THE ANALYSIS OF THE IN VIVO EFFECTS OF THE HIV-1 NEF AND TAT REGULATORY GENES USING A TRANSGENIC MOUSE SYSTEM

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Research Thesis Submitted in Partial Fulfilment of the Requirements of the University of London for the Degree of Doctor of Philosophy.

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To my Mum and Dad.
Abstract

Although it is widely accepted that the human immunodeficiency virus (HIV) is the aetiologic agent responsible for the acquired immunodeficiency syndrome (AIDS), the precise mechanisms that underlie the HIV-1-mediated destruction of the immune system are unclear. Nevertheless, one thing is certain, the breakdown of the host immune system must be caused by, or be the result of, the presence of a viral component(s). Two such viral components are the HIV-1 nef gene and the HIV-1 tat gene.

Transgenic mouse models were generated to examine the in vivo interaction of the nef gene and the tat gene with a dynamic mammalian immune system. These genes were expressed in the majority of thymocytes and T-cells using the human CD2 regulatory elements.

In CD2-nef transgenic mice, a downregulation of cell surface CD4 (to a region co-localising with a golgi-specific marker) in CD4/CD8 double positive thymocytes, and a significant loss of CD4 single positive thymocytes was observed. These mice also displayed a severe reduction in thymic cellularity, resulting from a partial block in the transition of CD44- CD25+ thymocytes to a CD44- CD25- phenotype during early thymic differentiation.

In CD2-tat transgenic mice activated T-cells were found to produce decreased levels of TNFβ, TGFβ and IL-4R mRNA. A decreased level of TNF protein was also detected. However, thymocyte development and mature T-cell proliferation responses appear normal, and no evidence of KS-like lesions or tumours were found in any mice at any age.

The CD2-nef and CD2-tat transgenic mouse models described and discussed in this thesis suggest that both the HIV-1 nef gene and the HIV-1 tat gene have a complex interaction with both developing thymocytes and mature T-cells. Therefore, these mouse models may provide an important tool for the detailed analysis of the in vivo effects that the expression of these HIV-1 regulatory genes have on the pathogenesis of an HIV-1 infection.
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<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>APS</td>
<td>Antigen presenting cell</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>CDC</td>
<td>Center for Disease Control</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CRS</td>
<td>Cis-acting repressor sequences</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-Cell</td>
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<tr>
<td>DN</td>
<td>double negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>dpc</td>
<td>days post coitus</td>
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<td>EBV</td>
<td>Epstein Barr Virus</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>FTOC</td>
<td>Fetal thymic organ cultures</td>
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<tr>
<td>GalC</td>
<td>Galactosyl ceramide</td>
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<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
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<tr>
<td>HBV</td>
<td>Hepatitis B Virus</td>
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<tr>
<td>hCD2</td>
<td>human CD2</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>HTLV</td>
<td>Human T-Lymphotrophic Virus</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>KLH</td>
<td>Keyhole limpet haemocyanin</td>
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<td>KS</td>
<td>Kaposi’s Sarcoma</td>
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<tr>
<td>LAV</td>
<td>Lymphadenopathy Associated Virus</td>
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<tr>
<td>LCMV</td>
<td>Lymphocytic Choriomeningitis Virus</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>LCR</td>
<td>Locus Control Region</td>
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<td>LTR</td>
<td>Long Terminal Repeat</td>
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<tr>
<td>mAB</td>
<td>monoclonal antibody</td>
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<tr>
<td>MCF</td>
<td>Mink Cell Foci (Virus)</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>Ms</td>
<td>Mouse lymphocyte stimulating (genes)</td>
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<td>MMTV</td>
<td>Mouse Mammary Tumour Virus</td>
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<td>MMWR</td>
<td>Morbidity and Mortality Weekly Report</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>MuLV</td>
<td>Murine Leukemia Virus</td>
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<td>NRE</td>
<td>Negative Response Element</td>
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<tr>
<td>PBL</td>
<td>Peripheral blood lymphocyte</td>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<tr>
<td>PBS</td>
<td>Primer binding site</td>
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<td>PCD</td>
<td>Programmed cell death</td>
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<td>PCP</td>
<td>Pneumocystis carinii pneumonia</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<td>PHA</td>
<td>Phytohemagglutinin A</td>
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<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate-13-acetate</td>
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<tr>
<td>RBC</td>
<td>Red blood cell</td>
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<tr>
<td>RNA</td>
<td>Ribose nucleic acid</td>
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<tr>
<td>RRE</td>
<td>Rev-responsive element</td>
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<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
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<tr>
<td>SAg</td>
<td>Superantigen</td>
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<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<tr>
<td>SH2/SH3</td>
<td>src homology 2/3 (domains)</td>
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<tr>
<td>SIV</td>
<td>Simian Immunodeficiency Virus</td>
</tr>
<tr>
<td>SP</td>
<td>single positive</td>
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<tr>
<td>TAR</td>
<td>Tat activation response element</td>
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<tr>
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<td>TdT</td>
<td>Terminal Transferase</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
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<tr>
<td>tRNA</td>
<td>transfer RNA</td>
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<tr>
<td>TUNEL</td>
<td>(TdT)-mediated dUTP-biotin nick end-labelling.</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>+ve</td>
<td>positive</td>
</tr>
<tr>
<td>-ve</td>
<td>negative</td>
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I would like to thank my supervisor Dr. Elaine Dzierzak for her excellent supervision. I would also like to especially thank Dr. Hugh Brady, Dr. David Abraham and Colin Miles for all their help, support, experience and guidance throughout my time at NIMR. In addition, thank you to Dr. Frank Grosveld, Dr. Dimitris Kioussis, Dr. Rose Zamoyska and Dr. Rod Daniels for their helpful discussions, Dr. Owen Williams for his help with the FTOCs, Dr. Mike Antoniou for his help with the immunofluorescence, Chirs Atkins for his help with the FACS sorting, and all the members of GSE, especially Bernadette, Alan, Albrecht, Maria-Jose, Alexander, Angus, Christophe, Jacky and Cora.

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Introduction

1. The history of AIDS and its association with HIV.

1.1 The discovery of a new disease - AIDS.

In June 1981, a short article appearing in the United States Center for Disease Control's (CDC) morbidity and mortality weekly report (MMWR) described a previously unrecognized immunodeficiency syndrome (CDC, 1981). Five previously healthy homosexual men from the Los Angeles area were diagnosed with *pneumocystis carinii* pneumonia (PCP), an infection highly indicative of chronic immunosuppression. No cause for this immunosuppression was evident. Over the next few months, several similar cases were documented (Gottlieb *et al.*, 1981, Masur *et al.*, 1981). In one of these reports, Gottlieb and co-workers described four previously healthy homosexual men with severe opportunistic infections, not normally associated with immunocompetence. Each patient had PCP, extensive mucosal candidiasis, and multiple viral infections, such as cytomegalovirus (CMV) and Epstein Barr virus (EBV). Three of the four men had prolonged fevers of unknown cause and one had Kaposi’s Sarcoma (KS). All patients were anergic and lymphopenic. In addition, their lymphocytes failed to proliferate in response to soluble antigen or phytohemagglutinin A (PHA). It was also noted that the helper T-cell population was severely depleted in all patients.

The conclusions to these studies were highly speculative. A link with lifestyle was an obvious candidate for the cause of this syndrome, as patients were exclusively either homosexual or intravenous drug users. However, no aetiologic agent was immediately apparent, although various pathogens, such as cytomegalovirus, were suggested (Masur *et al.* 1981).

The incidence of reported cases of this Acquired Immunodeficiency Syndrome (AIDS) grew rapidly through 1982. In the U.S., over 800 cases in over 30 states were described by the end of the year. In addition, it was becoming increasingly evident that the syndrome was not restricted to homosexual men and drug abusers. Figures published by the U.S. CDC in late 1982 (CDC, 1982), revealed 72% of diagnosed cases in either gay or bisexual men, and 17% of cases in drug abusers. However, a significant number of cases were also described in the heterosexual
partners of individuals previously identified with disease symptoms, and in haemophiliacs or the recipients of blood or blood products.

The mounting evidence suggested that the new syndrome was caused by a transmissible factor, passing horizontally in the population via intimate contact or contaminated blood products. An infectious agent, such as a virus, was a potential candidate, with the consistently depleted helper T-cell population a possible cellular target. In the years that preceded the onset of the AIDS epidemic, a family of human retroviruses had been described (the Human T-Lymphotrophic viruses (HTLV)), that seemed to possess many of the characteristics expected of such an infectious agent (Yoshida et al., 1982, Reitz et al., 1983). Members of this family are T-cell tropic, cytopathic in culture, and can cause T-cell dysfunction. They are also transmissible in blood products or via intimate contact. Therefore, it came as no surprise when initial studies with tissue from affected individuals detected reverse transcriptase activity (highly predictive for these viruses), and mAb reactivity directed against HTLV proteins, suggesting that previously identified members of the HTLV family, HTLV-1 and HTLV-2, could be the aetiologic agent responsible for AIDS (Gallo et al., 1983). However, Barre-Sinoussi and co-workers (1983), in Luc Montagnier's lab. at the Pasteur Institute in Paris, subsequently demonstrated that HTLV-1 and HTLV-2 were not responsible for AIDS, by isolating a novel closely related but distinct virus that they named Lymphadenopathy Associated Virus (LAV). Six months later, in the spring of 1984, a group at the NIH in the United States, headed by Robert Gallo, confirmed the isolation of this new virus (referred to as HTLV-III in these studies) and strongly suggested an aetiologic role for the virus in AIDS (Gallo et al., 1984, Popovic et al., 1984). Since then, several strains of the virus, now known as Human Immunodeficiency Virus Type-1 (HIV-1), have been isolated.

In 1986, a second human immunodeficiency virus was described in two AIDS patients in West Africa (Clavel et al., 1986). This virus, HIV-2, is closely related but genetically distinct from HIV-1. It shares biological and morphological properties with HIV-1, but differs in some antigenic components, especially the viral envelope glycoproteins. HIV-2 can also be recognised by serum from a macaque monkey infected with Simian Immunodeficiency Virus (SIV), suggesting it to be a close relative of primate retroviruses, as well as HIV-1.

1.2 Does HIV cause AIDS?

Since their isolation, there has been strong evidence to suggest that HIV-1 and HIV-2 are the aetiologic agents responsible for AIDS. However, the precise mechanism by which HIV
causes AIDS is not yet known. Until this link is firmly established, the cause of AIDS will remain a topic of intense debate. Peter Duesberg, a professor of Molecular Biology at the University of California, Berkeley, has maintained that HIV has no role in AIDS (Duesberg, 1987, 1989, 1991). His theories (sensationalised in the popular press) propose that HIV does not fulfil the criteria expected of the aetiologic agent of AIDS. Furthermore, he argues that the syndrome is the consequence of repeated and abusive use of psychoactive, aphrodisiac, and medical drugs. This he has termed the drug-AIDS hypothesis (Duesberg, 1991).

1.2.1 Duesberg's argument

The basis of Duesberg's argument is that HIV does not fulfil Koch's postulates for identifying the cause of an infectious disease. Koch's three postulates for an infectious agent state that; i) the agent occurs in each case of the disease and in amounts sufficient to cause pathological effects; ii) the agent is not found in other diseases, or as a non-pathological parasite; and iii) after isolation and serial propagation in pure culture, the agent can induce disease anew (Koch, 1884, Evans, 1976).

Duesberg's evidence for non-compliance with these postulates argues that; i) free virus cannot be detected in most HIV positive cases. In the cases it can be isolated, virus is only obtainable from re-activating a few infected lymphocytes amongst thousands of uninfected ones; ii) the virus is maintained in a non-pathogenic or latent state for up to 10 years, during which it is very difficult to isolate or propagate (Albert et al., 1987); and iii) animals and humans (accidentally) inoculated with HIV do not develop disease (Duesberg, 1987).

Duesberg also claims that HIV violates classical conditions of viral pathology. These arguments include; i) no other human retrovirus is pathogenic, yet HIV is claimed to be 50-100% fatal (Gallo & Montagnier, 1988). This would be higher than any other known human virus; ii) retroviruses typically do not kill cells. In AIDS, a decrease in CD4+ T-cells is seen, but only 1 in 1000 of these cells are infected by HIV in the peripheral blood (Harper et al., 1986); iii) progression to AIDS is slow, with long and unpredictable asymptomatic intervals. This, claims Duesberg, is not compatible with an infectious disease caused by a virus that takes 1-2 days to replicate, and 1-2 months to establish an immune response; iv) HIV is relatively closely related to other lentiviruses which are not pathogenic. However, HIV contains no additional late-expressing "AIDS" gene; v) Duesberg thinks it unlikely that HIV-1 and HIV-2, with only 40% homology at the nucleotide sequence level, would have independently evolved to cause AIDS
(Gallo & Montagnier, 1988); and vi) it is improbable that HIV would kill its only known host with a frequency of between 50-100% (Gallo & Montagnier, 1988).

Duesberg also claims to have highlighted certain non-correlations between HIV and AIDS; (i) The ratio of HIV-positive individuals versus diagnosed AIDS cases varies dramatically depending on the country studied. In the United States, the ratio of AIDS patients versus HIV positive individuals is 1 in 10, but only 1 in 120 in Uganda, and 1 in 675 in Zaire (CDC, 1990b, Goodgame et al., 1990). If AIDS is caused by a common agent, these frequencies should be more or less equivalent; (ii) A difference in the nature of AIDS disorders also exists. In the U.S., 90% of AIDS cases are diagnosed with PCP or KS (Selik et al., 1987), yet Africans tend to develop Slim disease, chronic fever, or diarrhoea (Colebunders et al., 1987); (iii) HIV testing in the U.S. army has revealed a 0.03% incidence of seropositive individuals in both male and female new recruits (Burke et al., 1990). This, Duesberg claims, indicates that the incidence of HIV infection in the general population is roughly equivalent between the sexes. However, ten times more males (91%) have developed AIDS than females, even though no AIDS illnesses are male specific (CDC, 1990a); (iv) Duesberg argues that HIV has not spread exponentially as would be expected of a new virus responsible for a new syndrome, especially one that has a sexually transmitted route of infection; and finally (v) Duesberg contests the theory that the principal method of HFV transmission is actually a sexual one. He states that a far more likely mechanism of HIV transmission is via a perinatal route, in a manner similar to animal retroviruses, where transmission rates can be as high as 50%.

Although Duesberg initially had no alternative hypothesis, he recently proposed that AIDS is the result of frequent and abusive use of drugs, such as cocaine, heroin, amphetamines, nitrites, and AZT (Duesberg, 1991). The use of these drugs has risen sharply throughout the 1960-70's, in line Duesberg states, with the occurrence of AIDS. This hypothesis claims to account for the 10 to 1 ratio of male to female AIDS cases, as 20-40 year old men are known to consume 80% of psychoactive drugs. In addition, it is also claimed to account for the vastly divergent AIDS diseases, these being the result of different pathogenic conditions and the method by which these conditions are treated using certain potent medical drugs.

1.2.2 Evidence for HIV

The Scientific community have responded forcefully to the claims of Peter Duesberg, with convincing commentaries outlining the evidence suggesting AIDS is caused by HIV (Weiss &
Jaffe, 1990). Duesberg's main argument states that HIV does not comply with Koch's postulates. Koch's postulates were formulated 100 years ago, and Koch himself expressed doubts over his third postulate, when working on TB and Cholera (Koch, 1884). Furthermore, he later abandoned his second postulate after discovering asymptomatic carriers of Cholera and Typhoid (Koch, 1893). However, the principle of having a set criteria to define infectious disease is a valuable one. With this in mind, Alfred Evans (amongst others) has revised Koch's postulates and has concluded that HIV is consistent with the causative agent of AIDS (Evans, 1976). In addition, the Simian Immunodeficiency Virus (SIV) of macaque monkeys (Letvin & King, 1990), a closely related cousin of HIV, satisfies Koch's postulates in both the old and new format.

To address Duesberg's other arguments, the following points have been presented; (i) True latency is never established in an HIV infection. Individual cells can harbour latent non-productive provirus, but a more accurate description of the overall viral state is one of persistent infection, in which HIV replication continues in the lymphoid tissues throughout the course of disease (Panteleo et al., 1993a, Embretson et al., 1993b); (ii) Persistent infections, such as tuberculosis, leprosy, syphilis, and mononucleosis, all display disease after similar periods of what could be termed "latency", often in the presence of a humoral immune response; and (iii) Although the number of infected peripheral blood lymphocytes may be as infrequent as 1:1000, it has been demonstrated that tissues such as the lymph node, gut, and brain are infected at a far greater frequency (Panteleo et al., 1993b, Embretson et al., 1993a). In addition, a rapid turnover of viral particles and CD4+ lymphocytes has recently been described in HIV seropositive individuals (Wei et al., 1995, Ho et al., 1995).

However, the most convincing evidence to suggest HIV is the aetiologic agent responsible for AIDS has come from several studies in which large numbers of patients have been followed over a period of years. Ascher and co-workers (1993) recruited the services of 1027 single men from the San Francisco area, aged between 25-54 years, following them with six-monthly check-ups for 96 months. Drug use, both type and frequency, HIV sero-status, and disease progression were monitored. Of these men, 215 were heterosexual and 812 were either homosexual or bisexual. Self confessed drug use was uniform across both groups, with the exception of amyl nitrite, (18% of homosexuals, but no heterosexuals, reported heavy use). During this study, no AIDS cases were detected in the heterosexual group, with just one patient becoming HFV positive. In the homosexual group, 445 were, or became HIV positive, with 215 AIDS cases and 174 deaths. All AIDS cases appeared in HFV seropositive individuals. If AIDS was linked to drug abuse, one would have expected an equivalent proportion (~56) of the heterosexual population to have developed AIDS in this study. This was not the case. The involvement of amyl nitrite in
the progression of KS was also investigated. No correlation was found. KS occurred independently of drug use but correlated with HIV seropositivity. Likewise CD4+ cell counts remained constant in HIV negative homosexual men but decreased steadily in the HIV seropositive group.

A complementary study by Schechter and co-workers (1993) described comparable results. They followed 715 homosexual men for an average of 9.5 years. All 316 AIDS cases occurred in the HIV antibody positive group. No correlation between AIDS cases and risk behaviour or drug use was observed, and CD4+ cell counts fell only in those patients known to be infected with HIV.

In conclusion, the consensus of opinion in the scientific community strongly supports a role for HIV as the aetiologic agent responsible for AIDS. Duesberg’s arguments are largely anecdotal, and have attracted many critics. However, Peter Duesberg has highlighted an important complacency in AIDS research. The cause of AIDS is still not known, and until unequivocal proof that HIV causes AIDS is demonstrated, an open mind must be maintained. It is a fair assumption that for a disease with a suspected incubation or latency period of 8-10 years co-factors such as age, environment, sexual behaviour, risk of infection, and drug use will be crucial elements in disease progression. However, the important question of what is both necessary and sufficient for AIDS must not be forgotten.

For the purposes of this thesis, it will be assumed that HIV is the aetiologic agent of AIDS.

1.3 AIDS Epidemiology

The World Health Organisation (WHO) and the U.S. CDC continually monitor HIV infection and diagnosed cases of AIDS throughout the world ("AIDS" Journal - monthly reports published). In developed countries, the diagnosis of AIDS is based primarily on the laboratory detection of the presence of HIV (ie HIV antibody) (table 1). If this test is either positive or cannot provide a conclusively negative result, a diagnosis of AIDS may be reached if one of the major indicator diseases is also definitively diagnosed. If no HIV infection is detected in the laboratory the diagnosis of AIDS if more strictly defined. First, no other cause of immunodeficiency must be present. If this is the case, there then must be the diagnosis of a disease state that would not be expected in an immuno-competent individual, in addition to the presence of a T-helper cell count of less then 400/mm³ (table 1).
Flow diagram for the revised CDC case definition of AIDS.

Flow diagram for revised CDC case definition of AIDS, September 1 1987. This method of defining clinical AIDS is based around the laboratory detection of HIV. In addition, the presence of certain indicator diseases, not normally associated with an immunocompetent host, allow a diagnosis of AIDS to be made.
Section A
History of immunosuppressive therapy such as systemic corticosteroid treatment
Any of the following diseases
- Hodgkin's disease
- Non-Hodgkin's lymphoma
- Lymphocytic leukemia
- Multiple myeloma
- Other cancer of the lymphoreticular tissue
- A congenital immunodeficiency syndrome

Section B
Candidiasis
- Extrapulmonary cryptococcosis
- Diarrhoea >1 month
- Cytomegalovirus disease in organ other than the spleen, liver, or LN.
- Kaposi's sarcoma, age < 60yr.
- HSV infection of extreme severity > 1 month
- Lymphoma of the brain, age < 60yr.
- Pneumocystis carinii pneumonia
- Progressive multifocal leukoencephalopathy
- Toxoplasmosis of the brain

Section C
Any disease in section B or D
- Disseminated coccidiomycosis
- Disseminated histoplasmosis
- HIV encephalopathy (dementia)
- Isosporiasis with diarrhoea >1 month
- Kaposi's sarcoma
- Lymphoma of the brain
- Non-Hodgkin lymphomas of B-cell immunologic type
- M. tuberculosis outside the lungs
- HIV wasting syndrome
- Recurrent Salmonella septicemia

Section D
Candidiasis of the esophagus
- Disseminated mycobacterial disease (acid-fast bacteria not identified by culture)
- Cytomegalovirus retinitis/loss of vision
- Kaposi's sarcoma
- Pneumocystis carinii pneumonia
- Toxoplasmosis of the brain

Flow diagram for revised CDC case definition of AIDS, September 1 1987
The diagnosis of AIDS using the criteria listed in table 1 is readily workable in the developed world. However, the resources and availability of advanced medical and scientific equipment in the developing world is severely restricted. The HIV lab-based "AIDS test" cannot always be used, and the definitive diagnosis of the more complex major indicator diseases of AIDS is difficult. Therefore, in an attempt to obtain an accurate standard of AIDS diagnosis, a Third World WHO case definition for AIDS has been established (WHO, 1986). This clinical case definition for adults looks for the existence of at least two of the "major" signs in association with at least one "minor" sign in the absence of other known causes of immunosuppression such as cancer or severe malnutrition. The "major" signs are defined as; i) weight loss greater than 10% of body weight; ii) chronic diarrhoea for more than one month; or iii) fever for longer than one month. The "minor" signs are; i) a persistent cough for longer than one month; ii) general pruritic dermatitis; iii) recurrent Herpes zoster; iv) oropharyngeal candidiasis; v) chronic progressive and disseminated Herpes simplex; or vi) general lymphadenopathy. The presence of disseminated Kaposi's Sarcoma or cryptococcal meningitis are sufficient alone to result in a diagnosis of AIDS.

In a study performed in Kinshasa, Zaire (Colebunders et al., 1987), this clinical case definition for AIDS had a specificity of 90%, a sensitivity of 59%, and a predictive value of 74%. Therefore, this WHO case definition is an acceptable way to monitor AIDS cases in less developed regions of the world, although laboratory identification of HIV is advised wherever possible.

The number of diagnosed AIDS cases are used as an estimate of the number of HIV-infected individuals in various regions of the world. These figures are obviously prone to error, but are based not only on the known incidence of AIDS, but also the estimated rate of local conversion from HIV seropositivity to AIDS, and the ratio of HIV seropositive versus AIDS cases in the major cities of the region in question. The WHO can therefore provide valuable data for disease surveillance and epidemiological analysis.

The WHO figures for mid-1993 estimate more than 13 million HIV-infected individuals worldwide (figure 1) (Merson, 1993). The majority of these infections were via a heterosexual route, although one million children are estimated to have become infected perinatally. Of the 13 million HIV infections more than 8 million have occurred in sub-Saharan Africa, where in some major cities as high as one in three adults are infected. In Europe and North America 1.5 million people have become infected, although the increase in the rate of infection has slowed. A similar number of HIV infections are estimated in South/Latin America/the Caribbean (ie 1.5 million), where the figures are rising steeply. Infections are also on the rise in North Africa/Middle East and Eastern Europe/Central Asia where levels stand at 75,000 and 50,000 respectively. The most
alarming increase in the estimated number of HIV-infected individuals is in South and Southeast Asia, rising from almost zero to 1.5 million in three years.

Finally, of the 13 million cases of HIV infected individuals reported by mid-1993, 2 million had already developed AIDS, with well over 1 million deaths.
Figure 1  Estimated distribution of cumulative HIV infections in adults, by continent or region mid-1993. (From WHO figures and Merson, 1993).
I WESTERN EUROPE
500,000

EASTERN EUROPE AND CENTRAL ASIA
50,000

NORTH AMERICA
> 1 MILLION

NORTH AFRICA MIDDLE EAST
> 75,000

LATIN AMERICA AND THE CARIBBEAN
1.5 MILLION

SUB-SAHARAN AFRICA
> 8 MILLION

SOUTH/SOUTHEAST ASIA
> 1.5 MILLION

AUSTRALASIA
> 25,000

EAST ASIA PACIFIC
> 25,000

SOUTH/SOUTHEAST ASIA
> 1.5 MILLION

SUB-SAHARAN AFRICA
> 8 MILLION

AUSTRALASIA
> 25,000
2. The Human Immunodeficiency Virus (HIV) - a retrovirus.

2.1 The Retroviridae.

HIV belongs to the retroviridae (Fields, 1990). Retroviruses are associated with many diseases, including rapid and long latency malignancies, wasting diseases, neurological disorders and immunodeficiencies. A feature of the retroviruses is their ability to reverse the normal flow of genetic information. They convert their RNA genomes into DNA, which integrates into the cellular DNA of the host. This process allows retroviruses certain biological characteristics not common among other viral families. For example, the error rate of the viral polymerase, used to produce the DNA copy of the viral RNA genome, is much higher than cellular polymerases. This allows a high frequency of mutation and a large number of slightly different viruses to exist within a single host. The result is a flexible viral population with the ability to rapidly respond to fluctuations in environmental conditions.

Retroviruses constitute a clearly defined viral genus (Varmus, 1988). They have a distinctive morphology and composition. They are approximately 100 nm in diameter, with a single protein envelope and an internal nucleocapsid core, usually spherical or conical in shape. This core contains several viral enzymes, including a reverse transcriptase, a ribonuclease, a protease, and an integrase, that have important catalytic roles in replication. The viral genome consists of two positive sense single stranded RNA molecules ranging between 7-10 kilobases in size. The RNA is capped at the 5' end and has a poly-A tail, reminiscent of messenger RNA. The gene order is invariably gag (coding for the proteins of the viral core), pol (coding for the proteins that constitute the viral enzymes), env (coding for the proteins of the viral envelope), with a short repeated sequence known as the long terminal repeats (LTR) flanking either side. Additional regulatory genes may also be included.

Retroviruses have traditionally been divided into three sub-families, the oncoviridae, the lentiviridae, and the spumaviridae (Fields, 1990). These divisions are based primarily on observed modes of pathogenicity, rather than genomic relationships. However, when nucleotide sequence and genome structure are included, seven sub-families are apparent, the oncoviruses being split into five sub-groups with clearly distinct characteristics (Table 2).

In recent years, a new method of sub-classifying the retroviridae has been suggested with only two sub-groups; simple and complex retroviruses (Cullen, 1991). The simple retroviruses (which includes the majority of the oncoviridae) possess only the structural genes gag, pol, and
Table 2  The retroviridae.

This table displays the traditional divisions of the retroviridae into the oncoviridae, the lentiviridae and the spumaviridae, with the subsequent sub-divisions of these groups, and well-known examples. (Adapted from Fields, 1990).
### Retrovirus groups

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Group</th>
<th>Example isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oncovirinae</td>
<td>Avian leukemia sarcoma</td>
<td>Rous sarcoma virus (RSV)</td>
</tr>
<tr>
<td></td>
<td>Mammalian C-type</td>
<td>Moloney murine Leukemia virus (MuLV)</td>
</tr>
<tr>
<td></td>
<td>Mammalian B-type</td>
<td>Mouse mammary tumor virus (MMTV)</td>
</tr>
<tr>
<td></td>
<td>Mammalian D-type</td>
<td>Mason-Pfizer monkey virus (MPMV)</td>
</tr>
<tr>
<td></td>
<td>HTLV/BLV group</td>
<td>Human T-cell leukemia virus type I (HTLV-I)</td>
</tr>
<tr>
<td>Lentivirinae</td>
<td>Lentiviruses</td>
<td>Human immunodeficiency virus (HIV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Simian immunodeficiency virus (SIV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Feline immunodeficiency virus (FIV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Visna/maedi virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Equine infectious anemia virus (EIAV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Caprine arthritis-encephalitis (CAEV)</td>
</tr>
<tr>
<td>Spumavirinae</td>
<td>&quot;Foamy&quot; viruses</td>
<td>Simian foamy virus (SFV)</td>
</tr>
</tbody>
</table>
env. Proviral transcription results in only two major RNA molecules, a full length gag-pol transcript and a singly-spliced env transcript.

The complex retroviruses (which include the lentiviridae, the spumaviridae, and the HLTV sub-family of the oncoviridae) display a two phase temporal pattern of RNA expression, characterised by an early regulatory phase, and a late structural phase. An additional third class of multiply spliced transcripts are found, specifying a variety of regulatory molecules. These regulatory molecules are characterised by a sequence specific transactivator of LTR-driven viral gene expression (eg Tat, Tax, etc, see later) and a repressor of the expression of the early multiply-spliced transcripts (eg Rev, see later). The action of these gene products allows a switch from a non-productive early phase of the viral life cycle to a productive late phase of viral replication when cellular conditions are most favourable.

### 2.2 The Lentiviridae

HIV is a member of the lentiviridae (reviewed in Fields, 1990). In contrast to viruses that cause acute transient disease, lentiviruses do not always cause an acute illness on viral infection of the host. Long incubation periods of months to years precede the onset of disease, and persistent infection is often established in a number of tissues resulting in a slow progressive illness.

Although the first disease caused by a lentiviral agent was recognised in Icelandic sheep in the 1950s (Sigurðsson et al., 1954), the aetiologic agent, the Visna-maedi virus, was discovered much later (Nathanson et al., 1985). The syndrome caused by this virus manifests itself as a respiratory illness (maedi = laboured breathing), coupled with a neurological disorder (visna = paralysis and wasting). It results from the chronic progressive inflammation of infected tissues, and is often fatal.

Several additional lentiviruses have now been isolated from a number of mammalian species; (i) Caprine arthritis encephalitis; This syndrome is characterized by slow progressive synovitis, eventually culminating in a crippling arthritis. In some cases, a progressive neurological disorder is also observed. As with Visna-Maedi, chronic inflammation of infected tissues is the primary cause of illness (Narayan et al., 1978, 1982); (ii) Equine infectious anaemia; Infected animals develop an acute syndrome within one month characterized by anorexia, fever, and anaemia, which is often fatal. If the horse survives the initial syndrome it may remain clinically normal for months or years. Relapse with similar symptoms often occurs and may eventually lead to a chronic wasting syndrome with severe anaemia (Cheavers et al., 1985); (iii) Feline
Immunodeficiency Virus; This virus is distinct from the oncogenic retrovirus, feline leukaemia virus (FeLV). FIV infection is associated with lymphadenopathy and wasting. Immunodeficiency with opportunistic infections has also been observed (Pedersen et al., 1987); (i) Simian Immunodeficiency Virus; Simian Immunodeficiency Viruses (SIV) infect a range of primate species, including Macaques (SIV_{MAC}), African Green Monkeys (SIV_{AGM}), Sootev Mangabey monkeys (SIV_{SM}), and the Mandril (SIV_{Mandrillus}) (Daniel et al., 1985, Kanki et al., 1985, Fultz et al., 1986). SIV_{MAC} is closely related to HIV-2 (Chakrabati et al., 1987). It causes a syndrome in Macaques closely resembling AIDS in humans (Kestler et al., 1990). After experimental inoculation of Macaque monkeys, lymphadenopathy and focal cutaneous rashes are seen within weeks to months (Letvin & King, 1990). Clinical deterioration, accompanied by opportunistic infections are soon observed. A wasting disease, involution of the lymphatic system, and enteropathy, with life threatening dehydration, is also seen. In addition, infected animals develop neural lesions similar to those observed in the brains of patients with AIDS dementia (Desrosiers, 1990).
3. The structure of HIV.

3.1 Proviral genomic organization.

The genomic structure of HIV-1, HIV-2, and SIV\textsubscript{MAC} are illustrated in figure 2 (Greene, 1991, Chakrabati \textit{et al.}, 1987). HIV-1 has nine known genes. These include the structural genes; \textit{gag} and \textit{env}, the enzymatic gene; \textit{pol}, coding for four enzymes, and up to six regulatory genes; \textit{tat}, \textit{rev}, \textit{nef}, \textit{vif}, \textit{vpu}, and \textit{vpr}. The genome contains approximately 9,200 bases and is flanked by a 5' and a 3' long terminal repeat (LTR). These LTRs can be divided into three regions, known as the U3, R, and U5 domains (appearing in that order). The U3 sequence of the 5' LTR contains sequences recognized by cellular transcription factors, while the 5' R region includes a regulatory element known as the TAR sequence (Sodroski \textit{et al.}, 1985a). This element interacts with the viral Tat protein and is essential for the efficient regulation of viral transcription (Selby \textit{et al.}, 1989, see chapter 4.6). Other important elements in the HIV-1 genome include the viral packaging signal (\textit{ψ}), the primer binding site (PBS) which binds a tRNA primer to initiate reverse transcription, the RRE element which interacts with the viral Rev protein, and cis-acting repressor sequences (CRS) important for the correct splicing and transport of viral transcripts (see chapter 4.8).

HIV-1 and HIV-2 have a similar genomic organization (Guyader \textit{et al.}, 1987) with the exception of the presence of the \textit{vpx} gene in HIV-2, but not in HIV-1. The biological properties of their viral elements are conserved despite the fact that there is significant divergence at the nucleotide and amino acid level. The more conserved \textit{gag} and \textit{pol} genes show 56\% and 60\% nucleotide sequence homology respectively, with both proteins sharing less than 60\% homology at the amino acid level. The other viral genes differ more extensively, resulting in an overall HIV-1/HIV-2 homology of 42\% (Guyader \textit{et al.}, 1987). This level of diversity indicates that HIV-1 and HIV-2 are not genetically close enough to be considered isolates of one virus.

\textit{SIV\textsubscript{MAC}} is also closely related to HIV-1 and HIV-2. Sequence analysis of \textit{SIV\textsubscript{MAC}} (Chakrabarti \textit{et al.}, 1987) shows an overall nucleotide homology of 75\% with HIV-2, but only 40\% with HIV-1. Furthermore, the \textit{gag}, \textit{pol}, and \textit{env} proteins of \textit{SIV\textsubscript{MAC}} and HIV-2 are antigenically cross-reactive, whereas HIV-1 cross-reactivity with \textit{SIV\textsubscript{MAC}} or HIV-2 is restricted to the \textit{gag} and \textit{pol} gene products. Using the NUCALN program, sequence analysis of the \textit{pol} gene from a number of animal retroviruses has demonstrated that HIV-2\textsubscript{ROD} is 84\%, 59\%, 44\%, 35\%, and 36\% homologous to \textit{SIV\textsubscript{MAC}}, HIV-1\textsubscript{BRU}, Visna, HTLV-1, and RSV respectively.
Figure 2  Proviral organisation of HIV-1, HIV-2 and SIVmac.

Shaded areas represent long terminal repeats (LTR); env - gene coding for the gp120 and gp41 proteins of the viral envelope; gag - gene coding for the p24, p17, p9 and p6 proteins of the viral core; pol - gene coding for four viral enzymes, reverse transcriptase (RT), protease (PROT), integrase (INT) and ribonuclease H (RibH); tat, rev, nef, vpr, vif, vpu and vpx - genes coding for viral regulatory proteins (Adapted from Greene, 1991, Chakrabati et al., 1987).
However, although a high degree of homology exists, HIV-2 and SIV are distinct viruses, as their divergence is greater than that previously observed for independent isolates of HIV-1.

3.2 The HIV Virion.

Electron microscopy has revealed the HIV virion (figure 3) to be approximately 100nm in diameter. It is icosahedral in shape, with 72 external spikes, 9nm high, and 15nm wide (Gelderblom et al., 1987). These spikes are formed by the two major envelope proteins; gp120 (thought to occur in a tetramer), and gp41 (Greene, 1991). The gp120 and gp41 proteins are made as a gp160 precursor. This precursor is modified by the addition of complex carbohydrates, and transferred to the surface of the host cell where it is cleaved by cellular enzymes. The amino terminal portion, gp120, is located completely external to the cell, but is covalently linked to the transmembrane gp41. The viral lipid bilayer also includes various host proteins, such as MHC class-I and MHC class-II molecules, acquired during the process of viral budding (Greene, 1991).

The HIV core consists of four nucleocapsid proteins; p24, p17, p9 and p6 (figure 3). Each protein is produced by the proteolytic cleavage of a 55kDa gag precursor by the HIV protease (Wlodawer et al., 1989). The myristylated p17 polypeptide is associated with the inner surface of the viral lipid bilayer, stabilising both the exterior and interior components of the virion. Interior to this, the phosphorylated p24 polypeptide forms the inner shelf of the nucleocapsid. The p9 and p6 peptides make up the nucleoid core, with the p6 component directly binding the genomic RNA via a zinc-finger structural motif (Lever et al., 1989).

Each virion contains two positive sense single-stranded RNA genomes (Rabson & Martin, 1985). The RNA genome and nucleoid core are associated with the Vpr and Vif gene products, as well as with the viral enzymes. These enzymes include a reverse transcriptase, an integrase, a protease and a ribonuclease. They are generated from an elongated gag-pol polypeptide, the result of a shift in the translational reading frame that bypasses the usual gag stop codon approximately once in twenty translation events (Jacks et al., 1988).
Figure 3 The HIV-1 virion.

Diagram of the HIV-1 virion and provirus, indicating the position of the genes which code for various structural elements. The Tat, Rev, Nef, Vpu and Vif proteins are not associated with the virion. The lipid bilayer is derived from the host cell membrane and hence contains a number of host cell-surface proteins. Int - integrase; Prot - protease; RT/rib H - reverse transcriptase, ribonuclease H complex.
HIV-1 genome

HIV-1 virion

RNA genome

viral lipid membrane

LTR
4. The HIV life cycle.

4.1 HIV target cells.

The major target for HIV infection are cells expressing the surface glycoprotein CD4 (McDougal et al., 1986). A high affinity interaction between the HIV gp120 envelope protein and the CD4 molecule has been demonstrated. This interaction can be blocked using monoclonal antibodies to CD4. By mapping the binding sites of these antibodies, the gp120 binding site has been localised to the first immunoglobulin-like domain of the CD4 protein, between residues 42 and 55 (the CDR-2 region) (Arthos et al., 1989).

The CD4 positive T-helper subset appear to be the principal population of cells that become infected with HIV. This population is severely depleted during the course of the disease, and is the subset of cells generally thought to be involved in HIV-mediated pathogenesis. However, CD4 is also expressed on the cells of the macrophage/monocyte lineage, with HIV infection being detected in cell types such as blood monocytes (Ho et al., 1986), Langerhan's cells of the skin, alveolar macrophages of the lung, spinocerebral fluid macrophages (Eilbott et al., 1989) and microglial macrophages in the brain (Koeing et al., 1986).

In addition to these two major CD4 positive targets for HIV infection, more recent studies have suggested that CD4 negative cell types can also become infected with HIV, both in vivo (Gyorkey et al., 1987), and in vitro (Chiodi et al., 1987). The neutral glycosphingolipid, galactosyl ceramide (GalC) has a relatively high binding affinity for HIV-1 gp120. As antibodies against GalC can block HIV infection of certain CD4 negative cells, it has been suggested that GalC can act as an alternative receptor for HIV. GalC-mediated infection has been demonstrated in neural cell lines (Harouse et al., 1991), neuronal cells (Bhat et al., 1991) and human colonic HT29 epithelial cells (Fantini et al., 1993). It has also been proposed that GalC on the apical membrane of epithelial mucosal cells may facilitate HIV infection during sexual intercourse (Yahi et al., 1992). However, GalC is not restricted to human cells, and is present on the U373 human glioma cell line which is not permissive for HIV (Chesebro et al., 1990). This suggests that GalC alone is not sufficient for HIV infection.

HIV infection of other cell types has also been demonstrated. These include human fetal astrocytes via a novel receptor (Ma et al., 1994), and a placental cell line, via a membrane-type C-lectin. As free virus will be bound by antibodies, a route of virus internalisation via Fc receptors has also been proposed (McKeating et al., 1990, Takeda et al., 1988). Gp120 has also been shown to bind CD4 negative glial cells activating a cellular tyrosine kinase (Schneider-
4.2 The HIV co-receptor.

The presence of CD4 was originally thought sufficient for HIV infection. However, human CD4-expressing murine NIH3T3 cells bind HIV but do not become infected. In addition, the human CD4 +ve cell lines U87MG, U373 (both glioma cell lines) (Aishorn et al., 1990) and SCL1 (a human squamous cell carcinoma line) (Chesebro et al., 1990) are not permissive for HIV infection. This evidence suggests that either a co-factor for HIV entry/infection is necessary, or a suppressor of HIV entry/infection is present in the non-permissive cell types (Maddon, et al., 1986). Surprisingly, HIV-2 will infect U87MG cells, suggesting different entry requirements to that of HIV-1.

The fusion of a human CD4 +ve HIV-resistant cell with a human cell that will permit HIV infection forms a hybrid that can also become infected with HIV. This suggests that there is an accessory factor(s) for HIV infection rather than the presence of some kind of suppressor (Broder et al., 1993, Dragic et al., 1992, Harrington et al., 1993). A number of candidates for this co-factor or co-receptor have been suggested. These include MHC-I (Grassi et al., 1991), MHC-II (Mann et al., 1988), LFA-1 (Hildreth et al., 1989), CD7 (Sato et al., 1994), a protease known as tryptase-2, and several novel proteins of various sizes (Henderson et al., 1993, Ebenbichler et al., 1993). More recently, the cell surface molecule CD26 was implicated as the HIV-1 co-receptor (Callebaut et al., 1993). It was shown that an enzymatic inhibitor of CD26, and anti-CD26 monoclonal antibodies, blocked HIV entry, while transfection of CD26 into murine cells expressing human CD4, rendered them permissive for HIV entry. However, this result has been refuted by a series of papers, which have now effectively dismissed any role for CD26 as the co-receptor for HIV infection on CD4 positive cells (Stamatatos & Levy, 1994, Lazaro et al., 1994).

An alternative possibility for the HIV co-receptor has now been proposed. Fusion of a human red blood cell (RBC) to a CD4 + ve HIV non-permissive cell permits subsequent infection. Furthermore, the RBC can be restricted to just its plasma membrane, and extensively digested with proteinase-K, yet still provide a co-receptor function (Dragic et al., 1995). These results suggest that the HIV co-receptor is not a protein, although certain domains of a tightly-folded protein could be extensively protected from proteinase-K treatment. However, it seems likely that a molecule such as a phospholipid, a neutral lipid, cholesterol or a glycolipid (reviewed in Varki,
1993) would be a more likely candidate. An interaction between HIV-1 gp41 and a lipid-anchored sugar residue could therefore initiate lipid mixing of the viral and cellular membranes, allowing formation of a fusion pore, and the subsequent entry of the viral RNA genome and nucleoid core.

4.3 Viral entry.

Viral entry was originally thought to be the result of receptor-mediated endocytosis, HIV being transported into the cell in association with internalised CD4 molecules (Maddon et al., 1988). However, it was found that in the presence of inhibitors of endocytosis, HIV infectivity was not significantly reduced (Stein et al., 1987, McClure et al., 1988). In addition, a truncated or chimeric CD4 molecule, lacking the endocytotic signals necessary for phorbol ester induced internalisation, was shown to function as a receptor for HIV infection (Bedinger et al., 1988). Furthermore, it has been demonstrated that HIV does not enter the cell in association with CD4 (Maddon et al., 1988).

Evidence now suggests that viral entry occurs by a mechanism known as pH-independent receptor mediated activation of virus fusion (Bedinger et al., 1988, Stein et al., 1987, Allan et al., 1991). On binding to CD4, gp120 undergoes a series of conformational changes (Sattentau et al., 1991). This may result in the dissociation of gp120 from membrane bound gp41, exposing previously hidden residues on the latter (Hart et al., 1991, Moore et al., 1990). Virions and infected cells can be induced to shed gp120 on addition of soluble CD4 in culture. However, it is not clear whether gp120 completely dissociates from gp41 in virus/cell interactions (Allan et al., 1990, Sekigawa et al., 1990). The conformational changes in gp120 are known to expose a region of the molecule known as the V3 loop (Clements et al., 1991). This region is strongly implicated in the process of viral fusion (Moore et al., 1991). The exposed residues have certain conserved motifs, even though the majority of the V3 loop is hyper-variable (Clements et al., 1991). These motifs correspond to cleavage sites for tryptic proteases, and their disruption destroys virus infectivity (Page et al., 1992). As yet, there is no direct evidence for proteolytic cleavage in the process of HIV entry, although proteolytic cleavage is implicated in the entry of certain other retroviruses.

The partial or full dissociation of gp120 from gp41, and the presence or action of the V3 loop, facilitate the fusion of viral and cellular membranes. A fusogenic domain of gp41, resembling the F proteins of the paramyxoviruses, is central to this process. The phenomenon of membrane fusion allows the internalization of the viral nucleocapsid.
4.4 Viral DNA synthesis.

The life cycle of HIV involves the integration of viral genetic information into the cellular genome. However, before this can occur, a DNA copy of the internalised viral RNA genome must be generated. This is achieved by the virally encoded reverse transcriptase (RT) enzyme (Goff, 1990, Hu & Temin, 1990, Hsieh et al., 1993), and the p24, p17 and p6 components of the viral core (Barat et al., 1993). However, this presents two problems. First, the method of copying the RNA genome using the virally-encoded RT enzyme requires the need for an RNA primer to initiate polymerase elongation. However, as the viral RNA template is linear, a mechanism must be employed that allows the RNA sequence that binds the primer to be copied also. Second, viral RNA genomes are generated by the host/cellular RNA polymerase II. To initiate such transcription, a viral promoter element is necessary. This is located upstream of the viral transcriptional start site, but still within the viral genome. As these upstream sequences are not represented in the viral transcripts, they would be lost if no mechanism existed to retrieve this missing information. The retrovirus solves these problems by employing an identical sequence at each end of the integrated proviral genome, known as the long terminal repeats (LTR). During the RNA stages of the viral life cycle, these sequences are present in reduced form. However, all the information to reproduce two complete LTRs at either end of the DNA provirus still remain.

4.4.1 Synthesis of negative strand DNA.

A full length LTR is comprised of three regions; U3, R and U5. The 5' end of the viral RNA genome, by definition, marks the start of the R region of the 5' LTR. This is then followed by the U5 region and a region known as the primer binding site (PBS) (Li et al., 1994). The gag, pol, env and regulatory genes follow, with the U3 and R region of the 3' LTR at the 3' end of the RNA molecule, immediately before the poly-A tail (figure 4A).

To initiate reverse transcription, a tRNA primer (also packaged in the mature virion, (Jiang et al., 1993)) binds to the primer binding site, providing a 3'-OH end from which the viral RT enzyme can start to generate DNA (figure 4A) (Kohlstaedt & Steitz, 1992). This elongation continues in the direction of the 5' end of the RNA molecule through the U5 and R regions. On reaching the end, the RT enzyme stops momentarily, ready for its first "jump". The last DNA sequence that was generated, complementary to the 5' R region, is also complementary to the R
Figure 4  The stepwise progression of the reverse transcription process.

Reverse transcription generates a double stranded proviral DNA copy of the viral RNA genome. Unfilled line - RNA; heavy line - DNA; PBS - primer binding site; U5, R, U3 - regions of the HIV LTR. (Adapted from Fields, 1990).

A. A tRNA primer (included in the virion), binds to the PBS located immediately downstream of the 5' U5 region of the RNA genome. This allows the reverse transcriptase complex to generate a DNA copy of the 5' RNA U5/R region.

B. The tRNA primer, and DNA strand corresponding to 5' RNA U5/R region, jumps to the 3' end of the RNA genome. The complementary R regions of the 3' RNA genome and the recently copied DNA allow the new DNA strand to bind in the correct position. During the process of the jump, the 5' U5/R region of the RNA is degraded by the ribonuclease H activity of the reverse transcriptase complex.

C. The positive (lower) strand of the DNA copy of the RNA genome is generated by the reverse transcriptase complex. A stop is reached at the 5' end of the RNA genome, corresponding to the 5' end of the PBS.

D. The ribonuclease H activity of the RT complex removes the 3' U3/R region of the RNA genome. This generates a 3'-OH primer for the DNA polymerase activity of the RT complex, allowing the negative strand of the DNA to be generated. This DNA polymerisation reaction comes to a temporary stop at the 5' end of the positive DNA strand, corresponding to a sequence complementary to the PBS, located in the original tRNA primer.

E. The final jump involves the complementary base pairing of the two DNA copies of the PBS. The PBS of the positive (lower) DNA strand in effect loops around to displace the tRNA primer from the PBS region of the negative (upper) DNA strand. This generates a 3'-OH end for both strands, allowing the DNA polymerase activity of the RT complex to complete a full length double stranded DNA copy of the original RNA genome.

F. This proviral DNA molecule, with a full length LTR at both ends, can then integrate into the host genome.
A. Transfer of primer and positive strand DNA to 3' end of RNA genome

B. RNA degradation by Ribonuclease H

C. RNA degradation by Ribonuclease H

D. Negative strand DNA

E. Pull length viral DNA genome
region at the 3' end of the RNA template, allowing the RT replication machinery to move to the 3' end of the RNA template to continue elongation (figure 4B) (Gotte et al., 1995). It has been demonstrated that the viral p24 capsid protein is important in this first "jump", as the transfer is often directed to the wrong position in its absence. The driving force for the "jump" is the RT affinity for its template (Peliska & Benkovic, 1992), while the correct alignment for the continuation of elongation is dependent on the complementary base pairing between the two R regions (Klaver & Berkhout, 1994). The RT complex completes the negative DNA strand with the tRNA primer still attached to its 5' end (figure 4C) (Wakefield et al., 1994).

4.4.2 Synthesis of positive strand DNA.

The positive strand of the viral DNA genome is generated using the newly formed negative strand as a template. This requires a second primer to initiate elongation, which is created by the RT-complex ribonuclease H-mediated cleavage of the RNA genome (Smith & Roth, 1992). Cleavage occurs at the 5' end of the 3' U3 region (figure 4D), but is unlike that normally observed with ribonuclease H. No ends need be present, a DNA/RNA hybrid must act as substrate, and the cleavage occurs at a very precise position in the RNA genome, in a polypurine tract that is conserved in many retroviruses and retrotransposons (Finston et al., 1984).

The RT molecule is now able to elongate the positive DNA strand towards the original tRNA primer that is still attached to the 5' end of the negative DNA strand (figure 4D). The RT enzyme copies approximately twenty bases of the primer binding site region of the tRNA primer before coming to another stop. This stop is not determined by the end of the tRNA primer, but by the presence of a modified base in the tRNA which does not permit itself to be copied (Fields, 1990). The purpose of copying this last twenty bases provides a complementary sequence that can base pair with the primer binding site at the 3' end of the negative DNA strand (figure 4E). Once this transfer has occurred, both DNA strands are copied to their logical ends and the tRNA primer is removed (figure 4F).

