Role and Specificity of Regulatory T Cells During Retroviral Infection

Inês Lopes Antunes

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Supervisor: Dr George Kassiotis

Division of Immunoregulation
MRC National Institute for Medical Research
Mill Hill, London NW7 1AA

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I, Inês Lopes Antunes, 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

During chronic viral infection of the hematopoietic system, both virus-mediated and immune-mediated effects may cause bone marrow dysfunction leading to cytopenias. To study the pathological consequences of the T helper response to unresolving chronic infection, a system was developed using infection of immunodeficient mice with Friend virus (FV). FV is a murine retroviral complex, which causes non-cytopathic persistent infection of hematopoietic cells. The results obtained suggested that unregulated $CD4^+$ T cell response to FV causes bone marrow pathology and anaemia.

Regulatory T (Treg) cells are a subset of $CD4^+$ T cells which have been shown to suppress immune responses and to have protective roles in other models of bone marrow pathology. Therefore, the role of Treg cells in the model of FV-induced immune pathology was addressed. Bone marrow pathology was triggered by local gamma interferon (IFN-γ) production by FV-specific $CD4^+$ T cells and was associated with relative low numbers of Treg cells, while enrichment of the Treg cell population protected against development of the immune pathology, by suppressing IFN-γ production by pathogenic $CD4^+$ T cells.

The specificity of Treg cells is still a matter of controversy, with studies suggesting they are mainly self-reactive while other studies indicate they can be reactive to foreign antigens. The issue of Treg cell specificity was addressed in the FV-induced immune

pathology model. Analysis of mice transgenically expressing the TCRβ chain of a FV-specific $CD4^+$ T cell clone, which harbor a polyclonal TCR repertoire with increased frequency of FV-specific $CD4^+$ T cells, indicated that the TCR repertoire of Treg cells in virus-naïve mice was virtually devoid of FV-specific clones. Moreover, FV infection did not cause expansion of a small number of virus-specific Treg cells or conversion of virus-specific effector T cells into Forkhead box P3 (FoxP3)-expressing Treg cells. Importantly, pathogenic $CD4^+$ T cells and Treg cells differed dramatically in their requirements for direct recognition of viral antigens, since bone marrow pathology driven by FV-specific TCRβ-transgenic $CD4^+$ T cells was efficiently suppressed by virus-nonspecific Treg cells. Therefore, protection from bone marrow pathology in chronic viral infections may be provided by sufficient numbers of polyclonal Treg cells.

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To my grandfather, who will always be part of who I am.

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Abbreviations

aa	Amino acid
AB	Air-Buffered
Ab	Antibody
ACK	Ammonium-chloride-potassium
AIDS	Acquired immunodeficiency syndrome
AIRE	Autoimmune regulator
APC	Antigen presenting cells
B6	C57BL/6
BFU-E	Burst-forming units-erythroid
BM	Bone marrow
cAMP	Cyclic adenosine monophosphate
CCR	Chemokine (C-C motif) receptor
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
cfu	Colony-forming units
CFU-GM	Colony-forming units-granulocyte/macrophage
CIA	Collagen-induced arthritis
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CTLL	Cytotoxic T lymphocyte lines
CXCL	Chemokine (C-X-C motif) ligand
DC	Dendritic cell
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco's Phosphate Buffered Saline
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveoretinitis
EBV	Epstein-Barr virus
EGFP	Enhanced green fluorescent protein

env	Envelope
FACS	Fluorescence activated cell sorter
FcR	Crystallizable fragment receptor
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FIV	Feline immunodeficiency virus
F-MuLV	Friend murine leukaemia virus
FoxP3	Forkhead box P3
FR4	Folate receptor 4
FV	Friend virus
GFP	Green fluorescent protein
glyco-Gag	Glycosylated product of the viral gag gene
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft-versus-host disease
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HSV	Herpes Simplex virus
i.p.	Intra-peritoneally
i.v.	Intravenously
IBD	Inflammatory bowel disease
ICOS	Inducible costimulatory
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IPEX	Immunodysregulation, polyendocrinopathy and enteropathy X-linked
iu	Infectious units
L. major	*Leishmania major*
LAG-3	Lymphocyte activation gene 3
LDV	Lactate dehydrogenase-elevating virus

LN	Lymph nodes
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MCP	Monocyte chemotactic protein
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MIG	Monokine induced by IFNγ
MIP	Macrophage inflammatory protein
MMTV	Mouse mammary tumour viruses
NK	Natural killer
NOD	Nonobese diabetic
OVA	Ovalbumin
PBS	Phosphate-buffered saline
PC	*Pneumocystis carinii*
RA	Retinoic acid
RAG	Recombination activating gene
RNA	Ribonucleic acid
RT	Room temperature
SCID	Severe combined immunodeficiency
SEM	Standard error of the mean
SFFU	Spleen focus-forming units
SFFV	Spleen focus-forming virus
SIV	Simian immunodeficiency viruses
SP	Spleen
T1D	Type 1 diabetes
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper type cell
TLR	Toll-like receptor

TNF	Tumour necrosis factor
Tr1	T regulatory type 1
Treg	Regulatory T
U	Units
v/v	Volume/volume
VEGF	Vascular endothelial growth factor
w/v	Weight/volume
WT	Wild-type

Chapter 1

Introduction

1.1 Historical perspective on regulatory T cells

The idea of suppressor T cells has been present in immunology since the early 1970s, when it was published that a specific subset of T cells (at that time called suppressor T cells) was able to suppress immune responses (Gershon and Kondo, 1970).

In 1969 a seminal study showed the existence of T cells with autoimmune-suppressive activity: nenonatal thymectomy between day 2 and 4 after birth resulted in destruction of the ovaries (Nishizuka and Sakakura, 1969), which was later found to be the result of an autoimmune reaction. Subsequent studies have shown that thymectomy on day 3 after birth leads to the development of thyroiditis, gastritis, orchitis, prostatitis, and sialadenitis (Kojima and Prehn, 1981). It was also shown in similar experimental systems that reconstitution of thymectomized rats with normal syngeneic T cells ($CD4^+$ T cells) would prevent the development of the diseases (Penhale et al., 1976). Conversely, transfer of $CD4^+$ T cells from mice which had developed autoimmunity was able to induce the disease in T cell-deficient hosts (Sakaguchi et al., 1982). Together, these findings suggested that the thymus normally produces different populations of $CD4^+$ T cells which co-exist in the periphery in normal mice. Thymectomy shortly after birth abrogates the

development of the suppressor cells, allowing for spontaneous activation of self-reactive T cells (Sakaguchi et al., 2007). However, by the 1980s, the notion of suppressor T cells attracted criticism due to the lack of reliable markers for their identification and the poor knowledge of the molecular mechanisms of their function. The existence of a specialized suppressor T cell population was then faced with scepticism and gradually the interest in the suppressor T cell field was lost.

It was owing to advances in identification of distinct $CD4^+$ T cells populations that the suppressor T cells were reborn again, gaining a more subtle name as regulatory T cells (Maloy and Powrie, 2001). CD5 and CD45RB (Sakaguchi et al., 1985; Powrie et al., 1993) were the first markers shown to distinguish the pathogenic and regulatory $CD4^+$ T cells subpopulations. Soon after, CD25 proved to be a unique surface marker able to define a pure suppressive T cell population (Sakaguchi et al., 1995) and it is still today the best extracellular marker available to identify Treg cells. A recent milestone in the field of Treg cells was the discovery of the transcription factor Forkhead box P3 (FoxP3), which is considered the best marker for Treg cells and seems to be the main determinant in their development and function (Tang and Bluestone, 2008). The reliable identification of Treg cells by expression of CD25 and FoxP3 has allowed intensive studies on the role and mechanisms of function of these crucial regulators of immune responses.

During evolution, the high versatility and potency of the immune responses lead to the development of mechanisms that tightly regulate the immune system. It is therefore not

surprising that different populations with suppressive functions are found in the organisms. They include FoxP3$^+$ CD4$^+$ T cells but also FoxP3$^-$ CD4$^+$ T cells, namely T helper type 3 (Th3) and T regulatory type 1 (Tr1) cells (O'Garra and Vieira, 2004). In addition, suppressor CD8$^+$ T cells, natural killer T cells and γ/δ T cells have also been shown to regulate immunity (Shevach, 2006).

This study focused on FoxP3$^+$ CD4$^+$ regulatory T cells present in normal conditions, which make up between 5-10% of total CD4$^+$ T cells in mice and humans. The definition used for regulatory T cells was of a specialized FoxP3-expressing CD4$^+$ T cell population with suppressive functions obtained from naïve, unmanipulated mice.

1.2 Role of regulatory T cells in controlling immune responses

1.2.1 Regulatory T cells in autoreactive immune responses

Protection from infections is essential for the survival of all animals and is achieved through the immune system, which has evolved both innate and adaptive mechanisms to deal with invading pathogens. The high variability of unanticipated pathogens that need recognition demands the immune system to maintain a high degree of flexibility in the targets it recognizes. This is achieved by developing a diverse receptor repertoire, capable of recognizing a multitude of antigens and then expanding as effector cell populations those that can recognize molecules from the pathogens. With such a low stringency, potentially self-reactive T cells capable of causing autoimmunity develop in the organisms, creating the need for mechanisms limiting inflammation in this setting (Cohn, 2004). Several mechanisms have been proposed that deal with these pathogenic self-reactive lymphocytes. For example, they may be rendered anergic or further deleted upon encounter with self-antigens (Zajac et al., 1998; Moskophidis et al., 1993). Furthermore, self-reactive T cells may fail to be activated (i.e., they ignore self-antigens) because of low avidities of their TCRs for self-antigens, lack of costimulation from antigen-presenting cells (APCs), or their seclusion from the target self-antigens (Zinkernagel and Hengartner, 2001).

In addition to the above mechanisms, the reactivity of antigen-specific cells can also be regulated by suppressive cell types, such as Treg cells, which are responsible for restraining the activation and expansion of autoreactive lymphocytes (Sakaguchi et al., 2008).

The concept that Treg cells have an important role in protection from autoimmune reactions has been present since the initial studies of suppressor cells, where neonatal thymectomy resulted in a fatal autoimmune reaction (Nishizuka and Sakakura, 1969) until the more recent analysis of the Scurfy mice, which develop fatal autoimmune disease due to the lack of Treg cells (Brunkow et al., 2001).

A vast amount of evidence has been accumulated showing an important role for Treg cells in the prevention of autoimmune reactions. The nonobese diabetic (NOD) mouse represents a model of immune dysregulation since it spontaneously develops several autoimmune diseases, including type 1 diabetes (T1D) (Delovitch and Singh, 1997). It was shown that block of Treg cell development leads to accelerated onset of diabetes, which can be reversed by transfer of Treg cells (Salomon et al., 2000). Collagen-induced arthritis (CIA) is a T cell-mediated autoimmune disease. This model was used to demonstrate that depletion of Treg cells prior to collagen immunization increased the severity of the disease and was associated with increased anti-collagen response. Adoptive transfer of Treg cells into CD25-depleted mice reversed the severity of the disease, proving a protective role for Treg cells in this autoimmune disease (Morgan et al., 2003). In experimental autoimmune encephalomyelitis (EAE) Treg cells have been

shown to limit several stages of the disease. It was shown that enrichment of Treg cells could limit the disease (Kohm et al., 2002) and, conversely, Treg cell depletion would increase the severity of disease due to expansion of autoreactive effectors (Reddy et al., 2004). A similar scenario was observed in the mouse model of experimental autoimmune uveoretinitis (EAU), where supplementation of Treg cells suppressed the development of the disease (Keino et al., 2007).

These, and numerous similar studies, on the function of Treg cells in limiting autoimmune reactions, led to a general belief that Treg cells had evolved as a mechanism to suppress autoimmune responses and they would not be involved in the immune responses to pathogens. This idea gained support from studies unveiling the process of T cell thymic selection, which suggest Treg cells have the highest affinities for self-ligands (Jordan et al., 2001) (see chapter 1.5 Thymic selection of regulatory T cells) and would therefore be activated through TCR stimulation by self antigens (Hsieh et al., 2004).

1.2.2 Regulatory T cells in immune responses to foreign antigens

However, all immune responses, not just the autoreactive ones, need to be regulated; damage to the host's own cells and tissues is an inevitable side effect of immunity (therefore called immune pathology) and its extent depends on the strength and duration of the response. During infection, immune regulation can arise as a result of the host

response to the infection in an effort to maintain or restore a homeostatic environment and/or it can be actively induced by the pathogen to diminish the host response and promote its survival. The mechanisms described above for controlling inflammation during autoreactive responses also apply to responses against foreign antigens, are partly effective and act at different stages of the immune responses. These checkpoints need to be at a balance between preventing inflammation and not impairing immunity. Importantly, the immune system is unable to distinguish between benign and harmful signals and it is rather the quantity, quality and location of the stimulus, either self or nonself, that determines the strength of the response (Silverstein and Rose, 1997). For instance, exposure of T cells to high and persistent self or foreign antigen loads has been shown to result in functional exhaustion or physical loss of antigen-specific T cells (Moskophidis et al., 1993; Zajac et al., 1998). Given the inability of the immune system to discriminate between benign and harmful signals, it would be expected that Treg cells suppressing immune responses would also not make the distinction. This implies that the balance between effector and regulatory populations is critical for the proper control of the quality and magnitude of all adaptive immune responses.

It is now clearly established that Treg cells have important functions in a variety of immune responses to foreign antigens and pathogens, from viruses to parasites, where they may affect the magnitude of immunity and influence the course of infection. Treg cells have been shown to affect, among others, the immune pathology, the immune response and also pathogen clearance (Rouse et al., 2006; Suvas et al., 2003; Dittmer et al., 2004).

Treg cells have different potential effects during the course of infection. The interactions between a host and the pathogen range from uncontrolled pathogen growth to pathogen clearance. Excessive control of effector immune responses by Treg cells can lead to uncontrolled growth of the pathogen and eventual death of the host. At the other extreme of the host-pathogen interaction, the effector immune responses are high enough to efficiently eliminate the pathogen, but can lead to immune pathology. In this case, Treg cells may be beneficial to the host by controlling the strength of the immune responses and preventing immune pathology. Persistent infections are in the middle of this range. In these situations, Treg cells may be beneficial by controlling immune pathology, but they may also be detrimental by inducing systemic immunosuppression (Rouse et al., 2006; Belkaid et al., 2006; Suvas et al., 2003).

1.2.2.1 Models related to inflammatory bowel diseases

Inflammatory bowel diseases (IBD) are thought to arise due to dysregulated immune responses towards components of the intestinal bacterial flora (Pacholczyk et al., 2007), since susceptible mice kept under pathogen-free conditions fail to develop inflammatory diseases (Gad, 2005). In an experimental model of IBD, reconstitution of immunodeficient severe-combined immunodeficient (SCID) recipients with $CD4^{+}CD45RB^{high}$ T cells from wild-type mice leads to the development of colitis,

whereas cotransfer of the reciprocal $CD4^+CD45RB^{low}$ Treg cell population inhibits disease development (Powrie et al., 1993). Suppression was later shown to be mediated by IL-10, TGF-β and CTLA-4 (Maloy et al., 2003; Powrie et al., 1996; Read et al., 2000; Read et al., 2006).

Based on work from the Powrie laboratory, several groups have developed models in which inflammation is induced by oral infection of mice with defined bacterial species such as *Helicobacter hepaticus*. In this model it was shown that Treg cells would limit inflammation and colitis severity (Kullberg et al., 2002) and this result seems to apply to other bacteria-induced colitis models (Maloy et al., 2005).

1.2.2.2 Immune responses to other infections

1.2.2.2.1 Fungal infections

Pneumocystis carinii (PC) is a fungus which naturally infects laboratory mice. PC-infected T and B cell-immunodeficient mice do not display clinical signs of disease, but transfer of wild-type $CD4^+$ $CD25^-$ T cells leads to lethal pneumonia, which can be prevented by cotransfer of $CD4^+$ $CD25^+$ Treg cells although at the expense of higher pathogen load (Hori et al., 2002a). These results proved a role for Treg cells in the control of PC-mediated immune pathology. *Candida albicans* infection of mice seems to induce a Treg cell population which produces IL-4, IL-10 and TGF-β and is able to suppress Th1

responses against the infection. In the absence of Treg cells, mice are able to mount enhanced protective responses that are, however, accompanied by severe pathology (Montagnoli et al., 2002).

1.2.2.2.2 Bacterial infections

Induced Treg cells were found in the lungs of *Bordetella pertussis*-infected mice. These Treg cells secrete IL-10 and are able to suppress T cells responses *in vitro* and *in vivo* against the bacteria (McGuirk et al., 2002). In humans, $CD25^+$ Treg cells seem to be increased in numbers in *Mycobacterium tuberculosis*–infected patients (Guyot-Revol et al., 2006) and to suppress the immune responses direct against the bacterial antigens (Chen et al., 2007b). The induction of Treg cells during infection seems to be a common strategy exploited by the infectious pathogen to subvert immune responses *in vivo*.

1.2.2.2.3 Parasitic infections

Intradermal infection with *Leishmania major* (*L. major*) provokes strong local inflammation and accumulation of $CD4^+$ T cells in skin lesions and draining lymph nodes of mice. A Treg cell population producing IL-10 and with *in vitro* suppressive capacity was shown to be present in the skin lesions (Belkaid et al., 2002). These Treg cells are able to suppress the ability of effector T cells to eliminate the parasite from site, in an IL-

10-dependent and independent way. However, the sterilizing immunity achieved in mice with Treg cells with impaired function is followed by the loss of immunity to reinfection, indicating that the equilibrium between effector and Treg cells in sites of chronic infection might reflect both parasite and host survival strategies (Belkaid et al., 2006).

1.2.2.2.4 Viral infections

A role for Treg cells in viral infections has been clearly shown in both mice and humans. Hepatitis C virus (HCV) causes persistent chronic infection in humans. A recent study with HCV-infected patients has shown that this infection induces virus-specific $FoxP3^+$ Treg cells (Ebinuma et al., 2008). Furthermore, Treg cells from infected patients where shown to suppress the expansion and perforin expression of HCV-specific $CD8^+$ T cells *in vitro* (Rushbrook et al., 2005), suggesting a role for Treg cells in viral persistence.

Mice that were depleted of Treg cells and infected with Herpes Simplex virus (HSV) showed elevated $CD8^+$ T cell responses, which persisted for longer *in vivo*. Furthermore, Treg cells from HSV-infected mice were able to suppress $CD8^+$ T cells proliferation *in vitro* (Suvas et al., 2003), confirming the capacity of Treg cells to suppress virus-specific $CD8^+$ T cell responses. Furthermore, in a model of immunological lesion induced by HSV infection in the eye it was shown that the presence of Treg cells had a protective effect against development of disease (Suvas et al., 2004).

Although Treg cells function in the immune responses to infection has always been associated with a suppressive role of effector T cells and consequently diminished immune responses and increased pathogen load, a recent study has unveiled a novel role for Treg cells in the fight against pathogens during the early stages of infection. The Rudensky laboratory has shown that Treg cells present in the lymph nodes of HSV-infected mice suppress proinflammatory cytokines and chemokines and prevent influx and activation of effector cells, such as NK cells and DCs. This allows migration of these cells out of the lymph nodes and into the site of infection, where an effective response is initiated (Lund et al., 2008). Therefore this study suggests for the first time that the suppressive effect of Treg cells in the lymph nodes may potentiate and optimize the immune responses at the site of infection.

Studies so far suggest that Treg cells may also play an important role in infections by retroviruses. In the Friend virus (FV) mouse model of retroviral infection, Treg cells seem to be induced in the periphery and to suppress the $CD8^+$ T cell function, which can be related to viral persistence (Dittmer et al., 2004; Robertson et al., 2006) (see chapter 1.7.2 Regulatory T cells in Friend virus infection). Human Immunodeficiency virus (HIV) infection is characterized by a chronic state of T-cell hyperactivation, viral persistence and T cell depletion, leading to severe immunodeficiency (Nixon et al., 2005). HIV infection studies have yielded conflicting results regarding Treg cell function. Some suggest that Treg cells are depleted during infection and that this is a major contributor to the immune activation observed, suggesting an association between Treg cell depletion and HIV disease progression (Eggena et al., 2005; Oswald-Richter et al.,

2004). Other studies suggest that Treg cells mediate suppression of HIV-specific responses throughout the course of infection, thus impairing the ability to control viral replication (Epple et al., 2006; Kinter et al., 2007).

1.3 Mechanisms of regulatory T cell function

1.3.1 Cellular types regulated by regulatory T cells

Treg cells can have both direct suppressive effects over effector cells or they can modulate the maturation and/or function of APCs, which will affect effector function. Treg cells have been most thoroughly studied with regard to their effects on either $CD4^+$ or $CD8^+$ T cells, where they have been implicated in the control of proliferation, differentiation and effector function (Thornton and Shevach, 1998; Piccirillo and Shevach, 2001; Chen et al., 2005). However, Treg cell action goes beyond other T cells and they can influence other cells of the adaptive or innate immune system. A direct effect of Treg cells on B cells by inhibiting immunoglobulin production and class switch (Lim et al., 2005) and by increasing cell death of antigen-presenting B cells has been shown (Zhao et al., 2006). In an innate immunity-dependent model of colitis, infection of B and T cell immunodeficient *Rag*$^{-/-}$ mice with *Helicobacter hepaticus* caused IBD, which could be prevented by transfer of Treg cells (Maloy et al., 2003), showing their capacity to act on the innate immune system. Treg cells have been shown to directly interact with dendritic cells (Tang et al., 2006) and downregulate the expression of costimulatory molecules, thus reducing their stimulatory capacity (Misra et al., 2004; Tang et al., 2006).

1.3.2 Cellular functions regulated by regulatory T cells

Not only do Treg cells act on different immune cell subsets, but they can also inhibit multiple stages of target cell activity, such as proliferation, differentiation and effector function. The standard *in vitro* suppression assays have shown that Treg cells are able to suppress proliferation and IL-2 production by T cells (Takahashi et al., 1998). Furthermore, it was shown that target T cells undergo initial activation (upregulation of CD69 and CD25) and secrete IL-2, but the presence of Treg cells leads to the premature termination of the activation programme (Sojka et al., 2005). Recent data have also shown *in vitro* and *in vivo* that Treg cells reduce the duration of the contacts between the antigen-specific $CD4^+$ T cells and the antigen-loaded DCs (Tang et al., 2006). In addition to disruption of early activation events, Treg cells can also block T and B cell differentiation and effector function. In fact, many *in vivo* studies have associated Treg cell function with suppression of effector function at the inflammation site, while observing no differences in the expansion of the antigen-specific cells (DiPaolo et al., 2005; Chen et al., 2005; Sarween et al., 2004; Xu et al., 2003; Mempel et al., 2006; Dittmer et al., 2004). DiPaolo et al. have analyzed the effects of Treg cells on the development of gastritis and found they did not inhibit the expansion of the reactive T cells but their primary effect appeared to be inhibition of differentiation into effector T cells, which reflected in a decrease in IFN-γ production and Th1 differentiation (DiPaolo et al., 2005). Using a mouse model of $CD8^+$ T cell-mediated tumour rejection, it was found that $CD8^+$ T cells expanded to the same extent and produced similar levels of IFN-

γ in the presence of Treg cells but their cytotoxic capacity was specifically suppressed (Chen et al., 2005). Both in *Leishmaina major* infection and in the colitis model, Treg cells suppress differentiation and function of both Th1 and Th2 cells (Xu et al., 2003). Another study analyzed how $CD8^+$ T cells interact with APCs in the presence or absence of Treg cells and found that the proliferation, induction of effector molecules and ability to form conjugates with target cells by the $CD8^+$ T cells was unchanged and only granule exocytosis was impaired in the presence of Treg cells (Mempel et al., 2006). In the FV model of retroviral infection, it was shown that virus-specific $CD8^+$ T cells were able to proliferate and appeared activated, but failed to produce IFN-γ in the presence of Treg cells (Dittmer et al., 2004).

Together, these studies suggested that the stage of target cell activity which is inhibited by Treg cells maybe dependent on the context in which Treg cells encounter their target cells and is probably influenced by the anatomical location, the inflammatory milieu, the antigen load and the target cell number and activation status.

1.3.3 Mechanisms of regulatory T cell suppression

The mechanisms of action of Treg cells remain poorly understood and controversial, mainly due to differences between *in vitro* and *in vivo* requirements. For instance, *in vitro* studies unequivocally demonstrated a requirement for cell-to-cell contact for Treg cell-

mediated suppression and excluded a role for cytokine secretion, which has been shown to be essential for Treg cell function in some *in vivo* inflammatory settings. Treg cells suppressive mechanisms can be broadly subdivided in three categories: cell-to-cell contact, secretion of inhibitory cytokines and local competition for growth factors (Levings et al., 2006).

1.3.3.1 Cell-to-cell contact-dependent mechanisms

Several molecules have been shown to be important for Treg cell function in a cell-to-cell contact-dependent way. Membrane-bound TGF-β is important for Treg cell suppression of *in vitro* proliferation (Nakamura et al., 2001) and this may relate to the important role for TGF-β for Treg cell function in some *in vivo* settings (Li et al., 2006). Fas-Fas ligand interaction has been shown to be involved in B cell killing by Treg cells (Janssens et al., 2003), while granzyme B and perforin seem to be involved in tumour clearance by Treg cells (Cao et al., 2007). Huang et al. have provided evidence for LAG-3 expression upon Treg cell activation and antibody depletion of LAG-3 lead to inhibition of Treg cells suppression both *in vitro* and *in vivo* (Huang et al., 2004). Interestingly, some studies suggest that cyclic adenosine monophosphate (cAMP) can be expressed by Treg cells and directly delivered to target T cells inhibiting cytokine production, proliferation and differentiation (Bopp et al., 2007).

The Powrie laboratory has shown that the immunosuppressive function of Treg cells controlling intestinal inflammation *in vivo* is dependent on cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (Read et al., 2000; Read et al., 2006). In a recent study, it was shown that deficiency of CTLA-4 specifically in Treg cells resulted in fatal autoimmune diseases in mice 7 weeks old. CTLA-4 deficiency impaired *in vivo* and *in vitro* suppressive function of Treg cells, namely down-regulation of CD80 and CD86 expression in dendritic cells, which suppresses the capacity of denditric cells to activate T cells (Wing et al., 2008).

1.3.3.2 Secretion of inhibitory cytokines

In vivo Treg cell activity has been reported to be cytokine dependent, with IL-10 and TGF-β assuming key roles on Treg cells function, although *in vitro* studies favour a cytokine-independent mode of action. In the colitis model, TGF-β and IL-10 seem to be critical for suppression of Th1 response (Powrie et al., 1996; Maloy et al., 2003) although it has been suggested these suppressive cytokines do not need to be produced by Treg cells (Fahlen et al., 2005). Suppression via IL-10 is also important for Treg cell function in *L. major* infection (Belkaid et al., 2002). In a recent study where IL-10 was specifically depleted in Treg cells, it was shown that IL-10 produced by Treg cells was essential for keeping immune responses in check at environmental interfaces such as the colon and the lungs but was not required for the control of systemic autoimmunity (Rubtsov et al.,

2008), suggesting Treg cells may use different mechanisms to control inflammation mediated by distinct immune cell types in different settings.

