A mouse model for the pathogenesis of immunodeficiency virus infection

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I, Rute Leal de Sousa Marques, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

T lymphocyte numbers in the human body are kept constant by homeostatic mechanisms balancing cell gain and loss. These mechanisms eventually fail in HIV infection, which is characterized by progressive immune deficiency attributable to a slow but relentless depletion of $CD4^+$ T cells, the main viral targets. HIV infection is also associated with increased T cell turnover and a state of generalized immune activation. One of the fundamental questions in the field of HIV research is the relation between $CD4^+$ T cell depletion and immune activation. It has been suggested that the virus has a direct effect by killing $CD4^+$ T cells and increased T cell turnover reflects a homeostatic response to $CD4^+$ T cell depletion. Alternatively, chronic immune activation may lead to enhanced turnover of T cells by ongoing proliferation-differentiation and cell death. In both cases, AIDS is the result of an exhaustion of the regenerative capacity of the immune system.

To address these questions we examined the consequences of activated $CD4^+$ T cell killing in a virus-free mouse model. Immunodeficiency viruses are highly selective for activated/memory and regulatory $CD4^+$ T cells due to restricted expression of CCR5, the co-receptor for HIV and SIV, or CD134 (OX40, TNFRSF4), the cellular receptor for FIV. Activated $CD4^+$ T cells were depleted by conditional reactivation of diphtheria toxin gene mediated by *Tnfrsf4*-driven Cre recombinase expression.
Conditional ablation of activated $CD4^+$ T cells resulted in accelerated turnover, with only a minimal apparent effect on their numbers, and was associated with a reduction in CD4:CD8 ratio and development of $CD4^+$ T cell immune deficiency, resembling HIV infection. Importantly, activated $CD4^+$ T cell killing also resulted in generalized

immune activation, including lymph node enlargement, B cell expansion, elevated serum levels of proinflammatory cytokines and increased turnover and activation of $CD8^+$ T cells, characteristic of HIV infection. $CD8^+$ T cell activation correlated with lack of regulatory $CD4^+$ T cell function and was prevented upon regulatory $CD4^+$ T cell reconstitution.

We therefore propose a causal link between memory and regulatory T cell depletion and immune deficiency and immune activation, respectively.

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Abbreviations

7-AAD	7-amino-actinomycin D
AB	Air-Buffered
AIDS	Acquired immunodeficiency syndrome
AP	Alkaline phosphatase
APC	Antigen presenting cells
APC	Allophycocyanin
β2m	Beta-2 microglobulin
B6	C57BL/6
BM	Bone marrow
BrdU	Bromodeoxyuridine
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T lymphocyte Antigen 4
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DC	Dendritic cell
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
EBV	Epstein-Barr virus
ELISA	Enzyme-linked-immunosorbent-assay
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
Floxed	l*oxP*-flanked

F-MuLV-B	B-tropic helper murine leukaemia virus
FoxP3	Forkhead box P3
FV	Friend virus
GFP	Green fluorescent protein
GITR	Glucocorticoide induced tumour necrosis factor receptor related protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H&E	Haematoxylin and eosin
HAART	Highly active anti-retroviral therapy
HAU	Hemagglutinin units
HB-EGF	Heparin-binding epidermal growth factor
HEV	High endothelial venules
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HTLV	Human T cell leukemia/lymphotropic virus
i.p.	Intra-peritoneally
i.v.	Intravenously
IAV	Influenza A virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IPEX	Immunodysregulation, polyendocrinopathy and enteropathy X-linked
iu	Infectious units
LDV	Lactate dehydrogenase-elevating virus
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph nodes
loxP	Locus of crossing over
LPS	Lipopolysaccharide
LTR	Long terminal repeat
mAb	Monoclonal antibody
MCP	Monocyte chemotactic protein
MDCK	Madin Darby canine kidney
MFI	Median fluorescence intensity

MHC	Major histocompatibility complex
MIG	Monokine induced by IFNγ
MIP	Macrophage inflammatory protein
MyD88	Myeloid differentiation primary response gene (88)
nAb	Neutralizing antibody
NEMO	NF-kappaB essential modulator
P. murina	*Pneumocystis murina*
PBS	Phosphate-buffered saline
PC	Peritoneal cavity
PCP	*Pneumocystis carinii* pneumonia
PCR	Polymerase chain reaction
PDBu	Phorbol 12, 13-dibutyrate
pDCs	Plasmacytoid dendritc cells
PE	Phycoerythrine
Poly-IC	Polyriboinosinic:polyribocytidylic acid
R26	Rosa 26
RAG	Recombination activating gene
RANTES	Regulated upon activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Room temperature
SDF	Stromal cell-derived factor
SEM	Standard error of the mean
SFFU	Spleen focus-forming units
SFFVp	polycythemia-inducing spleen focus-forming virus
SIV	Simian immunodeficiency viruses
SP	Single positive
SP	Spleen
SPF	Specific pathogen free
TCID	Tissue culture infective dose
T_{CM}	Central memory T cells
TCR	T cell receptor

T_{EM}	Effector memory T cells
T_H	T helper type cell
THY	Thymus
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TNFRSF	Tumour necrosis factor receptor superfamily
VEGF	Vascular endothelial growth factor
w/v	Weight/volume
WT	Wild-type
YFP	Yellow fluorescent protein

1. Introduction

1.1. Infection with HIV

1.1.1. Introduction

Acquired immunodeficiency syndrome (AIDS) was first described in 1981 in two papers in the New England Journal of Medicine. These papers described *Pneumocystis carinii* pneumonia in individuals with low $CD4^+$ T cell counts (Masur et al., 1981; Gottlieb et al., 1981). Two years later, the etiological agent of AIDS was isolated and shown to be a retrovirus, now known as human immunodeficiency virus type 1 (HIV-1) (Barre-Sinoussi et al., 1983). In 1986, a second retrovirus strain, known as HIV-2, was isolated from patients with AIDS from West Africa (Clavel et al., 1986). HIV-2 has approximately 40-60% homology with HIV-1, but the majority of infected people do not progress to disease and die of unrelated causes (Rowland-Jones and Whittle, 2007; Leligdowicz et al., 2007).

HIV-1 has spread rapidly and the HIV epidemic has become a major global health problem. According to the global summary of the AIDS epidemic published by UNAIDS in December 2007, 33.2 million people worldwide are living with HIV and 2.1 million died as a result of AIDS in that year. The situation is particularly serious

in Sub-Saharan Africa, where the same report states an adult prevalence of 5% (http://data.unaids.org/pub/EPISlides/2007/2007_epiupdate_en.pdf).

At present there is no cure for AIDS. Although highly active anti-retroviral therapy (HAART) - a combination of anti-retroviral drugs - can suppress HIV-1 replication and delay illness for many years, it does not clear the virus completely. Moreover, access to antiretroviral treatment is limited in many parts of the world and even when antiretroviral treatment is available, it is associated with serious side effects and problems with adherence to therapy. Although much research has been done in order to develop a vaccine against HIV-1, none of the candidate vaccines that have been tested in clinical trials has been successful. This highlights the necessity for more basic research to understand the complex relationship between HIV and the immune system.

1.1.2. Origin of HIV strains

HIV strains have evolved in multiple cross-species transmissions from simian immunodeficiency viruses (SIVs) that infect nonhuman primates in Africa. HIV-1 evolved from a strain of SIV of chimpanzee (*Pan troglodytes troglodytes*) (Gao et al., 1999), whereas HIV-2 originated in SIV found naturally in sooty mangabey monkeys (*Cercocebus atys*) (Hirsch et al., 1989).

SIVs do not appear to cause AIDS in their natural African host: infected sooty mangabeys, African green monkeys, mandrils, sun-tailed monkeys, just to mention a few, are free of clinical disease and have a normal life span (Hirsch, 2004). In contrast, several species of Asian macaques (*Macaca spp*), like the rhesus macaque

(*Macaca mulatta*), develop AIDS when infected with a common non-pathogenic virus of African sooty mangabeys, albeit in a relatively short time when compared to HIV infection in humans (Heeney et al., 2006). Nevertheless, SIV infection of macaques has provided a powerful experimental model system to study the pathogenesis of AIDS. Additionally, studies of the differences between non-pathogenic SIV infections as well as of the less pathogenic HIV-2 infection and HIV-1 infection have also provided important clues on the determinants of immune deficiency in HIV-1 infected humans.

1.1.3. $CD4^+$ T cell depletion in HIV disease

The major hallmark of HIV-1 infection is the progressive $CD4^+$ T cell destruction and subsequent loss of immune function, which manifests clinically as an increased susceptibility to opportunistic infections and tumours. What drives the loss of $CD4^+$ T cells in this infection is still unclear.

Interestingly, idiopathic $CD4^+$ lymphocytopenia which is a rare immunodeficiency of unknown cause, clinically very much resembles HIV infection. Patients have persistently low $CD4^+$ T cell counts and experience opportunistic infections, autoimmune disease and hematologic malignancies (Bonilla and Geha, 2006). Thus, the immunodeficiency seen in HIV infection resembles the $CD4^+$ T cells primary immunodeficiencies (Carneiro-Sampaio and Coutinho, 2007).

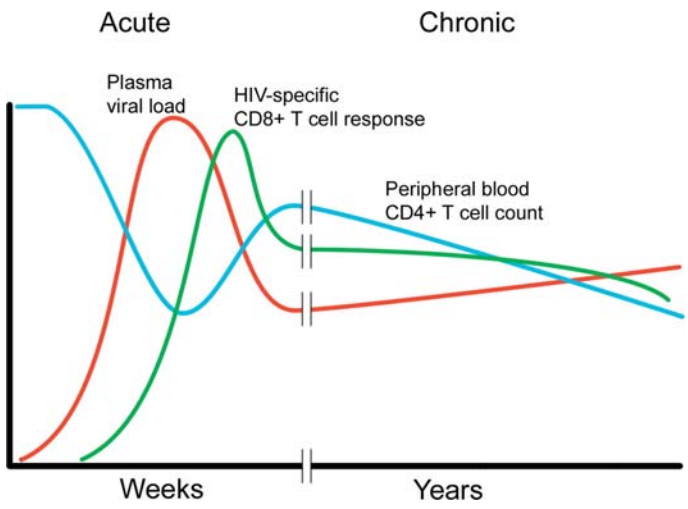
No human virus other than HIV-1 causes such extensive and unavoidable $CD4^+$ T cell loss. HIV-2 targets the same cells as HIV-1 (i.e. $CD4^+$ T cells) and has the same capacity to infect and replicate as suggested by the fact that proviral loads of HIV-2

and HIV-1 infected patients at the same disease stage are similar (Popper et al., 2000). Nevertheless, HIV-2 does not usually cause high viremia or such severe $CD4^+$ T cell depletion. Another retrovirus that exhibits the same tropism for $CD4^+$ T cells, the human T cell leukemia/lymphotropic virus 1 (HTLV-1), results in $CD4^+$ T cell lymphocytosis rather than lymphopenia (Matsuoka and Jeang, 2007).

1.1.4. Clinical stages of HIV disease

The typical course of untreated HIV-1 infection can be artificially divided in distinct phases. Primary infection occurs via the mucosal (gastrointestinal or reproductive) or parenteral route and is often associated with a febrile illness and clinical signs of viral dissemination to lymphoid tissues, central nervous system and other sites and may last from a few days to 4 weeks and then subside. This stage is characterized by active viral replication and high viremia and a sharp drop in peripheral blood $CD4^+$ T cell counts (**Fig. 1**). Viral replication then falls concurrently with the appearance in circulation of virus-specific $CD8^+$ T cells (Koup et al., 1994). This lower viral load set point is predictive of the rate of progression to AIDS in untreated individuals: the higher the level, the worse the prognosis (Mellors et al., 1996).

Recently it has been shown that very early after infection there is a rapid and dramatic depletion of mucosal-associated memory $CD4^+$ T cells, which is not reflected in blood cell counts (Brenchley et al., 2004; Mehandru et al., 2004). This is also observed in pathogenic (Mattapallil et al., 2005; Li et al., 2005) and non-pathogenic infections with SIV (Pandrea et al., 2007; Gordon et al., 2007; Milush et al., 2007).



From (Douek et al., 2003)

Figure 1. Changes in plasma viral load, peripheral blood $CD4^+$ T cell counts and HIV-1 specific $CD8^+$ T cell counts in the course of a typical untreated HIV-1 infection.

Following the acute infection, there is a partial rise in blood $CD4^+$ T cell counts and although there may be no further evidence of illness for the next decade, peripheral blood $CD4^+$ T cell counts slowly decline and viremia slowly rises (**Fig. 1**).

Circulating antibodies to HIV antigens appear within a few weeks of infection, usually after viral levels have begun to fall to the steady-state level. Although some of these antibodies may have strong neutralizing activity, rapid viral escape is characteristic.

This long clinically asymptomatic period is misleading, because HIV infection is not latent. The steady-state in $CD4^+$ T cell counts actually represents a balance between rapid cell depletion and powerful restoration within the immune system. Eventually, the regenerative capacity of the immune system becomes exhausted and $CD4^+$ T cell counts drop below a threshold of about 200 cells/μl, when opportunistic infections occur (Douek et al., 2003; Weiss, 2008).

Despite 25 years of research and many important events in HIV immunology (reviewed in (Rowland-Jones, 2003)), the precise mechanisms responsible for the unrelenting decline of $CD4^+$ T cell function and number are still unclear. In healthy individuals there is a rigorous control of the number of T cells in the body, but in HIV-1 infection these dynamics are affected. It is thus crucially important to understand how $CD4^+$ T cells are regulated to be able to understand what is happening in HIV-1 infection.

1.2. Regulation of CD4$^+$ T cells

1.2.1. Peripheral CD4$^+$ T cell subsets

T cell progenitors are generated in the bone marrow and migrate to the thymus where they rearrange their T cell receptors (TCRs) and undergo a process of selection. The TCR is designed to recognise antigens on cell surfaces and ignore structures that are soluble or free-floating (such as virus particles outside infected cells). TCRs recognise small peptide fragments on cell surfaces, bound to major histocompatibility complex (MHC) molecules. T cells with TCRs specific for self antigen are deleted through a process of negative selection, whereas T cells with TCRs specific for foreign antigens are retained by positive selection. T cells that survive selection differentiate to either CD4$^+$ or CD8$^+$ mature T cells and are exported to peripheral lymphoid tissues.

1.2.1.1. Naïve and memory CD4$^+$ T cells

T cells that leave the thymus are called naïve (**Fig. 2**) because they have not yet encountered their cognate antigen, and are thus in a resting state. They have a diverse distribution of TCRs with specificities capable of recognizing a broad array of peptides bound to MHC molecules.

Naïve T cells home to secondary lymphoid tissues (spleen, lymph nodes and Peyer's patches) and migrate continuously from one lymphoid organ to another, via blood and lymph. Entry to lymph nodes and Peyer patches is highly specific and occurs through high endothelial venules (HEV).

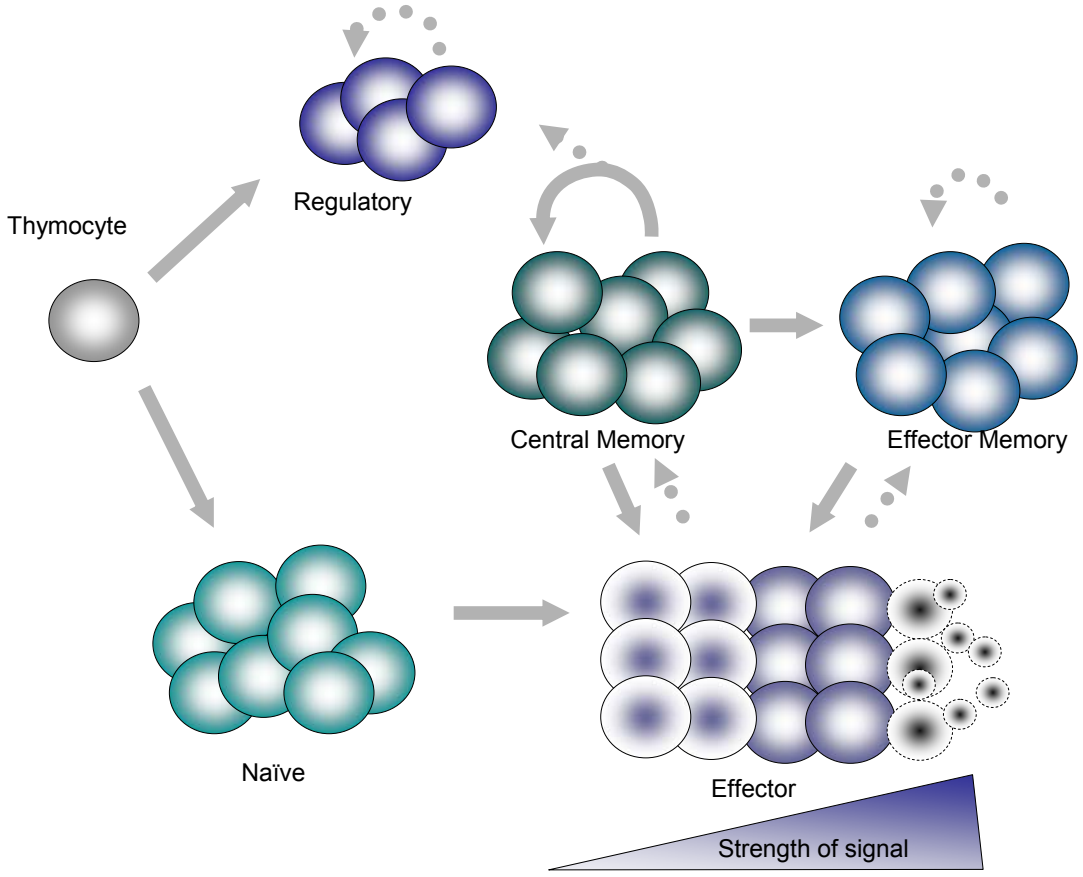


Figure 2. Schematic representation of peripheral CD4$^+$ T cell pools. Naïve CD4$^+$ T cells migrate from the thymus to the periphery. Upon encounter with antigen presented on the surface of MHCII molecules on antigen presenting cells, naïve cells differentiate into effector cells, some of which are selected to enter the memory pool once the infection is cleared. The memory pool is a heterogeneous population composed of central memory and effector memory cells. Regulatory T cells are a distinct lineage originated in the thymus, although under certain conditions they can also be induced in the periphery.

Naïve T cells express L-selectin (CD62L), chemokines (C-C motif) receptor 7 (CCR7) and lymphocyte function-associated antigen 1 (LFA-1) which interact with molecules on HEV. This combination of molecules is uniquely found on HEV and is essential for efficient homing of naïve T cells in lymphoid tissues. In these organs, naïve T cells travel to the T cell areas in search of antigen presented by antigen presenting cells (APCs). Continuous migration of naïve T cells through secondary lymphoid tissues is thus highly important for allowing T cells to make rapid contact with antigens released from pathogens.

When naïve lymphocytes in T cell areas of secondary lymphoid organs encounter their cognate antigen presented on the surface of specialized APCs they become activated, clonally expand and differentiate into effector lymphocytes that can migrate to B cell areas or to inflamed tissues where they mediate clearance of the pathogen.

After resolution of the infection, 90%–95% of antigen-specific T cells die by programmed cell death, leaving behind a long-lived population of resting T cells, called memory T cells, which provide protection upon re-infection (**Fig. 2**). Memory T cells form the cellular basis of immunological memory which can last for decades and is dependent on cell division (Bellier et al., 2003).

Memory T cells possess several properties crucial for their function, including higher frequencies than naïve precursors, the ability to rapidly reactivate upon antigen stimulation, the ability to survive and self-renew for long periods in the absence of cognate antigen and wide tissue distribution. Indeed, whereas only a minority of naïve

T cells can be found in peripheral sites as part of their recirculation (Cose et al., 2006), memory cells can be found in virtually all tissues of the body (Reinhardt et al., 2001; Stockinger et al., 2004). Importantly for HIV-1 pathogenesis, a large population of memory $CD4^+$ T cells resides in mucosal sites (particularly the lamina propria) (Paiardini et al., 2008).

Naïve and memory $CD4^+$ T cells can be distinguished by expression of distinct surface markers. In mice, naïve cells express CD62L and low levels of CD44, whereas memory cells lose CD62L and gain high levels of CD44 expression. Other markers are transiently expressed by memory cells upon activation, such as CD25, CD69 and CD43.

Memory T cells are heterogeneous in terms of both homing capacity and effector function and are classified as central memory (T_{CM}) and effector memory T cells (T_{EM}) (Sallusto et al., 1999). T_{CM} cells home to lymph nodes and have limited effector function, but they proliferate and become effector cells upon secondary stimulation. By contrast, T_{EM} cells are able to enter peripheral non-lymphoid tissues, can rapidly produce effector cytokines such as interferon-γ (IFN-γ) upon antigenic stimulation, but have limited proliferative capacity (Bouneaud et al., 2005; Lanzavecchia and Sallusto, 2005). It has been suggested that T_{CM} cells act as memory stem cells, self renewing and at the same time continuously replenishing the T_{EM} pool (Lanzavecchia and Sallusto, 2002).

Although several models have been proposed, there is good evidence that T cells differentiate progressively according to a linear sequence: from naïve T cell to central

memory cell to effector memory cell. Lanzavecchia and Sallusto have proposed that these two memory populations result from survival of effector cells and intermediate cells generated in a primary infection that are arrested at different stages of differentiation. This progressive differentiation theory is based on the fact that these stages depend on the strength of antigenic and cytokine stimulation that a cell has received (Lanzavecchia et al., 2002).

1.2.1.2. Regulatory $CD4^+$ T cells

Naturally-occurring regulatory $CD4^+$ T cells originate in the thymus and migrate to the periphery as a different lineage of $CD4^+$ T cells (**Fig. 2**). They are called regulatory because they are committed to suppressing both physiological and pathological immune responses and are essential in establishing and maintaining self-tolerance and immune homeostasis. In mice, depletion of regulatory $CD4^+$ T cells activates self-reactive T cell clones inducing widespread autoimmune/inflammatory diseases in otherwise normal animals. Humans suffering from IPEX (immunodysregulation, polyendocrinopathy and enteropathy X-linked) syndrome lack regulatory T cells and develop autoimmune diseases and severe allergies (Sakaguchi et al., 2006).

Naturally-occurring regulatory $CD4^+$ T cells have several features that make them unique among other T cells. Most regulatory $CD4^+$ T cells are produced by the thymus as a functionally distinct and mature T cell subpopulation, i.e. they are already specialized for suppressive function even before antigen encounter. Their TCR repertoire is as broad and diverse as that of naïve cells but it is more self-reactive and

only partially overlapping with that of naïve T cells (at least in mice) (Sakaguchi et al., 2006).

Regulatory $CD4^+$ T cells do not produce proinflammatory cytokines upon antigenic stimulation and therefore do not cause harm to the host despite high self-reactivity. Finally, they potently suppress the activation, proliferation and/or effector function of other $CD4^+$ and $CD8^+$ T cells, and possibly NK cells, NK T cells, B cells and dendritic cells (DCs) (Sakaguchi et al., 2006).

Regulatory $CD4^+$ T cells account for approximately 10% of peripheral $CD4^+$ T cells and most express the CD25 marker on the cell surface. Indeed regulatory $CD4^+$ T cells have an activated phenotype, with expression of a number of activation markers including cytotoxic T lymphocyte Ag 4 (CTLA-4), glucocorticoide induced tumor necrosis factor receptor related protein (GITR), CD134 and LFA-1. Regulatory $CD4^+$ T cells are also characterized by lower levels of the α chain of the IL-7 receptor (CD127) (Liu et al., 2006; Seddiki et al., 2006).

The most reliable marker of regulatory $CD4^+$ T cells at present is however the expression of the transcription factor forkhead box P3 (FoxP3) (Hori et al., 2003; Fontenot et al., 2003; Khattri et al., 2003). It is becoming clear that FoxP3 can also be transiently expressed in peripheral naïve T cells without inducing suppressor function. It has been proposed that regulatory $CD4^+$ T cells lineage is rather determined by a high-order regulatory system that sustains high levels of FoxP3 (Hori, 2008).

In the mouse, it has been shown in a number of experimental settings that regulatory $CD4^+$ T cells can also be induced by antigen in the periphery. These cells are called adaptive regulatory $CD4^+$ T cells and are phenotypically and functionally indistinguishable from the thymus-derived naturally occurring regulatory $CD4^+$ T cells (Thorstenson and Khoruts, 2001; Cobbold et al., 2004; Apostolou and von Boehmer, 2004). However it is questionable whether these cells are generated in physiological situations.

Regulatory $CD4^+$ T cells are not anergic but they have proliferative capacity *in vivo*. They expand when transferred into lymphopenic hosts (Annacker et al., 2001; Gavin et al., 2002) and in response to immunization (Walker et al., 2003). In normal unmanipulated mice, regulatory T cells are heterogeneous: some are quiescent while others are constantly cycling and have an activated phenotype (Fisson et al., 2003). For this reason, these subsets are sometimes called 'naïve' and 'memory' regulatory T cells, respectively.

In humans it has been shown that regulatory T cells are highly proliferative but very susceptible to apoptosis resulting in a limited self-renewal capacity. It has been proposed that they are continuously recruited from the memory $CD4^+$ T cell pool (Vukmanovic-Stejic et al., 2006) and various studies showing homology of TCR repertoire strongly support this idea (Kasow et al., 2004; Scheinberg et al., 2007; Ebinuma et al., 2008; Vukmanovic-Stejic et al., 2006).

1.2.2. Lymphocyte homeostasis

Lymphocyte homeostasis is the process by which appropriate numbers of lymphocytes are maintained. Indeed, except fluctuations during infection or stress and predictable age-related changes, the number of lymphocytes stays within a relatively narrow range (Grossman et al., 2004). Homeostatic regulation occurs to maintain total numbers of lymphocytes but also diverse subpopulations of lymphocytes.

1.2.2.1. $CD4^+$ and $CD8^+$ T cell homeostasis

$CD4^+$ T cells are key players in the adaptive immune system being involved in both cellular and humoral responses whereas $CD8^+$ T cells are involved in response to viruses and tumours. In mice, the number of $CD4^+$ and $CD8^+$ T cells is co-regulated, i.e., in the absence of either subset the other will compensate to achieve the same total number of T cells (Freitas and Rocha, 2000). In MHC-$I^{-/-}$ and MHC-$II^{-/-}$ or $CD4^{-/-}$ and $CD8^{-/-}$ mice, the absence of either of the two T cell subsets can be compensated by the remaining subset and the total number of T cells remains similar to that of normal mice (Tanchot et al., 1997).

Similarly, most people with primary MHC-II deficiency, also known as the Bare Lymphocyte Syndrome, have normal number of circulating total T cells, but the number of $CD4^+$ T lymphocytes is reduced (Reith and Mach, 2001). Conversely, patients with absence of $CD8^+$ T cells due to mutations in the *CD8α* gene display normal numbers of $CD3^+$ T cells (Calle-Martin et al., 2001).

With age, the CD4:CD8 ratio in mice is reduced. This reduction is due to oligoclonal expansion of $CD8^+$ T cells responding to environmental antigens (Callahan et al., 1993) and it is therefore dependent on the health status of the mice. In contrast, mice kept in specific pathogen free (SPF) facilities, the CD4:CD8 ratio remains constant or even increases with time (Bourgeois et al., 2005).

Similar to mice housed in conventional facilities, the ratio CD4:CD8 drops in ageing humans. This is often due to expansion of cytomegalovirus (CMV)-specific $CD8^+$ T cells (Khan et al., 2002). Interestingly, the changes seen in ageing humans show many common features with changes seen in untreated HIV-1 infected patients, for example drop in CD4:CD8 ratio, decreased thymic output and reduced naïve cell numbers (Appay and Rowland-Jones, 2002).

1.2.2.2. Naïve and memory T cell homeostasis

The maintenance of balanced naïve and memory compartments is critical for immune function: diverse naïve T cell repertoire ensures adequate responses against new pathogens while memory T cells respond rapidly on re-encountering the same pathogens (Ge et al., 2002).

The size of the naïve T cell pool is much more dependent on the input of new thymus emigrants than the size of the memory pool (Freitas et al., 2000) and in normal mice naïve T cells divide infrequently (La Gruta et al., 2000).

Pivotal studies on the kinetics of bromodeoxyuridine (BrdU) labelling of T cells in BrdU-treated mice have shown that memory cells have a much higher rate of division

(turnover) than naïve cells (Tough and Sprent, 1994) and the same has been confirmed in humans by using different methods such as *ex vivo* BrdU incorporation (Geginat et al., 2003), Ki67 expression (Champagne et al., 2001), and *in vivo* incorporation with deuterated glucose into DNA (Macallan et al., 2003; Macallan et al., 2004).

The pools of naïve and memory T cells are maintained independently, at least in steady state conditions (Stockinger et al., 2004), implying that the generation of new memory cells results in competition for survival with existing memory cells, but not with naïve T cells (Tanchot et al., 1997; Bourgeois et al., 2005). Consequently, the sensors for space must differ between naïve and memory cells, and these pools are often referred to as occupying distinct 'niches' (Freitas et al., 2000).

Because naïve T cells depend on replenishment from the thymus, with ageing and thymic atrophy, their numbers are reduced. Memory cell numbers, on the other hand, are not reduced in ageing animals (Bourgeois et al., 2005). The naïve T cell pool in humans is thought to be less reliant on thymic function than in mice, and displays a certain degree of self-renewal capacity. As a result, numbers of peripheral T cells in ageing humans decline less than in ageing mice (Goronzy and Weyand, 2005; Akbar et al., 2004).

1.2.2.3. Lymphopenia driven proliferation

T cell lymphopenia can occur after chemo- or radiotherapy or after certain infections, for instance transiently in measles (Okada et al., 2000) and highly virulent Influenza A infection (Tumpey et al., 2000) and chronically in HIV-1 infection.

In lymphopenic conditions, the requirements for homeostatic proliferation differ from those of an immune response. Indeed, both naïve and memory cells extensively divide in the absence of their nominal antigen (Jameson, 2002). Naïve $CD4^+$ and $CD8^+$ T cells that proliferate under these conditions convert to a memory-like phenotype and lose their naïve status (Murali-Krishna and Ahmed, 2000; Tanchot et al., 2001). Interestingly, although the existence of memory $CD4^+$ cells of broad repertoire efficiently inhibits proliferation/differentiation of naïve $CD4^+$ T cells, a memory population of similar size but with a repertoire of limited diversity fails to do so (Min et al., 2004).

1.2.2.4. Shared resources: the niche effect

One of the hypothesized mechanisms by which the homeostatic control of cell numbers is achieved is that lymphocyte populations must compete for survival signals and resources are limited. To avoid competition, exclusion and to maintain diversity, cell populations have different requirements for resources defining different 'ecological niches' (Tanchot et al., 1997).

The survival of naïve T cells requires continuous contact with self peptides bound to MHC molecules together with interleukin (IL)-7. In contrast, survival of memory T cells is less reliant on TCR ligation. At least for memory $CD8^+$ T cells, the turnover and survival is controlled mainly by cytokines, namely IL-7 which enhances survival, and IL-15, which mediates proliferation (Goldrath et al., 2002; Tan et al., 2002; Kieper et al., 2002). What controls turnover and survival of $CD4^+$ memory T cells is

not clear but IL-7 and IL-15 have also been implicated, as has been TCR signalling (Li et al., 2003; Seddon et al., 2003).

1.3. Selectivity of HIV/SIV/FIV for memory and regulatory CD4$^+$ T cells

1.3.1. Cellular tropism of HIV and SIV

The key event in the course of viral entry into a target cell is the interaction between the virus and its cellular receptor(s). Both viral cell tropism and pathogenicity are determined by the specificity of these interactions.

The CD4 molecule is the primary cellular receptor for HIV-1 and HIV-2 (Dalgleish et al., 1984; Klatzmann et al., 1984). These viruses bind CD4, which causes a change in the viral entry proteins, allowing them to bind the co-receptor and enter the cell (Doms and Moore, 2000).

HIV-1 predominantly uses two different major co-receptors: CCR5 (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996) and CXC chemokines receptor (CXCR4) (Feng et al., 1996). These are members of the family of seven-transmembrane spanning chemokines receptors. CCR5 is almost always the initial target co-receptor for naturally transmitted virus. However, in about 50% of HIV-infected individuals progressing to AIDS, CXCR4-using viruses emerge late in the course of disease (Scarlatti et al., 1997). These 'X4' strains are more virulent than the initial 'R5' strains and probably accelerate the depletion of CD4$^+$ T cells and the onset of disease. However, switch to CXCR4 is not necessary for disease progression. Certain variant strains are dual tropic and exhibit features of both groups. There are more than a

dozen of other minor co-receptors that HIV can use *in vitro*, but their significance *in vivo* is not clear (Clapham and McKnight, 2001).

Accordingly, HIV mainly infects hematopoietic cells that express CD4 and CCR5 or, in late stages of disease of some individuals, CXCR4. It is interesting to note that a broader use of receptors and co-receptors would mean a wider cell tropism which could be advantageous for the virus. However HIV has not evolved variants capable of using alternative receptors/co-receptors. The factors that restrict the use of co-receptors other than CCR5 and CXCR4 *in vivo* are not known (Clapham et al., 2001).

CD4 is expressed predominantly in lymphocytes but can also be expressed by cells of the monocyte-macrophage lineage, dendritic cells and microglia cells in the brain.