The result of this rather complex process is a linear DNA molecule with a complete and identical LTR sequence at both ends of the genome. Although the linear form of this DNA molecule is thought to be biologically important, circular molecules with either one or two LTRs have also been observed in vitro. A large amount of unintegrated DNA has been shown to accumulate on HIV infection of a cell. This is probably due to the infection of the cell by
numerous viral particles, and may have a role in viral pathogenesis as observed in other viral infections.

4.5 Proviral integration.

The synthesis of viral DNA occurs 4-8 hr post-infection. Once complete, the DNA genome is transported to the nucleus. The mechanism of this transfer is unknown, but in T-cells it may be linked to cell proliferation (Stevenson et al., 1990). However, the infection of non-proliferating cells such as macrophages suggests that an active transport process could occur in certain cell types. A nuclear localisation signal found in the myristylated p17 matrix protein supports this hypothesis.

Once in the nucleus, the proviral DNA is inserted into the host genome. After integration a deletion of two bases at both the 5' and 3' end of the provirus, and a duplication in the host genome at the point of insertion, is observed. The ends of the integrated provirus have the sequence 5'TG......CA3'. This is highly conserved throughout the retroviridae, suggesting that these specific bases have an indispensable role in integration (Pauza et al., 1990).

The process of integration involves a ribonucleoprotein complex derived from the viral core, which includes the viral integrase protein (Cannon et al., 1994). It does not appear to involve ATP or any other energy providing source, and therefore may resemble the sort of breakage/rejoining reaction catalysed by topoisomerases. The first step in the integration process is the integrase-mediated removal of the 3' terminal two bases at either end of the linear viral DNA molecule (Brown et al., 1989). A staggered cut is then made in a target sequence of the host DNA. This sequence is basically random, although the structural context of the chromatin, an association with nucleosomes, and the use of DNA that is extensively bent, seem to influence this process (Pruss et al., 1994, Chow et al., 1994). The staggered cut in the host DNA leaves a four base 5' overhang. The integrase enzyme then catalyses the joining of the exposed 5' phosphorylated ends of the host DNA to the recessed 3'-hydroxy end of the viral DNA. The breaking of the host DNA and the joining of the viral DNA is probably a one-step process, again catalysed by the viral integrase enzyme. Finally, cellular repair mechanisms remove the two mismatched bases from the viral DNA (hence the two base deletion) and fill in and ligate the remaining exposed bases, creating the characteristic duplication at the site of integration (Fields, 1990).
4.6 Transcription from the provirus.

The proviral genome is transcribed by the cellular RNA polymerase II. By definition, transcription starts at the 5' end of the upstream R region, reading through the entire viral genome into the cellular DNA before being processed. Transcriptional initiation is also possible from the 3' LTR (reading in the same direction as that from the 5' LTR), but the absolute level of transcription is much lower (Klaver & Berkhout, 1994). This is presumably a result of the difficulty in forming a transcription initiation complex on a stretch of DNA that is being actively transcribed from the 5' LTR. The 3' LTR has an efficient poly-A signal, again by definition at the junction of the 3' R region and the 3' U5 region. The poly-A signal in the 5' LTR does not function efficiently (Bohnlein et al., 1989), as in vitro 3' poly-A processing has been shown to require an upstream element and an upstream TAR sequence (Gilmartin et al., 1992).

The viral promoter and enhancer elements lie in the upstream U3 region of the 5' LTR (figure 5), and can be divided into several regions. The first is known as the promoter core region. It is located in the 3' region of the U3 sequence and is crucial for the initiation of transcription. This region contains a TATA box element at position -28 to -24 (relative to the first base transcribed) (Rosen et al., 1985). This motif is present in many eukaryotic gene promoters and is known to bind the cellular transcription factor TFIID. A transcription initiation complex can form around this element, which will ultimately include the cellular RNA polymerase II. The presence of a number of other cellular factors, bound to elements on the DNA adjacent to the TATA box, facilitate the formation of the initiation complex. The transcription factor Sp1 is one such factor. Three Sp1 binding sites have been located in the core region of the HIV LTR at position -78 to -47 (Rosen et al., 1985).

Upstream of the core region is the promoter modulatory region. This contains several DNA sequence motifs that can bind cellular transcription factors, facilitating the formation of the transcription initiation complex. Factors can also bind that block the formation of this complex, decreasing the rate of transcription initiation. An important feature of the factors that bind to elements of the modulatory region is their responsiveness to cellular stimuli such as cellular activation. Therefore, proviral transcription can be linked to and respond to a specific cellular environment for the maximal production of virus.

The modulatory region of the HIV-1 LTR contains the following sites; AP-1 limited homology sequences at -347 to -329 (Franza et al., 1988); NF-AT sites at -292 to -255 (Siekevitz et al., 1987); USF site at -173 to -159 (Giacca et al., 1992); TCF-1α site at -139 to -124 (Waterman et al., 1991); and 2 NFκB sites at -104 to -81 (Nabel et al., 1987).
Figure 5  The HIV-1 LTR promoter.

This figure illustrates the array of transcription factor binding sites located in the core, modulatory, and TAR regions of the HIV-1 LTR, (Adapted from Rosen et al., 1985).
HIV-1 5'-LTR Promoter

Viral Transcription

AP-1  NF-AT  USF  TCF-1α  NF-κB  SP1  TATA  INT  UBP1/LBP1  UBP-2  CTF/NF1  PBS  SD1  Ψ

Modulatory  Core  +1  TAR

U3  R  U5  gag leader

(not to scale)

Core region  Modulatory region  TAR region  gag leader

TATA  (-28 to -24)  NF-κB  (-104 to -81)  UBP1/LBP1  (+1 to +59)  PBS - primer binding site

SPI  (-78 to -47)  TCF-1α  (-139 to -124)  UBP-2  (+1 to +59)  SD1 - major 5' splice donor

INT  (-5 to -15)  USF  (-159 to -173)  CTF/NF1  (+1 to +59)  Ψ - packaging signal

NF-AT  (-292 to -255)

AP-1  (-347 to -329)
The AP-1 like sequences allow the binding of the Fos transcription factor, which has been shown to increase after cellular activation. These sequences may also bind a transcription factor that has been shown to be a member of the steroid/thyroid hormone receptor subfamily (Orchard et al., 1990). The transcription factor NFκB is also important in the transcription of genes that respond to cell activation stimuli. In resting cells, NFκB is complexed with the inhibitor IκB (Baeuerle & Baltimore, 1988). In response to external stimuli such as phorbol esters or the cytokine TNFα, protein kinase A or protein kinase C can phosphorylate IκB. Phosphorylated IκB dissociates from NFκB, allowing NFκB to enter the nucleus and activate transcription of a range of host genes such as IL-2, as well as the HIV-1 LTR.

The modulatory region of the HIV-1 LTR between bases -420 and -154 is also known as the negative response element (NRE) (Siekevitz et al., 1987, Rosen et al., 1985). Sequences contained in this region are thought to exert a negative influence on HIV-mediated transcription, as deletion of the NRE resulted in a 3-fold increase in both HIV transcription and replication (Lu et al., 1989). However, the significance of this NRE element and its role in the viral cycle are still unclear.

The third region of the LTR is known as the TAR region. It is located from position +1 to +59 in the R region of the LTR, and is the first sequence to be transcribed. This element has been shown to confer transcriptional transactivating potential to the core promoter (Sodroski et al., 1985b). As the TAR region is transcribed it forms an RNA stem loop secondary structure. This structure has been shown to interact with the virally encoded Tat protein in association with cellular factors (Selby et al., 1989). In the absence of the Tat gene, the vast majority of transcripts terminate soon after reaching the TAR region (Kao et al., 1987, Feinberg et al., 1991). However, in the presence of a Tat/TAR interaction, a dramatic overall increase in the rate of transcription of full length genomic transcripts is observed (Lasplia et al., 1989).

4.7 Transcriptional regulation.

The analysis of LTR sequences from HIV-1-infected patients has revealed that a broad range of mutations and the presence or absence of methylation can effect the absolute levels of HIV-1 LTR-driven transcription (Michael et al., 1994, Gutekurst et al., 1993). In resting T-cells, a basal or constitutive level of transcription from the HIV-1 LTR has been observed. This results in predominantly short transcripts that terminate at the TAR region, with very few polymerase complexes reading through this block (Feinberg et al., 1991). In the presence of the Tat protein
however, there is a dramatic increase in the transcription of full length transcripts (Sharp & Marciniak, 1989).

The Tat protein binds to the 59 base RNA stem loop secondary structure of the TAR region in association with cellular factor(s) (Carroll et al., 1992, Cullen, 1992). Initial work suggested that Tat binding of the TAR region had little effect on the rate of transcription of sequences proximal or upstream of the TAR region, suggesting only a marginal Tat-induced increase in transcriptional initiation (Marciniak & Sharp, 1991, Kato et al., 1992). In contrast, a huge increase in the transcription of sequences distal or downstream of the TAR region was observed (Kao et al., 1987, Feinberg et al., 1991). This strongly implicated Tat as a viral RNA-sequence-specific elongation factor. However, several results did not appear consistent with this hypothesis. The first was the position dependence of the TAR region. If the TAR region was functioning as a premature transcription termination signal, this effect would not be related to the distance from the site of transcription initiation. However, the function of Tat dramatically decreased if the TAR region was moved further downstream of the 5' LTR. Second, a Tat/Gal4-DNA-binding-domain fusion protein was shown to transactivate full length transcription from the LTR when a Gal4 DNA binding site was located upstream of the HIV-1 LTR core promoter. This suggested that Tat could also transactivate LTR transcription when not associated with the TAR region (Kamine et al., 1991). Third, the strong transcriptional activator protein VP16 from the herpes simplex virus was demonstrated to also provide an increase in full length proviral transcription when binding upstream of the HIV-1 promoter, suggesting that such transactivation was a consequence of the action of a classical transcription factor-type mechanism, rather than the promotion of transcript elongation (Southgate & Green, 1991).

In the light of such data, an alternative theory for the action of Tat on HIV-1 LTR transcription has been proposed (Cullen, 1993) (figure 6). This hypothesis suggests that there are two distinct promoters in the HIV-1 LTR, initiating transcription from the same nucleotide, but forming phenotypically different transcription initiation complexes. The first "non-processive" promoter has a relatively high level of basal transcription initiation but forms a RNA polymerase II complex that does not read through the TAR region with great efficiency. Transcription therefore halts at this point with the transcription complex dissociating from the DNA (figure 6A). The second "processive" promoter can only efficiently form a transcription initiation complex in the presence of Tat, made available by Tat binding to the RNA TAR regions that are generated by non-processive promoter-mediated transcription. The initiation complex formed in the presence of Tat has the ability to read through the transcription block at the TAR region and produce full length transcripts (figure 6B). The fact that the rate of initiation of total transcripts from the LTR

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The proposed mechanism of action of the HIV-1 Tat protein.

A. Schematic diagram of transcriptional initiation in the absence of both cellular activation and the HIV-1 Tat protein. In this state, only basal transcription factors and those factors that bind to the "non-processive" promoter are present. The transcriptional initiation complex that is generated is not processive and tends to prematurely terminate at, or around, the TAR region.

B. Diagram of transcriptional initiation in an activated cell in the presence of an increased concentration of Tat protein. In this state, the Tat protein, in combination with additional cellular factors, can bind to the RNA stem loop structure of the TAR region. This orientates the Tat/cellular factor complex in a position that allows it (in combination with transcription factors that are induced by cellular activation) to influence the formation of a "processive" transcriptional initiation complex. This complex does not prematurely terminate and allows the transcription of full length proviral transcripts. (Adapted from Cullen, 1993).
A. No Tat - resting T-cell

B. Tat+ activated T-cell

**Legend:**
- **BASAL TRANSCRIPTION FACTORS**
- **CELLULAR FACTORS INITIATING NON-PROCESSIVE PROMOTER**
- **CELLULAR FACTORS INITIATING PROCESSIVE PROMOTER e.g. NFkB**
- **TAT BINDING PROTEIN**
- **NON-PROCESSIVE TRANSCRIPTION COMPLEX**
- **PROCESSIVE TRANSCRIPTION COMPLEX**
does not alter too much in the presence or absence of Tat can be explained by promoter exclusion (i.e., a downregulation of initiation from the non-processive promoter as the processive promoter is up-regulated). Therefore, this hypothesis proposes that Tat acts as an inducer of transcriptional elongation, but does so not by elongating transcripts that would have previously prematurely terminated, but by allowing the formation of a phenotypically distinct transcription complex that is not susceptible to the termination effects of the TAR region.

Evidence for the two promoter hypothesis has been found. It has been demonstrated that sequences in the HIV-1 LTR can be deleted between -5 and +26 that stop the production of the short transcripts from the non-processive promoter, but still allow a Tat-induced increase in full length transcription (Sheldon et al., 1993). Tat has also been shown to directly interact with the transcription factor TFIID that binds to the TATA box (Kashanchi et al., 1994).

4.8 The products of HIV transcription.

In cell culture HIV mRNA is detectable about 12 hr post-infection (Kim et al., 1989), with transcription proceeding in two distinct temporal phases. The first phase occurs before the viral proteins Tat and Rev accumulate to any appreciable level, with the majority of transcripts terminating prematurely. Any full length transcripts that are produced at this stage are rapidly spliced (Robert-Guroff et al., 1990). All retroviruses must splice their RNA to some extent, specifically to remove the downstream env sequence from the upstream gag/pol sequences. Lentiviruses such as HIV use more splicing events to generate transcripts coding for the array of regulatory proteins that they additionally possess. The products of the splicing events belong in two groups; the singularly spliced intermediate class of transcript (4-5kb) and the multiply-spliced small transcripts (1.8-2kb) (Schwartz et al., 1990b, Furtado et al., 1991). The splicing occurs between several consensus and cryptic splice donor and acceptor sites that are present in the viral genome (Chang et al., 1989). With multiple combinations of splice donor and acceptor, well over twenty major splice products have been observed (figure 7) (Purcell et al., 1993). Chimeric proteins have also been detected (Salfeld et al., 1990, Arrigo et al., 1991). In the absence of Rev and Tat, small multiply-spliced transcripts predominate. It has been demonstrated that of these transcripts, ~80% code for the Nef protein, ~18% code for the Rev protein, and ~2% code for the Tat protein. This distribution is HIV-strain and tissue independent (Robert-Guroff et al., 1990).

As discussed previously, the generation of full length transcripts greatly increases when
Figure 7 The three major classes of transcripts generated from the HIV-1 LTR.

This figure illustrates the major species of the three classes of transcripts that are generated from the HIV-1 LTR. The 1.8kb multiply-spliced class of transcripts code primarily for the regulatory proteins Nef, Tat and Rev. The single-spliced 4.0kb class of transcripts code for the envelope proteins gp120 and gp41, plus Tat, Vpr, Vpu and Vif. The full length transcript provides coding sequence for the gag and pol proteins, (Adapted from Purcell et al., 1993).
Major 1.8kb class of RNA
- Nef
- Nef
- Nef
- Rev
- Rev
- Rev
- Tat
- Tat
- Tat
- Tat
- Vpr
- Vpr

Major 4.0kb class of RNA
- Env
- Tat
- Tat
- Tat
- Tat
- Vpr
- Vif

Full length transcript
the concentration of the Tat protein is elevated (about 24 hr after HIV infection in T-cell lines (Kim et al., 1989)). However, the Rev protein also influences the number of full-length transcripts that are available for translation and packaging. Rev suppresses RNA splicing and promotes the transport and translation of the full length 9.2 kb viral transcript (Emerman et al., 1989, D'Agostino et al., 1992). Rev recognises and binds to a region of the full length transcript known as the Rev-response element (RRE) (Malim et al., 1989c, Dayton et al., 1989). This interaction depends crucially on the secondary structure of the RNA in this region (Malim et al., 1990, Holland et al., 1990). It involves the basic domain of the 19 kDa Rev protein, inhibiting splicing in a dominant manner (Malim et al., 1989b, Kjems et al., 1991). Rev has also been demonstrated to form multimers on the RRE (Malim et al., 1990), and may associate with the murine homolog of human p32, an acidic protein that co-purifies with splicing factors SF2/ASF (Krainer et al., 1991).

The translation of viral transcripts is also regulated. Many of the transcripts are multicistronic coding for more than one gene. Eukaryotic translation by polysomal ribosomes is thought to occur by a scanning mechanism from the 5' cap of the mRNA, with the first AUG used to initiate translation. However the context of this AUG is important. The +4 and -3 bases in relation to the A of the AUG are critical for it to be recognised as a strong or weak initiator of translation. This is known as a Kozak consensus. In HIV transcripts, the Tat gene has a strong consensus Kozak sequence, which does not allow the translational machinery to pass very often. However, the rev AUG and the AUG of the vpu gene have a weak Kozak sequence, allowing the frequent translation of downstream genes.

4.9 Viral assembly and budding.

The HIV gag transcript (coding for p24, p17, p9 and p6) is translated into a p55 precursor protein. This precursor protein is targeted to the cellular membrane by the myristylation of the p17 subunit. When expressed in isolation, p55 gag will self-assemble into a virion core without the aid of other virally encoded genes (Hu et al., 1990, Toh et al., 1985, Chazal et al., 1994). Self-assembly of viral particles will also occur intracellularly if the myristylation signal is removed from the p17 subunit. The C-terminal domain of the p24 subunit of p55 has a critical role in the folding of the virion (Chazal et al., 1994). Regions important in this process have been mapped to amino acids 341-346 and 350-352 (Von Poblotzki et al., 1993). It is thought that a number of p55 precursor proteins interact and undergo a series of conformational changes at the
cell surface. This allows hydrophobic residues of p55 to come to the surface, pulling the lipid membrane around the complex, forming a virion-like structure, and initiating viral budding (Rhee et al., 1994). The p6 C-terminal domain of the p55 precursor has been shown to have an important role in the correct closing and maturation of the virion (Gottlinger et al., 1991). Mutations in the p6 domain lead to inefficient release of budding virus, with accumulation of viral protein at the cell surface.

The p17 subunit of the gag precursor has been shown to associate with a region of the gp41 env protein. This allows the gp41 and gp120 proteins to be retained at the cell surface. The gp41 protein has been demonstrated to contain a polarisation signal (Lodge et al., 1994). This directs the budding of HIV-1 virions to the basolateral membrane, rather than the apical membrane, of cells such as the polarised epithelial MDCK line (Owens et al., 1989). Virions can bud in the absence of any envelope protein in MDCK cells, forming non-infectious lipid-enveloped virions that are released in a non-polarised fashion (Gheysen et al., 1989, Owens et al., 1991).

The p17 subunit of the p55 gag precursor also contains a nuclear localisation signal. Mature p17 is found in the nucleus, associated with the RNA genome and the mature p6 subunit of the p55 gag precursor (Bukrinskaya et al., 1992). The p6 subunit has a zinc-finger motif allowing it to interact with the encapsidation (or packaging) sequence of full length RNA genomes (Gorelick et al., 1988, Lever et al., 1989).

The virion is therefore formed at the inner surface of the plasma membrane by the combination of p55 gag self-assembly, gp41/gp120 association, plus the delivery of core proteins carrying the RNA genome with the viral enzymes such as RT and INT. Budding then occurs, releasing infectious virus from the cell. It was initially thought that the final steps in virion maturation occurred after virus release, with the HIV protease cleaving the p55 gag precursor into the appropriate subunit proteins (Wlodawer et al., 1989). HIV-1 virions can form and bud in the absence of the HIV protease, but these particles are non-infectious (Loeb et al., 1989, Kaplan et al., 1994). Overexpression of the pol gene (and hence the protease enzyme) in a ratio of 1:1 with the gag precursor, (instead of the usual 1:20 ratio), resulted in early processing of all gag transcripts and a block in virion formation (Karacostas et al., 1993). However, recent studies have implicated protease activity at an earlier stage in the viral assembly mechanism, before viral release (Kaplan et al., 1994).
4.10 The accessory genes vpr, vpu and vif.

The vpu gene is present in the majority of HIV-1, but not HIV-2 or SIV isolates. The HIV-1 vpu gene encodes a well characterised integral membrane phosphoprotein that forms homooligomeric structures both in vitro and in vivo (Cohen et al., 1988, Maldarelli et al., 1993). Vpu is not essential for in vitro HIV-1 replication, but has been demonstrated to enhance virion release in certain tissue culture systems (Terwilliger et al., 1986). In addition, Vpu has been shown to allow the efficient transport of gp160 to the surface of an infected cell. Gp160 has been demonstrated to form a complex with CD4 in the endoplasmic reticulum which inhibits the normal gp160 maturation pathway, and reduces cell-surface expression of CD4. Vpu has been proposed to play a role in the degradation of CD4 and the release of ER-trapped gp160 envelope protein (Willey et al., 1992a, Willey et al., 1992b). A direct interaction between Vpu and CD4 has recently been demonstrated (Bour et al., 1995). This interaction and the subsequent degradation of this ER-localised CD4 requires the presence of a region of the CD4 cytoplasmic tail between residues 402-420 (Chen et al., 1993). Such a release of bound gp160 has been proposed to allow the more efficient assembly and release of infectious viral particles, a process which may be far more important under the rigorous selection pressures of the in vivo environment.

The vpr gene is found in HIV-1, HIV-2 and SIV isolates (Cohen et al., 1990b). It codes for a 97 amino acid protein of between 12-15kDa. Vpr interacts with the 3' p6 domain of the gag core protein, resulting in the incorporation of Vpr into the virion (Cohen et al., 1990a, Paxton et al., 1993, Lavallee et al., 1994). Mutant HIV with a non-functional vpr gene replicate efficiently in rapidly dividing cells but display a significantly reduced rate of infection in terminally differentiated, non-proliferating macrophages (Westervelt et al., 1992). The mechanism of this Vpr-mediated effect could be via interaction with the gag proteins of the pre-integration complex. Such an interaction may allow HIV proviral DNA to enter the nucleus in the absence of mitosis (Heinzinger et al., 1994, Bukrinsky et al., 1993b).

The vif gene has been demonstrated to be conserved in most primate and non-primate lentiviruses (Oberste et al., 1992). It codes for a 23kDa virion-associated protein that is expressed from Rev-dependent single spliced HIV mRNA late in the viral cycle (Kim et al., 1993). HIV-1 vif -ve mutants have been shown to display impaired replication in primary lymphocytes and macrophages (Gabuzda et al., 1992, Sakai et al., 1991). This appears to be due to the decrease, by as much as 1000-fold, in the infectivity of the progeny virions (Fisher et al., 1987, Kishi et al., 1992, Strebel et al., 1987). Vif has been localised to the internal surface of HIV-infected cells (Goncalves et al., 1994). This location seems to allow Vif to play a role in the correct assembly
and maturation of the viral core, resulting in the production of a more infectious virion (Hoglund et al., 1994). However, the results of experiments performed in vitro seem to show a huge variability depending on the cell lines used, making the precise function(s) of Vif difficult to assess. In this light, it is still possible that Vif may also have additional roles in the viral cycle. These include the completion of RT (von Schwedler et al., 1993), the more efficient incorporation of env proteins into the virion (Sakai et al., 1991), the post-translational modification of gp41 (Guy et al., 1991) and the internalisation of the viral core (Sova et al., 1993).
5. Disease progression.

5.1 Primary HIV infection.

An acute clinical, immunological, and serological response to HIV infection is often observed in the days or weeks after initial exposure. This clinical illness, associated with the production of antibodies against HIV or seroconversion, was first described in 1985 for HIV-1 (Cooper et al., 1985), and later for HIV-2 (Besnier et al., 1990). Although originally described as an acute mononucleosis-like illness in eleven of twelve homosexual patients in Sydney (Cooper et al., 1985), subsequent investigation has characterised primary HIV infection as a distinct and unique clinical syndrome (Gaines et al., 1988b, Gaines et al., 1990). This syndrome has been shown to occur in between 50-70% of cases (Pederson et al., 1989, Lange et al., 1986), although it is rarely observed in paediatric AIDS (Tindall & Cooper, 1991).

The syndrome associated with primary HIV infection is of sudden onset. The incubation period in a care worker infected via a needlestick incident was just 13 days, and subsequent work has indicated that incubation periods of between 2-4 weeks are common (L’age-Stehr et al., 1985, Stricof et al., 1986). The syndrome is characterised by general symptoms such as fever, sweats, malaise, lethargy, anorexia, temporary weight loss, nausea, vomiting, diarrhoea, myalgias, arthralgias, and generalised lymphadenopathy (although lymph node structure is not affected), (Tindall & Cooper, 1991). In addition, neuropathic symptoms such as aseptic meningoencephalitis (Ho et al., 1985), peripheral neuropathy, facial palsy, headaches, retro-orbital pain, photophobia and myelopathy are often observed (Tindall & Cooper, 1991). Dermatological signs including an erythematous maculopapular rash and mucocutaneous ulceration of gingiva, palate, oesophagus, anus and penis are also frequently seen (Gaines et al., 1988b). A combination of these illnesses lasts for between two and four weeks, and in the vast majority of cases is entirely self-limiting (Pederson et al., 1989). The patients recover fully, entering an asymptomatic stage of the illness that can last from a few months to many years before the onset of AIDS.

A characteristic response to primary HIV infection is also revealed in the laboratory (figure 8) (Cooper et al., 1985). Early in infection, there is a widespread, intense, but short viraemia, with dissemination of virus to many tissues. HIV is readily isolated from peripheral blood mononuclear cells and cell-free plasma (Albert et al., 1987), cerebrospinal fluid (Ho et al., 1985) and cells of the bone-marrow. The cellular immune system of the CNS is also activated, indicating HIV infection, even in the absence of overt neurological symptoms or signs of disease.
Figure 8  The typical course of an HIV-1 infection.

In the first weeks after HIV-1 infection, a severe plasma viraemia is often detected, accompanied by a dramatic decrease in the CD4+ T-cell count in the peripheral blood. This period may be associated with a mononucleosis or flu-like syndrome.

Two months or so after the initial infection, the vast majority of HIV-1-infected individuals have made a full recovery from any symptoms associated with the primary syndrome. They then enter an asymptomatic phase of the disease which can last for longer than 10 years. During this period, the CD4+ T-cell count gradually falls, although virus is rarely detectable in the peripheral blood. The immune system of the infected individual progressively deteriorates until opportunistic diseases begin to develop. This characterises the onset of clinical AIDS, with death being an inevitable consequence usually within a year. (Adapted from Pantaleo *et al*., 1993).
Course of a typical HIV infection

- Primary infection
- Clinical latency
- Opportunistic diseases
- Death

CD4+ T-cells/mm^3

Plasma viraemia
Initially the total number of lymphocytes, (both CD4+ and CD8+), decrease dramatically in the peripheral blood. This may be due to the homing or re-distribution of these cells to secondary lymphoid organs as part of the HIV-associated immune response. However, the CD8+ cytotoxic T-cells increase to levels much higher than normal soon after infection. This is reminiscent of observations made in EBV-associated mononucleosis (De Waele et al., 1981), and leads to an inversion of the T4/T8 ratio (Tindall & Cooper, 1991). Levels of interferon-α, neopterin and β2-microglobulin are elevated in the blood and cerebrospinal fluid, supporting the hypothesis of an activated cellular immune response. The increase in CD8+ cells coincides with a decrease in HIV plasma antigen, viral clearance and a resolution of clinical symptoms (Tindall & Cooper, 1991). CD8+ cells return to normal levels, but are usually greater than the levels of circulating CD4+ cells, which rarely recover fully.

A significant humoral immune response to HIV infection is also detected (Gaines et al., 1987b). Increased levels of IgM and IgG, associated with immune complexes in the blood (Lange et al., 1986), and isolate-specific neutralising antibodies (Albert et al., 1990), are both observed. In all cases, HIV-1 infection can be confirmed serologically by the detection of serum antibodies (Cooper et al., 1985). IgM antibodies appear first, initially against the viral Gag protein. They are detectable at two weeks post-infection, peak at around five weeks, and decline to normal levels by about three months (Gaines et al., 1988a). IgG antibodies follow a week or two later, initially recognising gp41 and the product of the nef gene (De Ronde et al., 1988, Reiss et al., 1989). The HIV p24 antigen is also an early consistent marker for HIV infection. This has been detected as early as 24hr after the onset of acute illness, and is seen to fall as serum antibody levels rise.

The reasons why the primary HIV syndrome does not affect all infected individuals are not clear. However, it has been hypothesised that the appearance of primary symptoms is an indication that progression to full blown AIDS will occur more rapidly (Schechter et al., 1990). Petersen and co-workers (1989) observed that in a cohort study of 86 homosexual men, the three year progression rate to AIDS was eight times higher (78% versus 10%), in patients that had a primary illness lasting for longer than 14 days. They also demonstrated a more frequent progression rate to CD4+ cell counts of <500 x 10^6/litre (75% versus 42%), and a more frequent recurrence of antigenaemia over this period (55% versus 14%). A possible explanation for this could be that severe primary illness is an indication of an early and extensive spread of virus to many tissues.
5.2 Clinical Latency.

The asymptomatic period of clinical latency can last for several years. During this period, few symptoms are observed, although occasional peaks of viraemia, associated with night sweats, vomiting, fever, and diarrhoea have been reported (Tindall & Cooper, 1991).

The period of clinical latency is characterised by the slow deterioration and depletion of the host immune system but, paradoxically, the inability to obtain culturable virus from the peripheral blood. The early studies, conducted on peripheral blood mononuclear cells (PBMC) isolated from asymptomatic patients, revealed only 1 in 1000 cells harbouring HIV-1 DNA, and only 1 in 10 of these cells actually expressing HIV-1 mRNA (Harper et al., 1986, Psallidopouos et al., 1989, Shaw et al., 1984, Schnittman et al., 1989). Not only did this suggest that the percentage of HIV-1-infected peripheral blood cells was extremely low, it also suggested that the majority of HIV-1-infected cells were in a non-transcriptional or latent state. These observations raised doubts whether the depletion and dysfunction of the CD4+ T-helper population could be a direct consequence of viral infection of the cells that were to be affected. Recent work has however indicated a much greater viral burden than was previously considered. With sensitive in situ PCR techniques and histopathological analysis a much greater level of viral activity was demonstrated in the lymph nodes and the Peyer’s patches of the gut throughout the period of clinical latency (Panteleo et al., 1993a, Embretson et al., 1993a, Patterson et al., 1993, Embretson et al., 1993b, Fox et al., 1989). This was the case even in the absence of detectable viral activity in the blood, suggesting the peripheral blood to be a poor indicator of the state of HIV disease (Panteleo et al., 1993a). Using electron microscopy, it appeared that virus had been effectively filtered out of the blood and trapped in the follicular dendritic cell (FDC) network of the lymph node. Virus (often in immune complexes) was retained via association with Fc receptors and physically trapped on the villus processes of the follicular dendritic cells (Fox et al., 1991). Evidence also suggests that CD4+ cells are retained in the germinal centres of the lymph nodes, possibly explaining such low levels of infected cells in the peripheral blood (Panteleo et al., 1993a).

The consensus of opinion now views the so-called asymptomatic period of clinical latency from a different perspective. Instead of being thought of as a stage of disease with very low levels of virus and little viral replication, the available data now suggests that viral replication in the lymphoid organs is high. Evidence (Wei et al., 1995, Ho et al., 1995, see chapter 6.1) indicates a vast production of virus per day, massive viral clearance, and a rapid turnover of HIV-infected CD4+ cells. Therefore, the fact that clinical symptoms are not observed for the vast majority of
this asymptomatic period, now seems likely to be the consequence of the heroic efforts of the immune system in keeping a rapidly replicating virus in check, rather than the result of the HIV infection of CD4+ cells and the establishment of a true state of cellular latency.

5.3 Progressive impairment of the immune system.

Subtle defects in the immune system can be detected from early in the asymptomatic period of the disease, well before CD4+ cell numbers fall to critical levels. This dysfunction is progressive and is characterised by a general state of immune activation affecting a broad range of immunological cell types.

5.3.1 Haematopoietic progenitors.

HIV infection is associated with haematologic abnormalities such as thrombocytopenia, granulocytopenia, anemia, lymphopenia and pancytopenia (Zon et al., 1987). These abnormalities increase with disease progression, with an estimation that between 40%-70% of infected patients have one or more of these manifestations (Scadden et al., 1989). Abnormalities have also been observed in biopsies of bone marrow from HIV-infected individuals (Stutte et al., 1990). These include plasmocytosis, hypercellularity, benign-lymphoid aggregates, increased eosinophils, and megaloblastic changes.

Several studies have observed a decrease in the haematopoietic potential of both circulating and bone marrow progenitor cells in patients infected with HIV (Stella et al., 1987, Bagnara et al., 1990, Ganser et al., 1990). The suppression of in vitro haematopoiesis has also been observed (Donahue et al., 1987). Colony forming units for granulocytes, erythrocytes, monocytes and megakaryocytes were all less frequent in patients infected with HIV. In addition, normal bone marrow methylcellulose cultures produced less colonies in the presence of HIV (Steinberg et al., 1991), although a further study failed to reproduce this result (Molina et al., 1990).

The CD34+ cell subset of the bone marrow can be infected with HIV in vitro (Folks et al., 1988, Steinberg et al., 1991). The infection of these cells has also been observed in vivo (Stanley et al., 1992), with 36.5% of AIDS patients from Zaire and 14% from the U.S. demonstrating CD34+ HIV-1 infection. The frequency of infected CD34+ cells in these studies
was as high as 1:500 in some patients, but no correlation to a stage of disease, or any other laboratory indicator was observed.

5.3.2 Antigen-presenting cells.

Antigen-presenting cells (APC), such as macrophages, monocytes and dendritic cells can be infected with HIV (Ho et al., 1986). Macrophages are fully differentiated, non-proliferating mature cells. They appear to serve as long-lived reservoirs for HIV replication, as macrophages can sustain HIV infection for weeks or months in culture, without cell lysis or cytopathogenicity (Gendelman et al., 1988). Macrophages may avoid cell death from viral budding, as progeny virions were shown to assemble in intracytoplasmic vacuoles (Orenstein et al., 1988).

The infection of macrophages and their subsequent HIV-mediated dysfunction may be a crucial mechanism in AIDS pathogenesis. Macrophages are the primary cellular target for lentiviruses such as visna-maedi and equine infectious anemia (Narayan et al., 1982, Gorrell et al., 1992). In both diseases, a wasting syndrome and dementia complex is often observed resembling that seen in human AIDS but in the absence of a CD4+ T-cell depletion. In support of this, body weight loss of > 10% in AIDS patients was demonstrated to be more strongly associated with long-standing immune activation of macrophages, as detected by an increase in urinary neopterin levels, than a declining CD4+ cell count (Zangerle et al., 1993).

Aberrant cytokine production from infected macrophages (Guilian et al., 1990), and functional abnormalities such as defective monocytic chemotaxis (Smith et al., 1984), have also been demonstrated. This defect in APC function could have adverse effects on T-helper function, primarily those dependent on the presentation of antigen. However, experiments involving the mixing of T-helper cells and APCs from monozygotic twins, only one of which was asymptptomatically infected with HIV, demonstrated a defect only in the HIV-infected twin's T-helper subset but not the APC subset (Fauci et al., 1988). Although this suggests that defective APC function is not a characteristic of the asymptomatic period of HIV infection, an APC defect has been observed during clinical AIDS. Monocytic stimulation of the mixed lymphocyte reaction has been demonstrated to be defective in approximately 50% of symptomatic AIDS cases (Hofmann et al., 1986).
5.3.3 B-cells.

Complex B-cell functional disorders are a common feature in asymptomatic, seropositive HIV-infected individuals. Intense B-cell activation in the peripheral blood of infected patients (Lane et al., 1983, Mizuma et al., 1987) and an increased frequency of B-cell lymphomas in clinical AIDS (Ziegler et al., 1984) have both been observed. The HIV-mediated B-cell activation is characterised by early elevated serum IgD levels (Mizuma et al., 1987), but is not thought to be due to the re-activation of memory B-cells. There are also high numbers of B-cells spontaneously secreting anti-HIV antibody in the circulation (Amodori et al., 1989). This is a major contribution to the overall B-cell activation observed during an HIV infection (Amodori et al., 1989), and may be analogous to the polyclonal activation of B lymphocytes observed on the addition of HIV-1 in vitro (Schnittman et al., 1986, Pahwa et al., 1986). However conversely, the response of PBLs from HIV-infected asymptomatic individuals to mitogenic stimulation has been demonstrated to show a reduced capacity to produce antibody (Birx et al., 1986).

5.3.4 Natural killer cells (NK cells).

Natural killer (NK) cells are known to be involved in protection against tumours and viral infection (Herberman & Ortaldo et al., 1981). NK CD4-/CD16+ cells can be infected by HIV-1 in vitro (Ruscetti et al., 1986). NK cells are phenotypically and numerically normal, but functionally defective during clinical AIDS (Poli et al., 1985), being unable to lyse target cells (Bonavida et al., 1986). As yet, it is not certain whether these functional deficiencies are present during the asymptomatic period of HIV infection.

5.3.5 T-cells.

CD4+ T-helper cell function is defective in asymptomatic HIV-seropositive individuals. This defect is progressive, but is not due to a critical reduction in CD4+ T-cell numbers (Lane et al., 1983, Giorgi et al., 1987, Shearer & Clerici, 1991, Miedema et al., 1988). The major feature of this CD4+ T-cell dysfunction is a loss of T-cell responsiveness to recall antigen, a response that requires previous exposure, or priming, to the antigen in question (Garbrecht et al., 1987, Smolen et al., 1985, Shearer et al., 1986, Clerici et al., 1989b). The cells that are known
to preferentially respond to recall antigen are the CD45RA-/-CD29+ memory T-cells. A higher frequency of HIV infection has been demonstrated in this T-cell subset. (Schnittman et al., 1990b). This is probably a consequence of cellular activation and proliferation as such events provide the appropriate cellular environment for integration and replication of HIV (with T-cells that have yet to be activated not having stably integrated provirus). Memory T-cells have also been demonstrated to be selectively depleted in patients in the asymptomatic period of HIV-infection (Van Noesel et al., 1990). This depletion may explain the unresponsiveness of the total T-cell population to antigen plus self-MHC.

At least four levels of progressive immune dysfunction have been demonstrated to exist in asymptomatic individuals with CD4+ T-cell counts above 400x10⁶/litre. Initially a patient's PBLs can respond to recall antigens, HLA alloantigens and T-cell mitogens (such as PHA). There is then first a loss of response to recall antigens, then subsequently a loss of response to HLA alloantigens, before a final state where the T-cells fail to respond to all the above T-cell stimuli (Clerici et al., 1989a, Clerici et al., 1989b). An extensive cohort study has shown that this progressive T-cell functional impairment is both predictive for T-cell depletion as well as the onset of AIDS.

The CD8+ cytotoxic T-cell population appear relatively normal throughout the asymptomatic period of HIV-infection. However, once AIDS has been diagnosed, CD8+ T-cells have a depressed potential to form clonal expanded populations in response to appropriate activation (Pantaleo et al., 1990). These cells also do not express detectable levels of the IL-2 receptor (CD25), although normal levels of the activation antigen HLA-DR are observed (Pantaleo et al., 1990). Pantaleo et al., (1990) have demonstrated that activated HLA-DR+ CD8+ T-cells from AIDS patients did not proliferate in response to anti-CD3, anti-CD2, anti-CD28 mAb or mitogen-mediated (PMA and PHA) stimulation. The addition of IL-2 did not rescue these cells. This suggests that the expansion of non-clonogenic activated CD8+ T-cells that are refractive to further in vitro stimulation may occur during the clinical period of AIDS.

5.4 The onset of clinical AIDS.

After a variable period of asymptomatic clinical latency, a variety of signs may herald clinical deterioration. This phase is often known as the AIDS-related complex (ARC), but this definition is not clear-cut and does not appear in the official CDC disease classification (CDC, 1987).
Chronic fevers, night sweats, diarrhoea, weight loss, oral thrush, Herpes zoster, or hairy leukoplakia, all signal the onset of clinical AIDS. After diagnosis of AIDS (diagnosis criteria discussed in chapter 1.3), death usually occurs within a year. This time frame is highly dependent on co-factors such as age, sex, risk group, tissue diagnosis, and type and nature of therapy (Rothenberg et al., 1987).

Death from AIDS is primarily the result of the opportunistic and neoplastic diseases that develop in the severely immuno-compromised host. As discussed earlier, this reduced immunological state is the consequence of the depletion and dysfunction of various components of the immune system. Understanding the mechanisms responsible for this immunodeficiency could ultimately lead to the development of effective therapeutic strategies. Until these mechanisms are fully understood, therapeutic intervention may prove impossible.

At the present time, the mechanism of immune destruction is not known. Several plausible theories exist, supported by both experimental and circumstantial evidence. These include; (i) Direct cell killing by HIV; (ii) HIV virulence and rapid viral evolution; (iii) Cytotoxic T-cell-mediated killing of CD4+ T-cells; (iv) HIV-induced autoimmunity, (v) Superantigen-mediated T-cell depletion; (vi) HIV-induced apoptosis; (vii) T-cell anergy; (viii) An HIV-induced cytokine imbalance; and (ix) The HIV-induced degeneration of the lymphoid organs. Many of these theories seemed initially to be mutually exclusive. However, in recent months the connection between theories has become increasingly evident, allowing the combination of the more relevant aspects of each hypothesis to provide an overall picture of virus-mediated pathogenesis.

Although each of the theories presented below will be discussed in their entirety, it must be emphasized that several, if not all of these mechanisms could be involved in the dramatic HIV-mediated destruction of the immune system.

6.1 Direct cell killing by HIV.

As the CD4+ T-cell population is a principal target for HIV infection (McDougal et al., 1986), a direct HIV-mediated cytopathic effect within these cells would offer a simple explanation for their depletion during the course of the disease. Such a cytopathic effect has been observed in vitro, with extensive cell death in HIV-infected CD4+ T-cell lines such as CEM A3.01 (Barre-Sinoussi et al., 1983, Popovic et al., 1984, Gallo et al., 1984, Levy et al., 1984, Sarngadharan et al., 1984, Brun-Vezinet et al., 1984). Several groups have demonstrated that this poor cell viability is the consequence of the formation of multi-nucleated syncytia (Sodroski et al., 1986, Lifson et al., 1986a, Lifson et al., 1986b). The fusion process that forms these structures is the result of the interaction of HIV gp120 on the surface of an infected cell with the CD4
glycoprotein on the surface of neighbouring cells (Figure 9). The generation of syncytia could therefore represent a mechanism of T-cell depletion in HIV-infected individuals. However, as yet, syncytia have not been observed in vivo, although syncytia-like structures have been noted in the grey and white matter of the brains of AIDS patients with dementia complex. In addition, Lo et al. (1991) have demonstrated that syncytia-induced cell death is not the only mechanism of HIV-mediated cytopathicity. They showed that, in the presence of Mycoplasma fermentans (incognitos strain), HIV-1 infection of the syncytia-inducible CEM A3.01 T-cell line caused an increase in cell death, but no longer resulted in syncytia formation. A mycoplasma infection alone did not affect cell viability, and the level of viral replication with and without the mycoplasma was comparable by a p24 ELISA assay.

Evidence supporting the hypothesis of a direct HIV-mediated cytopathic effect is not strong. Virus isolated from patients with significantly reduced CD4+ T-cell counts failed to show any signs of a cytopathic effect in cultured T-cells (Evans et al., 1989). Furthermore, it has been argued that if the CD4+ T-cell depletion is directly related to killing of infected cells, the observed decline in CD4+ T-cells should increase with viral burden. However, although the viral burden when CD4+ T-cell levels are 200mm³ can be as much as 100-fold greater than when CD4+ T-cell levels are 500mm³, the rate of CD4+ T-cell depletion is comparable at both time points (Phillips et al., 1994).

An important consideration concerning the feasibility of a direct HIV-mediated cytopathic effect in vivo, is the level of viral burden. Initial studies using an in situ RNA hybridisation technique, indicated that in both the lymph nodes and the peripheral blood, as few as 1 in 10,000 mononuclear cells were actually expressing HIV mRNA (Harper et al., 1986). However, this figure has now been dismissed as a gross underestimate, with subsequent analysis of lymph node tissue using more sensitive techniques suggesting a frequency of HIV infection near 1% (Embretson et al., 1993b, Pantaleo et al., 1993a). Even so, Sheppard et al. (1993) argued that if the number of HIV-infected cells was this low, the regenerative capacity of the immune system would more than compensate for the loss of cells due to a direct cytopathic effect. However, the dynamics of the human immune system are not as well characterised as those of the mouse, in which 30-40% of peripheral T and B-cells are renewed every three days (Freitas et al., 1993). Moreover, from the analysis of human radiation patients, the regenerative capacity of the immune system appears to be far less efficient (Mclean & Mitchie, 1993). Therefore, it has been argued that even if very few CD4+ cells are infected at any one time, but the turnover rate of these cells is rapid and the regeneration rate low, a depletion of the CD4+ T-helper population could occur over an extended period of time (McClean & Mitchie, 1993).
Figure 9  Mechanisms of direct cell killing by HIV-1 in vitro.

A. Single cell killing. This involves the interaction of cell-surface CD4 with membrane-localised viral gp120 in an HIV-1-infected cell. This generates small vacuoles, resulting in the breakdown of the cell membrane, and subsequent cell death.

B. Syncytia formation. In this model, membrane-localised gp120 on an HIV-infected cell interacts with CD4 molecules on uninfected neighbouring cells. The result of this interaction is the fusing of the two cellular membranes, and the formation of a giant multi-nucleated short-lived syncytia. (Adapted from Haseltine, 1991).
A: Single-cell killing

Interaction between gp120 and CD4 in a single cell causing disintegration of the cellular membrane

Holes punched in cellular membrane leading to cell death

B: Syncytia formation

uninfected cell

HIV infected cell

CD4 cell surface molecule

gp120 viral protein

membrane fusion

Formation of multinucleated syncytia
The extent of the viral burden and the turnover of HIV-infected cells has been mathematically estimated using a number of different approaches (Wain-Hobson et al., 1993, Wei et al., 1995, Ho et al., 1995). The argument developed by Wain-Hobson used a number of assumptions based on data from experiments analysing the genetic diversity of viral isolates at various stages of an HIV infection. He argued that at the time of primary HIV infection the virus population is known to be homogenous (Zhu et al., 1993). However, a 10% variation from homogeneity is observed in isolates obtained from patients approaching clinical AIDS (Holland et al., 1992). As the viral RT enzyme is known to have an error rate of approximately 1 base per viral genome, the observed variation would have to be the consequence of between 500-5000 rounds of viral replication. If the viral burst size per infection was as small as two, 500-5000 rounds of viral replication would generate in the region of $10^{30} - 10^{300}$ virions, (more than the number of hydrogen atoms in the universe!). As the serum virus levels during AIDS are in the $10^{6} - 10^{11}$ range, the actual burst size must be at least 100-fold less. Therefore, it is possible that 99% of infected cells are destroyed before they are able to produce virus. Alternatively, the majority of progeny virus may be non-infectious. This is supported by the observation that 99.9% of virus in the serum is unculturable (Piatak et al., 1993), although HIV is probably highly unstable in the blood in comparison with the lymphoid tissue.

The prediction of a high viral load and a rapid turnover of CD4+ T-cells has been supported by the work of two independent groups (Wei et al., 1995, Ho et al., 1995). In these studies, an HIV protease inhibitor was administered to patients in the early stages of clinical AIDS. This type of drug effectively inhibits viral replication. The consequence is a decrease in the amount of virus in the serum, and an increase in the number of circulating CD4+ T-cells. Unfortunately, this effect is only transient, with resistant virus being detectable within a week (Richman et al., 1994). However, the observed changes in the number of both virus and CD4+ T-cells, has allowed the kinetics of an HIV infection to be estimated. Serum virus levels do not usually fluctuate significantly in the absence of the drug. In this equilibrium, viral production from infected cells is approximately equivalent to viral clearance by the immune system. In the presence of the drug, viral replication (and hence virus production) effectively ceases. Therefore, the observed decrease in plasma virus is an indication of the rate of viral clearance. This in turn should be an accurate estimate of the usual level of viral production when viral replication is unchecked. This analysis revealed that approximately $10^8 - 10^9$ virions, representing one third of the total virus population, were being generated and cleared each day. This is probably an underestimate, as the viral load in the blood probably represents an overflow of virus from the lymphoid tissues.
Using a similar argument, the rate of recovery in the CD4+ T-cell population after administration of the drug is a good estimate of the usual loss of CD4+ T-cells due to the presence of the virus. This analysis revealed that approximately $2 \times 10^6$ cells were being lost each day. In comparison, an HIV-infected patient permanently loses about 1% (~$2 \times 10^7$ cells) of this figure per day during the progressive depletion of the CD4+ T-helper population.

These calculations reveal that the number of virions produced per day is lower than the number of cells cleared per day. If this clearance is due to a CD4+ cell becoming infected, this result may indicate that very few infected CD4+ cells reach a state of full virus production.

All these calculations, based on fairly solid assumptions, argue against a very low frequency of HIV infection, even though very few cells can be detected as infected at any one time. They also suggest that HIV infection is very dynamic. The viral burden is high and the rate of CD4+ T-cell turnover is rapid. If correct, it is not unfeasible that a direct HIV-mediated cytopathic effect could be the major mechanism of CD4+ T-cell depletion. However, due to the considerations discussed at the start of this section, this is still considered unlikely.

### 6.2 HIV virulence.

Lines of evidence have suggested that the characteristics of an HIV viral isolate in T-cell culture can be directly related to the stage of HIV-induced disease from which the isolate was obtained. Asjo et al. (1986) first documented the different in vitro properties of HIV isolates, by classifying them according to their growth rate in T-cell lines, and the titres they could attain. Slow (growing), low (titre) isolates were obtained from asymptomatic HIV seropositive patients, while rapid (growing), high (titre) isolates were obtained from patients with AIDS. This analysis was extended by correlating the stage of viral isolation with the ability of those HIV isolates to form syncytia in vitro. Syncytia-inducing (SI) isolates were preferentially observed at or around the progression to AIDS, while the majority of isolates from asymptomatic patients were non-syncytia-inducing (NSI) (Tersmette et al., 1989, Nielsen et al., 1993). The appearance of SI viral isolates was calculated to precede clinical progression by a median of 2 years, with the switch linked to a fall in CD4+ T-cell numbers to below 500 mm$^3$ (Tersmette & Schuitemaker, 1993). Follow up studies have indicated that SI isolates develop from NSI isolates in vivo (Andeweg et al., 1992), and that NSI isolates are either preferentially transmitted to a new host, or that SI isolates are suppressed until later stages of HIV-disease (Keet et al., 1993). It was also claimed that isolates obtained from time points close to AIDS progression could replicate to higher titres.
in a wider variety of cells (Cheng-Mayer et al., 1988).

Although definable HIV isolate characteristics have been identified, the relevance of these factors to the disease state in vivo and the consistency with which they are observed are variable. SI isolates have been observed very soon after primary HIV infection, without subsequent progression to AIDS (Nielsen et al., 1993), and only about half the number of patients display SI isolate characteristics at AIDS diagnosis (Koot et al., 1993). In addition, Mosier et al. (1993) looked at the cytopathic effect of viral isolates with different in vitro syncytia-inducing capacities in the SCID-hu mouse model. SCID mice have no T or B-cells, but can be reconstituted with human lymphoid tissue or PBMCs (Bonyhadi et al., 1993). These human cells can develop relatively normally for several months and are susceptible to HIV infection. Mosier and co-workers found that a NSI macrophage-tropic isolate, shown to be non-cytopathic in cultured T-cell lines, induced a marked decrease in the SCID-hu CD4+ human T-cell population. In contrast, HIV-1\textsubscript{sf3}, an SI isolate highly cytopathic for T-cell lines in vitro caused little in vivo T-cell depletion at an equivalent viral burden.