Recently IL-35 was identified as a novel inhibitory cytokine specifically expressed by Treg cells which, unlike TGF-β and IL-10, is required for both *in vitro* and *in vivo* suppressive function in mice (Collison et al., 2007). It is now clear that these three inhibitory cytokines, IL-10, TGF-β and IL-35, are key mediators of Treg cell function, but the extent to which they are used in distinct settings differs, suggesting non-overlapping functions.

1.3.3.3 Competition for growth factors

Competition for resources is a mechanism for homeostasis maintenance in the immune system. Treg cell-mediated competition for growth factors leads to cytokine deprivation-induced apoptosis in the target effector cells both *in vitro* and *in vivo* (Pandiyan et al., 2007). A well studied example is the IL-2 cytokine, which is required for Treg cell survival and function (Barthlott et al., 2005). CD25, the IL-2 receptor, is constitutively expressed by Treg cells and is expressed by activated T cells, giving Treg cells a competitive advantage for IL-2 consumption. Studies showing Treg cell suppression by cytokine deprivation challenge the *in vitro* transwell studies demonstrating cell-to-cell contact-dependent suppression, since Treg cell suppression may be dependent on local

cytokine delivery or uptake in a balanced environment. Therefore, the *in vitro* transwell assays may be more a measurement of proximity rather than contact.

Together, these studies suggest that the suppressive mechanisms used by Treg cells are likely to be nonredundant, or only partially redundant, with individual suppressor mechanisms operating in a particular tissue or inflammatory setting.

1.3.4 Requirements for regulatory T cell function

1.3.4.1 Characteristics of regulatory T cell function

The analysis of *in vitro* and *in vivo* suppression of inflammatory diseases in rodents has revealed several requirements for Treg cell-mediated suppression. First, stimulation via TCR is thought to be essential for Treg cells to exert suppression (Thornton and Shevach, 2000; Takahashi et al., 1998). Antigen-specific as well as polyclonal TCR stimulation can activate Treg cells to exert *in vitro* suppression, whereas irrelevant antigens are incapable of activating Treg cells (Thornton and Shevach, 2000; Takahashi et al., 1998). Second, Treg cell-mediated suppression is highly sensitive to antigenic stimulation: much lower concentration of antigen can stimulate Treg cells to exert suppressive activity than the antigen concentration required for the activation/proliferation of naïve T cells with the same antigen specificity. This high antigen sensitivity of Treg cells may be partly

attributed to their high expression of accessory molecules (such as CTLA-4 and adhesion molecules) and possibly to their specific mode of signal transduction via TCR and/or accessory molecules. Third, once Treg cells are stimulated by a specific antigen, they can suppress cells from both the innate and adaptive immune system and the suppression they mediate is antigen-nonspecific (Thornton and Shevach, 2000; Takahashi et al., 1998). Finally, Treg cells are highly differentiated and ready to conduct their specific function upon encountering stimulating antigens (Levings et al., 2006).

1.3.4.2 Factors controlling regulatory T cell survival and activation

Although Treg cells require antigenic stimulation for their functional activation, they are anergic to *in vitro* antigenic stimulation, if one defines anergy as an antiproliferative state (Takahashi et al., 1998; Klein et al., 2003). However, specific *in vitro* conditions, such as TCR stimulation and high dose of IL-2 in the presence of dendritic cells can revert their anergic state and lead Treg cells to expand (Yamazaki et al., 2003). In contrast to their *in vitro* anergic condition, *in vivo* Treg cells can vigorously expand in response to antigen and homeostatic proliferative signals (Klein et al., 2003). Furthermore, Treg cells seem to have a constitutively activated phenotype and to be constantly dividing in the periphery, where it is possible to distinguish a naïve and a memory Treg cell subset (Fisson et al., 2003; Fritzsching et al., 2006).

In addition to signalling through TCR, which has been shown to be essential for T cell survival and homeostatic expansion (Seddon and Zamoyska, 2002; Polic et al., 2001), signals via accessory molecules, such as CD25, CTLA-4, CD28 and TLRs, contribute to the activation and proliferation of Treg cells and hence the tuning of the intensity of suppression (Levings et al., 2006).

Mice deficient for the IL-2 receptor (CD25) develop uncontrolled accumulation of activated T and B cells and autoimmune diseases (Suzuki et al., 1995). The defects found in IL-2 deficient mice were latter attributed to the lack of a population of Treg cells and reconstitution of these mice with Treg cells rescued the phenotype (Almeida et al., 2002). These studies suggest that Treg cells have important functions in controlling lymphocyte homeostasis and that IL-2 signalling in the thymus is required for Treg cell development. Treg cells are also dependent on IL-2 for their *in vitro* and *in vivo* expansion and survival (Papiernik et al., 1998). The levels of IL-2 may determine the size of the Treg cell pool in the periphery, which is therefore kept as a constant fraction of the number of activated IL-2 producing effector T cells. Therefore, activated $CD4^+$ T cells may contribute directly to their own regulation by providing the IL-2 that controls the number of suppressive Treg cells. This would constitute a feedback mechanism controlling T cell expansion during immune responses (Almeida et al., 2006).

TGF-β is another cytokine with a putative role in the development, expansion and survival and function of Treg cells. TGF-β seems to be important for inducing and/or

stabilizing FoxP3 expression, and has therefore a role in maintaining Treg cell peripheral homeostasis (Pyzik and Piccirillo, 2007). In fact, in mice expressing a dominant form of TGF-β, Treg cells fail to expand *in vivo* and do not suppress the development of diabetes (Peng et al., 2004).

CD28 seems to play a key role in the generation of Treg cells in the thymus and presumably in their survival in the periphery (Salomon et al., 2000). Signaling through CD28 in Treg cells abrogates their anergic and suppressive state, although it has no apparent effect in their *in vitro* suppressive activity (Thornton et al., 2004).

In contrast to naïve cells, Treg cells in normal mice constitutively express CTLA-4, which seems to be induced by FoxP3 expression. Signals through CTLA-4 may activate Treg cells to exert suppression and blockage of CTLA-4 signalling leads to impairment of their *in vivo* and *in vitro* function. As noted before (see section 1.3.3.1 Cell-to-cell contact-dependent mechanisms), signalling through CTLA-4 seems to be associated with the capacity of Treg cells to suppress the activation of effector T cells by affecting the potency of DCs (Wing et al., 2008).

A growing number of reports suggest that Toll-like receptor (TLRs) agonists can directly or indirectly influence the induction or function of effector and Treg cells. For instance, it was recently reported that activation of TLR9 on lamina propria DCs by gut flora DNA can disrupt intestinal homeostasis by inhibiting Treg cell conversion and promoting Th1

and Th17 responses (Hall et al., 2008). Moreover, TLRs may have a direct effect on Treg cells, since Treg cells have been shown to selectively express several members of the TLR family, such as TLR4 (Caramalho et al., 2003). Treg cell activation thorough lipopolysaccharide (LPS) via TLR may augment their suppressive capacity and thereby prevent local or systemic immunopathology (Caramalho et al., 2003).

Chemokine receptors may also play a role in Treg cell activation, since Treg cells express a particular set of chemokine receptors which may direct them to lymphoid organs or sites of inflammation. In humans, Treg cells constitutively express CCR4 and CCR8 (Levings et al., 2006), while murine Treg cells express CCR5 that directs them to sites of *L. major* infection, where they suppress effector T cells (Yurchenko et al., 2006).

1.4 Origin of regulatory T cells

FoxP3$^+$ regulatory T cells can originate in the thymus or be induced in the periphery. Studies have suggested that the majority of FoxP3$^+$ Treg cells in naïve, unmanipulated mice seem to originate in the thymus. This hypothesis originated from studies comparing the TCR repertoire of thymic and peripheral Treg cells, which were found to be highly similar, suggesting that Treg cells in the periphery originate directly from the thymic output (Hsieh et al., 2006; Pacholczyk et al., 2006). However, FoxP3$^+$ Treg cells found in the periphery can also result from expansion of pre-existing ones, since in the periphery the Treg cell population contains an actively dividing Treg cell subset (Fisson et al., 2003; Fritzsching et al., 2006). Moreover, several studies have now shown that FoxP3$^-$CD4$^+$ effector cells can be induced in the periphery to express FoxP3 and acquire suppressive functions (Chen et al., 2003; Apostolou and von Boehmer, 2004; Knoechel et al., 2005; Kretschmer et al., 2005), making them indistinguishable from the FoxP3$^+$ Treg cells that originated directly from the thymus.

1.4.1 Central versus peripheral generation of FoxP3$^+$ regulatory T cells

Two major factors have been shown to be involved in peripheral FoxP3$^+$ Treg conversion from FoxP3$^-$ CD4$^+$ T cells: the cytokine TGF-β and the mode of antigen presentation.
Some studies have shown that CD4$^+$CD25$^-$FoxP3$^-$ T cells can be converted to CD4$^+$CD25$^+$FoxP3$^+$ Treg cells by stimulation via the TCR in the presence of TGF-β (Chen et al., 2003).

The second major factor able to induce FoxP3 expression and suppressor functions seems to be the antigen dose and the mode it is presented. Stimulation *in vivo* of peptide-specific T cells from TCR transgenic mice on a recombination activation gene deficient (*Rag*$^{-/-}$) background with a continuous low antigen dose via an osmotic pump leads to the induction of FoxP3$^+$ T cells with suppressive capacities (Apostolou and von Boehmer, 2004). Similar conversion was observed after exposure of TCR transgenic T cells to their target antigen expressed endogenously in soluble form, which resulted in the sequential induction of IFN-γ-producing effector T cells and FoxP3$^+$ Treg cells (Knoechel et al., 2005).

Dendritic cells (DCs) seem to be excellent Treg cell inducers in the correct conditions. It was shown that low antigen dose together with suboptimal DC activation leads to Treg cell conversion *in vivo* (Kretschmer et al., 2005). Four different laboratories have also

shown that retinoic acid (RA) made by gut-associated DCs enhances the conversion of T cells into Treg cells through TGF-β (Coombes et al., 2007; Sun et al., 2007; Benson et al., 2007; Mucida et al., 2007), in what seems to be a process essential for achieving oral tolerance. These studies show that low antigen dose or presentation results in the generation of $FoxP3^+$ suppressor rather than effector T cells, suggesting that the milieu in which T cells are primed plays an important role in determining whether effector or suppressor cells are generated.

The physiological relevance of the peripheral induction of $FoxP3^+$ Tregs is not clear, since the majority of $FoxP3^+$ Treg cells in the periphery seem to originate directly from the thymus and not to be the result of peripheral conversion (Hsieh et al., 2006; Pacholczyk et al., 2006). Moreover, immunization of DO11.10 mice (which express a transgenic TCR specific for OVA) in a $Rag^{-/-}$ background (which do not develop Treg cells) with the OVA peptide does not lead to FoxP3 expression, showing the absence of conversion in these conditions (Yamaguchi et al., 2007).

1.5 Thymic selection of regulatory T cells

The existence of phenotypically and functionally distinct $CD4^+$ T cell populations developing in the thymus (Itoh et al., 1999) raises obvious questions regarding the principles and mechanisms governing the development of each of the $CD4^+$ T cell subsets.

T cell development takes place in the thymus, where developing thymocytes rearrange their TCR genes. It has been shown that thymic epithelial cells express the transcription factor autoimmune regulator (AIRE), which directs expression of many tissue-specific proteins in the medullary epithelial cells (Peterson et al., 2008). This promiscuous gene expression seems to be responsible for the central mechanism to ensure peripheral tolerance to self antigens, since disruption of AIRE reduces tolerance to tissue-specific antigens (Peterson et al., 2008).

During T cell maturation in the thymus, immature T cells express an enormously diverse range of TCRs formed by random rearrangements of TCRα and β chain segments, but only T cells expressing TCRs that recognize major histocompatibility complex (MHC) and associated self-peptides with moderate affinity can differentiate (positive selection). T cells whose TCR fails to bind the MHC/self peptide complex and T cells expressing TCRs that bind the complex too strongly are subjected to programmed cell death (death by neglect and negative selection, respectively) (Picca et al., 2006).

Recent data has shown that FoxP3-expressing thymocytes start being produced later when compared to effector T cells (Fontenot et al., 2005), confirming the original finding that thymectomized mice at day 3 would develop autoimmune diseases due to the lack of Treg cells. However, other studies have demonstrated the presence of functional Treg cells in the periphery already at the 3rd day of life (Dujardin et al., 2004), indicating that TCR repertoire diversity might be as important as absolute numbers of Treg cells.

1.5.1 Model of thymic selection based on the TCR avidity for self ligands

In addition to differences in developmental timing, studies of TCR transgenic mice by the Caton and von Boehmer laboratories have shown that thymocytes with higher avidity for self which survive the negative selection process are recruited into the regulatory T cell lineage (Jordan et al., 2001; Apostolou et al., 2002). Double transgenic mice expressing a transgene-encoded peptide in thymic stromal cells, have a significant increase in the proportion of peptide-specific transgenic T cells which differentiate into $CD4^+CD25^+$ Treg cells (Jordan et al., 2001; Apostolou et al., 2002). Whether the increase in the proportion of Treg cells is due to their increased resistance to clonal deletion than their $CD4^+CD25^-$ counterparts or due to diversion of $CD4^+CD25^-$ T cells differentiation into Treg cells is still a matter of debate (van Santen et al., 2004). It was also shown that Treg cells fail to develop in TCRα/β transgenic mice expressing either a low-affinity

transgenic TCR or a high concentration of the peptide, presumably due to insufficient positive selection or strong negative selection, respectively (van Santen et al., 2004).

For Treg cell selection in the thymus, expression of TCRs which allow recognition of self peptide/MHCII complexes with high avidity seems to be essential. In TCRα/β transgenic mice, which have the same numbers of Treg cells as the wild-type controls, Treg cells express two TCRs: the transgenic TCRα/β and an additional TCR composed of an endogenous TCRα chain paired with the transgenic TCRβ chain; while the former allows recognition of the antigen for which the transgenic TCRα/β chains confers specificity, the latter may allow Treg cell selection and development in the thymus by providing recognition of self peptide/MHCII with high avidity (Itoh et al., 1999). This is possible since the TCRα chains are not subject to allelic exclusion and therefore more than one TCRα chain maybe expressed in the same cell. When the transgenic mice are in a $Rag^{-/-}$ background which does not allow for TCR chains rearrangements, Treg cells do not develop, indicating that rearrangements of the endogenous TCRα chains are required for Treg cell development in TCR transgenic mice (Hori et al., 2002b). Together these findings support a model for Treg cell thymic selection based on the TCR avidity for self ligands.

A recent study analyzed the mechanism by which self-reactive thymocytes are diverted into the Treg cell subset and a two-step instructive model for Treg cell development has been proposed. According to this model, higher avidity for self ligands expressed in the thymus would signal through the TCR, resulting in upregulation of CD25. CD25

expression would then offer competitive advantage for IL-2, which would directly induce FoxP3 expression and finalize Treg cell development (Lio and Hsieh, 2008; Burchill et al., 2008).

1.5.2 Role of FoxP3 in the development and function of regulatory T cells

Treg cells specifically express the transcription factor FoxP3 (forkhead box P3), a member of the fork-head/winged-helix family of transcription factors. A mutation in the *FoxP3* gene was first identified as the cause of the Scurfy mouse phenotype. Scurfy is an X-linked recessive mutant that is lethal in hemizygous males within a month after birth, and is characterized by an inability to regulate $CD4^+$ T cell activity and proliferation, leading to extensive multiorgan infiltration and overproduction of proinflammatory cytokines (Brunkow et al., 2001). Similarly, several distinct mutations of the human gene *FoxP3* are the cause of the genetic disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is the human counterpart of Scurfy (Bennett et al., 2001). The similarities between the rodent and human diseases produced by the absence of $CD4^+CD25^+$ Treg cells prompted researchers to investigate the possible contribution of FoxP3 in Treg cell development and function (Hori et al., 2003; Khattri et al., 2003; Fontenot et al., 2003). Studies revealed that FoxP3 expression

is predominantly restricted to $CD4^+CD8^-CD25^+$ thymocytes and peripheral T cells, although a small percentage of $CD8^+$ T cells also express FoxP3.

In mice, there is a good correlation between FoxP3 and CD25, although a minor proportion (10%) of $FoxP3^+$ cells are $CD25^-$. Conversely, about 10% of $CD25^+$ T cells are $FoxP3^-$ and likely represent activated effector cells. Contrary to CD25, FoxP3 is not expressed in activated T cells, proving it a better marker for Treg cell identification (Khattri et al., 2003). In humans the situation is more complex, since FoxP3 is induced by both $CD4^+$ and $CD8^+$ T cells upon TCR stimulation, but this population is not suppressive or anergic *in vitro*. Therefore, the reliability of FoxP3 as a marker for human Treg cells is questionable (Levings et al., 2006).

FoxP3 was proved to be a crucial molecule for Treg cell function, since ectopic FoxP3 expression could render naïve $CD4^+$ T cells hyporesponsive to TCR stimulation, the cells would upregulate surface markers (CD25, CTLA-4, GITR and CD103) associated with Treg cell function and acquire suppressive capacities both *in vitro* and *in vivo* (Hori et al., 2003; Fontenot et al., 2003). Furthermore, *FoxP3*$^{-/-}$ mice do not develop Treg cells (Fontenot et al., 2003), proving an essential role for FoxP3 in Treg cell development. These findings lead to the concept that FoxP3 is a "master" regulator of Treg cells, influencing both their development and function. More recent work has shown that in the absence of a functional FoxP3 protein, thymocytes still develop into mature T cells with

Treg cell characteristics which, however, do not exhibit regulatory function, suggesting that Treg cell commitment occurs before FoxP3 expression (Kinter et al., 2007).

Mechanistically, FoxP3 has been shown to control hundreds of genes. FoxP3 interacts with transcription factors that otherwise facilitate the activation of non-Treg cells and their differentiation into effector T cells, suggesting that FoxP3 functions by overriding the transcription machinery for effector T cells, converting them to Treg cells (Bettelli et al., 2005).

1.6 TCR specificity of regulatory T cells

1.6.1 TCR usage of regulatory T cells

Although the Treg cell field has expanded enormously since the 1990's, many fundamental questions about Treg cell function remain unknown. One of the issues that yields contradictory results concerns the TCR usage and antigen specificity of Treg cells. *In vitro* it has been shown that Treg cells need to be stimulated through their TCR in an antigen-specific way and they can then suppress the immune responses in an antigen-nonspecific way (Sakaguchi et al., 1995; Thornton and Shevach, 2000). Specificity is also thought to play a critical role in Treg cell development and function (Picca et al., 2006; Shevach et al., 2006). So far it is still not known what the antigenic specificities of Treg cells are and what molecules activate them during inflammation *in vivo*. From the model of thymic selection described above where Treg cells are thought to develop from the thymocytes with the highest avidity for self antigens (Jordan et al., 2001; Apostolou et al., 2002), one would predict that the repertoire of conventional and regulatory T cells is distinct and that the latter is comprised of self-reactive TCRs. Measurement of the TCR repertoire is an extraordinary challenge due to its diversity. In studies using TCRβ-transgenic mice to compare the TCR repertoire of naïve and regulatory T cells, the TCR α and β chains of both subsets were sequenced and it was found that they have a comparable degree of diversity but express largely distinct repertoires of receptors, with only around 20% of overlap (Hsieh et al., 2004; Pacholczyk et al., 2006). However, these results have been contested and the overlap of the TCR repertoires may be greater than

initially reported (Wong et al., 2007; Pacholczyk et al., 2007), arguing against a model of Treg cell development in which the expression of a specific TCR determines the commitment to the FoxP3$^+$ lineage. An alternative model was suggested, where commitment to the Treg cell lineage would occur prior to thymic selection by the TCR (Pacholczyk et al., 2007).

1.6.2 TCR specificity of regulatory T cells

Given the model of thymic selection where Treg cells have the highest affinity for self ligands presented in the thymus and the fact that Treg cells have always been associated with the suppression of autoimmune reactions (Seddon and Mason, 1999), Treg cells have been thought for a long time to be self-specific.

A more recent analysis showed that T cells transduced with TCR from CD25$^+$ Treg cells had a higher capacity to expand in lymphopenic mice than the ones expressing TCRs from naïve cells. Furthermore, cells expressing Treg cell-derived TCR would induce wasting disease in lymphopenic conditions (Hsieh et al., 2006). These results were used to claim that the TCRs from Treg cells have higher affinities for self ligands. However, the idea that Treg cells are specific for self is controversial and it has been shown that self-specific cells are very rare among both the FoxP3$^-$ and the FoxP3$^+$ populations and a substantial proportion of Treg and T conventional cell-derived hybridomas showed reactivity towards APCs displaying foreign peptides (Pacholczyk et al., 2007).

Some studies have addressed Treg specificity during infection, showing that Treg cells which are induced during infection, presumably effector T cells which acquired suppressive functions, are pathogen-specific (McGuirk et al., 2002; Cong et al., 2002). Suffia et al. addressed the specificity of naturally-occurring Treg cells during *L. major* infection, and showed that parasite-specific Treg cells are present at the sites of infection (Suffia et al., 2006), supporting the idea that Treg cells are equipped to recognize foreign ligands.

1.7 The Friend virus model

1.7.1 Immune responses to Friend virus infection

Knowledge of the protective immune responses to retroviral infections is essential for the development of vaccines for human retroviral infections such as HIV. However, little is known about what these responses might be. The Friend virus was chosen in the laboratory for studies of the immune responses required for protection from retroviral infection in mice. The Friend virus is a viral complex containing a replication-competent retrovirus named Friend murine leukemia virus (F-MuLV) and a replication defective retrovirus named spleen focus-forming virus (SFFV) (Hoatlin and Kabat, 1995).

The outcome of FV infection is influenced by a number of genetic and extrinsic factors (Hasenkrug and Chesebro, 1997). Infection of adult immunocompetent mice leads to acute infection which is resolved by virus-specific cytotoxic T lymphocyte, T helper, and B cell responses. Despite the recovery from the acute phase, the virus is never completely cleared and the mice remain persistently infected throughout life (Marques et al., 2008; Hasenkrug and Chesebro, 1997). Many types of cells, including erythroid precursors, monocytes and lymphocytes are initially infected with FV during the acute phase of disease (Hasenkrug et al., 1998). During the chronic phase, the infection in the spleen is reduced and appears to be primarily restricted to a very small population of B cells (Hasenkrug et al., 1998).

Some studies have addressed the role of $CD4^+$ T cells in FV infection, showing that $CD4^+$ T cells can have several important functions: they provide help for $CD8^+$ T and B cell responses and they also seem to have direct antiviral activity, particularly during persistent infection (Hasenkrug and Chesebro, 1997; Hasenkrug et al., 1998). $CD4^+$ T cells have two separate mechanisms of antiviral activity *in vitro*, they can lyse infected target cells and they can also suppress virus replication by production of IFN-γ. *In vivo*, neutralization of IFN-γ increases the levels of viral load in persistently infected mice (Iwashiro et al., 2001b), showing that production of IFN-γ plays an important role in the long term control of FV infection *in vivo*.

1.7.2 Regulatory T cells in Friend virus infection

Previous studies have addressed the role of regulatory T cells in FV infection (Iwashiro et al., 2001a; Dittmer et al., 2004; Robertson et al., 2006; Robertson et al., 2008). Iwashiro et al. have shown that FV infection induces or expands a population of inhibitory $CD4^+$ T cells expressing markers associated with Treg cells (Iwashiro et al., 2001a). It was later shown that $CD4^+$ T cells from persistently FV-infected mice were able to diminished $CD8^+$ T cell IFN-γ responses *in vitro* and *in vivo* (Dittmer et al., 2004).

However, it was not clear from the above studies if the suppressive Treg cells resulted from the expansion of previously existing ones or if they were effector cells induced to

acquire a suppressive phenotype. Furthermore, recent evidence has shown that the FV stock commonly used in the laboratories was contaminated with lactate dehydrogenase-elevating virus (LDV) (Robertson et al., 2008). LDV coinfection leads to a delayed $CD8^+$ T cell response to FV and a change in the time course of Treg cells induction (Robertson et al., 2008).

1.8 Aims

Studies using infection models have addressed the presence of Treg cells in the anatomical location of infection and their suppressive function (Dittmer et al., 2004; Belkaid et al., 2002), but fundamental questions regarding the ontogeny of these cells in the thymus and their antigen specificity remain unclear. Recent studies have shown that the TCR repertoires of Treg cells and conventional T cells are largely non-overlapping (Hsieh et al., 2006; Pacholczyk et al., 2006) and it has been suggested Treg cells (Hsieh et al., 2004) show higher average affinity for self-MHC than conventional T cells (Jordan et al., 2001). These findings raise the hypothesis that the spectrum of antigens recognized by conventional and Treg cells might not necessarily be the same.

In distinct models of infection it has been shown the role of Treg cells in suppressing immune responses from both the innate and adaptive immune system using distinct mechanisms. This study is focused on the analysis of the role and mechanisms of function of Treg cells during chronic FV retroviral infection, using a model of FV-induced T cell-mediated immune pathology.

The TCR specificity and the mechanisms of activation of Treg cells during FV infection are addressed and several hypotheses are raised and investigated. Treg cells can be activated via the TCR by recognizing either pathogen-derived antigens directly or self antigens. Alternatively, they can be activated by non-TCR receptors like TLRs

(Caramalho et al., 2003) or cytokine receptors (Almeida et al., 2002). Given the possibility that viral epitopes may be inducing Treg cells, the role of endogenous retroviruses in the selection of virus-specific Treg cells is also addressed. Furthermore, conversion of virus-specific effector cells into Treg cells during infection is analyzed.

Chapter 2

Materials and Methods

2.1 Mice

Mouse strains used in the experiments are listed in **Table 1** and were maintained at the National Institute for Medical Research (NIMR) animal facilities. All animal experiments were conducted according to the UK Home Office regulations and local guidelines.

Table 1: Name, targeted gene and source (or reference) of mouse strains used in this study.