CCR5 is expressed in memory/activated and regulatory $CD4^+$ T cells (Bleul et al., 1997; Oswald-Richter et al., 2004) and directs their migration along CCL3 (macrophage inflammatory protein 1α- MIP-1α), CCL4 (MIP-1β) and CCL5 (regulated upon activation, normal T cell expressed and secreted (RANTES)) gradients (Samson et al., 1996; Raport et al., 1996). $CD4^+$ $CCR5^+$ T cells are infrequent in the peripheral blood, lymph nodes and spleen but account for almost all $CD4^+$ T cells in other tissues including the mucosal surfaces of the intestine, respiratory and reproductive tract. CCR5 expression has also been reported (primarily from mouse studies) on multiple other cell types including macrophages, dendritic cells, neutrophils and hepatic stellate cells.

CXCR4 is more widely expressed in cells of both the immune, particularly by naïve T cells (Bleul et al., 1997) and the central nervous systems and regulates cell migration along gradients of stromal cell-derived factor-1 (SDF-1 or CXCL12) (Oberlin et al., 1996; Bleul et al., 1996).

The importance of CCR5 as primary co-receptor for the pathogenesis of AIDS is illustrated by the fact that individuals with polymorphisms in the *CCR5* gene show increased resistance to HIV infection and/or slower disease progression (Michael, 1999).

Similar to HIV, primary SIV isolates use CD4 as the main receptor and mainly CCR5 as co-receptor for host cell entry (Chen et al., 1998; Gautam et al., 2007). However, unlike HIV, co-receptor switching form CCR5 to CXCR4 has not been demonstrated *in vivo* for SIV. A switch from macrophage-tropism to lymphocyte tropism has been shown to be due not to co-receptor switching but to the way CCR5 is exploited as co-receptor (Mori et al., 1992).

1.3.2. Receptors for FIV

FIV, the feline immunodeficiency virus, is the only non primate lentivirus that causes a disease similar to AIDS in its natural host species, the domestic cat (Pedersen et al., 1987).

The feline immunodeficiency syndrome is characterized by a progressive decline in the number of circulating $CD4^+$ T cells and an associated inversion of the CD4:CD8 ratio, activated $CD8^+$ T cells, polyclonal B cell activation and

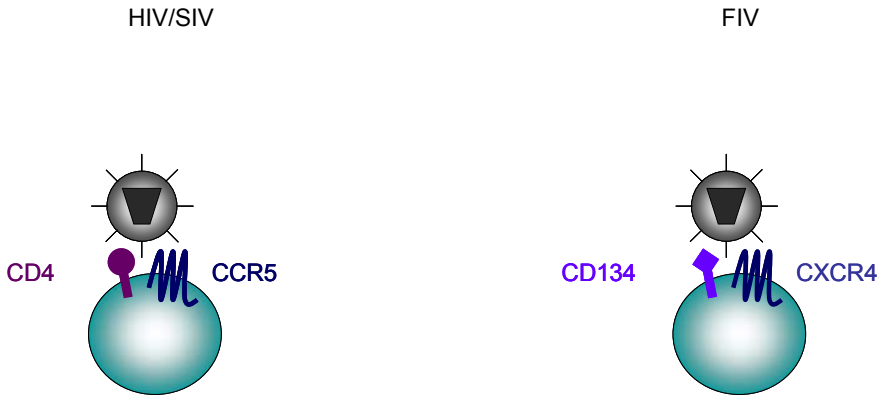
hypergammaglobulinemia (Willett et al., 1997b; Novotney et al., 1990). Clinical manifestations include wasting, neurological disturbances, chronic stomatitis and gingivitis and increased incidence of lymphoma.

In contrast to HIV and SIV, however, FIV does not use CD4 as its viral receptor. The primary receptor for FIV is CD134 (or OX40) (Shimojima et al., 2004), a T cell activation antigen and co-stimulatory molecule. CXCR4 is the sole co-receptor for infection (Willett et al., 1997a) although with disease progression, the viral cell tropism becomes broader (English et al., 1993; Dean et al., 1996).

CD134 is a member of the tumour necrosis factor receptor (TNFR) superfamily (TNFRSF4) (Watts, 2005). It is a co-stimulatory receptor that, unlike CD28, is not constitutively expressed on naïve $CD4^+$ T cells but is induced within 12-24 hours after antigen recognition, peaking at 48 hours and declining after 72 to 96 hours (Gramaglia et al., 1998). In regulatory $CD4^+$ T cells, expression of CD134 is constitutive (Takeda et al., 2004; McHugh et al., 2002). Although largely restricted to $CD4^+$ T cells, under conditions of strong antigenic stimulation, CD134 can also be induced in $CD8^+$ T cells (Baum et al., 1994).

CD134 ligand is normally expressed on APCs such as B cells, dendritic cells, macrophages and endothelial cells when activated (Watts, 2005).

Thus, although using different receptor/co-receptor combinations, all retroviruses that cause immunodeficiency predominantly target the same type of cells, i.e. activated/memory $CD4^+$ T cells and regulatory $CD4^+$ T cells (**Fig. 3**).



	Naïve CD4	T_{CM} CD4	T_{EM} CD4	Regulatory CD4
CD4	+	+	+	+
CCR5	-	+/-	++	++
CD134	-	+/-	++	++
CXCR4	+	+	+	+

Figure 3. Immunodeficiency viruses target memory and regulatory $CD4^+$ T cells. Viral cellular tropism is dictated by receptor/co-receptor usage: HIV and SIV use the broadly expressed CD4 molecule as receptor for host cell entry, while the specificity of the target cells is given by the more strictly expressed co-receptor, CCR5, which is solely expressed in activated/ memory and regulatory $CD4^+$ T cells. FIV, on the other hand, utilizes the broadly expressed co-receptor CXCR4. Specificity for target cells in this case is given by the usage of CD134 as receptor.

1.4. Pathogenesis of HIV/SIV infections

Virtually every arm of the immune response is affected by HIV, but the precise mechanisms that lead to immune dysfunction are still poorly understood. While it is now clear that $CD4^+$ T cell numbers decrease because there is a failure to replenish them, what drives their loss in the first place is unclear.

1.4.1. Tap and drain hypothesis

Early studies done even before the HIV-1 receptor was discovered showed that infected $CD4^+$ T cells are directly killed by the virus, providing a logical explanation for the progressive decline of these cells in HIV-infected individuals (Ho et al., 1995; Wei et al., 1995).

According to this 'tap and drain' model of $CD4^+$ T cell depletion, the rate of $CD4^+$ T cell destruction by HIV is too fast to be compensated for by natural $CD4^+$ T cell production by the immune system, ultimately resulting in $CD4^+$ T depletion (Ho et al., 1995).

The impact of HIV-1 on the human immune system could be solely explained by the crucial role $CD4^+$ T cells have in coordinating a range of immune functions. However, there was a discrepancy between the number of infected $CD4^+$ cells in the circulation and extend of T cell dysfunction. This issue was clarified when it was shown that HIV infected T cells are trapped in the follicular dendritic cells network in the lymph nodes (Spiegel et al., 1992). Indeed we now know that in the early stages of

infection 1,000-times more infected cells are present in lymphoid tissue that can be found in the blood (Pantaleo et al., 1993; Embretson et al., 1993).

1.4.2. Immune activation hypothesis

While some recent studies (Okoye et al., 2007) continue to indicate a role for viral destruction in $CD4^+$ T cell depletion, other important experimental observations suggest that factors others than (or in addition to) destruction by viral infection are responsible for $CD4^+$ T cell depletion in the setting of HIV-1 infection.

It has been shown that during chronic HIV infection, more $CD4^+$ T cells die that can be accounted for by direct infection, and that, in fact, an increased death rate is observed in non-target cells, such as $CD8^+$ T cells (Finkel et al., 1995). This bystander loss of uninfected T cells is thought to be related to a state of generalized immune activation that is consistently associated with HIV infection.

This immune activation is characterized by increased frequencies of B and T cells expressing activation markers and exhibiting a memory phenotype, increased plasma levels of proinflammatory cyokines and increased turnover of T and B and NK cells and accessory cells (Grossman et al., 2006).

Based on these findings, it has been proposed that chronic immune activation plays a key role in the HIV-associated $CD4^+$ T cell depletion. Consistent with this model is the finding that the expression of activation markers on T cells is a strong predictor of disease progression in HIV-infected individuals, independently of viral loads (Muro-Cacho et al., 1995; Giorgi et al., 1999; Leng et al., 2001; Sousa et al., 2002).

Thus, HIV-associated chronic immune activation results in a constant recruitment of $CD4^+$ T cells from the central memory and naïve pools to the effector pool, thus creating a strain on the homeostatic mechanisms of $CD4^+$ T cell maintenance.

The causes of this generalized immune activation are however not well understood. Possible proposed causes include HIV antigens and non-HIV antigens such as translocated microbial products and impairment of regulatory T cells.

Recent studies have shown that HIV encodes single stranded RNA sequences that can directly activate the immune system via Toll-like receptor (TLR)7 and TLR8 (Meier et al., 2007). Another proposed cause of immune activation is based on the early direct cytopathic effects of HIV on the gastrointestinal tract $CD4^+$ T cells. As a consequence of the depletion of these cells, the integrity of the mucosa is compromised, which in turn allows commensal bacterial to leak into the circulation (a phenomenon known as microbial translocation) (Brenchley et al., 2007). Chronic immune activation may also be due to antigen processing during cell death. In HIV infection, $CD4^+$ T cells die predominantly by apoptosis - the process of programmed cell death. Excessive levels of T cell apoptosis in HIV-1 infection release protein fragments that trigger the formation of autoreactive $CD8^+$ T cells. These $CD8^+$ T cells, that would normally not be activated, undergo clonal expansion and are primed by cross-presenting APCs, which in turn cause more apoptosis and results in chronic immune activation (Rawson et al., 2007). Eventhough this may occur, it is known that apoptotic cells are cleared by macrophages. Finally, the susceptibility of regulatory T

cells to HIV infection (Oswald-Richter et al., 2004) may remove the control of excessive immune activation.

The relationship between immune activation and progressive immune deficiency is supported by studies in non-human primates. Sooty mangabeys present high levels of SIV replication but manifests limited evidence of disease (Rey-Cuille et al., 1998). This lack of pathogenicity is accompanied by absence of the extensive immune activation and cellular proliferation that characterizes SIV infection of other primates such as the rhesus macaque, in which immune activation closely mimics the activation seen in HIV-infected humans (Chakrabarti et al., 2000). Moreover Sooty mangabeys seem to maintain thymic and bone marrow function and do not demonstrate bystander lymphocyte apoptosis (Silvestri et al., 2003).

1.4.3. Diminished production

Not only HIV-1 has an effect on $CD4^+$ T cell dynamics, but it is also responsible for alterations in the organs responsible for the primary production of these cells. Indeed late stages of HIV infection are often characterized by cellular hypoproductivity of the bone marrow, which is not limited to the $CD4^+$ T cells and leads to the pancytopenia that is often observed in advanced AIDS. The mechanisms responsible for this impairment of bone marrow productivity are not well understood but concurrent infections with opportunistic agents may play a role. Furthermore, hematopoietic progenitor cells in the bone marrow are susceptible to infection with HIV (Stanley et al., 1992) and their function is impaired (Marandin et al., 1996; Louache et al., 1992).

Although some studies show preservation of thymus size (particularly in older individuals) (McCune et al., 1998; Kalaylian et al., 2003) and suggest that thymic output is maintained in HIV infected individuals (Zhang et al., 1999), there is also evidence that HIV can infect thymic stromal cells and that HIV strains that use CXCR4 co-receptor can infect thymocytes as well (Berkowitz et al., 1998).

1.5. Mouse tools

Genetic manipulations of the mouse genome, such as random integration of transgenes or homologous recombination in embryonic stem cells, are widely used and have been instrumental for the understanding of gene function. These technologies allow the introduction of a permanent genetic alteration in the mouse germ line, but they do not allow manipulating the genome in a spatially or temporally controlled manner. For example, a knock-out of a given gene results in the absence of its expression in all cells of the animal and throughout pre- and post-natal development, thus precluding the analysis of the gene's function in a specific cell type or at a given time.

These conventional genome modifications can be combined with site-specific recombination systems to generate tissue-specific, time-specific or inducible genetic alterations.

1.5.1. Conditional mutagenesis: the Cre-loxP system

The Cre/*loxP* recombination system has been successfully used as a tool for genome engineering (Sauer, 1998). Cre is a 38 kDA recombinase protein from bacteriophage P1 which catalizes site specific recombination between two *loxP* sites (locus of crossing over). A *loxP* site consists of two 13 basepair inverted repeats separated by an 8 basepair asymmetric spacer region (**Fig. 4**).

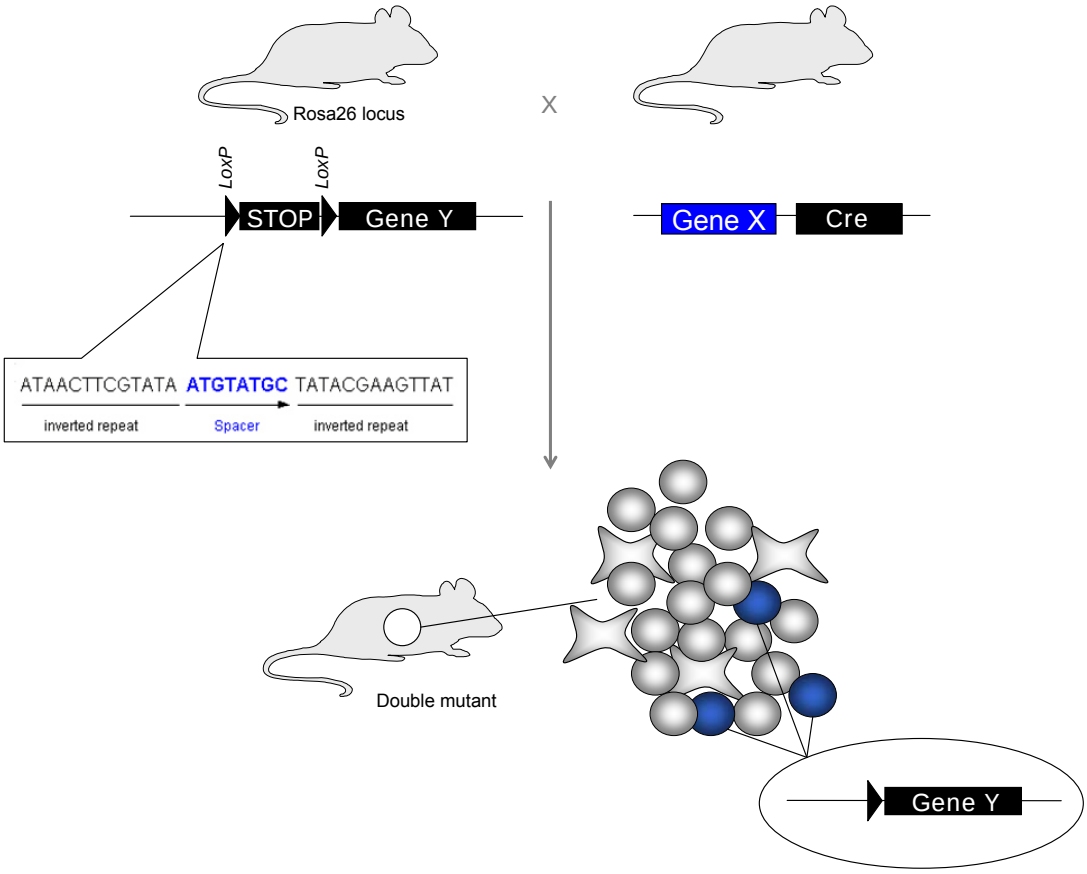


Figure 4. Application of Cre/*loxP* approach for cell type specific expression. Expression of Y gene in the double mutant is initiated by cell specific Cre-mediated recombination between two *loxP* sites and consequent excision of a STOP cassette. Sequence of a *loxP* site is depicted in callout balloon.

Depending on the relative orientation of the *loxP* sites, Cre-mediated recombination can result in DNA excision or inversion. Recombination between two *loxP* sites positioned in the same orientation excises the intervening DNA whereas recombination between *loxP* sites positioned in inverted orientations inverts the intervening DNA sequences. If the two l*oxP* sites are located in different chromosomes, the product is a interchromosomal translocation (reviewed in (Sauer, 1993)).

1.5.1.1. Conditional gene activation

The exact excision of DNA can be used to eliminate a gene (conditional gene deletion) or alternatively to activate a transgene (conditional gene activation) by excising an intervening stop sequence between the promoter and the coding region of the transgene (**Fig. 4**).

For this purpose, two mouse strains are required. One is a mouse line expressing Cre in a lineage-specific manner. Cre can either be introduced into the genome by conventional transgenesis or can be 'knocked in' into a defined locus to give the required expression.

The second mouse strain has a transgene incorporated whose 5' regulatory elements are separated from the coding region with a *loxP*-flanked (or floxed) stop sequence. The recombination, i.e. excision of the stop signal, occurs only in those cells expressing Cre depending on the specificity of the Cre construct. Therefore, the transgene can be transcribed in this special cell type whereas it remains inactive in all other Cre non-expressing cells.

This second strain can be used for gene activation in a number of different tissues or at different developmental times, by simply mating with a Cre strain that displays the desired tissue or temporal specificity of expression.

The Cre/*loxP* system can also be used to generate inducible gene (in)activation. For this purpose, the expression of Cre is controlled by a inducible promoter, for example, the promoter of the *Mx1* gene. This gene is not expressed in healthy animals, but it is transiently activated by interferon (IFN)α or IFNβ or poly-IC. The progeny of mice carrying the Mx1-cre transgene and mice carrying a floxed allele can be treated with IFN or poly-IC to inactivate the floxed target gene (Kuhn et al., 1995; Seibler et al., 2003).

1.5.1.2. Strain validation

The availability of Cre mouse strains in which Cre activity is tightly controlled in space and time is critical to the success of conditional mutagenesis. Indeed, leaky expression of Cre from a cell-type specific or inducible promoter can occur, resulting in recombination events in unwanted cell types (Gustafsson et al., 2001; de Boer et al., 2003) or at the wrong time (Kuhn et al., 1995). The expression of Cre can also be mosaic, in which case the excision will only occur in a subset of cells of a given tissue (Campsall et al., 2002).

Therefore it is essential to evaluate the pattern of Cre expression at the cellular level. RNA or DNA methods have the limitation of giving an estimate of the overall recombination rate of total populations of cells rather than of individual cells. On the other hand, reporter mouse strains allow monitoring of Cre-mediated recombination at

the single-cell level. In these lines, expression of a cellular marker (for example yellow fluorescence protein (YFP)) can be checked by flow cytometry.

One commonly used reporter mouse strain has the YFP gene targeted to the ubiquitously expressed Rosa26 gene locus (Srinivas et al., 2001). As expression from the R26 locus is ubiquitous, any leaky expression of Cre can be readily detected. Another advantage of this reporter over similar reporters, such as lacZ, is that visualization of YFP in living tissue does not require a substrate.

1.5.2. Cell ablation by Diphtheria toxin gene expression

Diphtheria toxin (DT) is secreted by *Corynebacterium diphtheriae,* the causative agent of diphtheria. Once internalized into a mammalian cell, diphtheria toxin blocks protein synthesis and causes rapid cell death. The toxin polypeptide is composed of two subunits: DTA and DTB. The DTB subunit binds to the receptor, a cell surface expressed heparin-binding epidermal growth factor (HB-EGF), facilitating the translocation of DTA into the cell. Once inside the cell, DTA is extremely toxic. Its enzymatic activity (ADP rybosylation of elongation factor 2) leads to the inhibition of protein synthesis and concomitant host cell death (Collier, 2001; Naglich et al., 1992).

Intracellular production of diphtheria toxin has been used to kill cells in a variety of systems. Killing of cells within a selected lineage can be achieved by transgenic mice that express the diphtheria toxin under the control of cell-specific regulatory elements or have diphtheria toxin 'knocked-in' into specific locus (Palmiter et al., 1987; Breitman et al., 1987; Kaplan et al., 2005). Alternatively, expression of diphtheria toxin in a cell type specific manner can be driven by Cre-expression by using the

Cre/*loxP* system described above (Matsumura et al., 2004; Brockschnieder et al., 2004). In this case, a floxed stop cassette is inserted between the promoter and the DTA gene, thereby preventing expression of the toxin independently of Cre.

1.5.2.1. DTA mode of action

DT induces apoptosis by inhibition of protein synthesis and activating components of the death receptor pathway, thus without inducing inflammation or other side effects (Thorburn et al., 2003; Bennett et al., 2005; Miyake et al., 2007).

Studies performed in the related DT receptor (DTR)/DT based system, have shown that DTA-mediated depletion occurs within 24 hours after injection of DT (Bennett et al., 2005).

Even if released from the cytoplasm of apoptotic cells, the DTA fragment is unable to enter neighbour cells in the absence of the DTB fragment, thus preventing non-specific cell death (Palmiter et al., 1987). In addition, sensitivity of mammalian cells to diphtheria toxin varies with the receptor. Rat and mouse cells are resistant because their receptor has very low affinity for the toxin (Cha et al., 1999).

1.6. Aim of this work

The aim of this work was to understand the contribution of HIV driven $CD4^+$ T cell depletion for AIDS pathogenesis. Due to the complexity of phenomena triggered by the virus during infection, it is very difficult to disentangle the relationship between cause and effect of $CD4^+$ T cell depletion and immune activation. For this reason, we have chosen a reductionist approach to deplete the same cells that HIV targets, namely activated $CD4^+$ T cells, in a virus-free system. We have chosen the mouse model due to the availability of genetically altered strains that allow us to specifically deplete the desired cell types. With this model, we intended to dissociate $CD4^+$ T cell depletion from any other effects the virus might have in the development of disease and observe whether $CD4^+$ T cell depletion of activated cells alone is sufficient to generate all or some of the homeostatic disturbances observed in HIV infection.

2. Materials & Methods

2.1. Animals

Inbred C57BL/6 (B6) and CD45.1-congenic B6 mice (B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ) were originally obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) and were subsequently maintained at NIMR. B6-backcrossed *Rag1*-deficient mice (B6.129S7-*Rag1*^{tm1Mom}/J or *Rag1*$^{-/-}$) (Mombaerts et al., 1992), *Rag2*-deficient mice (B6.129-*Rag2*^{tm1Shi} or *Rag2*$^{-/-}$) (Shinkai et al., 1992), MHCII-deficient mice (B6.129S2-*H2*$^{dlAb1\text{-}Ea}$/J or MHCII$^{-/-}$) (Madsen et al., 1999) and T cell receptor α (TCRα)-deficient mice (B6.129-*Tcra*^{tm1Phi} or *Tcra*$^{-/-}$) (Philpott et al., 1992) have been previously described and have also been maintained at NIMR.

Mice with an activatable gene encoding YFP targeted into the ubiquitously expressed *Gt(ROSA)26Sor* (*R26*) locus have been described (Srinivas et al., 2001) and were backcrossed onto the B6 genetic background for at least 10 generations.

Mice with an activatable gene encoding diphtheria toxin fragment A (DTA) targeted into the *R26* locus were kindly provided by Drs Adam Williams and Dimitris Kioussis, Division of Molecular Immunology, NIMR. They were generated by gene-targeting in embryonic stem cells which were subsequently injected into B6 blastocysts. Germ-line transmitting *R26*$^{Dta/+}$ mice were backcrossed onto the B6 genetic background for at least 6 generations.

Mice with a targeted insertion of Cre recombinase into the *Tnfrsf4* locus have been kindly provided by Dr Nigel Killeen, Department of Microbiology and Immunology, University of California, USA, and were backcrossed onto the B6 genetic background for at least 6 generations.

To obtain $Tnfrsf4^{Cre/+}$ $R26^{Dta/+}$ progeny, homozygous $Tnfrsf4^{Cre/Cre}$ mice were mated with heterozygous $R26^{Dta/+}$ mice. In all experiments $Tnfrsf4^{Cre/+}$ $R26^{+/+}$ littermates were included as controls for $Tnfrsf4^{Cre/+}$ $R26^{Dta/+}$ mice to control for any potential effects of CD134 hemizygosity.

Mice with intestinal epithelial cell-specific deletion of *Ikbkg* (encoding for IKKγ, also called NEMO) were obtained by crossing mice with a floxed *Ikbkg* allele ($Ikbkg^{fl/-}$) with mice expressing Cre under the intestinal epithelial-specific Villin promoter (*Vil-Cre*) and have been previously described (Nenci et al., 2007). $Ikbkg^{fl/-}$ *Vil-Cre* and control $Ikbkg^{fl/-}$ mice were bred and maintained at the Institute for Genetics, Cologne, Germany. Spleens and lymph nodes from $Ikbkg^{fl/-}$ *Vil-Cre* and control $Ikbkg^{fl/-}$ mice were harvested at Cologne and shipped to NIMR, where they were analysed the following day.

All animal experiments were conducted according to local government regulations and institutional guidelines.

2.2. Flow cytometry

2.2.1. Cell and tissue preparation

Single cell suspensions were prepared from thymus, spleen or lymph nodes of donor mice by mechanical disruption through a 70 μm cell strainer (Falcon, Becton Dickinson Labware). All cell suspensions were prepared and kept in Air-Buffered (AB) Iscove's Modified Dulbecco's Medium (IMDM) containing 25 mM HEPES buffer and L-glutamine and supplemented with 0.21% NaCl, 60 μg/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Life technologies), and 2% heat inactivated foetal calf serum (FCS) from BioSera.

Spleen cell suspensions were treated with ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2-7.4) for erythrocyte lysis. Bone marrow cell suspensions were prepared by flushing the bone cavities of femurs and tibiae from donor mice with AB IMDM and red blood cells were lysed with ACK lysis buffer. Peritoneal cells were obtained by carefully injecting 5 ml of AB IMDM intra-peritoneally (i.p.) in euthanized mice, followed by gentle abdominal massage and subsequent aspiration of medium-containing cells.

Cell numbers were determined by an automated cell counter (Casy1, Schaerfe Systems). Lymph node cellularity was calculated as the sum of the cellular contents of inguinal, axillary, brachial, superficial cervical and mesenteric lymph nodes. Total numbers of each cell type in each organ were calculated from the frequency of each

cell type determined by flow cytometry, and the total number of cells recovered from each organ.

2.2.2. Cell surface marker staining

Cell surface expression of lymphocyte antigens was measured by monoclonal antibody (mAb) staining of freshly isolated cells, followed by Fluorescence Activated Cell Sorter (FACS) analysis. Cell concentrations were adjusted to 10^7cells/ml for analysis. Cells were incubated with anti-FcR monoclonal antibody (2.4G2) to block non-specific binding through Fc receptors, and stained with directly-conjugated antibodies to surface markers or biotin-conjugated mAbs for 20-30 minutes, followed by PE-TexasRed-, APC-Cy7- or PerCP-streptavidin staining. All stainings were performed at 4^{o}C and cells were washed with FACS buffer (phosphate-buffered saline (PBS), 2% FCS, 0.1% azide). Antibodies used were obtained from eBiosciences, CALTAG/Invitrogen, BD Biosciences or prepared at NIMR and are summarized in **Table 1**.

Shedding of the adhesion molecule L-selectin (CD62L) was prevented by preparing cell suspensions in medium containing 50 μM of the inhibitor TAPI-2 (Peptides international, Inc) in an adaptation of a previously described procedure (Jabbari and Harty, 2006).

Up to 4-colour samples were acquired on an analytical flow cytometer (FACSCalibur, BD Biosciences) and analyzed with FlowJo v8.7 software (Tree Star Inc). Up to 8-colour cytometry was performed on a CyAn flow cytometer (Dako) and analyzed with Summit v4.3 analysis software (Dako).

Table 1: Specificity and clone name of anti-mouse Abs used for extra-cellular staining. Alternative names are show in parenthesis.

Specificity	**Clone name**	**Company**
Fcγ III/II R	2.4G2	Made at NIMR
CD4 (L3T4)	RM4-5	eBiosciences / Caltag
CD8*a* (Ly-2)	53-6.7	eBiosciences /BD Biosciences
CD11b (Mac1α; integrin α_M chain)	M1/70	eBiosciences
CD11c (integrin α_X chain)	N418	eBiosciences
CD19	MB19-1	eBiosciences
CD25 (IL-2 receptor α chain	PC61.5	eBiosciences
CD38	90	eBiosciences
CD43 (Ly-48; leukosialin)	1B11	BD Biosciences
CD44 (Pgp-1; H-CAM; Ly-24)	IM7	eBiosciences
CD45.1 (Ly-5.1)	A20	eBiosciences
CD45.2 (Ly-5.2)	104	eBiosciences
CD45R (B220)	RA3-6B2	eBiosciences
CD45RB	C363.16A	eBiosciences
CD49b (integrin α_2 chain)	HMα2	BD Biosciences
CD62L (L-selectin; LECAM-1; Ly-22)	MEL-14	eBiosciences
CD70	FR70	eBiosciences
CD103 (integrin α_{IEL} chain)	2E7	eBiosciences
CD127 (IL-7receptor α chain)	A7R34	eBiosciences
CD134 (Ox40)	OX-86	eBiosciences
TCR β	H57-597	eBiosciences
IgM	DS-1	BD Biosciences
IgD	11-26c	eBiosciences
MHCII (I-A^b)	M5/114.15.2	eBiosciences
Ter119/Erythroid cells (Ly-76)	TER-119	eBiosciences
Gr1 (Ly-6G)	RB6-8C5	eBiosciences
F4/80	BM8	eBiosciences
GL7 (Ly-77)	GL-7	BD Biosciences
Glyco-Gag	34	Made at NIMR
mIgG2b-FITC	R12-3	BD Biosciences
2^{nd} layers		
streptavidin-PETxR		Caltag
streptavidin-APCCy7		BD Biosciences
streptavidin-PerCp		BD Biosciences

2.2.3. Intracellular cytokine staining

For intracellular cytokine staining, cytokine production was induced by stimulating cells for 4 hours with phorbol 12, 13-dibutyrate (PDBu) and ionomycin (both at 0.5 μg/ml, from Sigma chemical Co) at 37°C, together with surface staining antibodies. One hour later, an inhibitor of intracellular transport, monensin (at 1μg/ml), was added to block secretion of cytokines. Three hours later, cells were washed and resuspended in fixation buffer (eBioscience) for 20 minutes at room temperature (RT) and then washed and incubated in permeabilization buffer (eBioscience) with antibodies for cytokine staining for one hour at RT. Antibodies used for cytokine intracellular staining are summarized in **Table 2**.

Table 2: Specificity and clone name of anti-mouse Abs used for cytokine intra-cellular staining.

Cytokines	Clone name	Company
IFN-γ	XMG1.2	eBiosciences
IL-10	JES5-16E3	eBiosciences
IL-2	JES6-5H4	eBiosciences
TNF-α	MP6-XT22	eBiosciences
IL-4	11B11	eBiosciences
IL-17A	TC11-18H10.1	eBiosciences

2.2.4. FoxP3 intranuclear staining

Intranuclear staining for Foxp3 was done with a Foxp3 staining kit purchased from eBioscience following manufacturer's instructions. Briefly, 10^6 cells were first stained for surface molecules. Cells were then washed and incubated for 1 hour at 4°C with fixation/permeabilization buffer and then washed 3 times with permeabilization buffer

followed by staining with anti-Foxp3 antibody (Clone FJK-16s) at 4°C for at least 30 minutes.

2.2.5. Analysis of cell turnover by BrdU

BrdU is a thymidine analogue that incorporates into dividing cells during DNA synthesis (Gratzner, 1982). Mice were given BrdU (Sigma chemical Co) -containing water (0.8 mg/ml) for 7 days following a single i.p. administration of 0.8 mg of BrdU. BrdU-containing water preparations were made fresh or kept frozen, changed daily and kept protected from light. Single cell suspensions of the spleen and lymph nodes were labelled with cell-surface antibodies, as usual, washed and fixed for one hour to overnight with fixation buffer (eBioscience) and washed. Afterwards they were incubated with 0.1% NP40 (Sigma chemical Co) in PBS-azide-free for 3 minutes on ice, washed twice and stained with 20-40 µl of FITC-conjugated anti-BrdU (clone B44) with DNase (FastImmune Anti-BrdU FITC with DNase) in the dark for one hour.

2.2.6. Analysis of cell turnover by Ki67

Ki67 is a nuclear cell proliferation-associated antigen expressed in all active stages of the cell cycle (Kubbutat et al., 1994) that was detected by intranuclear staining. The fixation protocol and reagents used were similar to the ones used for FoxP3 staining except that 20 µl of PE mouse anti-human Ki-67 or matched isotype control were used (clone B56 and clone MOPC-2, respectively, both provided in PE mouse anti-human Ki-67 set from BD Pharmingen).

2.2.7. Analysis of apoptosis by Annexin V staining

Apoptosis was assessed by Annexin V staining. In the early stages of apoptosis, cells expose phospholipid phosphatidylserine to the external cellular environment which is bound by Annexin V. Cells that are undergoing apoptosis can therefore be detected by flow cytometry using Annexin V conjugated to a fluorochrome (Vermes et al., 1995).

Apoptotic cells were stained using an AnnexinV-PE Apoptosis detection Kit (BD Biosciences) according to the manufacturer's instructions. Briefly, cells were stained with 5 µl of Annexin V-PE and 5 µl of 7-AAD in binding buffer provided for 15 minutes at RT in the dark and analysed within one hour.