These more recent results suggest that CD4+ T-cell death in vivo, certainly in the SCID-Hu model, is not directly related to the syncytia inducing and cytopathic characteristics of a viral isolate in cultured T-cells. This may be the consequence of the fact that the T-cell lines that are now routinely used to culture virus and to assay rates of viral infection have been highly selected for their sensitivity to HIV-mediated cell killing.

Although the syncytia-inducing potential of HIV isolates may not be a direct cause of disease progression, the extensive genetic variation within the virus population could effect the degree of virulence of the virus, the severity of the disease, and the ability of the host to mount an effective immune response (Meyerhans et al., 1989). Both the virulence and the tropism of a viral strain is a consistent, reproducible property. For example, the SIV\textsubscript{rhesus} strain of SIV will kill infected monkeys in 10-14 days due to a massive infiltration of lymphocytes into the gut, resulting in haemorrhaging diarrhoea (Dewhurst et al., 1990, Daniel et al., 1992). Animals given rehydration therapy can survive this crisis, and will then develop the classical symptoms of SIV-induced immunodeficiency. This is a consistent property of this strain of SIV, indicating a unique degree of virulence. Similarly, occasional deaths from primary HIV infection, and long surviving clusters of HIV-infected patients, could be an indication of the inherent virulence of different strains of HIV (Learmont et al., 1992). It has been suggested that a possible component of this virulence may be the tissue tropism of the isolate in question (Gendelman et al., 1990).

In addition to possible fluctuations in virulence, the high mutation rate of HIV replication could introduce a significant degree of antigenic diversity into the viral population over the course
of an HIV-infection (McNearney et al., 1992). Wolfs et al. (1991) followed two HIV seropositive patients for a period of over 5 years. At each time point, beginning at primary HIV-infection, they cloned approximately 50 viral isolates. A highly homogenous population of isolates was observed around the time of seroconversion. As the infection progressed, the diversity in amino acid sequence increased, resulting in a dynamic interaction with the host. For example, a single amino acid change in gp120 resulted in a reduction in the efficiency with which the patients neutralising antibodies were able to bind. The generation of a new neutralising antibody population soon followed. Genetic diversity was greatest at the point when the patient was progressing to AIDS, although the concentration of neutralising antibodies against the V3 loop of gp120 were seen to fall dramatically during this period. After the onset of AIDS, a more homogenous population of viruses emerged. A possible explanation for this could be the selection of fast replicating strains of HIV in the virtual absence of an immune response. The emergence of immuno-escape mutants in this study has been supported by in vitro observations. A high selective pressure on virus during the HIV infection of a cultured T-cell line in the presence of a V3 neutralising antibody was demonstrated. This led to the generation of a new virus population that could efficiently replicate in the presence of the antibody (McKeating et al., 1989).

The increasing variation in the viral population and the subsequent evasion of host immune responses has led to a model of AIDS pathogenesis in which the antigenic diversity of HIV is the cause and not the consequence of immunodeficiency (Nowak et al., 1991). This hypothesis suggests that the continual increase in viral antigen diversity drives the immune system beyond its ability to control an HIV infection. It argues for an antigen diversity threshold, above which the immune system collapses. As the rate of diversity is due to a random process of mutation, this model could explain various characteristics of AIDS, such as the variable periods of clinical latency and the generation of a broad range of clinical symptoms.

6.3 Cytotoxic T-cell mediated killing of CD4+ T-cells.

The cytotoxic T-lymphocyte (CTL) response is a component of cell-mediated immunity directed against intracellular pathogens such as viruses. CD8+ CTLs have been demonstrated to lyse infected target cells both in vitro and in vivo, via the action of perforin, fas, or other lytic mechanisms (Podack et al., 1991). CTLs also produce a range of cytokines such as the interferons and TNF, which are known to have anti-viral properties (Ramsey et al., 1993).

Evidence suggests that the CTL response is critical in controlling an HIV infection. PBLs
isolated from HIV seropositive patients fail to support high titres of virus in culture unless CD8+ T-cells are first removed. Furthermore, the titres of virus from CD8 negative cultures are reduced if CD8+ cells are re-introduced (Walker et al., 1986). The mechanism of this protection is not fully understood, although it has been suggested that a soluble factor, secreted by the CD8+ cells, could inhibit viral infection (Levy et al., 1988).

In vivo, HIV-specific CTL activity has been isolated from blood, lymph nodes, spleen, and lungs of both HIV seropositive asymptomatic individuals and patients with AIDS (Plata et al., 1987, Walker et al., 1987, Hoffenbach et al., 1989, Mackewicz et al., 1991). PBMCs from HIV seropositive patients, and CD8+ T-cell clones derived from these PBMCs, have both been demonstrated to have CTL activity directed against T-cells, B-cells and macrophages presenting either the HIV env protein (Koenig et al., 1988, Langlade-Demoyen et al., 1988), the HIV p24 gag protein (Nixon et al., 1988), or the HIV RT enzyme (Walker et al., 1988). Although the HIV-specific CTL response remains strong during the asymptomatic period of HIV infection, HIV-specific CTL responses are significantly lower after the onset of clinical AIDS (Carmichael et al., 1993, Clerici et al., 1992). This loss of HIV-specific CTL activity may be the consequence of either an exhaustion of HIV-specific CTL precursors (Hoffenbach et al., 1989, Pantaleo et al., 1990), defective CD4+ T-cell help for a CTL response (Pantaleo et al., 1993b), cell-mediated suppression of HIV-specific CTLs by CD8+ CD57+ cells (Joly et al., 1989), or the in vivo selection for HIV mutants that are no longer recognised by the HIV-specific CTL precursors (Hahn et al., 1986, Phillips et al., 1991).

CD4+ CTLs have been identified in diseases such as HBV (Celis et al., 1988), measles (Jacobson et al., 1984) and influenza (Kaplan et al., 1984). CD4+ CTLs have also been demonstrated in HIV-infected individuals. The CD4+ CTL lysis of autologous uninfected CD4+ Ia+ T-cells was shown to be dependent on the CD4-mediated uptake of gp120 by these target cells in vitro (Siliciano et al., 1988). Class-II expressing "victims" could include HIV-infected monocytes/macrophages, B-cells, and activated T-cells, the loss of which would obviously cause immunoregulatory problems.

Evidence therefore exists to suggest that CTLs have a central role in the removal of HIV-infected cells in vivo. However, this strong CTL-based immune response may ultimately be the downfall of the immune system. As discussed in the previous section, the calculations by Wain-Hobson (1993), Wei et al. (1995), and Ho et al. (1995), have indicated that a large number of CD4+ T-cells are destroyed by some mechanism each day. While this T-cell clearance does not appear to be the consequence of a direct in vivo HIV-mediated cytopathic effect, it could be the result of a strong HIV-specific CTL response. This hypothesis has been supported by the
detection of HIV-specific CTL infiltration of lymphoid organs in HIV-infected patients (Tenner-Racz et al., 1993, Hadida et al., 1992, Devergne et al., 1991, Cheynier et al., 1994). In one such recent study, Cheynier et al. (1994) analysed numerous white pulp structures from the spleens of AIDS patients. These structures, which are primary sites of antigen presentation for T and B-cells, were shown to harbour virus. HIV clones isolated from different white pulps displayed up to 17% diversity at the amino acid level, while the isolates from a single white pulp were often the descendants of a single founder virus. This distribution pattern of HIV clones presumably results from an infected but resting T-cell carrying a founder virus into the white pulp structure as a latent provirus. The antigen-dependent activation of this cell would lead to viral replication and the subsequent HIV infection of many surrounding T-cells (also attracted into the white pulp structure by the presentation of a particular antigen). Significant CTL infiltration into these white pulps was observed, suggesting that HIV-specific CTLs may enter the lymphoid organs and actively remove HIV-infected cells.

In the light of these observations, a mechanism for CD4+ T-cell depletion has been proposed. As discussed earlier, it is thought that HIV infection of activated CD4+ T-cells in the lymphoid organs occurs at a significant level. In the absence of an inherent cytopathic effect, these cells rapidly produce HIV proteins which mark them as infected. The infected T-cells become a target for an efficient CTL immune response, often before they can produce infectious progeny. However, if this process continually removed more CD4+ T-cells than could be replaced by the regenerative capacity of the immune system, the result would be the gradual depletion of this CD4+ subset over an extended period of time. As HIV will preferentially infect activated cells, the generation and proliferation of memory T-cells will be severely reduced. This differential loss of memory T-cells as opposed to naive T-cells could therefore also help to explain the progressive immune dysfunction observed before the onset of AIDS.

If this hypothesis is correct, it would suggest that AIDS is the consequence of immunopathological mechanisms resulting from a conventional, antiviral, protective CTL-based cellular immune response directed against HIV-infected cells (Wain-Hobson, 1993). Precedents exist for the causation of a disease state by such a mechanism. Several non-cytopathic viruses such as Hepatitis B virus (HBV) (Peters et al., 1991) and Lymphocytic choriomeningitis virus (LCMV) (Buchmeier et al., 1980) can cause a range of tissue damage and chronic disease depending on the extent of viral infection and the corresponding severity of the CTL response. For example, low dose infection of HBV in humans leads to the rapid induction of a cellular immune response and elimination of the virus. However, if T-cell responses are generated slowly, severe chronic, subacute or acute aggressive hepatitis may develop. If the immune system is
suppressed or immature, a high dose of virus can lead to a virus-carrier state. Virus is not eliminated and infected cells are not destroyed. The host remains healthy in this condition, but may eventually develop an immune-complex disease or liver malignancy due to incomplete suppression of HBV-specific immune activity (Blum et al., 1991, Liang et al., 1991). LCMV infection of mice displays similar characteristics. Low dose or a slow dissemination of virus accompanied by a potent CTL response rapidly destroys infected cells, eliminating the virus with minimal tissue damage. If however, LCMV can spread widely in the presence of a slow CTL response, a generalised wasting disease, and/or aggressive hepatitis, and/or a severe immunodeficiency may develop. These disorders are a direct result of the destruction of large numbers of infected macrophages, liver cells or antigen-presenting cells, by a CTL immune response. Again a healthy virus-carrier state can be established if the spread of virus is very aggressive, exhausting or depleting the LCMV-specific CTL clones, or if the host is in some way immunocompromised at the time of infection (Moskophidis et al., 1993).

6.4 HIV-induced autoimmunity.

Even before the discovery of HIV, it had been noted that the symptoms of AIDS were very similar to graft-versus-host disease (GVHD) (Shearer et al., 1981). A graft-versus-host reaction is the consequence of recognition by host lymphocytes of non-self, or allogenic MHC molecules on the surface of foreign cells. The activation and proliferation of these host lymphocytes can cause antigen-specific dysfunction, B-cell hyper-reactivity leading to hypergammaglobulinaemia and auto-reactive antibody production, changes in the number of CD8+ CTLs, skin lesions, gastrointestinal abnormalities, lymphadenopathies, opportunistic infections, and lymphomas (Gleichmann et al., 1984).

Similarities between AIDS and an MHC class-II mismatched GVHD (rather than an MHC class-I mismatched GVHD) have been observed (Via et al., 1990, Moser et al., 1987). In addition, mice stimulated with allogenic cells have been demonstrated to possess antibodies that cross react with both env and gag peptides from HIV (Kion & Hoffmann, 1991).

In light of these observations, it has been proposed that the virus or a component of the virus can mimic, alone or in combination with host proteins, allogenic MHC molecules on the surface of antigen-presenting cells (ie function as an alloepitope) (Habeshaw et al., 1992). This would allow host T-cells to recognise HIV-infected cells as foreign, stimulating their clearance by the immune system. The chronic infection of HIV over a prolonged period of time could
therefore result in a GVHD-like syndrome.

In order for HIV to activate a GVHD-like response, some aspect of the virus must functionally resemble the TCR binding region of the MHC molecule. A number of reports have identified variable degrees of amino acid sequence homology between MHC-II and HIV proteins. These include gp120 (Young, 1988), gp41 (Golding et al., 1988), and the Nef protein (Vega et al., 1990). HIV-1 gp160 is a strong candidate to function as an alloepitope. MHC class-II molecules and gp160/gp120 both bind CD4 with high affinity. The CD4 binding site for these molecules display appreciable overlap (Sattanau et al., 1986), with certain CD4 point mutants losing the ability to bind both gp160 and MHC-II (Clayton et al., 1989). In addition, one study has proposed that the amino acid sequence leading up to the gp120/gp41 cleavage site of gp160 has a high probability of forming an α-helix with high homology to the MHC class-I α chain and the MHC class-II β chain (Hounsell et al., 1992). Such 3D homology is argued to be sufficient to allow the interaction of gp160 with a T-cell receptor, perhaps due to conformational changes induced by gp160/gp120 binding of CD4. Therefore gp160/gp120 is not being presented to the T-cell as a peptide in a usual TCR/MHC interaction, but is resembling the stimulatory epitope of an allo-MHC.

A prediction of this hypothesis is that certain T-cell receptor Vα and Vβ domains, those that recognise the HIV-alloepitope, would be more common on the T-cells of patients that rapidly progress to AIDS. A study by Dalgleish et al. (1992) has claimed to have found such a correlation, with HIV-infected individuals having a greater frequency of Vβ5.3 expressing T-cells. Other groups have failed to corroborate these findings and have questioned the suitability of the controls in this study (Bansal et al., 1992). However, it has been demonstrated that general Vβ expression on T-cells is altered in HIV-infected individuals (Imberti et al., 1991), thus supporting the alloepitope hypothesis.

HIV-infected cohort studies provide further, albeit rather circumstantial, evidence to support this theory. In the Edinburgh cohort of HIV-infected haemophiliacs, there is a clear difference in HLA haplotype between those individuals that have progressed slowly to AIDS and those that have progressed more rapidly (Simmonds et al., 1991). In particular, a close association between the HLA A1.B8.DR3 haplotype and progression to AIDS was demonstrated (Kaslow et al., 1990). In conjunction with these observations, the fact that approx. 10-20% of HIV-infected individuals have not yet progressed to AIDS after 10 years (Rutherford et al., 1990), suggests that progression to AIDS may have a large genetically-related component. Habeshaw et al. (1992) suggest that such a genetic factor could be the presence or absence of a TCR Vβ domain that recognises an HIV peptide as an alloantigenic factor.
6.5 Superantigen-mediated T-cell depletion.

The process of antigen recognition involves a highly specific interaction between the T-cell receptor (TCR) on the surface of a T-cell, and an antigen presented by an MHC class-I or class-II molecule on the surface of an antigen-presenting cell (APC) (figure 10A). The T-cell co-receptors, CD4 and CD8, stabilise the TCR/MHC interaction by binding to the appropriate MHC molecule; CD4 with class-II and CD8 with class-I. The TCR specificity for the vast array of possible antigen/MHC combinations is a result of the high degree of polymorphism in the variable (V) domain of the α and β chains that together comprise the TCR (Davis et al., 1985). This TCR specificity coupled with self-MHC antigen presentation allows a broad spectrum of antigen to be efficiently recognised and distinguished from self-antigen. A rapid expansion of the few T-cells that are specific for the particular antigen/MHC complex initiates the relevant immune response to efficiently deal with the invading pathogens or toxins.

Superantigens (SAgs) are molecules that can stimulate a T-cell response by cross-linking the TCR and MHC in a non-specific manner (figure 10B). SAgs bind to a particular subset of MHC molecules and the Vβ domain of the TCR. As there are only twenty or so different Vβ domains in the entire T-cell repertoire, the consequence of activation of a particular subset of Vβ-specific T-cells can be the proliferation of up to 15% of all T-cells at one time. This huge expansion is not only toxic in terms of massive cytokine production, but also results in the anergy and/or depletion of large numbers of T-cells bearing the SAg-specific Vβ domain (Kappler et al., 1988, Janeway et al., 1990).

SAgs were originally found in bacterial toxins (Janeway et al., 1990). Activated T-cells expressing the staphylococcus bacterial SAg were shown to induce inactivation of certain Vβ-specific T-cell subsets (O’Hehir et al., 1990). SAgs formally known as minor lymphocyte stimulating (mls) genes, have since been identified in the genomes of many different strains of mice, located in the proviral genomes of endogenous (ie non-replicating) retroviruses (Frankel et al., 1991, Woodland et al., 1991, Dyson et al., 1991, Janeway et al., 1991). The expression of these SAgs early in thymic ontogeny results in the deletion of all T-cells that carry the SAg-specific Vβ domain. Marrack et al. (1991) demonstrated the presence of a SAg in an exogenous mouse mammary tumour virus (MMTV). This virus, passed vertically from mother to infant via the milk, expressed a SAg that was able to activate T-cells bearing the Vβ14 domain of the TCR. Transgenic mice carrying a transgene that expressed this Vβ14-specific SAg (Golovkina et al., 1992) showed a Vβ14-specific deletion of T-cells during thymic ontogeny. These mice were then non-susceptible to infection by the exogenous Vβ14-specific-SAg expressing MMTV. Therefore,
A. T-cell

- Variable region of β chain
- CD4
- TCR
- Antigen
- MHC class II

Antigen presenting cell

B. T-cell

- Superantigen
- CD4
- TCR
- MHC class II

Antigen presenting cell

The inhibition of cellular growth of T cells with a combination of agonistic antibody and fusion of lymphoid cell lines (Ishizu et al., 1986) provides a potential target for viral suppression and prevention of viral infection.

The inhibition of cellular growth of T cells with a combination of agonistic antibody and fusion of lymphoid cell lines (Ishizu et al., 1986) provides a potential target for viral suppression and prevention of viral infection.
it appears that the activation of a subset of T-cells by the action of an exogenous viral SAg provides many potential target cells for viral integration and productive viral infection.

The infection of certain strains of mice with a combination of replication-defective and replication-competent murine leukaemia viruses (MuLV) results in murine acquired immunodeficiency syndrome (MAIDS) (Morse et al., 1992). MuLV is B-cell tropic, inducing an disorder characterised by the polyclonal activation of T-cells as well as various other immunological abnormalities (Morse et al., 1992). The replication-defective virus is both necessary and sufficient for disease progression (Chattopadhyay et al., 1989, Huang et al., 1989). B-cells which express the defective virus can polyclonally activate T-cells expressing the Vβ5 domain, implicating the involvement of a SAg-like factor (Hugin et al., 1991). To support this, a monoclonal antibody directed against the MuLV gag gene product of the defective virus blocks the SAg and disease effect (Hugin et al., 1991).

The above evidence demonstrates that both the MMTV-family and MuLV-family of retroviridae encode SAg-like factors that can polyclonally activate and deplete large numbers of T-cells. Therefore, it has been suggested that the severe depletion of CD4+ T-cells observed in AIDS may be due to the expression of an HIV-encoded SAg-like factor. This SAg would presumably be expressed on the surface of HIV-infected cells. It would then have to interact with both MHC class-II and a specific T-cell receptor Vβ domain on a T-cell. The progressive depletion of T-cell subsets bearing a number of Vβ domains over the extended course of an HIV infection would require the mutation or manipulation of the SAg in order for it to recognise several Vβ domains.

This hypothesis predicts that during the course of an HIV infection there will be the deletion of subsets of Vβ-bearing T-cells. Imberti et al. (1991) initially compared six symptomatic AIDS patients with non-HIV-infected controls. The AIDS patients had CD4+ cell counts of less than 200/mm³, and all had clinical signs of opportunistic infection. PCR analysis established that the Vα T-cell subset frequencies were normal. However, the analysis failed to detect Vβ14, Vβ15, Vβ16 and Vβ18 expression in any AIDS patient, while Vβ17, Vβ19 and Vβ20 were only seen in one of the six HIV-infected patients. Furthermore, asymptomatic individuals with CD4+ T-cell counts < 200/mm³ but having no malignancies, showed Vβ expression comparable to that of the AIDS patients, whereas HIV seropositive individuals infected for at least 5 years but with relatively normal numbers of T-cells showed Vβ usage similar to that of the control group. In a separate study, RT PCR was used to assess the Vβ usage in CD4+ and CD8+ T-cells from individuals at all stages of disease (Hodara et al., 1993). A deletion or underexpression of Vβ9-Vβ20 in CD4+, but not CD8+ cells was observed. Vβ3 was deleted in both CD4+ and CD8+
cells, while Vβ2 usage was increased in the HIV-infected T-helper population. These alterations in Vβ usage were more pronounced in individuals with AIDS.

It has been shown that steady-state levels of mRNA do not always correlate with cell surface expression of a particular TCR (Maguire et al., 1990). Therefore, the direct detection of the variable domains of the β chain of the TCR using monoclonal antibodies is potentially a far more accurate method of analysis. Using this technique, Bansal et al. (1992) demonstrated the significant reduction in the frequency of Vβ5.1, Vβ12 and Va2 usage on CD4+ T-cells from asymptomatic HIV-seropositive individuals. This supports the PCR data for an HIV-mediated superantigen effect.

In contrast, Posnett et al. (1993) while using monoclonal antibodies to detect a range of Vβ domains from HIV-infected individuals at all stages of disease progression, found no preferential usage or deletion of any Vβ-specific T-cell subsets. The antibodies used in this experiment detected Vβ3, Vβ5.1, Vβ5.2/3, Vβ6.7, Vβ8, Vβ12, Vβ13.3 and Vβ17, comprising approximately one third of all known Vβ domains.

The analysis of the role of Vβ domains in HIV infection has also been performed in vitro (Laurence et al., 1992). This study demonstrated the preferential stimulation of a Vβ12-bearing T-cell line in the presence of freshly irradiated APCs from HIV seropositive, but not normal donors. In addition, the replication of several isolates of HIV was shown to be approximately 100-fold higher in the Vβ12-bearing T-cell line than that seen in a T-cell line specific for Vβ6.7a. This effect was dependent on the presence of APCs expressing MHC class-II molecules. However, in vivo, the frequency of Vβ12, Vβ17 and Vβ6.7a expressing T-cells were no different from that of the controls.

Therefore, the available evidence does not appear to support the hypothesis that an HIV-encoded SAg plays a major role in the deletion of the CD4+ T-cell population over the course of an HIV infection. However, the recent results of Laurence et al. (1992) suggest that an HIV-mediated SAg-like effect could provide a greater frequency of activated target cells, increasing the efficiency of productive HIV infection in a manner analogous to that seen with MMTV, but without causing extensive deletions of T-cell subsets.

6.6 HIV-induced Apoptosis.

Apoptosis or programmed cell death (PCD) is a well recognised cellular phenomenon (McConkey et al., 1990b). Biologically distinct from necrosis, it is a highly regulated process that
occurs in the absence of bystander cell destruction (Duvall & Wyllie, 1986). Apoptosis requires protein synthesis and is associated with an endogenous endonuclease activity, cleaving the cellular genome into oligonucleosomal length DNA fragments of approximately 200 base pairs (McConkey et al., 1990b).

PCD is an important mechanism in several biological systems. For example, it has been implicated in haematopoietic stem cell growth, as bone marrow cultures will undergo apoptosis in response to a deficiency in IL-3, GM-CSF or erythropoietin (Williams et al., 1990, Koury et al., 1990). In thymic ontogeny, self-reactive or non-useful thymocytes will enter the apoptotic pathway and die (Smith et al., 1988, Shi et al., 1989). This PCD response is central to the effective selection of an efficient T-cell repertoire and to the establishment of self tolerance (Blackman et al., 1990). Mature CD4+ T-helper cells will enter the PCD pathway when activated through the T-cell receptor in the absence of a co-signal from an antigen presenting cell (Liu et al., 1990). It has also been demonstrated that the pre-incubation of mature splenic CD4+ T-cells with an anti-CD4 monoclonal antibody primes these cells to enter the PCD pathway upon subsequent activation through the T-cell receptor (Newell et al., 1990). Therefore, it is evident that programmed cell death is an established method of potentially deleting T-cells at certain stages of their development. This being so, it is possible that the inappropriate re-emergence of apoptosis during the course of an HIV infection, could account for the observed deletion and dysfunction of the CD4+ T-cell population.

A number of studies have linked HIV infection with the apoptosis of T-cells. Several strains of HIV-1 were shown to induce apoptotic cell death in culture (Terai et al., 1991), with apoptosis appearing before virus was produced at significant levels (Laurent-Crawford et al., 1991). T-cells from HIV-seropositive asymptomatic patients have also been demonstrated to strongly resemble the apoptotic phenotype of immature thymocytes. These thymocytes show intracellular mobilisation of Ca^{2+} ions and the release of a small amount of IL-2, yet the cells do not proliferate (McConkey et al., 1989, Ucker et al., 1989). PCD can be prevented in immature thymocytes by the administration of exogenous IL-1, IL-2 or phorbol esters (McConkey et al., 1990a, McConkey et al., 1990b). In asymptomatic HIV infection, the failure of CD4+ T-cells to proliferate in vitro in response to recall antigen is an early functional defect of the immune system (Clerici et al., 1989a). However, although no proliferation is observed the cells are activated, demonstrating elevated levels of intracellular Ca^{2+} (Van Noesel et al., 1990), and the transient secretion of low levels of IL-2 (Clerici et al., 1989a). Furthermore, in a manner similar to that observed for immature thymocytes, the addition of IL-2 (Gruters et al., 1990) or anti-CD28 antibody (Van Noesel et al., 1990) can partially restore the proliferation of apparently
anergic cells from an HIV-infected individual.

Clinical studies have also revealed a link between HIV infection and the induction of apoptosis. Groux et al. (1992) analysed T-cell populations from 59 asymptomatic patients compared with 58 non-infected controls. T-cells from the seropositive individuals did not proliferate in response to an MHC-II-dependent SAg or pokeweed mitogen. This failure to proliferate was shown to be due to the induction of apoptosis.

Therefore, apoptosis has been identified as a possible mechanism for the depletion and dysfunction of CD4+ T-cells during an HIV infection. This could involve the indirect priming of CD4+ T-cells by an HIV-encoded factor. On subsequent activation through the T-cell receptor, the cells would not proliferate as expected, instead entering the programmed cell death pathway (Ameisen & Capron, 1991). If this hypothesis is correct, T-cell depletion would be a function of T-cell activation rather than the absolute number of HIV-infected cells. Gp120 has been proposed as an HIV-encoded factor that could prime CD4+ T-cells for apoptosis on subsequent TCR-mediated activation. Gp120 is known to have a high binding affinity for CD4 (McDougal et al., 1986), and has been detected at levels of 12-92 ng/ml in the blood of patients with AIDS (Oh et al., 1992). The exogenous administration of gp120 to rodent retinal ganglion cells or hippocampal neurons in culture stimulates an increase in intracellular Ca2+ and cellular injury (Dreyer et al., 1990). Furthermore, the binding of gp120 to CD4 and the subsequent cross-linking by anti-gp120 antibodies, has been demonstrated to prime human CD4+ T-cell lines for apoptosis in vitro in response to a subsequent T-cell receptor stimulus (Banda et al., 1992). Circumstantial evidence to support such a process in vivo has been the detection of circulating antibodies to gp120 (Ho et al., 1987) and the antibody-dependent enhancement of HIV-1 infection (Robinson et al., 1988).

Therefore, evidence exists to suggest that activation induced apoptosis via the gp120-mediated priming of mature T-cells, could have a significant role in both the functional and numerical abnormalities observed in the CD4+ T-cell population during the course of an HIV infection. However the full extent of the influence of this process will not be realised until the role of HIV-mediated apoptosis in vivo is fully understood.

6.7 T-cell Anergy.

Anergy is a state in which viable T-cells fail to secrete the appropriate cytokines or proliferate in response to TCR engagement (Schwartz et al., 1990a). This mechanism is important
in the control of peripheral T-cell populations that have escaped negative selection in the thymus and therefore would have the potential to respond to self-antigen.

Normal T-cell anergy is primarily the result of the presentation of antigen in the context of MHC without a co-stimulatory signal from the APC (Quill et al., 1987). Professional APCs such as macrophages and dendritic cells can provide the required co-stimulation, in the form of cell surface molecules such as B7, thus initiating the correct T-cell responses (Harding et al., 1992).

T-cells isolated from HIV seropositive asymptomatic individuals resemble anergic lymphocytes, as both can be rescued by the addition of IL-2 (Bentin et al., 1989). This induction of an anergic state in the T-cell subset has been suggested to result from an HIV-induced defect in the APC population. Monocytes/macrophages and dendritic cells, both major APC populations, are known to be infected with HIV. However, although monocytes from AIDS patients display decreased accessory cell function in a monocyte-dependent T-cell proliferation assay (Goh et al., 1987), normal monocyte function has been reported well into the asymptomatic period of HIV infection (Clerici et al., 1990). In contrast, dendritic APCs have been demonstrated to lose their ability to present soluble antigen and to participate in alloantigenic stimulation from an early stage in HIV infection. This appears to occur before any inability of T-cells to respond to allogenic stimulation is observed (Macatonia et al., 1992). MHC-II levels on these cells have also been shown to decrease, which correlates with a reduction in accessory cell function (Macatonia et al., 1990). In addition, certain functional characteristics have been demonstrated to be altered in HIV-infected APC populations. TGFβ production, thought to have a immunosuppressive effect on T-cells, was increased (Kekow et al., 1990), while cysteine production, known to be limiting for the maximal function of T-cells (Gmunder et al., 1991), was decreased (Staal et al., 1992). The cell surface expression of B7 was also shown to be reduced on monocytes and B-cells from HIV-infected individuals (Odum et al., 1992).

Defective APC function may therefore be an important factor in AIDS pathogenesis, resulting in the induction of anergy or non-responsiveness in the T-cell population. Circumstantial evidence to support this has been observed in HIV-infected chimpanzees. Although these animals become infected they fail to develop an immunodeficiency. A possible explanation for this is the observation that the monocytes of chimpanzees are resistant to HIV infection (Watanabe et al., 1991, Gendelman et al., 1991).
6.8 HIV-induced cytokine imbalance.

From a fairly simplistic standpoint, the immune system can be separated into two major functional or effector pathways. The first, cell-mediated immunity, primarily protects the host against viruses and intracellular pathogens, while the second, the humoral response involving the induction of antibodies, protects the host against extracellular parasites and bacteria (Mosmann & Coffman 1987, Romagnani et al., 1991). The CD4+ T-helper cell population regulates both these effector arms of the immune response. In mice (Mosmann et al., 1986) and subsequently in humans (Maggi et al., 1991, Parronchi et al., 1991, Del Prete et al., 1991, Yssel et al., 1991), it was demonstrated that this CD4+ T-cell subset does not consist of a functionally homogenous population. CD4+ T-cell clones generated by limiting dilution in the presence of exogenous IL-2 and Concanavalin A, demonstrated one of three characteristic cytokine-secreting phenotypes. Th1 clones secreted IL-2, IFN-γ and IL-12, promoting cell-mediated immune responses such as macrophage activation, delayed type hypersensitivity and inflammation. Th2 clones secreted IL-4, IL-5, IL-6, IL-10 and IL-13. These cytokines influence B-cell development and augment the humoral immune response. A third type of clone, Th0, secreted IL-2, IFN-γ and IL-4. It was also shown that Th1-associated cytokines such as IL-12 and IFN-γ reinforce the generation of Th1 clones, but downregulate the generation of Th2 clones (Trinchieri et al., 1993). In contrast, IL-4 and IL-10 facilitate Th2 differentiation, but inhibit the development of Th1 clones (Fiorentino et al., 1989, Fiorentino et al., 1991).

As more information about the cytokine-mediated control of the immune response has become available, the Th1/Th2 nomenclature has become misleading. It is now known that non T-cells produce cytokines originally defined as Th1 or Th2 specific. Human monocytes/macrophages and B-lymphocytes produce IL-10 (Zlotnik et al., 1991) and IL-12 (Chehimi et al., 1994), while NK cells can produce IFN-γ. Therefore, it has become more accurate to refer to a strong cell-mediated immune response as a type 1 response, and a strong humoral immune response as a type 2 response (Clerici & Shearer, 1994). Type 1 cytokines, such as IFN-γ, IL-2 or IL-12, are defined as those that primarily stimulate cell-mediated immunity, while type 2 cytokines, IL-4, IL-5, IL-6, IL-10 and IL-13, are defined as those that primarily stimulate B-cell differentiation and facilitate a humoral immune response (figure 11) (Montaner et al., 1993, Zurawski et al., 1994).

The dominance of either a type 1 or a type 2 immune response has been demonstrated to control the elimination of certain infections. In addition, a particular type of immune response has also been shown to increase the severity of disease. For example, a MuLV infection in mice
Figure 11  The complex regulatory network of the type 1 and type 2 immune responses.

A fine balance exists between the dominance of a cell-mediated type 1 immune response and a humoral, antibody-based type 2 immune response. This dominance is controlled by the production of cytokines such as IL-12, IFN-γ, IL-4 and IL-10. Certain cells such as macrophages, cytotoxic T-cells, natural killer cells, and B-cells can produce these cytokines in response to either their infection or the presence of an invader. If the production of type 1 cytokines predominate, the generation of Th1 T-cell clones which can control the cell-mediated immune response is favoured and the generation of Th2 T-cell clones which control the humoral immune response is repressed. However, if type 2 cytokines predominate, the reverse situation occurs, with Th2 clones being favoured at the expense of Th1 clones.
Cells of the macrophage/monocyte lineage

NK cells

Cytotoxic T-cells

IFN-γ
IL-12

B-cells

IL-4

TYPE 1 cytokine profile

TYPE 2 cytokine profile

Generation of Th1 T-cell clones

Generation of Th2 T-cell clones

Dominant cellular immunity

Dominant humoral immunity

IFN-γ
IL-12

IFN-γ
IL-12

IFN-γ
IL-12

IL-10

IL-10

IL-2

IL-4

IL-5

IL-6

IL-10

IL-13

IL-10

IL-10

IL-4

IL-4

IL-4
results in a condition known as murine AIDS (MAIDS) with death after approximately 25 weeks (Morse et al., 1992). However, in mice homozygous for a non-functional IL-4 gene and hence unable to mount an effective type 2 response, MuLV infection causes no disease (Kanagawa et al., 1993). This would suggest that the presence of a type 2 response, rather than the virus itself, is actually detrimental to the survival of the host. In humans, leprosy is an example of a disease that has clear-cut type 1 (tuberculoid) and type 2 (lepromatous) phases. However, the division is not absolute, with three intermediate stages having been described (Yamamura et al., 1991). Therefore, it has become increasingly evident that the tightly regulated balance between the two effector arms of the immune response is important in establishing not only the efficiency with which an invading pathogen can be neutralised, but also the characteristics and severity of the disease.

Evidence exists to suggest that a type 1 immune response could be far more effective at controlling an established HIV infection than a type 2 immune response. Several studies have noted that multiply-exposed HIV-seronegative individuals have remained virus free, yet still exhibit strong HIV-specific T-cell responses (Clerici et al., 1994a). For example, the PBMCs from six out of six HIV seronegative gay men generated strong IL-2 responses when stimulated with env peptide (Clerici et al., 1992, Clerici et al., 1991). A further study, incorporating over one hundred high risk individuals including gay men, intravenous drug users, health workers involved in accidental needlestick injuries and newborn infants, found that between 39-75% of these HIV negative/provirus PCR negative individuals responded to env presentation by increased IL-2 production. In contrast, only 5% of unexposed low risk individuals responded in a similar manner, with only 2% of the control group responding to more than one env peptide (Clerici & Shearer, 1993). It has also been shown that Rhesus macaques exposed to sub-infectious doses of SIV exhibit a strong SIV-specific cellular, but not humoral immune response. These animals then demonstrated an increased resistance to infectious doses of SIV (Dittmer et al., 1994). This data suggests that successful priming and possible protection against HIV may be possible if a strong type 1 cellular immune response is rapidly mounted. This would suggest that HIV would be more virulent in individuals with either a natural bias toward type 2 responses or a less rapidly-acting type 1 response. In addition, the presence of infectious agents that promote type 2 responses, such as helminth parasites (Actor et al., 1993), certain mycoplasma and mycobacterial strains (Locksley et al., 1994), and STDs such as syphilis (Fitzgerald et al., 1992), may inhibit the efficiency of any type 1 response to HIV infection. If this were so, it could explain the increased frequency of seroconversion and progression to AIDS in areas of the world where parasitic infections, known to induce type 2 profiles, are endemic (Sher et al., 1992).
It has been proposed that an HIV-mediated switch from a type 1 immune response to a type 2 immune response may be occurring during the progression of an HIV infection (Clerici & Shearer, 1993). As described previously, CD4+ T-cells from seropositive asymptomatic individuals show a decrease in IL-2 production in response to recall antigen. B-cell activity has also been shown to increase, while hyper-IgE due to an increase in the production of IL-4 has been reported in certain AIDS patients (Wright et al., 1990). This latter observation was accompanied by a higher incidence of atopic diseases and allergic reactions to drugs, plus the correlation that progression to AIDS was significantly accelerated (Israel-Biet et al., 1992). In addition, recent studies have also indicated a strong correlation between the loss of type 1 cytokines in an HIV seropositive individual and the rapid decline of CD4+ T-cells, progression to AIDS, and time to death (Lucey et al., 1991).

Evidence for a type 1 to type 2 switch has also been demonstrated in vitro. While no definitive markers exist to distinguish Th1 and Th2 cells, it has been demonstrated that CD4+ CD7+ cells mainly produce type 1 cytokines, while CD4+ CD7- cells mainly produce type 2 cytokines. In HIV+ patients the proportion of CD4+ CD7+ cells was shown to decrease, while the proportion of CD4+ CD7- cells was shown to increase with progression to AIDS (Legac et al., 1992). In addition, defective in vitro type 1 function can be restored in HIV+ PBMCs by the addition of type 1 cytokines such as IL-12 (Clerici et al., 1993b), and/or by the addition of antibodies to type 2 cytokines such as anti-IL-4 or anti-IL-10 (Clerici et al., 1993a, Clerici et al., 1994b). HIV-specific CTL function can also be restored in vitro by the addition of IL-2 (Shearer et al., 1985), while the infusion of IL-2 into AIDS patients has seen a large increase in their CD4+ T-cell count (Fauci, 1988).

The above evidence suggests that; (i) the ability of the immune system to control an HIV infection is markedly reduced in the absence of cell-mediated type 1 immunity, and (ii) During an HIV infection, many of the characteristics of a type 1 immune response are lost, while type 2 responses are observed. With this in mind, several longitudinal studies have been conducted in an attempt to identify the actual switch from a type 1 to a type 2 immune response in a single HIV-infected individual. Clerici et al. (1993a) followed 45 asymptomatic seropositive individuals for 15 months, analysing PBMC cytokine production and proliferation in response to certain stimuli. During this period, IL-2 production in response to a recall antigen progressively deteriorated, while IL-4 production in response to PHA stimulation increased, but only after a decline in IL-2 was observed. Both IL-2 and IL-4 production were then seen to decrease to levels far lower than non-infected controls when the PBMCs failed to respond to allogenic stimuli and recall antigens. Therefore, an increase in IL-4 was only observed in the absence of self-MHC-
restricted T-helper cell function. A three year follow up to this study revealed the progression rate to AIDS was 8% in the patients whose T-cells had a strong IL-2 response, but 50% in those individuals who had poor production of IL-2 in response to recall antigen.

A number of additional studies have reported a range of evidence for and against this theoretical type 1 to type 2 switch including; (i) a type 1 downregulation accompanied by an upregulation in the type 2 response (Barcellini et al., 1994, Navikas et al., 1994), (ii) a type 2 upregulation in the absence of any change in the type 1 response (Maggi et al., 1994a), and (iii) no real change in either type of response (Graziozi et al., 1994). In these studies, Graziozi et al., (1994) directly assessed cytokine expression via mRNA levels in the LN of patients infected with HIV. Changes in cytokine levels were observed, but a bias toward the production of type 2 cytokines, or a major decrease in the production of type 1 cytokines, was not detected over an extended period of investigation. In addition, a significant degree of cytokine production from non T-cells was noted. Maggi et al. (1994a) also failed to observe a type 1 to type 2 switch in T-cell clones generated from PBMCs from patients progressing to AIDS compared to those generated from uninfected controls. However, clones generated from seropositive individuals in the presence of a recall antigen (ie memory cell clones), did display a switch from a type 1 profile (80:20 Th1:Th0 clones) to a IFN-γ/IL-4 secreting Th0 profile (29:71 Th1:Th0 clones). The preferential replication of various strains of HIV in clones expressing Type 2 cytokines (either Th0 or Th2 clones) was also observed, while HIV did not replicate in Th1 clones. This observation further complicates matters, as it could be argued that a switch to a type 2 profile would be masked by the preferential infection and clearance of type 2 cytokine secreting T-cells.

At the present time, few solid conclusions can be drawn from this data. However, a model of HIV infection incorporating most of these results has been proposed (Clerici & Shearer, 1994). On HIV inoculation, a strong cell-mediated or type 1 immune response may contain the infection. If the inoculating dose is low, or the type 1 response is rapid, virus may be effectively eliminated. However, higher doses of virus, or the lack of a rapid type 1 response, may allow dissemination of virus to many tissues and the establishment of a persistent infection. Infected T-cells producing virus as a result of cellular activation by environmental antigens would largely be controlled by type 1 responses such as CTL activity. This clearance of activated HIV-infected cells would progressively deplete memory T-cell responses relative to naive T-cells. The gradual increase in the production of type 2 cytokines by a whole range of cell types would promote the differentiation of Th2 cells in response to T-cell activation. As HIV may preferentially infect Th0 or Th2 cells (type 2 cytokine secreting), this differentiation would provide the virus with more target cells in which to replicate. The fact that these cells are then preferentially destroyed, may
mask a type 1 to type 2 switch if T-helper clones are analysed. The other consequence of a type 2 bias, would be the depression of the type 1 responses that would be most capable of fighting the virus, allowing a greater viral burden to be established. The logical conclusion to this scenario would be the depletion of Th2-mediated responses, the suppression of type 1-mediated responses, and the subsequent development of immunodeficiency.

A further level of complexity has been added to this model by Clerici & Shearer, (1994). They claim that type 1 cytokines such as IL-2, IL-12, and IFN-γ, can prevent the activation induced programmed cell death of PBMC from HIV seropositive individuals in vitro. In addition, they claim type 2 cytokines increase PCD. If this is correct, the predominance of a type 2 cytokine profile could also induce PCD in the T-cell population as a whole, further depleting type 1 specific T-cells.

Although much of the available data fits the above model, a substantial amount of work is needed to prove this hypothesis. The in vivo analysis of the cytokine profiles of T-cells responding to a range of antigenic stimuli over the extended course of an HIV infection should help to answer many of the questions raised.

6.9 The degeneration of the lymphoid organs.

HIV-induced abnormalities in the lymphoid organs such as the thymus, spleen or lymph nodes could play an important role in the degeneration of the immune system over the course of the disease.

The thymus is an important organ concerned with the replenishment of the peripheral T-cell compartment. The thymus is most active before adulthood, but is still thought to produce a significant number of T-cells late into life. Human thymocytes express CD4 from an early stage in their development and their HIV-infection has been demonstrated in vitro (DeRossi et al., 1990, Schnittman et al., 1990b, Tremblay et al., 1990). The infection of thymic epithelial cells has also been observed in culture (Numazaki et al., 1989), although this was not observed in an independent study (Schnittman et al., 1991), nor in vivo (Muller-Hermelink et al., 1987), even though abnormalities in this tissue type have been seen in AIDS patients (Savino et al., 1986, Papiernik et al., 1992).

Severe thymic atrophy has been observed in postmortems of AIDS victims, although the mechanisms involved could not be ascertained. This effect on the thymus has been compared to that seen in congenital immunodeficiency or to the result of an allogeneic bone marrow
transplantation (Schuurman et al., 1989). Thymic atrophy has also been observed in SIV infections of Rhesus monkeys. This atrophy is slow and progressive, preceding SIV AIDS, but complete only after AIDS has been diagnosed (Baskin et al., 1991). A narrowing of the thymic cortex is seen 12-24 weeks post infection. Immature double positive cells are decreased in number and vacuolisation, shrinkage and finally cytolysis of cortical epithelial cells and interdigitating dendritic cells is evident (Muller et al., 1993). Further evidence for an HIV-mediated effect on the thymus has come from the SCID-hu mouse model. Mice reconstituted with a section of fetal thymus show fairly normal thymocyte development for a number of months (Bonyhadi et al., 1993). However, such mice exhibit thymic tissue atrophy when infected by HIV (McCune, 1990), which includes the destruction of the thymic epithelium (Stanley et al., 1993).

Therefore, the available data does suggest that an HIV-mediated effect on the thymus could lead to thymic atrophy and a severely decreased capacity for the thymus to regenerate peripheral T-cell reserves. This process would be exaggerated in young patients whose thymuses are still very active, which could explain the shorter period of latency observed in paediatric AIDS cases.
7. The HIV-1 regulatory genes and possible links to pathogenesis.

At the present time, the mechanism of HIV-induced pathogenesis is still at a stage of intense debate. However, one thing is certain, the breakdown of the host immune system must be caused by, or be the result of, the presence of a particular viral component(s). The structural and functional characteristics of these viral components have been examined in great depth and a number of candidate genes thought to have a possible role in pathogenesis have been identified. Two such genes are the tat and nef regulatory genes. This work attempts to further characterise the role of these genes in an HIV infection, with specific attention to their interaction with the immune system.

7.1 The HIV-1 nef gene.

7.1.1 Characteristics of the nef gene and Nef protein.

The nef gene (negative factor, also known as 3'orf, F, orf B or E') is a highly conserved feature of the primate lentiviruses HIV-1, HIV-2, and SIV (Shugars et al., 1993). It is located at the 3' end of the viral genome overlapping the 3' LTR (Guy et al., 1987). Although a strong selective pressure exists for the nef gene in vivo, with 92% of primary HIV-1 isolates containing a nef ORF (Blumberg et al., 1992), a high degree of polymorphism exists between these isolates, with up to 17% non-homology at the amino acid level (Ratner et al., 1985). In addition, no nef gene sequences or motifs predominated or were preferentially selected during a four year in vivo analysis (Delassus et al., 1991).

Nef mRNA is produced early in HIV infection (Schwartz et al., 1990), with approximately 80% of the early multiply spliced species of mRNA coding for the Nef protein (Robert-Guroff et al., 1990). In culture, nef expression is observed during the lytic phase of HIV replication, but levels were demonstrated to decrease dramatically after the establishment of a persistent infection (Schneider et al., 1992). Two translation products of 27kDa and 25kDa predominate, the former being the major species (Guy et al., 1987, Guy et al., 1990). The 25kDa protein may be the result of initiation of translation at an internal ATG, (Kaminchik et al., 1991).
or be a product of an alternative folding pattern (Zazopoulos et al., 1993b). The 27kDa species is myristylated at the amino terminus, in accordance with the presence of the myristylation motif GXXXS commencing at the second residue (Franchini et al., 1986, Guy et al., 1987). Myristylation of the Nef protein allows it to interact with cell membranes (Yu et al., 1992). In vitro expression analysis of the p27 Nef protein has localised it predominantly to the cytoplasmic membrane fraction (Kaminchik et al., 1990, Guy et al., 1987), specifically in the golgi complex (Ovod et al., 1991). However, nuclear localisation of the Nef protein has been reported (Kienzle et al., 1992). Using electron microscopy, a small fraction of Nef protein was localised to specific curvilinear tracks that extended from the nuclear envelope through the nucleoplasm (Murti et al., 1993).

Autophosphorylation of the Nef protein at various serine residues has been observed upon incubation with both GTP (Guy et al., 1987, Nebreda et al., 1991) and ATP (Nebreda et al., 1991). Certain isolates, namely those with a threonine residue at position 15, can also be phosphorylated by protein kinase C, (Guy et al., 1987). As there is a high rate of mutation to alanine at this residue in both in vivo (Ratner et al., 1985; Alizon et al., 1986) and in vitro (Laurent et al., 1990) studies, there has been lengthy speculation that the phosphorylation state of residue 15 is critical in the function of the protein, although as yet the significance of this is not fully understood.

Proteolytic cleavage and H-NMR analysis of the Nef protein has identified two major domains (Freund et al., 1994a). Amino acids 2-65 form an N-terminal myristylated membrane-anchored domain, while the well-folded C-terminal core (amino acids 66-206) would be released from the membrane after cleavage by the HIV-1 protease (Freund et al., 1994b). Oligomerisation of the Nef protein into 2-, 3-, or 4-mers has also been demonstrated (Kienzle et al., 1993). The isolation of 54 nef sequences from a number of HIV-1-infected individuals allowed a consensus nef gene to be formulated (Shugars et al., 1993). Sequence analysis revealed a conserved myrisylation signal (residues 2-7), regions of high polymorphism (eg residues 7-23), a conserved acidic region (residues 62-65), a conserved β-turn motif (residues 130-133), a putative PKC recognition pattern (RPMTYK from residues 77-82), and a sequence containing a highly conserved proline followed by two other residues, repeated four times ((PXX), from residues 69-80). This last conserved region has subsequently been demonstrated to interact with the SH3 domains of a subset of src kinases, such as lyn and hck (Saksela et al., 1995).

The Nef protein shows limited amino acid sequence homologies with the GTP-binding and GTPase domains of known G-proteins (Guy et al., 1987). It has been shown to bind GTP and have GTPase activity when expressed in E. Coli (Guy et al., 1987, Guy et al., 1990), but these
findings have failed to be substantiated in a number of other studies (Backer et al., 1991, Wolber et al., 1992, Kaminchik et al., 1990, Matsura et al., 1991, Nebreda et al., 1991). Analysis of the primary structure of the Nef protein argues against a G-protein like function (Nebreda et al., 1991). G-proteins such as ras and EF-TU have a distinct 3D spacing of four consensus amino acid motifs (Dever et al., 1987, Santos & Nebreda, 1989). Two of these motifs, GXXXXGK and DXXG are recognised by the phosphate groups of the guanine nucleotides, while the NKXD and EXSAX domains form the pocket into which the guanine base fits (Milburn et al., 1990). The glycine rich KEKGLEG of Nef (Guy et al., 1990) resembles the GXGGXGK motif in the ATP binding site of proteins with kinase activity rather than the GXXXXGK of ras-like proteins. Nef also has a NKGE motif, somewhat resembling the NKXD motif of G-proteins. However, the D residue of the latter has been shown to be very important for GTP binding (Siegal et al., 1986, Feig et al., 1988). Therefore it seems unlikely from both experimental and theoretical evidence that Nef has G-protein like qualities, although Nef may have a potential for interaction with the gamma phosphate of nucleoside triphosphates analogous to ATP binding kinases.

In addition to membrane-associated G-proteins, sequence homology between the Nef protein and the MHC class-II β chain (Vega et al., 1990), the MHC class-I α chain (Hobohm et al., 1993), leucine zipper trans-activators (Samuel et al., 1991), scorpion neurotoxins (Werner et al., 1991), and superantigens (Haase et al., 1988) have all been claimed. However, none of the correlations are particularly strong, bringing into question the functional significance of these observations. Antibodies generated against amino acids 60-73 of the Nef protein have been found to cross-react with a 137kDa protein on the surface of uninfected human PBLs and human T-cell lines (Schneider et al., 1993). Antibodies against Nef may therefore contribute to the induction of HIV-induced autoimmune effects.