Common name/ Strain name	Targeted gene	Phenotype	Source/ Reference
B6/ C57BL/6		Wild-type mice	Jackson Laboratory (Bar Harbor, Maine, USA)
FoxP3EGFP/ B6-*Foxp3*^{tm1Mal}	*Foxp3*	Mice co-express EGFP and FoxP3, allowing the identification of FoxP3^{+} cells	(Wang et al., 2008)
B6 CD45.1/ B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ		Mice express the CD45.1 allele, which is atypical in the C57BL/6 background, and this marker can be used to track donor cells in cell transfer experiments	Jackson Laboratory (Bar Harbor, Maine, USA)
Rag1$^{-/-}$/ B6.129S7-	*Rag1*	Mice are deficient for recombination activation gene	(Mombaerts et al., 1992)

Rag1^{tm1Mom}/J		1, leading to impairment of T and B cell development	
IFNγR1$^{-/-}$/ B6.129S7-*Ifngr1*^{tm1Agt}/J	*Ifngr1*	Mice have a targeted disruption of the interferon gamma receptor gene	(Huang et al., 1993)
μMT or *Igh6*$^{-/-}$/ B6.129S2-*Igh-6*^{tm1Cgn}/J	*Igh6*	Mice have a disruption of the immunoglobulin heavy chain 6 gene, leading to impaired B cell development	(Kitamura et al., 1991)
IL10$^{-/-}$/ B6.129P2-*Il10*^{tm1Cgn}/JLt	*Il10*	Mice have a disruption of the IL10 gene	(Kuhn et al., 1993)
TCRα$^{-/-}$/ B6. TCRα$^{-/-}$	*TCRα*	Mice have a disruption of the TCRα gene, leading to impairment of T cell development	(Philpott et al., 1992)
OT-II/ A^b/$ova_{323-339}$-specific OT-II TCR-transgenic		Mice carry a transgenic TCRα and β chains, specific for the ova peptide	(Barnden et al., 1998)
EF4.1 TCRβ transgenic/ B6 EF4.1 TCRβ		Mice carry a transgenic TCRβ chain, specific for the env peptide	(Antunes et al., 2008)

2.1.1 Generation of EF4.1 TCRβ-transgenic mice

A transgenic mouse strain was previously generated in the laboratory, expressing the TCRβ chain of a $CD4^+$ T cell clone (clone SB14-31) specific to the N-terminal region (aa 122-141) of the gp70 envelope glycoprotein of FV (referred to as EF4.1 TCRβ-transgenic mice, and the antigen to which they are specific, the envelope peptide or $env_{122-141}$).

The $CD4^+$ T cell clone SB14-31 was previously published by Iwashiro M. et al. (Iwashiro et al., 1993). This clone was isolated from FV-induced tumour cells and was shown to be

reactive to the N-terminal region of the gp70 env protein of the FV, more specifically, to the amino-acid residues 122-141 ($env_{122-141}$): DEPLTSLTPRCNTAWNRLKL (Iwashiro et al., 1993). In a subsequent study, the amino-acid residues of $env_{122-141}$ involved in recognition by the TCR and binding to the MHC class II were identified (Shimizu et al., 1994): three of the residues (Leucine-128, Threonine-129 and Asparagine-133) were essential for recognition by the TCR of the clone SB14-31 specifically and two (Arginine-131 and Threonine-134) for binding to MHC class II (MHC II).

The Friend virus env-specific TCRβ-transgenic mice were generated by pronuclear microinjection of genes encoding the TCRα and TCRβ chains of an env-specific $CD4^+$ T cell clone, into fertilized B6 oocytes. Briefly, the SB14-31 T cell clone, specific to $env_{122-141}$ presented by MHC II A^b, was fused to TCRα/β-negative BW5147 thymoma cells to produce the 1A6 hybridoma cell line, which was then used as source for RNA. TCRα and TCRβ cDNAs were cloned and inserted into the hCD2-VA expression cassette (Zhumabekov et al., 1995), which directs expression in T cells. Specifically for the TCRβ chain, a cDNA-genomic DNA hybrid was used, in which intron 1 of the TCRVβ-encoding gene (*Tcrbv1s1*) was preserved. For the present study, a TCR-transgenic line (EF4.1) expressing only the TCRβ chain of the SB14-31 clone was selected, to maintain a polyclonal TCR repertoire. Lack of transgenic TCRα chain expression was confirmed by crossing to B6-*Rag1*$^{-/-}$ and B6-*Tcra*$^{-/-}$ mice.

2.2 Flow Cytometry Analysis

2.2.1 Tissue and cell preparation and assessment of organ cellularity

Single cell suspensions were prepared from spleen or lymph nodes of 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 ammonium-chloride-potassium (ACK) lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2-7.4) for erythrocyte lysis. After ACK treatment, cells were washed and resuspended in AB IMDM. Bone marrow cell suspensions were prepared by flushing the bone cavities of femurs and tibiae of B6 mice with AB IMDM followed by treatment with ACK lysis buffer.

For macrophages, cells from the peritoneal cavity were obtained by carefully injecting 5 ml of AB IMDM intra-peritoneally (i.p.) in euthanized B6 mice, followed by gentle abdominal massage and subsequent aspiration of cells-containing medium. The cells were placed in culture for 1 hour, allowing macrophages to adhere to tissue culture plastic plates. After this incubation period, non-adherent cells were removed by washing the plates with AB IMDM.

For bone marrow-derived dendritic cells, bone cavities of femurs and tibiae of B6 mice were flushed with AB IMDM. The cells were resuspended in culture medium supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF). GM-CSF was obtained from supernatant from X63 cells and was used at 1:10 dilution. $5x10^6$ cells were plated in 10cm petri dishes and incubated in culture conditions for 7 days. After this period, non-(or loosely) adherent cells (dendritic cells) were collected and a purity of 40-70% was usually obtained.

Culture medium, which was used for maintenance of cell lines and *in vitro* assays 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 from Sigma). Tissue culture conditions were 95% humidity, 5% CO2 and 37°C.

Cell numbers were determined using an automated cell counter (Casy1, Scharfe Systems) or a Neubauer hemocytometer (Marienfeld). 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 the various organs 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 for 30 minutes or, alternatively, with biotin-conjugated mAbs for 30 minutes, followed by PE-TexasRed-streptavidin staining for 20 minutes. All stainings were performed at 4^{o}C and cells were washed and stained in FACS buffer (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 2**.

Table 2: Specificity, clone name and source of anti-mouse Abs used for extra-cellular staining. Alternative names are show in parenthesis.

Specificity	**Clone name**	**Source**
Fcγ III/II R	2.4G2	NIMR
CD3	2C11	NIMR
CD4 (L3T4)	RM4-5	eBiosciences / Caltag
CD8a (Ly-2)	53-6.7	BD Biosciences
CD11b (Mac1α; integrin α_M chain)	M1/70	eBiosciences
CD11c (integrin α_X chain)	N418	eBiosciences
CD25 (IL-2 receptor α chain)	PC61.5	eBiosciences
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	DX5	eBiosciences
CD69	H1.2FE	eBiosciences
CD103 (integrin α_{IEL} chain)	2E7	eBiosciences
CD154 (CD40 ligand, gp39)	MR1	eBiosciences
FR4 (folate receptor 4, FBP, FRd)	12A5	eBiosciences
F4/80	BM8	eBiosciences
Glyco-Gag	34	NIMR
Gr1 (Ly-6G)	RB6-8C5	eBiosciences
ICOS (CD278)	7E.17G9	eBiosciences
MHCII ($I\text{-}A^b$)	M5/114.15.2	eBiosciences
TCR β	H57-597	eBiosciences
TCRVα2	B20.1	eBiosciences
TCRVβ 2	B20.6	BD Biosciences
TCRVβ 3	KJ25	BD Biosciences
TCRVβ 4	KT4	BD Biosciences
TCRVβ 5.1 and 5.2	MR9-4	BD Biosciences
TCRVβ 6	RR4-7	BD Biosciences
TCRVβ 7	TR310	BD Biosciences
TCRVβ 8.1 and 8.2	MR5-2	BD Biosciences
TCRVβ 8.3	1B3.3	BD Biosciences
TCRVβ 9	MR10-2	BD Biosciences
TCRVβ 10^b	B21.5	BD Biosciences
TCRVβ 11	RR3-15	BD Biosciences
TCRVβ 12	MR11-1	BD Biosciences
TCRVβ 13	MR12-3	BD Biosciences
TCRVβ 14	14-2	BD Biosciences
TCRVβ 17^a	KJ23	BD Biosciences
Ter119/Erythroid cells (Ly-76)	TER-119	eBiosciences
2^{nd} layers		
mIgG2b-FITC	R12-3	BD Biosciences
streptavidin-PETxR		Caltag

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, Fort Collins, CO) and analyzed with Summit v4.3 analysis software (Dako).

2.2.3 Intracellular cytokine staining

For intracellular cytokine staining, cytokine production was induced by stimulating cells with phorbol 12, 13-dibutyrate (PDBu) and ionomycin (both at 0.5 μg/ml) together with surface staining antibodies in tissue culture conditions for 1 hour. After this period, an inhibitor of intracellular transport, monensin (at 1μg/ml), was added to block secretion of cytokines and cells were incubated for an additional three hours in tissue culture conditions. Cells were then 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 3**.

Table 3: Specificity, clone name and source of anti-mouse Abs used for cytokine intracellular staining.

Cytokine	Clone	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

FoxP3 was detected by intranuclear staining using a FoxP3-staining kit (eBiosciences), according to the manufacturer's instructions. Briefly, 10^6 cells were stained for surface molecules during 30 minutes at 4°C. Cells were then washed and incubated for 1 hour at 4°C with fixation/permeabilization buffer (eBioscience) 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. Cells were washed and resuspended in FACS buffer before analysis.

2.2.5 Tetramer staining

$A^b/env_{122\text{-}141}$ tetramers (kindly provided by Prof. D. Gray, University of Edinburgh, UK) were prepared and used as previously described (Crawford et al., 1998; MacLeod et al., 2006). Briefly, cells were incubated with PE-labeled $A^b/env_{122\text{-}141}$ tetramers at 37°C in culture medium for 3 hours, mixing gently every 30 minutes. Cell surface antibodies were then added and the cells were incubated in the dark at RT for 20 minutes. Cells were washed and resuspended in FACS buffer before analysis.

2.2.6 Cell purification and sorting

Target cells were enriched in lymph node and spleen suspensions using immunomagnetic positive selection (EasySep beads, StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. For example, for $CD25^+$ T cell enrichment, single cell suspensions were diluted at a concentration of $1x10^8$ cells/ml. Positive selection was then performed by staining the cells with CD25 PE-conjugated antibody for 15 minutes at RT. Cells were washed and PE selection cocktail (50µl/ml) was added and cells were incubated at RT for 15 minutes. Magnetic beads (50µl/ml) were then added followed by an incubation of 10 minutes at RT. The tube containing the cells was placed on a magnet (EasySep Magnet, StemCell Technologies) for 5 minutes. The magnetic field allowed for the retention of the labeled cells, while the non-labeled ones remained in the supernatant. The supernatant was poured off and the tube was removed from the magnet. The walls of the tube, containing the selected cells were washed with AB IMDM. After two additional selection steps, positively-selected cells were collected. For cell sorting, enriched cell suspensions were stained with antibodies for 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 assays

2.3.1 CFSE labelling

For carboxyfluorescein succinimidyl ester (CFSE) labelling, cells were washed in Dulbecco's Phosphate Buffered Saline solution (D-PBS) (GIBCO) and resuspended in CFSE (Molecular Probes). For labeling more than $1x10^6$cells, CFSE was used at 2.5μM, while for lower cell numbers the concentration was 0.6μM. Cells were incubated for 10 minutes in tissue culture conditions and then washed in culture medium. Dividing cells were identified by CFSE dilution on FACS analysis.

2.3.2 *In vitro* T cell activation

Single cell suspensions were prepared from the spleen or lymph nodes of B6 wild-type or EF4.1 TCRβ-transgenic mice and 0.5×10^6 cells per well were plated in 96 well-plates and stimulated with the indicated amount of $env_{122-141}$. Peptides used in this study were all custom synthesized at NIMR and are listed in **Table 4**.

Table 4: Protein, aminoacid region and sequence of the peptides used in this study.

Protein	**Aminoacid region**	**Aminoacid sequence**
Friend virus envelope ($env_{122-141}$)	122-141	DEPLTSLTPRCNTAWNRLKL
Ovalbumin ($ova_{323-339}$)	323-339	ISQAVHAAHAEINEAGR

Endogenous envelope 2 (endogenous $env_{124-138}$)	124-138	PLTSYTPRCNTAWNR

T cell activation was assessed 18 hrs later by flow cytometric detection of CD69 or CD154 (CD40L) upregulation, with comparable results. For CD154 detection, the anti-CD154 antibody was added at the beginning of the culture. For assessment of T cell activation on day 3, cells were labeled with CFSE before stimulation and responding cells were identified by CFSE dilution on FACS analysis.

2.3.3 *In vitro* suppression assay

0.5×10^6 naïve $CD45RB^{hi}CD25^-CD4^+$ T cells or $CD25^+CD4^+$ Treg cells, purified by flow cytometric sorting, were stimulated separately or mixed together at 1:1 ratio, by added APCs. B cells, macrophages or denditric cells were used as APCs with comparable results. B cells were purified with immunomagnetic positive selection (EasySep beads, StemCell Technologies) from the spleen of donor B6 or *Tcra*$^{-/-}$ mice as previously described. Protocols for macrophages and dendritic cells isolation have been described in previous sections (see section 2.2.1 - tissue and cell preparation and assessment of organ cellularity). Cultures were stimulated with either anti-CD3 antibodies (0.5 μg/ml) or with titrated amounts of $env_{122-141}$ or $ova_{323-339}$ peptides. For assessment of T cell proliferation on day 3, naïve $CD45RB^{hi}CD25^-CD4^+$ T cells were labeled with CFSE before stimulation.

2.3.3.1 IL-2 assay

A CTLL-2-based assay was used to measure IL-2 produced by proliferating T cells. CTLL-2 cells are mouse-derived cytotoxic T cells which depend on IL-2 for their survival and growth. Therefore the growth rate of a CTLL-2 culture can be used as a measure of the IL-2 present in the culture. CTLL-2 cells were kept in culture medium supplemented with 10ng/ml of human recombinant IL-2 (Insight Biotechnology).

Culture supernatants were collected at 48 hrs into a flat bottom 96 well plate and frozen to eliminate any possible cell contaminants. CTLL-2 cells were washed and ressupended in culture medium in the absence of IL-2, plated at $5x10^3$ cells/well (100µl final volume) in the thawed 96 well plates and incubated in tissue culture conditions. On day 1, cells were washed and culture medium with AlamarBlue (Biosource) (1:10 dilution) was added to each well. Cellular growth causes a change from an oxidized to a reduced environment leading to a change of colour on the AlamarBlue reagent, allowing the use fluorescence intensity as a measure of cellular growth. On day 2 the plates were read on a fluorescence plate reader (PerkinElmer LS50B or Safire2, Tecan). Results were expressed as fluorescence units, with higher intensity indicating higher CTLL-2 growth and, therefore, higher IL-2 amounts.

2.3.4 *In vitro* expansion of antigen-specific T cells

For T cell expansion *in vitro*, $CD25^{-}CD4^{+}$ and $CD25^{+}CD4^{+}$ T cells were purified from the spleen and lymph nodes of donor mice by flow cytometric sorting. $CD45.1^{+}CD4^{+}$ effector T cells and $CD45.2^{+}CD4^{+}$ Treg cells were purified from bone marrow of FV-infected *Rag1*$^{-/-}$ recipients of $CD45.1^{+}$ EF4.1 TCRβ-transgenic $CD4^{+}$ T cells and $CD45.2^{+}$ wild-type $CD25^{+}$ $CD4^{+}$ Treg cells at day 21. Cells were stimulated in the presence of bone marrow-derived dendritic cells at a 1:1 ratio. Bone marrow-derived denditric cells isolation has been described (see section 2.2.1 - tissue and cell preparation and assessment of organ cellularity). Cultures were stimulated in the absence or in the presence of 20 U/ml recombinant IL-2, with similar results. Cultures were stimulated with either anti-CD3 antibodies (at 0.5 μg/ml), with titrated amounts of $env_{122-141}$, endogenous $env_{124-138}$ or $ova_{323-339}$ peptides or with FV-pulsed dendritic cells. For assessment of T cell proliferation on day 5, T cells were labeled with CFSE before stimulation and the proliferation profile was identified by CFSE dilution on FACS analysis.

2.3.4.1 Dendritic cell pulsing with FV

Dendritic cells were prepared from bone marrow cultures as previously described (see 2.2.1 tissue and cell preparation and assessment of organ cellularity). Cells were pelleted,

resuspended in undiluted Friend virus (FV) stock (10% w/v spleen homogenate) (see section 2.5 – Friend virus infection) and incubated at 37°C for 1 hour. Cells were subsequently washed extensively with culture medium to remove the excess FV and used for stimulation of T cells.

2.4 Adoptive transfer of T cells

$CD4^+$ T cells were isolated using immunomagnetic positive selection followed, in some experiments, by cell sorting. Naïve $CD45RB^{high}$ $CD4^+$ T cells and $CD25^{high}$ $CD4^+$ T cells were purified by cell sorting. For the *in vivo* FoxP3 conversion experiments, $FoxP3^-$ (GFP^-) $CD45.1^+$ EF4.1 TCRβ-transgenic $Foxp3^{egfp}$ $CD4^+$ T cells and $CD45.2^+$ wild-type $CD25^+$ $CD4^+$ Treg cells were purified by cell sorting. 1×10^6 effector cells, alone or together with the same numbers of Treg cells, were injected in recipient mice via the tail vein in 0.1 ml of AB IMDM.

2.5 Friend Virus infection

The Friend virus (FV) used in these studies was a retroviral complex of a replication-competent B-tropic helper murine leukemia virus (F-MuLV) and a replication-defective polycythemia-inducing spleen focus-forming virus (SFFV). 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 and was free of LDV. *Rag1*$^{-/-}$ mice received an inoculum of ~1,000 spleen focus-forming units (SFFU) of FV, injected via the tail vein in 0.1 ml of PBS. B6 and *Igh6*$^{-/-}$ mice received a higher dose of FV containing ~10,000 SFFU.

2.5.1 Friend virus detection by flow cytometry

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-specific monoclonal antibody 34 (mouse IgG2b) for 30 minutes at 4°C. Cells were washed with FACS buffer and incubated with an antimouse IgG2b-FITC secondary reagent (**Table 2**) for 20 minutes at 4°C. Cells were then washed and resuspended in FACS buffer before FACS analysis.

2.6 Assessment of immune pathology

2.6.1 Assessment of anaemia

Mice were bled weekly from week 1 until week 7. Blood was taken by making a small incision of the tail vein and approximately 50µl of blood was collected into heparinized capillary tubes and subsequently transferred to eppendorf tubes. Complete blood counts were measured on a VetScan HMII hematology analyzer (Abaxis, CA, USA), following the manufacturer's instructions.

2.6.2 Assessment of hematopoietic colony-forming cells

Bone marrow cells obtained from the femurs and tibiae of donor mice were treated with ACK lysis buffer and washed with culture medium. The cells were then plated in methylcellulose-containing IMDM (MethoCult media, StemCell Technologies, Vancouver, BC, Canada), according to manufacturer's instruction. Methylcellulose-containing IMDM was supplemented with erythropoietin or SCF, IL-3, IL-6 and GM-CSF were used to assess the numbers of burst-forming units-erythroid (BFU-E) and colony-forming units-granulocyte/macrophage (CFU-GM), respectively. Bone marrow cells were diluted in culture medium at $2x10^6$ or $2x10^5$ cells per ml for BFU-E and CFU-GM counts, respectively. 0.3ml of cells were added to 3ml of methylcellulose-containing IMDM. After vortexing, the tubes were left still to allow for bubble dissipation. Cell-containing medium was carefully dispensed with a blunt end syringe into 35mm plates

and incubated in tissue culture conditions. Colony identification and count was assessed using an inverted light microscope (Fisher Scientific) at day 6 for CFU-GM and day 8 for BFU-E.

2.6.3 Bone marrow histology

Histological examination of bone marrow biopsies was carried out by IZVG Pathology, Leeds, UK. Briefly, femurs were decalcified for 24 hours in 15% v/v formic acid/ 5% v/v formaldehyde solution and sent for histological analysis. Longitudinal sections were prepared, stained with haematoxylin and eosin and photographed under light microscopy.

2.7 Analysis of serum cytokine levels

2.7.1 Serum preparation

Serum was prepared by leaving non-heparinised blood to clot at room temperature for 2 hours. Clot was then detached from the sides of tubes which were then centrifuged at 3,000 rpm for 7 minutes. Clear serum was transferred to new tubes which were centrifuged at 12,000 rpm for 7 minutes. Clear serum was transferred to new tubes which were stored at -20°C.

2.7.2 Measurement of serum cytokine levels

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)), keratinocyte chemoattractant (KC or 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 interferon-gamma (MIG or CXCL9) was performed by using a multiplex cytokine bead array (20-plex Ab bead kit, BioSource), using the Luminex 100 System (Luminex).

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-labeled detection antibody. Following 30 minutes incubation at room temperature in plate shaker and 3 washes, 25 µl of SAV-PE were added and a further incubation step for 10 minutes at room temperature was performed. Following 3 washes, samples were resuspended in 150 µl of Bio-Plex assay buffer and analyzed on a Luminex 100 instrument (Bio-Rad Laboratories Ltd. UK) according to manufacturer's instruction.

2.8 Statistical analysis

Statistics were generated by Student's *t*-test performed using SigmaPlot v10 software (Systat Software Inc., San Jose, CA, USA).

Chapter 3

Results

3.1 Characterization of EF4.1 TCRβ-transgenic mice

The main focus of studies in the laboratory is the response of $CD4^+$ T cells to FV infection, but the frequency of FV-specific cells in the wild-type repertoire is too low to allow their detection, raising the need for a genetically modified mouse strain with increased frequency of virus-specific $CD4^+$ T cells. Thus, a transgenic mouse strain, expressing the TCR β chain of a $CD4^+$ T cell clone (clone SB14-31) specific to the N-terminal region (amino-acids 122-141) of the gp70 envelope glycoprotein of FV, was previously generated in the laboratory and used for the studies presented here. This strain is referred as EF4.1 TCRβ-transgenic mice, and the antigen to which they are specific, the envelope peptide or $env_{122\text{-}141}$.

In EF4.1 TCRβ-transgenic mice, the transgenic TCRβ chain is free to associate with any of the endogenous TCRα chains, generating a polyclonal TCR repertoire, which was of fundamental importance for the study of Treg cells. Numerous studies have shown that TCRα/β transgenic mice on a *Rag*$^{-/-}$ background lack Treg cells, suggesting that Treg cell development depends on expression of endogenous TCR chains. Furthermore, several

studies have validated the use of TCRβ-transgenic mice for the study of Treg cells, by showing that TCRβ-transgenes do not impair Treg cell development (Hsieh et al., 2004; Pacholczyk et al., 2006). In TCRβ. transgenic mice, TCRβ allelic exclusion is very efficient, and endogenous TCRα chains are responsible for the antigen specificity and development of Treg cells (Apostolou et al., 2002).

3.1.1 T and B cell development in EF4.1 TCRβ-transgenic mice

The EF4.1 TCRβ-transgenic strain of mice was characterized in terms of T and B cell numbers in the spleen and lymph nodes, to ensure that expression of the transgene did not affect lymphocyte development.

Typical gating for flow cytometric analysis of the T and B cell populations is shown (**Figure 1A**). The EF4.1 TCRβ-transgenic mice showed no difference compared with wild-type control mice in total cell numbers or in the B and T cell compartments (**Figure 1B**) either in spleen or lymph nodes. There was a small increase in the number of $CD4^+$ T cells and a decrease in $CD8^+$ T cell numbers in EF4.1 TCRβ-transgenic mice (**Figure 1C**). This bias towards the generation of $CD4^+$ T cells occurred because the TCRβ chain transgene was obtained from a $CD4^+$ T cell clone and it was therefore expected to confer preferential skewing towards $CD4^+$ T cells during T cell development.

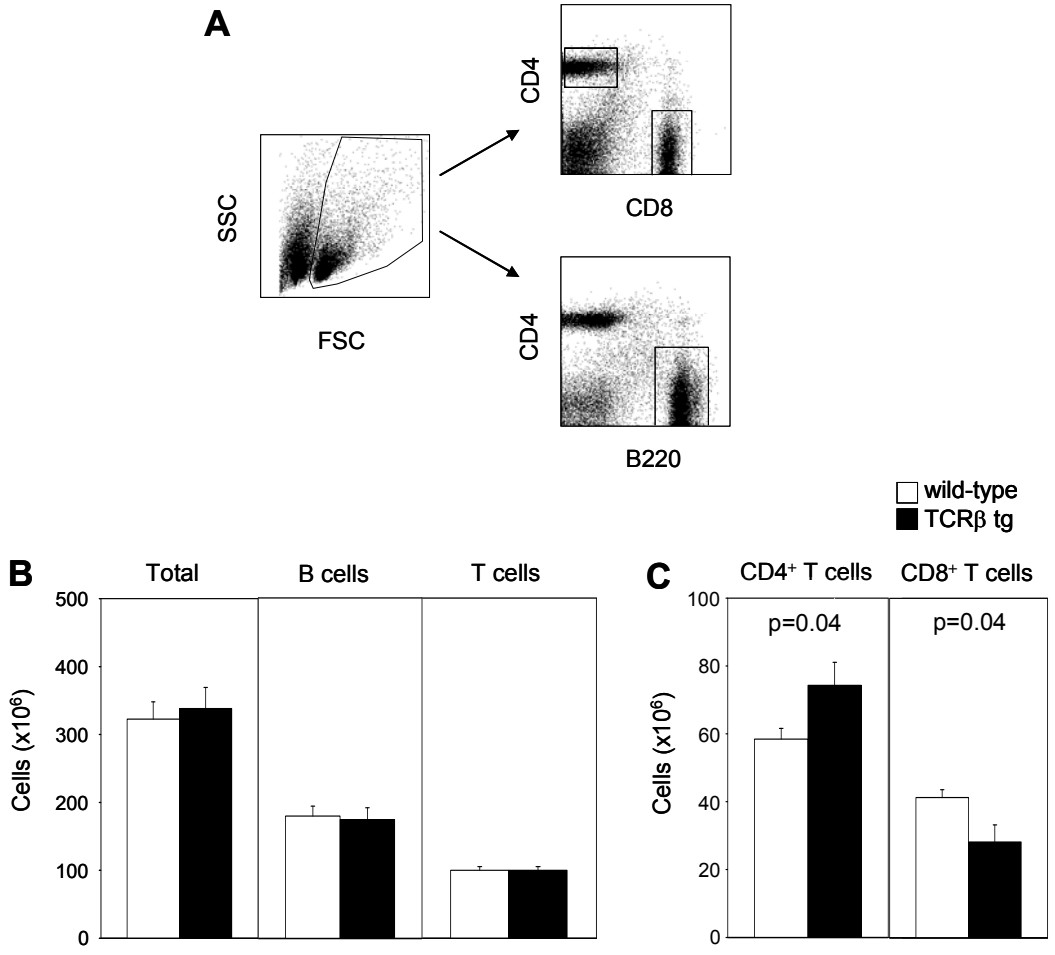

Figure 1. Normal lymphocyte development in EF4.1 TCRβ-transgenic mice.
(**A**) Flow cytometric example of the gating applied to define the $CD4^+$, $CD8^+$ T and B ($B220^+$) cells populations.
(**B-C**) Cellularity in the EF4.1 TCRβ-transgenic (TCRβ-tg) and wild-type control mice. Absolute number of splenic and lymph node total cells, B, T (**B**), $CD4^+$ and $CD8^+$ (**C**) cells. Values are the mean (±SEM) of 5-7 mice per group and numbers within the graphs denote the *P* value.

