit (Kulaga *et al.,* 1989), and SCID mice reconstituted with human tissues or cells (reviwed in Bonyhadi & Kaneshima, 1997; Mosier *et al.,* 1996). Further, mice infected with simple retroviruses such as LP-BM5 MuLV complex (Coffin *et al.,* 1997; Mosier, 1996) or FV (Bendinelli *et al.,* 1985; Hasenkrug & Dittmer, 2000) offers model systems for studies of more general aspects regarding retrovirus induced immune dysfunctions. The next few paragraphs briefly describe the SIV and FIV viruses as examples of complex retrovirus models. The Friend virus model is described as an example of immunosuppression induced by simple retroviruses in mice. These models are also used in vaccine research.

Complex retrovirus models

Simian immunodeficiency viruses (SIVs) are a group of HIV-related, but distinct, lentiviruses isolated from several different African primates including chimpanzees (SIV_{cpz}), sooty mangabeys (SIV_{sm}), mandarills (SIV_{mnd}), and African green monkeys (SIV_{agm}) (Holterman *et al.,* 1999 and references therein). The primate lentiviruses have little or no pathogenicity in their natural hosts, and disease appears to result only after transmission to another species. It has been confirmed that SIV_{cpz} is the progenitor of HIV-1 (Gao *et al.,* 1999). Some SIV strains, like pathogenic isolates of $SIV_{mac}251$, induce AIDS in macaques. Highly pathogenic SIV isolates results in loss of $CD4^+$ T-cells within six

months and death by one year. The availability of molecular clones of SIVs that vary in cellular tropism and virulence makes this experimental model particular useful (reviewed in Joag, 2000; Johnston, 2000). Further, infection of macaque monkeys with simian immunodeficiency virus is still the best model for HIV vaccine research (reviewed in Warren, 2002).

Feline immunodeficiency virus (FIV)

FIV, a *lentivirus* of cats that causes a disease similar to human AIDS (Pedersen *et al.,* 1989), has been developed as a naturally occurring small animal model for HIV infection and disease (Willett *et al.,* 1997). Cats infected with FIV progress from acute to asymptomatic infection, followed by increasing immune dysfunctions and finally an AIDS like disease. As with HIV infection, the course of disease development is prolonged, taking several years in most cats to reach the AIDS stage. During that time, the $CD4^+$ cell subset declines, cytokine profile changes, and macrophage functions are disrupted (Ackley *et al.,* 1990; Barlough *et al.,* 1991; Lawrence *et al.,* 1995; Willett *et al.,* 1997).

Simple retrovirus models

The Friend murine leukaemia virus complex (FV) is one of the classical murine models used to study immunosuppression associated with retrovirus infection (Bendinelli *et al*., 1985; Soldaini *et al.,* 1989). FV replicate in numerous cell types including macrophages, B-lymphocytes, and to a lesser extent, T lymphocytes (Soldaini *et al.,* 1989). Considerable immunosuppression, involving both humoral and cell-mediated responses, is strongly affected by FV infection. For example, the specific response of B-cells to certain T-cell dependent antigens such as sheep red blood cells (SRBC) is depressed severely early after infection in immunecompetent susceptible adult mice (Bendinelli *et al.,* 1985; Ceglowski & Fridman, 1970). Responses to T-independent antigen have also been reported (Morrison *et al.,* 1986). The ability of T-cells to mediate cytolysis of allogenic target cells is reduced (Mortensen *et al.,* 1974), and the generation of cytotoxic T-cells is impaired (Garaci *et al.,*

1981). Modulation of cytokine production, including increased IL-1 production and reduced IL-2 production are observed (Soldaini *et al.,* 1989). Further, decreased NK-cell activity (Lu *et al.,* 1992), as well as impaired antigen presentation by machrophages (Jones *et al.,* 1992) have been associated with FV-infection. The symptoms of a general immune suppression which are immediately associated with FV infection in some mouse strains have been considered very similar to those observed in AIDS patients (Morrison *et al.,* 1986; Soldaini *et al,* 1989). Many of the dysfunctions induced by FV are also observed in F-MuLV infected mice. However, the immunosuppressive effects of F-MuLV are less severe.

Friend immunosuppressive variant, FIS-2.

When HIV emerged on the scene, the FV-induced immunosuppression again gained attention and was suggested as a murine model of AIDS (Soldaini *et al.,* 1989). However, the immunosuppressive effects in FV infected adult mice are soon overwhelmed by the massive infiltration of leukaemia cells (see above). The initial goal of "The FIS-2 project" was to isolate an immunosuppressive but low oncogenic variant of FV, and to develop a more available murine model to study retrovirus induced dysfunctions. To obtain such a variant, lysates of T-helper cells from FV infected NMRI mice were passed to recipient uninfected mice. A group of these mice developed a condition distinct from the disease induced by FV, and a viral stock prepared from these mice induced a profound suppression of the primary antibody response without acute transformation (Faxvaag *et al.,* 1993a). This stock (initially named Fd-MIV for Friend derived murine immunodeficiency virus) was used to investigate retrovirus induced derangement of cytokine homeostasis (Faxvaag *et al.,* 1993b; Faxvaag *et al.,* 1995), polyclonal activation of B-lymphocytes, and induction of autoimmunity (Faxvaag *et al.,* 1993c).

Analysis of viral DNA and RNA from infected NIH 3T3 cells showed that the virus stock contained a mixture of at least two viral components, a replication-competent F-MuLV-related virus and a replication-defective mink cell focus-forming virus (MCF) related virus.

Since the MCF related genome was not detected in infected, immunocompromised mice, it was assumed that the F-MuLV related virus component was responsible for the disease (Faxvaag *et al.,* 1993a). In order to prove this, two biological clones designated Friend immunosuppressive variant-1 and -2 (FIS-1 and FIS-2), were obtained from NIH 3T3 cell inoculated with spleen extract from mice in early and advance stages of disease, respectively (Dai *et al.,* 1994). Southern blot analysis showed that the defective viral component was eliminated during the procedure of end-point dilution. Only mice inoculated with FIS-2 became immunosuppressed. FIS-2 was further molecularly cloned and characterized. Restriction mapping and nucleotide sequence analysis of FIS-2 showed a high degree (about 95%) of homology between FIS-2 and the prototype F-MuLV clone (cl.) 57 (Troxler *et al.,* 1980), suggesting that FIS-2 was a variant of F-MuLV. However, there were some striking differences between FIS-2 and F-MuLV cl. 57 (Dai *et al.,* 1994):

Differences in the LTRs:

- The second copy of the direct repeat (74 bp) in the F-MuLV LTR had been completely deleted from the FIS-2 LTR.
- The sequence of the enhancer core element of the F-MuLV, TGTGGTAA, was changed to TGTGGTGA in FIS-2.
- A single-nucleotide transition of G to A occurred in the binding site of factor FVa, and because of the deletion of the second copy of the direct repeat, the binding site for nuclear factor FVb1 was missing in the FIS-2 LTR.
- A binding site for the glucocorticoid receptor GR, a glucocorticoid response element (GRE), AGAACAGATGG, had been generated in the FIS-2 LTR.
- Analysis of the nucleotide sequences of the LTRs of FIS-2 and SFFVp indicated that the FIS-2 was generated by recombination between SFFVp and F-MuLV.

Differences in g*ag* - encoded proteins:

A total of 38 point mutation occurred scattered over the FIS-2 *gag* gene, and 24 mutations led to amino acid changes in the *gag* product. Two single point mutations led to the

appearance of two extra potential N glycosylation sites in the FIS-2 *gag*-encoded glycoprotein.

Differences in *Env* – encoded proteins:

Among the 23 amino acid mismatches found, 22 were distributed over the SU region.

Most newborn mice infected with the FIS-2 clone developed erythroleukaemia, but with an increased latency period compared with that of F-MuLV cl. 57. In contras, FIS-2 was shown to induce suppression of antibody response against sheep erythrocytes (SRBC) in adult NMRI mice more efficiently than the prototype F-MuLV cl. 57. Together these initial studies (Faxvaag *et al,* 1993, a, b, and c; Faxvaag *et al.,* 1995; Dai *et al.,* 1994) suggested that FIS-2 could represent an interesting murine model to study retrovirus-induced immunosuppression on the basis of its combined property of low leukaemogenicity and relatively strong immunosuppressive activity in adult mice.

IV: Virus-Host interactions

The overall course of a retrovirus infection is determined by a dynamic interaction between the virus and its host organism. As described above, small differences in viral gene sequences (e.g., in the LTRs) can have a dramatic effect on biological properties. Divergent patterns of disease progression following infection from a common source virus are thought to reflect different host responses. Some viral and host factors that may influence the susceptibility and/or outcome of a retrovirus infection are summarized in Table 8.

Virus Factors

As described above, the events occurring after HIV infection is divided into several phases. The **quantity of virus in the blood** is one of the major parameters characterizing the primary acute infection, the prolonged asymptomatic phase, and the final AIDS phase (Daar *et al*., 1991; D'Souza & Mathieson. 1996; Lyles *et al.,* 1999). A correlation between a high viremia level at the time of seroconversion (see Fig 6.) and early disease manifestation has been documented (Ho, 1996; Mellors *et al*., 1996). Therefore, identification of the factors affecting this viremia level has been an important objective in studies of experimental SIV (Watson *et al*., 1997) and FIV (Diehl *et al*., 1995) infections. Some studies in SIV infected monkeys demonstrated that **peak viremia** level correlated with the level at which viremia stabilized after seroconversion (Lifson *et al*., 1997; Marx *et al*., 1996). Other factors such as **route of virus administration** (Baba *et al*., 1996; Bosch *et al.,* 1997; Sodora *et al*., 1998; Triverdi *et al*., 1996) and **early virus replication** (Staprans *et al.,* 1999; ten Haaft *et al.,* 1998) has also been studied.

It has been hypothesised that AIDS-causing lentiviruses can become more **virulent** upon *in vivo* evolution and acquire the capacity to accelerate the progress to AIDS (Hirch, 1999; Kimata *et al.,* 1999). Holterman *et al.* (1999) did indeed show that selective transmission of SIV from late-stage cases of AIDS in rhesus monkeys resulted in a SIV strain that was

capable of inducing a highly accelerated AIDS-like syndrome with extremely high virus load and rapid loss of $CD4^+$ T-cells within weeks of infection. The animals progressed to AIDS before an effective immune response could be mounted.

In mice infected with the Friend virus complex, the severity of virus induced immunosuppression is directly correlated with the **virus dose** inoculated (Bendinelli *et al.,* 1985; Ceglowski & Friedman, 1970). The incubation period of CasBrE (MuLV like virus) induced spongiform neurodegenerative disease in mice can be dramatically shortened (from 150 days to 15 days) by concentrating the virus inoculum 1000-fold (Brooks *et al.,* 1979; Brooks *et al.,* 1980). Similar effects of the virus dose have also been shown by others (Czub *et al.,* 1992).

Host factors

With the identification of HIV as the cause of AIDS, it seemed that a vaccine would follow closely behind. However, despite a large concerted effort, the problem has proven more difficult than anticipated, and researchers are still struggling to design a protective AIDS vaccine. A remaining problem is a lack of basic knowledge about the immunological requirements for protection against retroviruses (reviewed in Gandhi & Walker, 2002; Johnson & Desrosiers, 2002; McMichael & Rowland-Jones, 2001; Nabel, 2001).