7.1.2 The function of the nef gene and Nef protein.

The function of the nef gene and its precise role in the viral life cycle is not understood. The HIV-1 nef gene product was initially reported to downregulate the level of viral replication. A recombinant HIV-1 virus with a defective nef gene was shown to replicate at a higher rate than its non-defective counterpart, (Luciw et al., 1987; Terwilliger et al., 1986). Further studies suggested Nef acted as a repressor of HIV-1 LTR transcription (Ahmad et al., 1989, Niederman et al., 1989, Maitra et al., 1991) and that this repression was due to the suppressive effect of Nef on the negative regulatory element (NRE) in the HIV-1 LTR (Ahmad & Venkatesan, 1988).
Based on these results, Nef was suggested to be an important factor in the establishment of viral latency (Ranki et al., 1987, Ameisen et al., 1989). The detection of antibodies against Nef before seroconversion to the structural proteins of HIV (Ranki et al., 1987; Ameisen et al., 1987) and the inability of Nef to repress transcription from the LTR of a rapid/high isolate obtained from the late stages of an HIV infection (Cheng-Mayer et al., 1989), provide circumstantial evidence to support this theory. However, various other groups have failed to demonstrate the negative effect of Nef on viral transcription and replication (Hammes et al., 1989, Kim et al., 1989; Bachelerie et al., 1990; Luria et al., 1991, Balliet et al., 1994). Furthermore, recent studies have shown that Nef can accelerate viral replication in primary lymphocytes and macrophages (de Ronde et al., 1992, Zazopoulos et al., 1993a, Murphy et al., 1993). This conflicting evidence is probably due to the method by which the nef gene has been studied. It is highly unlikely that the use of immortalised cell lines will mimic the characteristics of an in vivo system, and the resulting selective pressures on the passage of virus will not be comparable. Various cell lines were used in these studies, each differing with regard to the factors present. In addition, the isolates of HIV-1 and alleles of nef were not identical, and were not all obtained directly from infected tissue, bringing into question the physiological relevance of the experiments. For these reasons, the latest attempts to analyse the effect of the nef gene on viral replication have involved more sophisticated strategies. Low titre Nef +ve virus was observed to replicate in CEM cells with a 3-5 fold increase in p24 production per round of replication as compared to low titre Nef -ve virus (Chowers et al., 1994). This enhanced Nef +ve viral replication was not observed at high titres. In addition, the level of p24 production from Nef +ve and Nef -ve proviral transfections were similar, implying a role for Nef before proviral integration. Further investigation into this Nef-mediated increase in viral infectivity, demonstrated an intrinsic difference in virions generated in the presence of Nef. This difference was detectable after viral entry, but before viral gene expression had begun (Miller et al., 1995). The role of Nef in quiescent cells has also been analysed. Nef -ve virus (as compared to Nef +ve virus) demonstrated a severely reduced capacity to replicate in primary PBMCs that were PMA-activated a day after initial viral infection (Spina et al., 1994, Miller et al., 1994). In contrast, no difference in the rate of Nef -ve/Nef +ve viral replication was observed in pre-activated PBMCs. This initial infection of quiescent cells, with a subsequent cellular activation, is a more accurate model of an in vivo HIV infection. It strongly suggests a positive role for the nef gene during the process of cellular activation-induced viral replication, when the amount of infectious virus is limiting.

The study of nef in an in vivo system bears a much greater significance to the role of this
regulatory protein in an HIV infection. Therefore, a very important result concerning the role of Nef in the viral life cycle has come from work on SIV in Rhesus macaques. SIV closely resembles HIV and is a good animal model for HIV infection (Desrosiers & Ringler, 1989, Binninger et al., 1991). In the study by Kestler et al., (1991) the requirement of a full open reading frame of the SIV nef gene for efficient viral replication and pathogenesis in vivo was demonstrated. An SIV virus with a deletion in the nef gene did not replicate or produce any symptoms in the infected animals, while wild type SIV did. Furthermore, an SIV nef gene with a premature termination signal was found to revert to wild type within two weeks of the infection. All three types of recombinant SIV showed no differences in the rate of replication in a cultured T-cell line. These results and others (Ilyinskyi et al., 1994), indicate a strong selective pressure for SIV that encodes a full length Nef protein. This hypothesis is supported by experiments in the SCID-hu mouse model. An "in vivo" HIV infection of human PBMCs in these animals, revealed attenuated growth characteristics of Nef -ve virus when compared to Nef +ve virus, even though both showed similar replication kinetics in vitro. This evidence therefore suggests Nef plays a critical role in the in vivo life cycle of the virus.

The presence of the Nef protein in the cells of the immune system has been suggested to have an adverse effect on host immune responses. To address this point, a number of groups have expressed the nef gene in vitro in the absence of other HFV sequences. In such experiments, the Nef protein from HIV-1, HIV-2 and SIVmac have all been shown to downregulate the cell surface expression of the CD4 glycoprotein in T-cell, B-cell, and macrophage/monocyte lines (Guy et al., 1987, Garcia & Miller, 1991, Foster et al., 1994). This downregulation of CD4 was also demonstrated to be a reproducible, consistent property of nef genes from primary isolates of HIV (Anderson et al., 1993, Mariani & Skowronski, 1993). CD4 downregulation is not species specific, as human CD4, murine CD4 and chimpanzee CD4 all showed decreased surface expression (Garcia et al., 1993). No downregulation of human CD8 was observed, although murine CD8 is affected, but to a lesser extent than murine CD4. Downregulation is independent of human specific factors, or PKC-dependent phosphorylation of serine residues on CD4, and requires the cytoplasmic domain of the CD4 molecule (Garcia et al., 1993, Inoue et al., 1993). By using CD4 mutants, the region of CD4 necessary for Nef-mediated downregulation has been identified as twenty membrane-proximal amino acids (Aiken et al., 1994). This region of CD4 has also been demonstrated to overlap that involved in the CD4/p561ck interaction (Anderson et al., 1994). However, certain cysteine residues on CD4 crucial for the binding of p561ck appear unnecessary for Nef-mediated downregulation. A direct interaction between CD4 and Nef has been observed (Harris et al., 1994), yet a number of studies have failed to find such an
association (Anderson et al., 1994). The steady state levels of CD4 mRNA and protein in Nef-expressing cells have been shown to be comparable to control cells, suggesting a post-translational modification to the CD4 expression pathway (Garcia & Miller, 1991, Garcia et al., 1993). In support of this, recent reports have provided compelling evidence to suggest that Nef mediates the dissociation of CD4 from p561ck, allowing a rapid Nef-enhanced internalisation of CD4 by an endocytotic mechanism with subsequent lysosomal degradation (Aiken et al., 1994, Rhee et al., 1994).

In addition to the well documented effect on CD4, the Nef protein (alanine at residue 15) has also been reported to inhibit the DNA-binding activity of the inducible transcription factors NFkB (Niederman et al., 1992) and AP1 (Niederman et al., 1993a, Bandres et al., 1994a, Bandres et al., 1994b). Both NFkB and AP1 are induced upon T-cell activation and have binding sites located in the HIV-1 LTR as well as other cellular genes (Greene, 1991). One of these genes is the cytokine IL-2. In the presence of Nef (ala15), transcription from the IL-2 promoter was reduced by up to 20-fold (Luria et al., 1991), although this has not been confirmed in subsequent studies (Carreer et al., 1994). Further effects of Nef on cellular pathways have been demonstrated. A Nef-associated cellular serine kinase activity independent of PKA or PKC has been observed (Sawai et al., 1994), with phosphorylation of 62kDa and 72kDa proteins that co-immunoprecipitate with Nef. Nef-expressing NIH3T3 cells also fail to proliferate in response to bombesin or PDGF (De et al., 1994). Both Nef-expressing cells and controls were activated at the cell surface, but an increase in intracellular calcium was not observed in those cells containing Nef. Finally a Nef protein association with the cytoskeletal matrix has been demonstrated that could play a role in cellular activation in a manner similar to that observed with src (Niederman et al., 1993a).

7.2 The HIV-1 tat gene.

7.2.1 Characteristics of the tat gene and Tat protein.

The HIV-1 tat regulatory gene is a strong transactivator of transcription from the HIV-1 LTR. It is expressed early in lytic infection, predominantly from the multiply-spliced 2kb class of viral transcripts (Schwartz et al., 1990), of which approximately 2% code for Tat (Robert-Guroff et al., 1990). The Tat protein has been localised to the nucleus, specifically the nucleolus, of an HIV-infected cell (Hauber et al., 1989, Siomi et al., 1990). However, it has been shown
that Tat protein is also secreted (Frankel & Pabo, 1988) and taken up by non-infected cells (Fawell et al., 1994), either from free culture media (Frankel & Pabo, 1988) or via cell-to-cell contact (Helland et al., 1991).

The HIV-1 Tat gene has two exons. The first codes for 72 highly conserved amino acids, while the second specifies a further 14 amino acids that vary considerably between HIV strains. The first exon includes two domains that are functionally important in Tat transactivation. Domain 1, from amino acids 1-48, has transcription transactivating function. Within this first domain, residues 1-21 are acidic and proline-rich forming a structure consistent with an amphipathic α-helix (Rappaport et al., 1989). Residues 22-37 contain a conserved cysteine-rich region, thought to be involved in Zn²⁺ or Cd²⁺ metal binding (Frankel et al., 1988), stabilisation of protein structure (Frankel et al., 1988), and possible multimerisation into 2-, 4- or even 5-mers (Battaglia et al., 1994). Residues 38-48 are also highly conserved and have been strongly implicated in transcriptional activation. Various mutations have been introduced into the first domain of the Tat protein to assess the role of various residues in in vitro assays. Using this approach, substitution mutants of amino acids 2-5 or 1-13 have no transactivational function. These proteins accumulate in the nucleolus and have a recessive phenotype (Rappaport et al., 1989), possibly due to the mutated regions being involved in the interaction of Tat with cellular proteins. Substitution of six of the seven conserved cysteine residues with other amino acids also dramatically reduces Tat activity (Garcia et al., 1988, Sadaie et al., 1988), as does a glutamine to alanine substitution at residue 35, or a lysine to alanine substitution at residue 41 (Rice & Carlotti, 1990).

The second functional domain of the Tat protein (coded for by the first exon) stretches from residues 49-58. This contains an arginine-rich basic region which has been demonstrated to allow transport of the Tat protein from the cytoplasm into the nucleus and nucleolus (Siomi et al., 1990). In addition, this region has nucleic acid binding properties. A synthetic peptide, comprising of Tat amino acids 49-72, effectively competes with full length Tat protein for TAR RNA binding (Weeks et al., 1990). A single arginine residue has been demonstrated to provide the only sequence-specific contact with the TAR RNA, but the surrounding basic amino acids are needed to achieve high affinity RNA binding (Calnan et al., 1991a, Calnan et al., 1991b).

The second exon of tat, coding for residues 72-86, does not appear critical for transactivation of the HIV-1 LTR, as a protein encoded by just the first exon of tat has full transactivating activity (Sodroski et al., 1985a, Sodroski et al., 1986). However, this region does contain an RGD motif, which is a characteristic of extracellular matrix proteins that bind to cell surface receptors (Brake et al., 1990) and hence may be important for the uptake of the Tat protein by non-HIV-infected cells.
7.2.2 The role of the HIV-1 tat gene and Tat protein.

As discussed in a previous chapter 4.7, the Tat protein is able to induce a 100-fold plus increase in full length viral transcription and the production of viral proteins (Sodroski et al., 1985b, Jakobovits et al., 1988). It does this via an interaction with the RNA stem/loop structure of the TAR region (bases +19 to +42) in transcripts generated from the HIV-1 LTR (Rosen et al., 1985, Jakobovits et al., 1988, Hauber et al., 1989). The binding of recombinant Tat protein to synthesised TAR is RNA-specific and was observed in a 1:1 ratio (Dingwall et al., 1989). The TAR RNA forms a stable loop structure with several unpaired nucleotides. This secondary structure is critical for Tat function. It includes a three pyrimidine bulge (UCU), introducing a kink approximately half way up the stem, and a six nucleotide loop (CUGGGA) at the top of the stem. Deletion of the bulge, specifically the first U, dramatically reduces Tat binding in vitro (Rosen et al., 1985, Feng & Holland 1988, Berkhout & Jeang, 1989). Mutation of the loop does not affect Tat binding, although transactivating activity is significantly reduced (Dingwall et al., 1989, Dingwall et al., 1990). This suggests a role for the loop in the binding of cellular factors. Such factors have been isolated, and correlate with Tat function in vitro. These include a 68kDa protein (Marciniak et al., 1990) and the TRP-1 complex (consisting of a 90kDa and a 185kDa protein) (Sheline et al., 1991) that bind to the loop region, and the TRP-2 complex (consisting of four proteins of between 70 and 100kDa) that binds to the bulge region (Sheline et al., 1991).

A number of cellular proteins have also been demonstrated to bind Tat. These include a 50kDa protein known as TBP-1 that has an inhibitory effect on Tat function (Nelbock et al., 1990) and a 36kDa protein that binds to the carboxy-terminal residues of Tat and has a positive effect on Tat function (Desai et al., 1991). In addition, a direct interaction between Tat (amino acids 36-50, Lys 41 is critical) and the cellular transcription factor TFIID has been observed (Kashanchi et al., 1994).

The above data, coupled with the experimental evidence outlined in chapter 4.7, has led to a model for Tat transactivation (Cullen, 1993). Briefly, HIV transcription in resting T-cells is predominantly the result of initiation at the constitutive non-processive promoter that maps to the TATA box and LBP-1 binding site in the HIV-1 LTR. Full length transcription from this promoter is not efficient and the transcription complex terminates after 58-60 nucleotides before reaching the viral genes. On cellular activation, transcription factors such as NFκB are activated and translocated to the nucleus where they can initiate transcription from the processive promoter that maps to the SP1, NFκB and upstream sites in the HIV-1 LTR. Transcription from this promoter is far more efficient, reading through the full proviral genome. The multiply-spliced
transcripts that are generated encode in part for the Tat protein. Tat is then transported back to
the nucleus where, in combination with various cellular factors, it can bind to the TAR region
and rapidly increase transcription initiation from the processive promoter. This in turn boosts the
levels of the Rev protein, inhibiting further splicing of the viral transcripts and promoting the
translation of the viral structural proteins. This results in the eventual production of infectious
virions.

Further levels of Tat regulation may also operate. A TAR-independent mechanism of Tat
transactivation has also been observed that requires no RNA-binding activity (Bagasra et al.,
1992, Taylor et al., 1993). The NFκB binding sites have been implicated in this mechanism
suggesting that an activation of the processive HIV-1 promoter could be possible through an
interaction of Tat and NFκB. In addition, a post-transcriptional mechanism of Tat function has
been proposed. This was put forward to explain the observation that, after the Tat transactivation
of an HIV-1 LTR/reporter gene construct, an eight-fold increase in reporter mRNA was seen with
a 50-fold increase in reporter protein (Feinberg et al., 1986). The mechanism behind this is not
fully understood.

Tat has been demonstrated to interact with a number of other cellular factors and cellular
processes. Extracellular Tat protein has been demonstrated to stimulate the in vitro growth of cells
from Kaposi's sarcoma lesions from AIDS patients (Ensoli et al., 1990). This effect is blocked
by the addition of anti-Tat monoclonal antibodies, and may be the result of the specific interaction
of the Tat amino acid RGD sequence with integrin receptors on the KS cells (Barillari et al.,
1993). This observation was supported by work with transgenic mice that expressed the HIV-1
Tat gene from the HIV-1 LTR (Vogel et al., 1988). Although Tat expression was restricted to
the epidermis of these mice, male mice developed skin lesions, epidermal hyperplasia and
endothelial proliferation of the dermis, closely resembling that seen in AIDS-associated KS. Male
mice of greater than one year also developed liver cancers (Vogel et al., 1991). In addition,
transgenic mice expressing Tat under the control of the BK virus early region promoter display
KS-like lesions, skin tumours of different histotype, and B-cell lymphomas (Corallini et al.,
1993). However, the expression pattern of the Tat transgene is not clear in this model making the
interpretation of these results difficult.

The Tat protein has also been implicated as having growth factor-like properties. The
expression of Tat in lymphoid, epithelial and neuronal cells has been demonstrated to protect them
from apoptosis due to serum starvation, possibly due to the induction of Bcl-2 expression (Zauli
et al., 1993). An interaction of Tat with p53 has also been suggested, which could lead to cell
 transformation or apoptosis under certain conditions (Longo et al., 1995).
The Tat protein has been shown to interact with a number of cellular genes. Tat inhibits expression of the manganese-dependent superoxide dismutase gene (Mn-SOD) by direct interaction with Mn-SOD transcripts (Flores et al., 1993). Mn-SOD is a known tumour suppressor and senescence-inducing gene, and its inhibition by Tat could cause and maintain an environment of oxidative stress. Tat has also been demonstrated to upregulate the expression of TNF-β, via a Tat interaction with a TAR-like structure in its promoter (Sastry et al., 1990, Buonaguro et al., 1992, Buonaguro et al., 1994). It can also upregulate IL-6 in lymphoblastoid, epithelial and peripheral blood monocytic cells, through a direct interaction with a minimal region (-172 to -54) of the IL-6 promoter (Scala et al., 1994). In addition, Tat has been shown to upregulate TGF-β1 expression in human astroglial cells (Cupp et al., 1993), upregulate IL-4 receptor expression on a human B-cell line (Puri & Aggarwal, 1992), downregulate MHC class-I expression (Howcroft et al., 1993), and downregulate interferon-induced p68 kinase expression (Roy et al., 1990). Furthermore, Tat protein can activate heterologous viral promoters, such as the late promoter of the neurotropic JC virus in microglial cells (Tada et al., 1990), the major immediate-early promoter of the murine cytomegalovirus (Kim & Risser, 1993), and the early promoter of the human papillomavirus (Vernon et al., 1993).

Finally, a Tat-mediated effect on the antigen-specific response of T-cells has also been observed. Recombinant Tat protein was shown to inhibit proliferation of human peripheral blood lymphocytes in response to tetanus toxoid or candida antigens, although no effect was seen in response to polyclonal mitogens such as PHA (Viscidi et al., 1989, Subramanyam et al., 1993).
8. Animal models of HIV infection and AIDS.

The study of infectious human disease is continually limited by the fact that humans cannot be used as laboratory subjects. A certain amount of information regarding symptoms, disease progression, and disease kinetics can be obtained from the study of naturally occurring cases. However, it is not possible to dissect the causative agent into its component parts or introduce mutations, and then subsequently evaluate their effects by means of experimental inoculation of unexposed individuals. A possible solution to these problems is the utilisation of cell culture techniques, using the wide array of immortalised cell lines that now exist. Analysing the effect of the \textit{in vitro} infection of such cells can often provide an important insight into \textit{in vivo} mechanisms of pathogenesis. However, the use of cell culture will never mimic the exact characteristics of an \textit{in vivo} system. Factors such as selective pressures and an active host immune response are absent, and the nature of the cell lines can differ dramatically from the \textit{in vivo} cell types that they represent with regard to the genes that are being expressed. Therefore, it is possible that \textit{in vitro} investigations alone may result in the formulation of misleading conclusions, supporting the idea that these methods of analysis should not be used in isolation. Ideally, \textit{in vitro} analysis should be supplemented with experimental techniques which try to recreate certain aspects of human physiology. In this regard the use and generation of animal models of human disease are extremely important.

A number of animal models of HIV infection and AIDS pathogenesis have been generated or documented. The more useful examples are briefly described below.

8.1 HIV infection of chimpanzees.

The HIV infection of various primate species was an obvious candidate for the establishment of an animal model for AIDS. Animals including chimpanzees, Rhesus, Stumptailed, Cynomolgus, and Bonnet macaques, and Capuchin, Pata and Squirrel monkeys, were inoculated with blood plasma from human patients with either AIDS or pre-AIDS (Alter et al., 1984, Gajdusek et al., 1984, Gajdusek et al., 1985). Only the chimpanzees showed any signs of infection. All such animals in one study (totalling 23) seroconverted to the HTLV-III or LAV strain of HIV-1, displaying a prolonged viraemia. However, all the adult animals remained healthy over the period of investigation, with no tumours, very few cases of only mild
lymphadenopathy, and no severe opportunistic infections. Occasional and usually transient T-helper cell defects were observed, which included mild lymphocytosis and a partial inversion of the T4/T8 ratio.

The above results suggest that the HIV infection of primates is of limited use in the study of AIDS pathogenesis. However, understanding the reasons why chimpanzees do not develop disease symptoms even in the face of extensive HIV replication will be very important.

8.2 SIV infection of Rhesus macaques.

SIV is closely related to both HIV-1 and (particularly) HIV-2, sharing many biological properties and causing very similar syndromes (Letvin & King, 1990). In Rhesus macaques, SIV is T-cell tropic, being readily isolated from CD4+ rather than CD8+ T-lymphocytes (Daniel et al., 1985, Kannagi et al., 1985). SIV has a comparable genomic organisation to HIV-1 and HIV-2 (see chapter 3.1) and regulates viral replication in a similar manner (Malim et al., 1989a, Sakai et al., 1990, Viglianti et al., 1990). During the course of SIV infection, CD4+ T-cell numbers decrease progressively and a profound T-helper cell dysfunction is seen. After a period of clinical latency, an immunodeficiency syndrome results, with opportunistic infections, a multitude of lymphomas, a wasting syndrome, encephalitis, and death ~6 months after infection (Desrosiers, 1990). All these disease manifestations are characteristic of HIV-1 infection, making the SIV infection of Rhesus macaques an excellent model for HIV infection of humans. However, the maintenance and breeding of such animals is labour intensive and very costly. Gestation periods are long, and the course of disease is at least 6 months. Experiments can only usually be performed with a handful of animals, and the physiology of the macaque has not been well documented. Therefore, even though the syndrome caused by SIV infection of Rhesus macaques is of a very similar nature to AIDS, logistical problems can severely limit its use.

8.3 Mouse models of HIV infection and AIDS.

Mice are small laboratory animals with a well characterised immune system. They are relatively cheap to breed and maintain, and they have a gestation period of less than 3 weeks. This makes them good candidates to be used in the generation of an animal model for HIV infection and AIDS.
8.3.1 Murine Acquired Immunodeficiency Syndrome (MAIDS).

Mice suffer from a naturally occurring immunodeficiency syndrome known as MAIDS (Morse et al., 1992). The virus/host interactions in MAIDS and AIDS are quite different, yet the syndromes themselves share many common features, including immunodeficiency, enhanced susceptibility to infection and late stage lymphomas. Mice suffering from MAIDS show impaired T-cell function prior to a decrease in cell number, including defective responses to T-cell mitogens, soluble antigens, and the autologous mixed lymphocyte reaction, plus a decrease in T-cell help for CD8+ T-cells and B-cells. They also display polyclonal B-cell activation, hypergammaglobulinemia, auto-antibody production, impaired B-cell responses to mitogen, the development of B-cell lymphomas, and decreased NK cell responsiveness and function. Although there are many common features between the two syndromes, there are also important differences. HIV is a lentivirus, while the murine leukaemia virus (MuLV), the aetiologic agent in MAIDS, is a C-type retrovirus. CD4 is not the receptor for MuLV, B-cell and macrophages being predominantly infected. In addition, a neurodegenerative syndrome, Kaposi's sarcoma and extensive opportunistic infection are not observed in MAIDS. However, the study of the mechanism of disease and the analysis of methods of preventing various aspects of the syndrome could be valuable for the treatment of AIDS. With this in mind, the cause of MAIDS has been extensively studied.

MAIDS is caused by a unique mixture of murine leukaemia viruses (MuLV), that includes both replication competent and replication defective B-cell tropic viruses, and MCF viruses (Laterjet & Duplan, 1962). Subsequent analysis has demonstrated that the replication defective MuLVs are the cause of the immunodeficiency, with the other viruses acting as helper virus and greatly increasing the severity of the disease (Chattopadhyay et al., 1989). The defective MuLV have a major deletion in their env and pol genes (Aziz et al., 1989). They only code for one protein, pr60\(^{ps}\), which has significant changes and is slightly smaller than wild type. This protein is myristylated and phosphorylated, expressed highly on the surface of infected cells, and is not susceptible to proteolytic cleavage. It has been shown that this pr60\(^{ps}\) behaves as a SAg, recognising a broad range of V\(\beta\) chains including V\(\beta\)3, 5.1, 5.2, 7, 8, 11, 12, 13 and 17a (Hugin et al., 1991). Upon B-cell infection, it engages the V\(\beta\) domains of a number of T-cell receptors in association with MHC-II molecules, thus activating the T-cell. After an initial release of Th0-like cytokines, a type 2 immune profile predominates resulting in polyclonal B-cell activation and T-helper mediated defects.

In conclusion, the syndrome associated with MAIDS has a number of important
similarities to AIDS, even though the tissue tropism of the MuLVs and the virus/host interaction may differ. Understanding the mechanism of a SAg-mediated disease and the manipulation of the type 1/type 2 immune response by an infectious pathogen could reveal important insights into AIDS pathogenesis. In addition, it may also be possible to use this model to test various therapeutic agents to correct such immunological abnormalities.

8.3.2 The SCID-hu mouse model

The SCID-hu mouse model is generated by engrafting human fetal hematolymphoid organs into the mature T and B-cell deficient C.B-17 scid/scid (SCID) mouse (McCune et al., 1988). Co-engraftment of human fetal liver and thymus under the mouse kidney capsule results in the formation of a conjoint organ that is able to promote physiologically normal long-term human T-cell differentiation (Krowka et al., 1991, Namikawa et al., 1990). T-cell subsets are represented in the expected proportions, a normal Vβ T-cell repertoire is displayed (Vandekerckhove et al., 1992), and tolerance to both self-MHC antigens and exogenously provided SAgs is observed (Waller et al., 1992).

The SCID-hu mouse model is permissive for HIV-1 infection (Namikawa et al., 1988). This infection is dose, time and HIV-1 strain-dependent, and is blocked by the administration of AZT (McCune et al., 1990). Inoculation of HIV-1 into the Thy/Liv organ results in the progressive depletion of CD4+ thymocytes and an inversion of the T4/T8 ratio (Bonyhadi et al., 1993, Aldrovandi et al., 1993, Stanley et al., 1993). HIV replication, as monitored by p24 ELISA, peaked at ~350pg/10^6 cells at day 23 after infection and virus was seen throughout the thymic tissue after 5 weeks. Extensive thymocyte depletion has been observed, largely being attributed to the HIV-mediated induction of apoptosis, although CD3-/CD4+/CD8- T-cell progenitors are also affected (Su et al., 1995). Degeneration of thymic epithelium cells was seen, as has been noted in fetuses aborted from HIV-1 seropositive women (Papiernik et al., 1992), thymic biopsies of HIV-infected children (Joshi et al., 1984), and SIV-infected Rhesus macaques (Baskin et al., 1991). Finally, pathologic effects and HIV replication were observed to occur more efficiently when the inoculated virus had an intact nef ORF (Jamieson et al., 1994).

Therefore, it is apparent that this model of HIV infection reproduces many key aspects of HIV-mediated pathology in man, and will undoubtedly prove to be a very important small animal model to study HIV-1 induced pathogenesis.
8.3.3 Transgenic mice

The use of transgenic mice allows the analysis of single and combined gene products to be examined throughout the developmental and adult life of the animal. To generate such animals, DNA containing the transgene is directly microinjected into the pronucleus of a fertilised mouse egg (Hogan et al., 1986). The manipulated cell is then transferred to a foster mother, where uterine implantation allows development to term. If the injected DNA can stably integrate into the germline cells of the founder mouse the transgene will be transmitted to subsequent generations. The developmental and tissue expression of the transgene are controlled by the regulatory sequences that are included in the injected DNA construct. This expression pattern is also sensitive to the regulatory elements at or near the site of transgene integration (Dillon & Grosveld, 1993). These often undesirable position effects can be largely reduced by the presence of dominant control elements known as LCRs (Locus Control Regions). Such constructs have been demonstrated to confer tissue-specific, copy number dependent and position-independent expression to the transgenes in question (Grosveld et al., 1987, Greaves et al., 1989).

Transgenic mice have been used to study the effects of various gene products in a wide array of biological fields, including immunology, eg the role of the T-cell receptor in thymic ontogeny (Bonneville et al., 1989), organ development, eg the role of the homeobox gene loci in embryonic segmental development (Kessel et al., 1990), and oncogenesis, eg the synergistic effect of combining a number of oncogenes (Bems et al., 1991). Transgenic mice have also been used to assess the individual in vivo effects of viral proteins. For example the Tax protein of human T-cell leukaemia virus (HTLV)-1 was shown in vitro to be a potent transactivator of cellular genes such as IL-2, IL-2R, c-fos, GM-CSF and several others (Sodroski et al., 1992). When expressed in transgenic mice under the control of the HTLV-1 LTR, the animals developed thymic aplasia, neurofibromas and an exocrinopathy of the salivary and lacrimal glands thought to resemble Sjogren's syndrome (Green et al., 1989).

Transgenic mice thus offer distinct advantages for the in vivo analysis of genes from infectious agents such as HIV. They by-pass species and tissue infectability barriers and pose little or no risk in terms of biosafety. Individual viral genes can be expressed under the control of various regulatory elements to direct expression of the viral proteins to certain tissue types and to certain stages of animal or organ development. Separate lines of mice can be generated and crossed to observe the synergistic effect of combining the expression of viral proteins. All results can be measured in a workable time course and in a statistically significant manner with a large number of animals. Therefore, the use of transgenic mice to examine the interactions of HIV gene
products in the presence of a functional and dynamic immune system, may provide important
insights into AIDS pathogenesis that could not be obtained from in vitro studies.

As has been outlined in the preceding chapters, HIV infection and the mechanisms that underlie the pathogenesis of AIDS are complex. Many theories exist that attempt to explain such phenomena as the gradual depletion of the T-helper cell population and the progressive dysfunction of the immune system. However, as yet no single hypothesis has provided an adequate answer as to why an individual who becomes infected with HIV eventually dies from a severe immunodeficiency syndrome.

The work described in this thesis makes an attempt to further understand the mechanisms of HIV-induced immune dysfunction by focusing on the role that the HIV-1 regulatory genes nef and tat have on the cells of a dynamic mammalian immune system. In order to achieve this, nef or tat are expressed (in isolation from other genes of the virus) in an in vivo transgenic mouse model. Transgene expression is specifically directed to thymocytes and mature T-cells, cells which are known to be infected by HIV in humans, by using the human CD2 regulatory elements, including the hCD2 locus control region.

This study attempts to investigate; (i) the effect that nef and tat expression have on the development and differentiation of the immature thymocyte subset and mature T-cell compartment, (ii) the interaction of Nef and Tat with host cell genes such as cell surface antigens and immunoregulatory cytokines, and (iii) the influence that Nef and Tat have on a number of T-cell responses to a variety of activation stimuli.

Briefly, the analysis of the CD2-nef and CD2-tat transgenic mice has revealed a dramatic and reproducible phenotype in both systems. The CD2-nef transgenic animals display a severe reduction in thymic cellularity, resulting from an early block in thymocyte development. In addition, CD4/CD8 double positive thymocytes display a significant decrease in the expression of cell surface CD4, and the absolute number and percentage of CD4 single positive thymocytes is dramatically reduced. In contrast, the CD2-tat transgenic mice show a normal pattern of thymic ontogeny and have the expected distribution of thymocyte and T-cell subsets. However, transgenic thymocytes and splenocytes display a significantly altered cytokine profile in response to a range of activation stimuli.

This thesis describes the analysis of both the CD2-nef and CD2-tat transgenic mice and discusses the implications of the results with reference to an HIV infection and the mechanisms that induce AIDS pathogenesis.
Materials and Methods

1. Nucleic acids - DNA.

1.1 Genomic DNA preparation.

Tissue samples (~100mg) were carefully homogenised with a glass mortar and eppendorf tube in 430μl of tail mix buffer. To this, 50μl of 10% SDS, and 20μl of proteinase-K (10mg/ml) were added with gentle mixing. The samples were incubated at 55°C overnight, followed by a 1hr incubation at 37°C with 10μl of 10mg/ml DNase-free RNAse. The DNA was cleaned by 2x phenol/chloroform extractions, plus one chloroform extraction, and precipitated from the aqueous layer with 0.6 volumes of isopropanol. The DNA was hooked out using a sterile glass pasteur pipette, washed in 70% ethanol, and air-dried for 5 minutes. It was then resuspended in 50-200μl of TE pH 7.4, and stored at -20°C until needed.

DNA from tail cuts of mice was obtained as above, with the exception of the homogenisation step, while cells from culture were resuspended in tail mix, before preceding as described.

1.2 DNA fragment purification.

All DNA fragments for either cloning or DNA probes were isolated using the GELase™ (Cambio, Cambridge) or QIAEX™ (QIAGEN) protocols.

QIAEX™ method: The DNA was run on an ethidium bromide (1μg/ml) stained, 1xTAE/agarose (0.8-1.5%) gel, and the desired band removed with a sterile scalpel blade. The gel slice was weighed in an eppendorf tube, and 300μl of QX1™ solution per 100mg of gel was added. To this, 10-20μl of QIAEX™ resin was added and the sample was incubated at 50°C for 10 min. After centrifugation at 14K rpm for 30 seconds the supernatant was removed. 2x washes with QX2™ solution and QX3™ solution followed, with centrifugation at 14K rpm after each wash. The QIAEX™ resin pellet was then air-dried, before the DNA was eluted in 20-50μl of TE pH 7.4.

GELase™ method: The DNA was run on an ethidium bromide (1μg/ml) stained,
1xTAE/low melting point agarose (0.8-1.5%) gel, and the desired band removed with a sterile scalpel blade. The gel slice was weighed in an eppendorf tube and 1μl of 50x GELase™ buffer added per 50mg of gel. The gel slice was then completely melted at 70°C (approx. 20 min). The sample was then equilibrated to 42°C for 10 min., before 1 unit of GELase™ enzyme was added per 300mg of 1% LMP agarose gel. Incubation was from 1hr to overnight. One volume of 5M ammonium acetate, followed by 4x the original volume of room temperature absolute ethanol was then added. The DNA was pelleted by centrifugation for 30 min. at 14k rpm, and the supernatant removed. After air-drying the DNA was resuspended in 10-50μl of TE pH7.4.

1.3 DNA quantitation.

For concentrations of DNA thought to be in excess of 300ng/μl, the sample was diluted 1:200 and the absorbance was read at 260/280nm on a spectrophotometer. For samples less than 300ng/μl, 1-2μl of the DNA was run on a 1% agarose/1xTAE ethidium bromide (1μg/μl) stained gel, alongside known amounts of standard DNA. The concentration of the sample was then estimated by comparing the intensity of its fluorescence to that of the standards, under a UV light.

1.4 DNA restriction digests.

DNA digestion with restriction endonucleases were performed using the optimal conditions as recommended by the particular manufacturer. Digests were performed using 1x buffer, DNA (10ng to 1μg/μl) and 0.5-5 units of enzyme per μg of DNA. The total volume of enzyme was always limited to no more than 10% of the reaction volume to avoid glycerol inhibition of digestion. Incubations were at 37°C, unless otherwise instructed, for periods from 1hr to overnight.

1.5 Slot blot analysis of DNA.

Slot blot analysis of DNA from tissue samples. 5μg of DNA was added to dH2O, to a final volume of 180μl. To this 20μl of 4M NaOH was added, mixed and left at room temperature for 5min. The slot blot manifold was prepared by laying 2 sheets of 2x SSC soaked Watman
3MM paper onto the lower half of the apparatus. On this was placed a pre-wet nitrocellulose filter (0.45-micron pore size) that had been soaked in 1M ammonium acetate for 10min. The top manifold was fitted and clamped in place. The vacuum line was attached and turned on. Under suction, each slot was filled with 1M ammonium acetate and allowed to empty. To the DNA samples 200μl of 2M ammonium acetate was added and mixed. The 400μl sample was then added to the slot and allowed to pass through onto the filter. Known amounts of plasmid DNA added to 5μg of normal mouse genomic DNA were used as copy number controls. After the last sample the filter was allowed to dry under suction for 5min. The vacuum line was then removed. The filter was air-dried then baked in an 80°C oven for 2hr. The blots were hybridised in conditions as used for Southern blot analysis.

1.6 Southern blot analysis of DNA.

Between 5-20μg of genomic DNA was digested overnight with the appropriate restriction enzyme(s). The sample was then mixed with 0.2 volumes of orange-G loading dye, and loaded onto an ethidium bromide (1μg/μl) stained agarose (0.8%-1.2%)/TAE (1x) gel. Bacteriophage Lambda DNA, digested with BstEII, was used as DNA size markers, and plasmid DNA of known concentration was added to normal non-transgenic mouse DNA as copy number controls. The samples were run through the gel at between 1-5 V/cm, until the orange G front had reached the end of the gel. The gel was photographed and the position of the Lambda size markers were noted. The gel was inverted and soaked in 0.25M HCl for 20 min at room temperature, with gentle agitation. The gel was then washed twice with 0.5M NaOH/1.5M NaCl for 20min., followed by 2x 20min. washes in 0.5M Tris pH 7.4/1.5M NaCl. The inverted gel was then placed onto a wick comprised of a piece of Watman 3MM paper dipped into a tray of 20x SSC. The following was placed on top of the gel ensuring that no air bubbles were trapped; 1 piece of nitrocellulose or nylon (Nytran) filter soaked initially in H2O then in 20x SSC for 20 min., 2-4 20x SSC-soaked pieces of 3MM paper, 20-40 sheets of dry 3MM paper, a 5cm stack of dry paper towels, a glass plate and two 500ml bottles half filled with water (weight approx. 0.5kg). This blotting set-up was left for 12-16hr. The apparatus was dismantled and the position of the wells were marked on the filter with a water-proof pen. The filter was then baked at 80°C for 2hr. and hybridised as described below.
1.7 Oligo-labelling of DNA probes (random priming).

DNA fragments from 100-5000bp were used as DNA probes. 100ng of DNA in 7μl of ddH₂O was denatured by boiling at 100°C for 5min, then placed on ice. To this, 12μl of 2xOLB mix, 1μl of 1mg/ml BSA, 3μl of ²³P α-dATP and 1μl of Klenow enzyme (5U/μl) were added. The mix was incubated at 37°C for 30min, with the reaction terminated by the addition of 10 μl of 0.25M EDTA. The labelled DNA was separated from unincorporated nucleotides by spinning the DNA through a 2x SSC-equilibrated G50 sephadex column. Probes with a specific activity above 1x10⁶ cpm/μg of DNA were used.

1.8 End-labelling of DNA probes.

DNA probes were end-labelled using T4 polynucleotide kinase. The DNA fragment was prepared by leaving a 5' overhang at the end of the DNA that is to be labelled, by digestion with an appropriate restriction endonuclease. The phosphate was removed from this 5' end by incubation with 1μl of calf intestine alkaline phosphotase (1U/μl) for 30 min at 37°C. The DNA was cleaned using 1x phenol/chloroform plus 1x chloroform extraction, followed by an ethanol precipitation. The labelling reaction involved 100 ng of this prepared DNA in 10μl. To this 4μl of 5x kinase buffer, 5μl of ²³P γdATP, and 1μl of T4 polynucleotide kinase were added, and the mixture incubated at 37°C for 30 min. The reaction was terminated by adding 1μl of 0.5M EDTA and 1μl of 10% SDS and the labelled DNA separated from the unincorporated nucleotide by spinning it through a G50 sephadex column. Probes with a specific activity greater than 1x10⁷ cpm/μg were used.

1.9 Hybridisation of DNA filters.

DNA filters were pre-wet in 2x SSC then placed in Hybaid hybridisation bottles. The filters were pre-hybridised for 2hr at 68°C in 20ml of DNA pre-hybridisation solution without the radio-labelled probe. The pre-hybridisation solution was removed. The probe was then added to 20ml of DNA hybridisation solution and incubated overnight at 68°C. The filters were then washed twice for 20 min in 2x SSC/0.1% SDS and twice for 20 min in 0.2x SSC/0.1% SDS. The filter was then covered in Saran wrap and exposed on film (4hr-1wk) or on the Molecular Dynamics
Phosphorimager.

1.10 Polymerase Chain Reaction (PCR).

Great care was taken to avoid DNA or RNA contamination, the reaction usually being set up in a different lab or tissue culture hood. 5μl of cDNA sample (see above), 200ng of genomic DNA or 0.2pg of plasmid DNA was used per reaction. To this 100ng of sense and antisense primer, and 25μl of 2x PCR mix was added and made up to 50μl with H2O. The PCR conditions were 5 min at 94°C, then, 40 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min, with a 72°C incubation for 10 min to end. This was performed by a Techne PHC-2 PCR machine, and the results were visualised on an ethidium bromide staining agarose/TAE gel.

**Primers:**

Nef sense - 5'-GCCTGTACTGGTCTCTCGG-3'
Nef antisense - 5'-CCCAGGGTTTCCAAGGCATTCA-3'
HPRT sense - 5'-CAGAGGACTAGAACCTGC-3'
HPRT antisense - 5'-GCTGGTGAAAAGGACCTCT-3' (Keller et al., 1993)

HPRT - Hypoxanthine phosphoribosyl transferase gene.
2. Nucleic acids - RNA.

2.1 RNA preparation.

For the preparation of RNA, gloves, sterile plastic, and fresh DEPC-treated and autoclaved solutions were used, and the samples were kept at 4°C or below at all times. Tissue samples or cell pellets were placed directly into 3ml of 3M LiCl/6M urea on ice. The tissue samples were homogenised with the ultra-turrax on full power for 1 min. Samples were then sonicated for 1-2 min to shear the genomic DNA and then left at 4°C overnight. The solution was centrifuged at 14K rpm at 4°C for 30 min. The supernatant was removed and the pellet pipetted back and forth in 500μl of 3M LiCl/6M urea for 1-2 min. The sample was again centrifuged at 4°C for 30min, with the supernatant being removed. The pellet was then resuspended in 300μl of 10mM Tris/0.5% SDS. Following this, 2x phenol/chloroform, plus 1x chloroform extractions were performed. The RNA was precipitated with 30μl of NaOAc and 800μl of ethanol. The sample was left on dry ice for 30 min, before it was pelleted at 4°C, 14K rpm, for 30 min. The pellet was washed with 70% ethanol and resuspended in 20-100μl of DEPC-treated water. The samples were stored at -70°C until required.

2.2 Slot blot analysis of RNA.

The slot blot manifold was prepared by soaking it in 0.1M NaOH for 30 min, then washing well with DEPC-treated water. The nitrocellulose filter (0.45-micron pore size) was placed in DEPC-treated H₂O for 2min, then soaked in 20x SSC for 1hr at room temperature. As for DNA slot blotting, the 2 sheets of 20x SSC-soaked Watman 3MM paper were placed on the lower half of the manifold with the filter on top. The upper half of the manifold was then fitted and clamped in place. The suction line was attached and the vacuum turned on. All the slots were then washed with 10x SSC under suction. Between 5-20μg of RNA was made up to 10μl in DEPC-treated H₂O. To this, 20μl of 100% formamide, 7μl of 37% formaldehyde and 2μl of 20x SSC were then added. The samples were mixed well, and incubated at 68°C for 15min. The samples were removed to ice, mixed with 2 volumes of 20x SSC, and applied to the appropriate slot. After the last sample was loaded, the vacuum line was removed and the filter was allowed to air-dry for 5min. The filter was then baked in an 80°C oven for 2hr, before being hybridised.
in conditions as used for a northern blot.

2.3 **Northern blot analysis of RNA.**

The gel tank and apparatus was first washed well with 0.1M NaOH and rinsed in DEPC-treated H₂O. RNA samples (5-30µg) were made up to 4.5µl in DEPC-treated H₂O. To this, 2µl of 10x MOPS, 3.5µl of 37% formaldehyde, and 10µl of 100% formamide were added and mixed well. The samples were then incubated at 68°C for 15min before being removed to ice. Northern loading dye (0.2 vol.) was added, and the samples were loaded onto an agarose (0.8%-1.2%)/MOPS (1x)/formaldehyde (6%) gel. The samples were run through the gel at 1-5 V/cm until the furthest blue dye front had reached the end of the gel. Boehringer RNA size standards were used. The gel was then soaked in 50mM NaOH/100mM NaCl for 20min, 100mM Tris pH 7.6 for 20 min, and 20x SSC for 20 min. The gel was then blotted to 20x SSC soaked nitrocellulose of Hybond-N (Amersham) filters in the manner described for Southern blot transfer. After 12-16hr the filter was removed and baked in an 80°C oven for 2hr, and hybridised as described below.

2.4 **Hybridisation of RNA filters.**

RNA filters were pre-wet in 2x SSC then placed in Hybaid hybridisation bottles. The filters were pre-hybridised for 2hr at 42°C in 20ml of RNA hybridisation solution without the radio-labelled probe. The pre-hybridisation solution was removed. The probe was then added to 20ml of fresh RNA hybridisation solution and incubated overnight at 42°C. The filters were then washed twice for 20 min in 2x SSC/0.1% SDS and twice for 20 min in 0.2x SSC/0.1% SDS. The filter was then covered in Saran wrap and exposed on film (4hr-1wk) or on the Molecular Dynamics Phosphorimager.

2.5 **SI nuclease protection analysis.**

SI nuclease protection analysis was used to detect RNA in the tissues of transgenic mice. 5-30µg of RNA and 10ng of end-labelled probe were mixed, made up to 300µl with H₂O, and
precipitated using 30μl of 2M NaOAc and 800μl of 96% ethanol (10 min dry ice). The nucleic acid was pelleted by centrifugation at 14k rpm, air-dried for 5min, and resuspended well in 15μl of S1 nuclease hybridisation buffer. The samples were then incubated at 90°C for 5 min to denature the RNA, and then swiftly transferred to a water bath at 50-55°C. The probe and RNA were allowed to anneal in these conditions for at least 16hr. To each sample 200μl of ice cold S1 digestion mix, containing 1x digestion buffer, 10μg of tRNA carrier, and 100 units of S1 nuclease, was added as the tubes were removed from the water bath. They were quickly sealed, vortexed and placed on ice. After each had been removed from the bath, all the samples were placed at 23°C for 2hr 15min. The digestion reaction was terminated by placing the samples on ice. The remaining nucleic acid were phenol/chloroform extracted and ethanol precipitated in the presence of 0.1 vol. of 2M NaOAC. After centrifugation the pellet was resuspended in 5μl of S1 loading buffer. The samples were then denatured for 5 min at 90°C, placed on ice, and loaded onto a 7% denaturing polyacrylamide gel. The gel was electrophoresed and visualised as described above.

**S1 probes:**

**Tat** - 610bp StyI fragment from pTZ19 (gift from Dr. E. Blair, Wellcome). Protected fragment is 498bp at 5’ end of tat gene.

**U6** - 326bp FokI-Eael fragment from pGEM3Z (gift from Dr. M. Antoniou). Protected fragments of 79bp and 87bp at 5’ end of U6 gene.

### 2.6 Reverse transcription.

Reverse transcription of RNA (and PCR amplification of the resulting cDNA fragments - see above) was used to analyse the presence of transgenic transcripts in various mouse tissues. 1-10μg of RNA in 5μl of H₂O was heat denatured for 5 min at 65°C then placed on ice. To this, 2μl of 10x RT buffer, and 10 units of Super RT (HT biotechnology) were added and made up to 20μl with H₂O. The mix was incubated at 42°C for 2hr. 30μl of 1mM Tris pH 7.5 was then added and the resulting cDNA solution stored at -20°C until required.
3. Protein Analysis.

3.1 Protein Purification - total cell extracts.

Total cell protein extracts were generated using a high salt lysis buffer. A single cell suspension (10⁷-10⁸ cells) from tissues or from cell culture was first washed in ice cold PBSa. The cells were pelleted by centrifugation at 14k rpm for 10 seconds, and the supernatant was removed. The cell pellet was then resuspended in 300μl of protein extraction buffer I. The suspension was then frozen on dry-ice and thawed at room temperature three times, followed by a centrifugation at 14k rpm at 4°C for 10 min. The supernatant was taken and stored at -70°C until required.

3.2 Protein purification - nuclear/cytoplasmic extracts.

A single cell suspension (10⁷-10⁸ cells) was washed with ice cold PBSa. The cells were then pelleted (14k rpm 15 sec), and the supernatant removed to leave a combined cell/supernatant volume of 100μl. The cells were then snap frozen in liquid nitrogen. On thawing, the cells were gently resuspended in 300μl of ice cold protein extraction buffer II. The suspension was centrifuged at 14k rpm for 15 seconds and the supernatant removed. This supernatant contains cytoplasmic proteins and can be stored at -70°C if required. The pelleted cell nuclei were then gently resuspended in 400μl of protein extraction buffer II, and centrifuged at 14k rpm for 15 seconds at 4°C, the supernatant being discarded. Again the pellet was resuspended in 200μl of protein extraction buffer II. To this, 10μl of 5M NaCl was added. After mixing well the sample was left on ice for 45 min. The sample was then centrifuged at 14k rpm at 4°C for 25 min. The supernatant, containing nuclear proteins, was removed and stored at -70°C until required.

3.3 Immunoprecipitation.

Single cell suspensions from thymus and spleen of transgenic and non-transgenic mice were prepared. Erythrocytes were removed by lysis in tris-buffered ammonium chloride (Sigma). Extracts were prepared by lysing cells in 1ml of immunoprecipitation buffer I on ice for 15 min. The extracts were centrifuged at 14k rpm for 5 min at 4°C and the supernatant was removed. To this, 50μl of normal rabbit serum was added for 1 hr at 4°C, followed by 100μl of a 10%
suspension of protein A-sepharose beads in lysis buffer for 30 min, and centrifugation at 14k rpm for 15 min at 4°C. Anti-Nef antibodies (HIV-1 HXB3 Nef antisera, to the N and C termini (Hammes et al., 1989) were then added at a 1:250 dilution to the cell lysate (supernatant), and incubated overnight on ice. Following this, the extracts were incubated again with 100μl of 10% suspension of protein-A-sepharose beads for 1 hr. The beads were collected by centrifugation for 15 min. at 4°C and washed three times in lysis buffer. Pellets were resuspended in reducing sample buffer, heated at 100°C for 5 min. and the supernatants recovered. They were stored at -70°C until required.

3.4 Protein Quantitation.

Using non-acetylated BSA (2mg/ml), standard protein concentrations of 0, 100, 200, 300, 500 and 1000μg/ml were made in H₂O. 5μl of the protein samples, or 20μl of the protein standards, were added to 1ml of Coomassie protein assay reagent (Pierce), and mixed well. An H₂O blank was then inserted into the spectrophotometer and the absorbance at 595nm was set to zero. The absorbance of the protein samples was then read at 595nm, with the absorbance of the 0μg/ml standard being subtracted from each reading. Using a standard curve the concentration of the samples can then be calculated.