3.1.2 Expression of the transgenic TCRβ chain in EF4.1 TCRβ-transgenic CD4$^+$ T cells

Expression of the transgenic TCRβ chain in the EF4.1 TCRβ-transgenic CD4$^+$ T cell population was next addressed. A panel of antibodies for several of the endogenous TCR Vβ chains was used (which did not include the transgenic TCRβ chain, for which an antibody is not available), covering around 75% of the TCR Vβ families in wild-type mice. Since expression of the TCRβ chain is subject to allelic exclusion, cells expressing the transgene will not express endogenous TCRβ chains. Therefore the percentage of expression of the transgenic TCRβ chain could be extrapolated from the percentage of expression of endogenous TCRβ chains.

The percentage of EF4.1 TCRβ-transgenic CD4$^+$ T cells expressing any of the endogenous TCR Vβ chains was substantially reduced compared with wild-type CD4$^+$ T cells (**Figure 2A**). The sum of the percentages for the endogenous TCR Vβ chains was calculated (**Figure 2B measured**) showing that endogenous TCR Vβ chains were used by approximately 75% of CD4$^+$ T cells in wild-type B6 mice. By setting the sum of measured TCR Vβ expression in wild-type B6 mice to 100% and assuming that allelic exclusion by the transgenic TCRβ chain was similar for the remaining 25% of endogenous TCR Vβ chains not covered by the antibody panel, the residual expression of endogenous TCR Vβ expression in EF4.1 TCRβ-transgenic CD4$^+$ T cells was calculated (**Figure 2B projected**). In EF4.1 TCRβ-transgenic CD4$^+$ T cells exclusion of the

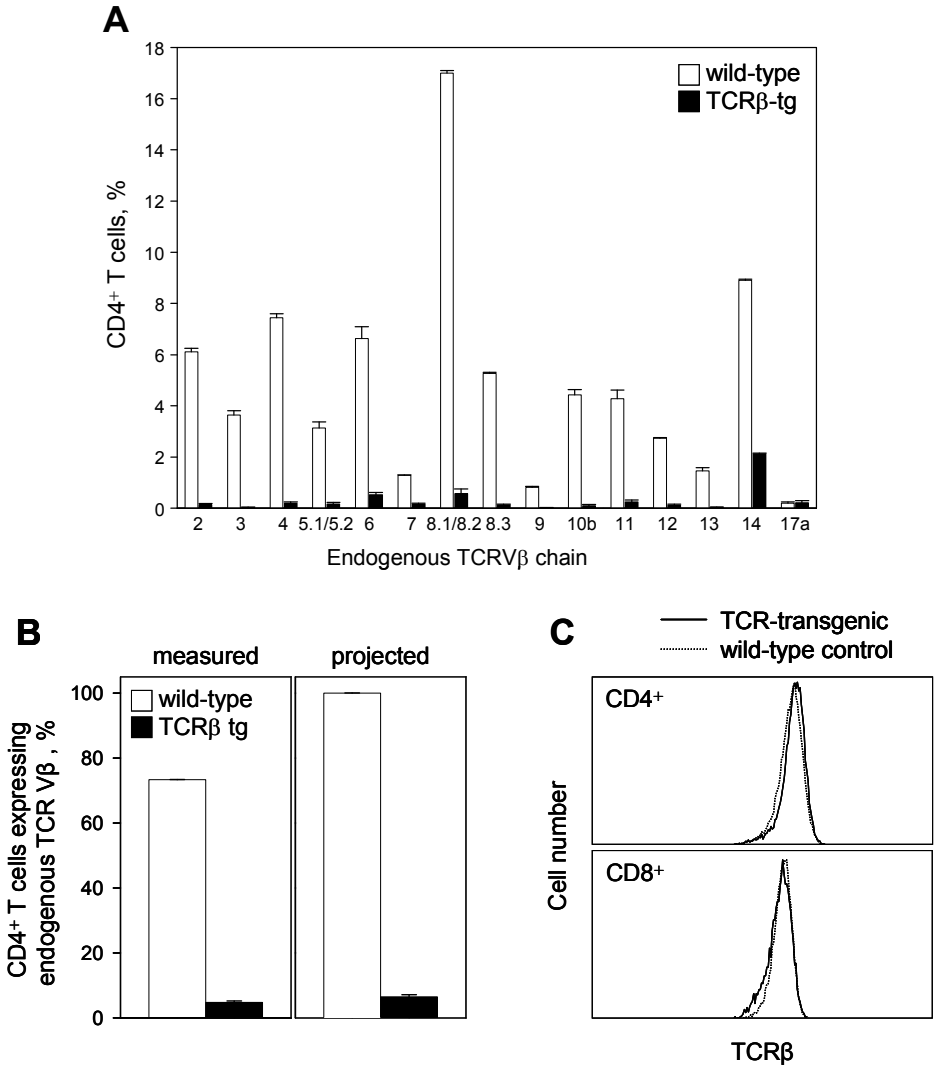

Figure 2. Exclusion of endogenous TCRVβ chains in EF4.1 TCRβ-transgenic mice.
(**A-B**) Expression of endogenous TCR Vβ chains in EF4.1 TCRβ-transgenic CD4$^+$ T cells. The percentage of T cells expressing TCR Vβ2, Vβ3, Vβ4, Vβ5.1/5.2, Vβ6, Vβ7, Vβ8.1/8.2, Vβ8.3, Vβ9, Vβ10b, Vβ11, Vβ12, Vβ13, Vβ14 or Vβ17a in CD4$^+$ T cells from EF4.1 TCRβ-transgenic (TCRβ-tg) and wild-type control mice is shown (**A**). Sum of the percentages for the endogenous TCR Vβ chains shown in **A** (measured) (**B**). These TCR Vβ chains are used by ~ 75% of CD4$^+$ T cells in wild-type B6 mice. The sum of measured TCR Vβ expression in wild-type B6 mice was set to 100% and, assuming that allelic exclusion by the transgenic TCRβ chain is similar for the remaining ~25% of endogenous TCR Vβ chains not covered by the antibody panel, the residual expression of endogenous TCR Vβ expression in EF4.1 TCRβ-transgenic CD4$^+$ T cells was then calculated (projected) (**B**)**.** Values are the mean (±SEM) of 3 mice per group from one experiment, representative of 3 similar experiments.
(**C**) Level of TCRβ chain expression in CD4$^+$ and CD8$^+$ T cells in EF4.1 TCRβ-transgenic and wild-type control mice. Values are representative of more than 3 mice per group.

endogenous TCRβ chain was 95% (**Figure 2B projected**), while the majority of the remaining 5% $CD4^+$ T cells were composed of endogenous TCRβ chains from the families TCR Vβ6, 8.1/2 and 14 (**Figure 2A**). Moreover, in both $CD4^+$ and $CD8^+$ T cells EF4.1 TCRβ-transgenic, the levels of TCR expression were similar to the wild-type control, revealed by staining for a common region of the TCRβ chain (**Figure 2C**).

3.1.3 Reactivity of EF4.1 TCRβ-transgenic $CD4^+$ T cells to the Friend virus envelope

3.1.3.1 Assessment of EF4.1 TCRβ-transgenic $CD4^+$ T cell reactivity to $env_{122-141}$

EF4.1 TCRβ-transgenic mice bear a polyclonal TCR repertoire with the majority (95%) of $CD4^+$ T cells expressing the transgenic TCRβ chain, and a proportion of them should be able to recognize the viral peptide $env_{122-141}$. To address this question lymphocytes were stimulated *in vitro* with $env_{122-141}$ and their activation status was analyzed using two readouts: the expression of the activation markers CD69 and CD40L and the amount of IL-2 secretion in the culture. A fraction of the EF4.1 TCRβ-transgenic $CD4^+$ T cells up-regulated CD69 and CD40L upon $env_{122-141}$-stimulation (**Figure 3A**), in contrast to B6 wild-type $CD4^+$ T cells which did not respond. Titration of $env_{122-141}$ gave rise to a linear response, with increasing concentrations of the peptide leading to an increase in the percentage of activated $CD4^+$ T cells, reaching on average a maximum of 4% (**Figure**

3B). Similarly, an increase in the amount of IL-2 produced by EF4.1 TCRβ-transgenic $CD4^+$ T cells with higher concentrations of the peptide was also detected (**Figure 3C**). In contrast, in wild-type controls no $env_{122-141}$-specific activation with any of the readouts was detected (**Figure 3B-3C**).These results showed enrichment of $env_{122-141}$-specific $CD4^+$ T cells in EF4.1 TCRβ-transgenic mice compared with wild-type controls. The $CD4^+$ T cell population was activated at different $env_{122-141}$ doses, leading to a linear response indicative of a polyclonal TCR composition. As noted before, this polyclonal repertoire resulted from the pairing of the transgenic TCRβ chain with endogenous TCRα chains.

3.1.3.2 Expression of TCR Vα chains in $env_{122-141}$–specific EF4.1 TCRβ-transgenic $CD4^+$ T cells

To further characterize $env_{122-141}$-specific EF4.1 TCRβ-transgenic $CD4^+$ T cells, an attempt was made to identify endogenous TCR Vα chains that would confer env_{122-14}-specificity when paired with the transgenic TCRβ chain. For this analysis EF4.1 TCRβ-transgenic lymphocytes were stimulated with $env_{122-141}$ and stained for several of the endogenous TCR Vα chains while assessing the activation status by CD69 expression. It was found that around 15% of the TCR $V\alpha2^+$ $CD4^+$ T cells up-regulated CD69, while the same happened in only 3% of TCR $V\alpha2^-$ $CD4^+$ T cells (**Figure 4A**). This suggested that pairing of TCR Vα2 chains with the transgenic TCRβ chain gave rise to a TCR with 15%

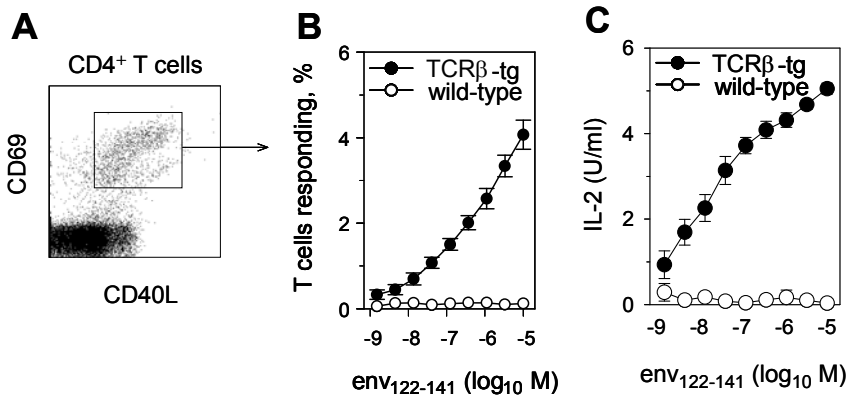

Figure 3. T cell reactivity to $env_{122-141}$ in EF4.1 TCRβ-transgenic mice.
(**A**) Flow cytometric example of CD69 and CD40L induction in EF4.1 TCRβ-transgenic $CD4^+$ T cells 18 hrs post stimulation with the $env_{122-141}$ peptide.
(**B**) Mean (±SEM) percentage of responding cells ($CD69^+$ $CD40L^+$) in total $CD4^+$ T cells from EF4.1 TCRβ-transgenic or wild-type control mice 18 hrs post stimulation.
(**C**) Mean (±SEM) IL-2 concentration in supernatants from single cell suspensions of spleen and lymph nodes of EF4.1 TCRβ-transgenic or wild-type control mice two days post stimulation. Values in **A-C** are representative of 5 independent experiments.

probability of being $env_{122-141}$-specific, which was significantly higher when compared with the 3% probability for chains other than Vα2. Furthermore, TCR $V\alpha2^+$ env-specific $CD4^+$ T cells required a lower peptide concentration for their activation (ED_{50} =0.04μM), showing that they were 30-fold more sensitive to $env_{122-141}$ than their TCR Vα2 counterparts (ED_{50}>1.24μM) (**Figure 4B**). To confirm these differences in frequency and avidity between TCR $V\alpha2^+$ and $V\alpha2^-$ clones, the frequency of these subsets was analyzed in CFSE-labeled EF4.1 TCRβ-transgenic $CD4^+$ T cells activated *in vitro* with $env_{122-141}$ for 3 days. While the TCR $V\alpha2^+$ clones represented 12-14% of all $CD4^+$ T cells in the undivided population, this frequency increased to 40-60% in the responding population (**Figure 4C**), confirming that TCR $V\alpha2^+$ cells had a higher frequency and avidity for $env_{122-141}$ than TCR $V\alpha2^-$ cells.

The increased percentage of TCR $V\alpha2^+$ clones in the $env_{122-141}$-activated population could be due to a higher expression of TCR $V\alpha2^+$ chains in the EF4.1 TCRβ-transgenic $CD4^+$ T cells in general. To address this possibility, expression of TCR Vα2 chains in the T cell populations of EF4.1 TCRβ-transgenic and B6 wild-type control mice was compared and it was found that EF4.1 TCRβ-transgenic mice have a decreased percentage of TCR $V\alpha2^+$ $CD4^+$ T cells (**Figure 4D**) and an increase in the percentage of TCR $V\alpha2^+$ $CD8^+$ T cells (**Figure 4E**). Overall, in total T cells, there was only a modest increase in the percentage of expression of TCR $V\alpha2^+$ (**Figure 4F**), which could not

account for the high percentage of TCR V$\alpha 2^+$ clones in the $env_{122\text{-}141}$-specific activated $CD4^+$ T cell population.

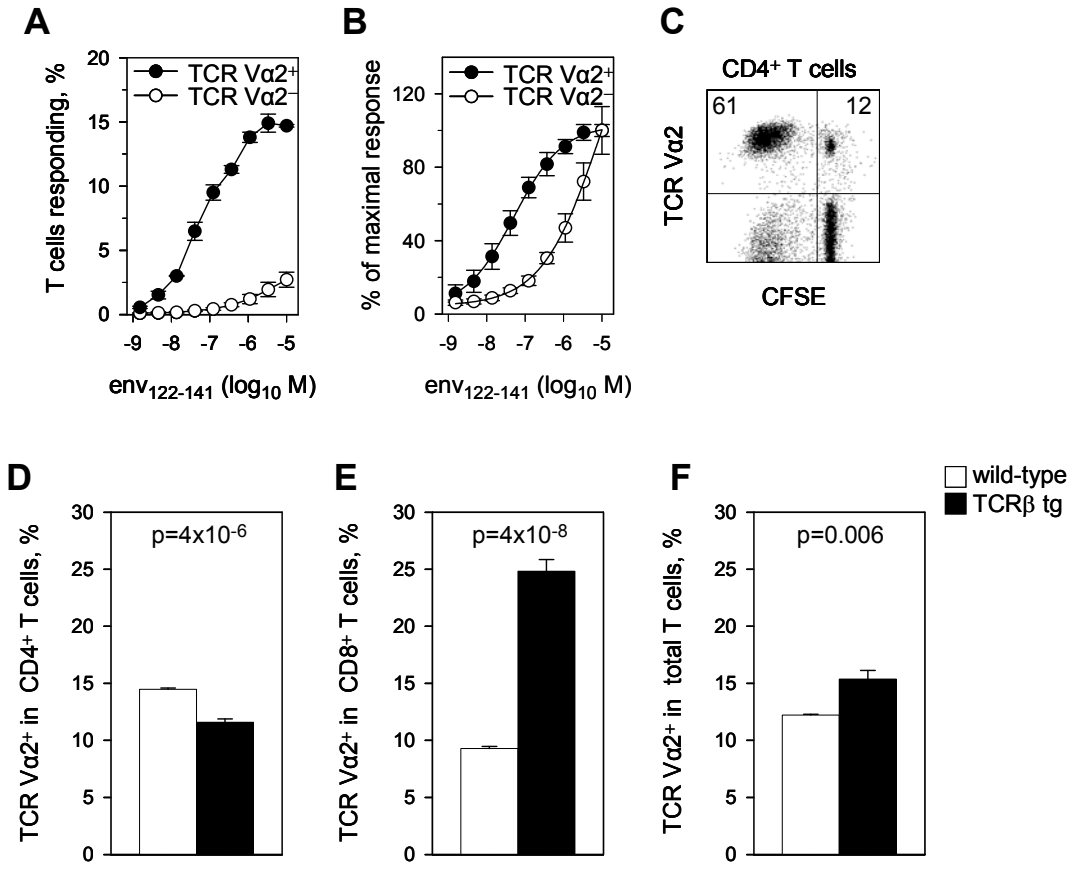

Figure 4. TCR Vα2 usage in $env_{122-141}$–specific EF4.1 TCRβ-transgenic $CD4^+$ T cells.
(**A**) Mean (±SEM) percentage of responding cells in gated TCR $V\alpha2^+$ or $V\alpha2^-$ $CD4^+$ T cells from EF4.1 TCRβ-transgenic mice 18 hrs post stimulation with the $env_{122-141}$ peptide.
(**B**) Mean (±SEM) percentage of responding cells, plotted as a fraction of the maximal response, in gated TCR $V\alpha2^+$ or $V\alpha2^-$ $CD4^+$ T cells from EF4.1 TCRβ-transgenic mice 18 hrs post stimulation. Values in **A** and **B** are representative of 5 independent experiments.
(**C**) TCR $V\alpha2^+$ T cells in responding ($CFSE^-$) or non-responding ($CFSE^+$) $CD4^+$ T cells from EF4.1 TCRβ-transgenic mice 3 days post stimulation. Numbers within the quadrants denote the percentages of $V\alpha2^+$ cells in the divided (left quadrant) and undivided (right quadrant) $CD4^+$ T cells populations.
(**D-F**) Mean (±SEM) percentage of TCR $V\alpha2^+$ clones in $CD4^+$ (**D**), $CD8^+$ (**E**) or total T cells (**F**) from EF4.1 TCRβ-transgenic or wild-type control mice. Values in **D-F** are representative of 3 independent experiments and numbers within the graphs denote the *P* value.

3.2 Model of Friend virus-induced immune pathology

3.2.1 Pathogenic effect of EF4.1 TCRβ-transgenic CD4$^+$ T cells in FV-infected immunodeficient mice

To follow the immune pathological consequences of a continuous FV-specific CD4$^+$ T cell response, EF4.1 TCRβ-transgenic CD4$^+$ T cells were adoptively transferred into FV-infected immunodeficient *Rag1*$^{-/-}$ hosts, in which the absence of T and B cells prevents virus control. The mice developed symptoms of anaemia from the second week after transfer, which was reflected in reduction of red blood cells (RBC) counts in peripheral blood (**Figure 5**). By following these mice throughout the infection it was found that the reduction in RBC counts peaked on day 21 and remained chronic during the timeframe of the experiment (maximum 70 days after transfer). In contrast, mice receiving EF4.1 TCRβ-transgenic CD4$^+$ T cells or FV alone did not develop any signs of anaemia at any time point in these experiments (**Figure 5**), suggesting that anaemia was a pathological consequence of the CD4$^+$ T cell response to FV infection and not directly caused by FV infection.

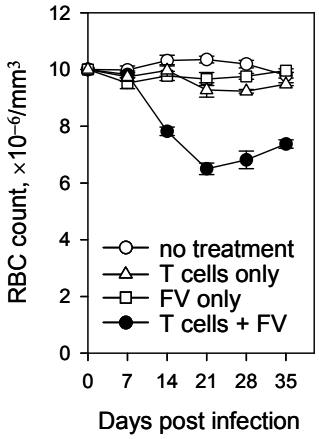

Figure 5. FV-infected *Rag1*$^{-/-}$ mice receiving EF4.1 TCRβ-transgenic CD4^{+} T cells develop anaemia.
Changes in RBC counts in *Rag1*$^{-/-}$ mice, which were infected with FV (FV only), or received EF4.1 TCRβ-transgenic CD4^{+} T cells (T cells only) or both (T cells + FV). Values represent the mean (±SEM) of 8-18 mice per group per time point, analyzed in more than 3 independent experiments. $P < 0.001$ between 'T cells + FV' group and all other groups for days 14-35.

3.2.2 Assessment of bone marrow function in FV-infected immunodeficient recipients of EF4.1 TCRβ-transgenic CD4$^+$ T cells

To further study the causes of disease development, the anaemic mice were examined for signs of bone marrow dysfunction. The cellularity and composition of the bone marrow and the frequency of erythroid precursors (Ter119$^+$), which are the preferential targets of FV infection, and myeloid precursors (CD11b$^+$ Gr1$^+$), which, together with Ter119$^+$ cells, make up for about 70-80% of total bone marrow cellularity in *Rag1*$^{-/-}$ mice, was examined.

No difference was found between mice infected with FV or receiving EF4.1 TCRβ-transgenic CD4$^+$ T cells alone and *Rag1*$^{-/-}$ controls in terms of bone marrow cellularity (**Figure 6A-6C**) or composition (**Figure 6D-6F**). In sharp contrast, mice receiving FV together with EF4.1 TCRβ-transgenic CD4$^+$ T cells showed significant reduction of bone marrow cellularity compared with any of the other groups (**Figure 6A**). This effect was more pronounced in the Ter119$^+$ compartment (**Figure 6B**) but the CD11b$^+$ Gr1$^+$ subset was also significantly affected (**Figure 6C**). Furthermore, EF4.1 TCRβ-transgenic CD4$^+$ T cell transfer in FV-infected recipients was associated with significant functional reduction in hematopoietic colony forming cells, either from erythroid (**Figure 6G**) or granulocyte/macrophage (**Figure 6H**) precursors. Histological analysis revealed that, while bone marrow from *Rag1*$^{-/-}$ recipients of EF4.1 TCRβ-transgenic CD4$^+$ T cells or FV alone was histologically similar to that of *Rag1*$^{-/-}$ controls, bone marrow from mice

receiving the combination of cells together with the virus showed significant hipocellularity with pronounced reduction in hyperchromatic normoblasts (**Figure 6I**), consistent with generalized myelosuppression.

3.2.3 Effect of Friend virus infection on immune pathology

To test if bone marrow dysfunction was associated with FV infection of bone marrow cells, the extent of infection between the groups of mice was compared. This analysis was made possible by the expression of the viral protein Gag in a glycosylated form (Glyco-Gag) at the membrane of the infected cells. Therefore, the levels of infection in the bone marrow could be analyzed by surface staining with an antibody for the Glyco-Gag protein. Approximately two-thirds of $Ter119^+$ cells were infected (**Figure 6E**) in mice infected with FV, showing these cells are preferential targets for the virus, as previously described (Hasenkrug et al., 1998). Approximately one-third of the $CD11b^+$ $Gr1^+$ compartment was infected (**Figure 6E**), suggesting that FV can infect most bone marrow cell types in immunodeficient mice. Moreover, in both compartments, the high percentage of infected cells was not associated with any bone marrow dysfunction (**Figure 6A-6C**), confirming that FV is non-cytopathic (Hasenkrug et al., 1998). In FV-infected mice receiving EF4.1 TCRβ-transgenic $CD4^+$ T cells, a significant decreased in the percentage of infected cells in both cellular subsets was observed (**Figure 6F**), indicating that the presence of FV-specific $CD4^+$ T cells during FV infection has strong

antiviral activity. However, substantial numbers of FV-infected cells remained present, in agreement with incomplete virus control in the absence of $CD8^+$ T cells and B cells (Zelinskyy et al., 2006).

Together, these results confirmed that the loss of bone marrow cellularity and the subsequent anaemia in FV-infected *Rag1*$^{-/-}$ recipients of EF4.1 TCRβ-transgenic $CD4^+$ T cells were not the result of FV infection itself but rather seemed to be caused by the activation of FV-specific $CD4^+$ T cells.

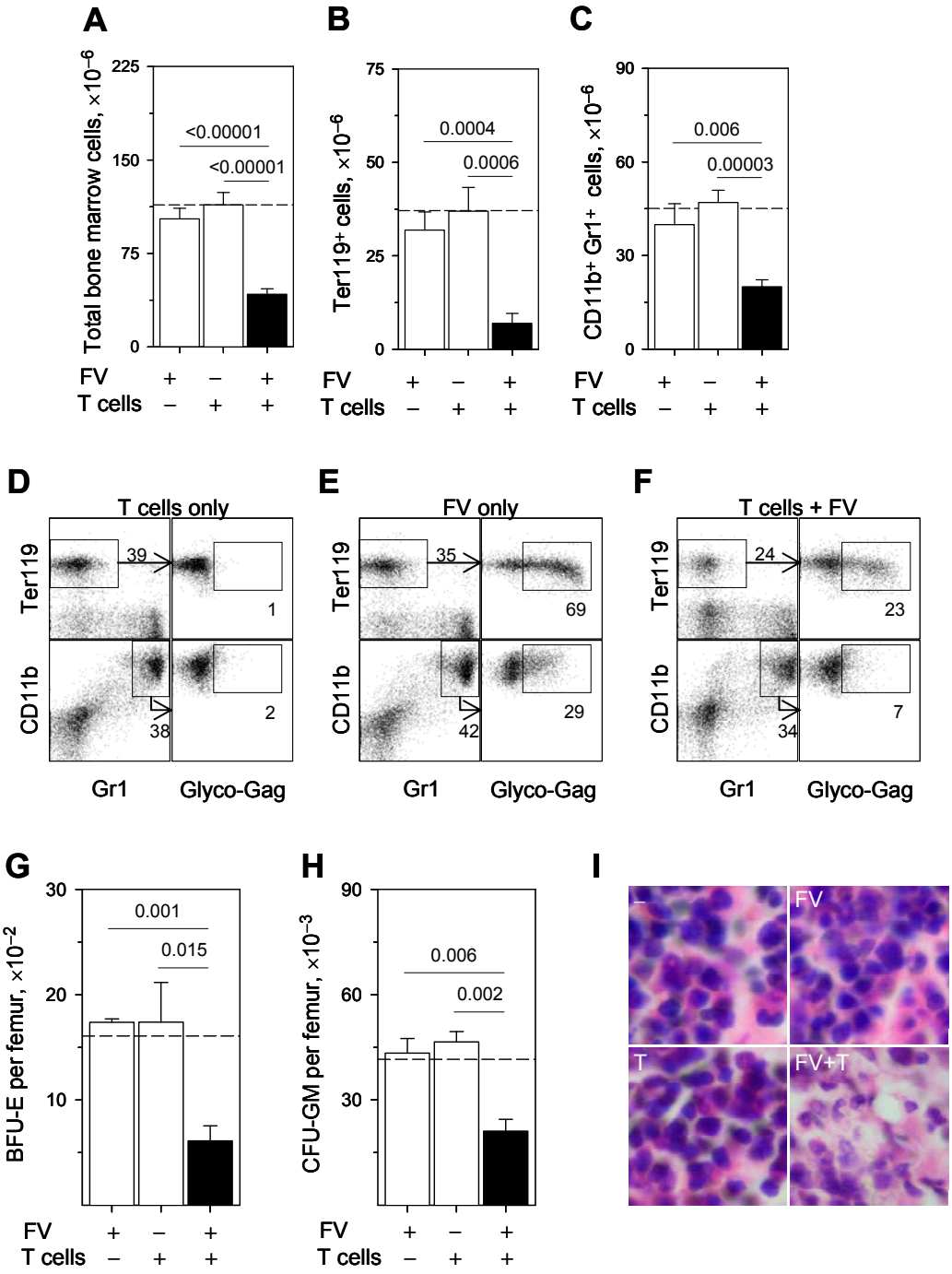
A
B
C
Total bone marrow cells, ×10−6
Ter119+ cells, ×10−6
CD11b+ Gr1+ cells, ×10−6
<0.00001
<0.00001
0.0004
0.0006
0.006
0.00003
FV
T cells
D
E
F
T cells only
FV only
T cells + FV
Ter119
CD11b
Gr1
Glyco-Gag
39
1
38
2
35
69
42
29
24
23
34
7
G
H
I
BFU-E per femur, ×10−2
CFU-GM per femur, ×10−3
0.001
0.015
0.006
0.002
T
FV+T

Figure 6. Anaemia in FV-infected immunodeficient mice receiving EF4.1 TCRβ-transgenic CD4$^+$ T cells is caused by bone marrow pathology.
(**A-C**) total bone marrow cellularity (**A**) and numbers of Ter119$^+$ erythroid precursor cells (**B**) and CD11b$^+$Gr1$^+$ bone marrow cells (**C**) in *Rag1*$^{-/-}$ mice, which were infected with FV, or received EF4.1 TCRβ-transgenic CD4$^+$ T cells or both. The dashed line represents the average cell numbers of untreated *Rag1*$^{-/-}$ mice. Values are the mean (±SEM) of 5-13 mice per group on day 21. Numbers within the graphs denote the *P* values.
(**D-F**) Percentages of uninfected and FV-infected (glyco-Gag$^+$) Ter119$^+$ and CD11b$^+$Gr1$^+$ cells in the bone marrow of the same groups of mice described in **A-C**. Numbers within the plots represent the percentage of positive cells and are the average of more than 5 mice per group analyzed on day 21.
(**G-H**) Numbers of burst-forming units-erythroid (BFU-E) (**G**) and colony-forming units-granulocyte/macrophage (CFU-GM) (**H**) in the bone marrow of the same groups of mice described in **A-C**. The dashed line represents the average colony-forming cell number of untreated *Rag1*$^{-/-}$ mice. Values are the mean (±SEM) number of colony-forming cells per femur of 3-5 mice per group on day 21. Numbers within the graphs denote the *P* values.
(**I**) Haematoxylin and eosin stained femur sections from the same groups of mice described in **A-C**. Compact hyperchromatic nuclei (blue) are predominantly normoblasts. Sections are 600x magnified and represent 3-5 mice per group.