2.2.8. Cell purification and sorting

Target cells were enriched in lymph node and spleen suspensions using immunomagnetic positive or negative selection (EasySep beads, StemCell Technologies) according to the manufacturer's instructions. For example, for *in vitro* T cell activation experiments, negative selection was performed by first staining the cells with CD8α- and B220-PE conjugated antibodies. Then, PE selection cocktail was added and single cell suspension incubated at RT for 20 minutes. Magnetic beads were added and incubated for a further 15 minutes at RT. The tube containing the cells was then placed on a magnet (EasySep Magnet) for 10 minutes and supernatant was poured off to a new tube which was loaded on the magnet. After repeating this procedure two times, negatively-selected cells were collected, centrifuged and counted.

For *in vivo* regulatory $CD4^+$ T cell transfers, positive selection was done following the same purification procedure as described above, with the exception that cells were initially stained with CD25 PE-conjugated antibody and in the last step, tube with cells was placed on the magnet for 5 minutes, and supernatant was poured off. After two washes, positively-selected cells were collected by removing the tube from the magnet.

Enriched cell suspensions were counted and when necessary stained with antibodies to surface markers and then further purified by cell sorting, performed on MoFlo cell sorters (Dako) by the NIMR Cell Sorting facility. Typical cell purity following cell sorting was higher than 98%.

2.3. In vitro T cell activation

Culture medium used for *in vitro* cultures and cell lines was IMDM supplemented with 5% heat inactivated FCS (BioSera) and 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10^{-5} M mercaptoethanol (all Sigma Chemicals Co).

For YFP *in vitro* kinetics, single cell suspensions were prepared from the spleen and lymph nodes of $Tnfrsf4^{Cre/+}$ $R26^{Yfp/+}$ mice. $CD4^+$ T cells were purified by using CD8/ B220 negative selection by Easysep beads and subsequently sorted as $CD4^+YFP^-$. Depending on the experiment, 0.25×10^6, 0.5×10^6 or 4.5×10^6 sorted T cells per well were stimulated in 96 well-plates with CD3/CD28 coated beads (Dynal Mouse CD3/CD28 T Cell Expander, Invitrogen Dynal AS), at 1:1 ratio for the indicated length of time in IL-2 (10 ng/ml) containing IMDM medium.

For *in vitro* apoptosis analysis, single cell suspensions were prepared from the spleen and lymph nodes of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. CD4^{+} T cells were purified as described for YFP analysis and sorted as CD4^{+}CD25^{-}CD45RB^{high} cells. Purity after sort was 98% in one experiment and 99% in another experiment. Depending on the experiment, 0.75-1 × 10^{6} sorted naïve T cells per well were stimulated as described for YFP analysis.

2.4. Histology

Organs were collected in formalin immediately after donor death and sent for histological analysis (Dr Mark Stidworthy at IZVG Pathology, Leeds, UK). For the analysis of growth retarded and normal *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice the following organs were collected: skin, kidney, lung, brain, salivary glands, stomach, liver, gall bladder, pancreas, small and large intestines, spleen and lymph nodes. Histological sections were stained with haematoxylin and eosin (H&E) unless otherwise stated.

2.5. Serum preparation

Serum was prepared by leaving non-heparinised blood to clot at room temperature for 2 hours or at 4°C overnight. Clot was then detached from the sides of tubes which were then centrifuged at 3,000 rpm for 5 minutes. Clear sera were transferred to new tubes which were centrifuged at 12,000 rpm for 5 minutes. Clear sera were transferred to new tubes. For luminex assay and detection of serum immunoglobulins, sera were stored at -20°C. For neutralizing antibody (nAb) assays, sera were first heat inactivated at 56°C for 10 to 15 minutes and then stored at -20°C.

2.6. Analysis of serum cytokines by luminex assay

Simultaneous measurement of serum levels of mouse fibroblast growth factor (FGF) basic, vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), tumour necrosis factor (TNFα), IL (interleukin) -1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40⁄p70, IL-13, IL-17, IP-10 (or chemokine (C-X-C motif) ligand 10 (CXCL10)), KC (CXCL1), monocyte chemotactic protein-1 (MCP-1 or Chemokine (C-C motif) ligand 2 (CCL2)), macrophage inflammatory protein-1 α (MIP-1 α or CCL3) and monokine induced by IFNγ (MIG or CXCL9) was performed by using a multiplex cytokine bead array (20-plex ab bead kit, BioSource), using the Luminex 100 System (Luminex). A second experiment was done using a multiplex cytokine bead array (BioRad) allowing the detection of seven additional factors: IL-3, IL-9, IL12p70, Eotaxin (CCL11), G-CSF, MIP-1β (CCL4), regulated upon activation, normal T cell expressed and secreted (RANTES or CCL5).

Serum samples were diluted two fold in PBS, and 50 µl transferred to 96 well plates pre-wet in Bio-Plex assay buffer and containing 50 µl of multiplex beads. Samples were incubated for 60 minutes at room temperature in a plate shaker (300 rpm) followed by 3 washes with Bio-Plex wash buffer and the addition of 50 µl of biotin-labelled detection antibody. Following 30 minutes incubation at room temperature in plate shaker and 3 washes, 25 µl of Streptavidin-PE were added and a further incubation step for 10 minutes at room temperature was performed. Following 3 washes, samples were ressuspended in 150 µl of Bio-Plex assay buffer and analysed on the luminex 100 System.

2.7. Bone marrow chimeras

Whole bone marrow cells (after red blood cell lysis) were injected intravenously (i.v.) into non-irradiated *Rag*-deficient mice via the tail vein in 0.1-0.2 ml of AB IMDM. Recipient mice were periodically bled via the tail vein to analyse peripheral blood for reconstitution and were killed and analysed twelve to fourteen weeks after transfer. Donor- and recipient-derived cells were distinguished by cell surface expression of CD45.1 or CD45.2 allotypes. In mixed bone marrow chimeras experiments, mixed populations were injected at a 1:1 ratio.

2.8. Adoptive transfer of cells

For transfers into *TCRα*$^{-/-}$ recipients, titrating amounts of CD4^{+} T cells (purified by positive selection) were injected i.v. via the tail vein in 0.1 ml of AB IMDM. Total amount of CD4^{+} T was considered to be ~6x10^{7}/mouse (Bourgeois et al., 2005) and percentage of 'take' in cell transfer ~20%. Accordingly 3x10^{7}, 3x10^{6}, 3x10^{5} and 3x10^{4} cells were transferred to represent 10%, 1%, 0.1% and 0.01% of total CD4^{+} T cells of B6 mice.

For regulatory T cell transfer experiments, 1-3x10^{6} sorted CD4^{+}CD25^{bright} cells **(Fig. 5)** from spleen and lymph nodes of WT mice were injected i.v. via the tail vein in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ and control mice and periodically tail bled. Mice were killed and analysed 7-10 weeks post transfer.

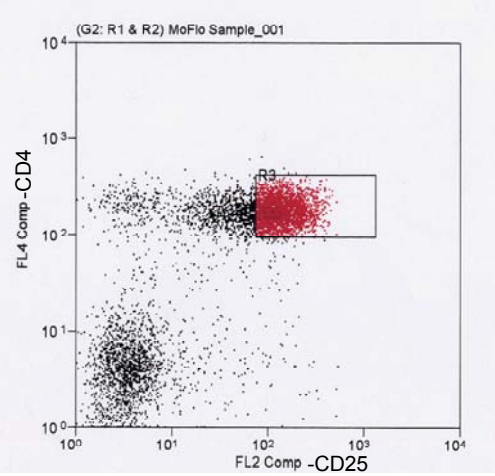


Figure 5. Sort profile of $CD4^{+}CD25^{bright}$ cells. Cell suspension from spleen and lymph nodes from 30 B6.CD45.1 mice were enriched with immunomagnetic positive selection for CD25 and further purified by cell sorting. The flow cytometry plot shows the positioning of the gate used to sort $CD4^{+}CD25^{bright}$ cells. The plot is of one out of 3 independent experiments. In all 3 experiments, purity was ≥98%.

2.9. Experimental infections in mice

2.9.1. Friend virus infection

The Friend virus (FV) used in this study is a retroviral complex of a replication-competent B-tropic helper murine leukaemia virus (F-MuLV-B) and a replication-defective polycythemia-inducing spleen focus-forming virus (SFFVp). The FV stock (kindly provided by Dr. Kim Hasenkrug, Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT, USA) was free of lactate dehydrogenase-elevating virus (LDV) and was obtained as previously described (Robertson et al., 2008). FV was propagated *in vivo* and prepared as 10% w/v homogenate from the spleen of 12-day infected BALB/c mice. Mice received an inoculum of FV complex containing between 1,000-2,000 spleen focus-forming units (SFFU) injected via the tail vein in 0.1 ml of PBS.

2.9.1.1. Friend virus detection by FACS

Cell-associated virus in infected mice was estimated by flow cytometric detection of infected cells using surface staining for the glycosylated product of the viral *gag* gene (glyco-Gag), using the matrix (MA)-specific monoclonal antibody 34 (mouse IgG2b), followed by an antimouse IgG2b-FITC secondary reagent (Table 1).

2.9.1.2. Friend virus nAb titre assay

FV-neutralizing antibodies in the sera of infected mice were measured using a modification of a previously described viral titre assay (Bock et al., 2000). *Mus dunni*

cells (Lander and Chattopadhyay, 1984) were transduced with the XG7 replication-defective retroviral vector, expressing green fluorescent protein (GFP) from a human cytomegalovirus (hCMV) promoter and a neomycin-resistance gene under the control of the LTR (Bock et al., 2000). *Mus dunni* and *Mus dunni*-XG7 cells were both kindly provided by Dr J. Stoye, Division of Virology, NIMR. Maintenance of GFP expression was ensured by constant selection with 1 mg/ml G418. *Mus dunni*-XG7 cells were then infected with F-MuLV-B and supernatant, which contained the pseudotyped XG7 vector, was harvested. Serial dilutions of sera from infected mice were mixed with ~1,500 infectious units (iu)/ml pseudotyped XG7 vector and allowed to incubate for 30 minutes at 37°C in IMDM cell culture medium containing 5% FCS. Mixtures were then added to untransduced *Mus dunni* cells and incubated for 3 days. The percentage of GFP^+ *Mus dunni* cells at the end of the incubation period was assessed by flow cytometry and the dilution of serum which resulted in 75% neutralization (i.e. 75% reduction in the percentage of GFP^+ *Mus dunni* cells) was taken as the neutralizing titre.

2.9.2. Influenza virus infection

The A/PR/8/34 (PR8) strain of influenza A virus (IAV) (kindly provided by Ms Rose Gonsalves, Division of Virology, NIMR) was an allantoic fluid preparation from PR8-infected embryonated eggs. Non-anaesthetized mice were infected with 250 hemagglutinin units (HAU) of PR8 by instillation onto their nasal cavities. After 6, 12 and 18 days, non-heparinised blood was collected by tail bleeding.

2.9.2.1. Influenza nAb titre assay

Serum titres of PR8-neutralizing antibodies were measured using a modified Madin Darby canine kidney (MDCK)-based assay. Serial dilutions of the sera were added to monolayers of MDCK cells in 96-well COSTAR cell culture plates (Corning, Inc), which were subsequently infected with a tissue culture infective dose ($TCID_{95}$) of PR8. MDCK cell viability was measured with an Alamar blue (Biosource) based assay 3 days after infection. Cultures were washed and pulsed with Alamar blue for 1-2 hours and fluorescence was measured with a fluorescence plate reader (Safire, Tecan). The dilution of serum which resulted in 50% neutralization of MDCK death was taken as the neutralizing titre.

2.9.3. *Pneumocystis murina* infection

Pneumocystis murina (*P. murina*) was obtained from ATCC/LGC Promochem (stock PRA-111) as lung tissue homogenate from *P. murina* infected rats. Non-anaesthetized mice were administered *P. murina* by instillation onto their nasal cavities. Mice were killed 17 weeks later, and left lower lung lobe was removed and frozen and subsequently used to extract genomic DNA. The remaining lung tissue was gently inflated via the tracheae with formalin and sent for histological analysis. Histological sections were examined for general architecture (haematoxylin and eosin (H&E) stain) and *Pneumocystis* organisms (Gomori's silver stain).

2.9.3.1. Detection of *Pneumocystis* in lungs by PCR

Lungs were incubated overnight with 0.5 ml of lysis buffer (100 mM Tris-HCl (pH7.5), 5 mM EDTA, 200 mM NaCl, 0.2% SDS) and 200 µg/ml proteinase K

(Roche diagnostics GmbH). The extracts were treated with buffered phenol and DNA was precipitated with ethanol and dissolved in water. To detect *P. murina* in the lung, DNA samples were subjected to nested PCR using specific primers (MGW biotech AG) for the mitochondrial large-subunit ribosomal RNA (rRNA) of *P. murina*. PCR reactions contained ~50 ng of the genomic DNA, 2 mM $MgCl_2$ (Abgene Ltd), 0.2 mM dNTP (G.E. Healthcare), 0.4 pmol/µl sense and antisense primer, and 0.1 U *Taq* DNA polymerase (Abgene Ltd) in the supplier's buffer in a volume of 20 µl, and were amplified in an Eppendorf mastercycler termocycler. PCR amplifications were carried out with a 2.5 minutes denaturation step at 94˚C and then 30 cycles of denaturation at 94˚C for 30 seconds, annealing at 55˚C for 30 seconds and extension at 72˚C for 30 seconds, followed by a final 5 minutes extension at 72˚C. Nested PCR amplifications were performed using 1 µl of this PCR product following the same protocol except for the annealing temperature which was 50˚C. The outer primers were pAZ102-H (5'-GTG TAC GTT GCA AAG TAC TC-3') and pAZ102-E (5'-GAT GGC TGT TTC CAA GCC CA-3'). The internal primers were pAZ102-X (5'-GTG AAA TAC AAA TCG GAC TA-3') and pAZ102-Z (5'-CCC ACT TCT T(A/G)A CTG TC-3') as described elsewhere (Hori et al., 2002). The expected product size is 346 bp for the first PCR and 263 for the nested PCR. As a control, PCR amplifications of mouse *Rag-1* gene were also performed. The primers were 5'-CTG TAG GAT CTG CAT TCT CAG ATG TC-3' and 5'-CAG CCT TCA CAT CTC CAC CTT CTT C-3', and the PCR condition were the same as described above. PCR products were separated on a 2% agarose gel containing ethidium bromide.

2.10. Analysis of serum immunoglobulins by ELISA

Serum immunoglobulin (Ig) levels were determined by a standard enzyme-linked-immunosorbent-assay (ELISA) technique. 50 µl of test supernatants were added to 96-well polyvinyl flexiplates (Thermo Electron Corporation) coated overnight with 50 µl of unlabeled goat anti-mouse Ig (H+L) (Southern Biotech) at 5µg/ml in coating buffer (0.2 M borate buffer, pH 8.5) and blocked with PBS supplemented with 10% FCS for at least 1 hour at RT. Following 3 washes, 50 µl of secondary antibody (IgG-Alkaline phosphatase (AP) or IgM-AP, 1:1,000) (Southern Biotech) were added and incubated for 1 hour at RT. Wells were extensively washed and 200 µl of alkaline phosphate yellow liquid substrate (Sigma Chemicals Co) was added. Absorbance was read at 405 nm when yellow reaction products were formed. All washes were done with PBS supplemented with 0.05% tween20 (Sigma Chemicals Co).

2.11. Statistical analysis

Statistics were generated by Student's *t*-test performed using SigmaPlot v10 software (Systat Software Inc). p values <0.05 were considered to be statistically significant.

3. Results

3.1. Specific targeting of memory and regulatory CD4$^+$ T cells by Tnfrsf4*-driven Cre expression*

Immunodeficiency viruses are highly selective for activated/memory CD4$^+$ T cells due to restricted expression of their receptors/co-receptors. One of the most specific markers of activation in CD4$^+$ T cells is CD134, the cellular receptor for FIV. We generated a mouse model in which activated CD4$^+$ T cells, i.e. CD134$^+$CD4$^+$ T cells are targeted.

Specificity of CD134 (encoded by *Tnfrsf4* gene) expression was evaluated using a reporter mouse strain. This strain was obtained by crossing the *Tnfrsf4*$^{Cre/Cre}$ knock-in strain in which Cre is knocked-in into the CD134 locus, with the *R26*$^{Yfp/Yfp}$ strain (Srinivas et al., 2001), in which expression of yellow fluorescent protein (YFP) under the ubiquitously expressed R26 promoter is prevented by a *loxP* flanked (floxed) stop cassette. We named this strain *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$. Cre mediates excision of the floxed stop cassette in the R26 locus activating the YFP gene. Consequently, in these animals, all cells that are expressing or have expressed CD134-driven Cre (as well as their daughter cells), will also express YFP.

We examined the frequency of YFP-expressing cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice by flow cytometry. The fluorescence intensity of YFP in these mice is very bright allowing an easy characterization of YFP-expressing cells (**Fig. 6**).

In the thymus, where only ~ 1% of all cells expressed YFP (**Fig 6**), the majority of these were CD4 single positive (SP) thymocytes and ~ 30% were double positive (DP) thymocytes (**Fig. 7-B**). YFP was hardly detected on CD8 SP cells (**Fig. 7-B**). Mature $CD4^+$ T cells can be subdivided into naïve, memory, and regulatory cells according to the expression of CD25 and CD44 markers. Naïve cells are $CD25^-$ $CD44^{low}$, memory cells are $CD25^-CD44^{high}$ and regulatory cells are $CD25^+CD44^{int}$ (**Fig. 7-C**). This has been the classification used throughout this work. While only approximately 14% of memory $CD4^+$ T cells expressed YFP, more than half of regulatory $CD4^+$ T cells (56%) expressed YFP (**Fig. 7-D**). Although it cannot be excluded that some of these cells could be re-circulating, this is consistent with the observation that the regulatory $CD4^+$ T cell subset is already activated in the thymus (Takeda et al., 2004).

In the spleen, the number of YFP^+ cells increased with age until 8 weeks, possibly reflecting an age-related increase in antigen-experienced cells in this organ. In adult mice, lymph nodes contained approximately 6% of cells expressing YFP, while in the spleen these cells constituted around 4% of the total cells (**Fig. 8-A**).

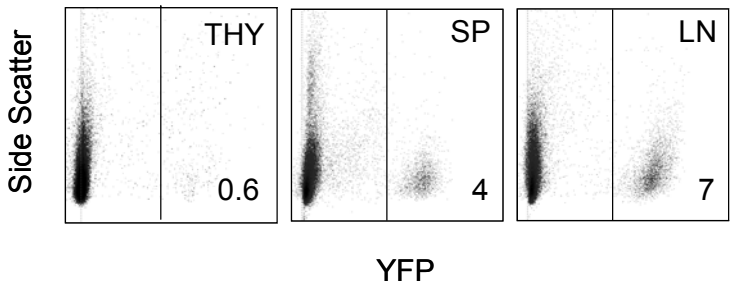


Figure 6. Intensity of YFP expression in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice. Flow cytometric analysis of YFP expression in thymus (THY), spleen (SP) and lymph nodes (LN) of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice at 8 weeks of age. Numbers within dot plots indicate the percentage of YFP^{+} cells.

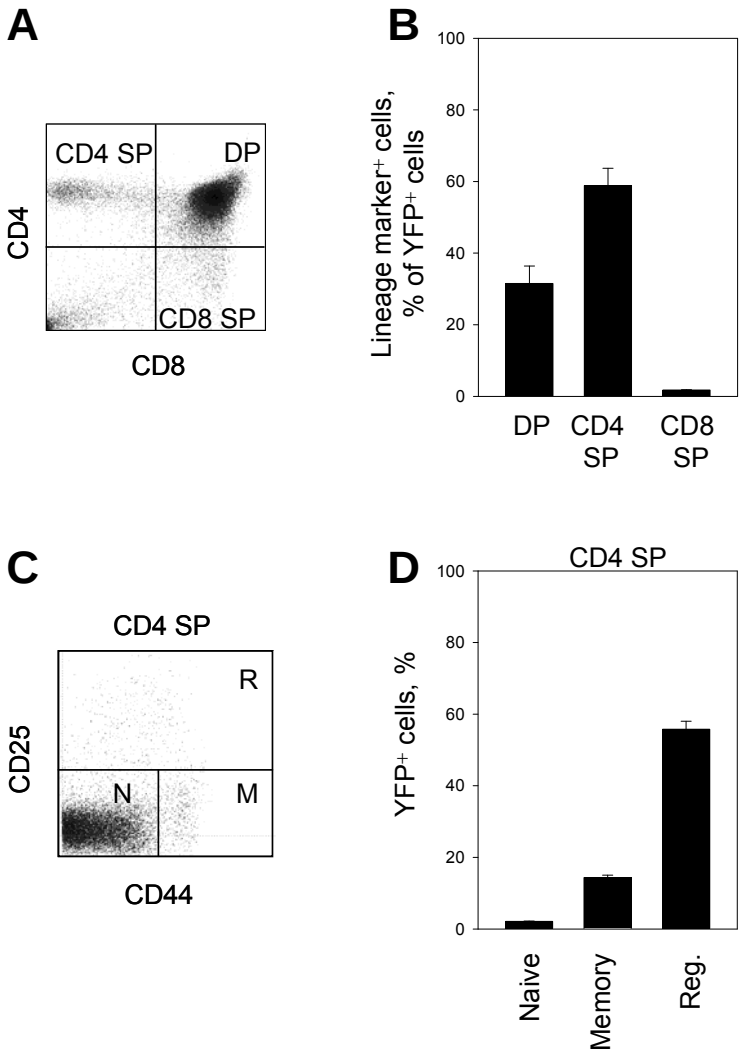


Figure 7. YFP expression in thymic subsets in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice. (**A**) Flow cytometric example of subdivision of thymocytes in CD4^{+}CD8^{+} (DP), CD4^{+}CD8^{-} (CD4 SP) and CD4^{-}CD8^{+} (CD8 SP) subsets. (**B**) Frequency of DP, CD4 SP and CD8 SP in gated YFP^{+} thymocytes in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice. **(C)** Flow cytometric example of subdivision of CD4 SP cells into naïve (N, CD44^{low}CD25^{-}), memory (M, CD44^{high}CD25) and regulatory (R, CD25^{+}) subsets. (**D**) Percentage of YFP^{+} cells in the indicated subsets. Mean values (±SEM) of 9 mice are shown.

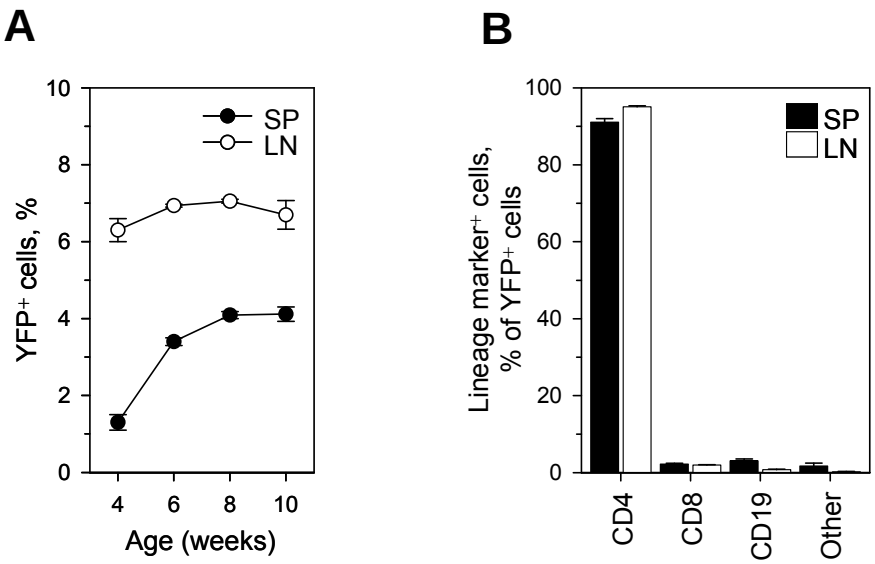


Figure 8. YFP expression in peripheral lymphoid organs of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice is largely restricted to the CD4$^+$ T cell lineage. (A) Relative accumulation of YFP expressing cells in secondary lymphoid organs of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice over time. Symbols represent the mean values (±SEM) of 2-3 mice per time point per organ. **(B)** Frequency of CD4$^+$, CD8$^+$ or CD19$^+$ cells or cells negative for all three markers (other) in gated YFP$^+$ cells from the spleen and lymph nodes of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice. Mean values (±SEM) of 9 mice are shown.

We next analysed which cell types in spleen and lymph nodes expressed YFP. Our analysis revealed that ~95% of YFP^+ cells were $CD4^+$ T cells whereas only 2% were $CD8^+$ T cells and 2% were B cells (**Fig. 8-B**), confirming the expected specificity of CD134 expression for $CD4^+$ T cells (Watts, 2005).

We looked at expression of YFP in $CD4^+$ T cell subsets in spleen and lymph nodes. YFP expression in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice marked ~ 55% and ~ 80% of memory and regulatory $CD4^+$ T cells, respectively. In contrast, the vast majority of naïve $CD4^+$ T cells (92%) were YFP^- (**Fig. 9-B**). In young adult mice, regulatory $CD4^+$ T cells constitute ~ 10% of total $CD4^+$ T, while the frequency of memory $CD4^+$ T is higher, particularly in the spleen, where they are twice as frequent as regulatory T cells, reaching more than 20% of total $CD4^+$ T cells. Thus, in total numbers, the majority of cells expressing YFP in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice were memory $CD4^+$ T cells.

We next looked at $CD8^+$ T cells in more detail in both spleen and lymph nodes. We subdivided $CD8^+$ T cells in naïve and memory subsets by low or high expression of CD44, respectively (**Fig. 9-C**). This has been the classification used throughout this work. Although CD44 expression does not distinguish between true memory cells, that is antigen experienced cells, and ones that acquired phenotypic properties of memory cells, it is normally accepted to use CD44 expression to classify memory cell subsets. We observed that both naïve and memory $CD8^+$ T cells (99% and 97%, respectively) were YFP^- (**Fig. 9-D**).

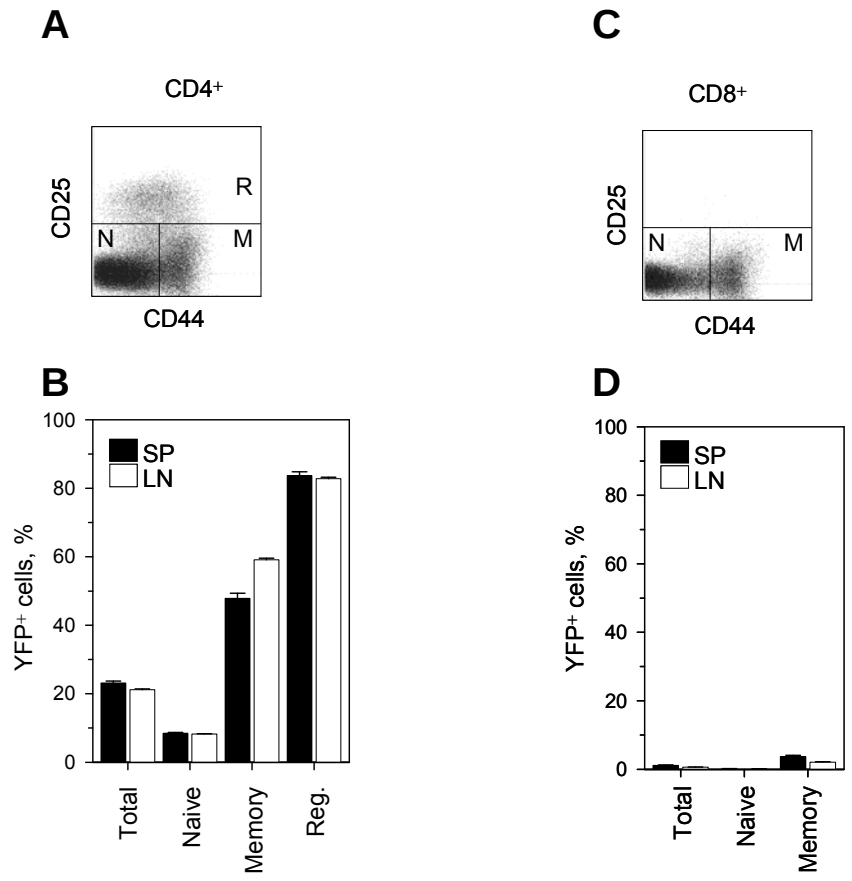


Figure 9. Specific targeting of memory and regulatory CD4$^+$ T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice. Flow cytometric example of subdivision of CD4$^+$ T cells into naïve (N, CD44^{low}CD25$^-$), memory (M, CD44^{high}CD25$^-$) and regulatory (R, CD25$^+$) subsets (**A**), and of CD8$^+$ T cells into naïve (N, CD44^{low}CD25$^-$) and memory phenotype (M, CD44^{high}CD25$^-$) cells (**C**). Percentage of YFP$^+$ cells in total, naïve, memory and regulatory CD4$^+$ (**B**) or in total, naïve and memory CD8$^+$ T cells (**D**) from the spleen and lymph nodes of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice. Mean values (±SEM) of 9 mice are shown.

We further investigated whether YFP was expressed in non-lymphoid organs. We looked at peritoneal cavity, a site normally populated by activated cells, many (~50%) of which are macrophages. Although we saw an increase with age in the frequency of YFP^+ cells it was always less than 5% of total cells, and the vast majority were $CD4^+$ T cells (not shown). Analysis of lung and liver showed no significant YFP expression (not shown).

In summary, using this reporter line we have identified the cell populations that express YFP upon CD134 upregulation. YFP expression was observed almost exclusively in memory and regulatory $CD4^+$ T cells, the major targets of immunodeficiency virus. We did not see significant YFP expression in other immune cells nor in non-lymphoid organs. This is in agreement with published results where expression of CD134 was assessed by surface staining with anti-CD134 antibody (Takeda et al., 2004).

3.2. DTA-mediated deletion of CD134^{+} CD4^{+} T cells

Having confirmed the suitability of the *Tnfrsf4*-Cre-mediated targeting of activated CD4^{+} T cells, we generated a mouse strain in which these cells were being deleted. This was achieved by crossing the *Tnfrsf4*$^{Cre/Cre}$ knock-in strain with the *R26*$^{Dta/+}$ transgenic strain, in which DTA expression under the R26 promoter is prevented by a floxed stop cassette. The progeny obtained was *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice and *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ littermate controls. In all experiments, littermate controls were included to rule out any potential *Tnfrsf4* gene dose effect.

We started by analyzing the efficiency of DTA-mediated deletion. For this purpose we generated mice expressing both DTA and YFP alleles in the R26 locus and named this heterozygous mice *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/Dta}$. Theoretically, if recombination and deletion are 100% efficient, these mice should have no YFP expressing cells.

Our analysis revealed a 2.3- and 3- fold decrease in the total number of YFP expressing splenic and lymph node cells, respectively, in mice with both YFP and DTA alleles when compared with mice with YFP allele only (**Fig. 10-A**). The reduction in CD4^{+} T cells was about 2.3- fold in both organs and paradoxically there were more YFP expressing CD8^{+} T cells in mice with both alleles than in mice with YFP allele only (**Fig. 10-A**). The proportion of YFP expressing CD8^{+} T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/Dta}$ was nevertheless very small.

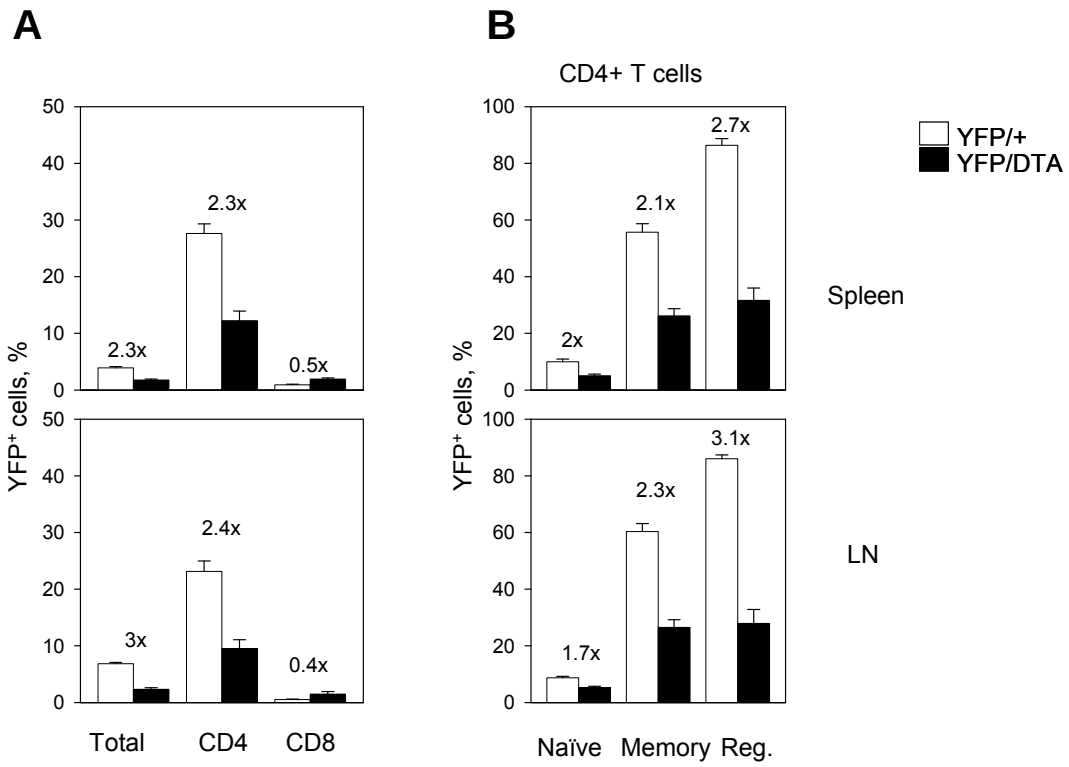


Figure 10. DTA-mediated deletion of YFP$^+$ CD4$^+$ T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/Dta}$ mice. Percentage of YFP$^+$ cells in total, CD4$^+$ and CD8$^+$ T cells **(A)** or naïve, memory and regulatory (Reg.) CD4$^+$ T cells **(B)** from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/Dta}$ (YFP/DTA) mice and littermates *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ (YFP/+). Numbers represent the fold difference in frequencies of YFP$^+$ cells between YFP/+ and YFP/DTA mice. Mean values (±SEM) of 4 mice per group from 2 separate experiments are shown.