3.5 Western blot analysis of protein.

Gels were cast and run using the Bio-rad Western apparatus. The plates were cleaned, one was siliconised, the spacers were introduced and the plates clamped in place. The resolving gel was poured first. For 10ml of a 10% gel, this consists of 4ml H₂O, 3.3ml of protogel acrylamide mix (30% acylamide and 0.8% bisacylamide), 2.5ml of 1.5M Tris pH8.8, 0.1ml of 10% SDS, 0.1ml of 10% ammonium persulphate, and 40μl of TEMED. After pouring, the gel was covered with 1:1 isopropanol/water (~0.5ml) to give a level surface to the top of the gel and to aid polymerisation. Once set the isopropanol/water is removed and the plates are dried. The stacking gel is then poured, and the comb is fitted. For 3ml of a 5% stacking gel, this consists of 2.1ml H₂O, 0.5ml of protogel acrylamide mix (30% acylamide and 0.8% bisacylamide), 0.38ml of 1.0M Tris pH 6.8, 0.03ml of 10% SDS, 0.03ml of 10% ammonium persulphate, and 30μl of TEMED. The gel is not pre-run.
The protein samples and molecular weight markers are diluted 1:1 in 2x Western sample buffer, boiled for 5 min, centrifuged for 5 seconds and chilled on ice. 10-50\(\mu\)g of protein is then run per lane. The gel is run at 5V/cm until the bottom dye reaches the bottom of the gel. After running the top plate is removed and the stacking gel is cut away. The gel is then floated off the bottom plate in Western transfer buffer, onto a piece of pre-wet Hybond C Extra (~10cm x 10cm). The transfer apparatus is then assembled. A pre-wet scotch-brite pad is covered with 2 sheets of 3MM paper, then the membrane and gel, 2 more sheets of 3MM paper and a second pre-wet scotch-brite pad. This sandwich is then placed in the transfer tank with approximately 3.5 litres of Western transfer buffer. The transfer of protein to the membrane for 3-5hr at 80V (~320mA) is then performed at 4°C, with continuous circulation of the buffer.

3.6 Western blot hybridisation.

The membrane is first blocked for 2hr at 37°C with 50ml of Western block buffer. After 3x washes for 10 min with 50ml of PBS/0.1% Tween 20, the primary antibody is added. 20ml of block, with 2\(\mu\)g/ml of the primary antibody is used, with an incubation at 37°C for 2hr. After 3x washes for 10 min with PBS/0.1% Tween 20, the secondary antibody (1:1000 - HRP conjugated), was added in 20ml of block solution. After 2hr at 37°C, 3x washes for 10 min with PBS/0.1% Tween 20 were performed, with the membrane stored under PBS/0.1% Tween 20 at 4°C until ECL™ detection.

3.7 ECL™ detection of Western blots.

The ECL™ detection system (Amersham) was used to visualise antibody binding to Western blots. In this protocol, 2ml of ECL™ solution 1 and 2 were mixed in a small container. The membrane was placed in this solution for 1 min, then blotted with 3MM paper to remove excess liquid. It is then covered with saran wrap and shielded from the light for 1 min. The membrane is then exposed to pieces of Kodak XAR5 film for 15 seconds upwards until the right exposure is found.

4.1 Generation of constructs.

CD2Nef constructs (see figure 13): The 800bp BamH1-Sma1 fragment from either pTG1147, pTG1191, pTG3191, pTG3132, or pTG2165 (gift from B. Guy) was blunted and ligated into a unique blunted EcoR1 site in the first exon of the p2629 CD2 expression plasmid (gift from D. Kioussis) to give either p2629N47, p2629N91, p2629N391, p2629N32, or p2629N65. The orientation of the insert was tested using an Asp718/HindIII and a Xho1/BamH1 digest (see map figure 13). A 4.5Kb BamH1-Not1 fragment containing the 3'-CD2 LCR from p2694 (gift from D. Kioussis) was then ligated to the BamH1-Not1 fragment from the p2629N... plasmids, resulting in either pCD2Nef1147, pCD2Nef1191, pCD2Nef3191, pCD2Nef3132, or pCD2Nef2165. A Sall-Not1 fragment was used to generate transgenic mice.

CD2Tat constructs (see figure 13): pCD2Tat72 was made as described for the CD2Nef constructs above. However, the original Tat fragment was excised using a Asp718/Sal1 digest from plasmid pTZ19 (gift from Dr. E. Blair, Wellcome), which was then blunted into the EcoR1 site of p2629. The orientation of the fragment was checked with a HindIII digest (see map figure 13).

IL-2Nef constructs (see figure 14): The 789bp BamH1-Sac1 fragment from pTG1147 was cloned into the BamHI-SacI sites of the bluescript vector. A 1.95kb PstI-HindIII fragment from the pATgMIL2-A plasmid (gift from Prof. W. Fiers - Uni. of Ghent, Belgium), containing the entire IL-2 promoter/enhancer and 44bp of untranslated 5' leader sequence, was then ligated into the PstI-HindIII fragment of the Nef/bluescript vector (this contained the bluescript backbone) to give pIL47. pIL47 was then cut with HindIII, blunted and then cut with EcoRI to generate a 2.75kb blunt/EcoRI IL-2 promoter/enhancer/nef gene fragment. This was ligated into the p2629 hCD2 expression cassette which had been prepared by first cutting with SalI, blunted and then cut with EcoRI. The resulting plasmid was pIL-2Nef1147. An ApaI-NotI fragment was used to generate transgenic mice.
4.2 Ligations.

In the cloning process, 10-50 ng of linearised plasmid and 100 ng of the insert fragment were mixed together in 8 µl of H₂O. To this, 1 µl of 10x ligation buffer and 1 µl of T4 DNA ligase (1 U/µl) were added. The reaction was incubated overnight at 16°C. For transformation into E. coli 2 µl of these samples were used.

4.3 Competent bacteria.

For transformation, CaCl₂ competent E. Coli (strains DH5α or DH10β), were prepared. A single E. Coli colony was inoculated into a 20 ml LB media culture and grown overnight at 37°C with vigorous shaking. 100 µl of this culture was then added to a fresh flask containing 250 ml of pre-warmed LB media and incubated at 37°C. The OD₆₀₀ was monitored at regular intervals until it reached 0.5. The culture was then cooled rapidly in an ice bath, followed by the centrifugation of the bacteria at 4k rpm for 10 min at 4°C. The cells were then resuspended in 62.5 ml of ice cold sterile 0.1M MgCl₂, and re-pelleted by centrifugation at 4k rpm at 4°C for 10 min. The cell pellet was resuspended in 31.25 ml ice cold sterile 0.1M CaCl₂ and left on ice for 20 min. After centrifugation at 4k rpm at 4°C for 10 min, the cells were resuspended in 26.5 ml of ice cold 0.1M CaCl₂ and 3.5 ml of glycerol. 200 µl aliquots of this solution were then snap frozen in eppendorf tubes and stored at -70°C until required.

4.4 Bacterial transformations.

The DNA to be transformed was mixed with 100 µl of rapidly thawed competent cells and left on ice for 30 min. The mixture was then transferred to a 42°C water bath for 90 seconds, then onto ice for 5 min. 1 ml of LB media was then added and the culture allowed to incubate at 37°C for 1 hr. The cells were pelleted by centrifugation for 10 sec and all the supernatant, with the exception of 50 µl, removed. The cells were resuspended in this volume, then spread onto LB plates containing 100 µg/ml ampicillin. After overnight incubation at 37°C, single colonies were picked and inoculated to 5 ml LB/ampicillin (100 µg/ml) cultures for miniprep purification of their plasmid DNA.
4.5 Plasmid miniprep purification.

Single plasmid-containing E. Coli colonies were inoculated into 5ml of LB media with 100μg/ml ampicillin. The cultures were incubated overnight at 37°C. 1.5ml of the culture was transferred to an eppendorf and centrifuged at 14k for 30 sec, the supernatant being discarded. 270μl of TEN buffer was added, with the pellet resuspended by vortexing. To this, 30μl of 10% SDS was added, vortexed, followed by 150μl of 2M NaOAc pH 5.2. After mixing well 1x phenol/chloroform and 1x chloroform extractions were performed. To the final aqueous layer 0.9ml of 96% ethanol was then added, the sample placed on ice for 5 min, and centrifuged at 14k rpm for 10 min to pellet the DNA. The pellet was washed with 70% ethanol, air-dried and resuspended in 50μl of TE. RNAse treatment using 1μl of 10mg/ml RNAse A was then carried out for 30 min at 37°C, followed by a phenol/chloroform extraction, ethanol precipitation and resuspension of the final DNA pellet in 50μl of TE pH 7.4.

4.6 Plasmid maxiprep purification.

A single bacterial colony, grown up in 5ml cultures as described for miniprep purification, was used to inoculate a 1 litre culture of LB media with 100μg/ml ampicillin. This was incubated overnight at 37°C. The culture was then centrifuged at 4k rpm in a 1 litre bottle for 20 min, the supernatant being discarded. The bacterial pellet was then resuspended in 40ml of 1x glucomix by vortexing. To this, 80ml of 0.2M NaOH/1% SDS was added, mixing gently, and left to stand for 5 min. 40ml of ice-cold 5M KOAc pH 4.8 was then added, again mixing gently and leaving to stand for 5 min. The mixture is then centrifuged at 4k rpm for 20 min, then poured through 8 layers of cheesecloth to remove the debris. To the remaining supernatant, 0.6 vol. of isopropanol is added, with gentle mixing to precipitate the DNA. The DNA is then pelleted by 20 min centrifugation at 4k rpm, washed with 70% ethanol, air-dried for 30 min, and resuspended in 5ml TE pH 8.0. The solution of DNA was then transferred to a pre-weighed Falcon 50ml tube, and adjusted to 9g with TE pH8.0. To this, 10.2g of CsCl and 1ml of 5mg/ml ethidium bromide was added. The solution was then transferred to Quick-seal™ centrifuge tubes and sealed with a heat clamp. The samples were then centrifuged for 24hr at 56,000 rpm in a 70.1 Ti Beckman rotor. The lower supercoiled band of plasmid DNA was removed using a syringe and needle. This was transferred to a 15ml Falcon tube, made up to 4ml with H2O, and then mixed with 8ml of 96% ethanol. The DNA was pelleted by centrifugation at 4k rpm for 10
min, followed by a wash in 70% ethanol and resuspension in 500μl of TE pH 8.0. To this, 10μl of 0.5M EDTA and 5μl of 10% SDS were added, followed by 2x phenol/chloroform extractions and a chloroform extraction. To the final aqueous phase, 50μl of 2M NaOAc pH 5.5 and 1ml of 96% ethanol were added to precipitate the DNA. After 5 min on dry ice, the sample was centrifuged for 10 min at 14k rpm. The DNA pellet was washed in 70% ethanol, and resuspended in 450μl TE pH 8.0, plus 50μl of 1M NaCl. To this 2μl of 10mg/ml RNAse A was added, and incubated at 37°C for 30 min. Then followed 2x phenol/chloroform extractions, 1x chloroform extractions, the addition of 50μl of 2M NaOAc pH 5.5 to the final aqueous phase, and the precipitation of the DNA by the addition of 1ml of 96% ethanol. After pelleting the DNA by centrifugation at 14k rpm for 10 min, a 70% ethanol wash was followed by the resuspension of the DNA in 500μl of TE pH 8.0. The plasmid DNA was then stored at -20°C until needed.

5.1 FACS analysis.

FACS analysis was used to detect cell surface markers on lymphocytes from transgenic mice. The thymus, spleen and lymph nodes were removed and homogenised to single cell suspension in ice-cold FACS media. Accurate cell counts were obtained. 10⁶ cells were then washed in 5ml of FACS media, pelleted and the supernatant removed. Antibodies were added at a dilution of 1:200 in FACS media and incubated for 30 min on ice. Cells were washed once with 5ml of cold FACS media, washed once with 5ml of cold PBSa, fixed in 1% formaldehyde/PBS and filtered through nylon mesh. Stained cells were analysed with a Becton Dickinson FACSCAN cell sorter and the LYSIS II software package.

Antibodies used were a PE-conjugated rat monoclonal antibody against murine CD4; a FITC-conjugated rat monoclonal antibody against murine CD8 (both Becton Dickinson Immunocytometry Systems, San Jose, CA); a PE-conjugated rat monoclonal antibody against murine CD8; a FITC-conjugated rat monoclonal antibody against murine CD25 (IL-2 receptor); a CY-chrome-conjugated rat monoclonal antibody against murine CD44; a FITC-conjugated hamster monoclonal antibody against murine CD3 (all Pharmingen, San Diego, CA); a FITC-conjugated rat monoclonal antibody against murine Thy-1.2 (Sigma Chemical Co., St. Louis, MO); a rabbit polyclonal serum against p561ck (gift from Dr. S Ley).

For the FACS analysis of intracellular protein, 10⁶ lymphocytes were washed twice with PBSa, and resuspended in 100μl PBSa containing 1% paraformaldehyde for 10min at 20°C. After two further PBSa washes, the cells were permeabilised in PBSa containing 0.1% saponin, 20% fetal calf serum and 1mM EGTA for 20 min at 20°C. Primary antibodies were added directly to this suspension of cells in saponin, and the staining procedure described above was followed.

For the analysis of apoptosis, 10⁶ cells were washed in PBS, and then incubated overnight at 4°C in 1.5ml of PI buffer. The cells were then run through the FACScan machine and acquired on the FL-3 channel.

5.2 Cell sorting.

Cell sorting was performed to isolate populations of cells displaying particular surface
characteristics. The cells were prepared from mouse tissues in exactly the same manner as for FACS analysis, with the exception that the FACS media used did not contain sodium azide. The cells were run through a Becton Dickinson cell sorter and the desired cell populations were collected into tubes containing FACS media minus azide. The accuracy of the sort was assessed by running a small fraction of the sorted cells back through the machine.

5.3 Immunofluorescence.

Indirect immunofluorescence was used to detect the intracellular or surface distribution of CD4 and p56lck on lymphocytes from transgenic and non-transgenic mice. Whole thymus and lymph nodes were homogenised to a single cell suspension in PBS and filtered through nylon. Approximately 3x10⁶ cells were spread on 10mm poly-lysine coated coverslips and were allowed to attach for 2-3 min. The cells were fixed with 3.7% paraformaldehyde in 1x CSK for 10 min with periodic swirling, followed by three 5 min washes with PBS. Cells were permeabilised with 0.5% Triton-X in 1x CSK for 15 min, followed by three 5 min washes with PBS.

To stain the cells the coverslips were first overlaid with 5μl of block solution (0.8% BSA, 0.1% gelatin in PBS) for 15 min. Diluted antibodies in the same block solution were then applied (10μl) in the following sequence: (1) anti-CD4 (1:200), or anti-p56lck (1:100); (2) FITC-anti-rat IgG (for CD4 staining) (1:100), or FITC-anti-rabbit IgG (for p56lck staining) 1:100; then for double staining either; (3) anti-Golgi marker (1:1000), or anti-ER marker (1:20), or anti-p56lck (1:100); (4) Texas red anti-rabbit IgG (1:100). The incubation period for each antibody was 30 min at room temperature in a humidified chamber with three 5 min washes with 0.05% Tween-20/PBS between each application. Coverslips were mounted with a drop of Univert (BDH, Poole, UK) containing 100mg/ml of DABCO (Sigma Chemical Co., St. Louis, MO) as anti-fading agent, and cells were viewed under a Zeiss Axiophot fluorescence microscope.

The antibodies used were a rat monoclonal antibody against murine CD4 (Pharmingen, San Diego, CA), detected via a FITC-conjugated goat anti-rat IgG (Calbiochem., La Jolla, CA); a rabbit antiserum against murine p56lck (gift from Dr. S. Ley), detected via a FITC-conjugated goat anti-rabbit IgG (Pharmingen, San Diego, CA); a Golgi-specific rabbit monoclonal anti-α mannosidase II antibody (gift from Dr. K. Moreman, University of Georgia); an endoplasmic reticulum-specific rabbit monoclonal antibody, anti-ERC55 (gift from K. Weis & A. Lamond, EMBL, Heidelberg) and; an anti-p56lck [RNGS] rabbit antiserum (gift from M. Marsh, Univ. Coll. London), all three detected via a Texas red-conjugated goat anti-rabbit IgG (Calbiochem.,
5.4 Thymic organ cultures.

Day 15 CBA embryos were dissected and the fetal thymus was removed. The lobes were separated, cleaned, washed in RPMI/10% FCS, and then placed on a filter. The filter was floated in a small petri dish filled with RPMI/10% FCS and 1.35mM deoxyguanosine. This was incubated in an humidified chamber at 37°C for 4-6 days to deplete the lobes of endogenous thymocytes.

After their thymocyte depletion, the thymic lobes were removed from the filter and washed twice in RPMI/10% FCS. The donor cells that were to repopulate the thymic organ cultures (between $3 \times 10^3$ and $3 \times 10^4$ from a cell sorting experiment) were pelleted and resuspended in $20\mu l$ of RPMI/10% FCS. This volume of cells was then transferred to one of the wells of a hanging drop Terasaki plate (Nunc). To this well, a depleted thymic lobe was then added. Once all lobes had been applied to their respective wells, the plate was carefully inverted, and the lobes were allowed to fall to the surface of the hanging drop. The lobes were then cultured in this manner for 10 days in an humidified chamber at 37°C. At day 4, day 6, and day 8, the lobes were fed by removing them from their drops and placing them in the wells of a Terasaki plate containing fresh RPMI/10% FCS media. At day 10 the lobes were harvested, converted to a single cell suspension by sieving, and the cells were analysed by FACS analysis.

5.5 Cryostat sections.

Cryostat sections were cut from the thymus, spleen and lymph nodes of transgenic and non-transgenic mice as follows. Tissues were dissected and carefully placed in a 1cm² mold containing O.C.T. compound (BDH Poole, England) mounting fluid. This was then left on dry ice until the OCT had solidified. These blocks were then mounted on the cryostat and 8 micron sections were cut at -20°C. The sections were touched onto slides and then air-dried for 1hr. The sections were fixed in acetone and stored at -70°C until required.
5.6 TUNEL staining for apoptotic cells.

Fixed sections obtained from the cryostat were first washed with TBS pH 7.4, then incubated in 0.2% H₂O₂ for 30 min to neutralise endogenous peroxidases. After 2x washes in TBS, the sections were washed in TdT buffer and then incubated for 1hr at 37°C with 4μM biotin-labelled dUTP (Boehringer Mannheim) and 10U TdT (Promega) in 25μl TdT buffer. Again the sections were twice washed with TBS, followed by a 1hr incubation at 37°C with a strepavidin-HRP complex (Vector) in TBS. To visualise the HRP staining pattern, 3,3'-diaminobenzidine was used as a substrate (DAB substrate kit for peroxidase - Vector laboratories), as instructed by the manufacturer. It was found that a 10 minute incubation with the substrate gave a good red/brown colour. After this incubation the sections were washed for 5 min in H₂O, before being examined under a Zeiss light microscope.

5.7 T-cell proliferation assays.

Thymocytes and erythrocyte depleted splenocytes were cultured in 200μl of αMEM, 10% FCS, 2mM glutamine, 10U/ml Penicillin, 100μg/ml Streptomycin and 50μM β-mercaptoethanol. Cells were cultured in microtiter wells from a density of 8x10⁵ to 1x10⁶ per well for thymocytes and 4x10³ to 5x10³ per well for splenocytes. Thymocytes were stimulated with anti-CD3e (145-2C11) monoclonal antibody (0.36μg/well) and 5ng/ml PMA (Sigma), or with 5 ng/ml PMA and 500ng/ml ionomycin (Sigma), or with 5ng/ml Con A (Sigma), or with 25ng/ml SEB (Sigma), or with 5ng/ml PMA alone as a control. Splenocytes were stimulated as above except no PMA was added with anti-CD3e antibodies. 48 hours after stimulation, cells were labelled for 16 hr with 1μCi/well of ³H thymidine (Amersham) before harvest. The incorporated radioactivity was precipitated on glass fiber filter paper and subsequently counted by liquid scintillation.

5.8 KLH recall antigen response.

The KLH was emulsified with an equal volume of complete Freund's adjuvant for 2 minutes in a sonicator. 40μl of this emulsion, containing 100μg of KLH was then subcutaneously injected into a mouse at the base of the tail. 5-7 days after this initial injection, the mice were sacrificed, and the inguinal and periaortic lymph nodes were removed. The lymph nodes were
sieved to make a single cell suspension, washed twice in PBS and then resuspended in 5ml of IMDM, 10% FCS, 2mM glutamine, 10U/ml Penicillin, 100μg/ml Streptomycin and 50μM β-mercaptoethanol. An accurate cell count was conducted and the cell concentration adjusted to 4x10⁶/ml. 0.1ml of this cell suspension was then added to each well of a 96-well microtiter plate, along with 0.1ml of the above media containing either 100, 50, 25, 12, 6, 3, 1.5, or 0μg of KLH. The cells and antigen were then cultured for 4 days at 37°C in a humidified atmosphere (5% CO₂). After 3 days in culture, the cells were labelled with 1μCi/well of ³H thymidine (Amersham), and left for 16hr before harvest. The incorporated radioactivity was precipitated on glass fiber filter paper and subsequently counted by liquid scintillation.

5.9 TNF bioassay.

Culture supernatants from PMA/ionomycin activated thymocytes (see 5.7 above) of transgenic and non-transegentic animals were examined for the production of TNF using the cytokine-sensitive cell line L929. The amount of biologically active TNF was determined relative to known amounts of recombinant cytokine (Sigma), and the L929 killing was shown to be TNF-specific by the addition of TNF neutralising antibodies. L929 cell number was assessed by absorbance at 540nm after dye solubilisation following staining with crystal violet.

6.1 Gel electrophoresis.

Agarose: Gel electrophoresis with normal or low melting point agarose from concentrations of 0.6%-2.0% were performed in 1x TAE. The mixture of agarose and TAE was boiled before the addition of 1μg/ml of ethidium bromide. The gels were cast in taped moulds at 4°C. The gels were run at between 1 and 10 volts/cm, and visualised by UV illumination. The images were either photographed onto polaroid film or transferred to heat sensitive paper using a video camera and image processing system (Cybertech CS1).

Denaturing polyacrylamide: Acrylamide gels of between 5% and 15% were made with Accugel 40™ acrylamide (40%) (National Diagnostics). The gels were made with 1xTBE, 6M urea, 0.1% ammonium persulphate and 0.1% TEMED. Gels were cast between glass plates, using 0.4mm spacers, and run in 1x TBE at a power of between 60-80 Watts. The gels were placed on Watman 3MM paper, covered with Saran wrap, and dried under vacuum at 80°C for 1 hr.

6.2 Autoradiography.

Filters were exposed to either Kodak XAR5 or Fuji RX 100 film at either room temperature or -70°C, with intensifying screens. Alignment of the image on the film was aided by phosphorescent markers. Alternatively, the filters were exposed for 4-6hr on a Molecular Dynamics Phosphorimaging screen. The image was then scanned into the Phosphorimager and analysed using the software package provided.

6.3 Generation of transgenic mice.

Fertilised (CBA x C57Bl/10)F2 mouse oocytes were microinjected with the 12kb SalI/Not1 fragment from the CD2Nef and CD2Tat plasmids. Positive founder animals were bred with (C57Bl/10)F1 mice and lines were maintained as heterozygotes, (Dr. H. J. Brady and C. G. Miles kindly generated the mice used in these experiments). DNA slot blot analysis or Southern analysis using Nde1, HindIII or Asp718 digests of DNA from tail clippings, was used to assess the integration patterns of the transgenes, and to identify the transgenic mice within a litter.
7. Reagent List.

CSK (2x): - 200mM NaCl, 600mM sucrose, 20mM PIPES pH 6.8, 6mM MgCl₂, 2mM EGTA pH 6.8.

Denhardt’s (100x): - 2% Ficoll, 2% BSA, 2% polyvinyl pyrrolidone.

DNA hyb. mix: - 3x SSC, 0.1% SDS, 10x Denhardts, 10% Dextran sulphate, 50μg/ml denatured salmon sperm DNA.

DNA pre-hyb. mix: - 3x SSC, 0.1% SDS, 10x Denhardts, 50μg/ml denatured salmon sperm DNA.

FACS fix: - 1% formaldehyde in PBSa.

FACS media: - 500ml αMEM, 5% fetal calf serum, 0.01% NaAzide.

Fluor. block buffer: - 0.8% BSA, 0.1% gelatin in PBSa.

Glucomix (10x): - 500mM glucose, 100mM EDTA, 250mM Tris pH 8.0.

Immuno-ppt. buffer I: - 0.5% Triton x-100, 10mM Tris pH 7.5, 1mM EDTA, 0.15mM NaCl, 10mg/ml BSA, 200μM PMSF, 5mM iodoacetamide, 5μM leupeptin.

Kinase buffer (5x): - 250mM Tris pH 9.0, 250mM MgCl₂, 50mM DTT, 50μg/ml BSA.

LB broth: - 1% bactotryptone, 0.5% bacto-yeast extract, 1% NaCl, adjust final pH to 7.5 with NaOH.

Ligation buffer (10x): - 500mM Tris pH 7.4, 100mM MgCl₂, 10mM DTT, 10mM spermidine, 10mM ATP, 1mg/ml BSA.

MOPS (10x): - 0.2M MOPS, 80mM NaOAc, 10mM EDTA.

Northern loading dye: - bromophenol blue added to each sample.

OLB (2x): - 100mM Tris pH 7.6, 100mM NaCl, 20mM MgCl₂, 200μg/ml gelatin, 40μM each of dCTP, dGTP, dTTP, 20μg/ml random hexamers.

PBSa (PBS): - 171mM NaCl, 3.3mM KCl, 10.1mM Na₂HPO₄, 1.8mM KH₂PO₄.

PCR mix (2x): - 2x cambio PARR™ buffer, 0.4mM of each of dATP, dTTP, dCTP, & dGTP, add taq polymerase last 1U/reaction.

Phenol: - Phenol (equilibrated with 0.1M Tris pH 8.0), 0.1% 8-hydroxyquinoline.

Phenol/Chloroform: - 1:1 (v/v) ratio.

PI buffer: - 50μg/ml propidium iodide, 0.1% sodium citrate, 0.1% triton-X in H₂O.

Protein extract buf I: - 20mM HEPES pH 7.8, 450mM NaCl, 0.4mM EDTA, 25% glycerol, 0.5mM DTT, 0.5mM PMSF, aprotinin (10μg/ml).
Protein extract buf II: - 10mM HEPES pH 7.9, 1.5mM MgCl₂, 0.1M EGTA, 5% glycerol, 0.5mM DTT, 0.5mM PMSF, aprotinin (10μg/ml).

Proteinase K: - 10mg/ml proteinase K, 10mM Tris pH 8.0, 1mM EDTA, 0.5% SDS.

RNA hyb. mix: - 60% formamide, 5x SSC, 0.1% BSA, 0.1% Ficoll 400, 0.1% polyvinyl pyrrolidone, 20mM sodium phosphate buffer pH 6.8, 1% SDS, 7% dextran sulphate, 100μg/ml denatured salmon sperm DNA, 100μg/ml tRNA(bakers yeast), 10μg/ml poly A.

RT buffer (10x): - 50mM Tris pH 7.6, 60mM KCl, 10mM MgCl₂, 1mM dNTPs, 1mM DTT, 1U/μl RNasein, 50μg/ml actinomycin D.

S1 digestion buffer: - 0.28M NaCl, 50mM NaOAc, 4.5mM ZnSO₄.

S1 hyb. buffer: - 40mM PIPES pH 6.4, 1mM EDTA, 0.4M NaCl, 80% formamide.

S1 loading buffer: - 7M urea, 0.05% xylene cyanol, 0.05% bromophenol blue.

SSC (20x): - 3M NaCl, 0.3M Na₂Citrate

TAE (50x): - 2M Tris pH 8.0, 50mM EDTA, 1M glacial acetic acid.

Tail mix buffer: - 50mM Tris pH 8.0, 100mM EDTA, 100mM NaCl.

TBE (10x): - 0.89M Tris pH 7.4, 0.89M boric acid, 10mM EDTA.

TBS: - 25mM Tris base, 137mM NaCl, 2.5mM KCl, adjusted to pH 7.4 with HCl.

TdT buffer: - 0.5M cacodylate pH 6.8, 1mM CoCl₂, 0.5mM DTT, 0.05% BSA, 0.15M NaCl.

TE pH 7.4/8.0: - 10mM Tris pH 7.4/8.0, 1mM EDTA.

TEN: - 10mM Tris pH 7.4, 1mM EDTA, 0.1M NaOH.

Western block buffer: - 0.1% tween-20, 5% skim milk powder, 1% BSA, in PBSa.

West. running buf. 10x: - 250mM Tris pH 7.5, 192mM glycinie, 0.1% SDS.

West. sample buf. (2x): - 125mM Tris pH 6.8, 4% SDS, 20% glycerol, 10% 2-βME, 4mg BPB/10ml.

West. transfer buffer: - 25mM Tris base pH 8.3, 192mM glycinie, 20% methanol.
Results

1. The generation of transgenic mouse lines.

In the studies described in this thesis, transgenic mouse models were generated to investigate the effect of the expression of the HIV-1 nef and HIV-1 tat regulatory genes in a mammalian immune system. Similar transgenic mouse strategies have previously been used in an attempt to analyse the in vivo expression of both the HIV-1 gp120 gene and the HIV-1 tat gene. The gp120 protein was expressed in astrocytes of the CNS by using regulatory sequences from the murine glial fibrillary acidic protein gene (GFAP) in order to assess the role of gp120 in HIV-1-induced neurological disorders (Toggas et al., 1994). The tat gene has been expressed under the control of both the HIV-1 LTR (Vogel et al., 1988) and the BK polyoma virus early region promoter (Corallini et al., 1993). In the HIV-1 LTR-tat animals skin lesions with KS-like characteristics and an increased rate of liver cancer was observed, while in the BK promoter-tat mice endothelial cell proliferation and tumour formation were seen in older animals. However, the relevance of these tat transgenic models is difficult to assess as both fail to direct tat transgene expression to the cell types known to be the targets for HIV-1 infection in humans (ie CD4 positive T-cells, macrophages etc). With this consideration in mind, the main aim of this study is to specifically direct the expression of both the HIV-1 nef gene and the HIV-1 tat gene to cells of known tropism for HIV-1, these cells being immature thymocytes and mature T-cells. This has been achieved by using the regulatory elements from either the human CD2 gene or the murine IL-2 gene. The hCD2 sequences direct transgene expression to the majority of immature thymocytes, and all mature T-cells, while the IL-2 sequences permit only very low levels of transgene expression until mature T-cells are activated through a variety of stimuli.

Therefore, in these studies nef and tat expression can be analysed in vivo, in isolation from other viral sequences, and directly in the cells known to be infected by HIV in humans.

1.1 The human CD2 expression cassette.

A human CD2 expression cassette was used to generate transgenic mice expressing either the HIV-1 nef gene or the HIV-1 tat gene in the majority of immature thymocytes and peripheral
T-cells. This allows the effect of transgene expression to be analysed in both thymic ontogeny and mature T-cell responses. As illustrated in figure 12, this cassette consists of the hCD2 promoter, the hCD2 minigene, and the hCD2 locus control region (LCR). The hCD2 LCR, encompassed within a 5kb BamHI-NotI fragment, has been demonstrated to confer position independent and copy number dependent expression to a transgene introduced into the hCD2 expression cassette (Greaves et al., 1989). The hCD2 coding sequences have been reduced in the hCD2 expression cassette to a hCD2 minigene. This comprises exon 1 of the hCD2 gene, followed by intron 1, and then a fusion between exons 2-5. The inclusion of the intron has been demonstrated to greatly increase the level of transgene expression from the hCD2 cassette (R. Festenstein, personal communication). The hCD2 minigene has an ATG in exon 1 and a polyadenylation signal at its 3' end. The transgene is cloned into the EcoR1 site of exon 1, which lies upstream of the hCD2 ATG. The cloned transgenes have their own stop codon but do not possess a poly-adenylation signal. Therefore, transcription from the hCD2 promoter reads through the transgene and exons 1-5 of the hCD2 sequences before terminating at the end of the hCD2 minigene. After processing, the ATG of the transgene is the first to be encountered by the translation machinery. As all the transgenes have a stop codon fusion proteins with hCD2 sequences are not generated. Any translation from the downstream hCD2 ATG is prematurely terminated at the site of a former EcoRV site, which had previously been mutated to introduce a stop codon at this position. Any truncated hCD2 protein that is produced is not properly processed or transported and is rapidly degraded. FACS analysis of all transgenic lines generated using this hCD2 expression cassette has shown no cell surface expression of human CD2 (data not shown).

1.2 The murine IL-2 expression cassette.

A murine IL-2 expression cassette was used to generate transgenic mice with low levels of transgene expression during thymic ontogeny, but high inducible levels of transgene expression on activation of mature peripheral T-cells (figure 13). This allows the effects of the transgene in early thymocyte development to be bypassed (ie if transgene expression resulted in cell death at these stages), and allows the effects of high transgene expression to be analysed in activated peripheral T-cells in response to a variety of controlled stimuli.

The IL-2 expression cassette consists of a 1.95kb HindIII-PstI IL-2 promoter/enhancer fragment (containing all the sequences necessary to direct transcription, (Degraeve et al., 1986)) fused to the hCD2 minigene. The IL-2 sequences include the transcriptional start site and 11 bases
Figure 12  The CD2-\textit{nef} and CD2-\textit{tat} plasmid constructs.

\textit{Nef}-1147, \textit{nef}-1191, \textit{nef}-2165, \textit{nef}-3132, or \textit{nef}-3191 (all BamHI-SacI digests), or \textit{tat}(1-72) (Asp718-SalI digest), were blunted and ligated into the blunted EcoRI site of the human CD2 expression cassette. This expression cassette consists of the hCD2 promoter, the hCD2 minigene (exons 1-5, plus intron 1), and the hCD2 3' locus control region (LCR). The hCD2 minigene contains a poly-adenylation signal at its 3' end. All transgenes contain their own stop codons to ensure a fusion protein with hCD2 sequences does not occur. A functional hCD2 protein is not generated from this construct as a premature stop codon has been introduced at a former EcoRV restriction site. Translation from the hCD2 ATG therefore leads to the production of a truncated non-functional hCD2 protein that is rapidly degraded. For the generation of transgenic mice, a SalI-NotI fragment was used for microinjection.
Nef genes

Tat genes

623bp.
(first exon only)

Micro-injected fragment

Full length transgenic protein
Non-functional hCD2 protein
Figure 13  The IL2-nef plasmid construct.

Nef-1147 (BamHI-SacI digest) was subcloned into bluescript. A PstI-EcoRI fragment containing the nef-1147 gene was then ligated into a PstI-EcoRI cut plasmid containing the IL-2 enhancer/promoter. From this plasmid, a HindIII (blunted)-EcoRI fragment was ligated into the hCD2 minigene plasmid cut with SalI (blunted) and EcoRI. For the generation of transgenic mice an ApaI-NotI fragment was microinjected.
Full length transgenic protein

Non-functional hCD2 protein

mRNA

micro-injected fragment

hCD2 minigene

Nef 1147 transgene

IL-2 enhancer/promoter element

Nef 1147

Ligated

hCD2 minigene construct

(Blunted) HindIII Xbal

NdeI HindIII BstXI XhoI Sae II

Bam HI

Sall Xhol

Asp718

(Sal)

EcoRI

PstI

Bam HI

NdeI HindIII BstXI XhoI Sae II

(Blunted) HindIII Xbal

NdeI HindIII BstXI XhoI Sae II

Bam HI

Sall Xhol

Asp718

Apal

HindIII Xbal

PstI Sall AccI Xbal

Apal

HindIII Xbal

PstI Sall AccI Xbal

IL-2 enhancer/promoter element

mRNA

Full length transgenic protein

Non-functional hCD2 protein

Nef 1147 enhancer/promoter element

hCD2 minigene

hCD2 minigene construct

Bluescript

Nef 1147 transgene

mRNA
of exon 1 of the murine IL-2 gene, but not the ATG translation start codon. The hCD2 minigene is exactly as described above, and is included in this cassette in order to utilise the stabilising effect of both the intron and poly-A signal on the generated mRNA (R. Festenstein, personal communication).

1.3 CD2-Nef gene constructs.

A number of alleles of the nef gene from the LAV_{bu} isolate of HIV-1 were introduced into the hCD2 expression cassette (figure 12). These alleles (described below) differ at sites thought to be important for Nef function having been demonstrated to effect; (i) the localisation of the Nef protein, (ii) the phosphorylation state of the Nef protein, and (iii) the level of Nef-mediated CD4 downregulation in vitro (Guy et al., 1987, Guy et al., 1990). Therefore, by expressing these alleles in the thymocytes and mature T-cells of transgenic mice the effect of these nef mutations and the interaction of these alleles with host cell genes and immune responses can be directly assessed in vivo.

The nef alleles used in this analysis are as follows; (i) 1147-nef. This allele is the "native" nef gene that was cloned from the original LAV_{bu} isolate (Guy et al., 1987). It has been shown to downregulate CD4 in vitro, is phosphorylated at threonine 15 by protein kinase C (PKC), and can be autophosphorylated on a number of serine residues (Guy et al., 1990). This is the main allele used for these investigations. (ii) 1191-nef. This allele has a threonine to alanine amino acid substitution at position 15 that has been shown to occur both in vivo and in vitro (Alizon et al., 1986). This inhibits protein kinase C mediated phosphorylation, but preserves both in vitro CD4 downregulation and serine autophosphorylation. (iii) 3191-nef. This allele has a glycine to alanine amino acid substitution at position 2. This glycine residue is normally myristylated, allowing the Nef protein to interact with cell membranes. This allele can undergo autophosphorylation, but is barely phosphorylated by PKC and does not downregulate CD4 in vitro. (iv) 2165-nef. This allele has two amino acid substitutions in a sequence that has been proposed to have homology to the consensus GXXXXGK "P" site of GTP binding proteins (Dever et al., 1987). In all Nef isolates a conserved and related inverse sequence KGGLEG is found. In allele 2165-nef the sequence KGGLEG is changed to KGVLEA, removing the motif but preserving the secondary structure. This allele can downregulate CD4 in vitro, but only shows a low level of phosphorylation by PKC, and is not autophosphorylated. (v) 3132-nef. This allele has an four amino acid deletion at the RFDS peptide sequence (at residue 184) that resembles one of the two "G" domains (WRFD)
of GTP binding proteins (Dever et al., 1987). The 3132-nef allele does not downregulate CD4 in vitro, is not phosphorylated by PKC, and does not undergo autophosphorylation (Guy et al., 1990).

1.4 IL-2 nef gene constructs.

The wild type 1147-nef gene was also cloned into the murine IL-2 expression cassette (figure 13) in order to generate transgenic mice that would express very low levels of nef mRNA during thymic ontogeny, but would have high inducible levels of nef expression in activated peripheral T-cells. This was used to minimise the detrimental effects of nef gene expression during early thymocyte development (see results section 2.5), allowing nef gene expression in peripheral T-cells to be examined. The use of this cassette also allows a burst of nef gene expression to be generated on T-cell activation, a scenario which more accurately represents the expression pattern of the nef gene in an HIV infection (see introduction chapter 4.8).

1.5 Nef transgenic lines.

CD2-nef transgenic lines were generated by the microinjection of a 12.5kb SalI-NotI fragment into fertilised mouse oocytes. The eggs were then transferred to pseudo-pregnant females and allowed to develop to term. The offspring from these transfers were analysed by Southern blot for the presence of the transgene, and any such positive "founders" were bred on to establish non-chimeric lines. In total eleven CD2-nef transgenic lines were generated; A(1147), F(1147), Y(1147), Z(1147), B(1191), D(1191), J(3132), K(3132), M(2165), N(2165), and T(3191). DNA from these lines were analysed by Southern blot for full length transgene integration and for determination of transgene copy number. All blots were probed with an 800bp BamHI-SmaI nef fragment and a 1.2kb XbaI-NruI Thy-1 fragment as a loading control.

Figure 14 shows a Southern blot of DNA from heterozygous CD2-nef transgenic lines cut with Asp718. This enzyme cuts only once in the injected fragment approximately 220bp from the 5' end of the nef alleles. Therefore, if only one copy of the transgene integrates into the host genome two unique "end" fragments (5' and 3') would be expected. If more than one copy has integrated, in either a head-to-tail or head-to-head fashion, the Asp718 digest would generate either a single 12.5kb band or two bands of 10.2kb and 14.8kb, respectively (in addition to the
**Figure 14** Southern blot analysis of the CD2-*nef* transgenic mouse lines - Asp718.

10μg of DNA from lines A(1147), F(1147), Y(1147), Z(1147), B(1191), D(1191), K(3132), M(2165), N(2165) and T(3191), was cut with Asp718 and run on a 1% agarose/1xTAE gel. This enzyme cuts once in the *nef* transgene, enabling the full length integration of the transgene to be assessed. After blotting, the filter was hybridised with a 32P-labelled 800bp BamHI-SmaI *nef* fragment, and a 1.2kb Xbal-NruI *Thy-1* fragment (as a loading control). Bands of 12.5kb for the *nef* transgene and 10kb for the endogenous *Thy-1* gene were visualised by autoradiography. Known amounts of pCD2*nef*1147 in 10μg of non-transgenic DNA were used as copy number controls.
two "end" fragments). Figure 14 illustrates that all the lines have integrated at more than one copy, and, with the exception of line T, have all integrated in a head-to-tail fashion with bands of varying intensity at 12.5kb. The analysis of the T line shows a head-to-head orientation with a band at both 10.2kb and 14.8kb.

Figure 15 shows a Southern blot of DNA from all heterozygous CD2-nef transgenic lines digested with HindIII. This digest cuts either side of the cloned transgene, removing a 6.5kb fragment that can be detected by hybridisation with a nef-specific probe. Using a phosphorimager, the intensity of the nef bands can be adjusted for loading errors by normalisation to the 3.0kb endogenous HindIII-cut Thy-1 band. Using the copy number controls (which are known amounts of plasmid DNA that represent a specific number of transgene copies per genome) the absolute number of copies in the transgenic lines can be accurately determined. From such calculations the number of copies per genome were estimated for all heterozygous lines as follows: A(1147), 6; F(1147) 20; Y(1147) 8; Z(1147) 25; B(1191) 48; D(1191) 32; J(3132) 8; K(3132) 15; M(2165) 8; N(2165) 38; T(3191) 70.

An attempt was made to generate IL-2-1147-nef transgenic mice by the micro-injection of a 4.5kb Apal-NotI fragment from the plasmid pIL-2Nef1147 (figure 13). To date, only one transgenic line (P) of six copies has been established. However, peripheral T-cells from this line do not appear to express nef mRNA after induction with a variety of stimuli (data not shown). A plausible explanation for this lack of inducible expression may be transgene repression at the site of integration, as unlike the CD2 expression cassette, the IL-2 expression cassette does not contain a locus control region that confers position independent expression to the transgene. For this reason the injection of the IL-2-nef construct is being repeated to establish more transgenic lines.

1.6 CD2-Tat gene constructs.

The HIV-1 tat gene from the LAVren isolate was cloned into the human CD2 expression cassette in order to generate transgenic mice that expressed the Tat protein in the majority of immature thymocytes and all mature T-cells (figure 12). However, the tat gene used in these studies consists of only the first 72 amino acids out of the full 86 residues found in "native" Tat. This corresponds to the first exon of Tat, which has been demonstrated in a number of assays to retain full transactivational activity on the HIV-1 LTR and many of the observed in vitro effects on cellular genes (see introduction chapter 7.2).
10μg of DNA from lines A(1147), F(1147), Y(1147), Z(1147), B(1191), D(1191), J(3132), K(3132), M(2165), N(2165) and T(3191), was cut with HindIII and run on a 1% agarose/1xTAE gel. This enzyme removes an internal 6.5kb fragment, including the entire nef transgene, in order to accurately estimate copy number. After blotting, the filter was hybridised with a 32P-labelled 800bp BamHI-Smal nef fragment, and a 1.2kb Xbal-NruI Thy-I fragment (as a loading control). Bands of 6.5kb for the nef transgene and 3.0kb for the endogenous Thy-I gene were visualised by autoradiography. Known amounts of pCD2nef/147 in 10μg of non-transgenic DNA were used as copy number controls.
CD2-*nef* Transgenic Lines

<table>
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<tr>
<th>CD2-<em>nef</em> Transgenic Lines</th>
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**Copy Number Controls**

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**Thy I**

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1.7 *Tat* transgenic lines.

CD2-\textit{tat} transgenic lines were generated by the microinjection of a 12.5kb Sall-NotI fragment into fertilised mouse oocytes, as described above. In total, three heterozygous CD2-\textit{tat} transgenic lines were generated; C(1-72), E(1-72), and W(1-72). DNA from these lines were analysed by Southern blot for full length integration and for determination of transgene copy number. All blots were probed with an 623bp Sall-Asp718 \textit{tat} fragment and a 1.2kb Xbal-NruI Thy-1.2 fragment as a loading control.

Figure 16 shows a Southern blot of DNA from heterozygous CD2-\textit{tat} transgenic lines cut with an EcoRI/HindIII digest. These enzymes remove a 2.4kb fragment from the CD2-\textit{tat} cassette. Using the endogenous HindIII/EcoRI Thy-1.2 band (present at two copies per genome) as a loading control, and known amounts of pTZ19Tat 1-72 as copy number controls, the copy number of the three CD2-\textit{tat} transgenic lines can be determined. From such calculations the number of copies per genome were estimated for all the heterzygous lines as follows: C(1-72), 29; E(1-72), 14; W(1-72), 43.
**Figure 16**  Southern blot analysis of CD2-*tat* transgenic lines.

DNA from lines C, E, and W was digested with HindIII and EcoRI. After blotting, the filter was probed with a $^{32}$P-labelled 623bp Asp718-Sall *tat* fragment and a 1.2kb Xbal-NruI *Thy-1* fragment (the latter to identify the endogenous Thy-1 gene as a loading control). Transgenic (+) and non-transgenic (-) mice are shown for each line.
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<th>Line C</th>
<th>Line W</th>
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<td>+</td>
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</tbody>
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Thy - 1

Tat
2. The analysis of the nef-expressing transgenic lines.

2.1 The expression of the nef gene in the CD2-nef transgenic mice.

Although the microinjection of a DNA construct into a mouse oocyte can result in its integration into the host genome, this does not guarantee transgene expression. The site of integration may repress transcription due either to chromatin structure or the presence of a strong negative regulatory element. Therefore, it is important to first ascertain the level of transgene expression before the phenotype of the animals is analysed.

The expression of the CD2-nef constructs in the thymus of animals from the eleven lines of CD2-nef transgenic mice were analysed by northern blot. Figure 17A shows total thymus mRNA from lines F, Z, B, J, K, M, N, and T, probed with a 800bp nef-specific fragment. Using a phosphorimager, the intensity of the 2.2kb CD2-nef bands were quantitated and normalised for loading errors with a β-actin control (not shown). If the level of CD2-nef expression observed in line Z is given an arbitrary value of one, the relative values for transgene expression in the other CD2-nef transgenic lines are as follows; A - 1.3 (not shown), F - 10.5, Y - 2.1 (not shown), B - 3.5, D - 2.9 (not shown), J - 0.04, K - 8.7, M - 0.1, N - 0.28, T - 0.08. These values do not strictly represent the relative figures that would have been expected if CD2-nef expression had adhered tightly to transgene copy number, indicating that the hCD2 LCR effect is not 100% efficient in these transgenic lines. However, the 1147-nef and 1191-nef lines (A, F, Y, Z, B, and D) all display significant levels of transgene expression, with only line Z having an appreciably lower level of mRNA expression than would be predicted from the analysis of transgene copy number. Line K (3132-nef) also expresses to a high level, but lines J (3132-nef), M (2165-nef), N (2165-nef) and T (3191-nef) only have minimal expression of the transgene. The reason for this low level of CD2-nef mRNA expression in these latter four lines is not clear, although the Southern blot analysis of line T (figure 15 and 16), reveals a head-to-head and tail-to-tail pattern of integration. This could conceivably effect the generation of transcripts if transcription is active in both directions within the integrated transgenes.

In order to analyse the role of the nef gene during the early stages of thymic ontogeny, it was first necessary to establish that transgene expression was detectable in primitive thymocyte progenitor populations. A convenient way to analyse these populations (as low as 0.1% of total adult thymocytes) in the absence of the more mature cell types is to look at thymocyte subsets at very early stages of embryonic development. For example, at 15 days post coitus (dpc) CD4/CD8
**Figure 17** RNA expression analysis in the CD2-*nef* transgenic mice.

A. Northern blot analysis of the steady state level of *nef* mRNA in thymocytes of CD2-*nef* transgenic mice. 20μg of total thymic RNA from lines F (1147), Z (1147), B (1191), J (3132), K (3132), M (2165), N (2165), and T (3191) was separated, blotted, and hybridised with a 32P-labelled 800bp BamHI-Smal *nef* fragment. The intensity of the 2.2kb *nef* band was then quantitated using a phosphorimager, and the values normalised against β-actin as a loading control (not shown).

B. RT PCR on total thymic RNA from CD2-*nef* transgenic animals (line F) during development. The primers span intron 1 of the CD2-*nef* transgene, resulting in a 277bp fragment from the integrated transgene, and a 190bp fragment from the cDNA copy of the spliced CD2-*nef* mRNA. Time points were 14, 15, 16, and 18dpc, plus day +4 after birth and adult.
A

CD2-nef transgenic lines

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>Z</th>
<th>B</th>
<th>J</th>
<th>K</th>
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<td>2165</td>
<td>3191</td>
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Controls

Non-Tg | Nef Cell Line
-------|---------------
-VE    | +VE

CD2-nef mRNA (2.2 kb)

B

E14  E15  E16  E18  +4  AD  NTg  Tg DNA  Puc (MspI)

DNA (277bp)
RNA (190bp)
Figure 18  Western blot analysis of CD2-\textit{nef} transgenic lines.

C. Nef specific Western blot of spleen lysates from lines A, F, B and D plus a non-transgenic control, after immunoprecipitation with two Nef-specific antisera. The positive control is lysate from the Nef CRIP L cell line. After immunoprecipitation and SDS PAGE, the samples were transferred to nitrocellulose, probed with a monoclonal antibody to Nef (AE6), and visualised by ECL\textsuperscript{TM} and autoradiography.

A. Southern blot analysis of lines A, F, B and D - HindIII digest, \textit{nef} probe (see figure 15). B. RNA slot blot analysis of total thymic and splenic mRNA, \textit{nef} probe (see figure 16).
### 2.2 Thy-1 Expression

In adult CD2-nef transgenic animals, Thy-1 expression was examined and compared to non-transgenic controls. This type of investigation can highlight the presence of any developmental abnormalities in thymic ontogeny and can demonstrate whether certain cell subsets are under- or over-represented due to the expression of the transgene.

To analyze the thymocytes and peripheral T cell populations in the CD2-nef transgenic line, FACS analysis was performed on the thymus, spleen, and lymph nodes using a panel of specific fluorescent-conjugated monoclonal antibodies. Figure 19 illustrates representative...

---

**Table: Transgenic Line Copy Controls**

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<td>Thy-1</td>
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**A.**

Transgenic Line

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<tr>
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<th>Tg-</th>
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<tr>
<td>Thy-1</td>
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Thymus

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<td>Thymus</td>
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Spleen

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**C.**

Nef

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<tr>
<th>Line</th>
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<th>F</th>
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<th>D</th>
<th>Tg-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nef</td>
<td></td>
<td></td>
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</table>
double positive thymocytes have yet to be detected. Therefore, any transgene expression at this
time point must at least be from the most mature population that has developed by this stage of
thymic ontogeny, which in this case would be the CD44-, CD25- population of CD4/CD8 double
negative thymocytes.