3.2.4 Expansion of EF4.1 TCRβ-transgenic CD4$^+$ T cells in FV-infected immunodeficient recipients

The expansion and localization of EF4.1 TCRβ-transgenic CD4$^+$ T cells in FV-infected *Rag1*$^{-/-}$ recipients was analyzed. CD4$^+$ T cell numbers in the spleen of infected mice were marginally increased compared with the homeostatic expansion observed in uninfected controls (**Figure 7B**). In contrast, in the bone marrow of *Rag1*$^{-/-}$ hosts there was significant increase in EF4.1 TCRβ-transgenic CD4$^+$ T cells numbers in the presence of FV, compared with uninfected recipients (**Figure 7D**), indicating that migration and/or local expansion of these cells in the bone marrow is enhanced by the presence of FV-infected cells. Furthermore, we observed enrichment in FV-specific TCR Vα2$^+$ clones in both the spleen and bone marrow of FV-infected mice (**Figure 7A- 7C**).

Therefore, these results suggested that upon FV infection of *Rag1*$^{-/-}$ mice, FV-specific CD4$^+$ T cells migrate and/or expand in the bone marrow and these cells are probably responsible for the bone marrow pathology observed in these mice.

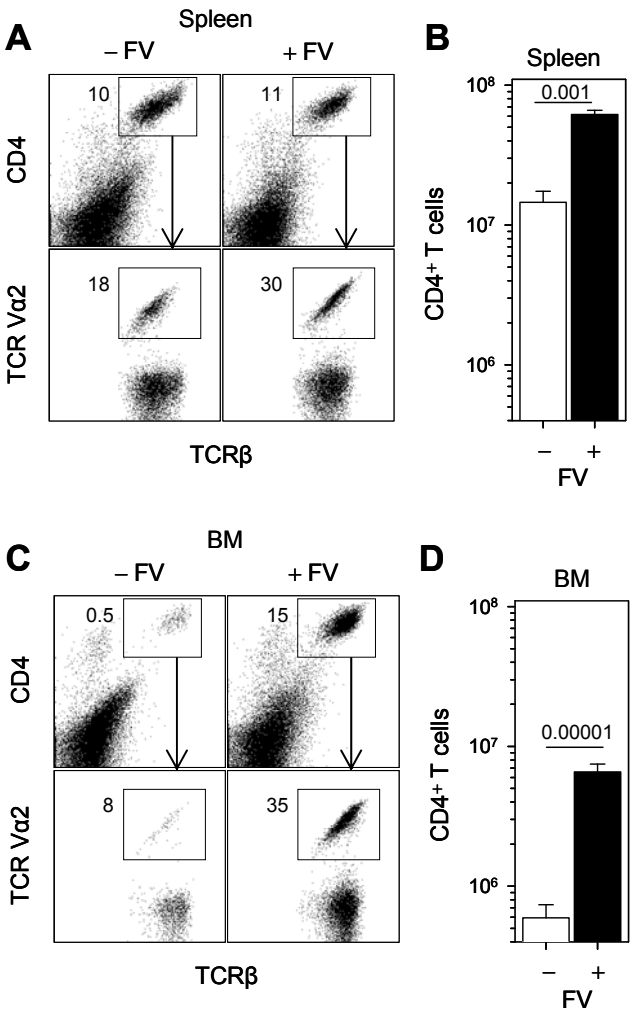

Figure 7. Enrichment in CD4$^+$ T cells in the bone marrow of FV-infected immunodeficient mice receiving EF4.1 TCRβ-transgenic CD4$^+$ T cells.
(**A-D**) Percentage (**A,C**) and absolute numbers (**B,D**) of CD4$^+$ T cells and percentage of TCR Vα2$^+$ T cells in total CD4$^+$ T cells (**A,C**) in the spleen (**A,B**) or the bone marrow (**C,D**) of uninfected (-FV) or FV-infected (+FV) recipients of EF4.1 TCRβ-transgenic CD4$^+$ T cells. Values are representative of 5-8 mice per group analyzed on day 21.

3.2.5 Potential contribution of lymphopenia in immune pathology

The model of FV-induced immunopathology was based on the use of *Rag1*$^{-/-}$ mice as hosts, raising the possibility that the disease could by influenced by the transfer of CD4^{+} T cells into the T cell-lymphopenic environment found in these mice. To exclude this possibility, B cell-deficient mice were used, which lack FV-neutralizing antibodies but are T cell-replete. A significant proportion of infected cells was found in the bone marrow of FV-infected B cell-deficient but not of wild-type control mice (**Figure 8A**). Furthermore, in contrast to wild-type mice, B cell-deficient mice developed significant anaemia during the course of infection (**Figure 8B**), suggesting that both EF4.1 TCRβ-transgenic and wild-type host CD4^{+} T cells were capable of mediating bone marrow pathology in the setting of immunodeficiency.

Thus, the contribution of immunodeficient hosts to anaemia development was to allow the replication and spread of FV to the bone marrow, rather than to provide a state of T cell-lymphopenia.

These results suggested a model for the development of anaemia where failure to contain FV replication and spread causes a systemic non-cytopathic infection which attracts the virus-specific (TCR Vα2^{+}) CD4^{+} T cell clones into the bone marrow, a location from which these cells would normally be excluded. Since *Rag1*$^{-/-}$ mice do not generate T or B cells, the antiviral activity of FV-specific CD4^{+} T cells must therefore be direct.

Furthermore, this activity of $CD4^+$ T cells seems to cause the loss of bone marrow cellularity, especially erythroid precursors, and is likely to be responsible for the development of anaemia observed in these conditions.

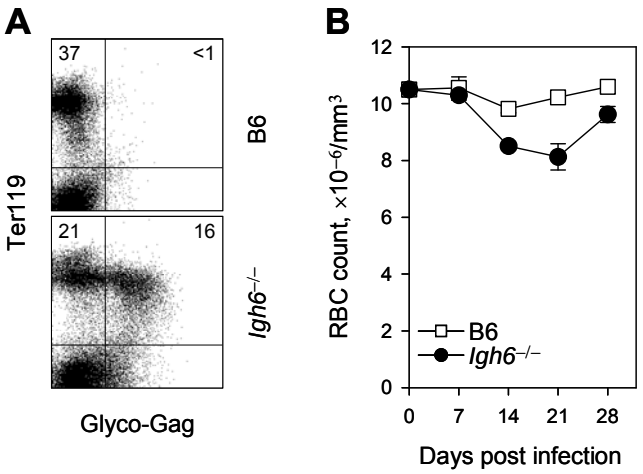

Figure 8. Bone marrow infection and anaemia development in FV-infected B cell-deficient mice.

(**A**) Percentages of uninfected and FV-infected (glyco-Gag$^+$) Ter119$^+$ cells in the bone marrow of wild-type B6 (B6) and B cell-deficient (*Igh6*$^{-/-}$) mice 28 days after FV infection. Numbers within the plots represent the percentage of positive cells and are the average of 4-5 mice per group.

(**B**) Changes in RBC counts in the same groups of mice described in **A**. Values represent the mean (±SEM) of 4-5 mice per group per time point, analyzed in 2 independent experiments. $P < 0.001$ between 'B6' and '*Igh6*$^{-/-}$' for days 14-21.

3.3 A role for Treg cells in FV-induced bone marrow pathology

Regulatory T cells have been shown to have a protective function in the development of immune pathologies (Sakaguchi et al., 2008) and certain studies have shown that in situations where anaemia is caused by graft-versus-host disease, Treg cells seem to be at a reduced frequency (Zorn et al., 2005; Solomou et al., 2007) and Treg cell transfer ameliorates the symptoms (Chen et al., 2007a). During FV-induced bone marrow pathology, the pathogenic potential of FV-specific $CD4^+$ T cells is highlighted by the development of anaemia. However, the $CD4^+$ T cell population should also contain Treg cells with the potential to counteract the pathogenic effect of excessive $CD4^+$ T cell response to FV infection. Therefore a role for Treg cells in the FV-induced bone marrow pathology was tested.

3.3.1 Development of Treg cells in EF4.1 TCRβ-transgenic mice

To address a possible role for Treg cells in the FV-induced immunopathology model it was first confirmed that Treg cells develop normally in EF4.1 TCRβ-transgenic mice. The distinct subpopulations of $CD4^+$ T cells from EF4.1 TCRβ-transgenic mice were identified based on the expression of CD44 and CD25. Naïve ($CD44^{low}$, $CD25^-$), memory ($CD44^{high}$, $CD25^-$) and regulatory ($CD44^{int}$, $CD25^+$) $CD4^+$ T cell subsets were all present in EF4.1 TCRβ-transgenic mice (**Figure 9A**). The number of naïve $CD4^+$ T

cells was increased in both the spleen and lymph nodes of these mice, while the numbers of memory and Treg cells were similar to wild-type controls (**Figure 9B-9D**). This increase in naïve $CD4^+$ T cell numbers was expected since the transgenic TCRβ chain was cloned from a $CD4^+$ T cell clone, which confers bias towards MHC class II recognition.

It is generally accepted that the best available marker for the identification of Treg cells is the transcription factor FoxP3, known to be important for Treg cell development and function (Hori et al., 2003; Khattri et al., 2003; Fontenot et al., 2003). In both wild-type and EF4.1 TCRβ-transgenic $CD4^+$ T cells, expression of CD25 overlapped with expression of the Treg cell-specific FoxP3 (**Figure 9E**). This result proved CD25 as a suitable marker for identifying Treg cells and therefore in subsequent experiments Treg cells from naïve EF4.1 TCRβ-transgenic mice were identified and isolated based on CD25 expression. Furthermore, the number of $FoxP3^+$ Treg cells was comparable between the spleen and lymph node of EF4.1 TCRβ-transgenic and wild-type controls (**Figure 9B-9D**), although EF4.1 TCRβ-transgenic mice had a lower frequency of Treg cells (**Figure 9E**), due to the higher numbers of naïve $CD4^+$ T cells.

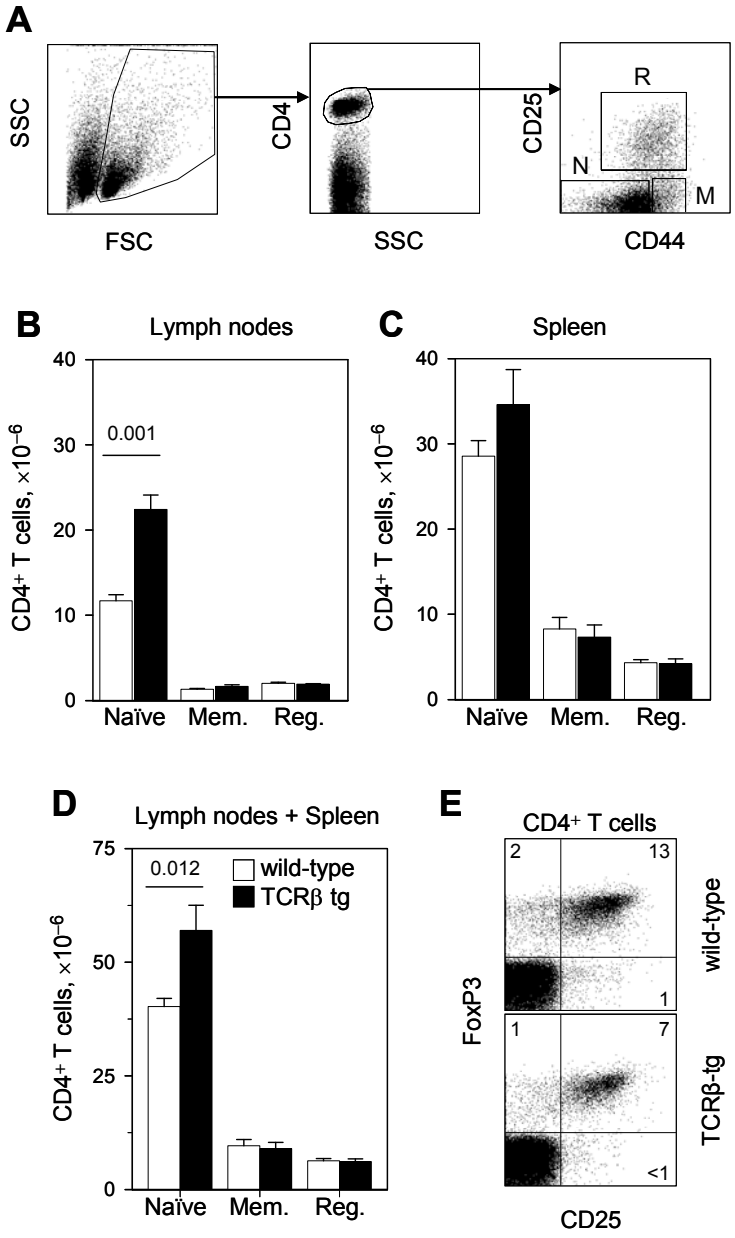

Figure 9. Treg cell development in EF4.1 TCRβ-transgenic mice.

(**A**) Flow cytometric example of the gating applied to define the total, $CD44^{lo}CD25^{-}$ naïve (N), $CD44^{hi}CD25^{-}$memory (M) and $CD44^{int}CD25^{+}$ regulatory (R) $CD4^{+}$ T cells populations.

(**B-D**) Absolute numbers of naïve, memory (Mem.) or regulatory (Reg.) $CD4^{+}$ T cells in the lymph nodes (**B**), spleen (**C**) or lymph nodes and spleen (**D**) of EF4.1 TCRβ-transgenic (TCRβ-tg) and wild-type control mice. Values are the mean (±SEM) of 6 mice per group and numbers within the graphs denote the *P* value.

(**E**) CD25 and FoxP3 staining in gated $CD4^{+}$ T cells from EF4.1 TCRβ-transgenic (TCRβ-tg) and wild-type control mice. Numbers within the quadrants denote the percentages of positive cells and are representative of 3 mice per group.

3.3.2 Effect of EF4.1 TCRβ-transgenic Treg cells in bone marrow pathology

To examine whether Treg cells contained in adoptively transferred EF4.1 TCRβ-transgenic $CD4^+$ T cells had a suppressive role in the development of immune pathology, the anaemic condition in transfers of EF4.1 TCRβ-transgenic total $CD4^+$ T cells or Treg cell-depleted naïve $CD4^+$ T cells ($CD25^-$ $CD45RB^{high}$) was compared.

Mice that received naïve $CD4^+$ T cells had significantly lower RBC counts when compared with mice receiving total $CD4^+$ T cells, reaching their lowest value at day 21 after T cell transfer (**Figure 10A**). This reduction in RBC counts was followed by partial recovery and by day 35 the RBC numbers were similar to those in recipients of total $CD4^+$ T cells (**Figure 10A**). Thus, there was an overall enhancement of the severity of anaemia in the absence of Treg cells, suggesting that Treg cells present in the pathogenic $CD4^+$ T cell population are suppressing the development of pathology, although they cannot totally prevent the anaemic condition. It was therefore reasoned that increasing the numbers of Treg cells in the total $CD4^+$ T cell population would have a protective effect in bone marrow pathology. Indeed, addition of extra EF4.1 TCRβ-transgenic Treg cells to total EF4.1 TCRβ-transgenic $CD4^+$ T cells at 1:1 ratio led to a significant, albeit incomplete, suppression of anaemia development throughout the infection (**Figure 10A**). This protective effect of Treg cells was the result of restoration of bone marrow cellularity (**Figure 10B**), in both the $Ter119^+$ (**Figure 10C**) and $CD11b^+Gr1^+$ (**Figure**

10D) compartments. Therefore, Treg cells seemed to be protecting from anaemia development by reducing the severity of bone marrow pathology. Together these results suggest a model where the severity of bone marrow pathology observed in FV-infected immunodeficient mice is proportional to the relative frequency of pathogenic $CD4^+$ T cells and Treg cells.

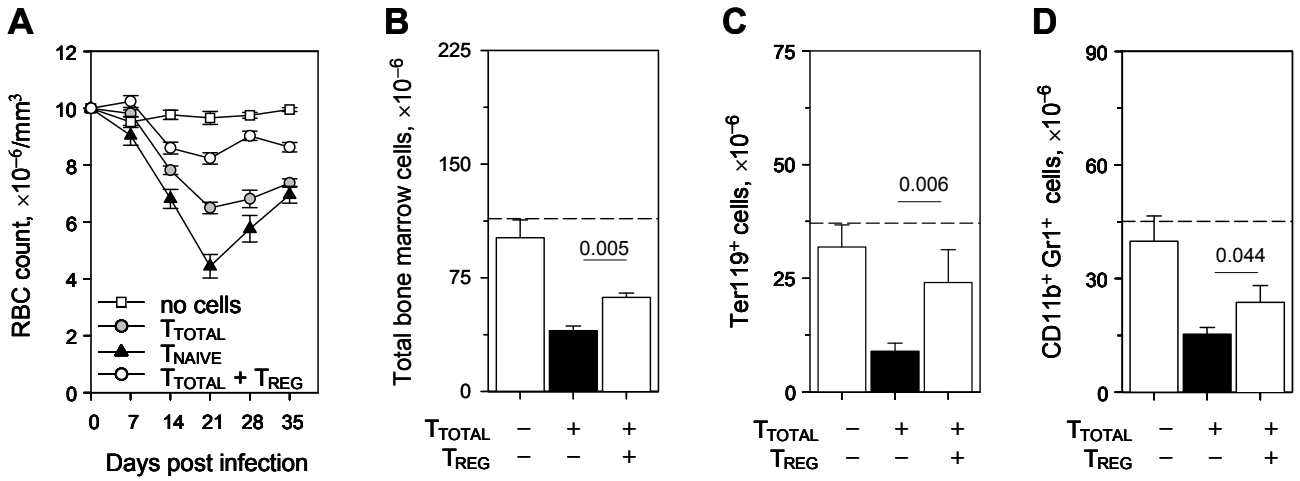

Figure 10. Suppression of bone marrow pathology by Treg cells in a dose-dependent manner.

(**A**) Changes in RBC counts in FV-infected *Rag1*$^{-/-}$ mice, which received total EF4.1 TCRβ-transgenic CD4$^+$ T cells (T_{TOTAL}), or FACS-purified Treg-depleted naïve CD45RB^{hi}CD25$^-$CD4$^+$ T cells (T_{NAIVE}) or total CD4$^+$ T cells together with FACS-purified CD25$^+$CD4$^+$ T cells at 1:1 ratio (T_{TOTAL} + T_{REG}). Values represent the mean (±SEM) of 8-12 mice per group per time point analysed in 3 independent experiments. *P* = 0.0002 between 'T_{TOTAL}' and 'T_{TOTAL} + T_{REG}', and *P* = 0.00002 between 'T_{TOTAL}' and 'T_{NAIVE}', on day 21.

(**B-D**) Total bone marrow cellularity (**B**) and numbers of Ter119$^+$ erythroid precursor cells (**C**) and CD11b$^+$Gr1$^+$ bone marrow cells (**D**) in FV-infected *Rag1*$^{-/-}$ mice, which received EF4.1 TCRβ-transgenic CD4$^+$ T cells, alone or together with EF4.1 TCRβ-transgenic Treg cells at 1:1 ratio. The dashed line represents the average cell numbers of untreated, uninfected *Rag1*$^{-/-}$ mice. Values are the mean (±SEM) of 5-8 mice per group on day 21. Numbers within the graphs denote the *P* values.

3.3.2.1 Pathogenic potential of wild-type $CD4^+$ T cells

The data indicated that anaemia was the result of chronic stimulation of FV-specific $CD4^+$ T cells and that Treg cells had a protective function by suppressing effector T cells. According to this model, wild-type $CD4^+$ T cells, where the frequency of virus-specific clones should be very low ($<10^5$), should not cause anaemia when transferred into FV-infected *Rag1*$^{-/-}$ recipients. However, removal of the Treg cell population should unmask the pathogenic potential of the few FV-specific $CD4^+$ T cell clones. Indeed, transfer of total wild-type $CD4^+$ T cells into FV-infected *Rag1*$^{-/-}$ mice did not cause anaemia (**Figure 11**), in contrast with transfer of total $CD4^+$ T cells from EF4.1 TCRβ-transgenic mice (**Figure 5**). However, transfer of Treg cell-depleted naïve wild-type $CD4^+$ T cells ($CD25^-$ $CD45RB^{high}$) induced anaemia in the recipient mice (**Figure 11**). The severity of anaemia induced by transfer of wild-type naïve $CD4^+$ T cells was milder than the one caused by EF4.1 TCRβ-transgenic naïve $CD4^+$ T cells, in agreement with the enrichment of FV-specific $CD4^+$ T cell clones in EF4.1 TCRβ-transgenic mice.

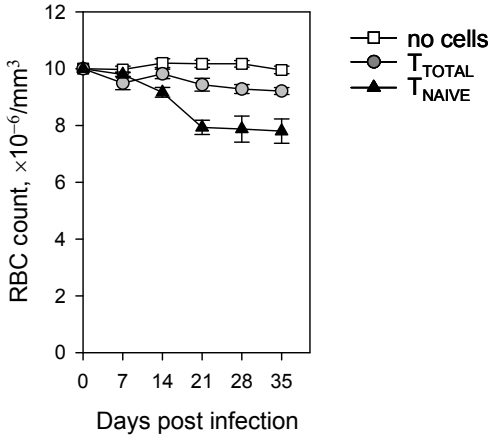

Figure 11. Induction of bone marrow pathology by wild-type non-transgenic CD4$^+$ T cells responding to FV infection in the absence of Treg cells.
Changes in RBC counts in FV-infected *Rag1*$^{-/-}$ mice, which received either total CD4$^+$ T cells (T_{TOTAL}) or FACS-purified Treg-depleted naïve CD45RB^{hi}CD25$^-$CD4$^+$ T cells (T_{NAIVE}) from wild-type B6 mice or no T cells (no cells). Values represent the mean (±SEM) of 9-10 mice per group per time point analyzed in 2 independent experiments. $P < 0.0003$ between 'T_{NAIVE}' and 'T_{TOTAL}', on day 21.

3.3.3 Mechanisms of Treg cell-mediated suppression of bone marrow pathology

To further investigate the mechanisms by which FV-induced bone marrow pathology was suppressed by Treg cells, their effect on the expansion and effector function of FV-specific pathogenic $CD4^+$ T cells was examined.

3.3.3.1 Effect of Treg cells on the expansion of FV-specific $CD4^+$ T cells

The effect of Treg cells on expansion of FV-specific pathogenic $CD4^+$ T cells was examined first. Addition of EF4.1 TCRβ-transgenic Treg cells caused a two-fold reduction in the number of $CD4^+$ T cells in the spleen of FV-infected recipients (**Figure 12A**). However, despite suppressing the proliferation of $CD4^+$ T cells, Treg cells did not prevent the expansion of FV-specific TCR $V\alpha2^+$ clones, the frequency of which rose from 32 to 42% in the spleen (**Figure 12B**). This increase was probably due to the loss of FV-nonspecific $CD4^+$ T cells since Treg cells would be preferentially suppressing the homeostatic expansion of the $CD4^+$ T cells, rather than FV-driven expansion. Moreover, the presence of Treg cells did not prevent the migration, accumulation or local expansion of $CD4^+$ T cells in the bone marrow (**Figure 12C**), where they cause pathology. Similar to the spleen, in the bone marrow of these mice there was enrichment in the frequency of TCR $V\alpha2^+$ virus-specific clones (**Figure 12D**). These results showed that Treg cells do not seem to be affecting the expansion or migration of the virus-specific $CD4^+$ T cell

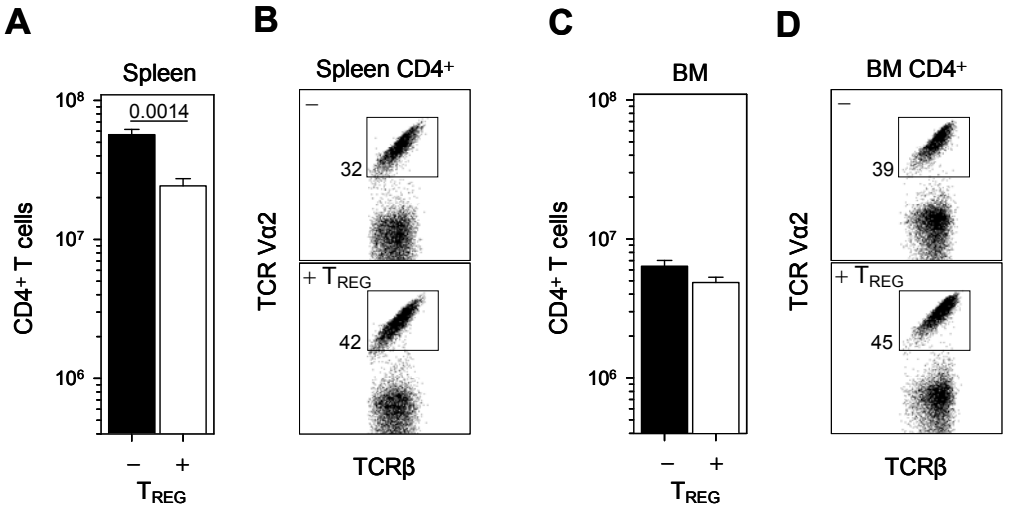

Figure 12. FV-specific EF4.1 TCRβ-transgenic CD4$^+$ T cell expansion in the presence of EF4.1 TCRβ-transgenic Treg cells.