Analysis of the major YFP expressing populations, ie, memory and regulatory $CD4^+$ T cells, showed that in comparison with *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice, the proportion of YFP^+ memory and regulatory $CD4^+$ T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/Dta}$ mice was reduced by more than half in both organs (**Fig. 10-B**).

This result suggests that more than 50% of the cells tagged with YFP in the absence of DTA expression were killed upon DTA reactivation. However, this analysis ignores the dynamic nature of T cell death and replacement. In *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice the immune system is at steady-state, but in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/Dta}$ mice the killing of activated $CD4^+$ T cells leads to changes in T cell dynamics for both $CD4^+$ and $CD8^+$ T cells that are described in the following chapters. Consequently, the relative presence of activated $CD4^+$ T cells and proportion of YFP^+ T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/Dta}$ mice reflect the balance between DTA-mediated killing, which reduces, and homeostatic replacement, which increases, the number of YFP^+ activated $CD4^+$ T cells.

In addition to T cell dynamics, the interpretation of the proportion of YFP expressing cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/Dta}$ mice may also be influenced by the relative kinetics of YFP and DTA induction following T cell activation. YFP and DTA are different proteins and consequently may have a different kinetic of expression. For YFP expression to be detected, YFP simply needs to be expressed in the cytoplasm in sufficient amount to be revealed by flow cytometry. Similarly, for DTA-mediated deletion to be detected, DTA needs to be expressed in sufficient amount to kill a cell. DTA causes cell death by triggering the apoptosis (or programmed cell death) pathway (Lessnick et al., 1992; Chang et al., 1989). Although it has been shown *in*

vitro that a single molecule is sufficient to kill a cell (Palmiter et al., 1987), it remains to be shown if this is true *in vivo* and for all cell types. In addition, cessation of protein synthesis and subsequent apoptosis are processes that take some time and thus the existence of YFP cells in the heterozygous mice could merely reflect the fact that cells have rearranged both alleles and are undergoing cell death but are not dead yet. In addition, it is plausible to consider that Cre-recombination could occur more easily in one allele than the other. These issues were addressed by comparing the kinetics of YFP and DTA induction following T cell activation in *in vitro* systems.

To investigate YFP induction following activation, naïve CD134$^-$ YFP$^-$ CD4$^+$ T cells from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice were sorted and stimulated *in vitro* with anti-CD3 and anti-CD28. We then compared the expression of CD134 and YFP by flow cytometry at different times. Cells begun to express YFP already in the first day of culture, with a delay of ~1 day in relation to CD134 induction (**Fig. 11-A and 11-B**), which achieved its maximal expression already at 24 hours and declined after 48 hours. This transient expression of CD134 *in vitro* is in agreement with previous studies (Gramaglia et al., 1998).

We tested the kinetics of DTA-mediated cell death by looking at Annexin V, a marker of early apoptosis, by flow cytometry. Sorted CD25$^-$ CD45RB^{high} naïve CD4$^+$ T cells from *Tnfrsf4*$^{Cre/+}$ *R26*$^{DTA/+}$ mice and littermate controls were stimulated *in vitro* with anti-CD3 and anti-CD28, and apoptosis was examined at different times. In the first 24 hours post activation both groups of cells behaved in similar way, indicating that DTA was not yet active (**Fig. 12-A and 12-B**).

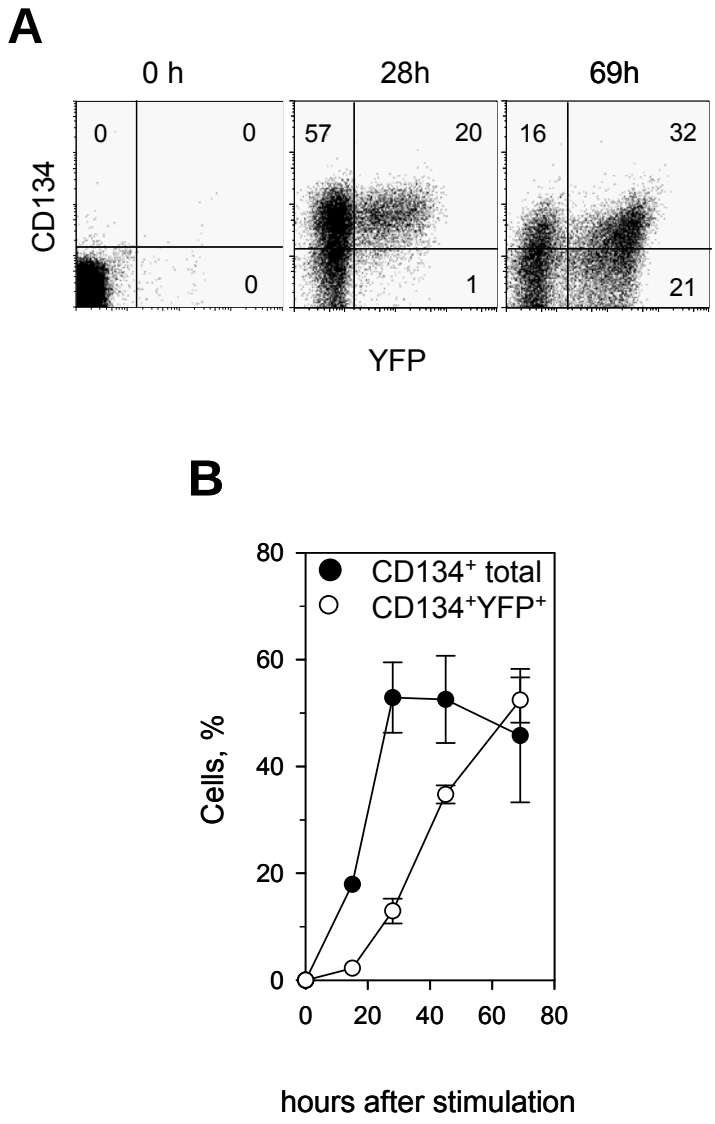


Figure 11. Kinetics of YPF and CD134 induction in CD4$^+$ T cells upon *in vitro* stimulation. (A) Flow cytometric example of YPF and CD134 expression in unstimulated cells (0h) or after *in vitro* stimulation of sorted naïve YFP$^-$ CD4$^+$ T cells from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice at the indicated time points after stimulation. Representative plots of 1 out of 3 separate experiments. **(B)** Kinetics of YPF and CD134 induction in CD4$^+$ T cells stimulated as in (A). The mean (±SEM) percentage CD134$^+$ and CD134$^+$YFP$^+$ cells in CD4$^+$ T cells from 4-6 mice from 3 separate experiments is shown.

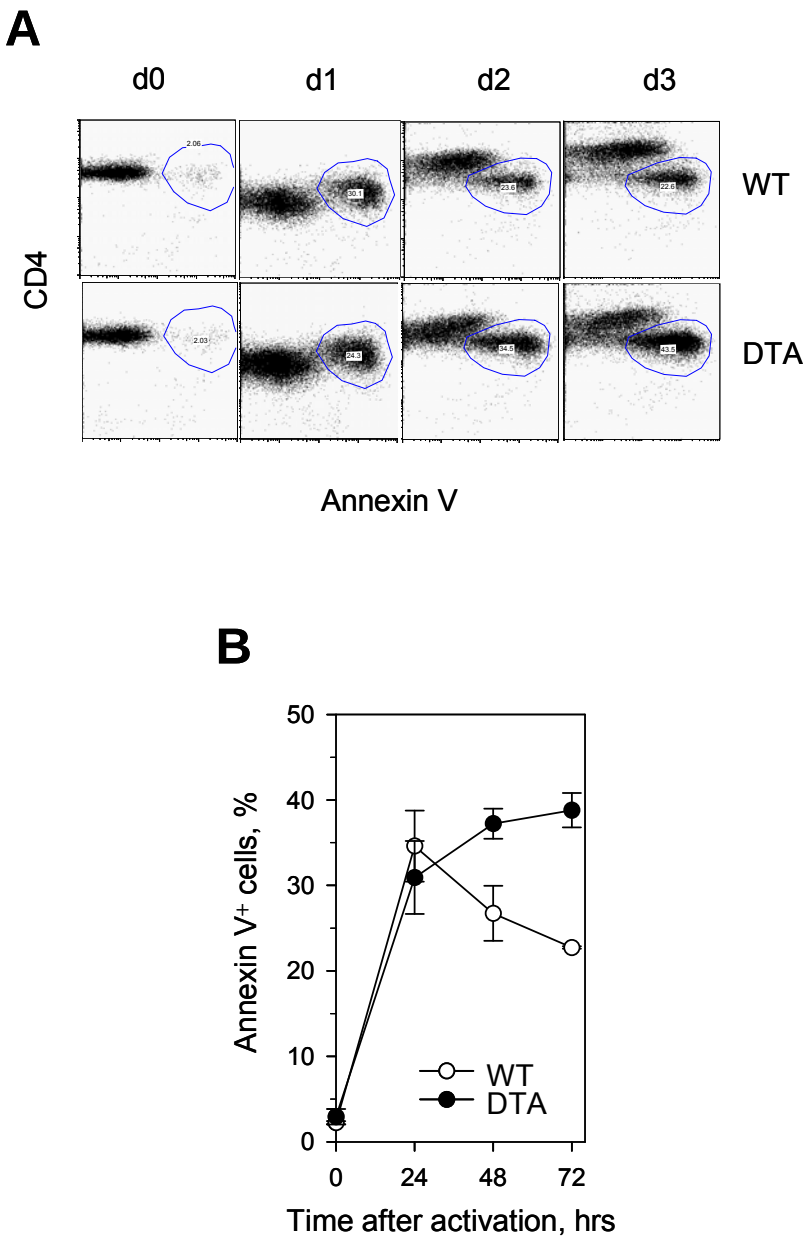


Figure 12. Kinetics of apoptosis induction in CD4$^+$ T cells by DTA expression upon *in vitro* stimulation. (A) Flow cytometric example of annexin V expression in unstimulated cells (d0) or following *in vitro* activation of sorted naïve CD4$^+$ T cells from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) or control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice at the indicated time points after stimulation. **(B)** Kinetics of apoptosis induction following in vitro activation as in (A). The mean (±SEM) percentage of annexin V$^+$ cells in CD4$^+$ T cells from 3-5 mice from 2 separate experiments is shown.

However, by day 2, we could see that DTA-originated cells had a higher percentage of Annexin V^+ cells, which increased only slightly by day 3. In contrast, cells of WT origin decreased their percentage of apoptotic cells in culture (**Fig. 12-A and 12-B**), which reflects the proliferation of viable cells in the well. We concluded that the effect of DTA activation on survival of *in vitro* activated naïve $CD4^+$ T cells from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice was not evident until the second day of culture.

In summary, DTA-mediated deletion is delayed by one day in relation to YFP induction. This has implications for the interpretation of the analysis of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/Dta}$ mice, because the percentage of YFP cells could reveal cells on their way to die.

Overall, we can conclude by the analysis of the two reporter lines described that in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice a majority of memory and regulatory cells are deleted. The fact that this deletion might not be absolute is not an obstacle. On the contrary, it makes the system more physiological, given that in *in vivo* infections, viruses only kill a proportion of these cells.

3.3. Immunological consequences of DTA-mediated deletion of CD134^{+}CD4^{+} T cells

The *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice are fertile and do not display any gross physical or behavioural abnormalities [1]. Upon macroscopic examination we observed significant lymph node enlargement in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice, in comparison with control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ mice (**Fig. 13**). Spleen size was not significantly different between the two groups. Microscopic examination of many non lymphoid tissues, including skin, kidney, lung, brain, salivary glands, stomach, liver, gall bladder, pancreas, small and large intestines showed no abnormalities, although mild lymphocytic infiltrates were sometimes observed in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. Lymph nodes and spleen had normal follicular structure with active germinal centres and were architecturally indistinguishable from control mice (not shown).

To see if the lymphoproliferation observed in lymph nodes was associated with a generalized proinflammatory status, we looked at serum levels of a panel of cytokines and chemokines by using a multiplex cytokine bead array. Indeed, we observed elevated serum levels of various proinflammatory cytokines (IL-1β, IL-5, Il-12p40, IL-13, IFNγ, TNFα and GM-CSF) and chemokines (IP-10, MIG, Eotaxin, MCP-1 and MIP-1α (**Fig. 14**).

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[1] Occasionally, a very small proportion of mice showed retarded development already evident at weaning. These mice were excluded from further analysis. Pathology analysis was done in several organs from 3 young littermate mice of very small size and revealed exocrine pancreatic hypoplasia in all 3 mice but unremarkable remaining organs including endocrine pancreas. The exocrine pancreas produces and secretes the digestive enzymes necessary for digestion which can explain the animals' small size. The cause of occasional developmental retardation in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice has not been further investigated.

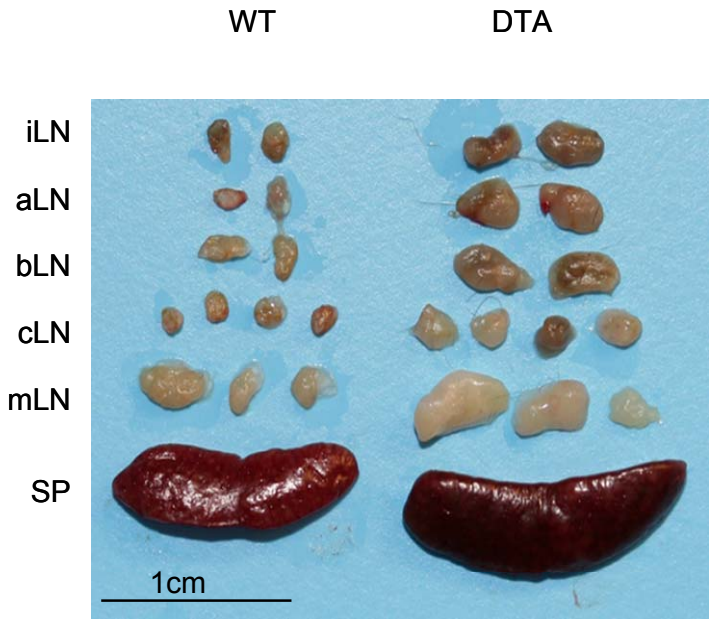

Figure 13. Macroscopic examination of lymphoid tissue reveals lymphoadenopathy in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. Size of inguinal (i), axillary (a), brachial (b), cervical (c), mesenteric (m) lymph nodes and spleen from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice is shown.

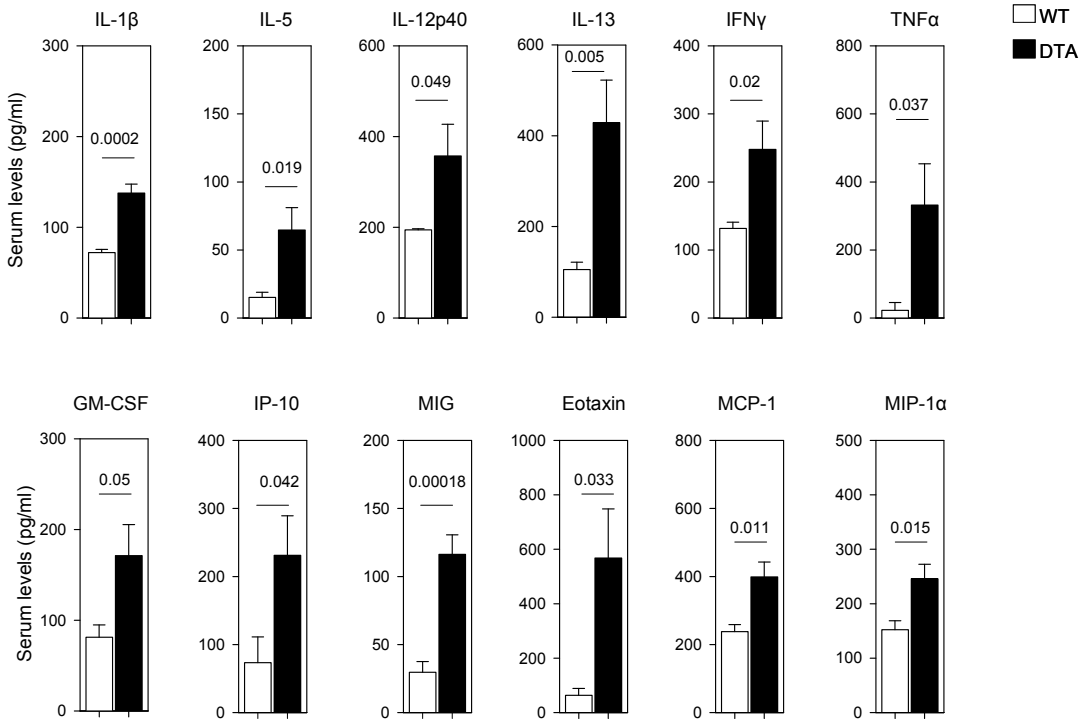

Figure 14. Increased levels of serum proinflammatory chemokines and cytokines in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. Serum levels of the cytokines IL-1β, IL-5, IL-12p40, IL-13, IFNγ, TNFα and GM-CSF and of the chemokines IP-10 (CXCL10), MIG (CXCL9), Eotaxin (CCL11), MCP-1 (CCL2) and MIP-1α (CCL3) in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice. Values are from two separate experiments and represent the mean (±SEM) of 5 or 7 mice per group, depending on the experiment.

To determine whether activated $CD4^+$ T cell deletion had any impact on lymphocyte population dynamics we compared lymphoid organ cellularity and composition in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ and control mice (**Fig. 15**). Consistent with the enlargement observed macroscopically, we saw an increase in absolute number of cells in lymph nodes. Spleen cellularity was not significantly different between the two groups (**Fig. 15-B**). To account for differential distribution between enlarged lymph nodes and spleen we calculated the sum of lymphocyte and myeloid populations in both organs. B cells, but not T cells or myeloid cells, were more numerous in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice than in control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ mice and lymph node B cells were primarily responsible for this increase (**Fig. 15-B**). An increase in lymph node T cells and myeloid cells was also observed, in proportion to the increase in total lymph node cellularity (**Fig. 15-B**).

Remarkably, total $CD4^+$ T cell numbers were only marginally reduced (**Fig. 16-A**), while total $CD8^+$ T cell numbers were elevated in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice, compared with *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ mice (**Fig. 16-A**). This became statistically significant when plotted as CD4:CD8 ratio (**Fig. 16-B**). This ratio is highly conserved between animals of the same inbred strain and shows effects masked by animal to animal variation.

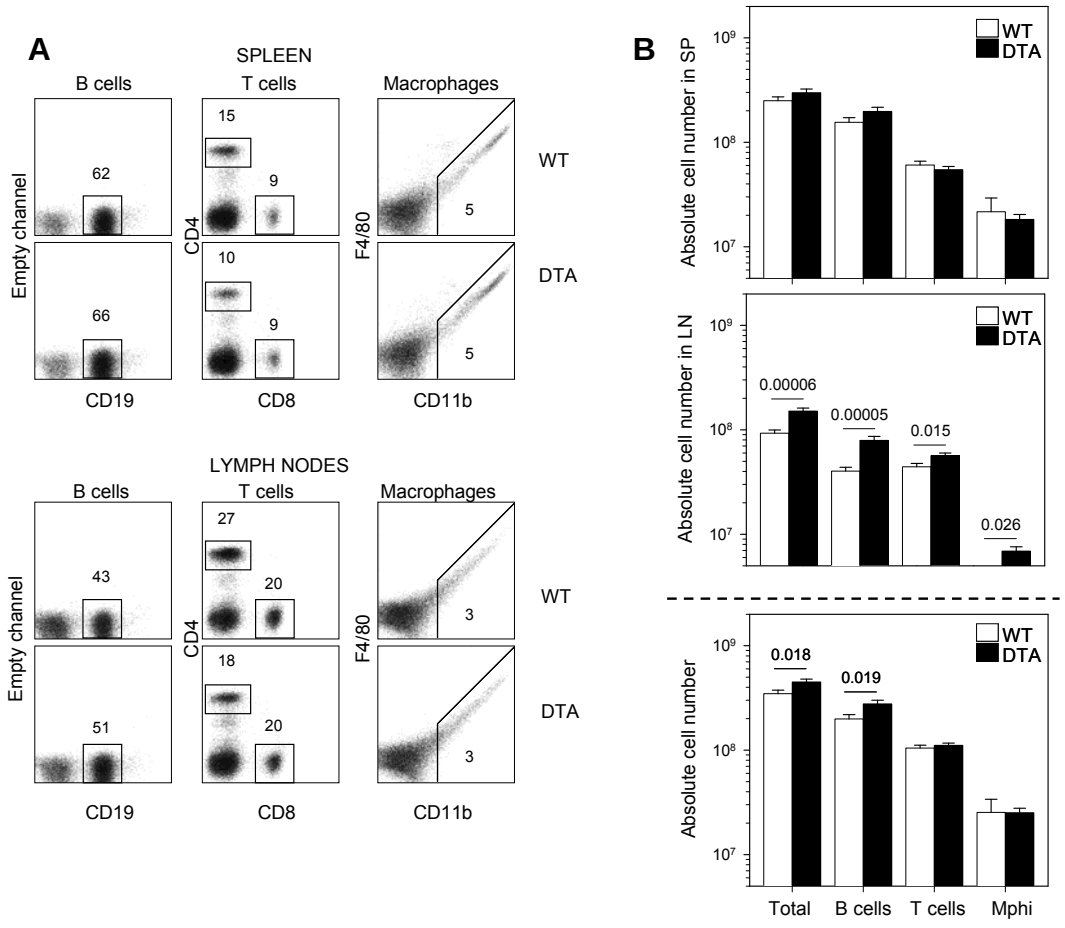

Figure 15. Lymphoid organ cellularity and composition in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. (A) Representative FACS plots showing gating strategy for analysis of B cells, T cells and macrophages in spleen (upper panel) and lymph nodes (lower panel) in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice. Numbers represent percentage of cells in gate. **(B)** Total cellularity and numbers of CD19^{+} (B cells), CD4^{+} and CD8^{+} (T cells) and CD11b^{+}F4/80^{+} (Mphi, macrophages) in the same mice, in spleen and lymph nodes in separate and pooled. Values represent the mean (±SEM) of 19-21 mice per group.

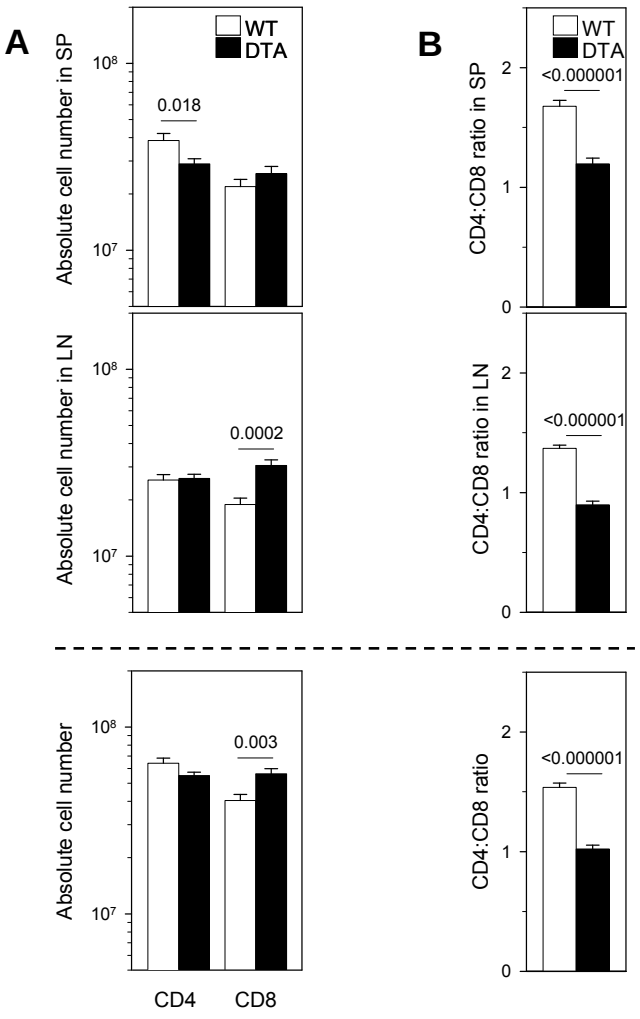

Figure 16. Decrease of CD4:CD8 ratio in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. Numbers of CD4^{+} and CD8^{+} T cells (**A**) and CD4:CD8 ratio (**B**) in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice in spleen and lymph nodes in separate and pooled. Values represent the mean (±SEM) of 19-21 mice per group.

In order to investigate whether there was any fluctuation of numbers over time, we analysed mice of different ages. We did not observe any variation throughout a 6-month observation period both in numbers of total $CD4^+$ (**Fig. 17-A**) and total $CD8^+$ (**Fig. 17-B**) T cell numbers, resulting in a stable CD4:CD8 ratio throughout the indicated period (**Fig. 17-C**) suggesting that total numbers were maintained irrespective of thymic output.

In summary, deletion of activated $CD4^+$ T cells resulted in a lymphoproliferative condition accompanied by a substantial systemic drop in CD4:CD8 ratio, mainly due to a paradoxical increase of $CD8^+$ T cell numbers and, to a lesser extend, reduction in $CD4^+$ T cell numbers.

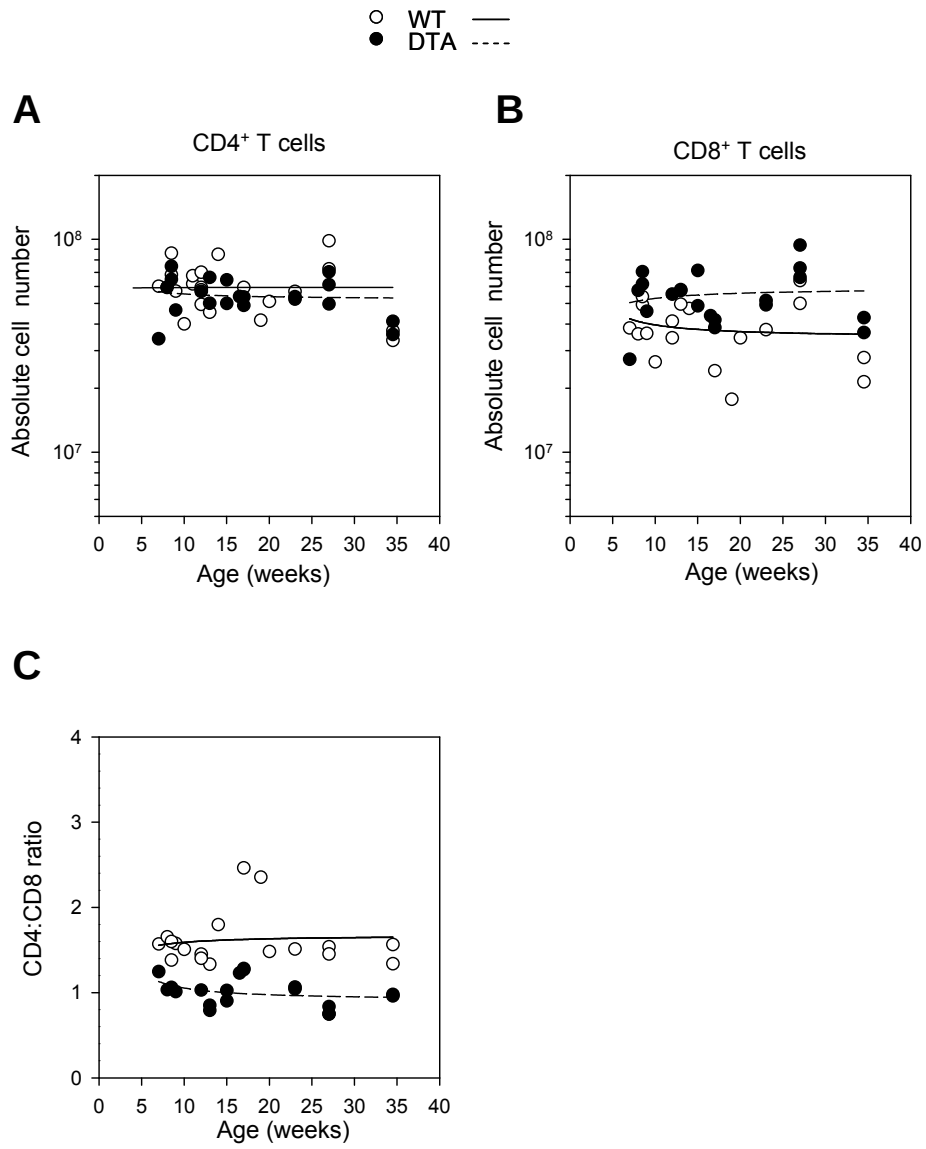

Figure 17. T cell cellularity in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice over time. Total numbers of CD4$^+$ **(A)**, CD8$^+$ T cells **(B)** or CD4:CD8 ratio **(C)** in pooled lymph nodes and spleen of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice at the indicated times after birth. Each symbol represents an individual mouse. Lines represent hyperbolic regression (DTA: dashed line, WT: solid line).

3.3.1. Effect of CD134^{+} CD4^{+} T cell-specific DTA induction on CD4^{+} T cell homeostasis

To assess whether activated CD4^{+} T cell numbers were selectively reduced in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice, we measured the numbers and proportion of naïve, memory and regulatory CD4^{+} T cells. Numbers of naïve CD4^{+} T cells were similar between *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ mice (**Fig. 18-A and 18-B**). Notably, numbers of memory CD4^{+} T cells were unchanged in the spleen and even elevated in the lymph nodes of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice, in comparison with control mice (**Fig. 18-A and 18-B**). Lastly, numbers of regulatory CD4^{+} T cells were reduced in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice by ~ 40%, compared with those in control mice (**Fig. 18-A and 18-B**).

Regulatory CD4^{+} T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice, identified by expression of CD25, expressed atypically high levels of CD44 (**Fig. 18-A**). CD25 is a marker that can be upregulated upon activation suggesting that these cells could be effectors rather than regulatory CD4^{+} T cells. To exclude this possibility, we further characterized phenotypically this cell population. Regulatory T cells are characterized by the expression of the FoxP3 transcription factor (Hori et al., 2003; Fontenot et al., 2003; Khattri et al., 2003) and lower levels of the surface molecule CD4 (Sakaguchi et al., 2006; Fontenot et al., 2003; Khattri et al., 2003). We have thus analysed these parameters in the CD4^{+}CD25^{+} population of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. Similarly to regulatory CD4^{+} T cells from normal mice, these cells expressed low levels of CD4 and homogeneously expressed the regulatory T cell-specific FoxP3 transcription factor (**Fig. 19-A and 19-B**).