A virus infection can activate both humoral and cellular arms of the immune system (Fig. 7). **Innate immunity**, such as phagocytes, natural killer (NK) cells, and complement, acts rapidly and has an important role in the initial control of acute viral infections. Further, cells of the innate immune system, such as dendritic cells are necessary to activate acquired immunity. **Acquired immunity** consist of humoral (B-lymphocyte mediated) and cellular (T-lymphocyte-mediated) responses. In addition, memory lymphocytes protect against re-exposure to the viral pathogen. **Antibodies** prevent infection of cells, by opsonisation or complement mediated lysis of the virus, and by antibody dependent cell-mediated

cytotoxicity. The **cellular immune responses** to virus consist of both $CD8^+$ cytotoxic T lymphocyes (CTLs) and $CD4^+$ T helper cells. Both CTL and T-helper cell recognize short peptide fragments of viral proteins that bind to MHC molecules. Recognition of viral peptide in conjunction with class I MHC on the surface of an infected cell by a $CD8^+$ cytotoxic T lymphocyte leads to lysis of the infected cell. A $CD4^+$ T-helper cell recognizes viral peptide in conjugation with class II MHC on the surface of an antigen-presenting cell. The T-helper cell is then activated to secrete lymphokines that coordinate $CD8^+$ T-cell and B-cell responses.

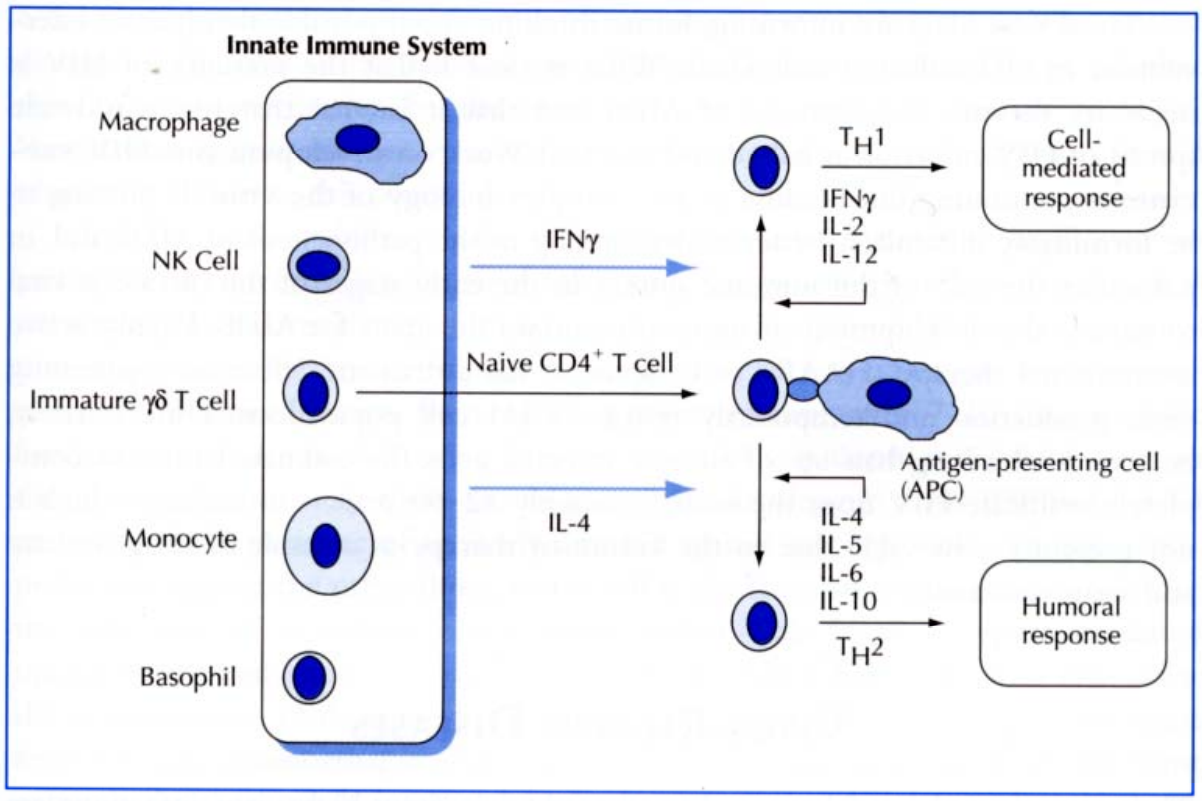

Figure 7: Regulation of cell-mediated and humoral immune responses.
Regulation of the immune system depends on a complex network of cells. $CD4^+$ T helper (T_H) cells have a central role in this process. Immunological theory suggests that there are two types of these: T_H1 cells, which promote the cell-mediated response, and T_H2 cells, which promote the humoral response. Protection against retrovirus infections which requires both cell-mediated and humoral effector mechanisms may be favoured by the development of a T_H1 rather than T_H2 $CD4^+$ T-cell response. The figure is from Cann, 2001.

In most virus infections where the immune response has been studied, it is clear that both humoral and cellular arms of the immune system are required for an optimum protective response. Nevertheless, many retroviruses replicates well in otherwise healthy individuals, despite strong, virus-specific immune responses (reviewed in Gandhi & Walker, 2002; Jonson & Desrosiers, 2002). However, it is well known that there is genetic variation among different individual hosts in their susceptibility to simple retroviruses like FV (Chesebro *et al.,* 1990; Hasenkrug & Chesebro, 1997; Ney & D' Andrea, 2000), and complex retroviruses like HIV-1 (Carrington *et al.,* 1999; Paxton *et al.,* 1996).

Host factors in FV infection.

As shown in Table 6, FV-induced diseases are genetically controlled by multiple non-H-2 linked virus susceptibility/resistance genes (*Fv-1, Fv-2, Fv-3, Fv-4, Fv-5*) as well as H-2 linked and non-linked immune response genes (*Rfv-1, Rfv-2, Rfv-3*).

Table. 6. Host genes that affect susceptibility to Friend virus-induced disease

		Gene	Function
I	Interference with retroviral infection	*Fv4* *Fv1*	Blocks retroviral cell surface receptors Interferes with retroviral life cycle
II	Altered immune response	*Rfv1, Rfv2* *Rfv3*	Susceptibility to immunosuppression Non-H-2-linked determinant of immune responses
III	Regulators of erythroid cell proliferation	*Fv5* *Kit, Mgf* *Fv2*	Determines anemia *versus* polycythemia Required for normal erythropoiesis Determines proliferative response to gp55

Generated from Ney and D'Andrea (2000).

Adult mice with appropriate susceptibility allels at the nonimmunological loci (e.g., Fv-$2^{s/s}$ or Fv-$2^{r/s}$) are infectable by FV and develop severe splenomegaly (described above). Their subsequent survival is dependent on specific major histocampatibility complex (MHC) class I and II allels, and a non-MHC gene Rfv-3, which controls virus-specific antibody

responses. Mice having high recovery MHC and *Rfv-3* genotypes, such as *H-2*$^{b/b}$ and *Rfv-3*$^{r/s}$, respectively, spontaneously recover to near normal spleen size within weeks and generally live out a normal life span. This phenomenon provides a system for the study of host and virus factors that are involved in the control and eventually elimination of retrovirus infection (selected references: Dittmer *et al.,* 1999, 2001, and 2002; Hasenkrug & Chesebro, 1997; Hasenkrug & Dittmer, 2000; Hasenkrugh *et al.,* 1998; Hasenkrug 1999; Stromnes *et al.,* 2002).

Host factors in HIV-1 infection.

As described above, the course of HIV infection varies widely among individuals. Immunologic and genetic studies of long-term nonprogressors and exposed, yet uninfected persons, have elucidated the complex interplay of host and virus factors that may determine the course of HIV infection, or even the risk for initial HIV acquisition (reviewed in Hogan & Hammer, 2001, a and b). Some host factors in HIV infection are summarized in Table 7.

The effects of sex and steroid hormones.

In addition to inter-individual host differences, gender related differences in susceptibility to virus-infection and disease outcome has been reported, but not intensively studied (for review and reports see, Weizemann & Pardue, 2001; Whitacre *et al.,* 1999). Sex-related differences in susceptibility to experimental virus infections have been reported for viruses like the encephalomyocarditis virus (Curiel *et al.,* 1993), the coxaxkievirus B3 (Huber *et al.,* 1999), the herpes simplex virus type 1 (Han *et al.,* 2001), the vesicular stomatitis virus (Barna *et al.,* 1996), the Theiler's murine encephalomyelitis virus (Hill *et al.,* 1998), and murine retroviruses (Gillespie & Rowson, 1968). Further, sex differences in HIV infected humans have been observed (reviewed in Gandhi *et al.,* 2002; and discussed below).

It is well known that there are sex differences in regard to immune functions, with females having generally higher immunoglobuline levels and mounting stronger immune responses following immunisation or infection than males (reviewed in Morell, 1995; Pelfrey, 2001;

Table 7: Host factors in HIV infection.

Host factors	Effect on HIV transmission and disease progression
Cell mediated immunity	
• Cytotoxic T-cells	Eliminates virions and virus infected cells; plays prominent role in initial control of viremia, slowing of disease progression, and perhaps prevention of infection.
• T-helper cell response	Preservation of this response may be vital to preservation of cytotoxic T-cell response, and its importance provides theoretic rationale for early treatment.
Humoral immunity	Role in prevention and control of disease is unclear.
Local factors	
• STDs	May upregulate HIV replication
• Mucosal immunity	Role in prevention of transmission and disease progression is unclear.
• Dendritic cells	Facilitate HIV infection of T-cells by capturing and transporting HIV to lymph nodes and activating T-cells. [1)]
Chemokine receptors	
• CCR5-Δ32	Homozygosity for this deletion is associated with decreased susceptibility to R5 virus infection; heterozygosity is associated with delayed progression to disease.
• CCR2-V641	Heterozygosity is associated with delayed progression to disease.
• CCR5 promotor	Several genetic polymorphisms that may affect transsmission or disease progression have been identified.
Chemokines	
• SDF-1 3'α	Homozygosity may be associated with delayed progression to disease
• Cytokines	Complex interplay of stimulatory and inhibitory cytokines affects HIV replication.
Other genetic factors	
• HLA alleles	Certain alleles are associated with differing susceptibility to infection and rates of disease progression.

The table is generated from Hogan & Hammer, (2001, a and b): Host determinants in HIV infection and disease, part I and part II. SDF= stroma cell-derived factor; STD = sexually transmitted disease

1) Many cell types, including erythrocytes (Hess *et al.,* 2002), can bind HIV directly through cell-surface receptors or via attachment of HIV immune complexes to cellular receptors. Infectious virus can then be transferred to various target cell (Levy, 2002).

Verthelyi, 2001; Whitacre *et al.,* 1999). Estrogens and progestins appear to have complex roles in regulating the balance between pro- and anti-inflammatory signals, primarily via the production of immunoregulatory cytokines (Correale *et al.,* 1998; Piccinni *et al.,* 2000). Progesterone, which is generally considered anti-inflammatory, often opposes estrogen effects (Hunt *et al.,* 1997). Further, estrogen have been demonstrated to induce a T_H1 type immune response, while progesterone, as well as glucocorticoids, induce a T_H2 shift *in vitro* (Miyaura & Iwata, 2002; Whitacre *et al.,* 1999).

A few studies have reported that sex-steroid hormones may influence retrovirus infections. For example, in ovulating women the HIV-1 load has been shown to fall during the early follicular phase through the midluteal phase (Greenblatt *et al.,* 2000). Further, cervicovaginal shedding of HIV has been reported to be higher among pregnant women than it is among non-pregnant women with more advanced HIV disease (Henin *et al.,* 1993). It has also been questioned whether use of contraceptives can influence the susceptibility to HIV in females. In particular, the use of progesterone-containing contraceptives (e.g., Depo-Provera and Norplant) have received attention (Bahamondes *et al,* 2000; Cohen, 1996; Miller *et al.,* 2000) after a study showed that monkeys treated with progesterone implants were at an increased risk for becoming infected with SIV (Marx *et al.,* 1996). Although it has been assumed that this enhanced SIV infection of the female genital tract after progesterone treatment was due to thinning of the vaginal barrier (Hild-Petito *et al.,* 1998; Sodora *et al*, 1998), other factors such as progesterone-induced immunomodulation, effects on target cells and receptor expression, or direct effect on virus transcription cannot be excluded in retrovirus infections. A study by Vassiliadou and coworkers (1999) suggested that progesterone could have negative effects on chemokine-mediated recruitment of lymphocytes and monocytes to mucosal epithelia. Further, use of oral contraceptive with equal levels of progesterone and estrogen induces up-regulation of the CCR5 chemokine receptor on $CD4^+$ T-cells in the cervical epithelium of healthy women, which could represent an increased risk of HIV-1 transmission via this route (Prakash *et al.,* 2002).