Thymic transgene expression in the CD2-nef transgenic mice was assessed at
developmental stages from 14dpc to adulthood. As the absolute number of thymocytes at these
eye developmental stages is as low as 10^6-10^7, Northern blot analysis was not used. Instead, total
mRNA was initially reverse transcribed and then subject to PCR with nef-specific primers. Figure
17B illustrates the RT/PCR of total mRNA from 14, 15, 16, and 18dpc, plus day +4 after birth,
and adult. The sense PCR primer used in this analysis was located in the nef gene while the
antisense primer was found in the hCD2 gene. These primers span intron 1 of the CD2 expression
cassette, enabling the PCR bands for the integrated transgene DNA (277bp) and the cDNA copy
of the CD2-nef mRNA (190bp) to be distinguished. This result clearly demonstrates that nef
mRNA is present in line F at 14dpc through to adult life.

In addition, to the detection of CD2-nef mRNA in the thymocytes of the eleven lines of
transgenic mice, Western blot analysis was used to confirm the expression of Nef protein in
peripheral T-cells (eg splenocytes) from lines A, F, B, and D. Figure 18 illustrates a Nef-specific
Western blot of spleen lysates from lines A, F, B, and D, plus a non-transgenic control,
immunoprecipitated with two Nef-specific anti-sera. The positive control is lysate from the Nef
CRIP L cell line. The Western blot was probed with a monoclonal antibody to Nef (AE6).
Although the bands on the Western are faint, a 27kDa band is visible in each of the four
transgenic lysates and not in the control.

2.2 Thymocyte and T-cell populations in CD2-nef transgenic mice.

The absolute number and distribution of thymocyte and peripheral T-cell populations in
adult CD2-nef transgenic animals was examined and compared to non-transgenic controls. This
type of investigation can highlight the presence of any developmental abnormalities in thymic
ontogeny and can demonstrate whether certain cell subsets are under or over represented due to
the expression of the transgene.

To analyse the thymocyte and peripheral T-cell populations in the CD2-nef transgenic
lines, FACS analysis was performed on the thymus, spleen, and lymph nodes using a number of
specific fluorescent-conjugated monoclonal antibodies. Figure 19 illustrates representative
Figure 19  Distribution of CD4/CD8 thymocyte and T-cell subsets in lymphoid tissues of CD2-nef transgenic mice.

Thymocytes, lymphocytes and splenocytes from transgenic and non-transgenic littermates from (A) line F(1147) and (B) line B(1191), were stained for CD4 (PE-ordinate) and CD8 (FITC-abscissa). 10^4 cells were analysed using a Becton Dickinson FACScan. Relative fluorescence intensities are shown on a logarithmic scale, with percentages of CD4/CD8 double negative, CD4/CD8 double positive, CD4 single positive and CD8 single positive populations indicated.
CD4/CD8 FACS plots of thymus, spleen, and lymph node for line F(1147) and line B(1191) compared with non-transgenic controls. This clearly illustrates that in the thymus of both lines of transgenic mice the percentage of mature CD4 and CD8 single positive (SP) thymocytes are reduced when compared with negative littermates. To fully examine the distribution of thymocyte cell subsets, CD4/CD8 subset analysis was performed on 27 litters from the eleven lines of mice, using at least three non-transgenic and three transgenic animals per experiment (tables 3 and 4). The analysis of lines M, N, J, K, and T showed no differences in the proportion of all thymic and peripheral T-cell subsets when compared with non-transgenic littermates (table 4). In the case of lines M, N, J, and T, this may be the consequence of the very low level of transgene expression. However, line K expresses the 3132-nef transgene at a high level, but no effect on the distribution or proportion of developing thymocytes is observed.

The six lines that express either the 1147-nef or 1191-nef alleles, A, F, Y, Z, B, and D, show distinct, reproducible and statistically significant differences in certain thymocyte populations when compared with the non-transgenic controls. Table 3 illustrates that when all litters are analysed, a statistically significant decrease (p < 0.05) in the percentage of transgenic CD4 SP thymocytes is observed. When the absolute number of cells in this thymic subset was calculated, the statistically significant depletion of this population was confirmed (table 5 for examples from line F and line D).

The results from this analysis also demonstrate (when compared with non-transgenic controls) a partial depletion in the CD8 SP thymocyte population in all 1147-nef and 1191-nef transgenic lines although this decrease is not as severe as that observed for the CD4 SP subset. The absolute number of CD4/CD8 double negative (DN) thymocytes remained similar in all lines, and the absolute number of CD4/CD8 double positive (DP) cells showed a small reduction in transgenic animals (table 5 for line D).

Line F is a notable exception to the above observations as this line displays a far more severe phenotype. The F line shows a severe reduction in thymic cellularity (discussed in more detail in section 2.5), having as few as 30% of the total number of thymocytes that are present in non-transgenic littermate controls. All other 1147-nef or 1191-nef lines do not have such a decrease in thymic cell number, although there is a consistent trend for transgenic thymi to display an approximate 10% reduction in size (table 5 example from line D). When the extent of the decrease in thymic cellularity in line F is examined, severe reductions in the percentage and absolute number of both the CD4 SP and CD8 SP thymocyte subsets, and a marked decrease in the absolute number of cells in the CD4/CD8 DP population, are observed. However, the number of cells in the transgenic CD4/CD8 DN cell population remains very similar to that observed in
Table 3  CD4/CD8 T-cell subset changes in CD2-\textit{nef} transgenic thymocytes (lines A, F, Y, Z, B, and D).

Thymocytes from three transgenic and three non-transgenic mice from various aged litters of lines A, F, B, D, Y, and Z, were stained with anti-CD4 and anti-CD8 mAbs. 10^4 cells were analysed on a Becton Dickinson FACScan. CD4/CD8 double negative, double positive and single positive populations were assessed as a percentage of total thymocytes. The mean values for the percentage of these populations is displayed in the table, with the standard deviation of the mean below.
CD4/CD8 SUBSET CHANGES IN CD2-NEF TRANSGENIC THYMOCYTES

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<th>%CD4 / CD8</th>
<th>%CD8 SP</th>
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<td>Nef Transgene</td>
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<tr>
<td><strong>Age</strong></td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17.5 wk.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1191-D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 wk.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0 wk.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0 wk.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

H 91-P

3.0 wk.

11.0 wk.

128
Table 4  CD4/CD8 T-cell subset changes in CD2-nef transgenic thymocytes (lines J, K, M, N, and T).

Thymocytes from three transgenic and three non-transgenic mice from various aged litters of lines M, N, J, K, and T, were stained with anti-CD4 and anti-CD8 mAbs. 10^4 cells were analysed on a Becton Dickinson FACScan. CD4/CD8 double negative, double positive and single positive populations were assessed as a percentage of total thymocytes. The mean values for the percentage of these populations is displayed in the table, with the standard deviation of the mean below.
### CD4/CD8 Subset Changes in CD2-NEF Transgenic Thymocytes

<table>
<thead>
<tr>
<th>Thymocyte Subset</th>
<th>Nef Transgene</th>
<th>%CD4 SP</th>
<th>%CD4 /CD8</th>
<th>%CD8 SP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2165-M</td>
<td>5.0 wk.</td>
<td>12.2±2.3</td>
<td>11.7±1.6</td>
</tr>
<tr>
<td></td>
<td>10.0 wk.</td>
<td>13.0±2.6</td>
<td>14.1±1.9</td>
<td>79.9±2.7</td>
</tr>
<tr>
<td></td>
<td>2165-N</td>
<td>3.0 wk.</td>
<td>18.9±4.2</td>
<td>13.0±2.7</td>
</tr>
<tr>
<td></td>
<td>9.0 wk.</td>
<td>12.9±2.0</td>
<td>13.3±0.4</td>
<td>78.9±23.2</td>
</tr>
<tr>
<td></td>
<td>3132-L</td>
<td>7.0 wk.</td>
<td>10.4±0.4</td>
<td>9.0±2.8</td>
</tr>
<tr>
<td></td>
<td>3132-K</td>
<td>9.0 wk.</td>
<td>7.7±0.7</td>
<td>9.9±1.2</td>
</tr>
<tr>
<td></td>
<td>12.0 wk.</td>
<td>11.0±3.1</td>
<td>10.2±0.9</td>
<td>77.0±4.0</td>
</tr>
<tr>
<td></td>
<td>3191-T</td>
<td>6.0 wk.</td>
<td>12.5±1.2</td>
<td>13.8±2.1</td>
</tr>
</tbody>
</table>

*Data presented as mean ± standard deviation.*
Table 5  Absolute number of thymocytes in CD4/CD8 T-cell subsets in representative litters of line F and line D.

Representative litters from line F(1147) and line D(1191) are shown in this table. Total thymocytes from three transgenic and three non-transgenic littermates were isolated and accurately counted. 10⁴ of these thymocytes were then analysed by CD4/CD8 FACScan analysis. Using the percentages of the various thymocyte populations, the absolute number of CD4/CD8 double negative, double positive and single positive cells were calculated for each individual mouse and averaged per litter (with the standard deviation of the mean indicated). Statistical analysis by two-paired t-test reveal significant differences between absolute numbers of non-transgenic and transgenic CD4 SP cells for line F (p < 0.001) and line D (P < 0.02), plus DP and CD8 SP cells for line F (both p < 0.05).
Absolute number of thymocytes (x10^6) in thymic subsets in adult CD2-nef transgenic mice

<table>
<thead>
<tr>
<th></th>
<th>TOTAL</th>
<th>CD4-/CD8- (DN)</th>
<th>CD4+/CD8+ (DP)</th>
<th>CD4+ (SP)</th>
<th>CD8+ (SP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1147-F Non-Tg</td>
<td>220 ±28</td>
<td>7.4 ±1.1</td>
<td>181 ±27</td>
<td>25.4 ±1.7</td>
<td>6.7 ±0.7</td>
</tr>
<tr>
<td>Tg</td>
<td>62 ±29</td>
<td>5.7 ±2.3</td>
<td>56 ±27</td>
<td>1.7 ±1.1</td>
<td>0.5 ±0.2</td>
</tr>
<tr>
<td>1191-D Non-Tg</td>
<td>218 ±23</td>
<td>14.2 ±1.8</td>
<td>173 ±21</td>
<td>24.4 ±3.4</td>
<td>6.5 ±1.3</td>
</tr>
<tr>
<td>Tg</td>
<td>184 ±34</td>
<td>11.0 ±2.9</td>
<td>154 ±28</td>
<td>15.5 ±2.4</td>
<td>4.2 ±1.2</td>
</tr>
</tbody>
</table>
littermate controls (table 5 and see later section, figures 25 and 26). The percentage and absolute number of cells in the peripheral lymphoid organs of line F were also dramatically reduced when compared with non-transgenic controls (figure 19 and table 6). In contrast to the other 1147-nef or 1191-nef lines of transgenic mice, the F line displayed a marked reduction in CD4+ peripheral T-cells, from an average of about 51% of total lymph node cells in the control animals to an average of approximately 15% in the transgenic animals (table 6). A decrease in the percentage and absolute number of peripheral CD8+ T-cells was also observed, from an average of 24% in the controls to an average of approximately 9% in the transgenic mice (table 6).

As discussed above, the expression of the transgene in line F is at least three times greater than the expression of the transgene in the other 1147-nef or 1191-nef transgenic lines, which may account for the dramatic phenotype of this 1147-nef transgenic mouse line.

2.3 Downregulation of cell surface CD4 during thymic ontogeny.

It has previously been demonstrated that the in vitro expression of the nef gene results in the downregulation of cell-surface CD4 (Guy et al., 1987, Guy et al., 1990). FACS analysis was therefore used to determine the level of cell surface expression of the T-cell surface antigens CD4, CD8, CD3ε, and Thy-1 on thymocytes, splenocytes and lymph node cells from the CD2-nef transgenic animals. Figure 20 shows representative histogram plots of thymocytes from line B and line F. Thymocytes from all of the 1147-nef and 1191-nef transgenic lines displayed a significant decrease in the cell surface expression of CD4 and a less pronounced decrease in the cell surface expression of CD8 (table 7). The cell surface levels of CD3ε and Thy-1 remained unchanged (data not shown), although the percentage of thymocytes displaying a CD3ε high phenotype was reduced in transgenic animals (corresponding to the loss of CD4 and CD8 SP cells, which both express high levels of CD3ε).

To further characterise the reduction in both cell surface CD4 and CD8, the CD4 SP, the CD8 SP, and the CD4/CD8 DP population of thymocytes from 27 litters of the eleven transgenic lines were independently analysed for cell surface expression of these two markers (table 7 and 8). The M, N, J, K, and T lines showed no consistent or significant difference in the cell surface level of either CD4 or CD8 expression (table 8). However, lines A, F, Y, Z, B, and D all showed a similar phenotype (table 7). All the lines displayed a dramatic cell surface downregulation of CD4 in the CD4/CD8 double positive population of thymocytes (ie an average 36%, 71%, 42%, 38%, 38% and 37% reduction of cell surface CD4 from non-transgenic levels
Figure 20  Levels of cell-surface expression of CD4, CD8, CD3ε, and Thy-1 on thymocytes from CD2-nef transgenic mice.

Thymocytes from transgenic and non-transgenic littermates from (A) line F(1147) and (B) line B(1191) were stained with fluorescent-labelled anti-CD4, anti-CD8, anti-CD3ε or anti-Thy-1 monoclonal antibodies, and analysed using a Becton Dickinson FACScan. Histogram plots show number of cells (ordinate) and intensity of fluorescence (log scale, abscissa) for non-transgenic cells (thin line), transgenic cells (thick line) and no stain controls (dotted line).
Table 6  
CD4/CD8 subset changes in CD2-\textit{nef} transgenic lymph node cells (lines A, F, B, and D).

Lymphocytes from three transgenic and three non-transgenic mice from various aged litters of lines A, F, B, and D, were stained with anti-CD4 and anti-CD8 mAbs. $10^4$ cells were analysed on a Becton Dickinson FACScan. The proportion of CD4 and CD8 single positive T-cells were determined as a percentage of total lymphocytes. The mean values for the percentage of these populations is displayed in the table, with the standard deviation of the mean below.
### CD4/CD8 Subset Changes in CD2-NEF Transgenic Lymphocytes

<table>
<thead>
<tr>
<th>Transgenic subset</th>
<th>CD4+ SP</th>
<th>CD8+ SP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1147-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 wk.</td>
<td>58.4%</td>
<td>54.8%</td>
</tr>
<tr>
<td></td>
<td>±4.9</td>
<td>±3.6</td>
</tr>
<tr>
<td>8.0 wk.</td>
<td>51.3%</td>
<td>47.8%</td>
</tr>
<tr>
<td></td>
<td>±4.4</td>
<td>±3.4</td>
</tr>
<tr>
<td>13.5 wk.</td>
<td>53.1%</td>
<td>44.2%</td>
</tr>
<tr>
<td></td>
<td>±3.3</td>
<td>±4.1</td>
</tr>
<tr>
<td>14.5 wk.</td>
<td>49.4%</td>
<td>47.0%</td>
</tr>
<tr>
<td></td>
<td>±3.8</td>
<td>±7.1</td>
</tr>
<tr>
<td>1147-B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 wk.</td>
<td>49.0%</td>
<td>10.0%</td>
</tr>
<tr>
<td></td>
<td>±4.6</td>
<td>±1.4</td>
</tr>
<tr>
<td>7.0 wk.</td>
<td>55.0%</td>
<td>11.8%</td>
</tr>
<tr>
<td></td>
<td>±4.0</td>
<td>±2.8</td>
</tr>
<tr>
<td>9.0 wk.</td>
<td>49.4%</td>
<td>22.4%</td>
</tr>
<tr>
<td></td>
<td>±3.9</td>
<td>±7.9</td>
</tr>
<tr>
<td>1191-B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 wk.</td>
<td>51.1%</td>
<td>43.1%</td>
</tr>
<tr>
<td></td>
<td>±4.6</td>
<td>±4.4</td>
</tr>
<tr>
<td>12.0 wk.</td>
<td>49.7%</td>
<td>49.3%</td>
</tr>
<tr>
<td></td>
<td>±2.1</td>
<td>±2.5</td>
</tr>
<tr>
<td>17.5 wk.</td>
<td>45.1%</td>
<td>30.0%</td>
</tr>
<tr>
<td></td>
<td>±4.4</td>
<td>±6.9</td>
</tr>
<tr>
<td>1191-D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 wk.</td>
<td>49.0%</td>
<td>50.0%</td>
</tr>
<tr>
<td></td>
<td>±3.3</td>
<td>±3.0</td>
</tr>
<tr>
<td>6.0 wk.</td>
<td>49.9%</td>
<td>37.8%</td>
</tr>
<tr>
<td></td>
<td>±4.1</td>
<td>±1.3</td>
</tr>
</tbody>
</table>
Table 7  Cell surface marker downregulation in CD2-nef transgenic thymocytes (lines A, F, Y, Z, B, and D).

10^4 thymocytes from three transgenic and three non-transgenic animals from various aged litters of lines A, F, B, D, Y, and Z, were analysed by CD4/CD8 FACScan analysis. CD4/CD8 double positive and single positive populations were isolated and then a single parameter (either CD4 or CD8) was displayed as a histogram. The average fluorescence intensity was obtained for each population and then averaged for transgenic or non-transgenic animals. This table shows the percentage reduction or increase in the average fluorescence intensity of the transgenic mice compared to the non-transgenic controls.
## Table: % Change in CD4 and CD8 Levels

<table>
<thead>
<tr>
<th>T cell Subset</th>
<th></th>
<th>% Change in CD4 Levels</th>
<th>% Change in CD8 Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age</td>
<td>SP</td>
<td>DP</td>
</tr>
<tr>
<td>1147-A</td>
<td>2.0 wk.</td>
<td>-25.4</td>
<td>-41.6</td>
</tr>
<tr>
<td></td>
<td>8.0 wk.</td>
<td>+2.7</td>
<td>-16.9</td>
</tr>
<tr>
<td></td>
<td>13.5 wk.</td>
<td>-21.8</td>
<td>-39.7</td>
</tr>
<tr>
<td></td>
<td>14.5 wk.</td>
<td>-21.2</td>
<td>-44.2</td>
</tr>
<tr>
<td>1147-F</td>
<td>5.0 wk.</td>
<td>-46.0</td>
<td>-72.1</td>
</tr>
<tr>
<td></td>
<td>7.0 wk.</td>
<td>-38.2</td>
<td>-73.4</td>
</tr>
<tr>
<td></td>
<td>9.0 wk.</td>
<td>-30.9</td>
<td>-68.9</td>
</tr>
<tr>
<td>1147-Y</td>
<td>6.0 wk.</td>
<td>-27.2</td>
<td>-47.9</td>
</tr>
<tr>
<td></td>
<td>10.0 wk.</td>
<td>+10.4</td>
<td>-27.3</td>
</tr>
<tr>
<td></td>
<td>14.0 wk.</td>
<td>-15.7</td>
<td>-51.0</td>
</tr>
<tr>
<td>1147-Z</td>
<td>6.0 wk.</td>
<td>-7.0</td>
<td>-33.0</td>
</tr>
<tr>
<td></td>
<td>11.0 wk.</td>
<td>-12.8</td>
<td>-42.0</td>
</tr>
<tr>
<td>1191-B</td>
<td>3.0 wk.</td>
<td>N/C</td>
<td>-33.8</td>
</tr>
<tr>
<td></td>
<td>6.0 wk.</td>
<td>-1.4</td>
<td>-29.4</td>
</tr>
<tr>
<td></td>
<td>12.0 wk.</td>
<td>-21.8</td>
<td>-42.8</td>
</tr>
<tr>
<td></td>
<td>17.5 wk.</td>
<td>-27.6</td>
<td>-47.2</td>
</tr>
<tr>
<td>1191-D</td>
<td>5.0 wk.</td>
<td>-0.01</td>
<td>-36.0</td>
</tr>
<tr>
<td></td>
<td>6.0 wk.</td>
<td>+5.0</td>
<td>-36.0</td>
</tr>
<tr>
<td></td>
<td>7.0 wk.</td>
<td>-16.9</td>
<td>-40.6</td>
</tr>
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</table>
Table 8  
Cell surface marker downregulation in CD2-\textit{nef} transgenic thymocytes (lines J, K, M, N, and T).

$10^4$ thymocytes from three transgenic and three non-transgenic animals from various aged litters of lines M, N, J, K, and T, were analysed by CD4/CD8 FACScan analysis. CD4/CD8 double positive and single positive populations were isolated and then a single parameter (either CD4 or CD8) was displayed as a histogram. The average fluorescence intensity was obtained for each population and then averaged for transgenic or non-transgenic animals. This table shows the percentage reduction or increase in the average fluorescence intensity of the transgenic mice compared to the non-transgenic controls.
### CELL SURFACE MARKER DOWNREGULATION IN CD2-NEF TRANSGENIC THYMOCYTES

<table>
<thead>
<tr>
<th>T cell Subset</th>
<th>% CHANGE IN CD4 LEVELS</th>
<th>% CHANGE IN CD8 LEVELS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SP</td>
<td>DP</td>
</tr>
<tr>
<td><strong>Ages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2165-M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 wk.</td>
<td>+2.5</td>
<td>+6.0</td>
</tr>
<tr>
<td>10.0 wk.</td>
<td>-4.0</td>
<td>-9.4</td>
</tr>
<tr>
<td>2165-N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 wk.</td>
<td>+27.0</td>
<td>-1.3</td>
</tr>
<tr>
<td>9.0 wk.</td>
<td>-12.8</td>
<td>-9.9</td>
</tr>
<tr>
<td>3132-L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0 wk.</td>
<td>-12.0</td>
<td>-5.6</td>
</tr>
<tr>
<td>3132-K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0 wk.</td>
<td>-1.0</td>
<td>-3.0</td>
</tr>
<tr>
<td>12.0 wk.</td>
<td>-11.0</td>
<td>+5.9</td>
</tr>
<tr>
<td>3191-T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0 wk.</td>
<td>+1.0</td>
<td>-10.9</td>
</tr>
</tbody>
</table>
Table 9  Cell surface marker downregulation in CD2-\textit{nef} transgenic lymph node cells (lines A, F, B, and D).

$10^4$ lymphocytes from three transgenic and three non-transgenic animals from various aged litters of lines A, F, B, and D, were analysed by CD4/CD8 FACScan analysis. After the CD4 and CD8 single positive populations were identified, a single parameter (either CD4 or CD8) was displayed as a histogram. The average fluorescence intensity was obtained for each population and then averaged for transgenic or non-transgenic animals. This table shows the percentage reduction or increase in the average fluorescence intensity of the transgenic mice compared to the non-transgenic controls.
## CELL MARKER DOWNREGULATION IN CD2-NEF TRANSGENIC LYMPHOCYTES

<table>
<thead>
<tr>
<th>Age</th>
<th>% Change in CD4 Levels on CD4+ T Cells</th>
<th>% Change in CD8 Levels on CD8+ T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1147-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 Wk.</td>
<td>+15.7%</td>
<td>+3.9%</td>
</tr>
<tr>
<td>8.0 Wk.</td>
<td>-10.9%</td>
<td>N/C</td>
</tr>
<tr>
<td>13.5 Wk.</td>
<td>-32.0%</td>
<td>-5.4%</td>
</tr>
<tr>
<td>14.5 Wk.</td>
<td>-5.5%</td>
<td>-6.5%</td>
</tr>
<tr>
<td>1147-P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 Wk.</td>
<td>-46.1%</td>
<td>-21.0%</td>
</tr>
<tr>
<td>7.0 Wk.</td>
<td>-12.7%</td>
<td>+9.0%</td>
</tr>
<tr>
<td>9.0 Wk.</td>
<td>-13.1%</td>
<td>N/C</td>
</tr>
<tr>
<td>1191-B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 Wk.</td>
<td>-12.8%</td>
<td>-1.9%</td>
</tr>
<tr>
<td>12.0 Wk.</td>
<td>+2.5%</td>
<td>+24.6%</td>
</tr>
<tr>
<td>17.5 Wk.</td>
<td>-5.4%</td>
<td>-4.2%</td>
</tr>
<tr>
<td>1191-D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 Wk.</td>
<td>-20.5%</td>
<td>-19.9%</td>
</tr>
<tr>
<td>6.0 Wk.</td>
<td>-11.6%</td>
<td>-23.6%</td>
</tr>
</tbody>
</table>
in CD4/CD8 DP thymocytes from line A, F, Y, Z, B and D respectively). In addition, the cell-surface level of CD8 expression was also reduced on the CD4/CD8 DP population. However, this decrease was not as dramatic as that observed for CD4 cell-surface downregulation in any of the six lines (ie an average 17%, 28%, 14%, 18%, 16% and 14% reduction of cell surface CD8 from non-transgenic levels in CD4/CD8 DP thymocytes from line A, F, Y, Z, B and D respectively).

The cell-surface expression of CD4 and CD8 in the single positive populations of thymocytes were also affected. In all the 1147-nef and 1191-nef transgenic lines, a reduction of cell-surface CD4 in the CD4 SP subset was observed, although this was not as severe as the cell-surface CD4 downregulation seen in the DP population of cells from the same litter (ie. an average 16%, 38%, 11%, 10%, 13% and 4% reduction of cell surface CD4 from non-transgenic levels in CD4 SP thymocytes from line A, F, Y, Z, B and D respectively). The level of cell-surface CD8 expression on CD8 SP thymocytes did not show a consistent decrease in any line except line F, where again the percentage reduction from littermate controls was less than that observed for CD8 on the corresponding DP population (an average 32% reduction in the DP population, compared with an average 21% reduction in the CD8 SP population).

The cell surface level of CD4 and CD8 expression was also examined on peripheral T-cells from the lymph nodes of lines A, F, B, and D (table 9). All the lines showed a decrease in peripheral CD4 cell-surface expression in the majority of litters analysed (eg an average 8%, 24%, 5% and 14% reduction of cell surface CD4 in peripheral CD4+ T-cells from lines A, F, B, and D respectively, when compared with non-transgenic controls). However, no consistent or significant decrease in the cell surface expression of CD8 in the peripheral CD8+ T-cell compartment was observed.

2.4 Intracellular sequestration of CD4

The Nef-mediated downregulation of CD4 has been suggested to occur at a post-translational level as steady state levels of CD4 mRNA and CD4 protein were shown to remain constant in the presence of Nef (Garcia & Miller 1991). These studies were performed in cell lines in vitro. Therefore, in order to assess the mechanism of CD4 downregulation in vivo, indirect immunofluorescence was used to determine the cellular location of CD4 in transgenic thymocytes. Figure 21 shows non-transgenic (A) and transgenic (B) permeablised thymocytes from line F, stained with an antibody specific for murine CD4. The non-transgenic thymocytes display an intense staining pattern as a ring around the surface of the cell. In contrast, the
Figure 21  Indirect immunofluorescence staining of permeabilised thymocytes from line F.

(A) Non-transgenic and (B) transgenic thymocytes from line F littermates were stained with (1) an anti-CD4 rat mAb, followed by (2) an anti-rat FITC-conjugated mAb. Cells were visualised using a Zeiss Axiophot fluorescence microscope and 15 second exposures were taken. (C) Control thymocytes from a non-transgenic littermate, carried through the staining procedure without the addition of either antibody and (D) control thymocytes from a transgenic littermate, stained with only the second layer antibody (FITC anti-rat Ig). Exposures of (C) and (D) were 30 seconds.
**Figure 22** Subcellular compartmentalisation of CD4 in transgenic thymocytes.

Permeabilised thymocytes from line F transgenic mice were double stained, first with an anti-CD4 rat mAb, detected via a FITC-conjugated anti-rat Ig mAb (A) and (C), followed by either (B) a rabbit anti-golgi (anti-α-mannosidase II) antibody or, (D) a rabbit anti-ER (anti-ERC55) antibody, detected via a Texas-red conjugated anti-rabbit Ig antibody. The cells were visualised using a Zeiss Axiophot fluorescence microscope under filters that activated either the FITC or Texas-red conjugate. The same field of cells was then photographed under both colours to reveal the independent staining patterns of CD4 and the Golgi or ER markers.
intensity of surface CD4 staining is significantly reduced in the transgenic thymocytes. Transgenic thymocytes display intracellular CD4 staining localising to a distinct compact region just under the plasma membrane. This confirms the FACS data indicating that CD4 is downregulated from the surface of these thymocytes and suggests that the CD4 is sequestered to an intracellular compartment.

The nature of this intracellular sequestration of CD4 was investigated further using double staining experiments. An anti-CD4 FITC-conjugated antibody was used in combination with Texas-red-conjugated antibodies specific for either the Golgi apparatus or the endoplasmic reticulum (ER). Figure 22 shows that the intracellular staining pattern of CD4 (A and C) colocalises with the specific region stained by the Golgi marker (B) but not with the marker for the ER (D). However, as the cytoplasm of these cells is small and the cellular organelles are closely packed in the perinuclear region, an endosomal location for the downregulated CD4 cannot be dismissed.

2.5 Severely reduced thymic cellularity in CD2-nef transgenic mice.

As described above, thymocytes from line F(1147) express high levels of the nef transgene resulting in a more pronounced phenotype. However, it is not a phenotype that is inconsistent with the phenotypes of the lines where nef expression is much lower. For example, CD4/CD8 FACS analysis on all 1147-nef and 1191-nef transgenic lines allows two general observations to be made; (i) there is a decrease in the average fluorescence intensity of cell-surface CD4 (and to a lesser extent CD8), on CD4/CD8 double positive thymocytes, and (ii) there is a dramatic reduction in the percentage and absolute number of the CD4 SP and the CD8 SP mature thymocyte populations. However, an additional phenotype, a significant decrease in thymic cellularity, was also observed in the high-expressing F line which was only barely evident in the lines of mice where nef expression was much lower. On average, the thymus of a transgenic animal from line F had between 30-40% of the total number of thymocytes that were observed in the thymus of a non-transgenic littermate. In lines A, B, D, Y, and Z, a decrease in total thymocyte cell number was barely detectable, with (on average) transgenic animals still generating approximately 90% of the number of thymocytes that were seen in non-transgenic controls (table 5 for an example from line D). Therefore, due to the very pronounced nature of this phenotype in line F, mice from this line were used to investigate the mechanisms underlying the loss of thymocytes in the CD2-nef transgenic mice.
An obvious first assumption would be that this reduction in thymic cell number was related to, or a consequence of, the downregulation of cell surface CD4 or CD8 expression. However, previous studies have indicated that mice that are homozygous for a non-functional CD4 or CD8 gene have a comparable thymic cell number to wild type mice (Rahemtulla et al., 1991). It is therefore reasonable to assume that the reduction of functional CD4 on the surface of thymocytes per se is not directly related to the severe reduction in thymic cellularity that is seen in the CD2-nef transgenic mice.

To further understand the nature of the decrease in thymic cellularity in the CD2-nef transgenic mice, thymocyte cell number was investigated at various stages of embryonic and neonatal development. Timed transgenic pregnancies were taken at 14, 15, 16, 17, and 18dpc, and at days +1, +7, and +16 after birth. At each time point the thymus and spleen of all littermates were removed, cell counts performed, and the cells examined by CD4/CD8 FACS analysis.

Figures 23 and 24 show the CD4/CD8 FACS plots obtained over this time course for thymocytes and splenocytes respectively. The CD4/CD8 FACS plots from transgenic and non-transgenic thymocytes from 14dpc and 15dpc are very similar. However, as soon as the CD4/CD8 double positive population is observed (ie between 15dpc and 16dpc), the level of cell surface CD4 is noticeably downregulated on these cells. This phenotype is then maintained throughout the remaining days of embryogenesis, through the neonatal stages, and on into adult life. Therefore, as nef expression has been observed as early as 14dpc (figure 17B), this suggests that Nef-mediated downregulation of CD4 occurs from the moment that the CD4 molecule first appears on the CD4/CD8 double positive thymic population.

The CD4/CD8 splenocyte plots for littermates from line F (figure 24) clearly indicate that for non-transgenic animals the spleen does not harbour mature CD4+ or CD8+ T-cells until birth. This is consistent with the observation that in the thymus mature CD4 and CD8 SP thymocytes are not observed until 18dpc (figure 23). After birth the T-cell compartment of the spleen of normal mice gradually increases until in the adult it reaches approximately 15% of the total number of splenocytes. In CD2-nef transgenic animals, the lack of mature CD4 and CD8 single positive cells being generated by the thymus results in only a few peripheral T-cells filling the spleen after birth. As the animal ages, this percentage does increase but never reaches the level observed in adult non-transgenic animals, the T-cell percentage in the spleen having a maximum of 2.5%.

In addition to the qualitative analysis of the CD4/CD8 FACS plots in figure 23 and 24, the proportion of cells in the CD4/CD8 double negative, CD4/CD8 double positive, CD4 single
Figure 23  Distribution of CD4/CD8 thymocyte subsets in CD2-nef transgenic mice during development.

Thymocytes from transgenic and non-transgenic littermates from line F(1147) were stained for CD4 (PE-ordinate) and CD8 (FITC-abscissa). 10⁶ cells were analysed using a Becton Dickinson FACScan. Relative fluorescence intensities are shown on a logarithmic scale. Quadrants have been set for the CD4/CD8 populations of the non-transgenic animals. These precise quadrants are also used on the transgenic plots as an indication of the downregulation of cell-surface expression of both CD4 and CD8 when compared to the controls. Time points used were 14, 15, 16, 17, and 18dpc, plus day +1, +7, and +16 after birth, and adult.
Figure 24  Distribution of CD4/CD8 splenocyte subsets in CD2-nef transgenic mice during development.

Splenocytes from transgenic and non-transgenic littermates from line F(1147) were stained for CD4 (PE-ordinate) and CD8 (FITC-abscissa). $10^6$ cells were analysed using a Becton Dickinson FACScan. Relative fluorescence intensities are shown on a logarithmic scale. Time points used were 14, 15, 16, 17, and 18dpc, plus day +1, +7, and +16 after birth, and adult.
positive and CD8 single positive populations at each time point during development were
determined. When this information is combined with the total cell number for the thymus, the
absolute number of thymocytes per mouse in each of these populations can be calculated (table
10 and figures 25 and 26). Figure 25 shows a graph of total thymic cell number against age of
litter from 14dpc through to adulthood. This demonstrates that a significant reduction in the
absolute number of thymocytes is first detectable at 16dpc. By the next day (17dpc) the total
thymocyte number in the transgenic animals is reduced to approximately 30% of the controls, a
level which is maintained into the adult life (table 10). Therefore, the point at which a difference
in thymic cell number in the transgenic animals is first observed (16dpc), coincides with the initial
appearance of the CD4/CD8 double positive thymocyte population.

The absolute number of the four major thymocyte populations; CD4/CD8 DN, CD4/CD8
DP, CD4 SP and CD8 SP, from 14dpc to adulthood are illustrated graphically in figure 26 (A-D)
(see also table 10). Figure 26A shows the CD4/CD8 double negative compartment. The absolute
number of DN cells in the transgenic animals is slightly lower than that observed for the
littermate controls throughout the time course of the experiment. This reduction is more
pronounced as the animal gets older. However, this decrease (to 80-90% of the controls) does not
even begin to approach the decrease (to 30-40% of the controls) observed for total thymocytes
(figure 25).

Figure 26B shows the CD4/CD8 double positive thymocyte compartment. These results
demonstrate that the transgenic animals display a severely reduced number of DP cells from the
earliest point at which they are observed (16dpc). This graph closely resembles the graph of total
cell number (figure 25), with the average number of transgenic DP thymocytes being equivalent
to approximately 30% of that observed in control animals. This phenotype is maintained from
16/17dpc right through to adult life.

Figures 26C and 26D show the absolute number of CD4 SP and CD8 SP thymocyte cell
populations respectively. The absolute cell number for both the CD4 and CD8 SP populations
show a very severe reduction in transgenic animals, from the time point at which they first appear
(18dpc) to adulthood. On average there are 10-fold fewer CD4 SP cells and 7.5-fold fewer CD8
SP cells in the transgenic thymi, with these values being maintained at more or less the same level
throughout the time points studied. The extent of this fold decrease in both these populations is
far higher than the fold decrease observed in the DP population (~ 3-fold, figure 26B). Therefore,
the fact that the fold reductions for the CD4 and CD8 SP subsets are 2-3 times greater, suggests
that either the depletion of DP cells is biased toward cells that would mature to the single positive
stages, or that a second deleterious effect is occurring at the transitional step between the DP
**Figure 25**  Total number of thymocytes in transgenic and non-transgenic littermates (line F) during development.

Total thymocytes from at least three transgenic and three non-transgenic littermates from line F(1147) were isolated and counted. The mean of these cell counts (ordinate) were plotted against the age of litter (abscissa) with the standard deviation of the mean displayed.
Figure 26  Absolute number of CD4/CD8 double negative, double positive and single positive thymocytes in transgenic and non-transgenic littermates (line F) during development.

Total thymocytes from at least three transgenic and three non-transgenic littermates from line F(1147) were isolated and counted. $10^4$ thymocytes per mouse were then analysed by CD4/CD8 FACScan analysis. The absolute number of CD4/CD8 double negative, double positive and single positive cells were calculated for each individual mouse and averaged per litter. These means were then plotted (ordinate) against the age of litter (abscissa) with the standard deviation of the mean displayed. (A) CD4/CD8 double negative thymocytes, (B) CD4/CD8 double positive thymocytes, (C) CD4 single positive thymocytes, (D) CD8 single positive thymocytes. Time points used were 14, 15, 16, 17, and 18dpc, plus day +1, +7, and +16 after birth, and adult.
A. Absolute number of DN

B. Absolute number of DP

C. Absolute number of CD4 SP

D. Absolute number of CD8 SP
Table 10  Absolute number of total thymocytes and CD4/CD8 thymocyte populations during development in CD2-nef (line F) transgenic mice.

Total thymocytes from three transgenic and three non-transgenic littermates were isolated and counted. 10^4 thymocytes per mouse were then analysed by CD4/CD8 FACScan analysis. The absolute number of CD4/CD8 double negative, double positive and single positive cells were calculated for each individual mouse and averaged per litter (with the standard deviation of the mean indicated). Time points used were 14, 15, 16, 17, and 18dpc, plus day +1, +7, and +16 after birth, and adult.
### Total Thymocytes

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<th>CD4+ CD8+ (DP)</th>
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<th>CD8+ (SP)</th>
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<td>0.18 ± 0.06</td>
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<td>53.0 ± 5.0</td>
<td>1.60 ± 1.5</td>
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(All values = cell number x 10^-6)
population and the two SP populations.

2.6 Is the reduction in thymic cellularity a consequence of an increase in apoptosis?

It is clear from the CD4/CD8 FACS analysis that a severe reduction in the absolute number of CD4/CD8 DP thymocytes is the major contributing factor to the dramatic decrease in thymic cellularity in the transgenic animals. A possible explanation for this reduction in the DP population would be an increase in the rate of programmed cell death (PCD) or apoptosis. Apoptosis is a well characterised cellular phenomenon (McConkey, 1990b). It requires protein synthesis and is associated with an endogenous endonuclease activity, cleaving the cellular genome into oligonucleosomal length DNA fragments of approximately 200bp (McConkey, 1990b). Apoptosis is a common process in the regulation of the development of the immune system. For example, PCD is the fate of CD4/CD8 DP thymocytes which are negatively selected during thymic ontogeny, or which are not positively selected (Blackman et al., 1990). Therefore, it is possible that the expression of the nef gene during thymocyte differentiation could mediate the inappropriate induction of apoptosis, leading to a decrease in thymic cellularity.

As apoptosis is a well regulated cellular process, the detection of events that are associated with this phenomenon allows an assessment of the level of PCD to be estimated. To analyse the level of apoptosis in the CD2-nef transgenic mice, two independent approaches have been used. The first is illustrated in figure 27 and uses FACS analysis to detect the level of intracellular propidium iodide (PI) staining. PI can pass into permeabilised cells and their nuclei where it associates with DNA. Therefore, the higher the level of PI fluorescence, the more DNA is present in the cell. This can be used to estimate the relative number of cells in the various stages of the cell cycle and the number of cells undergoing apoptosis. Figure 27A shows the PI profile of thymocytes that have been isolated from a non-transgenic mouse and then cultured for 20hr in the absence of serum (inducing thymocyte apoptosis), while figure 27B shows the PI profile of thymocytes from a non-transgenic mouse that had been injected with dexamethasone (an agent known to induce the apoptosis of thymocytes), 12hr previously. Three peaks are visible on each of these histograms. The central large, very thin peak, represents cells with the normal diploid complement of DNA (2n). The majority of the cells in the thymus will display this level of PI fluorescence. The much smaller peak at approximately twice the fluorescence intensity of the diploid peak, represents cells with twice the diploid complement of DNA (4n). Blast cells
undergoing DNA replication will display this level of PI fluorescence. Finally, the peak at a fluorescence intensity lower than the diploid peak represents cells that are undergoing apoptosis. This lower PI fluorescence is a consequence of the loss of DNA due to the induced endonuclease activity associated with PCD.

Using this PI staining technique, a number of litters from 15dpc to adult were examined for the presence of cells with a severely reduced complement of DNA indicative of apoptosis. Figure 27 (C-F) illustrates the PI profiles of thymocytes from non-transgenic and transgenic animals at 16dpc (C and D) and 18dpc (E and F). It is clear from these profiles that the proportion of total thymocytes that have a 2n and 4n complement of DNA are similar for both the transgenic and non-transgenic animals. In addition, there is no evidence of cells being present in the transgenic animals that would be consistent with those undergoing PCD.

To confirm these observations, a second method of assessing the level of apoptosis was used (figure 28). Cryostat sections from whole thymi from transgenic and non-transgenic animals were stained using the TUNEL method. This procedure involves the addition of terminal transferase (TdT) which adds nucleotides to DNA ends. Therefore, during apoptosis when the genomic DNA is cut into ~200bp fragments, many DNA ends will be available to act as the target for TdT. The increase in TdT activity can be detected by including biotin-labelled nucleotides in the TdT incubation, followed by the addition of a streptavidin-HRP complex. After the addition of a substrate for HRP, cells appearing brown represent those that are undergoing apoptosis.

Figure 28 A-D illustrates an example of this staining technique on a non-transgenic and transgenic mouse at day +6 after birth. Figure 28A is a control section incubated with just streptavidin-HRP and the HRP substrate, to demonstrate that in the absence of TdT the background level of brown staining is negligible. Figure 28B shows a thymic section from a mouse that had received a dexamethasone injection 12hr previously. A large number of the cells in the section are stained brown, confirming the presence of a high frequency of DNA ends, consistent with the widespread induction of PCD. Figures 28C and 28D show a non-transgenic thymic section and a transgenic thymic section respectively. The TdT staining on these two samples are very similar, with the frequency (2-5%) of brown cells almost identical. This evidence in combination with the results from the PI FACS analysis, would argue against there being an increased level of apoptosis in the thymocytes from the CD2-^ef transgenic animals. Therefore it is unlikely that the Nef-mediated induction of apoptosis could explain the reduced thymic cellularity that is observed.
Figure 27  FACS analysis for apoptosis using propidium iodide.

Thymocytes from transgenic and non-transgenic littermates from line F(1147) were incubated overnight in a hypotonic solution containing propidium iodide (PI), then run on a Becton Dickinson FACScan. Histogram plots were obtained of cell number (ordinate) against PI fluorescence (abscissa); (A) PI staining of non-transgenic thymocytes after 20hr in serum-free culture; (B) PI staining of thymocytes from a non-transgenic animal 12hr after the animal had been given a dexamethasone injection (dexamethasone is known to induce apoptosis); (C) A non-transgenic and (D) a transgenic animal from line F(1147) at 16dpc, stained with PI. (E) A non-transgenic and (F) a transgenic animal from line F(1147) at 18dpc, stained with PI.
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Cryostat sections of transgenic and non-transgenic littermates stained for apoptosis using the TUNEL method.

Cryostat sections of the thymus of day +6 (after birth) transgenic and non-transgenic littermates from line F(1147), stained for apoptotic cells using the TUNEL method. (A) Non-transgenic thymic section incubated with streptavidin-HRP and substrate only. (B) Non-transgenic thymic section, from a mouse that had received an injection of dexamethasone 20hr previously, stained using the TUNEL method. (C) Line F non-transgenic animal stained using the TUNEL method. (D) Line F transgenic animal (littermate of C) stained using the TUNEL method.
A. 2nd layer control

B. Dexamethasone treated thymus

C. Non-transgenic

D. Transgenic
2.7 The analysis of the frequency of thymic progenitors.

Since the reduced thymic cellularity in the CD2-\textit{nef} transgenic animals is not a consequence of the induction of an increased level of apoptosis, it is possible that a difference in the absolute number of cells entering the thymus from the bone marrow, or the failure of a particular progenitor population to progress to the subsequent stage of differentiation could be responsible for the reduced thymic output of more mature cell types.

With this in mind, the frequency, percentage and absolute number per thymus of the very early thymic progenitor populations were investigated. Figure 29 illustrates the stepwise developmental events that are believed to occur during thymic ontogeny, from the very earliest bone marrow-derived cells that seed the thymus, to the most mature CD4 or CD8 single positive thymocytes. The earliest populations of thymic progenitors do not express the lineage markers CD4 and CD8 (with the exception of the very earliest progenitor population that has a \textit{CD4}\textsuperscript{+} phenotype (Wu et al., 1991). This population of CD4/CD8 double negative cells can then be further divided into developmentally discrete populations depending on expression of the cell surface markers CD44 and CD25. For example, the earliest of these populations expresses only CD44 (CD44\textsuperscript{+} CD25\textsuperscript{-}). These cells then mature into a population which express no CD44 but have a high cell surface level of CD25 (CD44\textsuperscript{-} CD25\textsuperscript{+}). Finally, the population of cells that follow the CD44- CD25\textsuperscript{+} stage (and immediately precede the onset of CD4 and CD8 expression) has neither CD44 or CD25 (CD44\textsuperscript{-} CD25\textsuperscript{-}).

To analyse these progenitor populations the absolute number of cells per thymus was first calculated from CD2-\textit{nef} transgenic mice aged between 15dpc and adult. These thymocytes were then stained with a PE-conjugated anti-CD4 and anti-CD8 antibody, plus a FITC-conjugated anti-CD25 antibody and a Cy-chrome-conjugated anti-CD44 antibody. The cells were then examined by FACS analysis. Only cells that expressed neither CD4 or CD8 \textit{(ie. PE -ve cells)} were analysed. The proportion of the total cells that were included in this CD4/CD8 double negative gate was used to calculate the absolute number of these lineage -ve cells per thymus. These cells were then examined on the basis of size and granularity, with the cells displaying the characteristics associated with blast lymphocytes \textit{(ie cells larger than mature T-cells)} being retained (this was usually about 95\% of the lineage -ve cells, regardless of whether the cells were transgenic or not). This population was then analysed for CD44 and CD25 expression, resulting in its characterisation into three distinct subsets; (i) CD44\textsuperscript{+} CD25\textsuperscript{-} cells; (ii) CD44\textsuperscript{b/-} CD25\textsuperscript{+} cells; or (iii) CD44- CD25- cells (figure 30).

Table 11 displays the absolute number of cells in; (i) the total thymus; (ii) the CD4/CD8
Bone marrow-derived cells enter the thymus at the cortico-medullary junction. The earliest thymic progenitor so far isolated expresses low CD4, but no CD8. This cell is CD44+ CD25-. Surface expression of CD4 is then lost, and the developing thymocyte enters several CD4/CD8 double negative stages. These stages are characterised by the cell-surface expression of either CD44 or CD25, progressing in the order CD44+ CD25-, CD44+/- CD25+, CD44- CD25+, and finally CD44- CD25-. At this stage, CD4 and CD8 expression are upregulated, generating the huge CD4/CD8 double positive thymocyte population. This population then undergoes both positive and negative selection events to generate the mature CD4 and CD8 single positive thymocyte subsets.
BONE-MARROW PROGENITOR

THYMUS

CD4+CD8- Early thymic progenitor
CD4lo, CD8-, CD44+, CD25-

CD44 CD25-

CD4-CD8-

CD4+CD8- CD44+ CD25-

CD44lo CD25+

CD25

CD44 CD44-

CD4-CD8-

CD4-CD8-

CD4+CD8+ Double positive

CD4+ CD4+ Single positive

CD8+ CD8+ Single positive

PERIPHERY
**Figure 30** FACS plot analysis of CD4/CD8 double negative CD44/CD25 thymocyte subsets in CD2-^ nef transgenic mice during development.

Total thymocytes from at least three transgenic and three non-transgenic littermates from line F(1147) were isolated and counted. 10^4 thymocytes per mouse were then stained with anti-CD4-PE, anti-CD8-PE, anti-CD25-FITC and anti-CD44-Cy-chrome, and analysed by FACScan analysis. The CD4/CD8 positive PE-staining cells were removed from the analysis, with the remaining cells (the percentage of which was noted) being analysed for their expression of CD25 (FITC) and CD44 (Cy-chrome). This figure illustrates examples of the CD44 (ordinate)/CD25 (abscissa) FACSplots that were obtained for transgenic and non-transgenic animals at 15dpc, 18dpc, +6 after birth, and adult.
lineage negative compartment; (iii) the CD44+ CD25- population; (iv) the CD44+ CD25+ population; and (v) the CD44- CD25- population, for transgenic and non-transgenic mice from litters aged from 15dpc through to adult. Figure 31 displays in a graphical form the absolute number of cells from the above mentioned populations of transgenic thymocytes as a percentage of the absolute number of cells found in that population in the non-transgenic controls. A number of features stand out. First, in the CD2-nef transgenic animals (when compared to littermate controls) the absolute number of CD4/CD8 double negative thymocytes that express neither CD44 or CD25 (CD44- CD25-) are significantly reduced by a half to a third at all time points from 15/16dpc onwards. Figure 31 demonstrates that the plot for the absolute number of this CD44-CD25- population closely resembles the plot for total thymic cell number. Furthermore, this population of cells is the immediate precursor of the CD4/CD8 double positive population of thymocytes, which as described previously is also decreased by approximately three fold in the CD2-nef transgenic animals.

The subset of cells that immediately precedes the CD44- CD25- thymocyte population has a CD44-/lo CD25+ phenotype. As figure 31 illustrates, at all time points studied, the absolute number of thymocytes in this population in the transgenic animals is at least equivalent (or increased - up to almost 2-fold at day +6 and day +7) to the non-transgenic littermate controls. This observation demonstrates a clear transition between the CD44,+/- CD25+ population (where transgenic cell number is similar to controls) and the subsequent CD44- CD25- population and beyond (where transgenic cell number is three-fold down). This suggests that the expression of the nef gene could block the transition from the CD44,+/- CD25+ stage to the subsequent CD44+/CD25- stage of thymic ontogeny, or that nef gene expression in the cell populations from the CD44- CD25- stage onwards may have a detrimental effect on either the survival or expansion of these thymocytes.

To complete this analysis, the absolute number of the cell population immediately prior to the CD44,+/- CD25+ stage, the CD44+ CD25- population, was investigated. Figure 31 illustrates that this population is also decreased in the transgenic animals when compared to the littermate controls. However, this decrease (down to 65-70%) is much less than that observed for the CD44- CD25- thymocyte population, especially before birth (only down to 85-90%). As total thymocyte cell number and the physical size of the thymus is reduced to a third of the controls from just before birth to adult, this reduction in the CD44+ CD25- population may represent a decreased ability for this smaller thymus to accept bone-marrow derived progenitor cells. However, the expansion of the CD44+ CD25- population does not appear to be affected as the subsequent population, the CD44,+/- CD25+ subset, is represented in the transgenic animals by
Table 11  Absolute number of CD4/CD8 double negative CD44/CD25 thymic progenitor subsets in CD2-\textit{nef} (line F) transgenic mice during development.