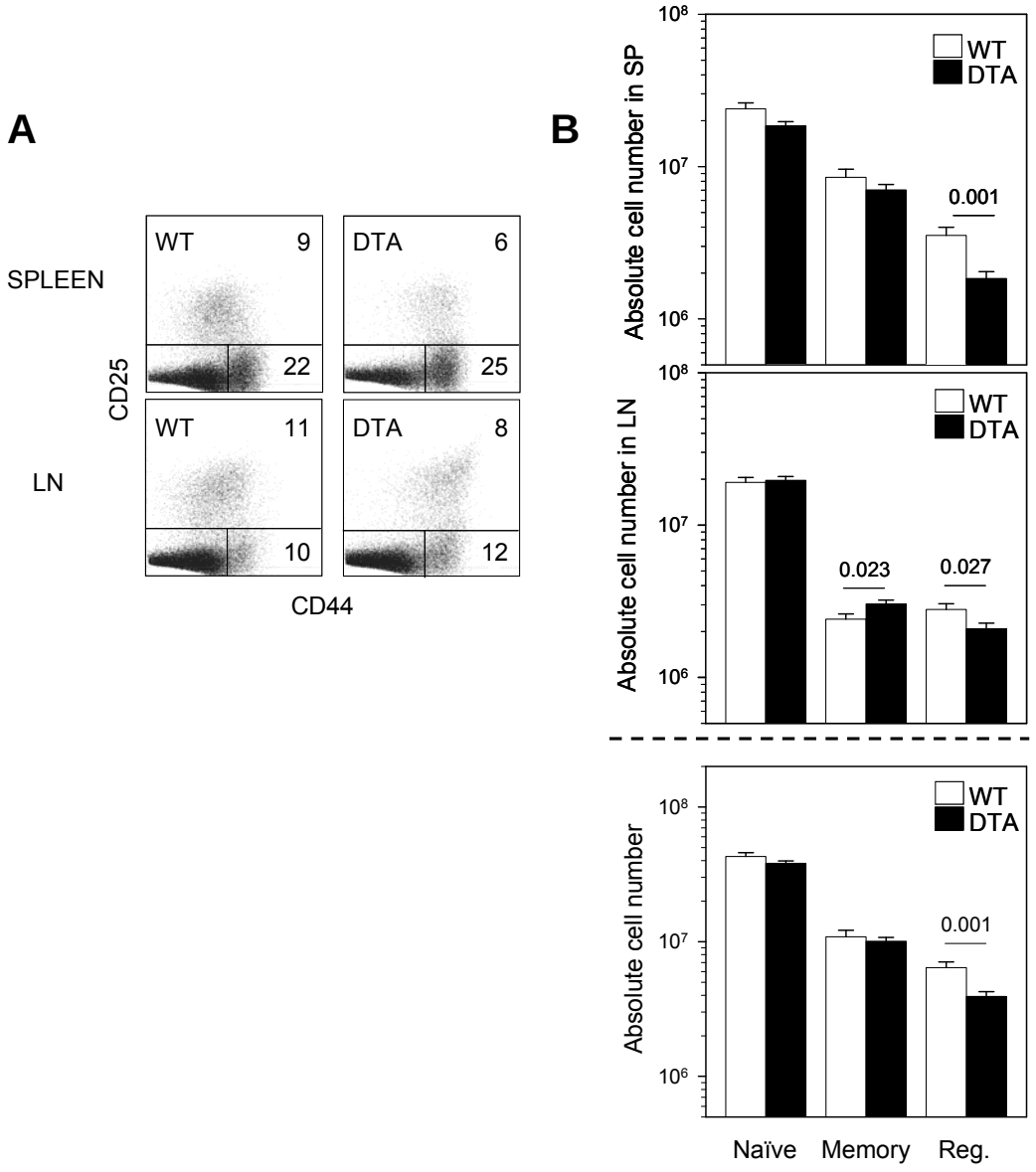

Figure 18. Cellularity of CD4$^+$ T cell subsets in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. (A) Flow cytometric profile and **(B)** absolute numbers of naïve, memory and regulatory (reg.) CD4$^+$ T cells from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice in spleen and lymph nodes, in separate and pooled. Values represent the mean (±SEM) of 15-18 mice per group.

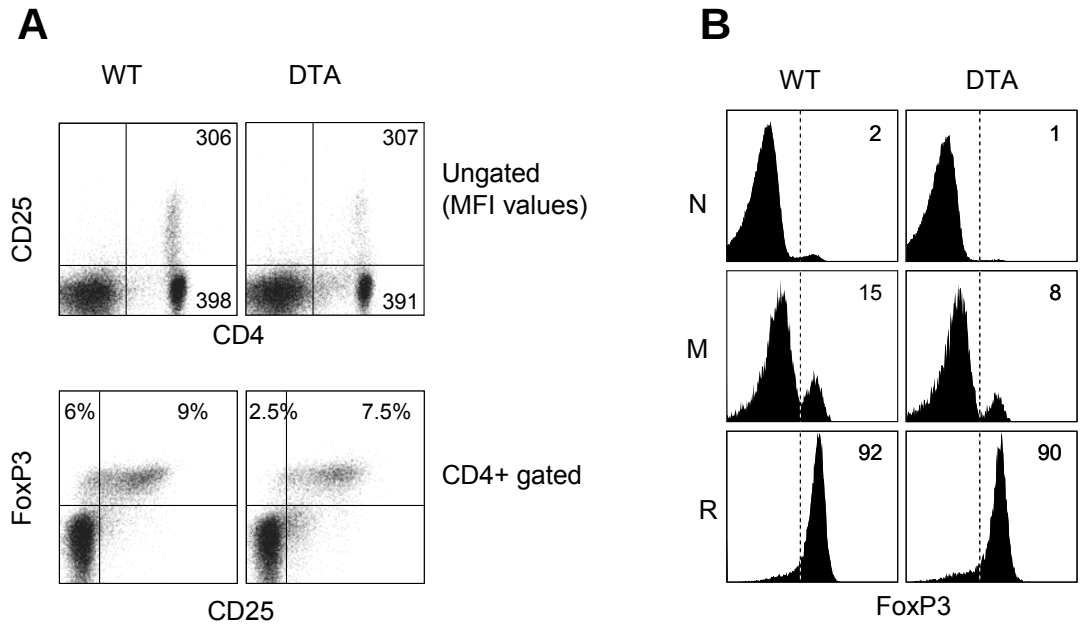

Figure 19. FoxP3 expression in CD4$^+$ T cells from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. (A) Illustrative FACS plot of CD4 and CD25 expression in splenocytes gate. Numbers indicate the median fluorescence intensity (MFI) value for CD4 staining in quadrants (top). Illustrative FACS plot of FoxP3 intracellular staining in CD4$^+$ T cell gate. Numbers indicate percentage of FoxP3 expressing cells in quadrants (bottom). **(B)** Flow cytometry histograms of FoxP3 expression in naïve (N), memory (M) and regulatory (R) CD4$^+$ T cells. Numbers indicate percentage of FoxP3$^+$ cells.

We further analyzed their phenotypic differences from memory $CD4^+$ T cells (**Fig. 20-B and 20-C**). Resting regulatory $CD4^+$ T cells are characterized by intermediate CD44 levels and the majority show high CD62L expression (Fisson et al., 2003). We have looked at these and other activation markers in the $CD4^+CD25^+$ population of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. In contrast to regulatory $CD4^+$ T cells from control mice, those from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice displayed a highly activated phenotype, characterised by down-regulation of CD62L and up-regulation of CD43, CD49b, CD103 and CD127. This activation is even more pronounced than in the 'memory' regulatory population from control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ control mice (**Fig. 21**).

Differences between memory $CD4^+$ T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ and those in control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ mice were unremarkable with only modest increases in expression of CD43 and CD49b activation markers (**Fig. 20-B**). Regulatory T cells, in contrary to effector cells, are not expected to produce proinflammatory cytokines (Sakaguchi et al., 2006). We could hardly detect production of TNFα and IFNγ (**Fig. 20-C**) and IL-2, IL-4, IL-10 and IL-17 (not shown) in this population. Memory cells, on the other hand, produced more IFNγ than controls, maybe reflecting their activation status (**Fig. 20-B**).

In order to investigate the proliferative status of these cell populations, we analysed BrdU incorporation over a 6 days labelling period. Regulatory $CD4^+$ T cells showed an increased turnover in lymph nodes and spleen, whereas memory $CD4^+$ T cells in both organs were not significantly different when compared to controls (**Fig. 22-B, 22-C and 22-D**).

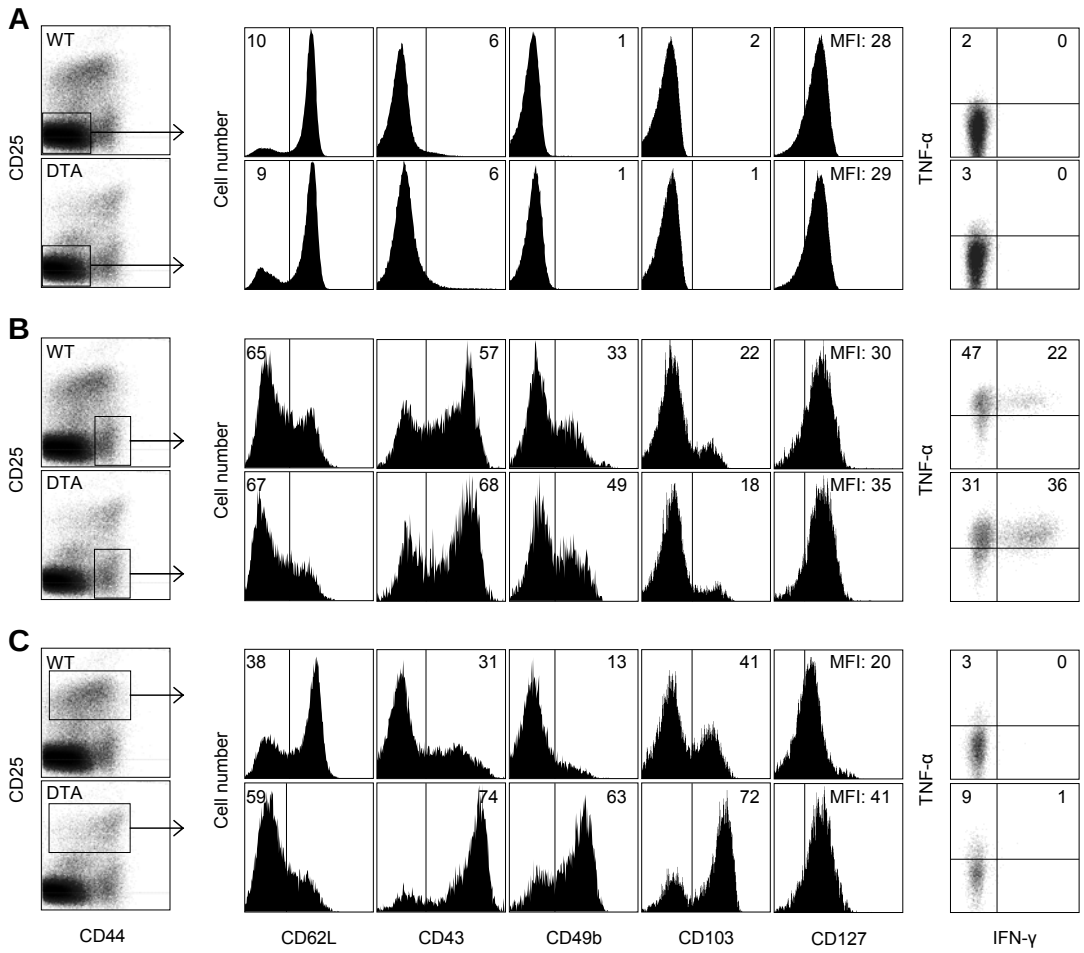

Figure 20. Phenotype of CD4$^+$ T cell subsets from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. Expression of activation markers and production of cytokines following *in vitro* restimulation in naïve **(A)**, memory **(B)** and regulatory **(C)** CD4$^+$ T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice and littermate controls. Numbers within the plots represent the percentage of CD4$^+$ T cells which are positive for each marker (or negative in case of CD62L). Specifically in the CD127 staining, numbers indicated the median fluorescence intensity (MFI). Plots are representative of 4-7 mice per group.

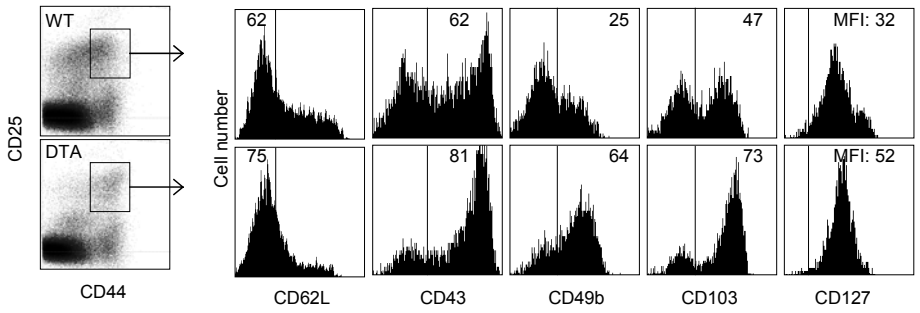

Figure 21. $CD4^{+}CD25^{+}CD44^{bright}$ regulatory T cells from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice are more activated than those of control mice. Expression of activation markers was analysed separately on $CD4^{+}CD25^{+}CD44^{bright}$ regulatory T cells. Numbers within the plots represent the percentage of cells that are positive for each marker (or negative in case of CD62L). Specifically for the CD127 staining numbers indicated the median fluorescence intensity (MFI). Plots are representative of 4-7 mice per group.

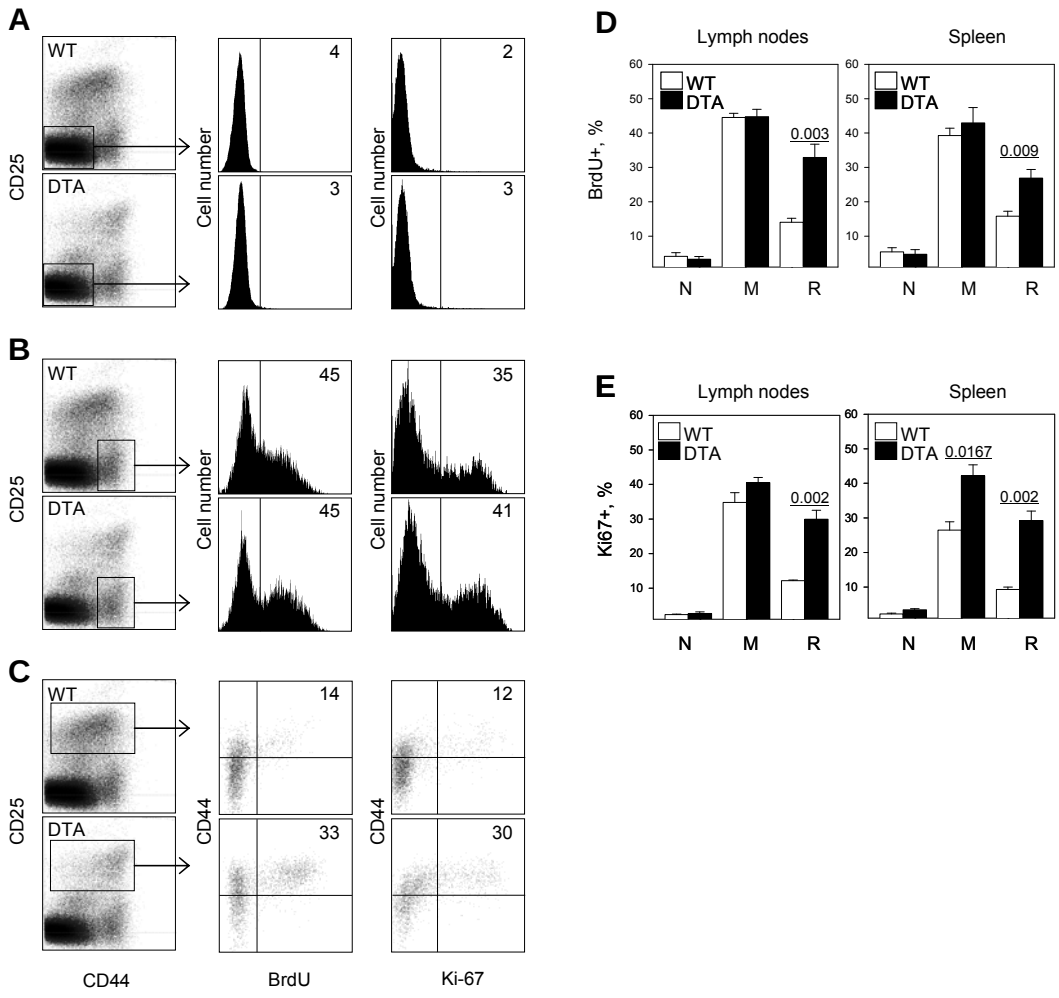

Figure 22. Kinetics of CD4$^+$ T cell subsets in *Tnfrsf4$^{Cre/+}$ R26$^{Dta/+}$* mice. Levels of BrdU incorporation after a 6-day administration period and expression of Ki67 nuclear antigen on naïve (**A**), memory (**B**) and regulatory (**C**) CD4$^+$ T cells from *Tnfrsf4$^{Cre/+}$ R26$^{Dta/+}$* (DTA) and control *Tnfrsf4$^{Cre/+}$ R26$^{+/+}$* (WT) mice. BrdU$^+$ cells were gated according to staining levels in untreated mice. Ki67$^+$ cells were gated on MIF obtained with isotypic control. Each plot is representative of 6 mice (2 independent experiments) for BrdU and 3 mice for Ki67 expression. Frequencies of BrdU$^+$ (**D**) and Ki67$^+$ (**E**) cells in lymph nodes and spleen. Values are the mean of 6 (**D**) and 3 (**E**) mice per group.

To investigate if the apparent lack of accelerated turnover in the memory population was due to killing of dividing cells, cell division was analysed by the expression of Ki67 cell cycle antigen which gives a snapshot of cell turnover. Memory $CD4^+$ T cells of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice contained more Ki67+ cells then control mice, although the difference was only significant in the spleen (**Fig. 22-B and 22-E**). Regulatory $CD4^+$ T cells had considerably higher frequencies of dividing cells when compared to controls in both organs (**Fig. 22-C and 22-E**).

In conclusion, despite higher expression of CD44, $CD25^+CD4^+$ T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice were similar to regulatory $CD25^+CD4^+$ T cells in control mice but were more activated and had higher turnover rate. Furthermore, DTA-mediated destruction of activated $CD4^+$ T cells had a significant effect on regulatory $CD4^+$ T cell numbers and activation state, but little apparent effect on memory $CD4^+$ T cells.

Memory $CD4^+$ T cells were deleted by DTA expression (by extrapolation of observations with *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice) as efficiently as regulatory $CD4^+$ T cells; one would therefore expect their numbers to be decreased but we observed the opposite. Memory $CD4^+$ T cells, under physiological conditions, display higher turnover rate, self-renewal potential and activation profile than either naïve or regulatory $CD4^+$ T cells, which could compensate for or mask DTA-mediated loss. To reveal the full extent of memory $CD4^+$ T cell killing in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice we set up mixed bone marrow (BM) chimeras.

Rag-deficient mice produce no mature B or T cells due to the deficiency in recombination activating gene (RAG), the enzyme necessary for antigen receptor gene rearrangement, which does not affect the development of other cell lineages. In theory, when bone marrow cells from $Rag^{+/+}$ mice are transferred into $Rag^{-/-}$ recipients, lymphocyte precursors from both types of mice will have to compete with each other until the developmental stage at which RAG enzymes are expressed which is double negative 4 (DN4) stage – very early in development of these cells (Shinkai et al., 1992). Afterwards, Rag-sufficient cells have no competition and should be able to develop normally. For all other cell lineages, the transferred RAG-sufficient cells face a full host and therefore will have a competitive disadvantage and will not be reconstituted. Large numbers of BM cells (~$20x10^6$ cells) were transferred into non-irradiated *Rag2*-deficient mice which were killed after 17 weeks and number of cells recovered calculated (**Fig. 23-A**). Donor and host-origin cells were discriminated by expression of CD45.2 and CD45.1 congenic markers, respectively. As shown in **Fig. 23-B and 23-C**, reconstitution occurred exclusively in lymphoid lineages but not in myeloid lineages.

Thus, although irradiation is common practice in bone marrow transplantation experiments, we show here that it is not necessary and that transferring high numbers of BM cells and giving enough time for reconstitution results in exclusive expansion of lymphocytes. The experiments described below were thus performed in non-irradiated hosts.

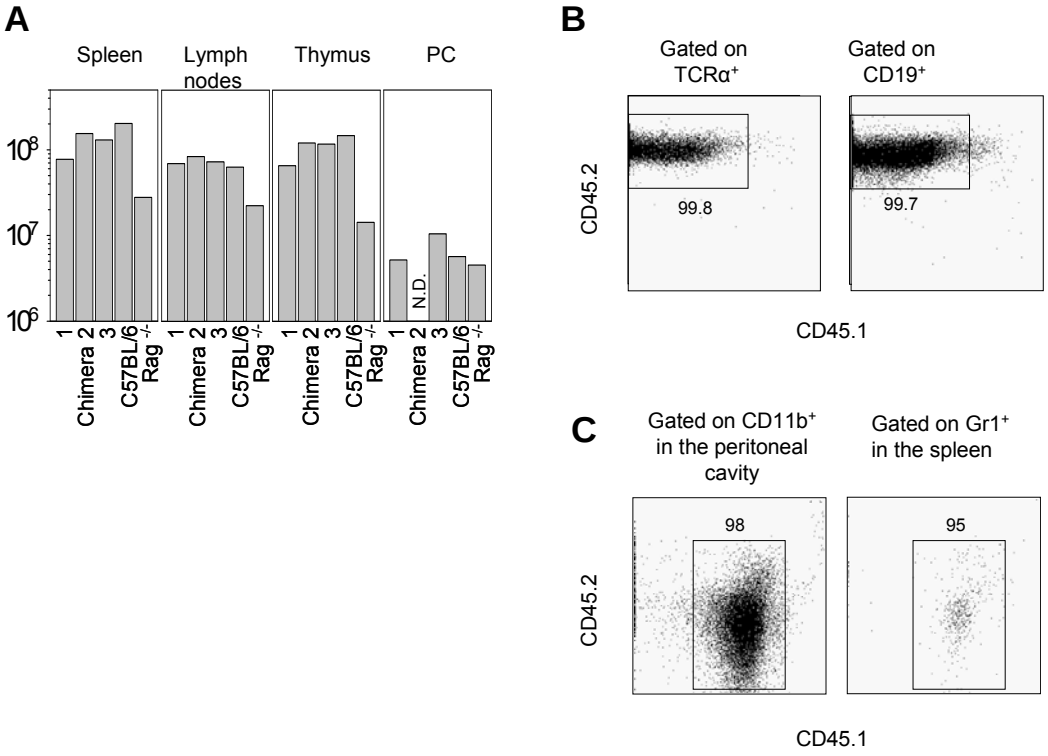

Figure 23. Exclusive reconstitution of lymphoid lineages in non-irradiated *Rag*-deficient recipients reconstituted with WT bone marrow. (**A**) Total cell numbers recovered from the spleen, lymph nodes, thymus and peritoneal cavity (PC) of CD45.1 *Rag2*-deficient recipients 16 weeks after reconstitution with $20x10^6$ BM cells from B6 mice. B6 and *Rag1*$^{-/-}$ cell counts are shown for comparison. Flow cytometry plots showing reconstitution of lymphoid lineages (**B**) but not of myeloid lineages (**C**) in same chimeras as in A. Plots are representative of 3 mice. N.D: not done.

To address the issue of memory $CD4^+$ T cell killing in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice, BM cells from CD45.1 wild-type and CD45.2 *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice, mixed or on their own, were transferred into non-irradiated CD45.2 *Rag1*$^{-/-}$ hosts. Reconstitution was monitored in the blood and twelve weeks post transfer mice were killed and analysed. Compared with recipients reconstituted with wild-type BM alone, those reconstituted with *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ BM alone showed a paradoxical increase in activated $CD43^+$ $CD44^+$ $CD4^+$ T cells and a small reduction in regulatory $CD25^+CD4^+$ T cells (**Fig. 24-B**). This is reminiscent of the phenotype observed in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. For the analysis of mixed BM chimeras, we assessed the proportion of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ -derived cells by the lack of expression of CD45.1 marker. We extrapolated the ratio of injection/efficiency of reconstitution by the *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$: wild-type ratio in the thymus or in peripheral B cells and $CD8^+$ T cells (which should not be affected). As shown in **Fig. 24-A**, ~35% of cells in thymus and peripheral B cells and CD8 cells are of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ origin and ~65% are of WT origin. Notably, we observed a significant selective loss of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$-origin memory and regulatory $CD4^+$ T cells, but not in the naïve pool, in which both *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$-origin and WT –origin contributed in the expected proportion (**Fig. 24-A**).

Thus, by co-transferring *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ and WT cells in the same host, we were able to reveal the full extend of the DTA-mediated deletion that occurs preferentially in memory and regulatory $CD4^+$ T cells.

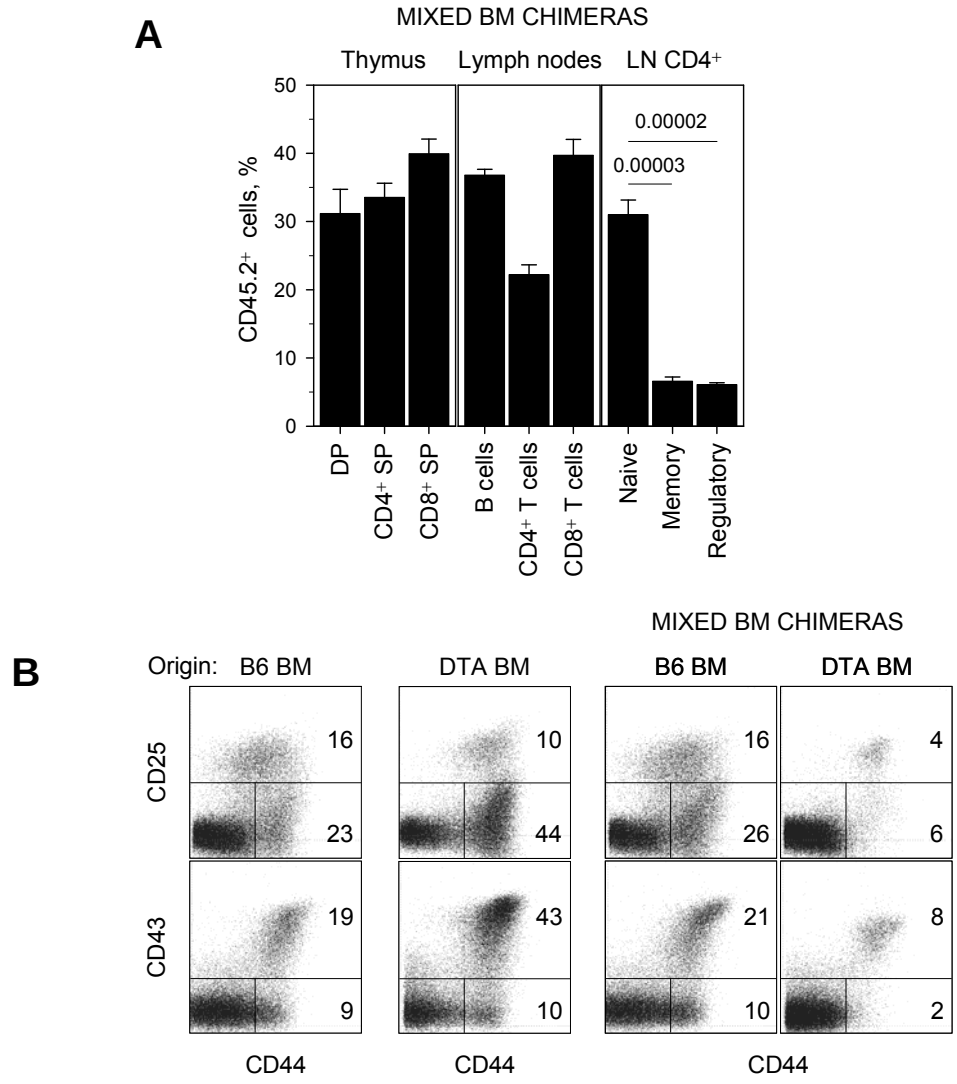

Figure 24. Loss of memory and regulatory CD4$^+$ T cells of DTA origin revealed in mixed bone marrow chimeras. CD45.2$^+$ *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and CD45.1$^+$ C57BL/6 (B6) wild-type (B6) bone marrow (BM) cells were injected separately or mixed together (mixed BM chimeras) into non-irradiated *Rag1*$^{-/-}$ recipients and lymphoid organs were analyzed 12 weeks later. **(A)** Mean (±SEM) percentage of DTA-origin (CD45.2$^+$) cells in thymocyte and peripheral lymphocyte subsets in mixed BM chimeras. **(B)** Expression of CD25, CD43 and CD44 on gated CD4$^+$ T cells from these chimeras is shown. Numbers represent percentage of cells within the quadrants. Values are representative of 4-8 mice analyzed in 2 independent experiments.

Overall, our results showed that while both memory and regulatory $CD4^+$ T cell subsets were being killed with similar efficiency by DTA-activation, regulatory $CD4^+$ T cells were incompletely replenished while the loss of memory $CD4^+$ T cells was overcompensated.

The result obtained with the *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ BM chimeras has also excluded a potential role for ectopic or leaky expression of Cre or DTA in the phenotype of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. Indeed, lymphocytes alone were able to recapitulate the homeostatic features observed in unmanipulated *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice.

3.3.2. Partial CD4$^+$ T helper cell immune deficiency in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice

To investigate whether the accelerated death and replenishment of effector/memory CD4$^+$ T cells compromised immune competence, we evaluated CD4$^+$ T cell function in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice in a number of infections.

3.3.2.1. Immune response of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice to a chronic viral infection

Friend virus (FV) is a murine retrovirus that causes persistent infection in resistant strains of mice. It preferentially infects erythroid precursor cells (Ter119$^+$ cells) but can also infect B cells and macrophages. CD4$^+$ T cells have been shown to play an indispensable role in the control of chronic infection with FV (Super et al., 1998). We have experimentally infected *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ and control mice with FV and evaluated their ability to produce FV-specific neutralizing antibodies as an indirect measure of helper T cell function. In comparison to *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ littermates and B6 control mice, which showed a strong nAb response to and effectively contained infection with FV, the FV-specific nAb response was undetectable in 4 out of 7 *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice and was significantly delayed in the rest (**Fig. 25-A**). Cell-associated virus can be estimated by flow cytometric detection of infected cells using surface staining for the glycosylated product of the viral *gag* gene (glyco-Gag).

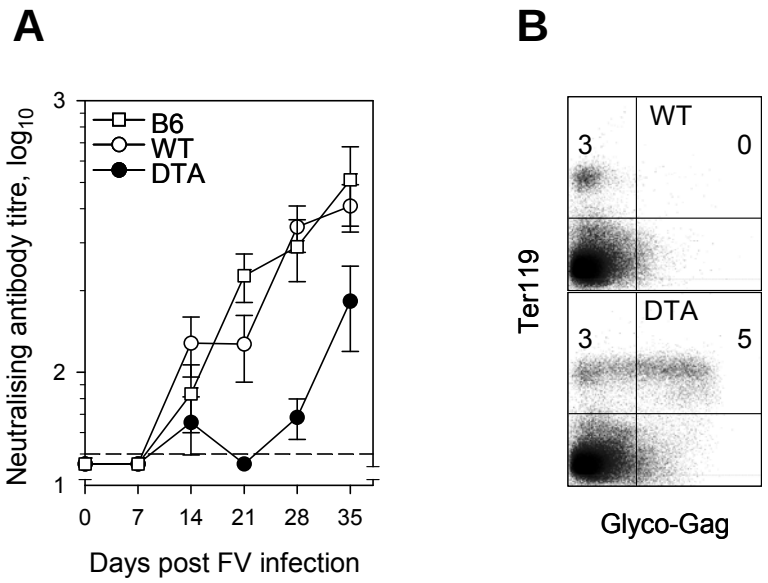

Figure 25. Impaired immune response to chronic viral infection in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and littermate control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) and B6 (B6) mice were infected with Friend virus (FV). **(A)** Serum titers of virus-neutralizing antibodies were measured at indicated time points. Values are the mean (±SEM) of 5-7 mice tested in 2 independent experiments. $P \leq 0.018$ and $P \leq 0.007$ on days 21 and 28, respectively, between DTA mice and either WT or B6 mice. **(B)** Cell-associated FV loads were determined 15 weeks after infection by staining for FV-encoded glyco-Gag on the surface of infected erythroid precursor (Ter119$^+$) cells. Numbers within quadrants represent the percentage of positive cells. Glyco-Gag$^+$ Ter119$^+$ cells were detected in 4 out of 7 *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice and in 0 out of 6 *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ mice.

As shown in **Fig. 25-B,** infected cells could be detected in some *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice but not in control mice 15 weeks after infection. The same mice that were unable to mount a strong Ab response were the ones that had not cleared the infection.

In summary *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice had defective FV-specific nAb response which correlated with inability to control FV replication.

3.3.2.2. Immune response of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice to an acute viral infection

Chronic viral infections, by their nature, create a continuous strain on the immune system which could have potentiated the effect of the deletion of activated cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. We therefore assessed the response of these mice to an acute viral infection with the influenza A virus (IAV). $CD4^+$ T helper cell is crucial for the production of neutralizing antibodies by B cells in IAV infection (Gerhard et al., 1997) and *TCR*$\alpha^{-/-}$ mice, which lack T cells, are unable to produce high titres of nAb (**Fig. 26-A**). Titres of virus-neutralizing antibodies in the serum of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice were 2-fold and 2.7-fold reduced, compared with *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ littermates and B6 mice, respectively, 18 days following IAV infection (**Fig. 26-A**).

In IAV infection, production of nAbs is dependent on the provision of $CD4^+$ T helper cells. We therefore determined the number of $CD4^+$ T cells that were necessary to produce a titre equivalent to the one produced by *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. We transferred titrating amounts: 10%, 1%, 0.1% and 0.01% of total $CD4^+$ T cells in normal B6 mice, as described in materials and methods, into *TCR*$\alpha^{-/-}$ and infected them with IAV.