(**A-D**) Absolute numbers of CD45.1$^+$ EF4.1 TCRβ-transgenic CD4$^+$ T cells (**A,C**) and percentage of TCR Vα2$^+$ T clones in these cells (**B, D**), isolated from the spleen (**A**, **B**) or the bone marrow (BM) (**C**, **D**) of FV-infected *Rag1*$^{-/-}$ mice, which received total CD45.1$^+$ EF4.1 TCRβ-transgenic CD4$^+$ T cells, alone (–) or together (+ T_{REG}) with CD45.2$^+$ EF4.1 TCRβ-transgenic Treg cells at 1:1 ratio. Values in **A** and **C** are the mean (±SEM) of 5-8 mice per group on day 21 analysed in more than 3 independent experiments. Numbers within the quadrants in **B** and **D** denote the percentages of positive cells and are representative of 5-8 mice per group on day 21.

clones and it was therefore reasoned that they could be acting on a subsequent step of $CD4^+$ T cell function, such as effector cytokine production.

3.3.3.2 Effect of Treg cells on the cytokine production of FV-specific $CD4^+$ T cells

To address the hypothesis that Treg cells were acting on effector T cell function, the cytokine production by pathogenic $CD4^+$ T cells from the spleen and bone marrow of FV-infected recipients was analyzed at day 21 and 35 after transfer. Specifically, the expression of IL-2, IL-4, IL-17 and IFN-γ was examined. No significant expression of IL-4 in the $CD4^+$ T cell population was detected. IL-17 production was detected in a small percentage of $CD4^+$ T cells in the spleen and bone marrow, but it was not affected by the presence of additional Treg cells in any of the locations (**Figure 13A-13C**). Similarly, IL-2 was detected in a small fraction of $CD4^+$ T cells and its production was not affected by the presence of Treg cells. In contrast, a significant proportion of $CD4^+$ T cells produced IFN-γ both in the spleen (42%) (**Figure 13A-13B**) and bone marrow (44%) (**Figure 13C-13D**). Significant amounts of IFN-γ were also detected in the serum of these mice (**Figure 13E**). These results suggested that under these experimental conditions, a significant proportion of the transferred $CD4^+$ T cells differentiate into Th1 helper cells. Importantly, the presence of additional Treg cells had a suppressive effect on IFN-γ production, reducing the percentage of IFN-γ-producers by 22% in the spleen (**Figure**

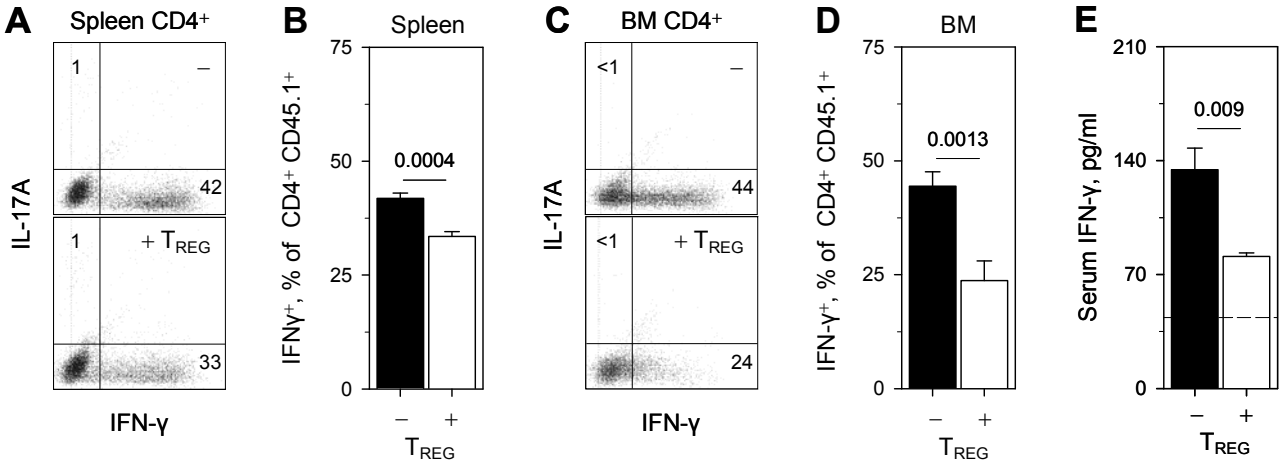

Figure 13. Treg cell-mediated suppression of local IFN-γ production by conventional EF4.1 TCRβ-transgenic CD4$^+$ T cells in the bone marrow.

(**A-D**) Percentage of IL-17A and IFN-γ producing cells in total CD45.1$^+$ EF4.1 TCRβ-transgenic CD4$^+$ T cells, isolated from the spleen (**A,B**) or the bone marrow (BM) (**C,D**) of FV-infected *Rag1*$^{-/-}$ mice, which received total CD45.1$^+$ EF4.1 TCRβ-transgenic CD4$^+$ T cells, alone (–) or together (+ T_{REG}) with CD45.2$^+$ EF4.1 TCRβ-transgenic Treg cells at 1:1 ratio. Values are representative of 6-9 mice per group, analysed either on day 21 or day 35 with identical results. Numbers within the bar graphs and dot plots denote the *P* values and percentage of positive cells, respectively. The median fluorescence intensity (MFI) ±SEM of IFN-γ staining in **C** was '- T_{REG}' = 160±19; '+ T_{REG}' = 105±15; P = 0.0003, Student's *t*-test, n = 6.

(**E**) Serum IFN-γ levels in FV-infected *Rag1*$^{-/-}$ mice, which received total CD45.1$^+$ EF4.1 TCRβ-transgenic CD4$^+$ T cells, alone (–) or together (+ T_{REG}) with CD45.2$^+$ EF4.1 TCRβ-transgenic Treg cells at 1:1 ratio. Values represent the mean (±SEM) of 4-5 mice per group analyzed on day 35. The dashed line depicts the basal levels of serum IFN-γ in unmanipulated *Rag1*$^{-/-}$ mice. Numbers within the graph denote the *P* values.

13A-13B) and, even more dramatically, by approximately 45% in the bone marrow (**Figure 13C-13D**), the place where the pathology develops. Furthermore, Treg cells had also a profound effect on the amount of IFN-γ produced by individual bone marrow $CD4^+$ T cells, based on the median fluorescence intensity (MFI) of the IFN-γ staining, which was reduced by around 34% (**Figure 13C**). Significant suppression of IFN-γ production by Treg cells could also be observed in the IFN-γ serum levels, which were reduced by around 40% (**Figure 13E**). Together these results suggested that Treg cells protect from immunopathology by suppressing IFN-γ production by a large proportion of the $CD4^+$ T cell population.

3.3.3.3 Role of IFN-γ in the development of immune pathology

The finding that Treg cells were inhibiting IFN-γ production by pathogenic $CD4^+$ T cells and the established role of IFN-γ in myelosuppression (Raefsky et al., 1985) raised the hypothesis that this cytokine could be responsible for the development of immune pathology. If this were the case, then mice which do not signal through the IFN-γ receptor should not develop the disease. INF-γ signals through a multimeric receptor complex consisting of two different chains: IFN-γ receptor 1 (IFN-γR1) and IFN-γ receptor 2 (IFN-γR2). IFN-γR1 is the primary binding subunit of the complex while IFN-γR2 is necessary for signal transduction of IFN-γ (Kotenko et al., 1995). *Ifngr1*$^{-/-}$ cells show a

major defect in IFN-γ signalling (Cantin et al., 1999) and *Ifngr1*$^{-/-}$ mice were used to test the role of IFN-γ in bone marrow pathology. In comparison with FV-infected *Rag1*$^{-/-}$ recipients, anaemia onset in FV-infected *Rag1*$^{-/-}$ *Ifngr1*$^{-/-}$ was significantly reduced or delayed (**Figure 14A**), demonstrating an essential role for IFN-γ signalling in this process. However, the development of anaemia in *Ifngr1*$^{-/-}$ mice could not be followed at later time points, as these mice developed severe pathology, characterized by expansion of the granulocyte population (**Figure 14B**), necessitating termination of the experiments. This granulocyte expansion peaked at week 2-3 after transfer and was followed by recovery at later time points (**Figure 14B**), suggesting it is a temporary effect caused by the lack of IFN-γ signalling in the recipient mice. This expanding population was further characterized and it was found to be positive for CD11b and Gr1 staining (**Figure 14C**), markers for neutrophil precursors.

Collectively, these results suggested a mechanism by which the IFN-γ produced by the CD4^{+} T cells in the bone marrow causes depletion of bone marrow precursors leading to the development of anaemia. They also implied that Treg cells protected from bone marrow pathology by suppressing IFN-γ produced by the pathogenic CD4^{+} T cells.

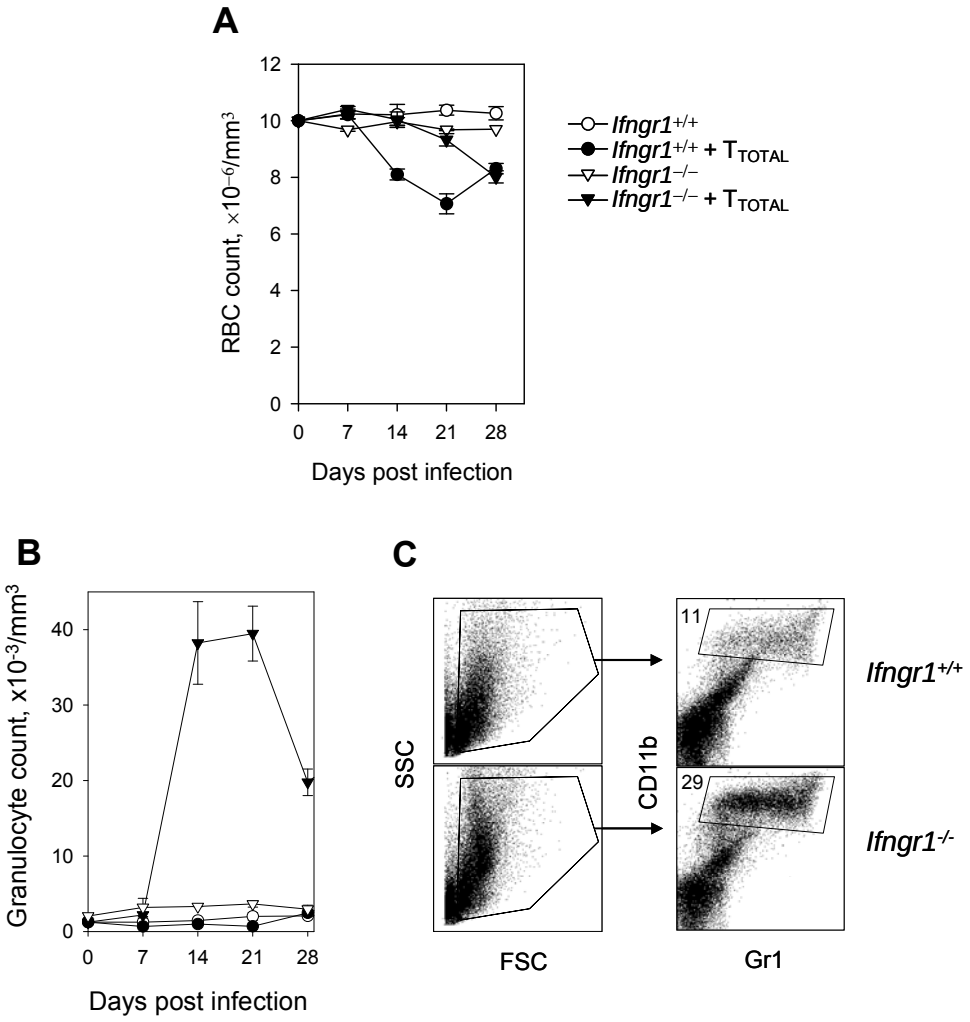

Figure 14. Delay in the onset of anaemia in FV-infected IFNγ receptor-deficient mice receiving EF4.1 TCRβ-transgenic CD4$^+$ T cells.

(**A-B**) Changes in RBC (**A**) and granulocyte counts (**B**) in *Rag1*$^{-/-}$*Ifngr1*$^{+/+}$ (*Ifngr1*$^{+/+}$) or *Rag1*$^{-/-}$*Ifngr1*$^{-/-}$ (*Ifngr1*$^{-/-}$) mice either infected with FV alone (*Ifngr1*$^{+/+}$; *Ifngr1*$^{-/-}$) or infected with FV and received CD4$^+$ T cells (*Ifngr1*$^{+/+}$ + T_{TOTAL}; *Ifngr1*$^{-/-}$ + T_{TOTAL}). Values represent the mean (±SEM) of 12-15 mice per group per time point analyzed in 3 independent experiments. $P < 0.0001$ between '*Ifngr1*$^{+/+}$ + T_{TOTAL}' and '*Ifngr1*$^{-/-}$ + T_{TOTAL}', on days 14 and 21 in **A** and $P < 0.0001$ between '*Ifngr1*$^{-/-}$ + T_{TOTAL}' and all other groups on days 14, 21 and 28 in **B**.

(**C**) Flow cytometric example of the CD11b$^+$GR1$^+$ population in the *Ifngr1*$^{+/+}$ + T_{TOTAL} and *Ifngr1*$^{-/-}$ + T_{TOTAL} mice. Numbers within the dot plots denote the percentage of positive cells and are representative of 3-5 mice per group analysed on day 21 from more than 3 independent experiments.

3.4 Envelope-reactivity of EF4.1 TCRβ-transgenic Treg cells from naïve mice

Development of FV-induced T cell-mediated immune pathology required activation of virus-specific $CD4^+$ T cells, as disease did not develop in uninfected controls (**Figure 5**). Studies on Treg cell reactivity have suggested their TCRs are biased towards self-reactivity (Hsieh et al., 2004) and that the TCR repertoire of regulatory and conventional T cells is largely non-overlapping (Pacholczyk et al., 2006; Hsieh et al., 2006). These studies raised the hypothesis that these two subsets may not be activated by the same ligands. Therefore, protective Treg cells could be specifically activated by viral antigens, as it was the case for pathogenic $CD4^+$ T cells, or alternatively, their protective effect could be mediated by Treg cells of unrelated FV-specificity.

3.4.1 Expression of the transgenic TCRβ chain in EF4.1 TCRβ-transgenic Treg cells

It was previously observed that normal numbers of Treg cells develop in EF4.1 TCRβ-transgenic mice, although with reduced frequency compared with wild-type controls (**Figure 9B-9E**). To establish the reactivity to FV of EF4.1 TCRβ-transgenic Treg cells, expression of the transgenic TCRβ chain in these cells was examined first. Although 95% of $CD4^+$ T cells from EF4.1 TCRβ-transgenic mice expressed the transgenic TCRβ chain

(**Figure 2B**), it was still possible that Treg cells were expressing an endogenous TCRβ chain. If this were the case, it would suggest that the expression of the transgene would impair the development of Treg cells. To address this question the degree of allelic exclusion of endogenous TCRβ chains, measured by a panel of antibodies for several of the TCR Vβ chains, was assessed in naïve, memory and regulatory subsets. In wild-type mice, TCR Vβ chains were equally expressed in the naïve, memory and regulatory $CD4^+$ T cell populations, with the exception of TCR Vβ5, which was preferentially expressed in the regulatory subset (**Figure 15A**). The EF4.1 TCRβ-transgenic $CD4^+$ T cells showed almost complete exclusion of endogenous TCR Vβ chains in the naïve compartment, highlighting the efficiency of allelic exclusion by the TCRβ transgene in these mice (**Figure 15A**). In sharp contrast, memory cells expressed many endogenous TCR Vβ chains at significant levels, sometimes higher than the wild-type controls (for the TCR Vβ7, Vβ14 and Vβ17 chains) (**Figure 15A**). The EF4.1 TCRβ-transgenic Treg cells showed frequencies of endogenous TCR Vβ expression between those observed for the naïve and memory subsets, indicating a reduced, but still considerable expression for most of the endogenous TCR Vβ chains. As seen in wild-type mice, TCR Vβ5 was also preferentially expressed by the regulatory T cells in EF4.1 TCRβ-transgenic mice, although at significantly lower levels (**Figure 15A**). As before (**Figure 2B**), the percentages obtained for all endogenous TCR Vβ chains were summed up (**Figure 15B measured**) and extrapolated to 100% (**Figure 15B projected**), demonstrating that in the naïve subset there was almost complete exclusion of the endogenous TCR Vβ chains, while in the regulatory and memory compartments there was significant expression: 21%

of regulatory and more than 50% of memory EF4.1 TCRβ-transgenic $CD4^+$ T cells expressed endogenous TCR Vβ chains. The development of a memory $CD4^+$ T cell population in both wild-type and EF4.1 TCRβ-transgenic mice is probably due to their polyclonal TCR repertoire, since in TCRα/β-transgenic mice, which have a single antigen specificity, the memory population does not develop(Hataye et al., 2006).

The expression of endogenous TCR Vβ chains in the regulatory compartment was surprisingly high (21%). This relatively high percentage of endogenous TCRβ-expressing Treg cells in EF4.1 TCRβ-transgenic mice is indicative of a requirement for either Treg thymic selection or peripheral expansion or both. Recent reports (Fisson et al., 2003; Fritzsching et al., 2006) have suggested the presence of different regulatory populations in the periphery: a quiescent, resistant to apoptosis and with long lifespan (naïve) subset and an actively dividing, prone to apoptosis and expressing activation markers (memory) subpopulation. Given these reports and the fact that 50% of the memory $CD4^+$ T cells from the EF4.1 TCRβ-transgenic mice expressed an endogenous TCR Vβ chain, it was hypothesized that Treg cells expressing endogenous TCR Vβ chains could also have a memory phenotype. To test this hypothesis expression of the memory marker CD44 in Treg cells from EF4.1 TCRβ-transgenic mice was examined in conjunction with expression of endogenous TCR Vβ chains. The majority of $CD25^+$ $CD4^+$ T cells expressing an endogenous TCR Vβ chain (for TCR Vβ5, 6, 11 and 14) were also higher for the expression of the memory marker CD44 (**Figure 15C**). Therefore, these findings supported the idea that regulatory T cells in the periphery are not a homogeneous

population and that it is possible to distinguish a naïve and a memory subset (Fisson et al., 2003; Fritzsching et al., 2006). They also suggested that, similar to memory $CD4^+$ T cells, Treg cells with a memory phenotype may be the result of exposure to diverse environmental antigens (Fisson et al., 2003).

Importantly, together these results indicated that the majority of Treg cells in EF4.1 TCRβ-transgenic mice express the transgene and could therefore contain env-specific clones.

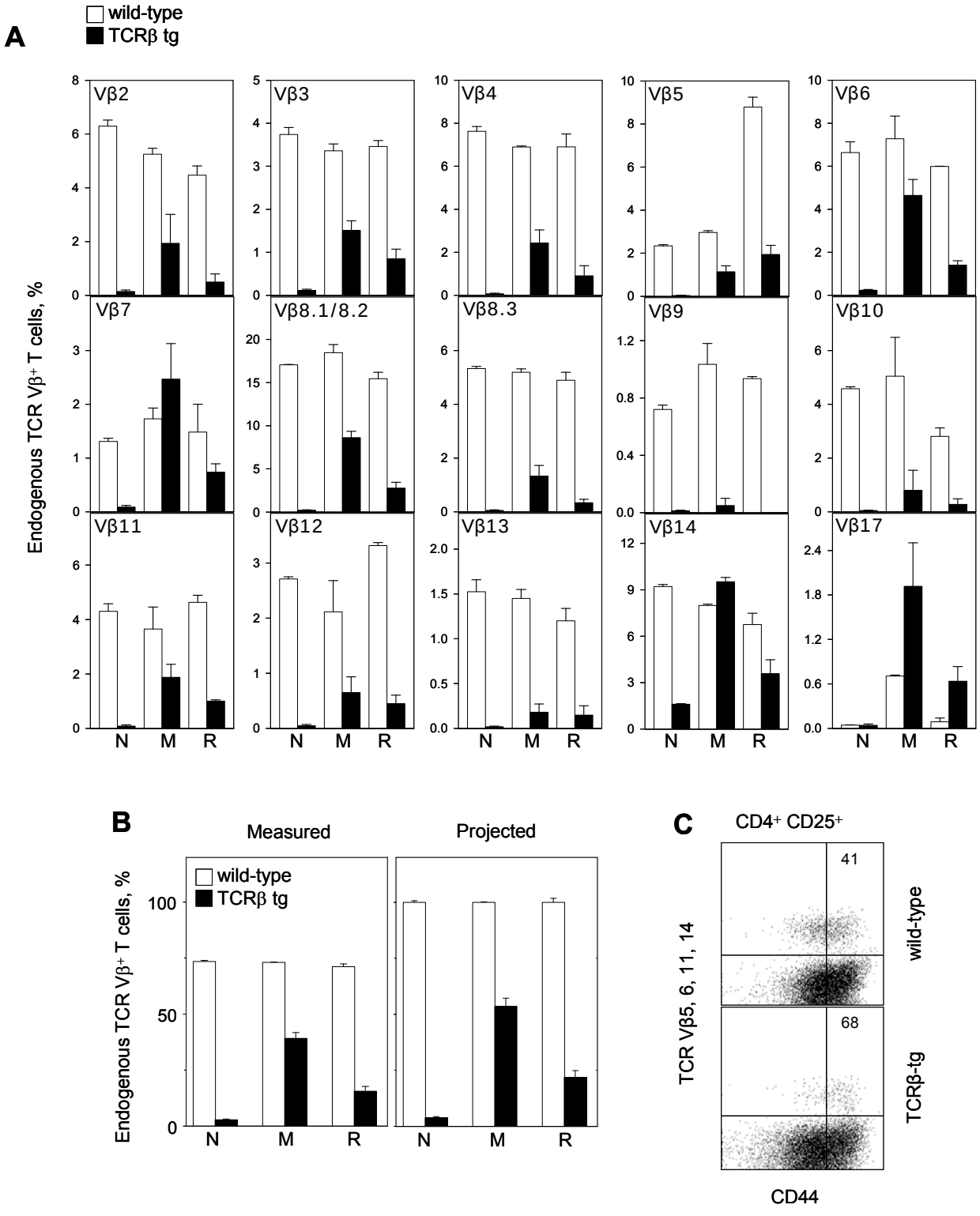
A
wild-type
TCRβ tg
Endogenous TCR Vβ+ T cells, %
Vβ2
Vβ3
Vβ4
Vβ5
Vβ6
Vβ7
Vβ8.1/8.2
Vβ8.3
Vβ9
Vβ10
Vβ11
Vβ12
Vβ13
Vβ14
Vβ17
N M R
B
Measured
Projected
wild-type
TCRβ tg
Endogenous TCR Vβ+ T cells, %
C
CD4+ CD25+
41
68
wild-type
TCRβ-tg
TCR Vβ5, 6, 11, 14
CD44

Figure 15. Expression of endogenous TCR Vβ chains in EF4.1 TCRβ-transgenic CD4$^+$ T cell subsets.
(**A-B**) The percentage of T cells expressing TCR Vβ2, Vβ3, Vβ4, Vβ5.1/5.2, Vβ6, Vβ7, Vβ8.1/8.2, Vβ8.3, Vβ9, Vβ10b, Vβ11, Vβ12, Vβ13, Vβ14 or Vβ17a, in naïve (N), memory (M) and regulatory (R) CD4$^+$ T cells from EF4.1 TCRβ-transgenic (TCRβ-tg) and wild-type control mice is shown (**A**). Sum of the percentages for the endogenous TCR Vβ chains shown in **A** (measured). These TCR Vβ chains are used by ~ 75% of CD4$^+$ T cells in wild-type B6 mice. The sum of measured TCR Vβ expression in wild-type B6 mice was set to 100% and, assuming that allelic exclusion by the transgenic TCRβ chain is similar for the remaining ~25% of endogenous TCR Vβ chains not covered by the antibody panel, the residual expression of endogenous TCR Vβ expression in EF4.1 TCRβ-transgenic CD4$^+$ T cells was then calculated (projected) (**B**)**.**
(**C**) CD44 expression by wild-type and EF4.1 TCRβ-transgenic (TCRβ-tg) Treg cells according to expression of endogenous TCR Vβ chain expression. Numbers within the quadrants denote the percentage of T cells which express high levels of CD44 in endogenous TCR Vβ5.1/5.2, Vβ6, Vβ11 or Vβ14-expressing CD25$^+$ CD4$^+$ T cells. Values in **A-C** are the mean (±SEM) of 3 mice per group from one experiment, representative of 3 similar experiments.

3.4.2 *In vitro* assessment of envelope-reactivity of EF4.1 TCRβ-transgenic Treg cells

The previous findings showed that 1) 4% of EF4.1 TCRβ-transgenic $CD4^+$ T cells respond to $env_{122-141}$ stimulation (**Figure 3B**), 2) that EF4.1 TCRβ-transgenic mice develop normal numbers of Treg cells (**Figure 9B-9D**) and 3) that 80% of these Treg cells express the transgenic TCR Vβ chain (**Figure 15A-15B**). Thus, the presence of $env_{122-141}$-specific clones in EF4.1 TCRβ-transgenic Treg cells was examined.

3.4.2.1 Assessment of envelope-reactivity of EF4.1 TCRβ-transgenic Treg cells using a suppression assay

The $env_{122-141}$ – reactivity of Treg cells was tested in an *in vitro* suppression assay, which is commonly used to study the specificity and function of Treg cells (Thornton and Shevach, 2000; Itoh et al., 1999; Takahashi et al., 1998), by analyzing their potential to suppress a responding $CD4^+$ T cell population in an antigen-specific manner. When Treg cells are stimulated by their cognate antigen *in vitro*, they become activated and suppress the proliferation (here assessed by CFSE labeling) and IL-2 production of a responding $CD4^+$ T cell population. Sorted $CD4^+CD25^+CD45RB^{low}$ T cells (Treg cell population) and CFSE-labeled $CD4^+CD25^-CD45RB^{high}$ naïve T cells (responding population) were

from EF4.1 TCRβ-transgenic mice. The cultures were stimulated either with α-CD3 (a general T cell activator used as positive control) or with $env_{122-141}$.

When EF4.1 TCRβ-transgenic Treg cells were stimulated with α-CD3, activation of these cells was detected by their ability to efficiently suppress the proliferation (**Figure 16A**) and completely inhibit IL-2 production (**Figure 16B**) of the responding $CD4^+$ T cell population, demonstrating the potent suppressive capacity of EF4.1 TCRβ-transgenic Treg cells. In contrast, when the same cultures were stimulated with $env_{122-141}$, EF4.1 TCRβ-transgenic Treg cells had no effect on the proliferation of (**Figure 16C**) and only a minor effect on IL-2 production by (**Figure 16D**) the responding population. The reduction in the amount of IL-2 present in the supernatant of these cultures was probably due to IL-2 consumption by Treg cells, which express CD25, the IL-2 receptor, resulting from the high ratio of total Treg cells (50% of the culture T cells) to env-specific T cells (2% of the culture T cells). As a specificity control, no activation of wild-type $CD25^+$ T cells stimulated with $env_{122-141}$ was detected. Therefore, using this *in vitro* assay, no activation of the EF4.1 TCRβ-transgenic Treg cells with $env_{122-141}$ was detected, suggesting lack of specific induction of suppressive function in EF4.1 TCRβ-transgenic Treg cells by the $env_{122-141}$ peptide.