A more direct effect of steroid hormones on retrovirus production is seen in **MMTV** infection were progesterone, in addition to glucocorticoids and androgens (but not estrogens), strongly stimulate the rate of MMTV transcription through the binding of hormone-receptor complexes to hormone regulatory elements (HRE) in the MMTV LTR (Beato *et al.,* 1989; Cato *et al.,* 1986; Otten *et al.,* 1988). The strong responsiveness of the MMTV LTR to steroid hormones has made the MMTV promoter the best-studied model for investigating the regulation of gene expression by steroid hormones (Beato, 1989; reviwed in Aranda & Pascual, 2001). General features of steroid-hormone mediated gene regulation are illustrated in Fig. 8, and are described in the correspondent legend.

Glucocorticoid response elements (GRE) are present in the LTR of several mammalian retroviruses, and hormonal regulations of virus replication through GRE are reported for both simple retroviruses (Beato *et al.*, 1989; Celander *et al.,* 1988; Miksicek *et al.*, 1986) and complex retroviruses (Niermann & Buehring, 1997; Kolesnitchenko & Snart, 1992; Mitra *et al.,* 1995; Russo *et al.,* 1999). In HIV, three potential regions are present in the LTR (Mitra *et al.,* 1995). Another GRE is located in the HIV-1 *vif* open reading frame (Sodeyns *et al.,* 1993). The role of these sites in HIV infection is still not clear, and studies performed *in vitro* have given diverse results. For example, dexamethasone inhibits LTR-driven gene expression in some T and B cell lines, but not in monocytic cells (Mitra *et al.,* 1995). Cortisol and dexamethasone increase HIV-1 production in some lymphoid and monocytic cell lines, apparently through interaction with response element located in *vif* (Sodeyns *et al.,* 1993).

Nelson *et al.* (1999) suggested that incorporation of hormone response elements in the LTR might be advantageous for extending the cellular host range of the virus to exploit a number of endocrine signaling pathways. This may be especially advantageous for the simple retroviruses which host cells are limited to actively dividing cells (see above). Further, the presence of HREs implies that physiological changes in the host, including hormonal fluctuations, can directly influence viral replication.

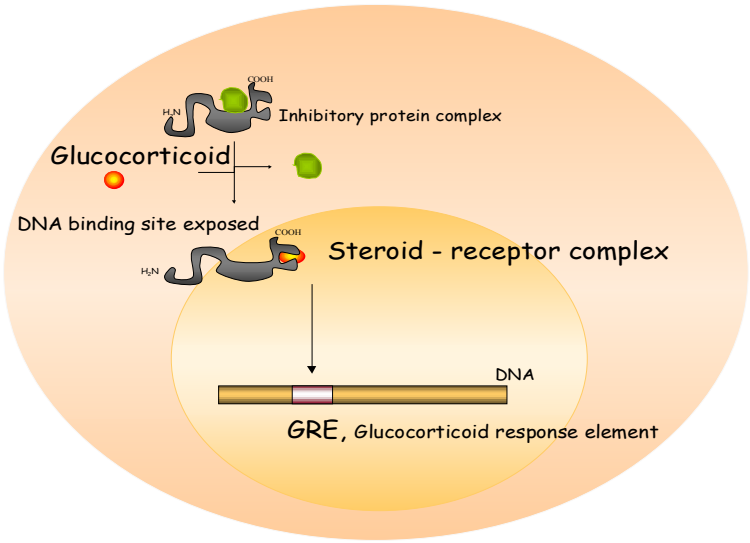

Figure. 8. Steroid hormones and gene regulation.

Steroid hormones enter cells passively and bind to receptors that are located in the cytoplasm or the nucleus. The DNA sequences that are recognized by steroid hormone-receptor complexes are termed hormone response elements (HREs). The first element to be identified was the glucocorticoid response element (GRE) in the MMTV LTR (Beato *et al.,* 1989 and references therein).

Glucocorticoids bind to the cytoplasmic form of the glucocorticoid receptor, which is associated with the heat chock protein, Hsp90 (Pratt & Welsh, 1994). Hormone bound glucocorticoid receptor dissociates from Hsp90 and is transported to the nucleus, where it binds with high affinity to DNA sites. Glucocorticoid receptor and other steroid receptors are competent to activate transcription of a basal promoter in the absence of other transcription factors. GRE mediates gene induction by glucocorticoids, progesterone, and androgens, but not by estrogens (Beato *et al.,* 1989; Cato *et al.,* 1986; Darbre *et al.,* 1986; Otten *et al.,* 1988; Scüle *et al.,* 1988). However, the efficiency of steroid hormone-mediated gene activation depends on several factors including the availability of receptors and hormones (Archer *et al.,* 1995; Nelson *et al.,* 1999). Further, coactivator complexes and other transcription factors influence hormone responsiveness of a particular gene (Deroo & Archer, 2001).

Nucleosomes, which fold chromosomal DNA, contain two molecules each of the core histones H2A, H2B, H3, and H4. Almost two turns of DNA are wrapped around this octameric core, which provides a major impediment to transcription. Glucocorticoid receptors (GR) in association with chromatin-remodelling complex can bind to target sites within promoter regions of genes assembled as chromatin. This interaction alters the nucleosome architecture to allow binding of other transcription factors like NF1 (see Fig. 3), that cannot bind an LTR assembled into chromatin (Archer, *et al.,* 1992, for review on GR-mediated chromatin remodelling see, Deroo & Archer, 2001). Only a single GR-binding site is sufficient for the structural transition to occur (Belikov *et al.,* 2000).

Early virus-host interactions

One of the main challenges that viruses present to the immune system is a kinetic one. The virus must replicate before the immune response is efficiently mobilized, and this race between the immune system and the virus in the early stage of infection often determines the severity of the infection (Bangham & Phillips, 1997). Overall, several studies show that there is an inverse correlation between plasma viremia and disease progression rate (described above). Further, the exact timing of peak viremia (see Fig. 6) varies with virus, route of inoculation, and virus dose inoculated (Table 8). Staprans *et al.,* (1999) suggested that a less vigorous early virus replication could give the immune response an advantage in the dynamic host-virus interaction that occurs very early after SIV infection. Studies in the mouse model of FV infection have also indicated that lower rate of virus spread may allow a weak immune response time to develop before being overpowered by high level of virus and virus infected cells (Peterson *et al.,* 2002, and references therein). It is also likely that the initial phase of infection is the crucial timeframe during which virus clearance or persistence is determined. Thus, small differences in the initial dynamic relationship between the host and the virus may influence the course of the infection. For example, virus factors (e.g., the envelope or LTR) and host factors (e.g., hormones) that exert their effects before full development of specific immune responses might be critical determinants for the immediate rate of virus dissemination and susceptibility to infection.

Exposure to HIV-1 may or may not result in infection, and the AIDS epidemic is characterized by extreme heterogeneity in the clinical course (see above). Identification of viral and host factors that determine the susceptibility to HIV infection, or the rate of progression to disease once infection is established, might lead to novel treatment paradigms. Unfortunately, the time of infection is often uncertain and the infectious dose conjectural in HIV infections, so the initial events may easily be missed. Therefore, investigations of early virus-host interactions must be performed in animal models.

Table 8.

Virus and host factors that may influence the overall course of a retrovirus infection.

	COMPLEX VIRUS	SIMPLE VIRUS	The FIS-2 model
VIRUS FACTORS			
inoculum size (dose)	Holterman *et al.,* 2000 Quinn *et al.,* 2000	Czub *et al.,* 1992	paper III
route of inoculation/ transmission	Burkhard *et al.,* 2002 Greenier *et al.,* 2001 Herz *et al.,* 2002	Okada *et al.,* 1998	
virulence	Asjo *et al.,* 1986 Holterman *et al.,* 1999	Czub *et al.,* 1992	papers II- IV
genetic determinants	Locher *et al.,* 2003 Coffin *et al.,* 1997*	Fan *et al.,* 1990* Coffin *et al.,* 1997*	papers I-IV; Dai *et al.,* 1994
HOST FACTORS			
host genetic immunity	Carrington *et al.,* 1999 Zagury *et al.,* 1998 Paxton *et al.,* 2001	Hasenkrug 1997*	paper V
receptors/co receptors	Hogan & Hammer 2001b*		
age	Chakrabarti *et al.,* 2000	Yamaguchi *et al.,* 2001	paper II
sex	Gandhi, 2002*	Gillespie & Rowson, 1968	papers III-V
hormones			
contraceptive use menstrual cycle	Prakash *et al.,* 2002 Greenblatt *et al.,* 2000		
stress hormones/ glucocorticoids	Corley, 1995 and 1996 Barr *et al.,* 2000	Beato *et al.,* 1989	paper IV
sex-hormones	Brabin, 2002 Marx *et al.,* 1996 Smith 2000	Karande *et al.,* 1975 Archer *et al.,* 1995*	paper V

*Reviews. The references listed in the table are representative, not exhaustive

AIMS OF THE STUDY

The overall objective of the present study was to investigate virus-host interactions in early murine retrovirus infection, and to define viral and host determinants responsible for retrovirus-induced erythroleukaemia and immunosuppression. In general, studies of biological variations can offer important insight into underlying biological mechanisms. This was used as an experimental approach throughout the study:

Viral determinants

Since FIS-2 is an immunosuppressive, low oncogenic, variant of the closely related F-MuLV, comparing the two variants may elucidate the roles of viral genes in the pathogenesis. At the start of this thesis, six chimeras between FIS-2 and F-MuLV had been generated (shown in paper I).

(1) We wanted to use these chimeras as a tool to study genetic (viral) determinant(s) responsible for the pathogenic properties of FIS-2 (**papers I-II**).

A major advantage with animal models for virus infections is that we can control for virus dose and time of infection, and thereby investigate early virus-host interactions that may be missed in the clinic (e.g., HIV infection).

(2) We therefore wanted to use our model system to examine whether the infective dose or early virus dissemination patterns could influence the course of infection (studied in **papers I-III**).

Host determinants

Besides inter-individual host differences, gender-related differences in susceptibility to virus infections and disease outcome had been reported, but not intensively studied.

(3) We therefore wanted to study the effects of host gender (sex) on susceptibility to murine retrovirus infection (**papers III-V**).

Virus-host interactions

In **paper III,** we reported that there indeed were gender differences in FIS-2 infection (susceptibility, early virus dissemination, and immunosuppression). We therefore decided to investigate this issue further. The main questions we wanted answers to were:

(4) Can the presence of a hormone response element (GRE) in the FIS-2 LTR mediate the sex-differences observed? This was investigated in **paper IV.**

(5) Can physiological conditions of the host, like the hormonal make up, influence the susceptibility to FIS-2 infection? This was investigated in **paper V.**

SUMMARY OF THE WORK

Paper I

Identification of genetic determinants responsible for the rapid immunosuppressive activity and the low leukemogenic potential of a variant of Friend leukemia virus, FIS-2: In this paper we focused solely on viral determinants for pathogenesis. We attempted to map the genetic factors responsible for the strong immunosuppressive activity and the low leukaemogenic potential of the F-MuLV variant, FIS-2. Six chimeric viruses between FIS-2 and F-MuLV cl. 57 were generated and used as experimental tools. The construction of recombinant viruses was performed before the onset of the present thesis.