Total thymocytes from three transgenic and three non-transgenic littermates were isolated and counted. $10^4$ thymocytes per mouse were then stained with anti-CD4-PE, anti-CD8-PE, anti-CD25-FITC and anti-CD44-Cy-chrome, and analysed by FACScan analysis. The CD4/CD8 positive PE-staining cells were removed from the analysis, with the remaining cells (the percentage of which was noted) being analysed for their expression of CD25 (FITC) and CD44 (Cy-chrome). The absolute number of CD44\textsuperscript+ CD25\textsuperscript-, CD44\textit{lo}/- CD25\textsuperscript+, and CD44\textsuperscript- CD25\textsuperscript- cells were calculated for each individual mouse and averaged per litter (with the standard deviation of the mean indicated). Time points used were 15, 16, 17, and 18dpc, plus day +1, +6, and +7 after birth, and adult.
<table>
<thead>
<tr>
<th></th>
<th>TOTAL THYMOCYTES</th>
<th>LINEAGE-VE</th>
<th>CD44+CD25-</th>
<th>CD44lo/-CD25+</th>
<th>CD44+CD25-</th>
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<td>+</td>
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<td>+</td>
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<tr>
<td>15 dpc</td>
<td>0.53 ±0.03</td>
<td>0.56 ±0.10</td>
<td>0.43 ±0.01</td>
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<td>16 dpc</td>
<td>3.14 ±0.08</td>
<td>1.45 ±0.21</td>
<td>0.84 ±0.15</td>
<td>0.73 ±0.09</td>
<td>0.068 ±0.007</td>
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<td>17 dpc</td>
<td>9.4 ±1.5</td>
<td>3.8 ±0.5</td>
<td>0.95 ±0.21</td>
<td>0.77 ±0.33</td>
<td>0.085 ±0.018</td>
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<tr>
<td>18 dpc</td>
<td>10.9 ±1.9</td>
<td>4.0 ±0.8</td>
<td>0.57 ±0.09</td>
<td>0.35 ±0.07</td>
<td>0.046 ±0.011</td>
</tr>
<tr>
<td>Day+1</td>
<td>14.8 ±1.4</td>
<td>6.8 ±0.7</td>
<td>1.19 ±0.19</td>
<td>0.61 ±0.21</td>
<td>0.077 ±0.017</td>
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<tr>
<td>Day+6</td>
<td>125.4 ±7.7</td>
<td>34.2 ±7.1</td>
<td>2.19 ±0.21</td>
<td>2.10 ±0.38</td>
<td>0.189 ±0.043</td>
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<tr>
<td>Day+7</td>
<td>152.6 ±27.0</td>
<td>50.5 ±15.0</td>
<td>6.30 ±1.70</td>
<td>6.20 ±1.30</td>
<td>0.490 ±0.11</td>
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<tr>
<td>Adult</td>
<td>259.0 ±36.9</td>
<td>113.3 ±31.6</td>
<td>9.16 ±1.07</td>
<td>6.79 ±1.59</td>
<td>1.210 ±0.15</td>
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</tbody>
</table>
Figure 31  Absolute number of CD4/CD8 double negative CD44/CD25 thymocyte subsets in CD2-nef transgenic mice during development.

Total thymocytes from at least three transgenic and three non-transgenic littermates from line F(1147) were isolated and counted. 10⁴ thymocytes per mouse were then stained with anti-CD4-PE, anti-CD8-PE, anti-CD25-FITC and anti-CD44-Cy-chrome, and analysed by FACScan analysis. The CD4/CD8 positive PE-staining cells were removed from the analysis, with the remaining cells (the percentage of which was noted) being analysed for their expression of CD25 (FITC) and CD44 (Cy-chrome). The absolute number of CD44+ CD25-, CD44/- CD25+, and CD44- CD25- cells were calculated for each individual mouse and averaged per litter. This graph illustrates the average absolute number of a particular transgenic thymocyte progenitor population, as a percentage of the average of that population in the non-transgenic littermate controls. The total number of transgenic thymocytes per thymus is also displayed as a percentage of the total number of thymocytes per thymus in the non-transgenic littermates (filled circles). Time points used were 15, 16, 17, and 18dpc, plus day +1, +6, and +7 after birth, and adult.
Earliest thymic progenitor

All CD4+ / CD8-

Early CD44+ / CD25- 

CD44- / CD25+

Double positive

CD4 Single positive

CD8 Single positive

Cell number as a percentage of non-transgenic

Total Thymocytes

Age of litter

157
a similar number of cells to that observed in the littermate controls.

### 2.8 Cell expansion in a thymic organ culture system.

The results from the thymic progenitor analysis suggest that in the CD2-\textit{nef} transgenic animals there is either an abnormality in the progression of CD44\textsubscript{w/-} CD25\textsuperscript{+} thymocytes to the CD44\textsuperscript{-} CD25\textsuperscript{-} stage, or(and) that the CD44\textsuperscript{-} CD25\textsuperscript{-} population of cells fail to expand at the normal rate or to the normal extent. To test this hypothesis, a fetal thymic organ culture (FTOC) system was set up to analyse the expansion potential of the CD44\textsuperscript{+} CD25\textsuperscript{-}, CD44\textsubscript{w/-} CD25\textsuperscript{+} and CD44\textsuperscript{-} CD25\textsuperscript{-} thymocyte populations. 15dpc thymic lobes from CBA inbred mice were removed and depleted of host lymphocytes using 2-deoxyguanosine. After a 4-6 day ablation period, an equal number of FACS-sorted transgenic and non-transgenic cells from either the CD44\textsuperscript{+} CD25\textsuperscript{-}, the CD44\textsubscript{w/-} CD25\textsuperscript{+} or the CD44\textsuperscript{-} CD25\textsuperscript{-} progenitor thymocyte populations were incubated with individual depleted lobes in hanging drop culture for ten days. Control lobes that did not receive donor cells did not show any repopulation in any experiment. At the end of ten day culture period, the progeny of the donor lymphocytes were analysed for CD4 and CD8 expression. As the phenotype of all donor cells was CD4/CD8 DN, the detection of CD4/CD8 DP and CD4 SP cells is indicative of the appropriate maturation of the donor cells in these thymic cultures.

At the time of writing this system is still being employed to analyse the expansion of the various thymic progenitor populations from transgenic and non-transgenic animals. However, three independent preliminary experiments are illustrated in figure 32. These graphs show the absolute number of total thymocytes, CD4/CD8 DN thymocytes, CD4/CD8 DP thymocytes and CD4 SP thymocytes, that were obtained after an equal number of either transgenic or non-transgenic CD44\textsuperscript{-} CD25\textsuperscript{+} progenitor donor cells, from various stages of development, were incubated with individual depleted thymic lobes for 10 days.

These results clearly demonstrate that the total number of cells obtained from ten day FTOCs is much greater when a certain number of non-transgenic thymocytes rather than transgenic thymocytes are used as donor cells. This suggests that either a proportion of the CD44- CD25\textsuperscript{+} thymocyte population from CD2-\textit{nef} transgenic mice fail to expand at all, or the population as a whole expands at a much slower rate when compared to non-transgenic controls. In addition this result implies that the reduction in thymic cellularity observed in CD2-\textit{nef} transgenic animals is an intrinsic property of the thymocytes that express the \textit{nef} gene, and not a consequence of the thymic environment in which they develop.
Figure 32  Ten day fetal thymic organ cultures, using CD4/CD8 double negative CD44-CD25+ thymocytes as donors.

Graphic illustration of three independent fetal thymic organ culture experiments. 15dpc CBA/CA thymic lobes were ablated for 4-6 days with 2-deoxyguanosine. A precise number of donor cells from transgenic or non-transgenic littermates (line F) were then cultured with these lobes for ten days in hanging drops. The bars represent the absolute number of total thymocytes, CD4/CD8 DN thymocytes, CD4/CD8 DP thymocytes, and CD4 SP thymocytes, recovered from each individual lobe at the end of the 10 day culture period.
A. Absolute cell number

Donor cells = day 16 - CD44+CD25+ (18000/thymic lobe)
Culture period = 10 days

B. Absolute cell number

Donor cells = day 5 - CD44+CD25+ (10000/thymic lobe)
Culture period = 10 days

C. Absolute cell number

Donor cells = day 6 - CD44+CD25+ (5000/thymic lobe)
Culture period = 10 days
The results from these preliminary FTOCs are encouraging. Ninety percent of the thymic lobes repopulate, and the cells that are recovered from non-transgenic animals closely resemble those that would be expected during in vivo thymocyte development. Therefore, further experiments are currently being conducted to analyse the other main CD4/CD8 DN progenitor populations, over various periods of culture, in order to assess the developmental stage at which the Nef-mediated retardation in thymocyte expansion first occurs.

2.9 The analysis of p561ck in CD2-nef transgenic mice.

The presence of the p561ck non-receptor tyrosine kinase has been implicated as a critical factor in the maturation of CD44-CD25+ thymocytes to the subsequent CD44-CD25- stage of thymic ontogeny and beyond (Molina et al., 1992).

In order to assess the amount of p561ck in the thymocytes of CD2-nef transgenic animals, Northern and Western blot analysis was performed on total thymocytes from transgenic and non-transgenic littermates at various stages during development. Figure 33A shows a Northern blot of total thymic mRNA from transgenic and non-transgenic animals at +7 days after birth, probed with a p561ck fragment (gift from R. Perlmutter), (the endogenous GAPDH gene was used as a loading control). It is clear from this figure that the level of p561ck mRNA in CD2-nef transgenic animals is not significantly different from the level observed in non-transgenic controls.

Figures 33B and 33C show representative Western blots of total thymic lysates from CD2-nef transgenic mice (line F) and non-transgenic controls from 18dpc and day +7 after birth stained with a rabbit polyclonal antibody against p561ck and a loading control antibody against β-actin. These blots demonstrate that the level of p561ck protein in transgenic total thymic lysates is very similar to that observed in the non-transgenic controls.

To complement this analysis, total thymocytes from transgenic and non-transgenic animals were permeabilised with a saponin buffer, and then stained intracellularly for the presence of the p561ck protein. The cells were also stained for CD4 and CD8, allowing only CD4/CD8 double negative thymocytes to be analysed for intracellular p561ck levels. Figure 34 shows representative FACS histograms of p561ck staining in CD4/CD8 DN thymocytes from transgenic and non-transgenic animals (line F) at 18dpc. This illustrates that the total amount of p561ck in the transgenic CD4/CD8 DN thymocyte population is very similar to that observed in the non-transgenic controls, confirming the results obtained from the Western blot analysis.

The above two assays do not identify the location of the detected p561ck protein. The
Figure 33  Northern and Western blot analysis of p56lck in CD2-nef transgenic mice.

The levels of p56lck mRNA and protein were analysed at various time points during the development of CD2-nef transgenic mice (line F); (A) Northern blot of total mRNA (20μg) from transgenic and non-transgenic mice at day +7 after birth; (B) Western blot analysis of total thymic lysates from transgenic and non-transgenic mice at day +7 after birth; (C) Western blot analysis of total thymic lysates from transgenic and non-transgenic mice at 18dpc.
A. p56lck Northern

F litter - Day + 7 after birth

Non Tg  Tg

p56lck  →
(2.3kb)

GAP DH  →
(1.6kb)

B. p56lck Western

F litter - Day + 7 after birth

Non Tg  Tg

p56lck  →
β Actin  →
(42kDa)

C. p56lck Western

F litter - Day 18pc

Non Tg  Tg

p56lck  →
β Actin  →
(42kDa)
Figure 34  Intracellular FACS analysis of p56lck in CD2-nef transgenic mice (line F).

FACS histogram plots of p56lck fluorescence against cell number for permeabilised transgenic and non-transgenic thymocytes from 18dpc; A. Non-transgenic thymocytes stained with only the second layer (anti-rabbit IgG, conjugated to a FITC label); B. CD4/CD8 double negative thymocytes from a non-transgenic animal stained with p56lck (plus second layer) after permeabilisation; C. CD4/CD8 double negative thymocytes from a transgenic animal stained with p56lck (plus second layer) after permeabilisation.
Control - second layer only, (FITC conjugated).

Cell number

$p56\text{ic}k$ fluorescence intensity

Intracellular $p56\text{ic}k$ staining

Non-transgenic

Cell number

$p56\text{ic}k$ fluorescence intensity

Intracellular $p56\text{ic}k$ staining

Transgenic

Cell number

$p56\text{ic}k$ fluorescence intensity
Figure 35  Indirect immunofluorescence for p56lck on permeabilised thymocytes from CD2-nef transgenic mice (line F).

A. Indirect immunofluorescence staining of 16dpc non-transgenic permeabilised thymocytes using an anti-p56lck mAb and a FITC-conjugated second layer. B. Indirect immunofluorescence staining of 16dpc transgenic permeabilised thymocytes using an anti-p56lck mAb and a FITC-conjugated second layer.
Intracellular p56lck staining
possibility therefore exists that even though there is an equivalent amount of p56lck in the cells of the CD4/CD8 DN compartment in both the transgenic and non-transgenic animals, the localisation of this p56lck protein may be altered. For example, cell-surface CD4 downregulation in these same mice results in the intracellular localisation of the CD4 in a compartment co-localising with a golgi-specific protein. Therefore, to analyse whether the cellular localisation of p56lck was equivalent in Nef-expressing and control cells, total permeabilised thymocytes were stained for p56lck (conjugated to a FITC label) and viewed using a Zeiss fluorescent microscope. Figure 35 shows the results of such staining on transgenic and non-transgenic thymocytes from 16dpc. This figure demonstrates that the staining pattern of p56lck is very similar in both Nef-expressing and control thymocytes, with a strong staining pattern at the surface of the cells (the no stain and second layer only controls did not show FITC fluorescence, data not shown).

At the time of writing, the intracellular localisation of p56lck is being further investigated. Thymocytes from 14 and 15dpc are being analysed with the fluorescent p56lck antibody and confocal microscopy. It is therefore hoped that a detailed three dimensional localisation pattern of p56lck protein can be obtained in Nef-expressing thymocytes from the early stages of thymic ontogeny.

### 2.10 T-cell activation in CD2-Nef transgenic mice

As expression of the Nef protein has been detected in the periphery (figure 18), peripheral T-cell responses in transgenic animals may be altered. Studies in vitro have demonstrated that certain Nef proteins can inhibit the DNA-binding activity of the inducible transcription factors NFκB and AP1 (Niederman et al., 1992, Niederman et al., 1993a, Bandres et al., 1994b). These factors are both induced upon T-cell activation and bind to the IL-2 promoter, thus influencing T-cell proliferation. Therefore, mitogen induced and anti-CD3ε antibody-mediated activation assays were performed on thymocytes and splenocytes from the A(1147), F(1147), B(1191), and D(1191) transgenic lines to assess whether the consequences of Nef expression had any effect on the proliferative capacity of thymic and peripheral T-cells.

Upon stimulation of the T-cell receptor/CD3 complex via anti-CD3ε antibodies, a measurable decrease in the proliferative capacity of transgenic thymocytes from lines A, B, D, and F was observed when compared with non-transgenic littermates (figure 36). This type of stimulus is specific for the TCR/CD3 complex, utilising signal transduction proteins such as lck and lyn.
The calcium ionophore (ionomycin) and phorbol ester (PMA) stimulation of cells results in a general activation through a number of signal transduction pathways. On such activation, transgenic thymocytes from lines A, B and D proliferated at similar rates to non-transgenic littermates (figure 37). However, transgenic thymocytes from line F again proliferated to a lesser extent than equivalent numbers of thymocytes from non-transgenic littermates.

Activation assays with ionomycin/PMA and anti-CD3e antibodies were also conducted on splenocytes from lines B and F (figure 38). On stimulation with ionomycin/PMA, proliferation of transgenic splenocytes from both line F and line B was very similar to non-transgenic controls. This was also the case after stimulation of line B splenocytes with anti-CD3e antibodies. However, splenocytes from transgenic animals of line F showed a reduction in cell proliferation when compared to non-transgenic littermates.

Therefore, it appears that when equal numbers of transgenic and non-transgenic thymocytes (from lines A, F, B, and D) are stimulated through the TCR complex by anti-CD3e antibodies, the transgenic cells fail to proliferate to the same extent as the non-transgenic controls. Such an effect was also observed for splenocytes from line F. However, this reduction in proliferation is not observed when a general activation stimulus (such as ionomycin and PMA) is used.
Figure 36  Activation assays using anti-CD3ε/PMA on thymocytes from CD2-nef transgenic mice.

Increasing numbers of thymocytes from non-transgenic (open symbols) and transgenic (solid symbols) littermates of line F, B, A, and D were stimulated with anti-CD3ε mAb/PMA, and proliferation was measured via $^3$H thymidine incorporation. PMA alone produced no proliferation (not shown). Proliferation is represented as $^3$H CPM incorporated versus initial cell number.
**Figure 37** Activation assays using ionomycin/PMA on thymocytes from CD2-nef transgenic mice.

Increasing numbers of thymocytes from non-transgenic (open symbols) and transgenic (solid symbols) littermates of line F, B, A, and D were stimulated with ionomycin/PMA, and proliferation was measured via $^3$H thymidine incorporation. PMA alone produced no proliferation (not shown). Cell proliferation is represented as $^3$H CPM incorporated versus initial cell number.
Figure 38  Activation assays using anti-CD3e/PMA or ionomycin/PMA on splenocytes from CD2-nef transgenic mice.

Increasing numbers of splenocytes from non-transgenic (open symbols) and transgenic (solid symbols) littermates of line F, B, A, and D were stimulated with either anti-CD3e mAb or ionomycin/PMA. Proliferation was measured via $^3$H thymidine incorporation, and is represented as $^3$H CPM incorporated versus initial cell number.
Figure 39  Activation assays using anti-CD3e/PMA on thymocytes from CD2-nef transgenic mice - corrected for SP cell number.

Corrected graphs for anti-CD3e antibody activation assays. Thymocyte proliferation was corrected according to the number of SP cells present in the assay, as demonstrated by CD4/CD8 FACS analysis. Corrected $^3$H CPM is plotted against initial cell number. Transgenic animals (solid symbols), non-transgenic animals (open symbols).
Line F

Line B

Line A

Line D

Cell Number
3. The analysis of the *tat*-expressing transgenic lines.

3.1 The expression of the *tat* gene in CD2-*tat* transgenic mice.

As described previously, three transgenic mouse lines were generated that carried the CD2-*tat*(1-72) transgene. The level and tissue specific distribution of CD2-*tat* expression in these lines was assessed by S1 nuclease protection analysis. Total mRNA from lymphoid and non-lymphoid tissues was examined using a 5' S1 probe to demonstrate the correct initiation of the transgenic transcripts from the CD2 promoter. As is shown in figure 40, expression of *tat* transcripts, confined to the lymphoid tissues, (thymus, lymph node, spleen, and peripheral blood), was detectable at high levels in all three transgenic lines. No transgene expression was detected in the RNA from skin, ovaries, muscle, lung, liver, kidney, heart or brain from any of these animals.

3.2 Thymocyte and T-cell populations in CD2-*tat* transgenic mice.

The absolute number and distribution of thymocyte and peripheral T-cell populations in adult CD2-*tat* transgenic animals was examined and compared to non-transgenic controls. This type of investigation can highlight the presence of any developmental abnormalities in thymic ontogeny and can demonstrate whether certain cell subsets are under or over represented due to the expression of the CD2-*tat* transgene.

To analyse the thymocyte and peripheral T-cell populations in the CD2-*tat* transgenic lines, FACS analysis was performed on the thymus, spleen, and lymph nodes using a number of fluorescent-conjugated monoclonal antibodies. Figure 41 illustrates representative CD4/CD8 FACS plots of thymus, lymph node and spleen from a non-transgenic and transgenic animal (line C). When a large number of animals were analysed, no differences in the distribution of the various populations of thymocytes, lymph node cells or splenocytes in the transgenic mice were observed. There were also no differences in the absolute cell number for each of these populations. In addition, the average fluorescent intensity of both CD4 and CD8 mAb staining was comparable between the *tat*-expressing animals and the non-transgenic controls. Therefore, in contrast to the transgenic animals that express the HIV-1 nef gene, transgenic mice that express the HIV-1 *tat* gene undergo normal thymic ontogeny and display the expected distribution of
Figure 40  Tissue specific expression of the CD2-tat transgene.

S1 nuclease protection analysis was performed using total RNA from various tissues of line C, line E and line W transgenic mice. The tissues were; thymus (T), spleen (S), skin (Sk), ovaries (Ov), muscle (M), lymph node (L), liver (Li), lung (Lu), kidney (K), heart (H), brain (Br) and blood (Bl). Tat and U6 DNA probes were end-labelled and protected fragments analysed on denaturing acrylamide gels.
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<th>Line</th>
<th>Tat transgene</th>
<th>β-actin</th>
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<td><strong>C</strong></td>
<td>![Image](U6 SnRNA)</td>
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<td><strong>W</strong></td>
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Figure 41  Distribution of CD4/CD8 thymocyte and T-cell subsets in lymphoid tissues of CD2-tat transgenic mice.

Thymocytes (A), lymphocytes (B), and splenocytes (C) from transgenic and non-transgenic animals from each of the CD2-tat lines were stained with fluorescent-conjugated mAbs against CD4 (PE ordinate) and CD8 (FITC abscissa). This figure illustrates an example of the FACS plots obtained from animals from line C. Relative fluorescent intensities are given on a logarithmic scale, with percentages of CD4/CD8 double negative, CD4/CD8 double positive and CD4 or CD8 single positive populations indicated. Analysis of $10^6$ cells was performed per sample.
peripheral T-cell populations.

3.3 Analysis of the cytokine profile in activated T-cells from CD2-tat transgenic mice.

3.3.1 The expression of TNF-β and TNF-α.

Evidence from a number of in vitro studies has demonstrated that the expression of the tat gene in a B-cell line (Sastry et al., 1990), T-cell line, or promonocytic cell line (Buonaguro et al., 1994), can upregulate TNF-β production. However, immortalised cell lines do not accurately reflect the characteristics of resting or activated primary cells. Therefore, in order to assess the effect of tat expression in primary cells from a mammalian immune system, the expression of TNF-β was examined in both activated primary thymocytes and activated peripheral T-cells from the CD2-tat transgenic mice.

Thymocytes were isolated from transgenic and non-transgenic littermates from line C and line E, then activated with the calcium ionophore, ionomycin and the phorbol ester, PMA. After 6-48hr, the culture supernatants were removed for a TNF-β bioassay and the cells were harvested for RNA and Northern blot analysis.

Northern blot analysis of steady state mRNA levels of TNF-α, TNF-β and β-actin (as a normalisation control) in mitogen induced transgenic and non-transgenic thymocytes is shown in figure 42. Levels of TNF-β mRNA were increased in the transgenic mice of both lines. When this signal was quantitated against β-actin controls, the level of TNF-β mRNA in the transgenic samples was, on average, 2.5-fold higher than in the non-transgenic animals (table 12). Levels of TNF-α were not increased in transgenic lines.

To establish whether this increase in the level of TNF-β mRNA was accompanied by an increase in the level of biologically active TNF protein, a TNF bioassay was performed on the culture supernatants from the activation of thymocytes of mice from line C and line E (figure 43). The amount of activity measured in the bioassay with the cytokine sensitive cell line L929 was significantly increased in the supernatants from the culture of thymocytes from both transgenic lines. This increase of approximately 3-4 fold in total TNF protein is slightly higher than the 2.5-fold increase observed in the levels of TNF-β mRNA (table 12).

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Figure 42  Northern blot analysis of cytokine expression in activated thymocytes from CD2-tat transgenic mice.

Thymocytes were isolated from transgenic and non-transgenic littermates (line C and line E), and activated in the presence of PMA and ionomycin for between 6-24hr. Total RNA was extracted and used for Northern blot analysis. Following transfer to nylon filters the RNA was hybridised with probes for either TNF-α, TNF-β, TGF-β, IL-4R and β-actin (the latter as a loading control). The relative intensity of the individual bands were quantitated using a phosphorimager. These values are shown in table 12.
Line C  |  +  |  +  |  +  
---|---|---|---
Line E  |  -  |  +  |  +  

- TNF-β
- TNF-α
- TGF-β
- IL-4R
- β-actin
Figure 43  Bioassay for TNF production in activated thymocytes from CD2-tat transgenic mice.

Cultured supernatants from activated transgenic and non-transgenic T-cells (littermates from line C or line E) were examined for the production of TNF using the cytokine-sensitive cell line L929. A standard curve was determined by using known quantities of recombinant titrated TNF. Inhibition of L929 cell death by TNF neutralising antibodies demonstrated that the assay activity was specifically due to TNF (not shown). L929 cell number was assessed by absorbance at 540nm following staining with crystal violet.
TNF activity (pg/ml)

- + +  
Line E

- + +  
Line C
Table 12  Quantitation of cytokine RNAs in CD2-tat transgenic mice.

Quantitation of cytokine RNAs in CD2-tat transgenic mice. After northern blot analysis of total RNA from PMA/ionomycin activated thymocytes from transgenic and non-transgenic mice, the filters were probed with radiolabelled probes for TNF-α, TNF-β, TGF-β, IL-4R and β-actin. Using a phosphorimager, the intensity of the bands were calculated and normalised against β-actin. Values are displayed in this table as fold RNA change in the transgenics as compared to non-transgenic controls.
Quantitation of Cytokine RNAs in CD2-Tat Transgenic Mice

<table>
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<th>Transgenic lines</th>
<th></th>
<th>Line E</th>
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<tbody>
<tr>
<td></td>
<td>Line C</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>TGF-β</td>
<td>2.2</td>
<td>3.3</td>
<td>2.7</td>
<td>2.9</td>
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<tr>
<td>IL-4R</td>
<td>3.4</td>
<td>2.8</td>
<td>4.0</td>
<td>3.8</td>
</tr>
<tr>
<td>TNF-β</td>
<td>2.3</td>
<td>2.3</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.81</td>
<td>0.67</td>
<td>0.74</td>
<td>0.85</td>
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Fold RNA change as compared with non-transgenic littermates.
3.3.2 The expression of other cytokines.

Evidence from in vitro studies suggests that the expression of Tat can effect cytokine and cellular genes such as TGF-β in human astrocytic glial cells (Cupp et al., 1993), and the IL-4 receptor on a human B-cell line (Puri et al., 1992). To assess the extent of these effects in vivo, the level of steady state TGF-β and IL-4R mRNA after PMA/ionomycin activation of transgenic thymocytes was analysed for littermates from lines C and E (figure 42). The level of steady state TGF-β mRNA was increased by approximately 3-fold in activated thymocytes from transgenic animals from both lines (normalisation to β-actin was performed - table 12). In a similar manner, the level of steady state IL-4R mRNA on activation of transgenic thymocytes was 3-4 fold greater than that observed on activation of non-transgenic controls (table 12).

At the time of writing a large panel of other cytokine probes, such as IL-2, IL-4, IL-6, IL-10, IL-12 and IFN-γ have been obtained and are currently being used to analyse the cytokine profiles of the CD2-tat transgenic animals in response to a wide array of activation stimuli.

3.4 T-cell activation in CD2-tat transgenic mice.

Since cytokine expression levels were altered in CD2-tat transgenic animals, the proliferation of tat-expressing primary thymocytes and splenocytes could also be affected. This would be of particular interest as recent evidence suggests that Tat inhibits antigen-mediated T-cell proliferation (Viscidi et al., 1989).

Transgenic and non-transgenic thymocytes from line C and line E were isolated and activated with either concanavalin A (ConA), anti-CD3ε antibody/PMA or ionomycin/PMA. After 72hr the cells were pulsed with tritiated thymidine in order to assess their rate of proliferation. Figure 44 illustrates that no significant differences were observed between the proliferation of the tat-expressing and the control cells for any of the activation stimuli used.

To examine whether CD2-tat transgenic lymphocytes had altered proliferation responses to recall antigen, transgenic and non-transgenic littermates were primed with Keyhole limpet haemocyanin (KLH) in Complete Freund’s Adjuvant. Five to eight days later, lymph nodes were isolated and re-stimulated in culture with various concentrations of KLH. After 72hr, tritiated thymidine was added and the proliferation of the samples assessed. Figure 45 shows two independent experiments and clearly demonstrates that there is no significant difference in the extent to which transgenic and non-transgenic lymphocytes proliferate in vitro in response to
Figure 44  Activation assays using anti-CD3ε/PMA, ConA, or ionomycin/PMA on thymocytes from CD2-tat transgenic mice.

Thymocytes from transgenic (closed circles) and non-transgenic (open circles) line C and line E littermates were cultured, in triplicate, at 10^6 cells/ml on 96-well tissue culture plates in RPMI/10% FCS/20mM 2, B-mercaptoethanol. T-cells were induced to proliferate in either the presence of Con A (2μg/ml), anti-CD3ε mAb (145-2C11, coated at 10μg/ml), or PMA in combination with ionomycin. After 48hr, the cells were pulsed for 16hr in ³H thymidine and cell proliferation was assessed.
Figure 45  T-cell proliferation in response to recall antigen in CD2-tat transgenic mice.

Peripheral lymph nodes (peri-aortic and sub-inguinal) from line C (circles) and line E (boxes) transgenic (open) and non-transgenic (closed) littermates, that had previously been immunised with KLH (50µg per mouse for 5-8 days), were isolated and cultured in vitro ($10^5$ cells/ml in triplicate), in the presence of increasing concentrations of KLH. After 4 days, cells were pulsed with $^3$H thymidine and cell proliferation was assessed 16hr later.
recall antigen.

3.5 CD2-tat transgenic mice and the onset of tumours/KS-like lesions.

It has been shown that transgenic mice expressing Tat under the transcriptional control of the HIV-1 LTR develop skin lesions (thought to resemble KS-like lesions), and have an increased incidence of liver cancer by 12 to 18 months of age (Vogel et al., 1988). However, in this study over 200 mice from both line C and line E have been observed for periods up to two years without any sign of dermal lesions or tumour of any sort. These old mice still express the tat gene at high levels in their lymphoid tissues as demonstrated by S1 nuclease protection analysis of tissue mRNA from a 18 month old transgenic animal from line C (figure 46). Therefore, tat expression when directed to thymocytes and peripheral T-cells does not lead to the onset of tumours or KS-like lesions.
The long-term expression of the CD2-tat transgene was examined in line C at the age of 18 months. The tissue designations, probes and protected fragments are as described in figure 40. Lanes 1, 2 and 5 show increasing amounts of control RNA (5, 10 and 25 μg respectively) from a stably transfected T-cell line expressing the pCD2tat(1-72) construct.
Discussion

1. CD2-nef transgenic mice.

To study the in vivo effect of the HIV-1 nef gene on a mammalian immune system, eleven transgenic mouse lines were generated that expressed one of five nef alleles in the T-cell compartment under the control of the human CD2 regulatory elements and locus control region (LCR).

As described in the results section, it is evident that there are both dramatic and severe developmental defects in thymic ontogeny in the CD2-nef (1147 and 1191 alleles) transgenic mice. Transgenic animals have a thymus that is reduced in both size and cellularity. All cell populations after the CD4/CD8 DN, CD44-CD25+ stage of thymocyte differentiation are under-represented in mice that express the nef gene. The cell-surface expression of CD4 (and to a lesser extent CD8) is significantly downregulated at the CD4/CD8 DP stage of thymocyte differentiation, and the percentage of CD4 and CD8 SP mature thymocytes developing from the CD4/CD8 DP population is dramatically reduced. In addition, mature thymocyte and peripheral T-cell proliferation in response to activation through the TCR/CD3 complex appears to be slightly reduced in CD2-nef transgenic mice. However, an attempt to express the nef gene at high levels in peripheral T-cells (but not in the thymus) under the control of the inducible IL-2 promoter is still ongoing and should more directly address whether mature T-cell responses are significantly affected by HIV-1 nef gene expression.

Clearly, the phenotype of the CD2-nef transgenic mice is complicated, with apparent Nef-mediated effects at a number of stages in T-cell maturation. Therefore, in order to fully understand these affected stages and to discuss the mechanisms involved, it is first necessary to provide a detailed overview on thymic ontogeny and the selection events that occur during this process.

1.1 An overview of thymic development.

The thymus is responsible for the generation of a rapidly functional and diverse T-cell repertoire. These T-cells are restricted by self-MHC (ie: only recognise antigen in association with
self-MHC class I and class II), but are tolerant to self antigens. Mature T-cells can be divided into two cell types based on their cell surface phenotype and functional properties. Cytotoxic T-lymphocytes express the cell-surface co-receptor CD8 and an αβ T-cell receptor (TCR) that is restricted by self-MHC class I molecules. These cells are primarily involved in destroying virus-infected cells as part of the cell-mediated immune response. Helper T-lymphocytes express the CD4 co-receptor and an αβ TCR that is restricted by self-MHC class II molecules. These cells are lymphokine-secreting, and orchestrate both humoral and cell-mediated immune responses.

Mature CD4 and CD8 single positive (SP) thymocytes develop from a very small number of bone marrow-derived lymphoid progenitors, that seed the thymus, rapidly expand, and mature. During this process, a huge number of potential T-cells are formed, each expressing a unique antigen-specific αβ TCR. This diverse TCR repertoire is generated by the random rearrangement of the V(variable), D(diversity), J(joining) and C(constant) regions of the TCRα and TCRβ gene loci. Such immature TCR-expressing thymocytes must then undergo a series of thymic selection events to ensure that only useful, non-self-reactive T-cells are allowed to develop. Although these selection events are not yet fully understood, the generation of various transgenic and gene "knockout" mouse models has enabled some of the mechanisms controlling these processes to be elucidated. In addition, the phenotypic changes of cell-surface marker expression during thymocyte maturation have been mapped out by adoptive transfer experiments. In such experiments, thymocytes from a particular step in thymic development are transferred to a recipient animal, genetically distinguishable by a surface allelic marker such as the bi-allelic Thy-1.1/Thy-1.2 gene loci (Guidos et al., 1989b). The phenotype and kinetics of the appearance of the progeny of these donor cells has allowed the sequential steps in thymic ontogeny to be determined (Fowlkes et al., 1985, Crispe et al., 1987, Scollay et al., 1988).

1.1.1 Early thymocyte differentiation and expansion.

The earliest thymic progenitors thus far isolated constitute a population of cells that have yet to rearrange their TCR loci. This population, first appearing at approximately 11/12dp, has a surface phenotype that is CD4<sup>−</sup>/−, CD8<sup>−</sup>, CD3<sup>−</sup>, TCRαβ<sup>−</sup>, HSA<sup>+</sup>, c-kit<sup>++</sup>, Thy-1<sup>−</sup>/−<sup>−</sup>, CD25(IL-2R)<sup>−</sup> and CD44(Pgp-1)<sup>+</sup> (Wu et al., 1991). These cells give the slowest but most extensive repopulation of the thymus in adoptive transfer experiments, with the appearance of CD4/CD8 DP thymocytes approximately 9 days post-transfer. As these cells start to mature, their surface phenotype changes (see figure 29). First CD4, and then CD44, are downregulated, while
the surface expression of Thy-1 is increased. As CD44 expression decreases, CD25 (IL-2R) cell surface expression is upregulated, the cells sequentially changing from a CD44+ CD25- phenotype to a CD44+ CD25+ phenotype and then on to a CD44- CD25+ phenotype. In addition, the TCRβ locus begins to rearrange. This is under the control of the recombination-activating genes 1 and 2 (RAG-1 and RAG-2). The TCRβ chain is known to be very unstable at the cell surface in T-cell lines that do not express the other components of the TCR complex. However, at this immature stage of development the TCRβ chain is stabilised by its association, via a disulphide linkage, with the developmentally regulated gp33 (Kishi et al., 1992, Groettrup et al., 1993).

The expression of a functional TCRβ chain at the CD4/CD8 DN, CD44- CD25+ stage of thymocyte development is critical for the subsequent maturation of these thymocytes into CD4/CD8 DP cells. Mice homozygous for a non-functional RAG-1 or RAG-2 gene are not able to rearrange the germline configuration of their TCR loci, and hence do not express a functional TCRβ protein. These mice have almost no thymocytes (1-3x10⁶ cells) compared to a wild type control (250x10⁶ cells), resulting from a developmental block at the CD4/CD8 DN, CD44-CD25+ stage of thymic differentiation. A similar and predicted phenotype was observed in mice homozygous for a disrupted TCRβ locus (Mombaerts et al., 1992). Furthermore, the phenotype of the RAG-2−/+ mice can be rescued by backcrossing these mice with mice carrying a transgene for a rearranged TCRβ chain (Shinkai et al., 1993). This data suggests that an important developmental selection event takes place at the CD4/CD8 DN, CD44- CD25+ stage of thymic ontogeny, that requires a thymocyte to express a functional TCRβ protein. The expression of a TCRβ protein has also been shown to induce a block in further TCRβ loci rearrangement (Uematsu et al., 1988). This TCRβ-mediated selection therefore avoids the unwanted accumulation of "useless" thymocytes with either no or non-productive TCRβ rearrangements, and ensures that a developing thymocyte expresses only one type of TCRβ chain.

The nature of the TCRβ chain/gp33 complex-mediated selection event is not fully understood, although it is likely to involve the transduction of a signal from the cell surface. In support of this, the 6 day co-culture of anti-CD3ε antibodies (known to transduce a signal via the TCR/CD3 complex) with day-14 fetal thymic organ cultures from RAG-1−/+ mice, restored the phenotypic maturation of the previously blocked CD4/CD8 DN, CD44- CD25+ population of thymocytes to the CD4/CD8 DP stage, increasing thymocyte cell number by 5-fold (Levelt et al., 1993). This suggests that CD3ε may also be associated with TCRβ and gp33 and that a signalling event through this complex (even in the absence of the TCRβ protein) can partially rescue the RAG-1−/+ phenotype.
The tyrosine kinase p561ck has also been implicated in these signalling events. Mice homozygous for a non-functional p561ck gene display a phenotype that shares many characteristics with that of the TCRβ- mice. These include almost no thymocytes (approx. 1-5x10⁶ cells - 1% of wild type), and an accumulation of cells at the CD4+ CD25+ stage of thymic ontogeny. However, CD4/CD8 DP cells are present in p561ck mice, suggesting that p561ck is involved in thymocyte expansion rather than the initiation of CD4 or CD8 expression.

Thymocytes progressing through the CD4/CD8 DN, CD4- CD25+ stage of thymic development immediately downregulate CD25 expression. CD4 and CD8 begin to be expressed (first seen at 15/16dpc), with CD8 observed slightly before CD4 (Guidos et al., 1989a). This is accompanied by a second wave of RAG-1 and RAG-2 expression, directing the rearrangement of the TCRα locus. The TCRα chain associates with both the TCRβ chain and the various subunits of CD3 which allows the low level expression of a functional αβ TCR/CD3 complex on the surface of DP thymocytes (Crispe et al., 1987, Guidos et al., 1990).

The most primitive subset of the CD4/CD8 DP population are large dividing blast cells which comprise approximately 20% of DP thymocytes. The other 80% consists of non-cycling cells that are considerably smaller. The developmental potential of these blast and "small" DP thymocyte populations differs greatly. For example, although the adoptive transfer and culture of DP blast thymocytes results in >95% "small" DP thymocytes being generated, a proportion of the blast cells develop into the precursors of either CD4+/CD8-/TCRα or CD4-/CD8+/TCRα SP thymocytes (Guidos et al., 1990). In the complementary experiment, the transfer of the "small" DP thymocytes results in no further differentiation and the gradual loss of these cells. The DP/TCRα blast cell population is therefore likely to be the major progenitor for TCR-mediated positive and negative selection events within the thymus.

1.1.2 Late thymocyte differentiation - positive and negative selection.

Kinetic experiments estimate that between 5-8x10⁷ CD4/CD8 DP thymocytes (~30% of the DP population) are produced in the adult mouse thymus each day. However, only between 2-4x10⁶ cells (1% of the DP population) actually mature to the SP stage and leave the thymus. The reason for this discrepancy is that immature CD4/CD8 DP thymocytes are subject to at least two selection events, resulting in the specific deletion of the majority of cells. Positive selection ensures that only thymocytes that express a functional αβTCR (capable of recognising foreign antigen in combination with host MHC complexes) are allowed to mature. Negative selection
removes the majority of thymocytes that possess an \( \alpha \beta \)TCR which would recognise self-antigen and thus could initiate auto-immune responses (Blackman et al., 1990).

1.1.2.1 Positive selection.

Positive selection is the process by which immature CD4/CD8 DP thymocytes, that would otherwise be relatively short-lived, are rescued to mature into either CD4 SP T-helper cells, or CD8 SP cytotoxic T-cells.

The signal for a thymocyte to undergo positive selection is based on the overall affinity of the interaction between the \( \alpha \beta \)TCR/CD3 complexes on the surface of a DP thymocyte and the polymorphic domain of a set of MHC molecules that are expressed on a wide variety of APCs (Gosgrove et al., 1991). If this overall affinity reaches a certain threshold, a signal for positive selection is delivered to the thymocyte. Only a minority of \( \alpha \beta \)TCRs will recognise a self-MHC molecule, and it is the thymocytes that express these particular TCRs that will be positively selected. MHC molecules rarely exist at the cell surface without peptide, yet it is evident that positive selection can occur in the absence of the specific peptide that will be later required for activation of the mature T-cell. For example, positive selection of a thymocyte expressing a TCR specific for a male-specific peptide can occur in female mice (Kisielow et al., 1988b).

The consequences of positive selection are numerous. Thymocytes are rescued from PCD, the level of a number of thymocyte cell surface markers such as CD69 are altered (Bendelac et al., 1992), and the cell can mature without further proliferation (Huesmann et al., 1991). In addition, the RAG-1 and RAG-2 genes are now repressed (Turka et al., 1991), effectively halting the further rearrangement of the TCR\( \alpha \) locus. Therefore, until the \( \alpha \beta \)TCR engagement of an intra-thymic MHC ligand (and positive selection) the thymocyte will continue to produce TCR\( \alpha \) chains until a productive \( \alpha \beta \)TCR is generated (Borgulya et al., 1992, Brandle et al., 1992). As no TCR\( \alpha \)-mediated feedback mechanism exists that prevents further TCR\( \alpha \) rearrangement (as for the TCR\( \beta \) protein), it is possible that mature T-cells may actually express two or more functional \( \alpha \beta \)TCR complexes (Borgulya et al., 1992, Padovan et al., 1993).

CD4 and CD8 are known to be essential in the process of positive selection. "Knockout" mice with either no CD4, or no CD8, do not produce mature T-helper cells, or cytotoxic T-cells, respectively (Rahemtulla et al., 1991, Fung Leung et al., 1991). In this context, CD4 and CD8 function by stabilising TCR/MHC interactions. CD4 specifically stabilises a TCR/MHC class II interaction, while CD8 will specifically stabilise a TCR/MHC class I interaction. However, as
yet it is unclear by what mechanism DP thymocytes that express an MHC class I-specific TCR become CD8+ cytotoxic T-cells, and DP thymocytes that express an MHC class II-specific TCR become CD4+ helper T-cells. Nevertheless, two possible models have been proposed; the instructive (von Boehmer et al., 1986), and the stochastic/selective (Robey et al., 1991). The instructive model predicts that on the interaction with an MHC class I molecule, an MHC class I-specific TCR in association with the CD8 co-receptor would generate a specific signal instructing the thymocyte to develop as a cytotoxic T-cell. Conversely, when an MHC class II-specific TCR plus CD4 interacts with an MHC class II ligand a different signal would be generated that instructs development of that thymocyte to a T-helper phenotype. In contrast, the stochastic/selective model argues that the decision to downregulate either CD4 or CD8 is random, regardless of the MHC specificity of the TCR. Further maturation of the cell then depends on the chance that the correct co-receptor has been retained. Therefore, thymocytes expressing an MHC class I-specific TCR and CD8 can mature further, but those that express CD4 will not. The same applies to thymocytes expressing an MHC class II-specific TCR, except that CD4 retention will allow further maturation, whereas CD8 expression would not. At the present time, a greater body of evidence argues for the stochastic/selective model of lineage determination. For example, this model predicts that if all developing DP thymocytes express a CD4 co-receptor transgene then cells that have downregulated endogenous CD4 (retaining CD8) but which express an MHC class II-specific TCR would be rescued to develop further. Consistent with this prediction mature CD4(transgene)/CD8(endogenous) T-helper cells have been observed in mice transgenic for murine CD4 (Chan et al., 1993, Davis et al., 1993). In addition, in mice that express no MHC class II (Gosgrove et al., 1991, Viville et al., 1993, Grusby et al., 1993), and hence have no mature CD4+ T-helper cells, a population of CD4+, CD8+ cells is observed, consistent with the hypothesis that these thymocytes have started to randomly downregulate CD8, but will not mature further due to the absence of MHC class II.

However, despite these observations further investigation is necessary to confirm or refute these theories and to fully understand the mechanisms involved in positive selection.

1.1.2.2 Negative selection

Due to the essentially random generation of the TCR, it is inevitable that certain thymocytes will express a TCR that will recognise self-antigen. To safeguard against the eventuality of an auto-immune response, a number of mechanisms exist that control the action of these self-specific T-cells. One such mechanism is the induction of peripheral T-cell anergy. This
occurs when a T-cell recognises a self-antigen, but fails to receive the co-stimulatory signals necessary to mediate T-cell activation. These co-stimulatory signals are generated only if the cell which displays the antigen is a "professional" APC. In the absence of these signals activation through the TCR complex results in the induction of a non-responsive state.

Although mechanisms exist that control the peripheral activation of self-reactive T-cells, a large proportion of potentially auto-reactive immature TCRαβ-expressing CD4/CD8 DP thymocytes are clonally deleted before leaving the thymus, in a process known as negative selection. For example, mice transgenic for an αβ TCR that is specific for the male transplantation antigen HY have been generated (Kisielow et al., 1988a). In female transgenic mice (HY -ve), virtually all mature T-cells expressed this TCR (being CD8+ and MHC class I restricted). However, in male mice (HY +ve) a massive deletion of CD4/CD8 DP thymocytes was observed, such that the thymus was only 5% of normal size.

Negative selection is thought to be the consequence of a strong affinity interaction between the TCR and CD4 (or CD8) co-receptor on an immature DP thymocyte and the MHC/self-peptide complex on an APC. The interaction between TCR, CD4/CD8 co-receptor and MHC/self-peptide seems highly sensitive. For example, transgenic mice have been generated expressing H-2kα at either a higher than normal level or a level 33% of that of wild type (Nossal et al., 1994). When these mice were crossed with transgenic mice for a H-2kα-specific TCR gene, the H-2kα low expressing mice displayed negative selection of immature thymocytes only in the presence of normal levels of CD8. However, in the mice that over-expressed H-2kα, negative selection was CD8 independent. This would suggest that there is a critical threshold of interaction for the induction of negative selection, to which the TCR, the MHC/peptide, and the co-receptor (CD4 or CD8) all contribute.

In summary, the thymus is the site of the development of a hugely diverse, self-restricted T-cell repertoire. From very few bone-marrow derived progenitors, a rapid expansion of thymocytes expressing a functional but randomly rearranged TCRβ-chain generates many millions of immature CD4/CD8 DP thymocytes each day. As this cell population expands, each of these thymocytes also begins to express a randomly rearranged TCRα chain, allowing the formation of a unique TCRαβ complex. Selection of those thymocytes expressing TCRαβ complexes capable of recognising foreign antigen in combination with self-MHC (positive selection) and elimination of those thymocytes expressing TCRαβ complexes that recognise self-antigen (negative selection), generates a highly specific and restricted population of mature T-cells capable of recognising and neutralising a vast array of invading antigens.
1.2 The effect of the HIV-1 nef gene on late thymic ontogeny.

1.2.1 CD4 downregulation and a decrease in thymic positive selection.

The analysis of the CD2-nef transgenic mice has revealed a dramatic perturbation in the late stages of thymocyte differentiation. In all 1147-nef and 1191-nef expressing lines, a marked downregulation of cell surface CD4 on the CD4/CD8 DP thymocyte population (and to a lesser extent on the CD4 SP thymocyte population), and a significant reduction in the absolute number and percentage of CD4 SP thymocytes (and to a lesser extent CD8 SP thymocytes), was observed. The cell surface levels of CD8 were also decreased on the CD4/CD8 DP population but to a lesser degree than that of CD4. This phenotype was consistent in all the 1147-nef and 1191-nef transgenic lines. However, line F(1147) displayed a more severe phenotype. In addition to a more pronounced downregulation of CD4 on DP cells and a greater loss of CD4 SP mature thymocytes, the CD4+ and CD8+ peripheral T-cell populations were also greatly depleted in both the lymph nodes and the spleens of transgenic animals. The cell-surface level of CD4 on the peripheral CD4+ T-cell population was also slightly reduced. The explanation for this severe phenotype in line F is probably the result of a high level of nef transgene expression, being considerably higher in this line than any other studied. This suggests that the effects of the expression of the HIV-1 nef gene on the events of late thymic ontogeny were dose-related.

A Nef-mediated decrease in the rate of thymic positive selection may be a possible explanation for the reduction in the percentage of CD4 and CD8 SP thymocytes that are generated from the CD4/CD8 DP population in CD2-nef transgenic animals. As discussed in section 1.1, positive selection depends on the overall affinity of the interaction between the CD4/CD8 DP thymocyte and an APC. The affinity of this interaction is dependent on several factors. These include the presence of an αβTCR that recognises a self-MHC molecule, and the cell surface concentration of the molecules involved; the TCR, the MHC and the co-receptor, be it CD4 or CD8 (Bogen et al., 1992). For example, mice whose thymocytes all express a transgenic TCR restricted by a self-MHC class II molecule, generate a massive increase in the number and proportion of mature CD4 SP cells (Berg et al., 1989, Kaye et al., 1989). Conversely, mice deficient in MHC class II molecules (Gosgrove et al., 1991) or mice regularly treated with anti-MHC class II antibodies (Kruisbeek et al., 1985) show a dramatic decrease in CD4 SP T-cell production. Finally, mice hemizygous for CD4 (Rahemtulla et al., 1991) or mice treated with anti-CD4 antibodies (Zuniga-Pflucker et al., 1989) also display a reduced number of mature CD4.
In the case of the CD2-\textit{nef} transgenic mice, the cell surface level of CD4 on the DP population of thymocytes is reduced to between 30-70\% of the littermate controls. This will effectively lower the affinity of the interaction between the thymocyte and the APC. If this affinity falls below a critical threshold level, the thymocyte will not receive the correct maturation signal to undergo positive selection. In such an hypothesis, DP thymocytes expressing very low levels of CD4 will not mature to the SP stage. This predicts that the mature CD4 SP population in the CD2-\textit{nef} transgenic mice would display a higher average level of cell surface CD4 than the CD4/CD8 DP population (as only CD4 high expressing cells are selected), which is exactly what is observed in all lines of mice (table 7). A further prediction of this hypothesis would argue that a greater downregulation of CD4 at the DP stage would result in a more dramatic reduction in the percentage (and absolute number) of mature CD4 SP cells. This is again seen, as highlighted by the severe phenotype of the F line of mice (cf. tables 3 and 7).

The decrease in the peripheral populations of CD4+ and CD8+ T-cells in the F line of CD2-\textit{nef} transgenic mice is most likely a consequence of the severely reduced thymic output of both CD4 and CD8 SP thymocytes. As the rate of production of these subsets is particularly reduced in this line, an incomplete seeding of the peripheral lymphoid organs would most likely result. The output of the thymus in the other lines of 1147-\textit{nef} and 1191-\textit{nef} transgenic mice is probably still sufficient to provide an adequate number of T-cell to both the lymph nodes and spleen in these animals.