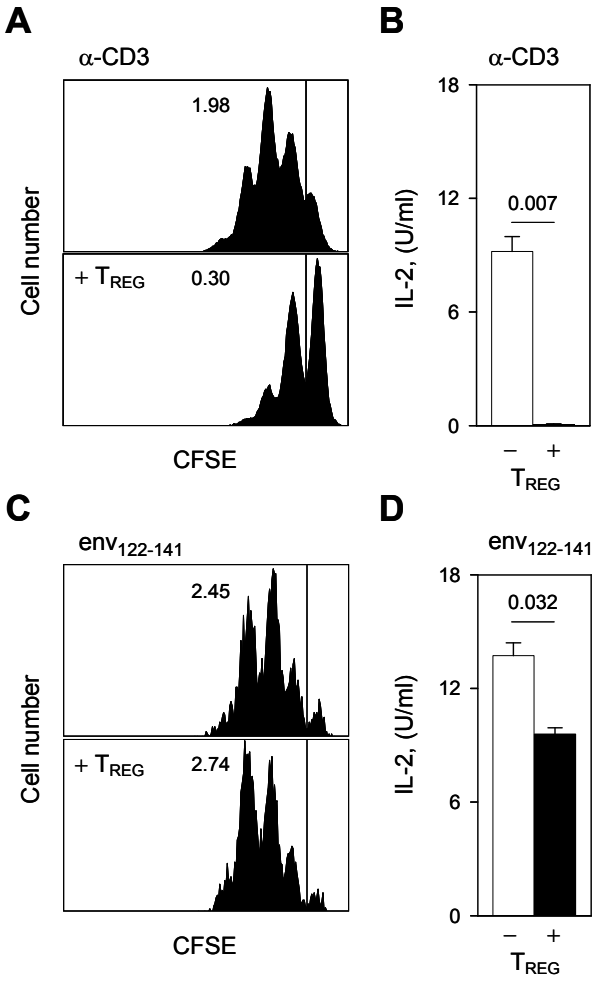

Figure 16. Lack of specific induction of *in vitro* suppressive function in EF4.1 TCRβ-transgenic CD4$^+$ Treg cells by the env$_{122\text{-}141}$ peptide.
(**A-D**) CFSE dilution profile of conventional EF4.1 TCRβ-transgenic CD4$^+$ T cells (**A, C**) and IL-2 concentration in supernatants (**B, D**) of cultures of CD45.1$^+$ CD45RB^{hi}CD25$^-$ EF4.1 TCRβ-transgenic CD4$^+$ naïve T cells, with (+ T_{REG}) or without an equal number of CD45.2$^+$ CD25$^+$ EF4.1 TCRβ-transgenic Treg cells, stimulated either with anti-CD3 antibody (**A, B**) or with the env$_{122\text{-}141}$ peptide (**C, D**) presented by bone marrow-derived dendritic cells. Similar results were obtained when using macrophages or B cells as antigen-presenting cells. IL-2 concentration and CFSE profiles were assayed on days 2 and 3 of culture, respectively. Specifically for env$_{122\text{-}141}$ peptide stimulation, the CFSE profile of responding cells only is shown. No differences in the fraction of responding cells were observed in the presence or absence of Treg cells. Numbers within the plots denote the mean division number of responding cells. One experiment, representative of 4 similar experiments, is shown.

3.4.2.2 Assessment of envelope-reactivity of EF4.1 TCRβ-transgenic Treg cells using a proliferation assay

To test the env-reactivity of TCRβ-transgenic Treg cells more directly, their proliferative response to $env_{122-141}$ presented by dendritic cells was measured under conditions previously shown to expand antigen-specific Treg cells (Kretschmer et al., 2005). Treg cell-depleted $CD4^+CD25^-$ EF4.1 TCRβ-transgenic T cells proliferated extensively when stimulated with $env_{122-141}$ (**Figure 17A**). This expansion was much more extensive than the spontaneous proliferation due to the removal of Treg cells in the absence of antigen or in the presence of the unrelated antigen $ova_{323-339}$ (**Figure 17A**), showing that proliferation was antigen-specific. In contrast, no proliferation of EF4.1 TCRβ-transgenic Treg cells was detected following stimulation with either peptide or in the absence of antigen (**Figure 17A**). As a control for the *in vitro* expansion capacity of antigen-specific Treg cells, $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells from ova-specific OT-II TCRα/β-transgenic mice (Barnden et al., 1998) were used. Both T cell populations expanded in the presence of the cognate antigen $ova_{323-339}$ but not when stimulated with $env_{122-141}$ under the same experimental conditions (**Figure 17B**).

These results suggested that EF4.1 TCRβ-transgenic Treg cell clones appeared unable to be stimulated *in vitro* by $env_{122-141}$. Both these results and the *in vitro* suppression assay are in agreement with previous studies suggesting that the TCR repertoire of Treg cells is

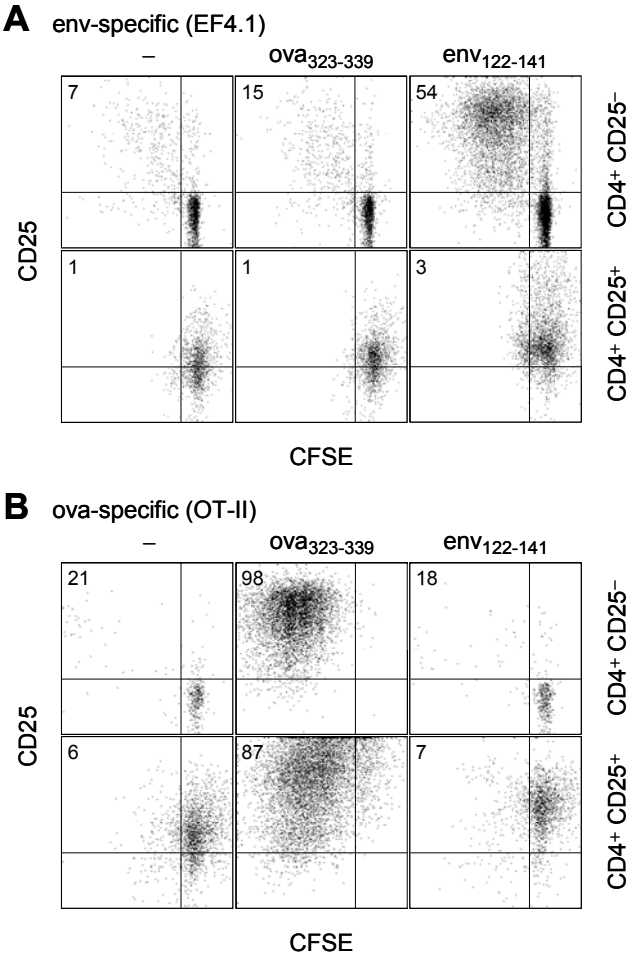

Figure 17. Lack of specific induction of *in vitro* proliferation in EF4.1 TCRβ-transgenic CD4$^+$ Treg cells by the env$_{122\text{-}141}$ peptide.
CFSE dilution profile of purified CD25$^-$ or CD25$^+$ EF4.1 TCRβ-transgenic (**A**) or OT-II TCRαβ-transgenic (**B**) CD4$^+$ T cells, 5 days following stimulation by bone marrow-derived dendritic cells in the absence of specific peptides (–) or presenting either ova$_{323\text{-}339}$ or env$_{122\text{-}141}$ peptides. Numbers within the quadrants represent the percentage of divided cells. One experiment, representative of 4 similar experiments, is shown.

biased towards recognition of self-antigens and therefore does not recognize foreign peptides (Hsieh et al., 2004).

The significant expansion observed in the OT-II TCRα/β-transgenic Treg cells in response to $ova_{323-339}$ stimulation may seem in disagreement with the findings of exclusion of env-specific clones from the EF4.1 TCRβ-transgenic Treg cell population, since similar exclusion should be expected in other TCR transgenic mouse strains. However, similarly to EF4.1 TCRβ-transgenic, OT-II TCRα/β-transgenic mice also showed exclusion of the TCR transgenic chains in both the memory (89% exclusion of TCR Vβ5Vα2) and Treg cell (78% exclusion of TCR Vβ5Vα2) populations (**Figure 18**). This is mainly due to reduced expression of the OT-II TCRβ-transgenic chain (TCR Vβ5) in memory and regulatory OT-II cells, compared with naïve OT-II T cells. Therefore, the expression of endogenous TCRβ chains in memory and regulatory OT-II TCRα/β-transgenic T cells is even more elevated than in memory and regulatory EF4.1 TCRβ-transgenic T cells. However, OT-II TCRα/β-transgenic mice also express a transgenic TCRα chain and since TCRα chains are not subject to allelic exclusion, OT-II Treg cells will express an endogenous TCRα chain, which allows for recognition of a selecting self-ligand in addition to the transgenic TCRα chain. This means that all the 22% of OT-II Treg cells which express TCR Vβ5Vα2 will be specific for $ova_{323-339}$, in addition to a selecting self-ligand. In contrast, 80% of EF4.1 TCRβ-transgenic Treg cells express the EF4.1 TCRβ-transgene (**Figure 15B**), but they are not necessarily specific for $env_{122-141}$ as that would require expression of an appropriate endogenous TCRα chain.

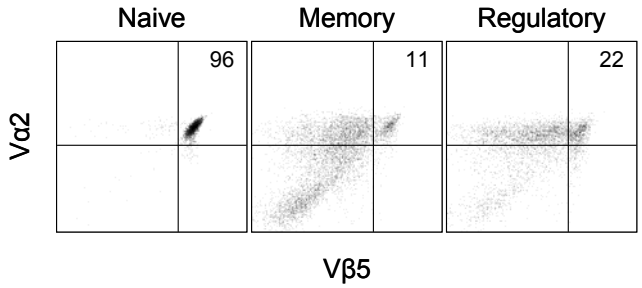

Figure 18. TCR allelic exclusion in OT-II TCRα/β transgenic CD4$^+$ T cell subsets. Expression of OT-II TCRα (Vα2) and TCRβ (Vβ5) transgenes in naïve, memory and regulatory gated CD4$^+$ T cells from OT-II (*Rag1*$^{+/+}$) mice. Numbers within the quadrants depict the percentage of Vα2$^+$ Vβ5$^+$ cells and represent the average values from 3 mice.

3.4.2.3 Assessment of envelope-reactivity of EF4.1 TCRβ-transgenic Treg cells by tetramer staining

To directly identify env-specific clones, EF4.1 TCRβ-transgenic Treg cells were stained with MHC class II $A^b/env_{122-141}$ tetramers ($A^b/env_{122-141}$). Although $A^b/env_{122-141}$ tetramers bind specifically to env-reactive clones (MacLeod et al., 2006), binding is also dependent on TCR affinity (Crawford et al., 1998), meaning that $A^b/env_{122-141}$ tetramer staining is restricted to env-specific T cell clones with a high TCR affinity and will not bind to env-specific T cell clones with low affinity. A small fraction (0.11%) of EF4.1 TCRβ-transgenic $CD4^+$ T cells were positive for $A^b/env_{122-141}$ and this population was absent from wild-type $CD4^+$ T cells (**Figure 19A**), demonstrating that $A^b/env_{122-141}$ tetramer staining can identify $env_{122-141}$–reactive clones. Importantly, $A^b/env_{122-141}$–positive clones were detected only in $CD25^-$ EF4.1 TCRβ-transgenic T cells and were absent from the $CD25^+$ EF4.1 TCRβ-transgenic Treg cell population (**Figure 19A**). To better visualize the distribution of tetramer-positive cells in EF4.1 TCRβ-transgenic $CD4^+$ T cells, the frequency of $A^b/env_{122-141}$ clones in the naïve, memory and regulatory compartments was analyzed. Compared with $A^b/env_{122-141}$–negative clones, $A^b/env_{122-141}$–positive clones were enriched in naïve cells, had fewer memory cells and were completely devoid of Treg cells (**Figure 19C**). These results indicated that env-specific clones with TCR affinity high enough for binding $A^b/env_{122-141,}$ were absent from the TCR repertoire of EF4.1 TCRβ-transgenic Treg cells.

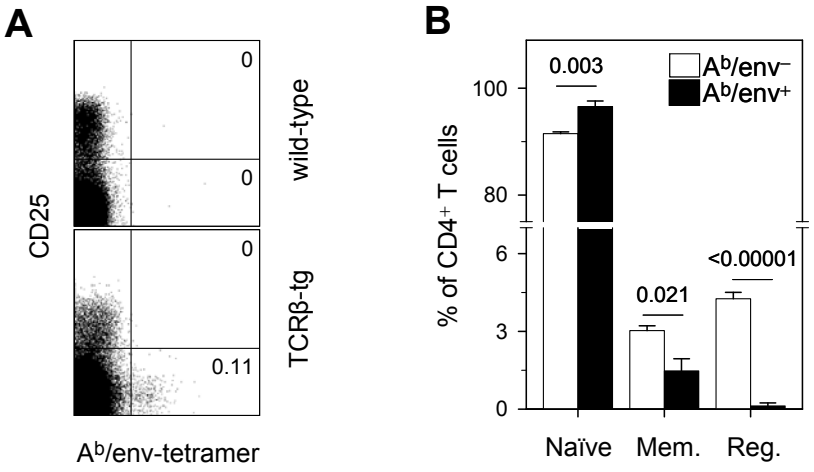

Figure 19. Absence of A^b/env tetramer-binding clones in EF4.1 TCRβ-transgenic $CD4^+$ Treg cells.

(**A**) A^b/env tetramer staining and CD25 expression in gated $CD4^+$ T cells from wild-type and EF4.1 TCRβ-transgenic mice (TCRβ tg). Numbers within the quadrants indicate the percentage of positive cells.

(**B**) Percentage of naïve, memory (Mem.) and regulatory (Reg.) cells in gated A^b/env^- or A^b/env^+ EF4.1 TCRβ-transgenic $CD4^+$ T cells. Numbers within the graph denote the *P* values. Data in **A-B** were obtained from 4 mice per group.

3.4.3 Reactivity of EF4.1 TCRβ-transgenic Treg cells to endogenous retroviruses

The previous results suggested that Treg cells do not express env-specific TCRs but they still have a protective function during FV infection. It was possible that endogenous retroviral sequences could be expressed during FV infection and specifically activated Treg cells. Endogenous retroviral sequences would thus provide an explanation for both Treg cell reactivity to self-antigens and specific activation during FV infection.

The possibility that the B6 mouse genome bears endogenous proviral sequences similar to the exogenous $env_{122\text{-}141}$ that could be recognized by EF4.1 TCRβ-transgenic Treg cells was explored. B6 mice have a single copy of an FV-related endogenous provirus that differs from the exogenous one at one amino-acid position within the epitope, with a tyrosine substituting a leucine at position 128 (Jenkins et al., 1982). Previous studies have shown that substitution of L-128 with an alanine disrupts the recognition of the env peptide by the SB14-31 TCR clone (Shimizu et al., 1994), highlighting its importance in the recognition of $env_{122\text{-}141}$ by the SB14-31 TCR and suggesting that EF4.1 TCRβ-transgenic $CD4^+$ T cells might not be able to recognize the endogenous envelope sequence.

To test the reactivity of EF4.1 TCRβ-transgenic Treg cells to the endogenous envelope sequence, their capacity to expand when stimulated *in vitro* with the endogenous

sequence was tested using the methods described before (**Figure 17**). After 5 days of *in vitro* stimulation $CD4^+$ $CD25^-$ T cells showed similar proliferation profiles with the exogenous and the endogenous envelope sequence (**Figure 20**), suggesting the single amino acid substitution at L-128 was not enough to completely abrogate recognition of the envelope sequence by EF4.1 TCRβ-transgenic $CD4^+$ T cells. However, no expansion of EF4.1 TCRβ-transgenic Treg cells stimulated with the endogenous envelope sequence was detected, similar to the absence of proliferation in the presence of the $env_{122\text{-}141}$ antigen (**Figure 20**). These results suggested that EF4.1 TCRβ-transgenic Treg cells protecting from disease in the FV-induced immune pathology model were recognizing neither the endogenous nor the exogenous envelope sequences.

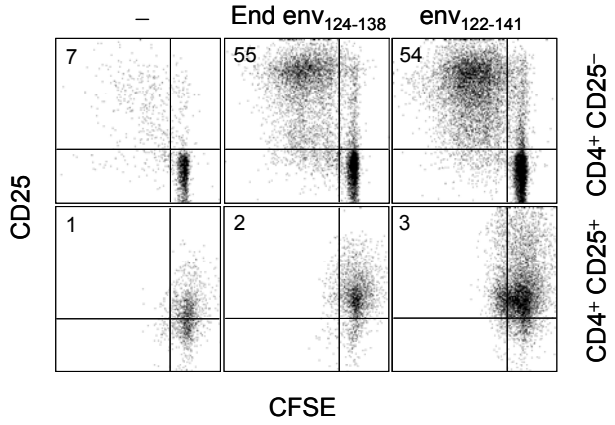

Figure 20. Lack of specific induction of *in vitro* proliferative response of EF4.1 TCRβ-transgenic CD4$^+$ Treg cells by the endogenous envelope peptide.
CFSE dilution profile of purified CD25$^-$ or CD25$^+$ EF4.1 TCRβ-transgenic CD4$^+$ T cells, 5 days following stimulation by bone marrow-derived dendritic cells in the absence of specific peptides (–) or presenting either env$_{122\text{-}141}$ or endogenous env$_{124\text{-}138}$ (End env$_{124\text{-}138}$) peptides. Numbers within the quadrants represent the percentage of divided cells. One experiment, representative of 3 similar experiments, is shown.

3.5 Role of wild-type, env-nonspecific Treg cells in the FV-induced immune pathology

Treg cells have been suggested to require antigen-specific TCR stimulation to function (Thornton and Shevach, 1998; Takahashi et al., 1998). Surprisingly, however, the previous experiments were unable to detect $env_{122-141}$-specific clones in the protective EF4.1 TCRβ-transgenic Treg cell population, raising the hypothesis that Treg cells protecting from FV-induced immune pathology are not activated by $env_{122-141}$.

To test this hypothesis the protective capacity of wild-type Treg cells, which should contain very few, if any, env-specific clones was examined. The expansion of these cells in FV-infected *Rag1*$^{-/-}$ recipients of CD45.2$^+$ EF4.1 TCRβ-transgenic CD4$^+$ T cells and either CD45.1$^+$ EF4.1 TCRβ-transgenic or CD45.1$^+$ wild-type Treg cells was compared. The frequency of Treg cells in the spleen (**Figure 21A**, top panel) and bone marrow (**Figure 21B**, top panel) was similar between EF4.1 TCRβ-transgenic and wild-type Treg cells. In contrast to EF4.1 TCRβ-transgenic CD4$^+$ T cells which showed enrichment of TCR Vα2$^+$ clones in the env-specific population (**Figure 7A-7C**), the frequency of TCR Vα2$^+$ clones remained low in both Treg cell populations in both the spleen (**Figure 21A**, lower panel) or bone marrow (**Figure 21B**, lower panel), indicating that their expansion was polyclonal and not the result of preferential selection of env-specific clones. Importantly, wild-type Treg cells were able to prevent the development of the pathology caused by FV-specific CD4$^+$ T cells in FV-infected hosts with similar efficiency to EF4.1

TCRβ-transgenic Treg cells (**Figure 21C**). This *in vivo* result supported the *in vitro* findings that EF4.1 TCRβ-transgenic mice do not develop $env_{122-141}$-specific Treg cells. Furthermore, it also suggested that Treg cells were able to mediate suppression of FV-specific $CD4^+$ T cell-induced bone marrow pathology independently of shared antigenic specificity.

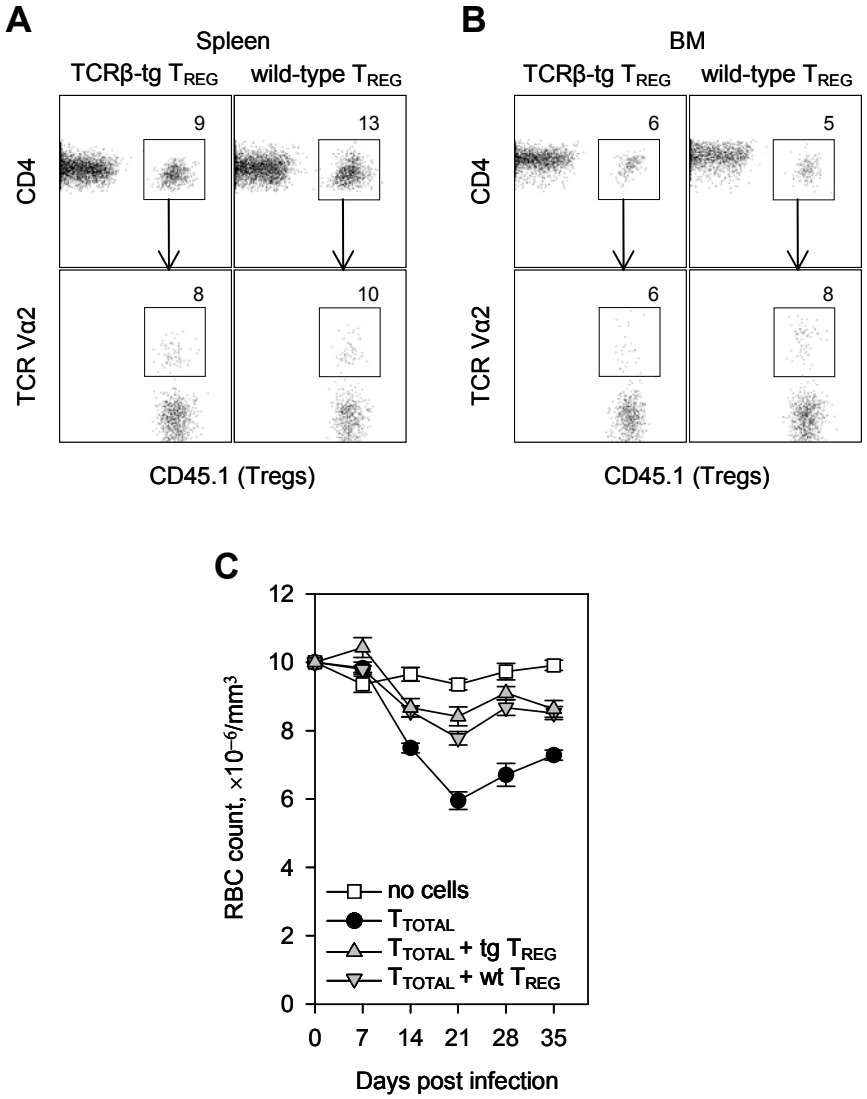

Figure 21. Suppression of env-specific CD4$^+$ T cell-induced bone marrow pathology by env-nonspecific Treg cells.

(**A**, **B**) Percentage of CD45.2$^+$ CD4$^+$ Treg cells in total CD4$^+$ T cells (top row) and percentage of TCR Vα2$^+$ T cells in CD45.2$^+$ CD4$^+$ Treg cells (bottom row), in the spleen (**A**) or the bone marrow (BM) (**B**) of FV-infected *Rag1*$^{-/-}$ recipients, following co-transfer of CD45.1$^+$ EF4.1 TCRβ-transgenic total CD4$^+$ T cells with either EF4.1 TCRβ-transgenic (TCRβ-tg T_{REG}) or wild-type (wild-type T_{REG}) CD45.2$^+$ CD25$^+$ CD4$^+$ Treg cells, at 1:1 ratio. Numbers within the plots indicate the percentage of positive cells and are representative of 6-8 mice per group, analyzed on day 21 after transfer.

(**C**) Changes in RBC counts in FV-infected *Rag1*$^{-/-}$ mice, which received total EF4.1 TCRβ-transgenic CD4$^+$ T cells, alone (T_{TOTAL}) or together with either EF4.1 TCRβ-transgenic (T_{TOTAL} + tg T_{REG}) or wild-type (T_{TOTAL} + wt T_{REG}) CD25$^+$ CD4$^+$ Treg cells. Values represent the mean (±SEM) of 6-10 mice per group per time point analysed in 3 independent experiments. $P < 0.0003$ between 'T_{TOTAL}' and either 'T_{TOTAL} + tg T_{REG}' or 'T_{TOTAL} + wt T_{REG}', on day 21.

3.6 Reactivity to viral epitopes of Treg cells from FV-infected mice

The previous experiments addressed the specificity of Treg cells obtained from naïve mice, since the objective was to study the Treg cell population existent in normal conditions in unmanipulated mice and not Treg cells induced by the infection. However, the presence of env-specific Treg cells after FV infection was not excluded in these analyses. Taking this into account, several possibilities for FV-reactivity of Treg cells during FV infection were raised. One possibility was the existence of a small population of env-specific Treg cells, which for technical limitations might not had been detected in the previous experiments, which could expand during FV infection and protect from anaemia development. Moreover, the analyses have been largely concentrated on envelope as a potential FV-derived epitope and did not address the capacity of Treg cells to recognize FV epitopes other than env. Additional epitopes from other FV polypeptides, such as gag and pol, would in principle be able to stimulate Treg cells. It was also possible that Treg cells might be expanding in response to FV-unrelated endogenous retroviruses sequences during infection. Finally, *de novo* generation of env-specific FoxP3$^+$ Treg cells from FV-specific CD4$^+$ T cell clones during infection was not addressed in the preceding experiments and these FV-specific Treg cells could contribute to disease suppression.

3.6.1 Assessment of envelope-reactivity of Treg cells from FV-infected mice by tetramer staining

The hypothesis that suppression of FV-induced pathology was mediated by *in vivo* expansion of a small number of FV-specific Treg cells, the initial frequency of which could be too low to be measured with the previous methods, was tested. $A^b/env_{122-141}$ tetramers were used to look for the presence of env-specific Treg cells at the peak of the pathology in FV-infected *Rag1*$^{-/-}$ mice following co-transfer of CD45.1$^+$ EF4.1 TCRβ-transgenic total CD4$^+$ T cells and CD45.2$^+$ wild-type Treg cells. Approximately half of the pathogenic EF4.1 TCRβ-transgenic CD4$^+$ T cells stained brightly with the $A^b/env_{122-141}$ tetramer in both the spleen and bone marrow (**Figure 22A**), indicating that virus-specific CD4$^+$ T cells experienced a significant expansion *in vivo* during FV-infection of *Rag1*$^{-/-}$ mice compared with the frequency of virus-specific CD4$^+$ T cells before transfer (**Figure 19A**). In contrast, no $A^b/env_{122-141}$-positive clones were found in the expanded Treg cell population (**Figure 22A**), indicating that suppression of bone marrow pathology by wild-type Treg cells was not the result of selective expansion of some few env-specific clones.