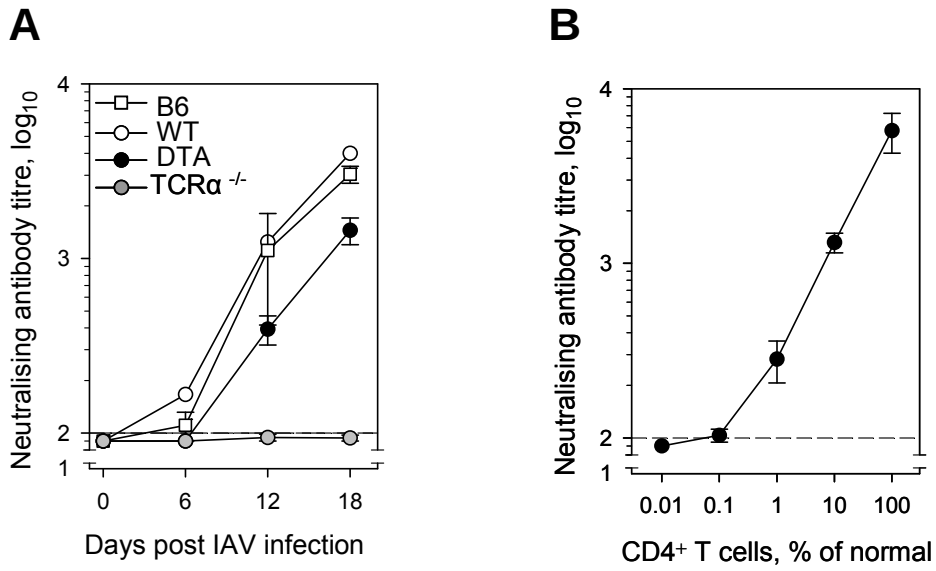

Figure 26. Impaired immune response to acute viral infection in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. (A) *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA), littermate controls *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT), B6 and TCRα$^{-/-}$ mice were infected with Influenza A virus (IAV). Serum titers of virus-neutralizing antibodies were measured at indicated time points. Values are the mean (±SEM) of 6-9 mice tested in 3 independent experiments. *P*=0.002 between DTA and WT mice and *P*=0.00003 between DTA and B6 mice on day 18. **(B)** Titers of IAV-neutralizing antibodies in the serum of T cell-deficient TCRα$^{-/-}$ mice, which received titrated numbers of CD4$^+$ T cells from wild-type B6 donor mice one day before IAV infection, are shown. Sera were tested 18 days post IAV infection. The X axis depicts the number of adoptively transferred CD4$^+$ T cells, on the day of the transfer, expressed as a percentage of the total number of CD4$^+$ T cells in wild-type B6 mice (100%). Symbols represent the mean (±SEM) of 3-4 mice.

Eighteen days later, we compared the nAb titres produced by each group receiving different numbers of $CD4^+$ T cells with the titres produced by *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice (**Fig. 26-A and 26-B**). This experiment revealed that the degree of reduction in IAV-specific nAb titers in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice corresponded to ~80% loss of $CD4^+$ T cells.

3.3.2.3. Immune response of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice to a fungal infection

In humans, *Pneumocystis carinii* pneumonia (PCP) is a common clinical problem in immune compromised individuals, including HIV patients and there is a clear correlation between $CD4^+$ T cell counts and the risk of PCP (Hanano and Kaufmann, 1998). In mice, *Pneumocyctis murina* (*P. murina*), an opportunistic pulmonary pathogen, can cause pneumonia in immune deficient mice with features resembling PCP in humans. Mutant mice deficient in lymphocytes (*Rag*$^{-/-}$), T cells (*TCRβ*$^{-/-}$) or $CD4^+$ T cells (MHC $II^{-/-}$) have been shown to be extremely susceptible to *P. murina* pneumonia (Hanano et al., 1996). In contrast, immunocompetent mice or mice deficient in $CD8^+$ T cells (*β2m*$^{-/-}$) are fully resistant to natural infection (Hanano et al., 1996), revealing the central role of $CD4^+$ T cells in controlling *P. murina* infection.

We compared the immune response of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice and *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ controls to experimental *P. murina* infection with respect to pulmonary inflammation and pathogen clearance. As positive and negative controls for the infection, we included MHC $II^{-/-}$ mice and B6 mice, respectively. Between week 10 and week 17 after infection, *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice failed to gain weight, while MHC $II^{-/-}$ mice had a more pronounced weight loss. Control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ and

B6 mice gained weight steadily during this period. Mice were killed and analysed 17 weeks post infection. Histologically, lung sections from B6 and *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ controls were unremarkable. All MHC II$^{-/-}$ mice analysed showed moderate to diffuse interstitial pneumonia with numerous intralesional organisms present whereas only one out of 4 *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice analysed showed a moderate multifocal interstitial pneumonia, without evidence of organisms present in H&E stain or Gomori's silver stain. PCR analysis was performed to detect numbers of organisms below histological detection levels. Using a standard PCR reaction, *Pneumocystis* specific DNA was detected exclusively in lung homogenates of MHC II$^{-/-}$ mice but not in other groups of mice. However a nested PCR reaction, setup with the first PCR product as template, revealed the presence of *Pneumocystis* in all *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ -origin DNA analysed. In summary, all four *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice were positive for *Pneumocystis* either by histology or by PCR.

Thus, even though *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice did not develop pneumonia as did MHC II$^{-/-}$mice (**Fig. 27-A**), they were not free of *P. murina* organisms (**Fig. 27-B**). This was in contrast to littermate controls and B6 mice, in which there was no evidence for the presence of *P. murina* organisms.

Taken together, these results showed that, despite the presence of normal numbers of CD4^{+} T cells, *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice were partially CD4^{+} T cell-immune deficient.

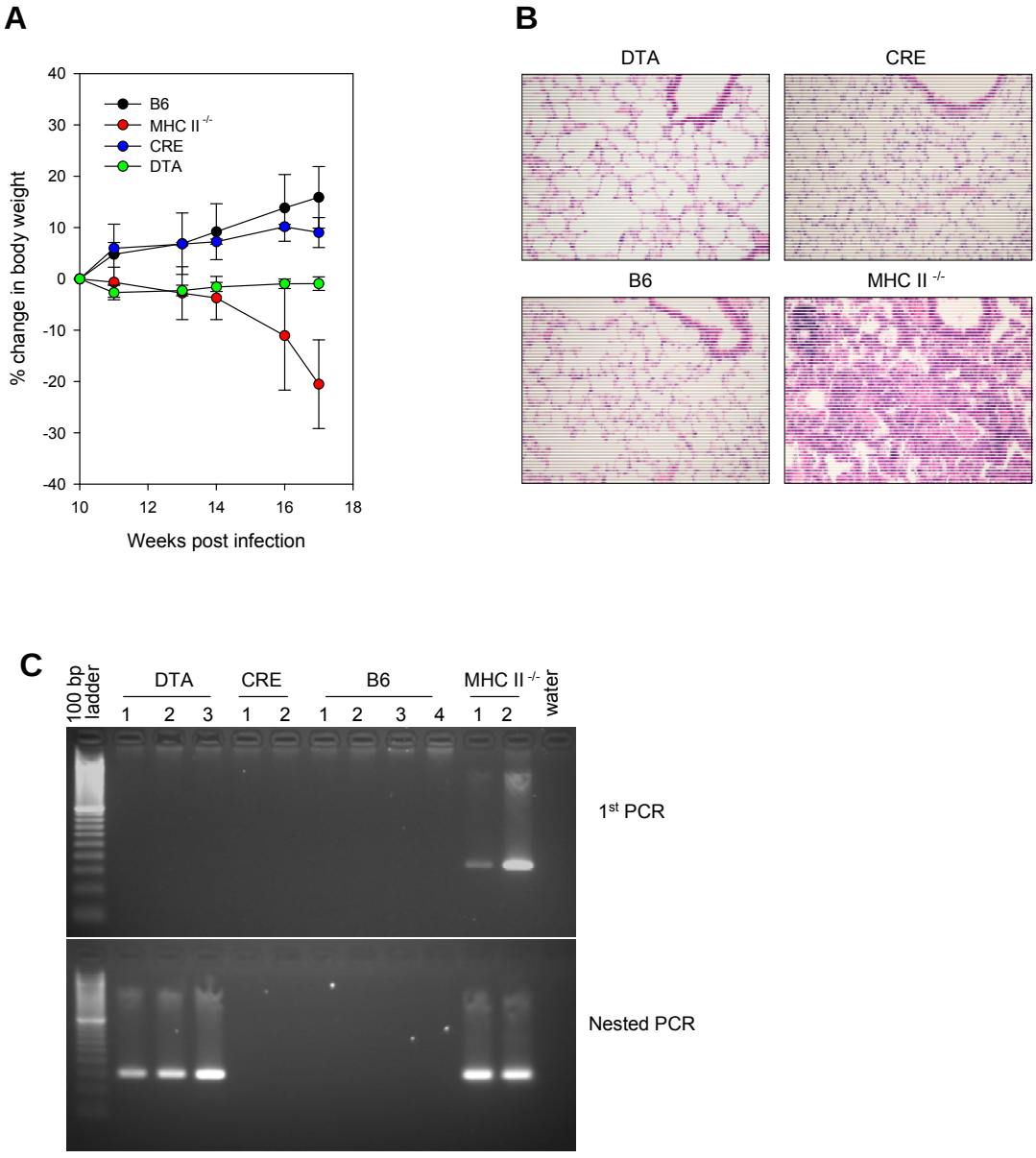

Figure 27. Immune response of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice to *Pneumocystis murina* infection. *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA), littermate control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (CRE), B6 and MHCII$^{-/-}$ mice were infected with *Pneumocystis murina*. (**A**) Body weight as percentage of change in relation to weight at 10 weeks post infection is shown (mean ±SEM). *P*=0.019 and *P*=0.033 between DTA and CRE or B6 mice, respectively, at week 17 (**B**) Representative lung histological sections after hematoxylin and eosin staining. 100x magnification. (**C**) Detection of *Pneumocystis murina* rRNA by standard and nested PCR.

3.3.3. Effect of DTA-mediated deletion of CD134^{+}CD4^{+} T cells on the homeostasis of B cells, macrophages and CD8^{+} T cells

3.3.3.1. B lymphocytes

HIV infection is associated with increased immune activation that affects all lymphocyte populations. Polyclonal hypergammaglobulinemia, defective humoral immunity, expansion in B-cell areas of lymphoid tissues and increased expression of activation, proliferation and differentiation markers are all features of B lymphocytes in HIV untreated individuals (Moir and Fauci, 2008).

We thus assessed whether B cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice showed signs of activation. We observed a comparable number of activated B cells (CD70^{+} cells) in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice and controls (data not shown) and both groups of mice expressed equivalent levels of MHC II as evaluated by MFI (data not shown), excluding B cell activation in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. Moreover, there was no detectable difference between serum levels of both IgM and IgG isotypes between both groups of mice (**Fig. 28-A**).

To determine whether the increased number of B cells observed in the lymph nodes of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice (**Fig. 15**) was associated with increased proliferation we measured BrdU incorporation. As shown in **Fig. 28-B**, the frequency of dividing B cells was similar in both groups. In addition, we did not observe any difference between the two groups with respect to the frequency of Ki67^{+} B cells (not shown).

As B cells were more numerous in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice than in controls, overall there was an increased number of dividing B cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice which however did not reach statistical significance.

To assess the composition of the B cell pool, we looked at germinal centre B cells. These are cells that proliferate rapidly and at the same time acquire the capacity to undergo somatic hypermutation and isotype switching. They can be phenotypically characterized as B220^{+} CD19^{+} IgD^{-} CD38^{low} GL7^{+}. *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice had a higher frequency of these cells (**Fig. 28-C**) and higher numbers (**Fig. 28-D**) in the lymph nodes but not in spleen.

In summary, immune activation in the B cell compartment of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice seemed to affect exclusively germinal centre B cells, but did not seem to impact on the production of immunoglobulins nor on the cell division rate of B cells.

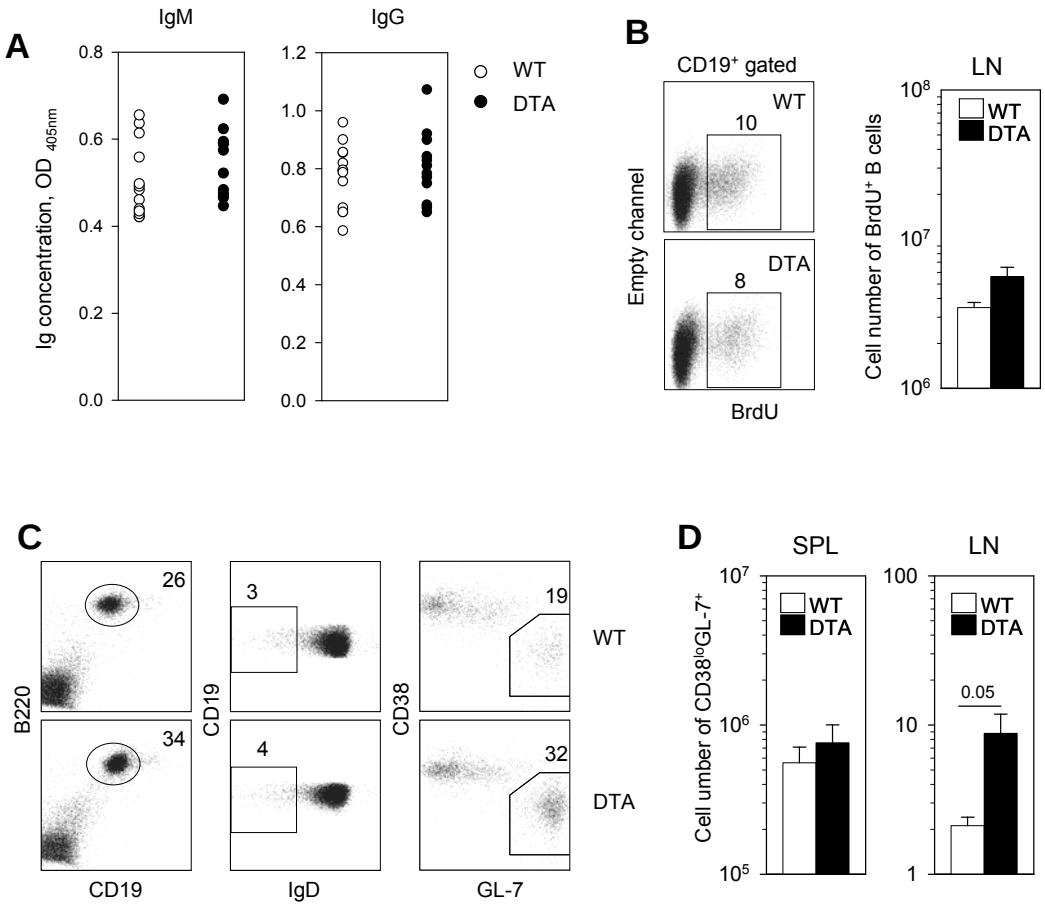

Figure 28. Analysis of B cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. (**A**) Serum levels of IgM and IgG in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice. Each symbol represents an individual mouse. (**B**) Frequency and absolute numbers of BrdU^{+} cells in lymph nodes from DTA and WT mice. (**C**) Flow cytometry of lymph node cells showing gating strategy to analyze germinal centre (CD38^{low} GL7^{+}) B cells. IgD^{-} cells gated on B220^{+}CD19^{+} were analyzed for cell expression of CD38 and GL7 (left). (**D**) Absolute numbers of germinal centre B cells in spleen and lymph node cells in DTA and WT mice. Numbers within the plots in (B and C) denote the percentage of positive cells in indicated gates and values in graphs (B and D) represent the mean (±SEM) of 4 mice or 6 mice per group, respectively.

3.3.3.2. Macrophages

Although the number of macrophages in lymph nodes of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ was increased compared to control mice (**Fig. 15-B**), this was proportional to the increase in the organ size. Examination of macrophages (CD11b^{+} F4/80^{+}) revealed no major differences between *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ and control mice in terms of expression of the activation marker CD70 and intensity of MHC-II staining (not shown).

3.3.3.3. CD8^{+} T lymphocytes

CD8^{+} T cells in HIV infection are increased in numbers and also display higher turnover rate and activation state (Papagno et al., 2004; Giorgi et al., 1999; Hazenberg et al., 2003; Deeks et al., 2004). We therefore assessed whether the increase in CD8^{+} T cell numbers in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice was also associated with higher activation and turnover.

Elevated numbers of total CD8^{+} T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice, compared with control mice, were due exclusively to a systemic expansion of memory CD8^{+} T cells (**Fig. 29**).

Notably, compared with the relatively homogeneous memory CD8^{+} T cell population in control mice, memory CD8^{+} T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice contained higher numbers of recently activated/effector CD8^{+} T cells, characterized by down-regulation of CD62L and up-regulation of CD43 expression (**Fig. 30-A**). The expanded memory CD8^{+} T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice showed a pattern of

cytokine production similar to those in memory $CD8^+$ T cells from control mice (**Fig. 30-B**). Moreover, the rate of turnover of this population, evaluated by BrdU incorporation and Ki67 expression, was similar to control mice (**Fig. 30-D**). Since cytokine-producing proliferating T cells are restricted in the memory T cell pool, overrepresentation of memory $CD8^+$ T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice was responsible for the higher activation state of the total $CD8^+$ T cell population in these mice (**Fig. 30-C**).

To examine whether expansion of memory $CD8^+$ T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice was a consequence of accelerated activated $CD4^+$ T cell turnover or a cell-autonomous effect of DTA expression, we examined $CD8^+$ T cells in the BM chimeras described in chapter 3.3.1.

Compared with mice reconstituted with wild-type BM alone, mice reconstituted with *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ BM alone had significantly elevated percentages of memory $CD8^+$ T cells, which also contained higher percentages of $CD62L^-$ and $CD25^+$ activated/effector $CD8^+$ T cells (**Fig. 31-A**), and reduced CD4:CD8 ratios (**Fig. 31-B**). In contrast, in mice reconstituted with a mixture of wild-type and *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ BM , $CD8^+$ T cells of either wild-type- or *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ BM-origin were comparable, with no signs of activation, and CD4:CD8 ratios were restored (**Fig. 31-A and 31-B**).

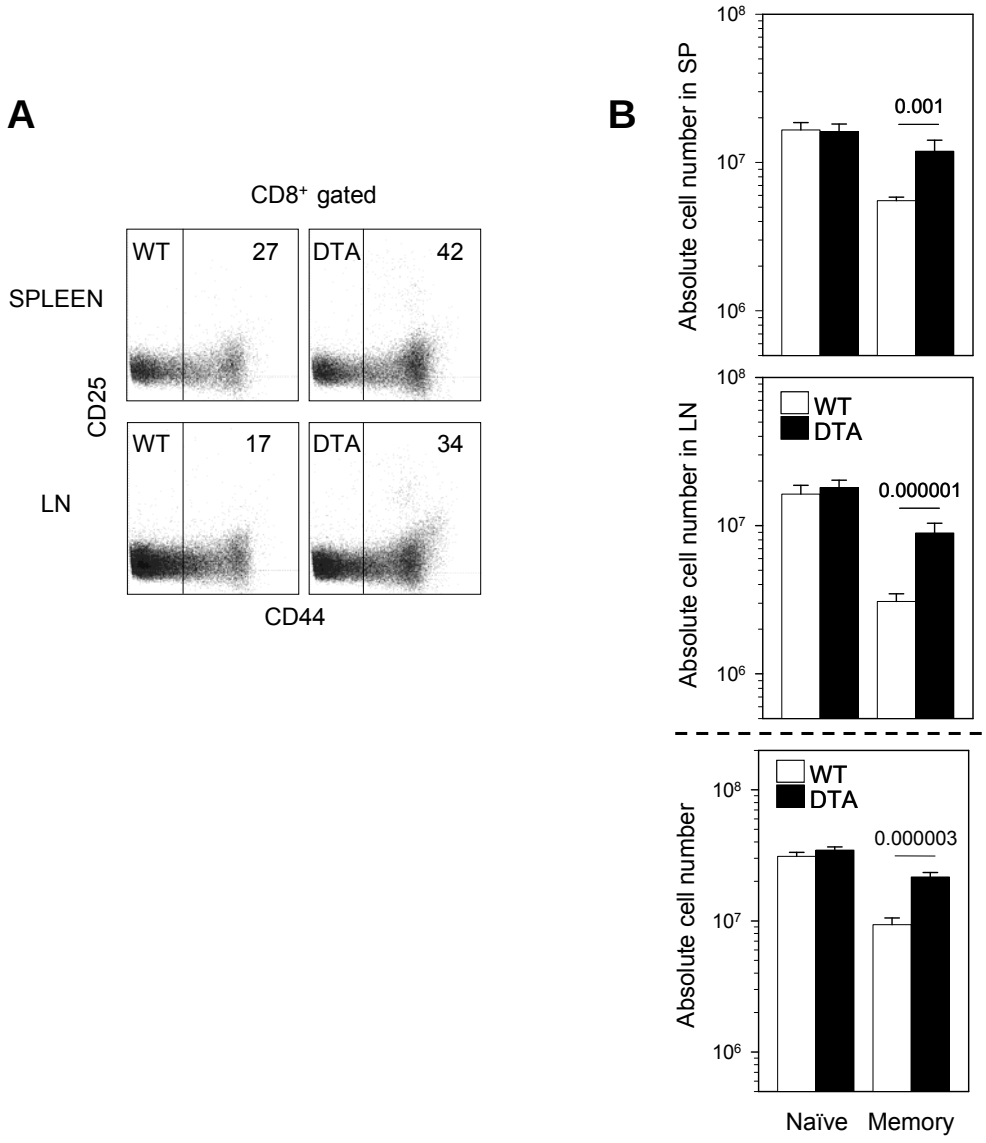

Figure 29. Composition of CD8$^+$ T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. (**A**) Flow cytometric profile and (**B**) absolute numbers of naïve and memory CD8$^+$ T cells from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice in lymph node and spleen, separate and pooled. Numbers within the plots in (A) denote the percentage of positive cells and values in (B) represent the mean (±SEM) of 15-18 mice per group.

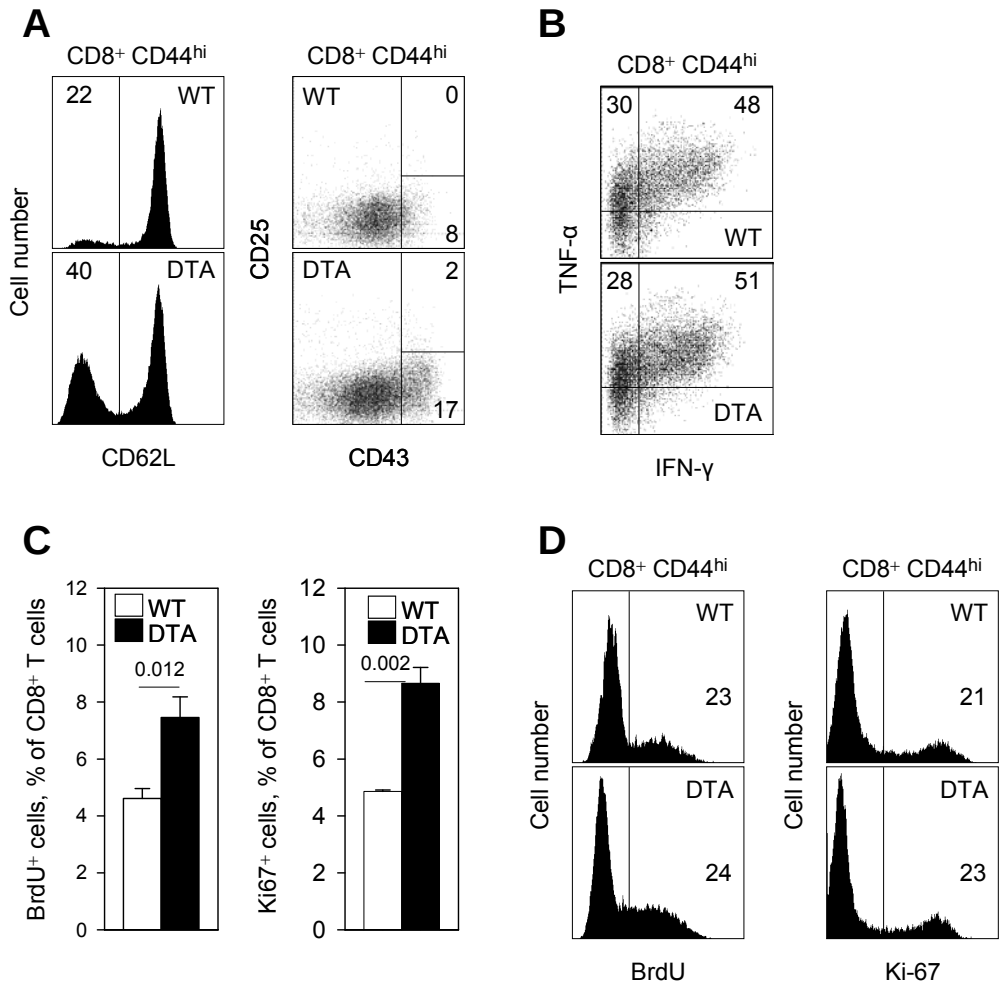

Figure 30. Phenotype and turnover of memory CD8$^+$ T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. (A) CD62L and CD43 expression in gated memory CD8$^+$CD44^{hi} T cells from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice. Numbers within the plots denote the percentage of positive cells. **(B)** TNF-α and IFN-γ production by memory CD8$^+$CD44^{hi} T cells from the same mice. Numbers within the quadrants represent the percentage of the respective cytokine secreting cells and are representative of 4 mice per group. **(C)** Percentage of BrdU$^+$ following a 6-day period of BrdU administration and Ki67$^+$ cells in total CD8$^+$ T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice. Values are the mean (±SEM) of 4 or 3 mice per group, respectively. **(D)** BrdU and Ki67 labelling in gated memory CD8$^+$CD44^{hi} T cells from the same mice. Numbers within the plots represent the percentage of positive cells for BrdU or Ki67 and are representative of 4 mice or 3 miceper group, respectively.

In conclusion, the expansion and activation of memory $CD8^+$ T cells seen in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice was an indirect result of altered $CD4^+$ T cell homeostasis. We should therefore expect to see a similar phenotype in mice with primary $CD4^+$ T cell immunodeficiency, such as MHC II-deficient mice. MHC II-deficient mice, in which generation of $CD4^+$ T cells is blocked in the thymus, exhibited a proportional increase in numbers of both naïve and memory $CD8^+$ T cells and the representation of memory $CD8^+$ T cells was therefore similar to wild-type mice (**Fig. 32-A and 32-B**). Nevertheless, as was the case with *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice, memory $CD8^+$ T cells in MHC II-deficient mice contained higher percentages of $CD62L^-$ and $CD43^+$ activated/effector $CD8^+$ T cells, than wild-type mice (**Fig. 32-C**).

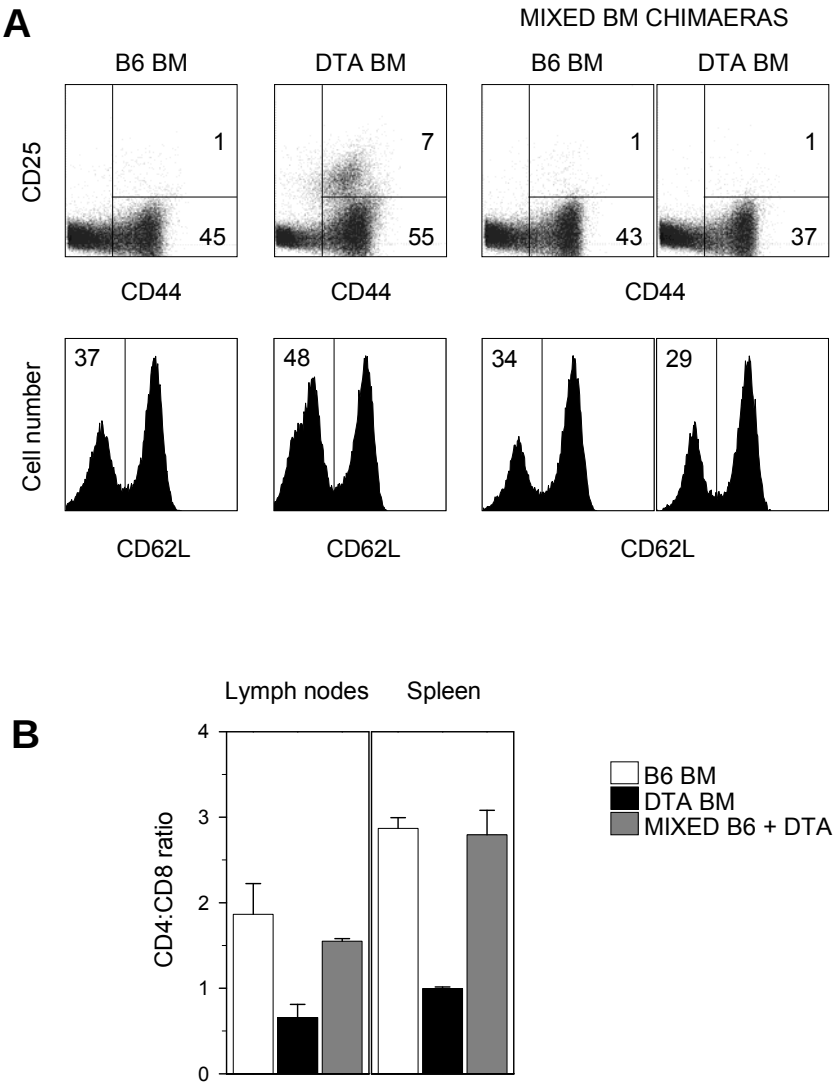

Figure 31. CD8 phenotype in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice is not cell intrinsic. Bone marrow chimeras, set up as in Fig. 18, were evaluated for the percentage of memory (CD44^{hi}) and effector (CD44^{hi}CD25^{+}) CD8^{+} T cells (**A**, top row) or the percentage of CD62L^{-} cells in gated CD44^{hi}CD8^{+} T cells (**A**, bottom row), or the mean (±SEM) CD4:CD8 ratio (**B**). Values are representative of 4-8 mice analyzed in 2 independent experiments.

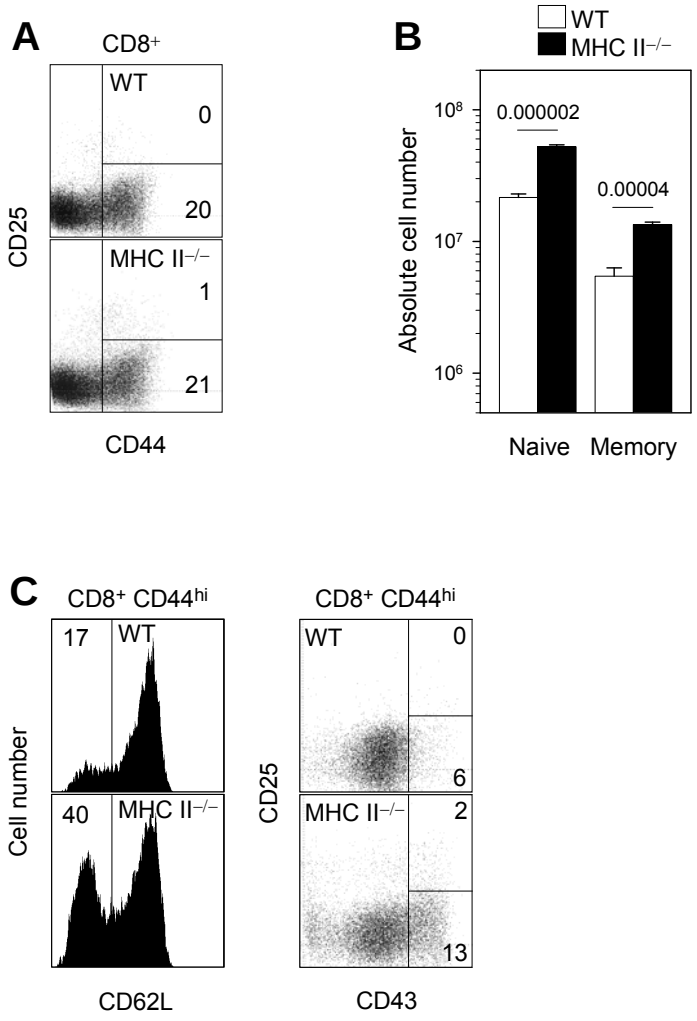

Figure 32. Composition and phenotype of CD8$^+$ T cells in mice with primary CD4$^+$ T cell immune deficiency. (A) Percentage and **(B)** absolute number (mean ±SEM of 4-6 mice) of naïve and memory CD8$^+$ T cells in MHC II$^{-/-}$ and wild-type control (WT) mice. **(C)** CD62L and CD43 expression in gated memory CD44^{hi}CD8$^+$ T cells from the same mice. Numbers within the plots in (A) and (C) represent the percentage of cells which are positive for each marker and are representative of 4-6 mice.

3.4. Immune activation is neither a consequence of self-reactivity to apoptotic T cells nor of bacterial translocation

Although the precise causes of immune activation in HIV infection are not entirely clear, two distinct mechanisms have recently been proposed, namely self-reactivity to apoptotic T cells (Rawson et al., 2007) and translocation of microbial products in the intestinal mucosa (Brenchley et al., 2007).

Reactivity to apoptosis-related self-peptides could be excluded as the cause of $CD8^+$ T cell activation in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice as activation of these cells was not observed in mixed BM chimeras in the presence of wild-type $CD4^+$ T cells (**Fig. 31-A**), despite continuous apoptosis of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ $CD4^+$ T cells.

Although we found no evidence for intestinal pathology in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice it could be possible that a small degree of histologically undetectable bacterial translocation was occurring. To address this issue, we examined the effect of microbial translocation on the immune system of mice. Villin promoter-driven Cre-mediated intestinal epithelial cell-specific deletion of NEMO (encoded by *Ikbkg*) leads to epithelial cell apoptosis, translocation of bacteria into the mucosa and TLR-dependent intestinal inflammation and colitis (Nenci et al., 2007). We therefore assessed the immune activation status in the *Ikbkg*$^{fl/-}$ *Vil-Cre* (NEMO) mice.