Since FIS-2 and F-MuLV have almost identical nucleotide sequences encoding the immunosuppressive transmembrane protein TM, we were interested in determine viral factors other than the TM which could underlie the enhanced immunosuppressive properties of FIS-2. The effect of virus infections (parental and recombinant viruses) on primary antibody response against a T-cell dependent antigen (SRBC) was used as a parameter for the immunosuppressive activity in adult NMRI mice. We found that a region encoding the envelope surface protein (SU) of FIS-2 was responsible and necessary for the enhanced immunosuppressive activity. This was a novel finding. The immunosuppressive activity in individual mice infected with F-MuLV or constructs that did not have FIS-2 SU varied greatly. Our results suggested that the suppressive effect of FIS-2 SU was overwhelming and dominated over various host genetic factors. In addition, we could not observe any direct correlation between the number of virus positive cells in the spleen and immunosuppressive activity. These results indicated that murine retroviruses might exert its immunosuppressive effect through other mechanisms than direct infection of immune cells in the spleen.

The leukaemogenic potential of each chimera was studied in newborn mice. Consistent with studies by others, the low leukaemogenic potential of FIS-2 was determined by the LTR. As described in the introduction, it is well accepted that sequences within the LTR contribute to cell-type specific transcription and acts as potentiators of leukaemogenicity.

Generally, MuLVs with one copy of a DR are thought to have lower transcriptional activity than MuLVs with two DRs. It was therefore of interest to study the transcriptional activity of the LTRs from FIS-2 (with one DR) and F-MuLV (with two DRs) in lymphoid cell lines. Different LTR-chloramphenicol acetyl transferase constructs were made and the CAT activity was monitored in different cell lines, including T (EL4.IL2 and L691-6), B (CH-1), monocyte-macrophage ($P388D_1$), and erythroleukaemia (SL9) cells. Although the FIS-2 LTR contained only one DR it had a transcriptional activity either higher than (L691-6, CH-1 and $P388D_1$) or similar to (EL4.IL2 and SL9) that of the F-MuLV LTR. To our knowledge, this was the first study showing that the transcriptional activity of a LTR *in vitro* did not correlate with the potential leukemogenicity of the corresponding virus *in vivo.* It was also shown that deletion of one direct repeat in the F-MuLV LTR caused a decrease in transcriptional activity in all cell lines except in the L691-6, where no effect was observed. These results indicated that a few single point mutations in the FIS-2 LTR had compensated for the loss of one direct repeat.

Paper II

Early dissemination rates of Friend murine leukaemia virus variants correlate with late pathogenesis: Since the mutations in the FIS-2 LTR did not reduce the transcriptional activity *in vitro,* we wanted to examine whether this observation could be confirmed *in vivo*. In **paper II** we also examined whether differences in virus dissemination rates or sites of replication could explain some of the biological features of FIS-2 and F-MuLV. We found that erythroblasts were the primary target cells for both F-MuLV and FIS-2, while B- and T-cells were infected later in the infection. Consistent with the *in vitro* experiments in **paper I**, FIS-2 replicated to similar or higher titres as F-MuLV *in vivo*. However, we did observe a delay in the initial spreading of FIS-2. Studies including the chimeras RE3 (FIS-2 LTR in a F-MuLV background) and RE4 (F-MuLV LTR in a FIS-2 background) indicated that the delay in dissemination was due to mutations in the FIS-2 LTR. The kinetic for early virus replication correlated with mean latency time for virus induced erythroleukaemia in mice inoculated as newborn (reported in **paper I)**. Early dissemination rate did also

correlate with the onset of immunosuppression and erythroleukaemia in adult mice. Early immunosuppression was induced in a phase with low levels of infected lymphocytes. This observation indicated once more that mechanisms other than direct infection of immune cells were involved in murine retrovirus induced immunosuppression. The results in **paper II** also confirmed that FIS-2 SU was responsible and necessary for giving FIS-2 enhanced immunosuppressive activity: The chimera RE4 with F-MuLV LTR and FIS-2 SU induced both an early and strong, persistent immunosuppression. F-MuLV-induced late immunosuppression coincided with signs of erythroleukaemia and persistent viremia. FIS-2 induced a more moderate late immunosuppression without persistent viremia or signs of erythroleukaemia. Overall, our results in **paper II** indicated that early viral replication is a prognostic factor in murine retrovirus induced pathogenesis.

Paper III

Gender-related differences in susceptibility, early virus dissemination and immunosuppression in mice infected with Friend murine leukaemia virus variant FIS-2: In paper III we used the FIS-2 model to study the effect of host gender (or strictly sex) on susceptibility to murine retrovirus infection. Our data showed a close relationship between virus dose, the onset of detectable viremia, and infection in the spleen in young adult, male and female mice. However, male mice were more susceptible to infection by low doses of FIS-2 than female mice were. Given equal, high FIS-2 doses we observed that the levels of virus in serum, bone marrow, and the spleen were initially higher in male mice. Male mice were also more susceptible to FIS-2 induced immunosuppression. These results indicated a more efficient virus replication and dissemination in male mice. Such gender differences were more profound in FIS-2 infection than in F-MuLV infection. Studies with LTR recombinants indicated that sex-related factors could have a more direct effect on the activity of FIS-2 LTR than on the activity of F-MuLV LTR.

Paper IV

A glucocorticoid response element in the LTR U3 region of the Friend murine leukaemia virus variant FIS-2 enhances virus production *in vitro* and is a major

determinant for sex differences in susceptibility to FIS-2 infection *in vivo*: As described in the introduction, the nucleotide sequence of the FIS-2 LTR shows a high homology with that of F-MuLV LTR, except for the deletion of one direct repeat, a few point mutations, and generation of a glucocorticoid response element (GRE) in the U3 region. GRE(s) can mediate gene induction by glucocorticoids, mineralcorticoids, progesterone and androgens, and it has been shown that incorporation of GRE(s) within the LTR can increase the transcriptional activity of retroviral enhancers. We therefore proposed in **paper III** that incorporation of a GRE in the FIS-2 LTR might contribute to the significant sex difference in FIS-2 infection. In order to investigate this hypothesis, we introduced a single point mutation in the GRE, and performed comparative studies in NIH 3T3 cells and in male and female NMRI mice. We found that significantly more virus were produced from NIH 3T3 cells infected with the wild type (wt) FIS-2 than from cells infected with the FIS-2 GRE mutant, and this difference was further augmented by glucocorticoids. Moreover, the progesterone/type II glucocorticoid antagonist RU486 inhibited virus production in a dose-dependent manner. Our previously observed sex-difference in early wt FIS-2 replication was confirmed in **paper IV**. In addition, wt FIS-2 disseminated significant faster than FIS-2 GRE mutant in male mice, but not in female mice. We found no significant difference in dissemination rate between male and female mice infected by FIS-2 GRE mutant. Based on these results, we concluded that sex-related factors could influence early FIS-2 replication via mechanisms involving the GRE.

Paper V

Identification of Friend murine retrovirus infected immune cells and studies of the effects of sex and steroid hormones in the early phase of infection. In paper V we investigated further the influence of sex and steroid hormones on early Friend leukaemia virus dissemination and immune functions. Consistent with former studies we showed that FIS-2 disseminates faster in male than in female mice. Lymphocyte subsets were more rapidly infected in males than in females, and some B- and T-cells, including both $CD4^+$ and $CD8^+$ cell subsets, were virus positive in male mice at 11 d.p.i. We could not find any

significant sex difference in the proportions of erythroblast or B- and T-cells in the spleen. However, an earlier expansion of the $CD8^+$ cell population was observed in infected male mice compared to female mice. This expansion paralleled viremia levels. Thus, one possible explanation for the sex differences in FIS-2 dissemination to lymphocytes, including $CD8^+$ cells, may be that in male mice high virus replication in the primary target cells (erythroblasts) and rapid rise in viremia titres are followed by a parallel expansion of the $CD8^+$ cell subset. Since murine leukaemia viruses only infect dividing cells, antigen-activated expansion of lymphocyte subsets subsequently increases the number of possible target cells.

Viremia and the number of detectable virus positive cells in the spleen declined faster in females than in males. These post peak declines coincided with a more rapidly generation of antibodies against virus positive cells. A rise in the $CD3e^+$ cell subset was most notable in female infected mice. Further, female derived T-cells responded better to *in vitro* mitogen stimuli (Concavalin A) than male derived T-cells. Physiological concentrations of testosterone or β-estradiol did not influence T-cell proliferation, while progesterone and dexamethasone induced a dose-dependent inhibition. Subsequently, we investigated the effects of progesterone administration and testosterone/glucocorticoid reduction on early virus dissemination in female and castrated male mice, respectively. Administration of the injectable contraceptive Depo-Provera® (depomedroxyprogesterone acetate) did not modify early FIS-2 production in female mice. Reduction of testosterone level in male castrated mice had significant impact on both early FIS-2 replication and early F-MuLV replication. Since F-MuLV does not have a GRE in its LTR, this result showed that androgens could influence on early virus replication at other levels besides that which involved the GRE. Castrated mice were notably calmer and less involved in fighting behaviour than male control mice. In **papers IV** and **V** we therefore discussed, in the context of the available literature and our observations, how behavioural factors like testosterone-dependent aggression (i.e., inter-male aggression) and subsequent stress-induced elevated levels of circulating cortisol might influence FIS-2 replication and immune responses during infection.

GENERAL DISCUSSION

The studies in the present thesis sought to define virus and host factors that can influence on the susceptibility to murine retrovirus infection. In addition, we wanted to study possible correlations between events of early infection and subsequent disease progression. For an extensive discussion of the major findings, the reader is referred to **papers I-IV**. The following section will give a general discussion concerning 1) some methodological aspects; 2) the course of FIS-2 infection; 3) determinants responsible for erythroleukaemia; 4) determinants responsible for immunosuppression; and, 5) does sex matter?

I: Methodological considerations

In vivo studies: The mouse model.

Infection of macaques with SIV is the major model in which to study the early phase of immunodeficiency retrovirus infection. However, problems with this model includes the limited number of available monkeys (Choen, 2001), the genetic heterogeneity of the available animals, the lack of certain immunological reagents, costs, and ethical considerations (Joag, 2000). Thus, fundamental concepts in retroviral immunology have to be defined in other ways such as in small animal models.

As described in the introduction, complex retroviruses encode and express additional proteins, and these proteins allow direct control over functions that in simple retroviruses are provided by the host. This additional control may be advantageous in dealing with the host immune responses; complex retroviruses infect adult, immunocompetent animals much more frequently than do simple retroviruses (Coffin *et al.,* 1997). Nevertheless, although complex retroviruses and the hosts they infect are different from the simple retroviruses, many structural and functional properties of complex retroviruses are common to all retroviruses, and initial studies on the "AIDS virus" benefited greatly from existing

knowledge on oncoretroviruses (see Table 3, and the introduction). Since the most fundamental features of complex and simple retroviruses are comparable, murine retroviruses offers inexpensive and less ethical controversial systems for studying general virus-host aspects of the early phase in retrovirus infections (Hasenkrug *et al*., 1997, Morrison *et al,* 1986; Mosier *et al*., 1996, Soldaine *et al.,* 1989). Further, mouse models have a special appeal because of (i) the ability to work in genetically defined mouse strains, transgenic, and knockout animals; (ii) the immune system in mice is known in detail and can easily be manipulated; and (iii) there are a variety of mouse immunological reagents available.