Finally, although murine CD8 displays a considerable degree of downregulation in the presence of Nef, the significance of this observation to an HIV infection is probably low. Garcia \textit{et al.} (1993) demonstrated that a number of Nef isolates were able to downregulate both human and murine CD4 to very similar extents. However, even though murine CD8 showed an appreciable level of Nef-mediated downregulation, human CD8 was unaffected. The explanation for this difference may be the amino acid sequence of the CD8 cytoplasmic tail, which in mice but not in humans strongly resembles that of CD4. Therefore, in the rest of the discussion, the emphasis of the Nef-mediated downregulation effects will be directed toward CD4.

\subsection*{1.2.2 Intracellular Sequestration of CD4}

The data from the CD2-\textit{nef} transgenic mice demonstrates that the expression of the \textit{nef} transgene results in a reduced expression of cell surface CD4 and CD8 on the CD4/CD8 DP
population of thymocytes. It also suggests that the CD4 is sequestered internally, co-localising with a marker specific for the Golgi apparatus, but not with a marker specific for the endoplasmic reticulum (figures 21 and 22). Thymocytes are known to possess large nuclei, with only a thin band of cytoplasm. As a result, many of the cellular organelles localise to the perinuclear region of the cell, which is the area highlighted by the Golgi stain. Consequently, it is possible that the downregulated CD4 could either be in the Golgi complex, or in an organelle also located in the perinuclear region, such as the endosome.

Previous work in T-cell lines has demonstrated that CD4 downregulation is not a consequence of the reduction of the steady state level of CD4 mRNA or protein (Garcia & Miller, 1991). Taken together with the data from the immunofluorescence staining, in which an intracellular accumulation of CD4 was observed (figure 21), this suggests that the HIV-1 nef gene modifies CD4 expression at a post-translational level. A Nef-mediated block in the intracellular pathway for modification or processing of the newly translated CD4 molecule could result in such an effect. As a result, CD4 may accumulate in an intracellular compartment on route to the surface. This could explain a Golgi localisation for the internally stained CD4 protein. Alternatively, CD4 may be internalised after its appearance at the cell surface. In other in vitro systems, CD4 downregulation can be obtained by exposing T-cells to antibodies against CD4 (Cole et al., 1989), CD2 (Blue et al., 1989), or the TCR/CD3 complex (Rivas et al., 1988). In addition, a reduction of cell surface CD4 expression can be generated by incubating T-cells with specific antigen (Acres et al., 1986), gangliosides (Offner et al., 1987), the soluble HIV-1 gp120 protein (Cefai et al., 1992), or phorbol myristate-13-acetate (PMA) (Acres et al., 1986). In the majority of the above cases, the mechanism of CD4 downregulation is thought to be the induction of an increased rate of endocytosis. In non-lymphoid cell lines such as HeLa or NIH-3T3, the cell surface expression of transfected CD4 only reaches 60% of the total amount of CD4 protein (Pelchen-Matthews et al., 1989). In these cells, CD4 is continually internalised via its association with the clathrin coated pits and vesicles of the endocytic pathway, and is recycled to the cell surface via the early endosomes. The cytoplasmic domain of the CD4 molecule has been shown to be necessary and sufficient for this CD4 endocytosis (Shin et al., 1991). This domain contains a di-leucine structural motif (Leu-413 and Leu-414) that has previously been demonstrated to be responsible for the endocytosis of the CD3γ and CD3δ proteins (Letourneur & Klausner, 1992). Upon addition of PMA, the rate of CD4 endocytosis is increased from 2%/min to approximately 12%/min, reducing the cell surface expression of CD4 to about 20% of the total CD4 protein. PMA induces the protein kinase C-mediated phosphorylation of three serine residues (at 408, 415 and 431) in the cytoplasmic domain of CD4 (Shin et al., 1991). This is thought to induce a
conformational change in the CD4 molecule, either facilitating the entry of CD4 into the coated pits, or allowing a more favourable interaction with factors involved in endocytosis.

In lymphoid cell lines such as CEM, the cell surface expression of CD4 is >95% of the total CD4 protein (Pelchen-Matthews et al., 1991). The tyrosine kinase p561ck is thought to be responsible for this increased stabilisation of surface CD4 expression (Pelchen-Matthews et al., 1992). p561ck is known to bind to a region which includes two cysteine residues (at 420 and 422) in the cytoplasmic domain of CD4 (Turner et al., 1990). This interaction effectively anchors the CD4 at the cell surface by not allowing the CD4 to enter the clathrin coated pits (Pelchen-Matthews et al., 1992). PMA addition has been shown to dissociate the p561ck/CD4 interaction, possibly as a consequence of the conformational changes induced by the phosphorylation of the serine residues on the CD4. This then allows the CD4 access to the coated pits via the di-leucine structural motif. The PMA-induced internalisation of CD4 in lymphoid cells is not as great as that observed in PMA treated non-lymphoid cells, but it is higher than the CD4 downregulation seen in resting non-lymphoid cell lines that lack p561ck.

The induction of a dramatic increase in the rate of endocytosis has also been strongly implicated in the Nef-mediated downregulation of CD4 (Aiken et al., 1994). The di-leucine motif in the cytoplasmic domain of CD4 was shown to be critical for this effect. However, the actual mechanism of the Nef-mediated induction of endocytosis is different from that observed with PMA. The Nef-mediated effect is independent of serine phosphorylation and only requires the 20 membrane-proximal amino acids of the cytoplasmic domain of CD4. Interestingly, this does not contain the two cysteine residues that are known to be involved in the binding of p561ck. Therefore, the Nef protein must either directly or indirectly induce a greater efficiency of interaction between the CD4 di-leucine motif and the endocytic machinery of the clathrin-coated pits. This may be analogous to the type of serine phosphorylation-induced conformational changes thought to be responsible for CD4 downregulation by PMA. Lastly, even though the cysteine residues that bind p561ck were shown not to be necessary for Nef-mediated CD4 downregulation, the Nef protein may first have to induce the dissociation of p561ck from wild type CD4 in order to induce the subsequent increase in CD4 endocytosis.

1.2.3 The role of CD4 downregulation in HIV-1 infection.

CD4 downregulation by the nef gene is likely to be critical in an HIV infection. This phenomenon is conserved in greater than 80% of nef genes from primary HIV isolates, indicating
that it is a strongly selected in vivo function of Nef (Mariani & Skowronska, 1993). The importance of CD4 downregulation in HIV infection has been highlighted by the fact that two additional mechanisms for this process have now been identified. The HIV gp160 Env precursor protein is known to bind CD4 in the endoplasmic reticulum (ER), preventing its transport to the cell surface (Crise et al., 1990), and the HIV-1 Vpu protein has been shown to induce the degradation of ER-localised CD4 by a process dependent on certain residues located in the cytoplasmic domain of the CD4 molecule (Willey et al., 1992b).

HIV-1 env and vpu are late viral genes. Their expression is dependent on the presence of significant levels of the HIV-1 Rev protein. This effectively suppresses RNA splicing events that would have previously removed both the env and vpu open reading frames. The result is that the Env and Vpu proteins only appear late in an acute HIV infection (Kim et al., 1989, Klotman et al., 1991). Furthermore, their expression may not be observed at all if viral latency is established (Pomerantz et al., 1990). In contrast, the nef gene is expressed very early after HIV infection, in a Rev independent manner. After RNA processing, nef transcripts have been demonstrated to constitute approximately 80% of the products of immediate HIV transcription (Robert-Guroff et al., 1990). This strongly suggests that the importance of Nef-mediated CD4 downregulation could be most evident early in HIV infection, or during viral latency, before the late gene products are generated.

A comparison of the mechanisms of Nef-mediated and Env/Vpu-mediated CD4 downregulation supports this hypothesis. CD4 is relatively stable at the cell surface. Therefore, an endoplasmic reticulum-located, gp160-induced block in the transport of CD4 to the cell membrane would only alter surface CD4 expression at the rate of CD4 degradation. As the half-life of CD4 is at least 4 hours (Aiken et al., 1994), an Env/Vpu-mediated block on cell surface CD4 expression is likely to be a slow process. Nef-mediated downregulation of CD4 via an increase in the rate of endocytosis is rapid. Rates of CD4 internalisation have been estimated at up to 10% of surface CD4 protein per minute (Aiken et al., 1994). Therefore, as soon as the level of Nef protein reaches a certain threshold, the expression of CD4 at the cell surface can be significantly reduced in minutes rather than hours.

Thus Nef-expressing cells have the potential to rapidly downregulate the cell surface CD4 receptor for HIV infection. A possible advantage of this would be the prevention of potentially cytotoxic multiple superinfections. This process has been well characterised for a number of retroviruses and is known as interference (Stevenson et al., 1988, Crise et al., 1990). In addition, certain viruses such as the influenza virus (Muchmore & Varki, 1987), and the coronavirus (Vlasek et al., 1988) have been shown to encode an enzyme that specifically degrades their cell
surface receptor. Therefore, the downregulation of the cell surface expression of the viral receptor appears to be important in many viral life cycles, preventing multiple infections and the subsequent overloading of the cellular machinery involved in the production of progeny virus.

As well as guarding against disadvantageous superinfection events, the removal of CD4 from the cell surface may further improve the efficiency of the viral life cycle. As mentioned above, the HIV-1 Env protein binds to CD4 in the ER. This prevents the transport of gp160 to the cell surface, possibly reducing the effectiveness of virion assembly. For this reason the Vpu protein may have evolved to liberate CD4-associated gp160 by specifically degrading ER-localised CD4. If such a CD4/gp160 interaction is detrimental to virus production when occurring in the ER, it may also be detrimental when occurring at the cell surface. It has been demonstrated that the accumulation of cell surface CD4/gp160 complexes can interfere with the correct budding of the virus, as in T-cells transfected with an HIV-1 provirus and selected for the presence of high surface levels of CD4 virion particles fail to form efficiently, and aggregate under the cell surface in large complexes (Marshall et al., 1992). In addition, it is thought that the surface gp160/CD4 interaction can undermine the integrity of the plasma membrane, leading to cell death. Therefore, the Nef-mediated downregulation of CD4 into the endosomal pathway could reduce the number of CD4-associated gp160 env molecules at the cell surface, decreasing the possible cytotoxicity of this interaction and greatly increasing the efficiency of viral budding.

Although the reduction in the expression of the cellular HIV receptor may play an important role in the efficiency of the viral life cycle, evidence suggests that the role of Nef-mediated CD4 downregulation may be slightly more complex. For example, CEM T-cell lines have been shown to express CD4, demonstrate Nef-mediated downregulation of CD4, and are highly susceptible to HIV-induced cytopathicity. However, in such cells a wild type virus with a functional nef gene does not display a significant growth advantage over a nef-deficient mutant (Poulin et al., 1991). This may be a consequence of the immortalised phenotype of these CEM cells, or the non-physiological dose of HIV often used in these experiments. Alternatively, it could also indicate that Nef-mediated downregulation of CD4 has an important role in activated primary T-cells that cannot be fully appreciated in cultured T-cell lines.

CD4 can have a positive or negative role in T-cell activation responses depending on the nature and combination of the stimuli. A negative signal is transduced by CD4 in the absence of a signal through the T-cell receptor complex and other co-receptor molecules. For example, the cross-linking of CD4 with either anti-CD4 antibodies (Newell et al., 1990), or the HIV-1 gp120 env protein (Banda et al., 1992), has been demonstrated to prime T-cells for apoptosis on a subsequent TCR-mediated stimulus. Therefore, one could speculate that by reducing the level of
CD4 (by Nef expression) in the presence of systemic gp120 (due to HIV infection), the virus would decrease the possibility of an infected cell being induced to enter the apoptotic pathway, thereby maximising the potential for virus production.

In addition to the induction of apoptosis, a signal transduced through CD4 and p56lck has recently been demonstrated to significantly reduce transcription from an integrated HIV provirus (Tremblay et al., 1994). The CD4-mediated stimuli in these experiments were the consequence of HIV virion binding. Therefore, if this situation is relevant in vivo, the Nef-mediated downregulation of CD4 would effectively increase HIV transcription thereby increasing the potential for generating new virus.

CD4 not only transduces signals from the cell surface that have an inhibitory or negative effect. In antigen-specific T-cell activation, CD4 acts as a co-activator (Zuniga-Pflucker et al., 1991). Therefore, it could be argued that by reducing cell surface levels of CD4, T-cells may become hypo-responsive. As transcription from the HIV LTR is dependent on factors induced by T-cell activation, this hypo-responsive state may force the HIV provirus into latency. Latency may prevent the recognition of the HIV-infected cell by the immune system, possibly allowing a long-term replicative advantage for the virus.

Although this induction of viral latency may be important in the life cycle of HIV, it again appears unlikely to represent the sole function of Nef. In the SIV/macaque/rhesus monkey system, the infection of animals with a mutant SIV that contained a stop codon in the nef gene, resulted in a reversion to a wild type nef in less than two weeks. This strong selective pressure is evident before the action of the immune response would have any significant effect. Therefore, the selection of virus with full length nef sequences would not be the consequence of the escape of immune clearance via a Nef-mediated induction of viral latency.

In the light of this last observation, it has frequently been argued that the Nef-mediated downregulation of CD4 may actually increase the activation potential of T-cells. This hypothesis is based on the cell surface interaction of CD4 with the p56lck tyrosine kinase molecule (Veillette et al., 1988). It has been demonstrated that the increase in non-CD4-associated p56lck is accompanied by less stringent requirements for TCR-based T-cell activation (Haughn et al., 1992). Therefore, Nef-induced CD4 downregulation would increase p56lck activity and lead to the activation hyper-responsiveness of the T-cell. This would increase transcription from the HIV LTR and significantly enhance the generation of progeny virions. This hypothesis has received considerable support in recent months (Skowronski et al., 1993, Littman, 1994). However, the data from the work presented in this report does not support this theory. As discussed later, the activity of p56lck in these CD2-nef transgenic mice is not increased and the T-cells from these
animals are not hyper-responsive to either general activation stimuli or activation through the TCR/CD3 complex.

In conclusion, the role of Nef-mediated CD4 downregulation is still the subject of intense debate. Many of the afore-mentioned consequences of CD4 downregulation may be important at different stages in the HIV life cycle, or under certain physiological conditions. However, a considerable amount of investigation is still required using both in vitro and in vivo models of HIV infection and Nef function in order to fully understand the relevance of this process.

1.3 The effect of the HIV-1 nef gene on early thymic development - a reduction in thymic cellularity.

In addition to the Nef-mediated downregulation of cell surface CD4 and the subsequent effect on CD4/CD8 DP thymic selection, the results from the CD2-nef transgenic mice also suggest that there is a perturbation of thymocyte development at an earlier stage of thymic ontogeny. This phenotype is readily detectable as a decrease in the overall size of the thymus as compared to non-transgenic littermate controls, and is most severe in the F line of transgenic mice. This line also expresses the highest level of nef mRNA, shows the most dramatic decrease in the expression of cell surface CD4, and exhibits the greatest selective loss of the mature CD4 SP thymocyte population. Therefore, the fact that this decrease in thymic cellularity was more difficult to detect in the other lines of transgenic mice may be the consequence of the sensitivity of this phenotype to the level of transgene expression.

In the F line of mice, a significant difference in total thymic cellularity is first evident at 16dpc. This coincides with the first appearance of CD4/CD8-expressing DP thymocytes, and is also the first time point at which CD4 is seen to be downregulated by the HIV-1 nef transgene. This latter observation suggests that the amount of cell surface CD4 never actually reaches wild type levels. However, although it is tempting to relate CD4 downregulation to the profound Nef-mediated effect on thymus cell number, it has been demonstrated that in mice homozygous for a non-functional CD4 gene, the cellularity of the thymus is comparable to littermate controls (Rahemtulla et al., 1991). This implies that the dramatic decrease in thymocyte number in the CD2-nef transgenic mice is not a consequence of a simple lack of CD4 at the cell surface.

The decrease in the size of the developing thymus could be the consequence of a number of possible mechanisms. For example, there may be fewer bone-marrow derived progenitors seeding the thymus, even though the subsequent thymic development of these cells may occur
normally. This seems an unlikely explanation for the decrease in thymic cellularity in the CD2-nef transgenic mice. When analysing all cells which do not express lineage markers such as CD4 and CD8, the absolute number of early progenitors per transgenic thymus is not significantly lower than that of the non-transgenic controls, even though the nef-expressing thymus is on average a third to a half the size of normal (figure 25 and table 10).

A second possibility for reduced thymic cellularity, is that the same number of cells seed the thymus and expand normally, but die prematurely before they can fully mature. The most likely cause of thymocyte mortality is the induction of PCD or apoptosis. This has many precedents. For example, as described in section 1.1 of the discussion, those immature thymocytes that either fail to be positively selected or undergo negative selection, will receive a signal that initiates apoptosis (Smith et al., 1988). In addition, apoptosis has also been observed after the activation of immature thymocytes with a variety of stimuli, such as anti-CD3 mAbs, anti-CD4 mAbs, or PMA/ionomycin (McConkey et al., 1989, Ucker et al., 1989). In relation to HIV-1, the gp120 protein has been demonstrated to prime human T-cell lines for apoptosis upon subsequent activation (Banda et al., 1992), and the huge thymocyte destruction after an HIV-1 infection of the SCID-hu mouse model is thought to be the result of the induction of massive PCD (Jamieson et al., 1994).

In the CD2-nef transgenic mice there is no evidence to suggest apoptosis is increased (figures 27 and 28). The data using TdT staining of thymic cryostat sections, and the FACS analysis of thymocytes using propidium iodide, reveal no increase in the cellular events known to be associated with PCD. It is therefore reasonable to conclude that there is no detectable increase in the amount of apoptosis in the CD2-nef transgenic mice.

It is also plausible that the expression of the nef gene causes the premature death of thymocytes by a mechanism other than the induction of apoptosis. This again seems an unlikely explanation for the reduced cellularity in a transgenic thymus. A high level of Nef protein is still observed in the peripheral T-cells of these mice (figure 18), and the cells which mature past early stages of thymic development obviously express reasonable levels of Nef as judged by the still extensive level of cell surface CD4 downregulation. Although very high levels of Nef at certain stages of thymocyte development could be cytotoxic, this possibility is not consistent with the complex phenotype of the CD2-nef transgenic mice, with the severe downregulation of cell surface CD4 at the CD4/CD8 double positive stage, the decrease in the CD4 SP thymocyte population, and the high level of nef mRNA expression throughout thymic ontogeny.

A third possibility for the reduced size of the transgenic thymi is that the expansion of a certain population of thymic progenitors is blocked or retarded. If this were the case, cell
populations at developmental stages before the block would tend to accumulate, and those populations at developmental stages beyond the block would be decreased. As described above, a developmental block is responsible for the small thymi in mice that are deficient for the RAG-1, the RAG-2, or the p56lck gene, or for mice that carry a disrupted TCRβ locus (Shinkai et al., 1992, Mombaerts et al., 1992b, Molina et al., 1992).

The CD2-nef transgenic animals display a phenotype that shares a number of similarities with the phenotype of the p56lck−/− or TCRβ-deficient mice. The first is obviously the reduction in thymic cellularity. A normal adult thymus has approximately 250×10⁶ thymocytes, while that of the p56lck−/− and TCRβ-deficient mice, have only between 1-6×10⁶ cells (Mombaerts et al., 1992a, Molina et al., 1992). CD2-nef transgenic adults (from line F) have approximately 80×10⁶ thymocytes, 30% of the cells seen in non-transgenic animals (figure 25). This phenotype is not consistent with mouse models that are either CD4-deficient (Rahemtulla et al., 1991), CD8-deficient (Fung Leung et al., 1991), TCRα chain-deficient (Mombaerts et al., 1992a), or MHC class II-deficient (Gosgrove et al., 1991). In all these "knockout" systems thymic cellularity is not decreased.

The analysis of the absolute number and percentage of thymic progenitors in CD2-nef transgenic animals, suggests that a partial block or retardation of thymocyte development occurs between the CD4/CD8 DN, CD44− CD25+ stage, and the CD4/CD8 DN, CD44− CD25− stage of thymic ontogeny. The expression of the nef gene, under the control of the human CD2 promoter and LCR, has been demonstrated at 14dpc (figure 17B). At this stage, the CD44−CD25+ thymocyte population represents the most mature subset of cells in the thymus. Therefore, in the CD2-nef transgenic animals, the expression of the nef gene is evident at least as early as the CD44−CD25+ population of progenitor thymocytes.

In the CD2-nef transgenic mice, the CD4/CD8 DN, CD44−CD25+ subset of transgenic thymocytes appears to be the most differentiated population of cells that is represented at a level that is at least comparable to that observed in non-transgenic animals. After this stage, the absolute number of transgenic thymocytes in all subsequent developmental populations is equivalent to approximately one third of the number of cells observed in non-transgenic littermates (figure 31). In addition, the preliminary results from the fetal thymic organ cultures suggest that the CD44−CD25+ population of transgenic thymocytes expand at a far slower rate to that of non-transgenic controls. This again resembles the phenotype of the p56lck−/− or TCRβ-deficient mice. In both of these systems, thymocyte development proceeds normally until the CD4/CD8 DN, CD44−CD25+ stage of thymic ontogeny. The TCRβ-deficient mice show a complete block at this stage of differentiation, with no thymocyte expansion and no cells
progressing to the CD4/CD8 DP phenotype (Mombaerts et al., 1992a). However, although the p56lck⁻/⁻ mice also display a block at this stage with no thymocyte expansion (hence the small thymus), CD4 and CD8 expression is detected and CD4/CD8 DP cells are evident (Molina et al., 1992).

The CD2⁻nef transgenic animals do have CD4/CD8 DP thymocytes. This would suggest that these mice have a phenotype more closely resembling the p56lck⁻/⁻ mice than that of the TCRβ-deficient mice. However, if the function of the TCRβ chain at this stage in thymocyte development was only partially inhibited by the nef gene, CD4/CD8 double positive thymocytes may well be generated. Therefore, it is still too early to rule out either a direct or indirect Nef-mediated effect on TCRβ function as an explanation for the reduced thymic cellularity in the CD2⁻nef transgenic mice. With this in mind, experiments are now analysing the extent of TCRβ loci rearrangement and TCRβ gene expression during thymic development in CD2⁻nef transgenic animals.

Although the thymi of the CD2⁻nef transgenic mice is reduced in size, this effect is by no means as pronounced as that observed in mice with either no p56lck or TCRβ protein. This suggests, as mentioned above, that the nef gene may not completely inhibit p56lck or TCRβ function. A partial p56lck "knockout" transgenic mouse model has been developed that allows the effect of different concentrations of active p56lck protein to be assessed. This involves a catalytically inactive p56lck transgene, that has been used to generate a number of transgenic lines with increasing ratios of inactive versus active p56lck (Levin et al., 1993). The inactive p56lck acts as a dominant negative mutant, effectively reducing the working concentration of active p56lck at the cell membrane. At a ratio of inactive versus active p56lck of 12:1, a phenotype closely resembling the p56lck "knockout" mice was observed. However, at lower ratios the phenotype of the mice was not as pronounced. As the effective level of active p56lck increased, thymic cellularity was progressively restored to normal.

The CD2⁻nef transgenic mice display a phenotype that more closely resembles a partial "knockout" of the p56lck gene. In adult transgenic mice (line F), the absolute number of thymocytes is approximately 30-40% of that seen in non-transgenic controls, with the peripheral T-cell populations about 5-fold down. This compares very well to a transgenic mouse expressing an inactive versus active p56lck ratio of 3.6:1 (Levin et al., 1993). In this line of mice, the thymus was approximately 35% of the non-transgenic control, and the peripheral T-cell populations began to show a decrease in cell number (down to ~70-80% of controls).

It is therefore plausible, that the expression of the HIV-1 nef gene in the CD4/CD8 DN, CD44⁻CD25⁺ population of progenitor thymocytes could result in a phenotype that resembles
a partial "knockout" of the p56lck gene. This suggests that Nef may have either a direct or indirect effect on the activity of p56lck. However, the activity of the p56lck signalling pathway could be decreased in a number of the following ways; (i) Nef may induce a reduction in the absolute amount of p56lck mRNA or protein, (ii) Nef may downregulate the membrane expression of p56lck by sequestering it internally, (iii) Nef may render the kinase domain of p56lck inactive, or (iv) Nef may effect a downstream substrate in the p56lck signalling pathway, resulting in a phenotype that would be very similar to a p56lck "knockout".

Preliminary results investigating p56lck in CD2-nef transgenic mice have yet to reveal any differences between transgenic and non-transgenic animals. Levels of p56lck mRNA and protein appear normal, and at 16dpc the cellular localisation of p56lck in transgenic thymocytes is comparable to non-transgenic controls. However, further investigations are underway to assess the kinase activity of p56lck in the early CD4/CD8 DN, CD44- CD25+ population of progenitor thymocytes in the presence of Nef.

At this stage of the investigations, the significance of a Nef-mediated inhibition of the p56lck signal transduction pathway is unclear. However, by assuming that a Nef-mediated inhibition of p56lck is occurring (even though one has yet to be identified), a hypothesis can be suggested that links the phenotypes of the early and late Nef-mediated perturbations in thymic ontogeny. As discussed previously, Nef-mediated downregulation of CD4 may first require the dissociation of p56lck from the cytoplasmic domain of CD4. Therefore, it is possible to speculate that in order to disrupt this association and allow CD4 endocytosis, Nef may interact either directly or indirectly (via other cellular proteins) with a specific region of the p56lck molecule. If this interaction also affected the kinase activity of p56lck, a decrease in the activity of the p56lck signal transduction pathway would result. The consequence of such a Nef/p56lck interaction would be; (i) a decrease in cell surface CD4 expression, (ii) a reduction in the efficiency of positive selection, (iii) a decrease in p56lck kinase activity at the early CD44-CD25+ stage of thymocyte differentiation, (iv) less thymocyte expansion from the CD44-CD25+ stage onward, and (v) a possible block in p56lck-dependent proliferative responses in mature T-cells (see next section). Therefore, this hypothesis may well account for the vast majority of the characteristic of the phenotype observed in the CD2-nef transgenic animals.
1.4 Thymocyte activation and proliferation.

The expression of the nef transgene in CD2-nef transgenic mice results in dramatic perturbations in both early and late thymocyte development. These effects involve CD4 and possibly p56^ckk, both molecules that are known to have central roles in T-cell activation. Therefore, it is possible that the nef transgene could also disrupt functional T-cell responses.

Anti-CD3e antibody activation/proliferation assays indicated that equal numbers of total thymocytes from CD2-nef transgenic mice proliferate to a lesser degree than those from non-transgenic littermates. It has been previously demonstrated that anti-CD3e antibody stimulation of thymocytes results in the activation of both SP and DP cells (Havran et al., 1987). However, only SP thymocytes (CD4 or CD8) are able to proliferate in response to this activation signal (Weiss et al., 1987), thought to be via a p56^ckk-dependent signalling pathway. This suggests that the decrease in transgenic thymocyte activation could be due either to a decrease in the number of SP cells in the assay or to the fact that individual SP cells proliferate at a lower rate. When the number of SP cells in the total pool of transgenic thymocytes are taken into account and the figures from the activation assays corrected accordingly (figure 39), the rates of transgenic thymocyte proliferation in lines B and D (1191-nef) become fairly comparable to non-transgenic littermates. However, even after such a correction transgenic thymocytes from lines A and (especially) F (1147-nef) still appear to proliferate to a lower extent than the controls. Therefore, the analysis of the proliferation of an identical number of FACS sorted CD4 SP thymocytes from both transgenic and non-transgenic animals may discover whether the proliferative potential of an individual SP cell from the thymus of a CD2-nef-1147 transgenic mouse is comparable to a non-transgenic control.

The possible decrease in the proliferative capacity of SP thymocytes from CD2-nef transgenic animals after activation through the TCR/CD3 complex may well reflect a Nef-mediated inhibition of the p56^ckk signal transduction pathway. However, from the investigations presented in this thesis, the complex phenotype of the CD2-nef transgenic mice with the range of disturbances in T-cell development may effectively obscure the Nef effect on T-cell activation responses. For this reason, the analysis of transgenic animals that express the nef gene under the control of the murine IL-2 promoter/enhancer, enabling high levels of Nef expression only on T-cell activation, may help to fully evaluate the Nef-mediated effect on T-cell signal transduction via the TCR/CD3 complex.

The activation data obtained from these CD2-nef transgenic animals is in direct contrast to the recent work of others (Skowronski et al., 1993). This group have constructed HIV-1 nef
transgenic mice using the CD3 regulatory elements to direct expression of the *nef* transgene. Despite large losses of CD4 SP thymocytes, they observe massive "hyperactivation" of total transgenic thymocytes on anti-CD3ε activation. The CD3 cassette used in these experiments directs expression of the transgene later in T-cell ontogeny than the CD2 cassette, and does not include elements that confer copy number dependent or position independent expression. Therefore, the vastly different activation phenotypes of these mouse models, could be due to the different developmental expression of the *nef* gene or to the position effects at the site of the CD3-*nef* cassette integration.

1.5 The HIV-1 *nef* gene and the immune system in an HIV-1 infection.

1.5.1 Could Nef function in a similar manner in an HIV-1 infection?

Are the effects observed in the CD2-*nef* transgenic mice relevant to the likely expression pattern of *nef* in an HIV-1 infection? For example, does HIV-1 infect the same subsets of cells that show the severe phenotype in the CD2-*nef* transgenic mice?

It has long been known that the principal cellular receptor for HIV infection is the CD4 molecule. It is therefore possible for CD4-expressing DP thymocytes to readily become infected with HIV, allowing Nef-mediated CD4 downregulation and a subsequent effect on positive selection. However, CD4 is not expressed at the CD4/CD8 DN, CD44- CD25+ stage of thymic ontogeny, suggesting that this population of cells would not be a direct target for HIV infection. However, a recent study has identified a more primitive population of murine thymocytes that express a significant level of cell surface CD4. This population of CD4+, CD8-, CD3-, TCRαβ-, CD25-, HSA+, CD44+ cells is the earliest population of murine thymic progenitors thus far isolated (Wu *et al.*, 1991). If this population has a counterpart in human thymic ontogeny, HIV could infect these primitive cells, allowing expression of the Nef protein at the subsequent CD44-CD25+ stage of development. A Nef-mediated effect on p561ck could then significantly reduce the T-cell output of the developing thymus.

HIV has also been demonstrated to infect certain progenitor populations of the bone marrow. For example, cells within the CD34+ population can be infected with HIV *in vitro* (Folks *et al.*, 1988, Steinberg *et al.*, 1991), and *in vivo* (Stanley *et al.*, 1992). Therefore, if an early population of haematopoietic progenitors can become infected, and progeny of these infected cells eventually seed the thymus, thymocytes of the CD4/CD8 DN, CD44- CD25+ phenotype
could then express the HIV-1 * nef* gene.

### 1.5.2 Thymic HIV infection and the consequence for the immune system.

The thymus is known to be the site of the development of a diverse and complete T-cell repertoire. It is particularly active from late gestation to puberty gradually seeding the peripheral lymphoid organs with functional T-cells, although it is now also thought that T-cell production continues, albeit at a lesser degree, well into late adult life.

Human thymocytes express CD4 from a very early stage of development and their HIV infection has been demonstrated *in vitro* (DeRossi *et al.*, 1990, Tremblay *et al.*, 1990). In contrast, HIV infection of the thymus *in vivo* has only been observed at a low frequency (Schuurman *et al.*, 1989). However, a number of reports have documented a severely involuted aspect of postmortem thymus histology in patients with AIDS (Grody *et al.*, 1985). Several theories have been proposed to explain this high degree of thymocyte cell death in the absence of excessive HIV infection. These include the HIV-induced destruction of the thymic microenvironment (Savino *et al.*, 1986), the induction of apoptosis in uninfected thymocytes after priming by circulating gp120 (Su *et al.*, 1995), and the onset of an HIV-mediated graft-vs.-host disease. However, the results from the CD2-* nef* transgenic mice suggest another possible mechanism for massive thymocyte depletion. If a very early thymic progenitor became infected with HIV (either before it entered the thymus or after), the expression of the * nef* gene at the CD44- CD25+ stage of thymic ontogeny could result in the inhibition of its expansion into potentially 210 or more thymocytes. The result would be a very significant decrease in the output of the thymus, even though it would be possible to detect only one HIV-infected cell. In addition, this effect does not require the infected cell to produce progeny virus, as the * nef* gene is expressed at high levels early after infection even in the absence of the expression of the viral structural genes (Pomerantz *et al.*, 1990). Therefore, if an increasing proportion of these very early progenitors were to become infected over time, this Nef-mediated inhibition of thymocyte expansion may progressively lead to a reduction in the effective regenerative capacity of the thymus.

A reduction in the overall productive capacity of the thymus would have a serious effect on the kinetics of the progression to AIDS. Peripheral CD4+ T-cell death, by direct-cell killing, the induction of apoptosis, the CTL-mediated removal of infected cells, or an autoimmune mechanism, would make it necessary for a large number of new T-cells to be generated to replace those that were lost. However, if the productive capacity of the thymus falls slightly below the
HIV-mediated removal (by whatever mechanism) of CD4+ T-lymphocytes, there would be a small but significant overall net loss of this population of cells. Over an extended period of time this would result in the gradual depletion of the CD4+ T-cell repertoire eventually leading to the onset of full blown AIDS.

If correct, this mechanism of HIV pathogenesis would be of particular relevance in paediatric AIDS. HIV-infected children are known to develop AIDS on average two to three times more quickly than HIV-infected adults. They also display very severe involution and atrophy of the thymus (Joshi et al., 1984). The thymus is known to be very active in children until the end of puberty with a very significant contribution to the peripheral T-cell reservoir throughout this period. Therefore, if the thymic output is severely affected at an early stage of life the reservoir of peripheral T-cells would never be fully seeded. This would in turn leave the patient more vulnerable to the effect of HIV-mediated CD4+ T-cell depletion, and could result in a more rapid progression to AIDS.
2. CD2 HIV-1-tat transgenic mice.

To study the in vivo effect of the HIV-1 tat gene on a mammalian immune system, three transgenic mouse lines were generated expressing the tat gene under the control of the human CD2 regulatory elements and locus control region (LCR). These hCD2 elements direct transgene expression to the majority of thymocytes and mature T-cells. As has been demonstrated by S1 nuclease protection, the steady state levels of tat mRNA in all lines is consistent with copy number, and expression is specific for the tissues of the lymphoid system, namely the thymus, spleen, lymph nodes and peripheral blood.

Unlike the results obtained for the CD2-nef transgenic mice, the results from the experiments with the CD2-tat transgenic mice are at a relatively preliminary stage, and at the time of writing much effort is being directed into analysing the phenotype of these mice in much greater detail. However, it has already become clear that the phenotype of the CD2-tat transgenic mice differs markedly from the phenotype obtained from mice that used the same expression cassette to express the HIV-1 nef gene (strongly implying that these independent phenotypes are a specific result of the expression of the particular HIV-1 regulatory gene in question, and not the use of the hCD2 expression cassette). CD2-tat transgenic mice are overtly normal and live to the expected age. In addition, thymic ontogeny appears to be unaffected by the presence of the tat gene. However, the cytokine profile on activation of thymocytes and peripheral T-cells is significantly altered, with several cytokine genes showing a number of fold increase in the level of mRNA expression.

2.1 Normal thymocyte and T-cell development, and no evidence of tumours /KS-like lesions in CD2-tat transgenic mice.

The CD2-tat transgenic mice are overtly normal. The size and appearance of the thymus, spleen, and lymph nodes does not differ between transgenic and non-transgenic littermates. In addition, the CD4/CD8 thymocyte and mature T-cell subsets are not altered in the transgenic animals. The mice live to the expected age, and even though the tat transgene is still expressed at a high level after 18 months, no signs of an increased incidence of tumour or dermal lesion formation is seen. This is contrary to much evidence in the literature. For example, in vitro work suggests that Tat (Ensoli et al., 1990), TNF and other cytokines (Barillari et al., 1993) can promote the growth and proliferation of Kaposi's sarcoma-derived cells from AIDS patients.
(Ensoli et al., 1990). In addition, it was recently demonstrated that basic fibroblast growth factor and Tat could synergise to induce KS-like lesions in nude mice, even though Tat alone had no effect (Ensoli et al., 1994). This last study could provide an explanation for the lack of KS-like lesion development in the CD2-tat transgenic mice. For example, a co-factor such as basic fibroblast growth factor or another virus may be necessary to induce this neoplastic KS-like state.

An alternative reason for the absence of any KS-like lesions in the CD2-tat transgenic mice could be due to the restricted expression pattern of the CD2-tat transgene. In previous studies, transgenic mice were generated that expressed the tat gene under the control of the HIV-1 LTR (Vogel et al., 1988), and the BK polyoma virus promoter (Corallini et al., 1993). These transgenic animals developed skin lesions, endothelial proliferation and a variety of tumours of different histotypes. However, in both cases, the expression of the tat transgene was not detected in the CD4+ T-cell population, the population of cells that would be expected to harbour a tat gene in an HIV infection. Instead, transgene expression was only detected in non-lymphoid cell types (such as the epidermis), which would normally sustain only very infrequent HIV infection, if any at all. Therefore, the lack of appropriate transgene expression challenges the relevance of the obviously striking results that were obtained in these two systems.

At the time of writing, the serum from the three lines of CD2-tat transgenic mice is being analysed for the presence of extracellular Tat protein, as Tat is known to be secreted from HIV-infected cells and may be actively taken up by a variety of other cell types (Brake et al., 1990).

2.2 Altered cytokine expression in activated thymocytes from CD2-tat transgenic mice.

Northern analysis of total mRNA after the activation of CD2-tat transgenic thymocytes by PMA/ionomycin, indicates that Tat upregulates the level of expression of certain cytokines.

Tat-induced cytokine modulation has previously been demonstrated in vitro. For example, on transfection of the HIV-1 tat gene into a human B cell line, an 8-fold increase in TNF-β mRNA was observed (Sastry et al., 1990). This effect on TNF-β has also been subsequently observed on mitogen stimulated T cell lines, and HIV-1 infected primary T-cells (Buonaguro et al., 1992). The mechanism of this upregulation has been shown to involve the interaction of the Tat protein with a TAR-like structure in the TNF-β promoter (Buonaguro et al., 1994).

The results from the PMA/ionomycin activation of CD2-tat transgenic thymocytes,
demonstrates that both TNF-β mRNA (but not TNF-α mRNA) and TNF protein are upregulated in the transgenic animals. This finding suggests that the Tat protein has an in vivo effect on TNF-β, supporting the previous in vitro observations. The role of a Tat-induced increase in TNF-β production is not clear. TNF-β is a type 1 cytokine involved in cell-mediated immunity. Therefore, an increase in TNFβ production after T-cell activation would argue against there being an HIV-mediated type 1 to type 2 switch. However, the upregulation of TNFβ may provide a further, as yet undiscovered replicative advantage for HIV.

In addition to the in vivo effect of Tat on TNF-β, tat expression in activated thymocytes from CD2-tat transgenic animals also results in the upregulation of TGF-β mRNA and IL-4R mRNA. Elevated levels of TGF-β have previously been found in peripheral blood mononuclear cells (Kekow et al., 1990) and the brains of HIV-1 infected individuals (Wahl et al., 1991). Not all brain cells producing TGF-β were infected with HIV-1, and subsequently the addition of extracellular Tat to human astrocytic glial cells has been demonstrated to transactivate transcription from the TGF-β promoter (Cupp et al., 1993). At present, it is unclear as to what effect the Tat-induced upregulation of TGF-β has on the in vivo immune system, although TGF-β is known to be an immunosuppressive cytokine with an anti-proliferative effect on human T and B cells (Massague, 1990).

Tat-induced elevated levels of IL-4R have previously been demonstrated on a human B lymphoblastoid cell line (Puri & Aggarwal, 1992). However, the significance of an increase in IL-4R is difficult to assess. This upregulation may effectively increase the concentration of IL-4 that a cell would encounter. If this were the case, an increase in IL-4 activity would tend to dampen down cell-mediated (type 1) immunity and promote a humoral (type 2) immune response. As discussed previously this would tend to favour the establishment of an HIV infection. However, as the switch from a type 1 to a type 2 immune response involves many cytokines, the precise role of the Tat protein in influencing this process is as yet uncertain. For this reason, the interaction of the Tat protein with a host of other cytokine genes such as IL-2, IL-4, IL-6, IL-10, IL-12, IFN-α, IFN-β and IFN-γ is currently being investigated.

2.3 Activated T-cell proliferation in CD2-tat transgenic mice

Tat expression in the CD2-tat transgenic animals does not profoundly modulate T-cell proliferation by polyclonal mitogens or PMA. This is in agreement with previous work (Viscidi et al., 1989, Subramanyam et al., 1993). However, these investigations suggested that Tat
inhibited antigen induced T-cell proliferation. This is contrary to the evidence obtained in these experiments. The immunisation of transgenic mice with KLH protein antigen, and the subsequent \textit{in vitro} KLH stimulation of lymphocytes from these animals, revealed no such inhibition of antigen induced proliferation. Therefore, these experiments suggest that the expression of the tat gene does not effect mitogen or antigen-induced T-cell proliferation in CD2-tat transgenic thymocytes.
3. Further investigations.

At the time of writing the analysis of the phenotype of the CD2-nef and CD2-tat transgenic animals is still ongoing. This should allow not only the full identification of the range of Nef and Tat-mediated effects in these mice, but should also provide an insight into the underlying mechanisms that are involved. Briefly described below are a list of investigations that will be conducted on either the CD2-nef or CD2-tat transgenic mice;

3.1 CD2-nef transgenic mice (1147 allele).

(i) In order to better understand the mechanism of CD4 downregulation, the intracellular localisation of CD4 in Nef-expressing thymocytes is currently being examined using confocal microscopy. Markers for the endosome (the transferrin receptor) and the Golgi apparatus (α-mannosidase II) are being used to co-localise CD4 staining with a specific intracellular compartment. This technique should offer a greater degree of accuracy than is available with the fluorescent microscope.

(ii) Using a similar strategy to that mentioned in (i) above, the cellular localisation of p56lck in Nef-expressing thymocytes is being examined. A Nef-mediated re-distribution of p56lck in CD44-CD25+ thymocytes leading to a reduction in the activity of the p56lck signal transduction pathway may explain the decreased thymic cellularity observed in the CD2-nef transgenic mice.

(iii) The kinase activity of p56lck is being examined at the early CD4/CD8 DN stage of thymic ontogeny. An anti-p56lck antibody (gift from Dr. Veillet) will be used to immunoprecipitate p56lck from 15dpc transgenic and non-transgenic total thymic lysates. A kinase assay using the CD3 zeta peptide as a substrate (gift from Dr. Perlmutter, Seattle), will then be performed to assess the effect of Nef expression on p56lck kinase activity. A Nef-mediated decrease in p56lck kinase activity in the CD44-CD25+ thymocyte population could explain the decrease in thymic cellularity in the CD2-nef transgenic animals (Levin et al., 1993).

(iv) The rearrangement of the TCRβ chain is being examined by PCR in both transgenic and non-transgenic animals. An Nef-mediated inhibition of TCRβ rearrangement in the CD44-
CD25+ thymocyte subset could possibly explain the reduction in thymic cellularity in the CD2-nef transgenic animals (Mombaerts et al., 1992a). TCRβ cell surface expression in early subsets of thymic progenitors is also being assessed.

(v) In order to examine whether CD4 downregulation is linked to the decrease in thymic cellularity that is observed in CD2-nef transgenic animals, mice from the 1147-nef expressing F line will be crossed into a CD4− background (gift from Dr. Rahemtulla, Oxford). As CD4+ mice have a comparable number of thymocytes to wild type controls, the analysis of the size of the thymus in the progeny of the above cross will indicate whether the re-distribution of CD4 plays a role in the inhibition of thymocyte expansion in the existing lines of mice. The hypothesis presented for Nef function in the discussion of this thesis would predict that thymocyte cell number in a Nef-expressing CD4+ background would be similar to that observed in a Nef-expressing CD4+/+ background.

(vi) An attempt will be made to rescue the Nef-mediated decrease in thymic cellularity by breeding the F line of transgenic mice with mice transgenic for p56lck (gift from Dr. Perlmutter, Seattle). Such mice express approximately twice the amount of p56lck than wild type, resulting in the delayed phenotypic development of CD4 and CD8 expression but the correct expansion of CD4/CD8 DN thymocytes (Abraham et al., 1991). Therefore if the Nef protein is affecting the activity of p56lck at early time points in thymic development, it may be possible to titrate this effect away by over-expressing p56lck, thus restoring normal numbers of thymocytes to the CD2-nef transgenic animals.

(vii) An attempt will be made to rescue the reduced thymic cellularity phenotype of the CD2-nef transgenic mice in vitro. It has previously been demonstrated that thymocytes from a RAG-1− mouse are blocked at the CD44- CD25+ stage of thymic ontogeny due to their inability to rearrange the TCRβ locus. However, by incubating a 14dpc RAG-1− thymus in a FTOC with anti-CD3ε antibodies, a 5-fold increase in thymocyte cell number and a restoration of CD4 and CD8 expression have been shown (Levelt et al., 1993). This suggests that a signal through the CD3/p56lck complex can partially restore early thymocyte expansion in TCRβ-deficient mice. The thymocytes of a CD2-nef transgenic mouse also display a partial block at the CD44- CD25+ stage of differentiation. Therefore, it may be possible to rescue the phenotype of Nef-expressing thymocytes by incubating thymic lobes from 14dpc animals with anti-CD3ε antibodies. An increase in thymocyte expansion in the presence of these antibodies may suggest that signal
transduction through the TCRβ/CD3/p561ck complex in Nef-expressing thymocytes is normally decreased when compared to non-transgenic controls.

(viii) The transfer of FACS-sorted populations of progenitor thymocytes into 15dpc CBA abladed thymic lobes has been reported for CD44-CD25+ thymocytes in results section 2.8 of this thesis. This type of FTOC analysis will also be employed to examine the expansion potential of other early thymic progenitor populations such as the CD44+CD25- and CD44-CD25-subsets. It is hoped that this will characterise the extent of the block in thymocyte development that has currently been identified in the CD2-nef transgenic animals.

(ix) The further analysis of the proliferation of thymocytes and splenocytes from CD2-nef transgenic animals will be performed. This will primarily concentrate on activation through the TCR/CD3 p561ck-dependent pathway by the use of anti-CD3ε monoclonal antibodies. It is hoped that an equivalent number (from FACS sorting or panning etc) of CD4 SP thymocytes and splenocytes can be used for each experiment in order to directly compare the proliferative potential of Nef-expressing and non-transgenic cells. The cytokine profiles after such cell activation will also be assessed, particularly for the level of IL-2, IL-4 and IFN-γ mRNA.

(x) Additional transgenic lines expressing the nef gene under the control of the murine IL-2 promoter/enhancer will be generated to analyse the effect of high level Nef expression in activated T-cells. As the IL-2 promoter/enhancer will allow only low levels of transgene expression during thymic ontogeny, the Nef-mediated effects on CD4-based positive selection and thymocyte expansion should be avoided. The IL-2 promoter/enhancer should allow a burst of Nef expression only after T-cell activation. Such an expression pattern is a more accurate representation of the expression of Nef after an HIV-1 infection, as proviral integration and the expression of the early HIV-1 genes (including nef) is only possible after the activation of the target cell. In addition these mice could be used to assess the effect of Nef expression on memory T-cell responses. Transgenic mice could be primed with an antigen such as KLH, initiating a primary immune response and hence Nef expression. A few days later, the in vitro challenge with KLH of peripheral T-cells isolated from the primed animal should allow the effect of Nef on the T-cell response to recall antigen to be investigated.
3.2 CD2-tat transgenic mice.

(i) The work presented in this thesis demonstrates that Tat-expressing thymocytes produce significantly increased levels of TNFβ, TGFβ and IL-4R mRNA after cellular activation. In an attempt to understand the significance of this phenotype with regard to the control of cell-mediated and humoral immune responses, the level of expression of a range of other cytokines will be examined. These cytokines include IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, IFN-α and IFN-β. This should then allow the Tat-mediated manipulation of both the type 1 and type 2 immune response to be assessed.

(ii) Recent work has suggested a role for the Tat protein in the control of T-cell apoptosis. Tat-expressing Jurkat cells (a CD4+ human lymphoblastoid cell line) were shown to have an increased resistance to apoptosis induced by incubation with TNFα, anti-CD4 mAbs or anti-fas antibodies (Gibellini et al., 1995). In contrast, the addition of extracellular Tat to Jurkat cells was shown to strongly sensitise them to subsequent apoptosis via the TCR or CD4 (Li et al., 1995, Westendorp et al., 1995). It is therefore hoped that the effect of Tat expression on the induction of, or the resistance to, apoptosis in primary thymocytes and T-cells from the CD2-tat transgenic animals can be examined.

3.3 CD2-nef/CD2-tat transgenic mice.

The CD2-nef and CD2-tat transgenic mice are presently being crossed together in order to examine the synergistic effects of the expression of these two HIV-1 regulatory genes in thymocytes and peripheral T-cells. Thymic ontogeny, peripheral T-cell proliferation and cytokine production, and the induction of apoptosis will be assessed.
4. Summary.

In summary, the work presented in this thesis has studied the in vivo effect of the expression of the HIV-1 nef gene and the HIV-1 tat gene on a dynamic mammalian immune system. Transgenic mouse lines were generated that expressed these genes under the control of the human CD2 regulatory elements and locus control region (LCR). These hCD2 elements direct transgene expression to the majority of thymocytes and mature T-cells. Therefore, these mouse models allow the in vivo effects of Nef and Tat to be analysed in cells of known tropism for HIV-1.

The analysis of the CD2-nef (1147 and 1191 alleles) transgenic mice revealed a dramatic phenotype with the following characteristics; (i) Transgenic animals have a thymus that is reduced in both size and cellularity, (ii) all cell populations after the CD4/CD8 DN, CD44- CD25+ stage of thymocyte differentiation are under-represented in mice that express the nef gene, (iii) the cell-surface expression of CD4 (and to a lesser extent CD8) is significantly downregulated at the CD4/CD8 DP stage of thymocyte differentiation, (iv) the percentage of CD4 and CD8 SP mature thymocytes developing from the CD4/CD8 DP population in transgenic animals is dramatically reduced, possibly as a result of a decrease in the rate of thymic positive selection, and (v) mature thymocyte and peripheral T-cell proliferation in response to activation through the TCR/CD3 complex appears to be reduced in CD2-nef transgenic mice.

The analysis of the CD2-tat transgenic mice revealed a very different phenotype, the characteristics of which were; (i) Transgenic mice were overtly normal with no evidence of dermal lesions or tumours, (ii) thymic ontogeny appears to be unaffected by the presence of the tat gene, and (iii) the cytokine profile on activation of thymocytes and peripheral T-cells is significantly altered in transgenic animals, with the cytokine genes TNFβ, TGFβ and the IL-4R showing a number of fold increase in the level of mRNA expression.

Therefore, both the transgenic models described in this thesis not only recapitulate a number of observations that until now had only been observed in vitro, but also extend the understanding of these effects to the influence that they have on the dynamic relationships between the cells of the immune system. These transgenic models may therefore prove extremely valuable in understanding HIV-1 mediated pathogenesis and the mechanisms of HIV-1 induced immune dysfunction.
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