Lymph nodes in *Ikbkg*$^{fl/-}$ *Vil-Cre* mice were enlarged and contained increased numbers of both B and T lymphocytes, compared with *Ikbkg*$^{fl/-}$ littermate control mice

(**Fig. 33-A and 33-B**). This however, was at the expense of splenic lymphocytes (**Fig. 33-B**) and overall numbers of B cell and $CD4^+$ and $CD8^+$ T cell subsets and the CD4:CD8 ratio in *Ikbkg*$^{fl/-}$ *Vil-Cre* mice were normal (**Fig. 33-A, 34-A and 34-B**). Compared with *Ikbkg*$^{fl/-}$ control mice, increased percentages and absolute numbers of memory $CD4^+$ and $CD8^+$ T cells were observed in enlarged lymph nodes but not in the spleens of *Ikbkg*$^{fl/-}$ *Vil-Cre* mice (**Fig. 35-A, 35-B, 36-A and 36-B**). In contrast to lymphocytes, numbers of $CD11b^+F4/80^+$ myeloid cells were dramatically elevated in all secondary lymphoid organs of *Ikbkg*$^{fl/-}$ *Vil-Cre* mice and outnumbered B cells or T cells (**Fig. 33-B**). Thus, microbial translocation in this mouse model was associated with systemic expansion of myeloid cells, rather than memory $CD8^+$ T cells.

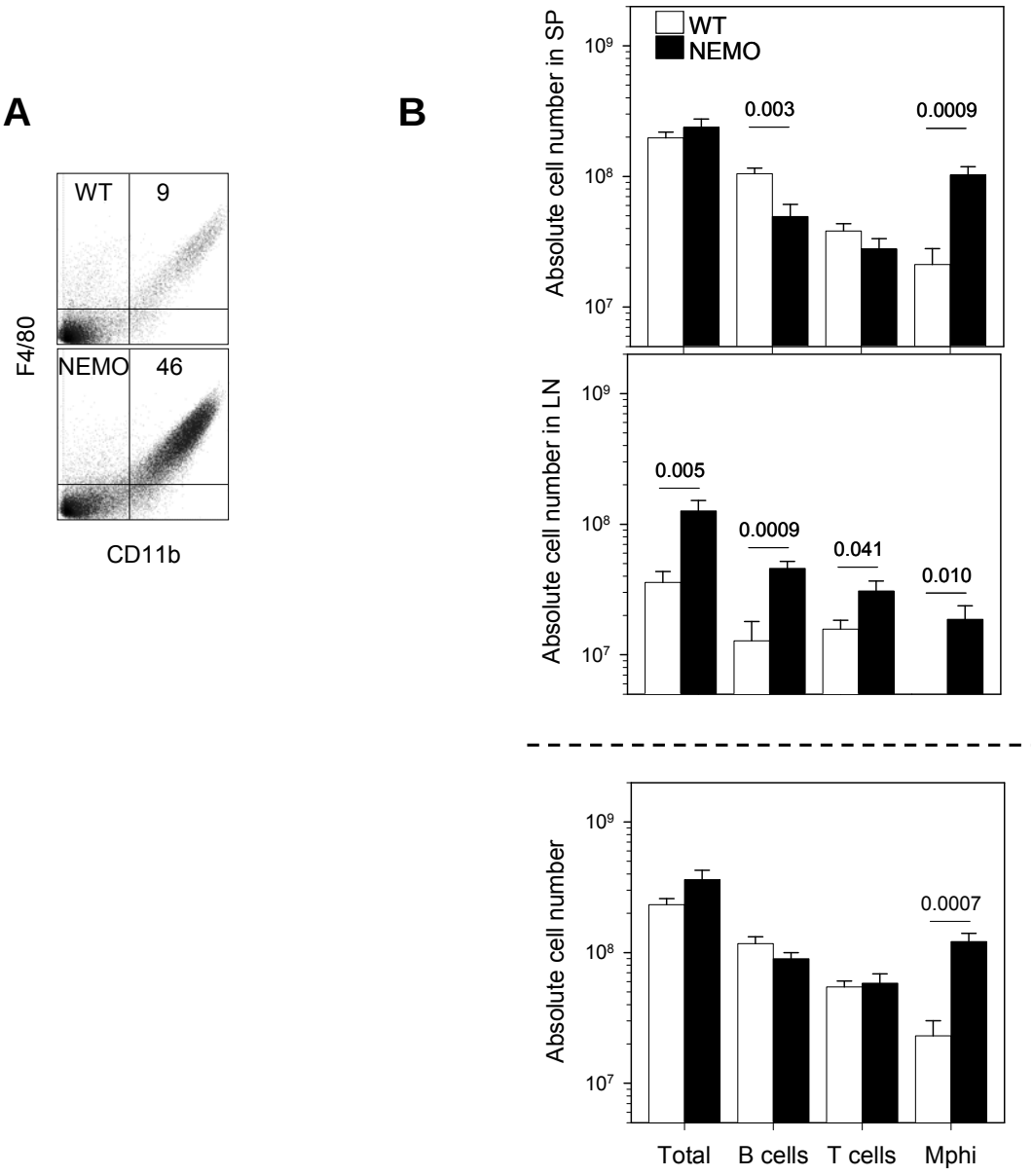

Figure 33. Effect of microbial translocation through the intestinal mucosa on lymphoid organ cellularity and composition. (**A**) Flow cytometric profile of $CD11b^{+}F4/80^{+}$ macrophages in *Ikbkg*$^{fl/-}$ *Vil-Cre* (NEMO) and control *Ikbkg*$^{fl/-}$ (WT) mice lymph nodes. **(B)** Total cellularity and numbers of $B220^{+}$ (B cells), $CD4^{+}$ and $CD8^{+}$ (T cells) and $CD11b^{+}F4/80^{+}$ (Mphi, macrophages) in spleen and lymph nodes separate and pooled. Values represent the mean (±SEM) of 9-11 mice per group.

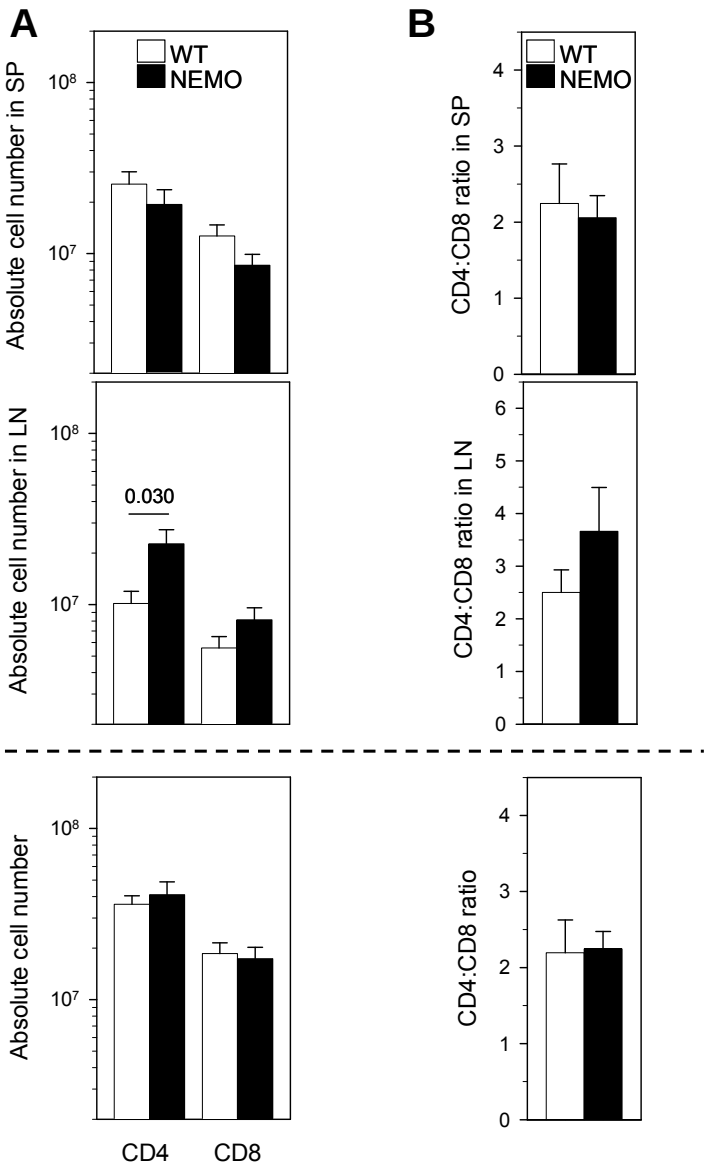

Figure 34. Effect of microbial translocation through the intestinal mucosa on cellularity of CD4$^+$ and CD8$^+$ T cells. Numbers of CD4$^+$ or CD8$^+$ T cells and CD4:CD8 ratio in *Ikbkg*$^{fl/-}$ *Vil-Cre* (NEMO) and control *Ikbkg*$^{fl/-}$ (WT) mice in spleen and lymph nodes, in separate and pooled. Values represent the mean (±SEM) of 9-11 mice per group.

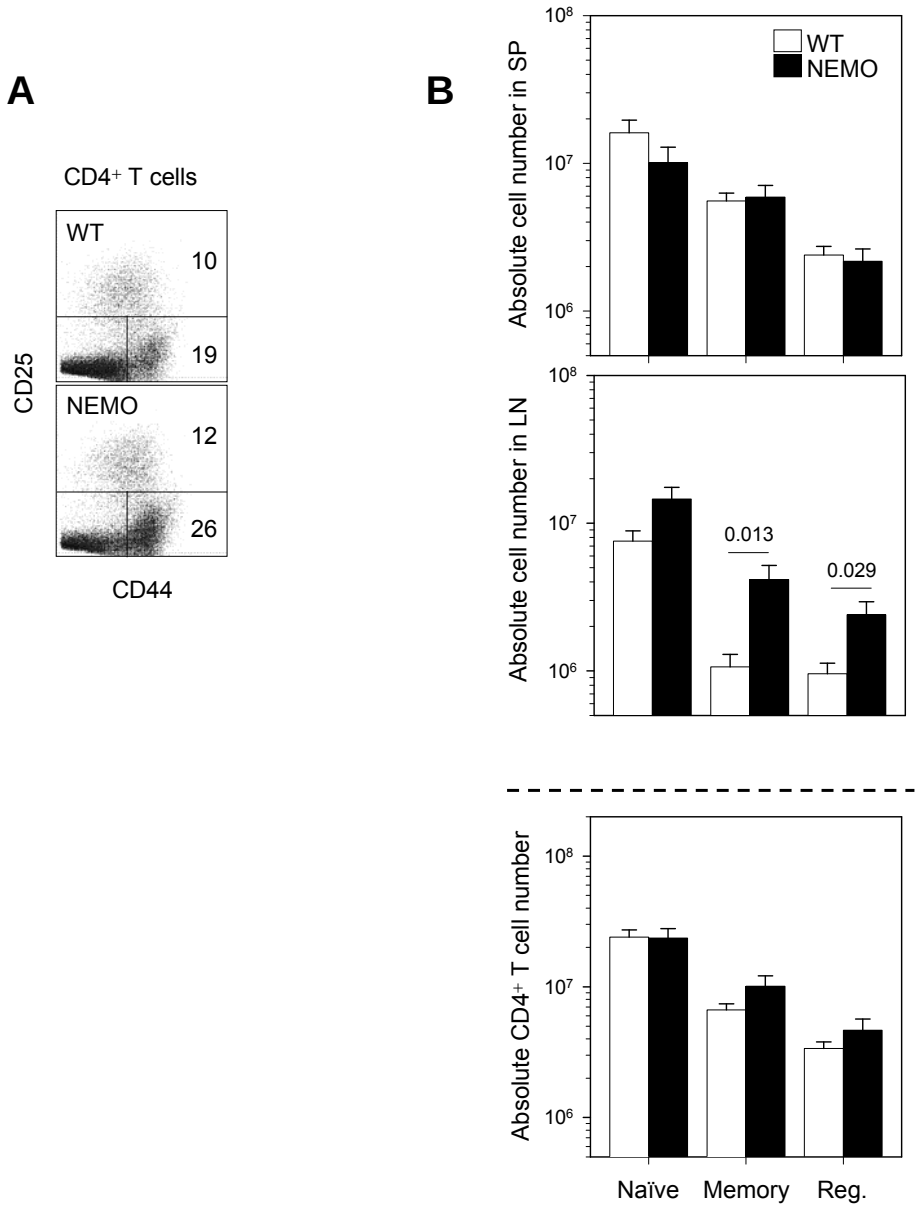

Figure 35. Effect of microbial translocation through the intestinal mucosa on composition of CD4$^+$ T cells. (A) Percentages and (**B**) absolute numbers of naïve, memory and regulatory (reg.) CD4$^+$ T cells from *Ikbkg*$^{fl/-}$ *Vil-Cre* (NEMO) and control *Ikbkg*$^{fl/-}$ (WT) mice in spleen and lymph nodes separate and pooled. Values represent the mean (±SEM) of 9-11 mice per group.

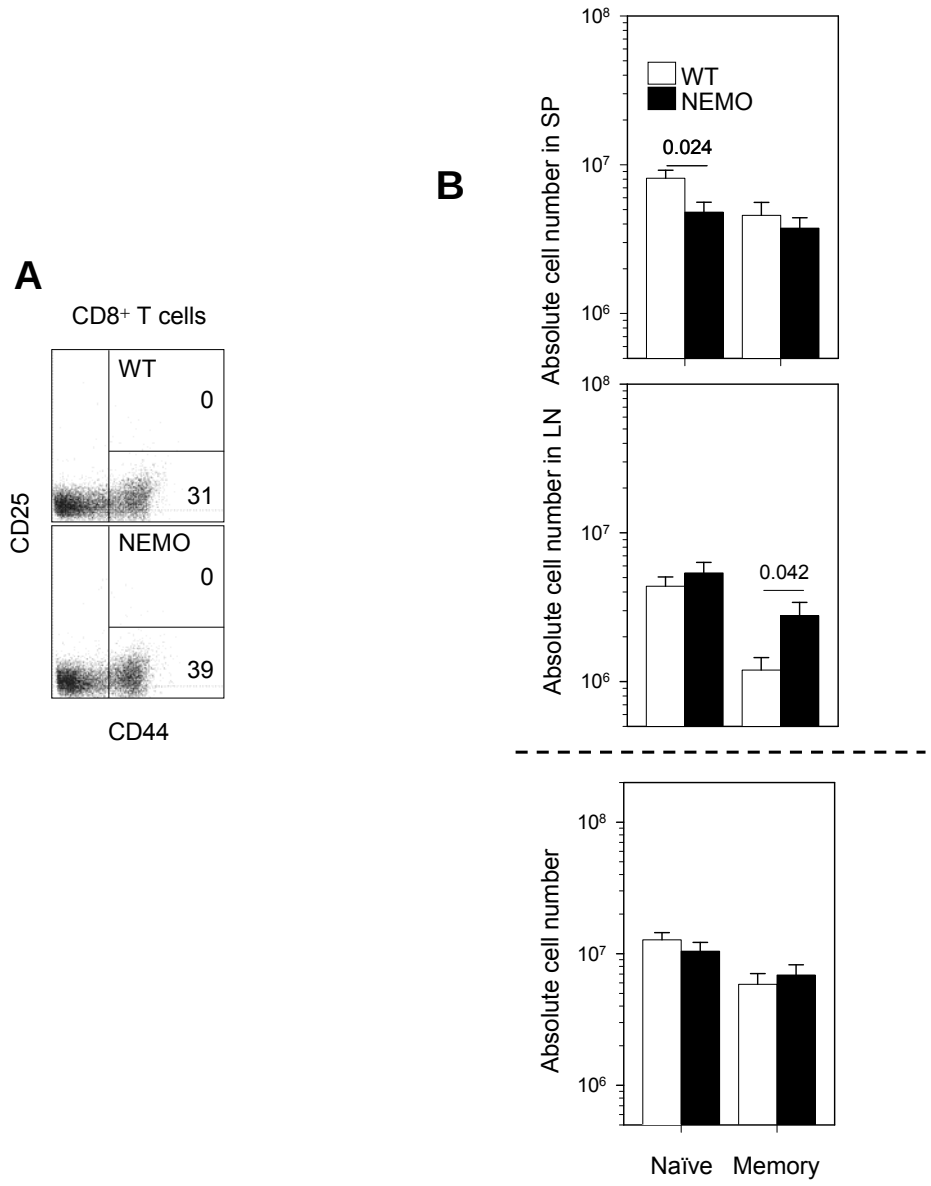

Figure 36. Effect of microbial translocation through the intestinal mucosa on composition of CD8$^+$ T cells. (A) Percentages and (**B**) absolute numbers of naïve and memory CD8$^+$ T cells from *Ikbkg$^{fl/-}$ Vil-Cre* (NEMO) and control *Ikbkg$^{fl/-}$* (WT) mice in spleen and lymph nodes separate and pooled. Values represent the mean (±SEM) of 9-11 mice per group.

3.5. Immune activation is a consequence of CD4$^+$ regulatory T cell homeostatic disturbances

Our results argued against self-reactivity to apoptotic products or translocation of microbial products as the causes of immune activation in *Tnfrsf4$^{Cre/+}$ R26$^{Dta/+}$* mice and instead supported immune activation as a direct consequence of CD4$^+$ T cell death.

To explore the mechanism by which activated CD4$^+$ T cell deficiency leads to CD8$^+$ T cell activation, we reconstituted selected subsets of CD4$^+$ T cells affected in *Tnfrsf4$^{Cre/+}$ R26$^{Dta/+}$* mice. The regulatory subset of CD4$^+$ T cells is targeted by HIV, SIV and FIV (Oswald-Richter et al., 2004; Pereira et al., 2007; Joshi et al., 2004), leading to enhanced turnover and activation, and a relative deficit in this subset has been linked by certain studies to immune activation and disease progression in HIV infection (Kinter et al., 2004; Eggena et al., 2005).

Purified wild-type regulatory CD4$^+$ T cells (CD4$^+$ CD25^{bright}) adoptively transferred into *Tnfrsf4$^{Cre/+}$ R26$^{Dta/+}$* mice expanded efficiently, reaching numbers comparable with those of regulatory CD4$^+$ T cells in wild-type mice, and maintained their FoxP3 expression (**Fig. 37-A and 37-B**). In contrast, the same cells transferred into regulatory CD4$^+$ T cell-replete control mice failed to expand (**Fig. 37-A and 37-B**).

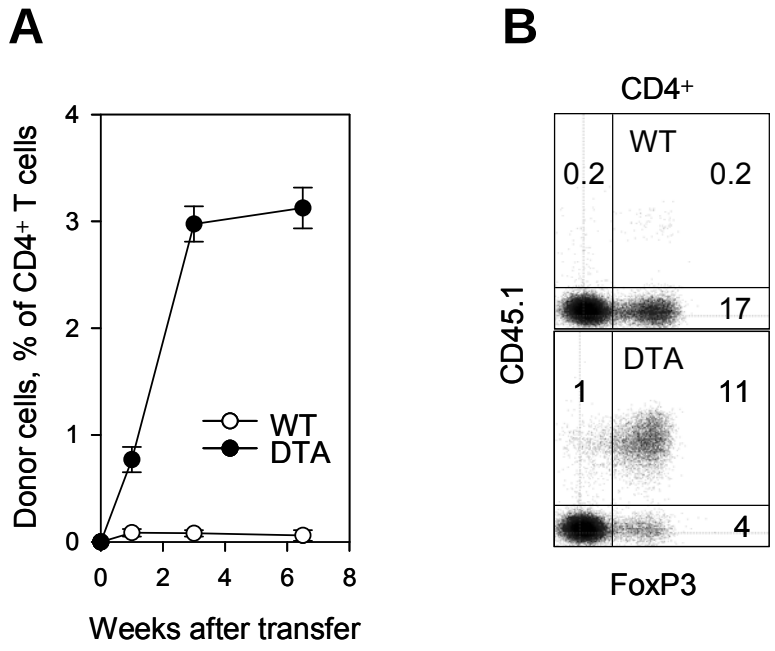

Figure 37. Adoptively transferred wild type regulatory CD4$^+$ T cells expand in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ but not in control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$mice. *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice received 1-3 × 10^6 purified regulatory CD25$^+$CD4$^+$ T cells from CD45.1-congenic B6 donor mice. Expansion of transferred cells was followed in the blood of recipient mice (**A**), and expansion and retention of Foxp3 expression in donor CD45.1$^+$CD4$^+$ T cells was assessed in lymphoid organs at the end of a 10-week observation period (**B**). Values represent the mean (±SEM) of 7-9 mice per group pooled from 3 independent experiments.

The adoptively transferred regulatory $CD4^+$ T cells had a significant effect on the proportion of memory $CD8^+$ T cells in the blood (**Fig. 38-A**) and lymphoid tissues (**Fig. 38-B**) of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice, particularly those with an activated $CD44^{hi}CD62L^-$ phenotype, which were reduced to wild-type levels over a period of three weeks (**Fig. 38-C and 38-D**). Thus, $CD8^+$ T cell immune activation in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice is due to a deficit in regulatory $CD4^+$ T cell function.

Furthermore, transfer of regulatory T cells had an effect on the serum levels of some, but not all, proinflammatory cytokines and chemokines. Serum levels of IL-1β, IFNγ, MCP-1 (CCL2) and MIP-1α (CCL3) were restored to levels in control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (**Fig. 39**).

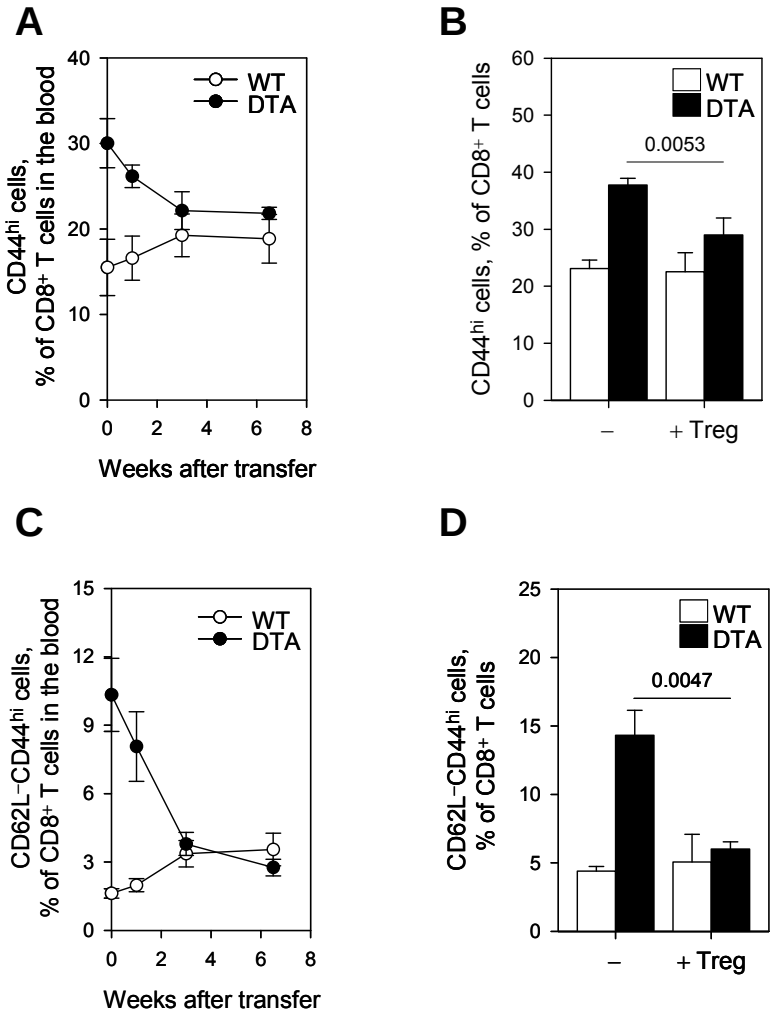

Figure 38. Effect of regulatory CD4$^+$ T cell transfer on proportion and phenotype of memory CD8$^+$ T cells. (**A**) Percentage of memory (CD44^{hi}) cells in CD8$^+$ T cells in the blood of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice, following transfer of 1-3 × 10^6 purified regulatory CD25$^+$CD4$^+$ T cells from CD45.1-congenic B6 donor mice. (**B**) Percentage of memory (CD44^{hi}) cells in CD8$^+$ T cells isolated from the spleen and lymph nodes of the same recipient mice (+ Treg), 10 weeks after transfer, or mice which did not receive CD25$^+$CD4$^+$ T cells (- Treg). (**C**) Percentage of effector/memory (CD62L$^-$CD44^{hi}) cells in CD8$^+$ T cells in the blood of the same recipients of regulatory CD25$^+$CD4$^+$ T cells. (**D**) Percentage of effector/memory (CD62L$^-$CD44^{hi}) cells in CD8$^+$ T cells isolated from the spleen and lymph nodes of the same recipient mice (+ Treg), 10 weeks after transfer, or mice which did not receive CD25$^+$CD4$^+$ T cells (- Treg). Values represent the mean (±SEM) of 7-9 mice per group pooled from 3 independent experiments.

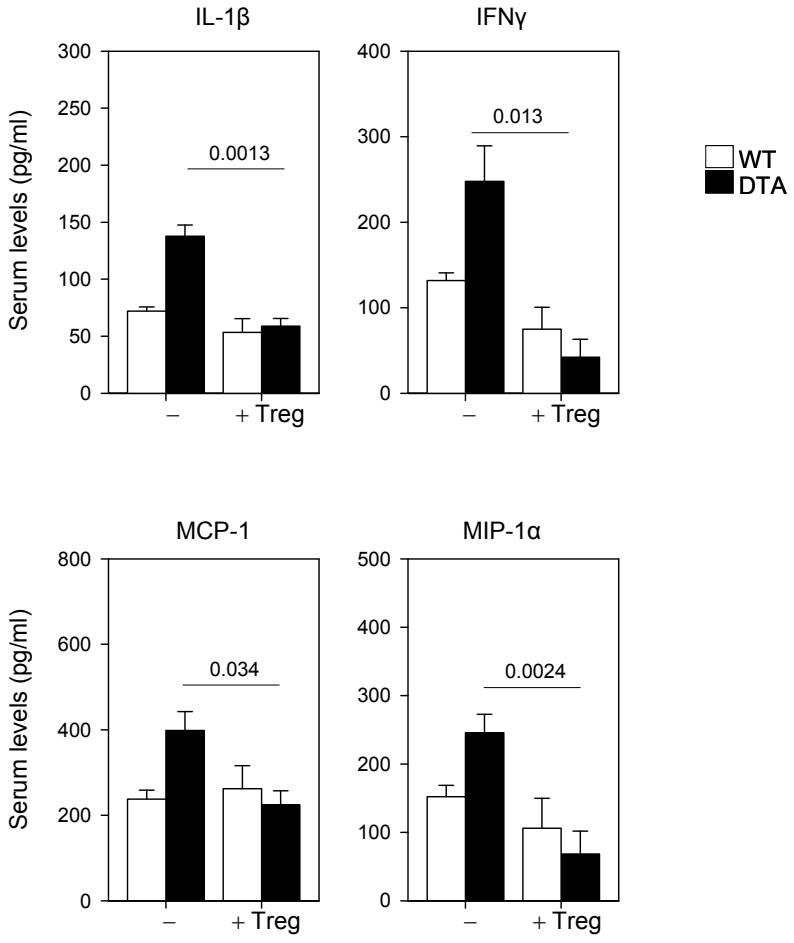

Figure 39. Effect of regulatory CD4$^+$ T cell transfer on serum levels of proinflammatory cytokines and chemokines in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice. Serum levels of the cytokines IL-1β and IFNγ, and of the chemokines MCP-1 (CCL2) and MIP-1α (CCL3) in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ (DTA) and control *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ (WT) mice and after transfer of purified regulatory T cells from CD45.1-congenic B6 donor mice. Values represent the mean (±SEM) of 5 mice per group, pooled from two independent experiments.

4. Discussion

In the present study we have induced conditional ablation of activated $CD4^+$ T cells, the targets of immunodeficiency viruses, in a virus-free murine system. Depletion of these cells affects memory/effector and regulatory $CD4^+$ T cells and leads to a disturbance in immune system homeostasis that shares many features with cell dynamics characteristic of HIV-1 infection. Specifically, partial deletion of memory/effector and regulatory $CD4^+$ T cells results in generalized immune activation characterized by enlarged lymph nodes, increased production of proinflammatory cytokines and chemokines, decrease in the CD4:CD8 ratio and increased activation and turnover of $CD4^+$ and $CD8^+$ T cells. Furthermore the immune response of these mice to a number of pathogens tested is impaired even though $CD4^+$ T cell numbers are relatively preserved. By separating killing from other viral-mediated effects, we show that deficiency in regulatory T cells is sufficient to cause $CD8^+$ T cell immune activation.

4.1. Conditional mutagenesis in the mouse

The success of the Cre-*loxP* technology is dependent upon tight regulation of Cre expression so that it is only expressed in the intended cells. In this respect, the *Tnfrsf4*$^{Cre/+}$ mouse line used in this study has the advantage of having Cre inserted into the endogenous CD134 locus, thereby eliminating position effect variegation sometimes observed in the classical transgenesis approach (Kioussis and Festenstein, 1997). In addition, by using a reporter line for Cre-mediated recombination, we were able to confirm that the expression of Cre in peripheral lymphoid tissues is restricted

almost exclusively to memory and regulatory $CD4^+$ T cell subsets, as expected. Because the removal of the stop signal is a permanent genetic modification all cells that have expressed but subsequently downregulated CD134 and all daughter cells will therefore express the reporter marker YFP. It is thus likely that the proportion of YFP^+ cells in the reporter line is an overrepresentation of the number of activated cells at any one moment. Importantly, we did not detect YFP expression in any other tissues analysed, indicating that Cre expression is tightly regulated in the *Tnfrsf4*$^{Cre/+}$ line. Although we cannot exclude the expression of Cre in other non-hematopoietic tissues that we did not analyse, we were able to show that the phenotype we are reporting is not due to ectopic expression but rather is immune cell-dependent, and could be reproduced by transfer of hematopoietic cells in *Rag*$^{-/-}$ mice.

It has been shown that CD134 can be up-regulated in $CD8^+$ T cells that are activated *in vitro* through TCR stimulation (Baum et al., 1994), but the absence of YFP^+ cells in the $CD8^+CD44^+$ fraction of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/+}$ mice indicates that, under physiological conditions, the majority of memory-phenotype $CD8^+$ T cells will not receive the amount of stimulation that *in vitro* TCR stimulation delivers. However, in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Yfp/Dta}$ mice, where the $CD8^+$ T cell population is activated, we did see a small fraction of $CD8^+$ YFP^+. Because these cells also express DTA, this reporter strain does not permit evaluation of the true extent of $CD8^+$ T cells that upregulated CD134. Importantly however, we have several lines of evidence that activation of $CD8^+$ T cells is secondary to the $CD4^+$ T cell phenotype we described. Firstly, $CD8^+$ T cells are maintained in mixed bone marrow chimeras and are not activated. Secondly, in MHCII$^{-/-}$ mice the absence of $CD4^+$ T cells results in $CD8^+$ T cell activation. Lastly, transfer of regulatory $CD4^+$ T cells is able to correct the $CD8^+$ T

cell activation phenotype. Thus we can rule out DTA-mediated depletion of $CD8^+$ T cells as contributing to their activated phenotype.

With respect to the relevance of this system in our attempts to mimic $CD4^+$ T cell-specific deletion by HIV, a number of points should be taken into account.

First, the kinetics of DTA-mediated depletion we observe is similar to the one estimated for cells infected with HIV. It is calculated that HIV has an eclipse time of ~1 day between cell entry and virion production (Perelson et al., 1996), and that productively infected cells have a lifespan of ~1 day (Markowitz et al., 2003). Thus it takes ~2 days for HIV to kill a cell from the moment of cell entry. Similarly, we have shown that it takes approximately one day for the cell to express CD134 after activation and that DTA-mediated cell death takes a further 24 hours.

Second, although we aimed at depleting 100% of activated CD134-expressing cells, this was not achieved either due to inefficiency of DTA-mediated killing or rapid replacement of cells, or both. This is to our advantage because it reproduces better the deletion of available target cells by HIV *in vivo*, where only a proportion of them are killed.

Finally, the genetic modification we have induced in the mouse genome is in place during the entire development of the mice, and therefore does not reproduce the acute onset of infection in adults. However it mimics situations of congenital infection.

4.2. $CD4^+$ T cell depletion and deficiency: homeostasis and function

The immune system has extraordinary self-regenerating capacity that allows the number of immune cells to be restored after serious insults, provided that the cues necessary for the maintenance of each subset are present. The proliferative capacity of each subset and its ability to self-regenerate are however different.

We show here that despite the fact that $CD4^+$ T cells are being depleted, the number of these cells in lymphoid tissues is not decreased. Furthermore, this depletion leads to changes in other immune populations by significantly increasing the numbers of B cells and $CD8^+$ T cells and results in a decrease of the CD4:CD8 ratio.

A decreased CD4:CD8 ratio is also a hallmark of HIV infection. In this case however, it reflects not only a similar increase in $CD8^+$ T cell counts but also a slow and progressive decrease in $CD4^+$ T cell counts.