Traditionally, random-bred laboratory animals have been said to have a degree of variance similar to what would be expected in the human population. The initial goal for the "FIS-2-project" was to develop a murine retroviral immunosuppressive system shearing central features with HIV-1 infection in humans. For that reason, an outbred mouse strain was chosen as model organism, and the low oncogenic and immunosuppressive variant of F-MuLV was isolated from a FV infected female NMRI mouse (Faxvaag *et al.,* 1993a). Murine leukaemia virus can be designated as either N-tropic or B-tropic, depending upon whether it replicates more efficiently on NIH Swiss (Fv-1^n) or BALB/c (Fv-1^b) fibroblast cells, respectively. A third group of MuLVs replicates equally well on both BALB/c and NHI Swiss fibroblasts, and they are designated NB-tropic (Troxler *et al.,* 1980). The prototype F-MuLV cl. 57 used in the present thesis is NB-tropic, while FIS-2 is N-tropic. Thus, FIS-2 does not replicate well in the commonly used inbred BALB/c mouse strain (data not shown). Therefore, outbred NMRI mice were used throughout the study.

Generally, the major disadvantage with outbred stocks is the necessity for more animals in the experiments. However, outbred NMRI mice are genetically variable to the extent that all individuals are genetically unique, but they tend to be relatively uniform in comparison with the variability within the species. Further, all NMRI mice have the same genotype of the MHC (H-2^q) (data sheet about NMRI mice supplied by Bomholdtgaard Breeding

Research Center). Nevertheless, pronounced inter-individual differences were observed in viral load, immunosuppression, and anti-virus responses in the populations examined (**papers I-V**). These findings demonstrate the significance of host-factors in murine retrovirus infection.

In vitro studies: Cell lines and primary cell cultures.

The ultimate test of pathogenic potential involves induction of disease in animals. Further, virus-host interactions must necessarily be studied *in vivo*. However, the ability to grow and quantitate retroviruses *in vitro* is important for several reasons, including the possibility to characterize viral mutants. In **paper IV** the replication of wt FIS-2 was initially compared to FIS-2 GRE mutant *in vitro*. Since more virus were indeed produced from NIH 3T3 cells infected with wt FIS-2 than from cells infected with FIS-2 GRE mutant, the virus replications were subsequently compared *in vivo*. Thus, by combining experiments *in vitro* and *in vivo*, the number of animals used in a study can be reduced (which is an important goal in all studies involving experimental animals).

Many cell types can be used to grow and assay retroviruses *in vitro.* For studies of murine retroviruses, immortalized rodent cell lines such as 3T3 cells have proven particular useful (Coffin *et al.,* 1997). However, fibroblastoid cells are usually not directly involved in retrovirus induced disease. We have therefore also used primary cultures of normal hematopoietic cell. Previously, an *in vitro* immunization culture system of splenic cells isolated from FIS-2 infected mice was used to study derangements of cytokine production (Faxvaag *et al.,* 1995). In **paper V**, a similar culture system was utilized to compare T-cell functions in male and female mice, and examine the influence of steroid hormones on mitogen induced T-cell proliferation. It was also our intention to use primary cell cultures to examine whether steroid hormones could influence FIS-2 production. However, as shown in **papers II, III,** and **V**, the amount of virus infected B– and T-cells are low in FIS-2 infected mice, making this approach rather cumbersome. In **paper IV**, NIH 3T3 cells were therefore used to study whether hormones could induce FIS-2 production.

NIH 3T3 cells were also used to quantify viral load in serum and organs, by co-culturing dilutions of e.g., serum or splenocytes with the cell-line. Such end-point dilution procedure is undeniably time consuming and cumbersome (described in **papers III** and **IV**). However, by this approach we could detect productive infection in e.g., the spleens. Further, the assay is rather sensitive, with detection limits on 50 IU per ml serum and one productively infected cell per 200 000 splenocytes. In comparison, the limit of HIV RNA detection in currently available assays is 50 copies per ml plasma (Richman, 2001).

Viruses used

Due to the low fidelity of the reverse transcriptase, **retrovirus stocks** amplified in cell culture or *in vivo* exist as quasispecies. Such high degree of variation poses problems of reproducibility and standardization. In contrast, a molecular cloned virus can be maintained as a DNA provirus in plasmids or lambda phages, and can be amplified with minimal or no changes. Molecular clones are therefore preferable to uncloned viruses for most experimental uses. In the present thesis, molecular cloned viruses (Dai *et al.,* 1994) were used in all experiment. Virus stocks were prepared and titrated from supernatants collected from transfected cells as described in **paper I**. The stocks were dispensed into small aliquots and stored at -80^{o}C until the start of each experiments. If possible, virus stock from the same "batch" was used in most studies. However, in **papers II** and **III,** two different stocks of FIS-2 were used in the experiments. We did not observe any striking difference in the dose-responses. Nevertheless, one should be cautious with direct comparison of the doses between the experiments.

As described in the introduction, chimeric viruses created by recombining segments of two strains of the same virus (e.g., Friend MuLV and Moloney MuLV) have proven very useful in defining amino acid or nucleotide sequence responsible for various biological properties. At the start of the present thesis, six chimeric viruses between FIS-2 and the prototype F-MuLV clone 57 had been generated (shown in **paper I**). Briefly, in the chimera RE1 and RE2 a fragment containing the whole *gag* and part of the *pol* gene was switched between

FIS-2 and F-MuLV. RE3 got FIS-2 LTR in a F-MuLV background and RE4 got F-MuLV LTR in a FIS-2 background. RE5 received FIS-2 LTR, *gag* and part of the *pol*. To study the pathogenic effect of the FIS-2 *env* gene, RE6 received a fragment of FIS-2 containing most of the *env* gene of FIS-2 in the F-MuLV background. In **paper I,** all chimeras were used to identify viral determinants responsible for virus induced erythroleukaemia and immunosuppression. In **papers II** and **III**, the chimeras RE3 and RE4 were used to examine the influence of FIS-2 LTR *versus* F-MuLV LTR on early virus replication and dissemination. Further, in **paper IV** we introduced a single point mutation in the glucocorticoid response element (GRE) presented in the FIS-2 LTR U3 region. It should be noted that these chimeras and the mutated virus do not constitute new models, but are specific application within the framework of the FIS-2 model.

The recombinant DNA techniques were performed by standard procedures, as described in **papers I** and **IV**. Since this (excellent) work was performed by my co-authors, no detailed description and discussion about this part of the study will be given here. However, when comparing virus variant with subtle differences, the stability of the changes performed have to be confirmed. In **paper I,** the chimeric viruses were confirmed by Southern blot analysis of Hirt extracts prepared from NIH 3T3 cell infected with the chimeric stocks. The stability of the single nucleotide exchange in the GRE core sequence after *in vitro* and *in vivo* replication was confirmed by RT-PCR and subsequent sequencing of the virus genome isolated from *in vitro*-infected NIH T3T cell culture supernatants and serum from infected mice, respectively (**paper IV**).

Detection of immunosuppressive activity.

The effect of viral infection of the primary antibody response against SRBC was previously used as a parameter of immunosuppressive activity of the FV (Bendinelli *et al.,* 1985; Ceglowski & Friedman, 1970; Mortensen *et al.,* 1974) and the primary stock of the FIS variant (Faxvaag et al., 1993a). This test system was therefore also used to examine the degree of immunosuppression in FIS-2 infected mice (Dai *et al.,* 1994; **papers I-III**). The

generation of an immune response to SRBC requires the interaction of T-cells, B-cell, and macrophages, and the assay, in which the number of B-cells producing anti-SRBC is determined by using a slide monolayer technique, is described in detail in **paper I.**

II: The course of FIS-2 infection.

Taken together, results from **papers I-V** show that the course of FIS-2 infection in adult mice has many features comparable to the courses in complex retrovirus infections. Similar to HIV infection (see Figure 6), the viremia peaks quite rapidly in the acute phase, and decline as immune responses develop and attain control over the infection. The kinetics of virus replication, immunosuppression, and some immune responses during FIS-2 infection in female NMRI mice are illustrated below:

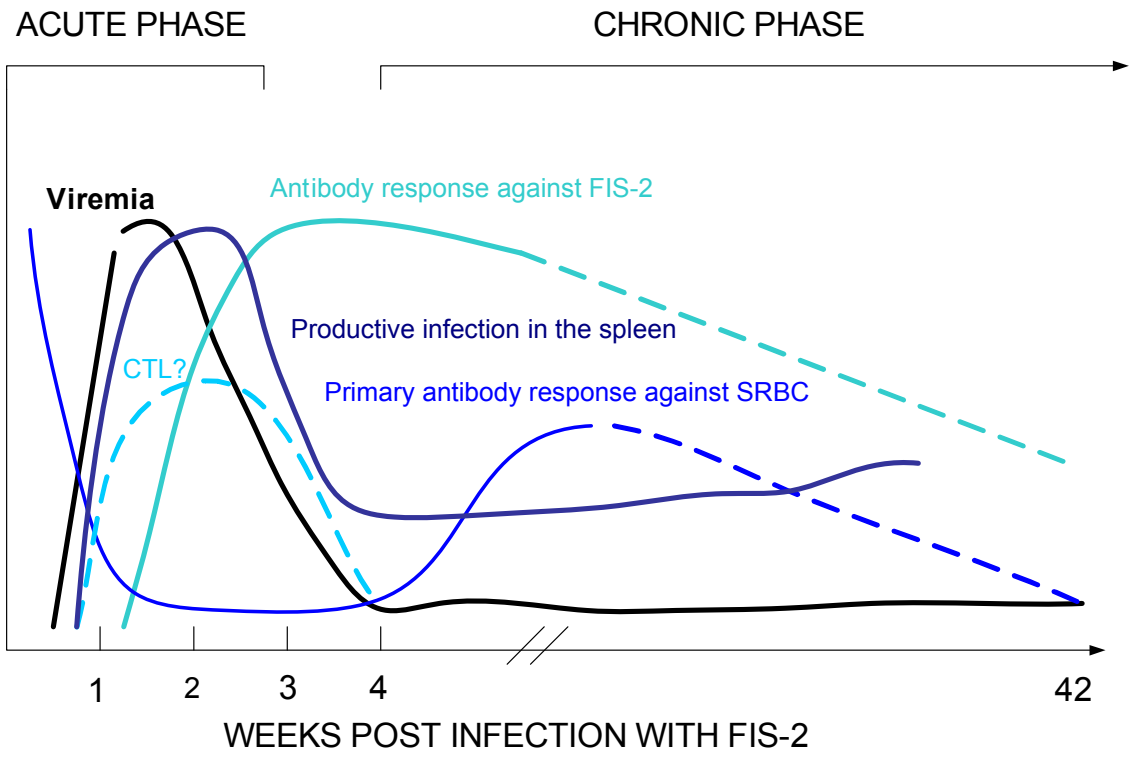

Figure 9. A schematic diagram of events occurring after FIS-2 infection in female NMRI mice. The figure is based on results presented in **papers I-V**. The dotted lines indicate unpublished or tentative observations. See text for details.

In FIS-2 infected mice, peak viremia were reached 5-11 dpi, depending on virus dose, virus variants, and host sex (**papers II** and **IV**). This event was followed by a decline in viremia that coincides wih the appearance of antibodies against virus infected cells (**papers IV** and **V**). In female mice the anti FIS-2 response was shown to be highly efficient, keeping the level of cell-free virus in the blood (viremia) below detectable limit, in spite the fact that productive virus infection was detected in the spleens during the same period of late infection (**paper II**). We observed expansions of the $CD8^+$ and $CD3^+$ cell subsets in the spleen during the early phase of infection (**paper V**), indicating involvement of cell-mediated immune responses in the initial control of FIS-2 infection. However, and extensive analyze of the immune responses against FIS-2 was not performed in the present study. Therefore, more investigation is needed to elucidate important immune parameters in FIS-2 infection.

FIS-2 induced a pronounced immunosuppression against SRBC at 14 dpi. The kinetic or severity of the immunosuppression was influenced by infective dose (**paper III**) and replication rate (**papers II-III**). An interesting phenomenon was noted in a study of the humoral immune responses in female mice inoculated with high FIS-2 dose (Standal *et al;* manuscript in preparations): Initially, high titer serum levels of neutralizing antibodies and antibodies directed against viral membrane proteins on infected cells were detected. However, as infection proceeds, these levels were drastically reduced (see Fig. 9). Further, the degree of the immunosuppression that developed was unrelated to the level of protective antibodies. Thus, antiviral antibodies does not protect against development of immune deficiency and antiviral humoral response, when initiated, is not significantly interfered by the immunosuppression that starts to develop the first week of infection. The FIS-2 induced immunosuppression was partly restored after primary infection, but seemed to become subsequently more severe.

The major clinical differences in FIS-2 *versus* F-MuLV cl. 57 inoculated adult mice were observed in the early and late phases of the infections. F-MuLV disseminated somewhat

faster than FIS-2, and induced a rapid, but transient and mild immunosuppression (**paper II**). At 14 dpi, all FIS-2 infected mice were totally immunosuppressed; whereas only 10% of the F-MuLV infected mice were immunosuppressed at this point (**paper I**). While FIS-2 seemed to be efficiently down regulated by the host immune system, some of the F-MuLV inoculated mice had detectable viremia 2-4 months post inoculation (**paper II**). Further, in contrast to FIS-2, several F-MuLV inoculated mice developed gross splenomegaly in this period. Thus, only FIS-2 induced late immunosuppression without any signs of erythroleukaemia in adult mice (**paper II**).

III: Determinants responsible for erythroleukaemia.

LTR and virulence

Consistent with previous studies (described in the introduction), the deletion of one direct repeat in the LTR was responsible for the long latent period of erythroleukaemia induced by FIS-2 in mice inoculated as newborne. Surprisingly, the mutations in the FIS-2 LTR did not seem to reduce transcriptional activity *in vitro* (**paper I**), and FIS-2 was shown to replicate to similar or higher titre than F-MuLV in the *in vivo* experiments (**paper II**). To our knowledge, this was the first evidence that high transcriptional activity of an LTR *in vitro*, or replication capacity *in vivo*, did not correlate with the potential leukemogenicity of the corresponding virus. However, we showed a correlation between **early virus replication rate** and subsequent virus induced erythroleukaemia. Initial virus dissemination was determined by the **LTR** (**paper II**). The **primary target cells** for both FIS-2 and F-MuLV seemed to be erythroblasts, while B- and T-cells were infected later in the course of infections. Thus, the biological difference between FIS-2 and the prototype F-MuLV cl. 57 was not due to replication in different target cells.

Age and viral load

Most studies of age-related changes in susceptibility to murine retrovirus have focused on the progression from young age to adulthood. During this period, mice generally become

more resistant with increasing age (Hoffmann *et al.,* 1984). This phenomenon is thought to reflect that young and adult mice are more immunocompetent than neonatal mice are. However, some reports have indicated that newborn mice are less immunology immature than previously thought (Forsthuber *et al.,* 1996, Ridge *et al.,* 1996), and that immunological responses in newborn mice are partly determined by the antigen level (Sarzotti *et al.,* 1996). The influence of the infecting virus dose in Friend virus (FV) infection has been previously investigated: Li *et al.* (1987) found that, given in high doses (from 10^5 PFU), a 100-fold dilution of input increased the latent period for erythroleukemia induction by 10 days (from 55 to 65 days). Chesebro and coworkers (1990) showed that in mouse strains with H-2 $D^{d/b}$ genotype the size of FV inoculum had strong influence on the incidence of spontaneous recovery from leukaemia in adult mice. Their experiments suggested that the anti-FV response was highly dependent on the initial dose of virus. Sarzotti *et al.* (1996) showed with another murine system that high doses of Cas-Br-M murine leukaemia virus induced a non protective T_H2-type response in newborn mice, while a very low dose of virus induced a protective cytotoxic T lymphocyte (CTL) response. A few studies have also studied the changes from adulthood to old age. In most species, there are age-dependent declines in the immune responses, which may result in an increased susceptibility to infectious agents (Hirokawa 1994). However, studies by Yamaguchi *et al.,* (2001), indicated that old target cells were more refractory to Friend leukaemia virus induced leukemogenesis than young target cells. Although the precise mechanism of this observation was not clearly shown, the authors suggested that old target cells might have lower proliferative potential and thus had less frequent chance for retroviral infection.

Since newborn mice have a substantial erythropoesis in bone marrow, liver, and spleen, we proposed in **paper II** that a high number of replicating target cells (i.e., erythroblasts) in newborn mice influence the susceptibility to Friend murine leukaemia infection. Further, our results indicated that the rate of replication or dissemination within the first few days of infection is a major determinant of subsequent pathogenesis. Our findings favor the view

that a high level of virus replication is required only transiently during the early pre-leukaemia stages of disease, and that the rate of infection of target tissue is more relevant to disease induction than the level of virus replication per se (Evans *et al.,* 1987). As described in the introduction, since murine leukaemia virus does not encode an oncogene and cause tumours by integrating near cellular oncogenes and activating or altering their transcription, the more cells that become infected, the greater the likelihood that an oncogenic integration will occur.

IV: Determinants responsible for immunosuppression

Initial virus dissemination rate or the virus dose inoculated also influenced virus-induced immunosuppression (**papers II** and **III,** respectively). Such a correlation between viral load and immune dysfunctions has been previously reported in FV infection (Bendinelli *et al.* 1985; Ceglowski & Friedman, 1970). The observation that viral uninfectious particles, and especially a conserved hydrophilic region of the TM protein, had immunosuppressive properties has been suggested as a possible explanation for the correlation between viral load and immunosuppression in retrovirus infection (Denner 1998). However, since TM is almost[1] unchanged in FIS-2 (Dai *et al.,* 1994), the difference in immunosuppressive properties between FIS-2 and F-MuLV must be determined by other sequences than TM. In **papers I** and **II,** we showed that the major determinant for the enhanced immunosuppression of the primary antibody response was the FIS-2 **SU** portion of the virus envelope. SU is composed of two domains that are linked by a short, proline-rich, "hinge" region (Lavillette *et al.,* 1998). In the SU of Friend MuLV the N-terminal 235 residues before the proline-rich region form a discrete domain (receptor binding domain, RBD) that binds to its receptor, murine CAT1 (mCAT1), with high affinity and 1:1 stoichiometry (Battini *et al.,* 1992; Battini *et al.,* 1995; Davey *et al.,* 1997; and see Fig. 10). Among the 22 amino acid changes found in the FIS-2 SU compared to the F-MuLV SU, 10 were

[1] There is one mismatch of an extra glutamine residue located in the carbocyl-terminal end of the FIS-2 TM protein.

shown to accumulate in a region of 100 amino acids (200-300), with three amino acids changes in region 282-290. Computer analysis showed that such changes gave rise to higher hydrophilicity in this region, which overlaps with the proline-rich region (Dai *et al.,* 1994).

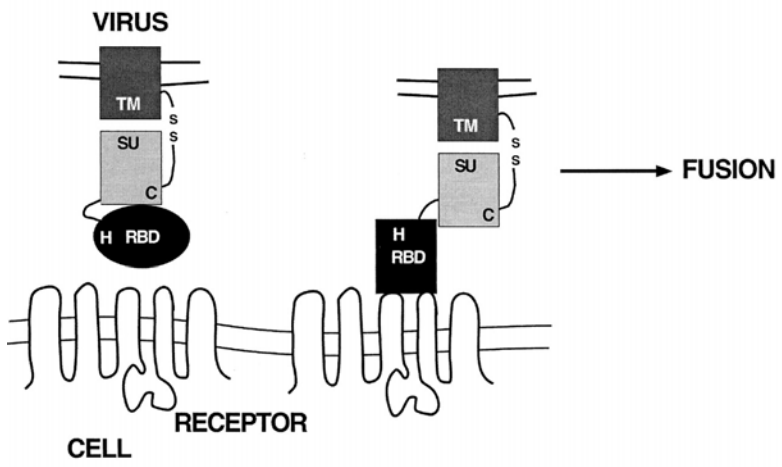

Figure. 10. The envelope protein of Friend MuLV and receptor binding. The viral membrane containing the Env protein is at the top. Env is depicted with the receptor binding domain, RBD (oval) connected by the proline-rich region (curved line) to the C-terminal segment (light shaded rectangle). The transmembrane domain (dark shaded rectangle) is connected to the C-terminal segment by a disulfide bond. The cell membrane containing the F-MuLV receptor (mCAT1) is at the bottom. From Barnett & Cunningham (2001).

Initially, we hypothesized that the difference in immunosuppressive properties between FIS-2 and F-MuLV clone 57 were due to different target cells infected by the two virus variants. However, in **paper II** we showed that erythroblasts were the primary target cells for both FIS-2 and F-MuLV in adult mice, while B-and T-cells were infected later. Our studies also showed that immunosuppression was induced in a phase with low levels of lymphocytes infected. This observation indicated that other mechanisms than direct infections of immune cells were involved in FIS-2 induced immunosuppression. Thus, it is still enigmatic how the FIS-2 SU envelope protein enhances the immunosuppressive properties of FIS-2.

More generally, soluble immune-regulatory factors induced by the virus infection itself or virus proteins (e.g., SU?) are known to constitute some of the immune cell abnormalities in retrovirus induced immunosuppression. It has been suggested that downregulation of immune function may be related to the increased production of interleukin (IL)-4, IL-10, and transforming growth factor-β (TGF-β) by T helper (T_H) cells of the T_H2 subset; all three cytokines are known to inhibit activation of macrophages and T_H1 cells mediated by interferon-γ (Joag & Narayan, 1993; Sher *et al.,* 1992).

There are indeed multiple derangements of cytokine homeostasis in FIS-variant[2] infected adult mice: A severe depression of immune reactivity towards neoantigens develops concomitant with suppression of IL-2 and TNF-α production and an increased production of TGF-β (Faxvaag *et al.,* 1993b, Faxvaag *et al*., 1995). These previous studies showed that the virus-induced immunosuppression involved both T- and B-cell functions. Disturbed cytokine homeostasis and a switch to a T_H2 immune cell response in infected mice may explain some of the observed changes in immune cell function (Faxvaag *et al.,* 1995). Further, production of TGF-β, a peptide with the ability to suppress immune cell functions (Letterio & Roberts, 1998), might be an important factor (Faxvaag *et al.,* 1993b).

An intriguing feature of TGF-β1 is that its inhibitions are not simply limited to the initial activation of the naïve T-cells: Lúdvíksson *et al.* (2000) found that TGF-β1 had a carry-over effect such that T-cells re-stimulated in secondary cultures were less reactive both with respect to proliferation and T_H1 or T_H2 cytokine secretion, even when they were stimulated secondarily in the absence og TGF-β1. Thus, strong stimulation of TGF-β1 production early in the infection could induce a long lasting immunosuppression (for review on regulation of immune response by TGF-β1, see Letterio & Roberts, 1998). Exclusive up-regulated expression of TGF-β1 from LP-BM5 MuLV infected stromal cell lines has been associated with impaired hematopoiesis (Gallicchio *et al.,* 1996).

[2] Isolated from spleen extract, not to be confused with the molecular cloned FIS-2.

Immunosuppression by TGF-β has been more clearly indicated in FV infected mice were chronic FV infection induced expansion of $CD4^+$ regulatory T-cells that suppressed the proliferation of $CD8^+$ T-cells *in vitro* by means of mechanisms involving TGF-β, but not IL-2 and IL-10 (Iwashiro *et al.,* 2001).