4.2.1. Memory and regulatory $CD4^+$ T cells

In our model, a significant proportion of memory and regulatory $CD4^+$ T cells are depleted. However, the number of memory T cells is unchanged and in lymph nodes is even increased, reflecting the powerful capacity for self-renewal of this population. In contrast, regulatory T cells are reduced in $Tnfrsf4^{Cre/+}$ $R26^{Dta/+}$ mice, indicating a breakdown in the homeostasis of this subset.

Regulatory T cells have been shown to divide homeostatically in lymphopenic conditions (Annacker et al., 2001; Gavin et al., 2002) and after antigenic stimulation (Walker et al., 2003). We analysed this population in search for clues as to why this population is not restoring itself in our system. The phenotype of these cells is markedly different from those of control mice. An increased proportion of regulatory T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice express activation markers such as low levels of CD62L and increased CD43, CD44 and CD49b expression. The increased activation of the regulatory T cell compartment in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice can be a consequence of a vicious cycle between killing of activated cells and elevated turnover of the remaining cells in order to replace the lost ones. The results obtained with BrdU and Ki67 labelling favour this hypothesis. Another possibility is that the regulatory population of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice is composed exclusively of the 'memory' population seen in WT mice (Fisson et al., 2003) due to the depletion in the thymus of the 'naïve' regulatory T cell population. We have shown that, in the thymus, expression of YFP by regulatory T cells was almost as high as in the periphery (60% vs 80%), in agreement with other studies that pointed out that regulatory T cells are already activated in the thymus. However CD134 is expressed preferentially in the 'memory' population. In addition, our observation that regulatory cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice are more activated than the 'memory' regulatory population of control mice strongly supports the first hypothesis. Therefore, it is unlikely that the regulatory population in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice appears activated due to selective loss of the 'naïve' regulatory subset.

Regulatory T cells from *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice show a higher than two fold increase in the turnover rate in comparison to control mice, as evaluated by BrdU

incorporation and Ki67 staining. The increased homeostatic proliferation is however insufficient to compensate for the loss of these cells, indicating that death occurs faster than cell proliferation. It is also possible that a fraction of regulatory T cells have been generated by conversion of memory $CD4^+$ T cells rather than by division of regulatory T cells (Akbar et al., 2007).

Contrasting with the higher levels of activation and proliferation of regulatory T cells, the memory T cell compartment in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice does not show such profound changes in their activation profile and turnover rate compared with those in control mice. Indeed, except for a small increase in the expression of CD43 and CD49b markers and in the production of IFNγ, they are comparable to memory cells in control mice. Their proliferation rate as assessed by BrdU incorporation was not significantly different when compared to controls and analysis of Ki67 revealed only a mild increase.

However, we demonstrated in the mixed bone marrow chimera experiments that *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$-origin memory cells are killed as efficiently as regulatory T cells.

In normal mice in steady-state conditions, the memory $CD4^+$ T cell pool contains a very high percentage of cells dividing at any one time (40% were labelled by Ki67) and consequently an equivalent proportion of cells must die as their absolute numbers do not change. Indeed, memory $CD4^+$ T cells have a much higher turnover rate and thus shorter life-span, at the single-cell level, than naïve $CD4^+$ T cells.

This means that the DTA-mediated depletion in memory $CD4^+$ T cells will probably have a minor impact on top of the already high death rate, because many of the cells targeted by DTA are pre-destined to die anyway. Thus, it is the difference between DTA-mediated killing and normal cell intrinsic death rate that dictates the impact DTA has on each population.

4.2.2. Preservation of $CD4^+$ T cell numbers

The mechanism responsible for the progressive decline of $CD4^+$ T cells in HIV infection is not completely understood nor is the long time scale of this process, often over a period of ten years or longer. One proposed mechanism for the $CD4^+$ T cell loss is that immune activation increases target cell availability and leads to ongoing cycles of infection and virus production resulting in depletion of more $CD4^+$ T cells. However, mathematical modelling has shown that if this 'runaway' hypothesis were correct, then $CD4^+$ T cells would fall to low levels in a matter of months, not years (Yates et al., 2007). Therefore, the slow decline of $CD4^+$ T cells in HIV infection needs to be better understood.

The average time to progress to AIDS is markedly shorter in SIV-infected Indian rhesus macaques than in untreated HIV-1 individuals. In contrast, in SIV-infected Chinese rhesus macaques the course of infection is slower, thus making them more similar to HIV-infected individuals (Ling et al., 2002). Interestingly, in SIV-infected Indian rhesus macaques, progression to AIDS is associated with a rapid depletion of circulating and mucosal $CD4^+CCR5^+$ memory T cells (Mattapallil et al., 2004) whereas Chinese rhesus macaques experience a relative expansion of the $CD4^+CCR5^+$ pool, the extent of which is clearly associated with markers of disease progression

(Monceaux et al., 2007). This is also the case for the asymptomatic phase of HIV-1 infection where there is an increase in the proportion of CCR5 expressing $CD4^+$ T cells (Ostrowski et al., 1998). Infection with HIV-2 which is characterized by a very slow disease progression, is also associated with an increase in the proportion of $CCR5^+$ within the memory /effector $CD4^+$ T cell pool which correlates with the degree of $CD4^+$ T cell depletion and immune activation (Soares et al., 2006).

Thus, progression to disease in slow SIV progressors (Chinese rhesus macaques), HIV-2 and asymptomatic HIV-1 is associated with a relative increase of $CD4^+CCR5^+$ in the periphery, whereas in the fast SIV progressors (Indian rhesus macaques) disease is associated with depletion of the same cells.

Interestingly, in our model, there is a relative increase in effector-memory $CD4^+$ T cells, the same cells that are being killed, similarly to what happens in HIV-1 infection. Our model is thus a good model for the asymptomatic phase of HIV-1.

$CD4^+$ T cells in $Tnfrsf4^{Cre/+}$ $R26^{Dta/+}$ mice, including the memory subset, are numerically stable over time, even in aged mice of 8 months of age, which was the latest time point analysed. It is therefore interesting to explore the mechanisms responsible for the memory $CD4^+$ T cell preservation in these mice.

Memory $CD4^+$ T cells can be renewed either by cell division (proliferation) or by differentiation of naïve T cells. For naïve T cells to differentiate into memory cells they need to be activated and divide. In this process they will become targets for HIV, and in our system they will express CD134 and die. This continuous pressure would

drain the naïve pool in situations of thymic involution. Thus it is tempting to speculate that in our model, the maintenance of a normal thymus contributes to the maintenance of the $CD4^+$ T cell numbers.

In HIV infection, depletion of naïve $CD4^+$ T cells can sometimes be observed, but whether it is critical for the decline of memory $CD4^+$ T cells is not known. In SIV infection of macaques, some studies report depletion of naïve T cells (Nishimura et al., 2007), whereas other studies report intact $CD4^+$ naïve cell compartment (Okoye et al., 2007). Observations that thymectomized rhesus macaques do not show significant differences in terms of T cell decay or disease progression (Arron et al., 2005), suggest that the naïve population is not a major determinant of memory $CD4^+$ T cell homeostasis in chronic SIV infection.

However, the age-associated thymic involution and HIV-mediated thymic inhibition are likely to make it more difficult for the thymus to keep up with the constant drain on the naïve T cell pool to flow into the memory T cell pool (Douek, 2003). Efficient thymopoiesis has been strongly associated with maintenance of circulating $CD4^+$ T cells in slow progressors (Dion et al., 2007) and has also been implicated in a better outcome of HIV-2 infection when compared with HIV-1 (Gautier et al., 2007).

4.2.3. Immunodeficiency

Tnfrsf4$^{Cre/+}$ *R26*$^{Dta/+}$ mice do not spontaneously develop any opportunistic infections, which is not surprising since they are housed in clean facilities, where even mice lacking innate (MyD88$^{-/-}$) or adaptive (Rag$^{-/-}$) immune systems live healthy. However, when submitted to experimental infection these mice proved to be immunodeficient. Thus, despite having normal numbers of CD4^{+} T cells, the accelerated turnover rate or the immune activation status of antigen experienced cells compromised their function.

The inability to mount a strong immune response can be due to insufficient precursor frequency of one or more of the effector populations. However, this is unlikely to be the case for the *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice, because naïve CD4^{+} or CD8^{+} T cells are not reduced. The accelerated turnover rate of effector/memory pool is more likely to be the cause, since cells respond to pathogens by becoming activated. A primary response peaks at ~7 days and by that time we have shown that cells are already affected by DTA-mediated killing.

Although a contribution of CD8^{+} T cell activation phenotype in the immunodeficiency of these mice is possible, we measured immune deficiency by specifically assessing CD4^{+} T cell function. In the experimental infection with Influenza virus, we looked at the production of nAb that is absolutely dependent on CD4^{+} T cells. In Friend virus infection, we assessed both the production of neutralizing antibodies and the control of viremia both of which have been shown to be dependent on CD4^{+} T cells in the chronic stage of infection, but not on CD8^{+} T cells (Super et al., 1998). The correlation observed between production of neutralizing antibodies and control of infection further support a role of CD4^{+} T cells, in the

resolution of this infection. Finally, $CD4^+$ T cells have been shown to be the critical subset in controlling *Pneumocystis* infection but also to be responsible for the deleterious effects of the inflammatory response. In this context, it was shown that regulatory T cells suppress the $CD4^+$ T cell mediated pulmonary hyper-inflammation in immune deficient mice (Hori et al., 2002).

In untreated HIV infection, there is a progressive loss of immune competence with some infections being more likely to occur at higher $CD4^+$ T cells counts than others. For instance, candidiasis and herpes simplex virus, both of which have increased incidence among individuals with HIV, can occur even at fairly high $CD4^+$ T cell counts. *Mycobacterium tuberculosis* carriers infected with HIV are more likely to develop tuberculosis than those without HIV, and this is also not necessarily associated to low $CD4^+$ T cell counts. Bacterial pneumonia, very frequent in HIV-positive individuals, occurs at all $CD4^+$ T cell counts, although it becomes more frequent as $CD4^+$ T cell counts go down (Hirschtick et al., 1995). Other infections however, develop only when $CD4^+$ T cell counts drop below a certain threshold: Kaposi's sarcoma, *Pneumocystis* pneumonia, toxoplasmosis and Cryptococcus, *Mycobacterium avium* complex and cytomegalovirus, all develop at < 250 $CD4^+$ T cells/µl.

Similarly, we find that *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice are immune deficient to an array of infections, but the degree of deficiency depends on the type of infection and its chronicity.

4.3. Immune activation

4.3.1. Immune activation in HIV

It has long been recognised that HIV infection is characterized not only by immunodeficiency but also by immune activation. In fact, elevated levels of CD38 expression in $CD8^+$ T cells were already described in the initial reports of AIDS in 1981 (Gottlieb et al., 1981; Masur et al., 1981).

Immune activation has recently been proposed as the major driving force behind the systemic decline of $CD4^+$ T cells in HIV infection (Grossman et al., 2006). The fact that activation marker expression correlates well with disease progression supports this idea (Silvestri and Feinberg, 2003; Sousa et al., 2002; Hazenberg et al., 2003). Moreover patients in whom highly active antiretroviral therapy fails to reduce viral loads, but who have low levels of T cell activation, show a continuous increase in $CD4^+$ T cells (Hazenberg et al., 2002; Deeks et al., 2002). Conversely, patients with high levels of immune activation despite viral load suppression have poor gains in $CD4^+$ T cell counts (Hunt et al., 2003). Additionally, SIV-infected sooty mangabeys and African green monkeys do not usually develop AIDS-like disease despite high viral loads. Contrary to SIV-infected macaques, they do not display marked immune activation (Silvestri et al., 2003; Kornfeld et al., 2005; Sumpter et al., 2007). Another piece of evidence comes from studies in the mouse. A transgenic mouse strain constitutively expressing the co-stimulatory molecule CD70 on B cells, shows persistent $CD4^+$ and $CD8^+$ T cell activation leading to excessive generation of

effector/memory $CD4^+$ and $CD8^+$ T cells and the gradual depletion of naïve $CD4^+$ and $CD8^+$ T cell pools. Notably, these mice develop lethal immunodeficiency (Tesselaar et al., 2003).

4.3.2. Immune activation in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice

Immune activation in HIV-infected individuals is manifested not only by an increase in activation and turnover of immune cells, but also by an enlargement of the lymph nodes and by increased serum levels of inflammatory factors.

Tnfrsf4$^{Cre/+}$ *R26*$^{Dta/+}$ mice show systemic and dramatic lymphadenopathy. This condition occurs in many inflammatory and infectious states and has been proposed to be dependent on dendritic cell-mediated endothelial cell proliferation and induction of high endothelial venules and consequent recruitment of circulating cells in the lymph nodes (Webster et al., 2006). However, mice lacking regulatory T cells such as *FoxP3*$^{-/-}$ (Fontenot et al., 2003) or the spontaneous mutant *Scurfy* (Brunkow et al., 2001) or mice with an inducible regulatory T cell deficiency (Kim et al., 2007; Lund et al., 2008) also show enlarged lymph nodes. Whether this enlargement is the result of the generalized inflammatory state of these mice, or is a direct consequence of the lack of regulation by regulatory T cells is open to debate.

Interestingly, although *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice have a deficit in the regulatory $CD4^+$ T cell compartment, they do not develop the severe pathology which includes wasting disease, failure to thrive, and inflammatory, autoimmune and allergic manifestations that is common in mice lacking regulatory T cells. Indeed *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice do not show developmental problems or obvious signs of autoimmunity or

inflammation. One obvious difference between *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ and regulatory T cell-deficient mice that could explain this finding is that in the latter all regulatory T cells are depleted, while in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice depletion is not complete but restricted to ~ 40% of these cells. The remaining cells however are activated and presumably have a strong suppressive effect. Furthermore, and perhaps more importantly, in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice both regulatory and activated/memory cells are targeted. In the *FoxP3*$^{-/-}$ or the natural mutant *Scurfy* it has been shown that the autoimmunity phenotype depends on a population of FoxP3^{-}CD4^{+} T cells with self-reactive TCR that act as effectors in the absence of regulation by regulatory T cells (Blair et al., 1994). It is thus possible that the effect of DTA depletion on possibly pathogenic non-regulatory T cells blocks the development of overt disease in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice.

Mice deficient in regulatory T cells have excessive T cell activation and consequent excessive secretion of proinflammatory mediators such as TNFα (Kanangat et al., 1996), IL-13, GM-CSF (Kim et al., 2007) and CCL2 (MCP-1), CXCL9 and CXCL10 (Lund et al., 2008). Similarly, *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice have increased levels of these and other proinflammatory cytokines and chemokines in serum. Cytokines and chemokines create complex networks of signals for cells and the increase in specific cytokine and chemokines in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice is probably only part of the picture, but it is nevertheless the reflection of a systemic increase in activation of the immune system.

Interestingly, HIV infection is also associated with increased levels of circulating inflammatory chemokines (Reinhart, 2003). Specifically, elevated levels of plasma

CXCL9 and CXCL10 have been observed in HIV-infected individuals with stable, chronic infection and with advanced disease when compared to uninfected controls (Brainard et al., 2007). IL-1α, IL-1β, IL-12 and IFNγ have been shown to be 4 to 12 times increased in HIV-infected tonsil tissue when compared to uninfected controls, and were reduced after 4 weeks of HAART (Andersson et al., 1998).

4.3.2.1. Immune activation of CD8 T cells

Cellular activation is mostly defined by the expression of markers on the cell surface that are indicative of activation and also by increased turnover rate.

In HIV infection the increase in total $CD8^+$ T cell counts in the asymptomatic period is primarily due to an expansion of memory cells (Roederer et al., 1995). In addition, $CD8^+$ T cells express activation markers and have higher turnover rates (Sachsenberg et al., 1998) and more recently it has been shown that the increased turnover of $CD8^+$ T cells involves effector-memory cells, while normal levels of turnover are observed for both naïve and central memory T cells (Hellerstein et al., 2003). Importantly, $CD8^+$ T cell activation is a better correlate for disease progression than viral loads or $CD4^+$ T cell counts.

We showed that the $CD8^+$ T cell population in the lymph nodes of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice was increased when compared with control mice, and this was also due to increased numbers of memory $CD8^+$ T cells. These cells express lower levels of CD62L, which is expressed in central memory but not effector memory $CD8^+$ T cells, and higher levels of CD43 activation marker consistent with a phenotype of activated/effector memory $CD8^+$ T cells. The $CD8^+$ T cell population of *Tnfrsf4*$^{Cre/+}$

R26$^{Dta/+}$ mice is thus activated, enriched in effector memory cells and with higher turnover rates, similar to the situation in HIV infection.

Causes of immune activation

Many factors have been proposed to drive immune activation, including HIV itself, microbes, cross reactivity to apoptotic cells and regulatory T cell depletion.

HIV

HIV-1 derived single-stranded RNA sequences have been implicated in the activation of the immune system. One way by which microbial products can activate the immune system is via interactions with toll-like receptors (TLRs) (Janeway, Jr. and Medzhitov, 2002). HIV-derived uridine-rich sequences have been shown to interact with TLRs. When $CD4^+$ and $CD8^+$ T cells were stimulated with these sequences *in vitro*, they upregulated CD69, a marker of recent activation (Meier et al., 2007). However the study did not include data on CD38 or HLA-DR markers, which are the activation markers usually reported to be increased in HIV studies. In addition, assuming that monkeys have a pattern of TLR expression similar to humans, the same activation should occur with SIV. However, natural hosts do not have immune activation. In this respect, it has been suggested that plasmacytoid dendritic cells (pDCs), the major IFN-α producing cells in response to viral infections, from sooty mangabeys stimulated with SIV failed to produce IFN-α, but were however responsive to influenza virus. In contrast, pDCs from rhesus macaques, were fully responsive to SIV. (Keystone Symposia, Determinants of Host resistance, Succeptibility or Immunopathology to Pathogens: Integrating knowledge from experimental models to human disease, 2006. Poster abstract 145, Klucking, S et al,

Inneficient production of IFN-α by pDCs is involved in the limited immune activation and AIDS-resistance in naturally SIV-infected SMs; Speaker abstract 006, Staprans S et al , Host-virus relationships in non-pathogenic SIV infections).

It is thus possible that the differential signalling by TLRs of sooty mangabeys may result in attenuated response of the innate system and may explain the low levels of immune activation in these animals. Interestingly, another SIV natural host, the African green monkey, has been shown to have an early anti-inflammatory response in response to SIV infection, by producing high levels of TGF-β and IL-10 which could as well explain the lack of immune activation in this species (Kornfeld et al., 2005).

In any case, a direct effect of viral infection in immune activation can be excluded in our virus-free model, indicating that other causes must be responsible for the immune activation in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice.

Other microbes

The drop in $CD4^+$ T cell counts and the inflammatory conditions occurring during the acute phase of HIV infection may favour the reactivation and replication of latent forms of CMV and Epstein-Barr virus (EBV) (Papagno et al., 2004). In addition, the chronic loss of $CD4^+$ in the asymptomatic phase of the disease renders the immune system less capable of fighting microbes and HIV individuals are more susceptible to widespread diseases such as malaria, tuberculosis, pneumonia, etc. This heightened exposure to microbes has been proposed to contribute to generalized immune activation.

To address this issue, cells were stimulated *in vitro* with several TLR ligands. Stimulation lead to expression of the activation marker CD38 in $CD4^+$ and $CD8^+$ T cells but not HLA-DR (Funderburg et al., 2008), suggesting that systemic exposure to a number of microbial TLR agonists could drive, at least partly, immune activation in HIV.

One way by which microbes can have access to the immune cells is through 'leaky' mucosas. It has been proposed that the early depletion of $CD4^+$ T cells from the gut mucosa in HIV infection is critical to disease pathogenesis (Brenchley et al., 2006). According to this study, the direct cytopathic effects of HIV on $CD4^+CCR5^+$ T cells are the cause of the depletion which in turn compromises the gut mucosa, allowing commensal bacteria and/or their products to leak into the circulation by a phenomenon known as microbial translocation, which then leads to systemic activation. In support of this, plasma levels of lipopolysaccharide (LPS), a component of Gram negative bacteria, which can be used as a quantitative indicator of microbial translocation, are significantly elevated in individuals with chronic HIV infection and AIDS and the same is true for SIV-infected macaques but not for sooty mangabeys. However, people with acute/early HIV infection did not show elevated LPS levels. This could be explained by the presence of anti-LPS antibodies which wane over time as individuals with HIV become more immunodeficient.

It has been argued that the depletion of $CD4^+$ T cells from the gut is unlikely to be very important because the majority of these cells are short-lived effectors and the ability to replenish the gut is maintained (Grossman et al., 2006). Supporting this

argument is the fact that a similar depletion occurs in SIV-infected sooty mangabeys and African green monkeys, but these animals almost never develop immunodeficiency or immune activation (Gordon et al., 2007; Pandrea et al., 2007).

More recently it has been shown that T_H17 cells, a recently described $CD4^+$ T cell subset that produces IL-17 and IL-22 and is involved in antimicrobial immunity at mucosal surfaces (Steinman, 2007), express CCR5 and are preferentially lost from the gastrointestinal tract of HIV-infected individuals and SIV-infected rhesus macaques but are maintained in SIV-infected sooty mangabeys (Brenchley et al., 2008; Raffatellu et al., 2008). Thus, depletion of T_H17 cells can be the critical mechanism to explain loss of bacteria control and microbial translocation in pathogenic lentiviral infections.

Although histological analysis performed on *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice did not reveal alterations in the morphology or cellular composition of the intestinal mucosa, we cannot definitely exclude that a small degree of microbial translocation is occurring in the *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice and that this is the mechanism involved in the $CD8^+$ T cell activation in these mice.

We do not favour this possibility because in a mouse model of severe microbial translocation, the *Ikbkg*$^{fl/-}$ *Vil-Cre* mice, the major immune compartment affected is the monocytic compartment rather than $CD8^+$ T cells. However to rule out a role of microbial translocation in the *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice, we are crossing them to $MyD88^{-/-}$ mice. MyD88 is an adaptor molecule essential for most TLR-mediated induction of inflammatory cytokines (Takeda and Akira, 2003). Although microbes

may trigger immune cells through pathways other than TLRs (Robinson et al., 2006), and a few TLRs can signal through Myd88-independent pathways (Kenny and O'Neill, 2008), MyD88 seems nevertheless to be essential for microbial translocation induced pathology, as evidenced by the fact that *Ikbkg*$^{fl/-}$ *Vil-Cre* mice rendered MyD88 deficient are free of any signs of disease. Ultimately, this issue can only be resolved by generating germ-free *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice.

Apoptotic/necrotic cells

The continuous turnover of apoptotic cells has been proposed to generate fragments of cellular proteins cleaved by caspases which are capable of priming self-reactive $CD8^+$ T cells. In HIV infection, the magnitude of the $CD8^+$ T cell response directed against this apoptosis-derived peptides correlates with the decline in $CD4^+$ T cell counts suggesting that these self-reactive $CD8^+$ T cells can contribute to the systemic chronic immune activation (Rawson et al., 2007).

In *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice this could be a potential cause of chronic immune activation, since in this system there is a continuous deletion of $CD4^+$ T cells by DTA-mediated apoptosis. However, in mixed bone marrow chimeras, where there is a continuous depletion of $CD4^+$ T cells, there is no evidence of $CD8^+$ T cell activation. Thus, it is unlikely that this mechanism is involved in the $CD8^+$ T cell immune activation seen in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice.

Regulatory T cells

Lack of immunosuppression by progressive loss of regulatory T cells during HIV infection has also been proposed as a possible mechanism of chronic immune

activation. Many studies have addressed the role of regulatory T cells in HIV and SIV infection but there is still controversy as to whether they are beneficial or detrimental. Although it is clear that regulatory T cells can be infected and killed by HIV (Oswald-Richter et al., 2004; Antons et al., 2008), there is no consensus as to whether they are increased (Weiss et al., 2004; Tsunemi et al., 2005; Tsunemi et al., 2005; Kinter et al., 2004; Montes et al., 2006), decreased (Oswald-Richter et al., 2004; Apoil et al., 2005) or unchanged (Aandahl et al., 2004) in the course of HIV infection. One of the reasons that could explain this discrepancy is the fact that some studies present data as proportion of $CD4^+$ T cells rather than absolute numbers. Since total $CD4^+$ T cells are diminished in HIV infection, the expression of regulatory T cells as percentage of $CD4^+$ T cells will not reflect their loss. Indeed one study reported decreased absolute numbers but increased numbers of regulatory T cells as percentage of $CD4^+$ T cells (Eggena et al., 2005). The inconsistency in the results may also be partly explained by the markers used to define this subset. Many studies have distinguished regulatory cells by the expression of CD25, but activated effector cells also express this marker transiently. Other studies have classified regulatory T cells according to the level of CD25 expression which has been shown to roughly divide activated T cells (low CD25 expression) from regulatory T cells (high CD25 expression) in the peripheral blood of healthy subjects (Baecher-Allan et al., 2001). Indeed, Foxp3 is expressed in more than 95% of peripheral blood $CD4^+CD25^{high}$ but in only 35% of $CD4^+CD25^{int}$ (Ziegler, 2006) supporting the notion that most regulatory T cells would be included in the fraction of $CD4^+$ T cells expressing high intensity of CD25. Another important point to consider is that changes observed in peripheral blood are not representative of what is happening in the lymphoid tissues or intestinal mucosa but rather are the consequence of redistribution of regulatory T cells to tissues with active replication

(Epple et al., 2006; Nilsson et al., 2006; Andersson et al., 2005; Kinter et al., 2007). Moreover, the stage of disease at the time of sampling can also contribute to the disparity. Finally, it should be pointed out that classification of cells according to surface markers not necessarily correlates with functional activity of these cells, and it has been shown that functional regulatory T cells decrease with disease progression and increasing viral loads (Kinter et al., 2004).

Considering all this, it is maybe not surprising that the role of regulatory T cells during HIV infection and pathogenesis is controversial. While some studies point to a beneficial role of regulatory T cells in limiting the overall immune activation (Kinter et al., 2004; Oswald-Richter et al., 2004; Tsunemi et al., 2005; Apoil et al., 2005; Eggena et al., 2005; Baker et al., 2007; Chase et al., 2008), others suggest that these cells are detrimental by impairing T cell responses and thus facilitating HIV persistence (Weiss et al., 2004; Aandahl et al., 2004; Andersson et al., 2005; Nilsson et al., 2006; Lim et al., 2007).

Studies in non-human primates have not been able to clarify this issue. Some studies point for a detrimental role (Boasso et al., 2007; Estes et al., 2006; Hartigan-O'Connor et al., 2007) while others point to a beneficial role (Li et al., 2005; Pereira et al., 2007; Kornfeld et al., 2005; Chase et al., 2007) of regulatory T cells in the control of SIV infection.

The decreased number of regulatory T cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice could imply less regulation and consequently activation of the $CD8^+$ population. If regulatory T cells are responsible for the $CD8^+$ T cells phenotype of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice,

then transfer of WT regulatory T cells should be able to revert the phenotype. Indeed, when we transferred WT regulatory T cells into *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ and control mice we observed first that these cells failed to proliferate when transferred to *Tnfrsf4*$^{Cre/+}$ *R26*$^{+/+}$ full recipient mice, but did so in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ recipients, confirming that these cells are capable of homeostatic proliferation in regulatory T cells-lymphopenic hosts (Annacker et al., 2001; Gavin et al., 2002). Second, the levels of $CD8^+$ memory cells in the blood started decreasing at 1 week and reached wild-type levels by week 3 which were maintained 4 weeks later. This was also true for the activated/effector memory $CD8^+$ T cells which reach percentages similar to control mice in lymphoid tissues. Thus, the immune activation seen in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice is a consequence of the deficit in the regulatory T cell population.

Some papers have implicated a role for CD25 cells on the suppression of memory $CD8^+$ T cells proliferation and activation (Murakami et al., 2002; Piccirillo and Shevach, 2001; Camara et al., 2003) while others pointed to a role of regulatory T cells in preventing $CD8^+$ T cell–mediated autoimmunity (Poitrasson-Riviere et al., 2008). Regulatory cells use multiple mechanisms to mediate their suppressive function, and specifically for what concerns their effect on $CD8^+$ T cells, the following mechanisms have been proposed: consumption of IL-2 (Murakami et al., 2002), suppression of DC maturation and function (Kim et al., 2007) and production of TGFβ (Mempel et al., 2006).

If immune activation in HIV and SIV is due to a progressive depletion of regulatory T cells, how can it be explained that non pathogenic SIV infected animals do not show immune activation? It is generally accepted that regulatory T cells are depleted during

HIV and SIV infection although to our knowledge there are no studies addressing if regulatory T cells from non-human primates are infected and killed by SIV in non-pathogenic natural infections. Interestingly, it has recently been show that T_H17 are depleted in pathogenic, but not in non-pathogenic SIV infections (Raffatellu et al., 2008; Brenchley et al., 2008). We could speculate that the same is true for regulatory T cells. It could be that regulatory T cells in natural hosts of SIV are more resistant to infection, perhaps due to reduced levels of CCR5 expression, or, to virus-mediated killing. Alternatively, it could be that regulatory T cells in these hosts are replenished more efficiently than those in humans. Indeed, it has been shown that SIV infection influences the level and function of regulatory T cells in rhesus macaques but not sooty mangabeys (Pereira et al., 2007).

4.3.2.2. B cells

Polyclonal hypergammaglobulinemia is a characteristic of chronic inflammatory conditions such as persisting viral infections and autoimmune diseases (Hunziker et al., 2003) and together with B cell hyperactivation is one of the hallmarks of HIV-1 infection. However we did not observe any evidence of B cell activation other than an apparently innocuous increase in the number of germinal centre B cells in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice, suggesting that $CD4^+$ depletion *per se* in not responsible or is not sufficient to activate this population.

Consistent with this hypothesis, a recent study has involved the HIV-1 Nef protein as the factor responsible for the B lymphocyte hyperactivation and hypergammaglobulinemia observed in HIV-1 patients (Swingler et al., 2008). The

study shows that Nef activates ferritin production in macrophages which is necessary and sufficient for the above mentioned effects on B cells. In HIV-1 infected individuals, plasma viral RNA load correlates well with ferritin and immunoglobulin levels but not with immune activation. In addition, similar correlations were observed in mice transgenic for HIV-1 Nef suggesting that the elevated levels of ferritin and consequent B cell dysfunction are a direct result of Nef expression due to viral replication and excludes a role of immune activation in the phenotype of B cells (Swingler et al., 2008).

Why are then B cell numbers increased in *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice? One possibility is the lack of negative regulation by regulatory T cells. In a study where regulatory T cells were depleted, B cells were shown to be increased by 3 fold in the spleen and 1.8 fold in the lymph nodes (Kim et al., 2007) but this was most likely due to the generalized inflammatory state. The increase in B cell numbers could thus be an indirect consequence of the increase in lymph nodes size that is regulated by DCs (Webster et al., 2006).

4.3.2.3. Macrophages

Cells of the monocyte/macrophage lineage disseminate the virus and serve as ubiquitous reservoirs for HIV-1. Certain tissue-specific macrophage populations have been shown to be increased in numbers and activation state in HIV-1 infection (Lee et al., 2003; Evans and Wansbrough-Jones, 1996) although this has not been confirmed at the systemic level. Macrophage numbers are thought to be stable during HIV-1 infection and to be a significant source of virus production when $CD4^+$ T cell counts

are low (Orenstein et al., 1997). Similarly, infection of rhesus macaques with a chimeric HIV/SIV was associated with a gradual increase in macrophages over time, which were responsible for virus production at the time of $CD4^+$ T cell loss (Igarashi et al., 2001).

Our analysis of *Tnfrsf4*$^{Cre/+}$ *R26*$^{Dta/+}$ mice did not reveal overt macrophage activation. The small increase in numbers of macrophages in lymph nodes of these mice is proportional to the increase in total cells and probably just reflects the fact that lymph nodes are enlarged. In contrast, macrophage numbers were dramatically elevated in mice with primary microbial translocation through the intestinal mucosa. It thus appears that numbers of macrophages are more responsive to microbial exposure than regulatory T cell function. Interestingly, increased viremia often seen during opportunistic infection of HIV-1 infected individuals has been attributed to macrophage activation (Orenstein et al., 1997).

5. Concluding remarks

The $CD4^+$ T cell compartment is complex and heterogeneous, comprising several lineages and maturation/differentiation states that differ in their life-spans, capacity for self-renewal, and functional properties in the immune system. At least three distinct major subsets can be identified: naïve, memory and regulatory T cells. Memory and the regulatory subsets are heterogeneous. Memory cells can be classified as T_{EM} and T_{CM} while regulatory T cells can also be divided in naïve and effector/memory. Additionally, a fourth population of effector cells is transiently produced in the course of an immune response.

These subsets are differentially targeted by HIV-1 infection and other immunodeficiency viruses. The fact that all retroviruses capable of causing immunodeficiency target the same cells indicates a central role of these cells in the pathogenesis of immunodeficiency disease. To test this hypothesis, our approach was to target the same cells in a virus-free murine model and assess whether specific depletion of these populations could cause disease. We were able to show a causal link between deletion of these cells and disease. Specifically, we were able to show that regulatory $CD4^+$ T cell killing leads to generalized immune activation and that effector/memory $CD4^+$ T cell killing results in immunodeficiency. Thus, the complexity of the dysfunction in HIV is a pure reflexion of the functional heterogeneity of its targets.

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