Another possible producer of TGF-β might be (virus-infected) erythroblast. Immature erythroide cells can suppress humoral immune response induced by both thymus dependent and thymus independent antigens. This immunoregulatory effect are mediated, at least in part, by soluble factors that can suppress B-cell proliferation (Mitasov *et al.,* 1991), including release of TGF-β (Sennikov *et al.,* 1996; Seledtsov *et al.,* 1998). In a preliminary study we have identified production of TGF-β in spleen reed pulpa as well as in spleen white pulpa from FIS-2 infected mice (data not shown). It would be of interest to determine whether the level of TGF-β production correlates with early viral load and/or if production of soluble immune-regulatory factors can be induced by virus infection of erythroide progenitors or activated by the SU portion of the FIS-2 envelope.

V: Does sex matter?

Besides **inter-individual** differences in the NMRI mice studied, we observed that **host gender** influenced the susceptibility to FIS-2 infection. Our results indicated a more efficient FIS-2 replication and dissemination in male mice compared to female mice (papers **III-V**), with subsequent more rapid immunosuppression induced (**papers III** and **V**). Male mice also showed impaired immune responses compared to female mice (**papers IV** and **V**). The presence of a **GRE** in the FIS-2 LTR enhanced initial virus dissemination in male mice, and was shown to be a major determinant for the observed sex differences (**paper IV**).

Are there any resemblances to these observations in humans? The answer is indeed yes. Despite reports to the contrary (Bush *et al.,* 1996, Fang *et al.,* 1997, Kalish *et al.,* 2000,

Moore *et al.,* 1999), several reports have suggested that early plasma HIV-1 RNA load (viremia) is lower in women than in men: Farzadegan *et al.,* (1998) showed that at a common threshold of CD4 cell count women had median viremia 38% to 65% of those in men. Other studies have shown sex difference at or near the time of seroconversion (Evans *et al.,* 1997, Lyles *et al.,* 1999, Sterling *et al.,* 1999). The difference seemed to decrease over time (Evans *et al.,* 1997, Sterling *et al.,* 1999), and no difference in viral load in men and women with very advanced HIV disease have been found (Kalish *et al.,* 2000).

In a critical epidemiological review of the available evidence concerning whether patient sex affect HIV virus levels, the authors demonstrated that there was a consistent association between female sex and lower HIV RNA level, even after adjustment for possible confounders such as age, race, mode of virus transmission, and antiretroviral therapy use (Gandhi *et al.,* 2002). A meta-analysis of published studies has also confirmed that women have lower plasma HIV RNA levels than men with similar CD4 lymphocyte counts and stage of disease (Napravnik *et al.,* 2002). Why women seem to progress to the clinical end points of AIDS and death as quickly as men, despite having lower levels of viremia, remains unexplained. Further, the biological mechanism for the observed gender effect on HIV RNA levels is unclear.

Gender differences in HIV-1 diversity at the time of infection has also been reported: Long *et al.,* (2000) found that women from Kenya were often infected by multiple virus variants, whereas men from Kenya were not. An Institute of Medicine report called "*Exploring the Biological Contributions to Human Health: Does Sex Matter?"* (Wizemann & Pardue, 2001) emphasized the impact of sex differences on disease from the cellular to the societal level. Overall, recent reports have shown that it is important to be cautious in extrapolating data obtained from men to understand HIV transmission and pathogenesis in women. Gandhi *et al*. (2002) suggested that because manifestations of HIV infections stem from the interplay between viral and host factors, sex differences in e.g., immune modulation will likely play instrumental roles in determining the course of disease.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In conclusion, the present study shows the following main results:

(1) Mutations in the FIS-2 LTR had made the virus variant less oncogenic, while mutations in the envelope surface protein (SU) gave FIS-2 enhanced immunosuppressive properties compared to the prototype F-MuLV cl. 57.

(2) Early viral dissemination rates of Friend murine leukaemia virus variants correlated with late pathogenesis. Thus, early viral replication seemed to be a prognostic factor in murine retrovirus infection.

(3) Host sex influenced susceptibility to FIS-2 infection, male mice being more susceptible than females.

(4) FIS-2 replication was enhanced by the presence of a hormone response element (GRE) in the LTR, and this element was a major determinant responsible for the observed sex differences in susceptibility, early virus dissemination, and virus-induced immunosuppression.

(5) Studies *in vitro* and *in vivo* suggested that stress hormones, like glucocorticoids, could influence retrovirus infection, directly through enhanced virus replication, and/or indirectly through regulation of immune responses.

Overall, our studies indicated cumulative effects of viral factors (e.g., LTR, GRE), sex, and hormones on murine retrovirus infections, and the hormonal background of the host might influence virus productions, as well as anti virus responses. However, the severity of immunosuppression in FIS-2 infected mice is determined by some virus factors, like the SU portion of the FIS-2 envelope protein, which is dominant over other viral factors as well as various genetic factors of individual mice. More research is needed to elucidate how the SU influence immunosuppression and how the GRE determines sex-differences in early FIS-2 dissemination.

Future perspectives

The scale of the HIV/AIDS epidemic has exceeded all expectations since its identification 20 years ago. Globally, an estimated 42 million people are currently (end 2002) living with HIV, and some 20 million people have already died (derived from http://www.unaids.org). Further, just as the spread of HIV has been greater than predicted, so too has its impact on social capital, population structure, and economic growth (reviewed in Piot *et al.,* 2001; Weiss, 2001). Highly active antiretroviral therapy (HAART) can block replication of HIV-1 and is associated with an improvement in the ability to resist opportunistic infections. However, because HIV-1 can establish a latent infection in long-lived memory $CD4^+$ T-cells, current HAART cannot eradicate HIV-1 (Finzi *et al.,* 1999). Therefore, life long HAART is necessary. Such therapy has several limitations, including long-term side effects and the requirement for strict adherence to the medication to prevent viral resistance (reviewed in Carr & Cooper, 2000; Richman 2001). Moreover, the current treatments are in many respects only for the economically privileged, and most developing countries cannot afford the drugs as well as the necessary monitoring and support of HIV care (Moore, 2000; Forsythe, 1998). Thus, new strategies to combat HIV are urgently needed.

The major challenge is to elucidate which types of immune responses are required for protection from retroviral infections. The FV mouse model has recently proven very valuable in this respect (reviewed in Hasenkrug and Dittmer, 2000). The FIS-2 model offers another approach to study retrovirus-host interactions in a small animal model. The major advantages with this model system are: (i) FIS-2 is immunosuppressive but low oncogenic. (ii) FIS-2 is molecular cloned and a well-defined virus. (iii) The course of FIS-2 infection shows some resemblance to the course of HIV infection; and (iv) in female mice, the anti FIS-2 response is highly efficient. Thus, the FIS-2 model may also be used to investigate basic mechanisms of retroviral immunity.

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Glossary of Virology:

Adapted from http://www.tulane.edu/~dmsander/WWW/MBChB/VirGloss.html, http://www.virology.net/ATVHIVGlossary.html , and Cann (2001).

Acquired immunodeficiency syndrome (AIDS): The most severe manifestation of infection with the human immunodeficiency virus (HIV). The Centers for Disease Control and Prevention list numerous opportunistic infections and neoplasms (cancers) which, in the presence of HIV infection, constitute an AIDS diagnosis. In addition, a $CD4^+$ T-cell count below $200/mm^3$ in the presence of HIV infection constitutes an AIDS diagnosis. The period between infection with HIV and the onset of AIDS averages 10 years in the USA. People with AIDS often suffer infections of the lungs, brain, eyes and other organs, and frequently suffer debilitating weight loss, diarrhoea and a type of cancer called Kaposi's sarcoma. Even with treatment, most people with AIDS die within two years of developing infections or cancers that take advantage of their weakened immune systems.

Acute infection: Relatively brief infections, i.e., a few days to a few weeks, following which the virus is usually eliminated completely from the body by the immune system (e.g., an infection causing disease with a sudden onset, severity and (often) short course). As related to HIV infection: Once the virus enters the body, HIV infects a large number of $CD4^+$ T-cells and replicates rapidly. During this acute or primary phase of infection, the blood contains many viral particles that spread throughout the body, seeding themselves in various organs, particularly the lymphoid tissues.

Chronic infection: The converse of acute infections, i.e., prolonged and stubborn. Caused by viruses that are able to persist in the body.

Complex retroviruses: Include *lentiviruses* that have multiple splice donors in the genome. This give rise to complex patterns of mRNA and a variety of gene products (up to six in addition to *gag*, *pro*, *pol* and *env* proteins in HIV and SIV), at least one of which has a virus-specific *trans*-activating function (see Transcription). HIV-1 DNA integration can take place in non-dividing cells.

Envelope: In virology, a protein covering that packages the virus's genetic information. The envelope of HIV is composed of two layers of fat-like molecules called lipids taken from the membranes of human cells. Embedded in the envelope are numerous cellular protein, as well as mushroom-shaped HIV proteins that protrude from the surface. Each mushroom is thought to consist of a cap made of four glycoproteinmolecules called gp120 and a stem consisting of four gp41 molecules embedded in the envelope. The virus uses these proteins to attach to and infect cells.

Fusion protein: The protein(s) on the surface of a virus particle responsible for fusion of the virus envelope with cellular membranes.

Gene expression: An important stage of viral replication at which virus genetic information is expressed: one of the major control points in replication.

Genome replication: The stage of viral replication at which the virus genome is copied to form new progeny genomes.

Inoculation: In the present thesis, the introduction of a substance (inoculum; e.g., virus) into the body to produce the disease or condition associated with the substance.

Latency: The period when e.g., a virus is in the body and not producing any ill effects.

Latent infection: Viruses that are able to down-regulate their gene expression can establish a truly latent state, i.e., with strictly limited gene expression and without ongoing virus replication. Latent virus infections typically persist for the entire life of the host.

Molecular epidemiology: The use of nucleotide sequence information to study the diversity and distribution of virus populations.

mRNA: Messenger RNA, translated on ribosomes to produce proteins.

Mutation: In biology, a sudden change in a gene or unit of hereditary material that results in a new inheritable characteristic. In higher animals and many higher plants, a mutation may be transmitted to future generations only if it occurs in germ or sex cell-tissue; body cell mutations cannot be inherited. Changes within the chemical structure of single genes may be induced by exposure to radiation, temperature extremes, and certain chemicals. The term mutation may also be used to include losses or rearrangements of segments of chromosomes, the long strands of genes. Drugs such as colchicine double the normal number of chromosomes in a cell by interfering with cell division. Mutation, which can establish new traits in a population, is important in evolution. As related to HIV: HIV mutates rapidly. During the course of HIV disease, viral strains may emerge in an infected individual that differ widely in their ability to infect and kill different cell types, as well as in their rate of replication. Strains of HIV from patients with advanced disease appear to be more virulent and infect more cell types than strains obtained earlier from the same individual.

Neonatal: Concerning the first four weeks of life after birth (in humans).

Neutralization: Blocking of virus infection by antibodies; also, an assay that measures this.

Open reading frame: protein-encoding domain within a gene.

Penetration: The stage of viral replication at which the virus genome enters the cell.

Persistent infection: Infections in which ongoing virus replication occurs, but the virus adjusts its replication and pathogenicity so as to avoid killing the host. They differ from chronic infections in that whereas in chronic infections, the virus is usually eventually cleared by the host (unless the infection proves fatal), in persistent infections, the virus may continue to be present and to replicate in the host for its entire lifetime.

Receptor: A specific molecule on the surface of a cell that is used by a virus for attachment.

Release: The stage of viral replication at which virus particles escape the infected cell.

Seroconversion: The development of antibodies to a particular antigen. When people develop antibodies to HIV or an experimental HIV vaccine, they "seroconvert" from antibody-negative to antibody-positive.

Simple retroviruses: Includes Murine leukaemia viruses. Simple retroviruses have what is called a "simple lifestyle" characterized by the presence of at the most one coding region in addition to those encoding the common virion proteins encoded by *gag*, *pro*, *pol* and *env*. F-MuLV DNA integration is currently thought to occur only after the nuclear membrane breaks down during mitosis.

Splenomegaly: An enlarged spleen.

Sterilizing immunity: An immune response that eliminates an infection.

Subcutaneous: Beneath or introduced beneath the skin (e.g., subcutaneous injections).

Susceptible: Vulnerable or predisposed to a disease.

Titer (also "titre"): A laboratory measurement of the amount (or concentration) of a given compound in solution.

Transcription: The process of constructing a messenger RNA molecule using a DNA molecule as a template with the resulting transfer of genetic information to the messenger RNA. As related to HIV: The process by which the provirus produces new viruses. RNA copies called messenger RNA must be made that can be read by the host cell's protein-making machinery. Transcription is facilitated by cellular enzymes, including RNA polymerase II. The viral genes may partly control this process: *tat*, for example, encodes a protein that accelerates the transcription process by binding to a section of the newly made viral RNA.

Transformation (e.g., cell transformation by virus)**:** A change in the morphological, biochemical, or growth parameters of a cell. Transformation may or may not result in cells able to produce tumours (neoplastic transformation). Oncogenesis is a complex, multi-step process in which cellular transformation may be only the first, although essential, step along the way.

Transmission: In the context of HIV disease: HIV is spread most commonly by sexual contact with an infected partner. The virus can enter the body through the mucosal lining of the vagina, vulva, penis, rectum, or the mouth during sex. The likelihood of transmission is increased by factors that may damage these linings, especially other sexually transmitted diseases that cause ulcers or inflammation. Studies of SIV infection of the genital membranes of nonhuman primates suggest that the cells known as mucosal dendritic cells may be the first cells infected. Infected dendritic cells may migrate to lymph nodes and infect other cells. HIV also is spread through contact with infected blood, most often by the sharing of drug needles or syringes contaminated with minute quantities of blood containing the virus. Children can contract HIV from their infected mothers either during pregnancy or birth, or postnatally, via breastfeeding. Current research indicates that the AIDS virus may be 100 to 1000 times more contagious during the first two months of infection, when routine AIDS tests are unable to tell whether people are infected.

Tropism: The ability of a virus to infect specific cells or tissue types.

Type C retrovirus: Simple retrovirus, frequently associated with cancer.

Uncoating: The stage of viral replication at which structural proteins are lost and the virus genome is exposed to the replication machinery.

Variant: A virus whose phenotype differs from the original wild type strain, but were the genetic basis for the difference is not known.

Viral burden (viral load): The amount of virus in the circulating blood. Monitoring a person's viral burden is important because of the apparent correlation between the amount of virus in the blood and the severity of the disease; sicker patients generally have more viruses than those with less advanced disease.

Viremia: The presence of virus in the bloodstream.

Virions: Structurally mature, extracellular virus particles.

Virus attachment protein: The protein on the surface of a virus particle responsible for binding the receptor.

(+) sense RNA (plus-sense RNA): A virus with a single-stranded RNA genome of the same polarity ('sense') as mRNA.

(-) sense RNA (minus-sense RNA): A virus with a single-stranded RNA genome of the opposite polarity ('sense') as mRNA.

Glossary of Immunology:

Adapted from http://www.tulane.edu/~dmsander/WWW/MBChB/ImmGloss.html and Cann (2001).

Adaptive immunity: Host immune response mediated by B- and T-cells, for example, antibodies, or cytotoxic (CD8+) T lymphocytes.

Anergy (e.g., T-cell anergy)**:** An immunologically unresponsive state in which lymphocytes are present but not functionally active.

Antibody: Serum protein formed in response to immunization; antibodies are generally defined in terms of their specific binding to the immunizing antigen.

Antibody-dependent, cell-mediated cytotoxicity (ADCC): A phenomenon in which target cells, coated with antibody, are destroyed by specialized killer cells (NK cells and macrophages), which bear receptors for the Fc portion of the coating antibody (Fc receptors). These receptors allow the killer cells to bind to the anti-body-coated target.

Antigen: Any foreign material that is specifically bound by specific antibody or specific lymphocytes; also used loosely to describe materials used for immunization. Antigens may also be immunogens if they are able to trigger an immune response, or haptens if not.

Antigen-binding site: The part of an immunoglobulin molecule that binds antigen specifically.

Antigen-presenting cell (APC): A specialized type of cell, bearing cell surface class II MHC (major histocompatibility complex) molecules, involved in processing and presentation of antigen to inducer, or helper, T-cells. Examples: macrophage, dendritic cells.

Antigen receptor: The specific antigen-binding receptor on T- or B-lymphocytes; these receptors are transcribed and translated from rearrangements of V genes.

Antigenic determinant: A single antigenic site or epitope on a complex antigenic molecule or particle.

Antigen processing: Large molecules are broken down (processed) within macrophages into peptides and presented within the groove of MHC molecules.

Autoimmunity (autoallergy): An immune response to "self" tissues or components. Such an immune response may have pathological consequences leading to autoimmune diseases.

B lymphocyte (B cell): The precursors of antibody-forming plasma cells; these cells carry immunoglobulin and class II MHC (major histocompatibility complex) antigens on their surfaces.

CD3 (clone 145-2C11; used in papers II and V)**:** antibody reacts with the 25-kDa chain of the T-cell receptor associated CD3 complex, which is expressed on thymocytes and mature T lymphocytes of all mouse strain tested. The cytoplasmic domain of CD3e participates in the signal transduction event, which activates several biochemical pathways as a result of antigen recognition. Also found

on some NK cell subsets (expression is activation dependent, e.g., antigen expression level changes on activated cells). Such cells might be more related to T-cells than NK-cell. See also NKT-cell.

Cell-mediated cytotoxicity (CMC): Killing (lysis) of a target cell by an effector lymphocyte.

Cell-mediated immunity (CMI): Immune reaction mediated by T-cells; in contrast to humoral immunity, which is antibody mediated. Also referred to as delayed-type hypersensitivity.

Class I and II MHC molecules: Proteins encoded by genes in the major histocompatibility complex (q.v.). Class I molecules are designated HLA-A, B, or C. Class II molecules are designated DP, DQ or DR.

Cytokines: Soluble substances secreted by cells, which have a variety of effects on other cells, e.g., interleukins.

Cytotoxic (cytolytic) T cell: Cell that kills target cells bearing appropriate antigen within the groove of a MHC class I molecule that is identical to that of the T cell.

Determinant: Part of the antigen molecule that binds to an antibody-combining site or to a receptor on T-cells.

Epitope: An alternative term for antigenic determinant.

Fluorescent antibody: An antibody coupled with a fluorescent dye, used with a fluorescence microscope to detect antigen on cells, tissues, or microorganisms.

H-2 complex: The major histocompatibility complex situated on chromosome 17 of the mouse; contains subregions K, I, and D.

Helper T-cells: A class of T-cells that help trigger B-cells to make antibody against thymus-dependent antigens. Helper T-cells also help generate cytotoxic T-cells.

Humoral immunity: Any immune reaction that can be transferred with immune serum is termed humoral immunity (as opposed to cell-mediated immunity). In general, this term refers to resistance that results from the presence of specific antibody.

Hybridoma: A hybrid cell that results from the fusions of an antibody secreting cell with a malignant cell; the progeny secrete antibody without stimulation and proliferate continuously both *in vivo* and *in vitro*.

Immunecompetent: Capable of developing an immune response (possessing a normal immune system).

Immunodeficiency: A deficiency of immune response or a disorder classified as antibody (B cell), cellular (T cell), combined deficiency or phagocytic dysfunction disorders.

Immunosuppression: A breakdown or inability of certain parts of the immune system to function, thus making a person susceptible to certain diseases that they would not ordinarily develop.

Immunosuppression may be induced by drugs or result from certain disease processes, such as HIV or FIS-2 infections.

Innate immunity: Host immune response mediated by macrophages, microglia, dendritic cells, mast cells and natural killer cells.

Interferon: A group of proteins having antiviral activity and capable of enhancing and modifying the immune response.

Interleukins: Glycoproteins secreted by a variety of leukocytes that have effects on other leukocytes.

Lymphocyte: Small cell with virtually no cytoplasm, found in blood, in all tissue, and in lymphoid organs, such as lymph nodes, spleen, and Peyer's patches, and bears antigen-specific receptors.

Macrophage: A large phagocytic cell of the mononuclear series found within tissues. Properties include phagocytosis, and antigen presentation to T-cells.

Major histocompatibility complex (MHC): A cluster of genes on chromosome 6 in humans, encoding cell surface molecules that are polymorphic and that code for antigens that lead to rapid graft rejection between members of a single species which differ at these loci. Several classes of protein such as MHC class I and II proteins are encoded in this region. These in humans, are known as 'Human leukocyte antigens' (HLA).

Memory: In the immune system, memory denotes an active state of immunity to a specific antigen, such that a second encounter with that antigen leads to a larger and more rapid response.

MHC class I molecule: A molecule encoded to genes of the MHC which participates in antigen presentation to cytotoxic T ($CD8^+$) cells.

MHC class II molecule: A molecule encoded by genes of the MHC which participates in antigen presentation to helper T ($CD4^+$) cells.

MHC restriction: The ability of T lymphocytes to respond only when they 'see' the appropriate antigen in association with "self" MHC classes I or II proteins on the antigen presenting cells.

Mitogen: A substance that stimulates the proliferation of many different clones of lymphocytes.

Monoclonal: Literally, coming from a single clone. A clone is the progeny of a single cell. In immunology, monoclonal generally describes a preparation of antibody that is monogenous, or cells of a single specificity.

Monocyte: Large circulating white cell, 2-10% of total white cells, phagocytic, indented nucleus. Migrates to tissues, where it is known as a macrophage.

NK cell: Naturally occurring, large, granular, lymphocyte-like killer cells. They may play a role in resistance to tumours and virus infection. In addition, they participate in ADCC. They do not exhibit antigenic specificity, and their number does not increase by immunization.

NKT-cells: A population of T-cells that share some characteristics with NK cell (see CD3). The expression of CD3/αβTCR by NKT-cells suggests that they require TCR-specific recognition to be activated. The function most characteristic of NKT-cells is the rapid production of high levels of immunoregulatory cytokines IL-4, IFN-γ, and TNF following stimulation *in vitro*. Further, NKT-cells exhibit potent lytic activity.

Phagocytosis: The engulfment of a particle or a microorganism by leukocytes.

Polyclonal activator: A substance that induces activation of many individual clones of either T or B-cells. See mitogen.

Primary responses: The immune response to a first encounter with antigen. The primary response is generally small, has a long induction phase or lag period, consists primarily of IgM antibodies, and generates immunologic memory.

Superantigen: Molecules which short-circuit the immune system, resulting in massive activation of T-cells, rather than the usual, carefully controlled response to foreign antigens.

Suppression: A mechanism for producing a specific state of immunologic unresponsiveness by the induction of suppressor T-cells. This type of unresponsiveness is passively transferable by suppressor T-cells or their soluble products.

T cell: A lymphocyte that undergoes a developmental stage in the thymus.

T-dependent antigen: An immunogen that is able to induce antibody synthesis only in the presence of lymphokines released by helper T-cells (e.g., sheep reed blood cells (SRBC) used in the plaque-forming cell assay for primary antibody response).

T-independent antigen: An immunogen which induces antibody synthesis in the absence of lymphokines released by T-cells; the antibodies are generally only of the IgM isotype.

Titre: The reciprocal of the last dilution of a titration giving a measurable effect; e.g., if the last dilution giving significant agglutination is 1:128, the titre is 128.

Vaccination: Originally referred to immunization against smallpox with the less virulent cowpox (vaccinia) virus; more loosely used for any immunization against a pathogen.