3.6.2 Assessment of reactivity to Friend virus epitopes of Treg cells from FV-infected mice

The question regarding the existence of FV-specific Treg cells that could recognize any FV-derived epitope after infection was then addressed. Treg cells were sorted from FV-infected mice and their *in vitro* reactivity to FV-infected dendritic cells, which would be presenting all possible FV-derived antigens, was tested. EF4.1 TCRβ-transgenic $CD4^+$ T cells and wild-type Treg cells were purified from the bone marrow of FV-infected *Rag1*$^{-/-}$ recipients 21 days after infection. $CD45.1^+$ EF4.1 TCRβ-transgenic $CD4^+$ T cells expanded considerably when stimulated with FV-pulsed dendritic cells but not in the absence of antigen (**Figure 22B**, top panel). This result indicated that $CD45.1^+$ EF4.1 TCRβ-transgenic $CD4^+$ T cells taken from mice with unresolving chronic FV infection were still able to react to FV. $CD45.2^+$ wild-type Treg cells showed some proliferative capacity even in the absence of antigen, which was not increased when the cells were stimulated with FV-pulsed dendritic cells (**Figure 22B**, lower panel), indicating that Treg cells in the bone marrow were in an activated proliferative state, which could not be further enhanced by stimulation with any FV epitopes.

To confirm the previous results, the same assay was repeated using Treg cells from the spleen of FV-infected B6 mice. Treg cells were stimulated with α-CD3, dendritic cells pulsed with $ova_{323-339}$ or $env_{122-141}$ peptides or FV. Naïve EF4.1 TCRβ-transgenic $CD4^+$ T cells, used as controls for the efficiency of stimulation, showed significant expansion

when stimulated both with $env_{122-141}$ and the FV-pulsed dendritic cells (**Figure 22C**, top panel). In contrast, no *in vitro* expansion of Treg cells from FV-infected mice was detected in any of the conditions (**Figure 22C**, lower panel), suggesting that even after FV infection no Treg cell population reactive to $env_{122-141}$ or any other FV epitope emerged. Together, these results showed that Treg cells protecting from disease were not reactive to FV-encoded env or other potential FV-derived epitopes.

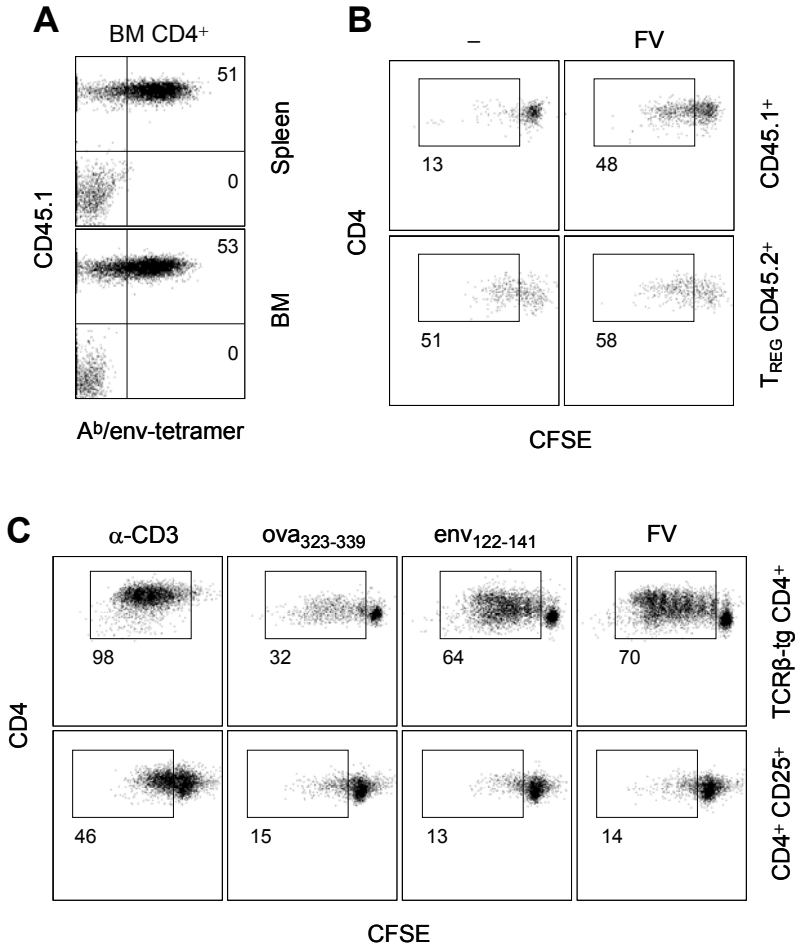

Figure 22. Absence of virus-reactivity of Treg cells from FV-infected mice.
(**A**) A^b/env tetramer staining in $CD4^+$ T cells from the spleen or the bone marrow (BM) of FV-infected *Rag1*$^{-/-}$ recipients, following co-transfer of $CD45.1^+$ EF4.1 TCRβ-transgenic total $CD4^+$ T cells with $CD45.2^+$ wild-type $CD25^+$ $CD4^+$ Treg cells, at 1:1 ratio. Numbers within the quadrants indicate the percentage of tetramer positive cells in either $CD45.1^+$ or $CD45.2^+$ $CD4^+$ T cells and are representative of 4 mice per group, analyzed on day 21 after transfer.
(**B**) CFSE dilution profile of purified $CD45.1^+$ $CD4^+$ effector T cells ($CD45.1^+$) or $CD45.2^+$ $CD4^+$ Treg cells (T_{REG} $CD45.2^+$) isolated from the bone marrow of FV-infected *Rag1*$^{-/-}$ recipients of $CD45.1^+$ EF4.1 TCRβ-transgenic $CD4^+$ T cells and $CD45.2^+$ wild-type $CD25^+$ $CD4^+$ Treg cells, 5 days following stimulation by dendritic cells in the absence (-) or the presence of whole FV (FV). Numbers within the plots depict the percentage of divided cells and are representative of 5 mice at day 21 after FV infection.
(**C**) CFSE dilution profile of $CD4^+$ T cells from virus-naïve EF4.1 TCRβ-transgenic mice (TCRβ-tg $CD4^+$) or $CD25^+$ Treg cells from FV-infected wild-type B6 mice ($CD25^+$ $CD4^+$) 5 days following stimulation by dendritic cells in the presence of anti-CD3 antibody, or $ova_{323-339}$ or $env_{122-141}$ peptides, or whole FV. Numbers within the plots depict the percentage of divided cells and are representative of 3 mice at day 35 after FV infection. Virus-naïve EF4.1 TCRβ-transgenic $CD4^+$ T cells were used as control for efficiency of FV-derived epitope presentation by FV-pulsed dendritic cells.

3.6.3 Reactivity of Treg cells from FV-infected mice to FV-unrelated endogenous retroviruses

Laboratory mice contain several mouse mammary tumour viruses (MMTV)-related proviral sequences which might be expressed upon FV infection. The superantigen expressed in the MMTV genome can be recognized by several TCR Vβ chains, one of which is TCR Vβ5 (Fink et al., 1994; Fink et al., 1992). As shown before, the frequency of TCR Vβ5$^+$ clones was enriched in the regulatory T cell compartment in wild-type mice and, albeit less pronounced, also in EF4.1 TCRβ-transgenic mice (**Figure 15A**). It was therefore possible that TCR Vβ5$^+$ Treg cells might be activated during FV infection by recognition of the MMTV-related superantigen. If this were the case, it should lead to relative enrichment of TCR Vβ5$^+$ clones in the Treg cell population after transfer into FV-infected *Rag1*$^{-/-}$ mice together with CD4$^+$ T cells. By staining Treg cells for TCR Vβ5 on day 21 after transfer, it was found that expression of TCR Vβ5 was reduced in all anatomical locations (lymph nodes, spleen and bone marrow) (**Figure 23**), suggesting that TCR Vβ5$^+$ Treg cells did not expand in response to the MMTV-related superantigen during FV infection.

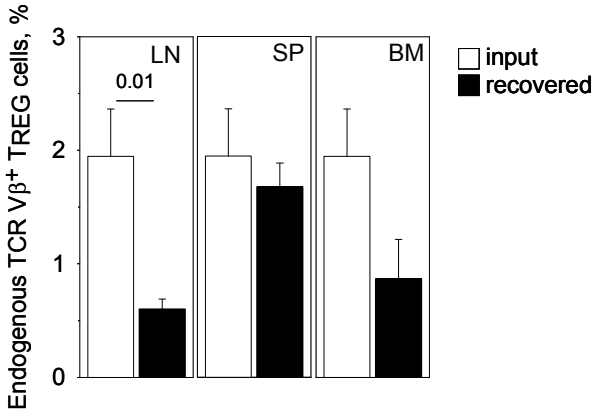

Figure 23. Enhanced exclusion of endogenous TCR Vβ5 chains in Treg cells from FV-infected mice.

Expression of TCR Vβ5.1/5.2 in EF4.1 TCRβ-transgenic Treg cells recovered from the lymph nodes (LN), the spleen (SP) or the bone marrow (BM) of FV-infected *Rag1*$^{-/-}$ recipients, which received CD45.2^{+} EF4.1 TCRβ-transgenic CD25^{+} CD4^{+} Treg cells together with CD45.1^{+} EF4.1 TCRβ-transgenic CD4^{+} T cells, at 1:1 ratio. Expression of EF4.1 TCRβ-transgenic Treg cells before (input) and 21 days after transfer (recovered) is shown. Values are the mean (±SEM) of 2-4 individual recipients from a single experiment. Numbers within the graphs denote the *P* values.

3.6.4 Assessment of conversion into FoxP3$^+$ Treg cells of FV-specific CD4$^+$ T cells during FV infection

To evaluate induction of FoxP3 expression in FV-specific CD4$^+$ T cells responding to FV infection, EF4.1 TCRβ-transgenic mice were crossed to a FoxP3EGFP reporter strain (**Figure 24A**), allowing the tracking of FoxP3-expressing cells (Wang et al., 2008). When CD45.1$^+$ EF4.1 TCRβ-transgenic CD4$^+$ T cells were transferred into FV-infected B6 wild-type mice they expanded significantly, peaking at day 7 after transfer. This was followed by a contraction phase and from day 21 the numbers of transferred CD4$^+$ T cell remained constant throughout the course of infection (**Figure 24B**). This indicated that CD45.1$^+$ EF4.1 TCRβ-transgenic CD4$^+$ T cells can persist in significant numbers in FV-infected B6 wild-type mice. To test the induction of FoxP3-expressing cells in these conditions, FoxP3$^-$ CD45.1$^+$ EF4.1 TCRβ-transgenic Foxp3^{egfp} CD4$^+$ T cells were sorted and transferred into FV-infected B6 wild-type mice. Significant numbers of transferred CD4$^+$ T cells were detected in the lymph nodes, spleen and bone marrow 35 days after transfer (**Figure 24C**, top panel). However, no FoxP3 expression was detected in the whole of CD45.1$^+$ CD4$^+$ T cell population or in the env-specific TCR Vα2$^+$ CD4$^+$ T cell clones (**Figure 24C**, lower panel), suggesting there was no FoxP3$^+$ Treg cell-conversion during FV infection, not even of the virus-specific TCR Vα2$^+$ CD4$^+$ T cell clones.

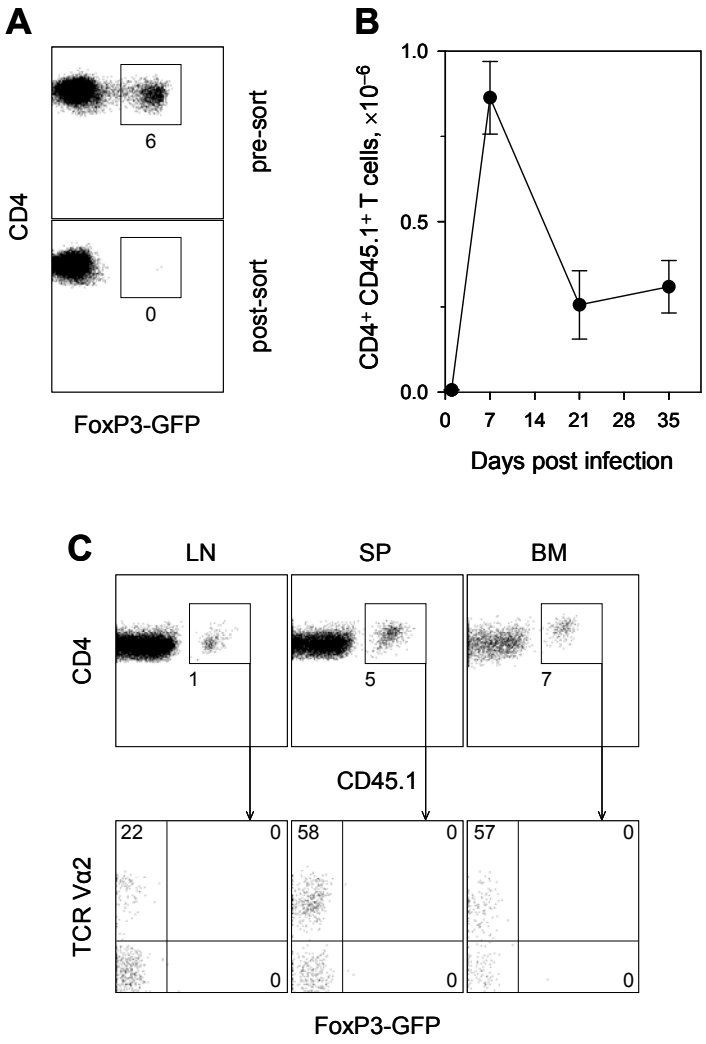

Figure 24. Lack of peripheral conversion of FV-specific EF4.1 TCRβ-transgenic CD4$^+$ T cells into FoxP3-expressing Treg cells.

(**A**) Flow cytometric example of GFP expression in EF4.1 TCRβ-transgenic Foxp3^{egfp} CD4$^+$ T cells before (pre-sort) and after (post-sort) isolation of FoxP3$^-$ (GFP$^-$) T cells.

(**B**) Absolute numbers of CD45.1$^+$ CD4$^+$ T cells in the spleen of wild-type B6 recipients of CD45.1$^+$ EF4.1 TCRβ-transgenic Foxp3^{egfp} CD4$^+$ T cells over the course of FV infection. Values are the mean (±SEM) of 5-7 mice per time point.

(**C**) Percentage of FoxP3$^+$ (GFP$^+$) cells in CD45.1$^+$ CD4$^+$ T cells isolated from the lymph nodes (LN), spleen (SP) or bone marrow (BM) of FV-infected wild-type B6 recipients of purified FoxP3$^-$ (GFP$^-$) CD45.1$^+$ EF4.1 TCRβ-transgenic Foxp3^{egfp} CD4$^+$ T cells, 35 days after infection. Numbers within the quadrants depict the percentage of positive cells and are representative of a total of 6 mice analysed in two different experiments.

FoxP3-conversion during FV infection was further investigated in the conditions that generated disease and at the place of pathology. FoxP3^{-} CD45.1^{+} EF4.1 TCRβ-transgenic Foxp3^{egfp} CD4^{+} T cells were transferred together with wild-type Treg cells into FV-infected *Rag1*$^{-/-}$ mice and FoxP3 expression was analyzed 35 days after transfer. Although there was significant expansion of CD45.1^{+} CD4^{+} T cells in all the locations (lymph nodes, spleen and bone marrow) (**Figure 25**, top panel), similarly to the previous results, no FoxP3 expression was detected in the recovered CD45.1^{+} CD4^{+} T cells (**Figure 25,** lower panel). Together, these results suggested that FoxP3^{+} Treg cells are not generated *de novo* from virus-specific CD4^{+} T cells during FV infection.

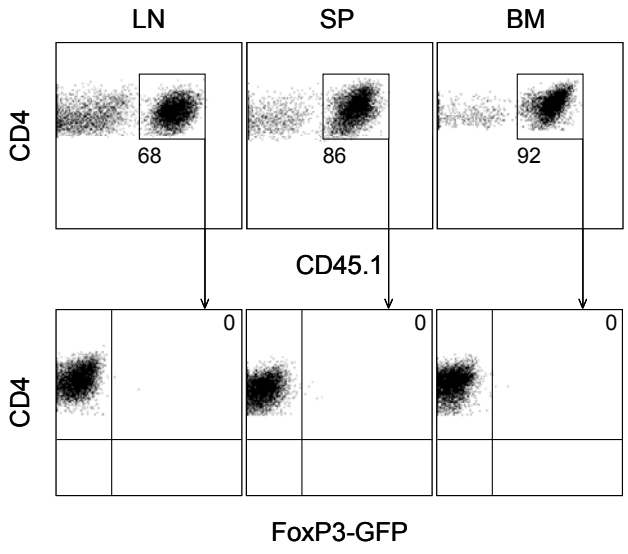

Figure 25. Lack of peripheral conversion of FV-specific EF4.1 TCRβ-transgenic CD4$^+$ T cells into FoxP3-expressing Treg cells in the bone marrow of FV-infected *Rag1*$^{-/-}$ mice.

Percentage of FoxP3$^+$ (GFP$^+$) cells in CD45.1$^+$ CD4$^+$ T cells isolated from the lymph nodes (LN), spleen (SP) or bone marrow (BM) of FV-infected *Rag1*$^{-/-}$ recipients of purified FoxP3$^-$ (GFP$^-$) CD45.1$^+$ EF4.1 TCRβ-transgenic Foxp3^{egfp} CD4$^+$ T cells and CD45.2$^+$ wild-type CD25$^+$ CD4$^+$ Treg cells, at 1:1 ratio, 35 days after transfer. Numbers within the quadrants depict the percentage of positive cells and are representative of a total of 4 mice from a single experiment.

3.7 Analysis of TCR expression, activation status and expansion of Treg cells during FV-infection

Lack of FV-specificity in Treg cells suppressing anaemia induction by pathogenic FV-specific $CD4^+$ T cells raised the important question of whether or how Treg cells were being activated to mediate this suppression. Furthermore, it was of interest to understand if Treg cell function was in anyway related to TCR or was a TCR-independent mechanism.

To test the status of Treg cell activation, Treg cells were transferred into FV-infected or uninfected *Rag1*$^{-/-}$ recipients. Treg cells from the lymph nodes, spleen and bone marrow from wild-type controls or FV-infected or uninfected *Rag1*$^{-/-}$ recipients were taken 21 days after transfer and stained for several activation markers: CD49b, CD103, FR4 and ICOS. No increased expression of CD49b or FR4 on Treg cells from any of the *Rag1*$^{-/-}$ groups of mice was detectable compared with Treg cells from uninfected wild-type controls. Elevated expression of ICOS and CD103 was detected in *Rag1*$^{-/-}$ recipients of Treg cells, which was not, however, further enhanced by FV infection (**Figure 26**), suggesting that transfer of Treg cells into lymphopenic *Rag1*$^{-/-}$ recipients had the potential to cause their activation irrespective of FV-infection and might have masked any further Treg cell activation caused by the infection.

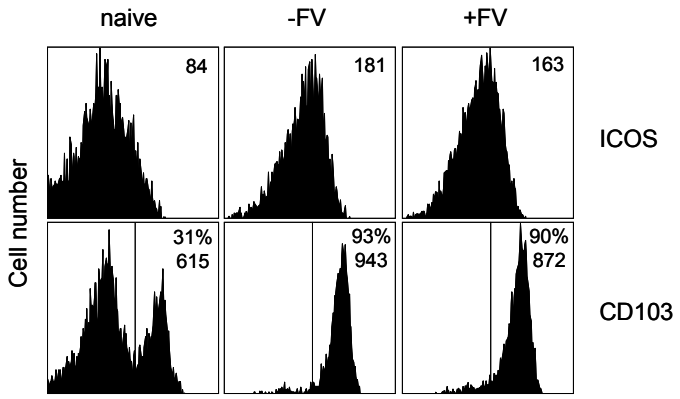

Figure 26. Activation profile of Treg cells from FV-infected or uninfected *Rag1*$^{-/-}$ mice.
Expression of ICOS and CD103 in CD45.2^{+} CD4^{+} Treg cells from the lymph nodes of B6 wild-type controls (naïve) or from bone marrow of FV-infected (+FV) or uninfected (-FV) *Rag1*$^{-/-}$ recipients, which received CD45.1^{+} EF4.1 TCRβ-transgenic total CD4^{+} T cells and CD45.2^{+} EF4.1 TCRβ-transgenic CD25^{+} CD4^{+} Treg cells. Numbers within the plots denote the percentage (%) and median fluorescent intensity of positive cells and are representative of 1 (naïve), 2 (-FV) or 4 mice (+FV) from one experiment.

As transfer of Treg cells into lymphopenic *Rag1*$^{-/-}$ recipients had the potential to cause their activation irrespective of FV-infection, the behaviour of Treg cells transferred into FV-infected T cell-replete hosts, mimicking the conditions present during anaemia development, was examined. To assess their proliferative response during the course of FV infection, a cohort of CFSE-labeled sensor Treg cells was adoptively transferred into *Rag1*$^{-/-}$ recipients 21 days after they were infected with FV and had received EF4.1 TCRβ-transgenic CD4^{+} T cells. At this time point after infection, T cell numbers in lymphoid organs of *Rag1*$^{-/-}$ recipients were equivalent to those in T cell-replete wild-type mice, due to significant expansion of the EF4.1 TCRβ-transgenic CD4^{+} T cells (**Figure 7B**). The CFSE-labeled Treg cells displayed extensive proliferation in the lymph nodes, spleen and bone marrow of recipient mice 3 days after transfer, with significantly fewer undivided cells in the bone marrow than in the spleen or lymph nodes (**Figure 27**). Although this analysis did not discriminate between enhanced division in the bone marrow or preferential migration of divided cells into the bone marrow, it revealed that Treg cell activation to enter cell division continued throughout the course of FV infection, despite the presence of significant numbers of T cells, and that Treg cells recovered from the bone marrow were associated with higher proliferative history.

To test the involvement of TCR specificity in the recruitment and/or expansion of Treg cells in the infected bone marrow, TCR usage was analyzed in the Treg cell population in lymph nodes, spleen and bone marrow of FV-infected or uninfected *Rag1*$^{-/-}$ recipients, which received CD45.2^{+} EF4.1 TCRβ-transgenic Treg cells together with CD45.1^{+} EF4.1

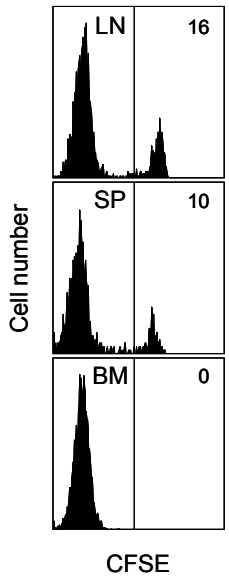

Figure 27. Proliferation profile of Treg cells from FV-infected *Rag1*$^{-/-}$ recipients of EF4.1 TCRβ-transgenic CD4^{+} T cells.
CFSE dilution profile of CD45.2^{+} CD4^{+} Treg cells in the lymph nodes (LN), spleen (SP) and bone marrow (BM) of FV-infected *Rag1*$^{-/-}$ recipients, which received CD45.1^{+} EF4.1 TCRβ-transgenic total CD4^{+} T cells at the time of FV infection, and a cohort of CFSE-labeled CD45.2^{+} wild-type CD25^{+} CD4^{+} Treg cells 21 days later. Organs were analyzed 3 days after transfer of the Treg cell cohort. Numbers within the plots denote the percentage of undivided cells and are representative of 4 mice analyzed in two separate experiments. $P < 0.007$ between 'BM' and either 'LN' or 'SP'.

TCRβ-transgenic $CD4^+$ T cells. Transfer of EF4.1 TCRβ-transgenic Treg cells into *Rag1*$^{-/-}$ recipients led to enrichment of clones expressing the transgenic, rather than endogenous TCRβ chains (**Figure 28**). However, the pattern of TCRβ expression in EF4.1 TCRβ-transgenic Treg cells was not affected by FV infection or the location, from which Treg cells were recovered (**Figure 28**), suggesting that Treg cell recruitment and/or expansion in the infected bone marrow was independent of TCR specificity.

To compare the degree of Treg cell activation directly by the infection, the presence of pathogenic $CD4^+$ T cells or the transfer into lymphopenic *Rag1*$^{-/-}$ recipients, their individual or combined effect on Treg cell expansion was further examined. Recovery of Treg cells from recipient mice was similar when Treg cells were transferred into uninfected *Rag1*$^{-/-}$ recipients either alone or together with EF4.1 TCRβ-transgenic $CD4^+$ T cells or alone into FV-infected *Rag1*$^{-/-}$ recipients (**Figure 29A-29B**). In contrast, recovery of Treg cells was 3 and 6 times higher in the spleen and bone marrow, respectively, when Treg cells were transferred together with EF4.1 TCRβ-transgenic $CD4^+$ T cells into FV-infected *Rag1*$^{-/-}$ recipients, than in any other combination (**Figure 29A-Figure 29B**). These results argued against a direct response of Treg cells to FV infection or transfer into *Rag1*$^{-/-}$ recipients and instead indicated that Treg cell expansion and/or survival depended on the presence of $CD4^+$ T cells responding to FV infection.

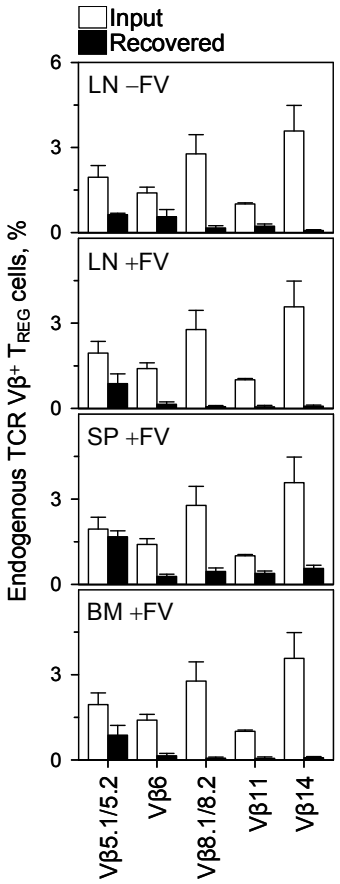

Figure 28. Enhanced exclusion of endogenous TCR Vβ chains in EF4.1 TCRβ-transgenic Treg cells, independently of FV infection.
Expression of individual endogenous TCR Vβ5.1/5.2, Vβ6, Vβ8.1/8.2, Vβ11 and Vβ14 in EF4.1 TCRβ-transgenic Treg cells recovered from the lymph nodes (LN), the spleen (SP) or the bone marrow (BM) of uninfected (–FV) or FV-infected (+FV) *Rag1*$^{-/-}$ recipients, which received CD45.2^{+} EF4.1 TCRβ-transgenic CD25^{+} CD4^{+} Treg cells together with CD45.1^{+} EF4.1 TCRβ-transgenic CD4^{+} T cells, at 1:1 ratio. Expression of EF4.1 TCRβ-transgenic Treg cells before (input) and 21 days after transfer (recovered) is shown. Values are the mean (±SEM) of 2-4 individual recipients from a single experiment.

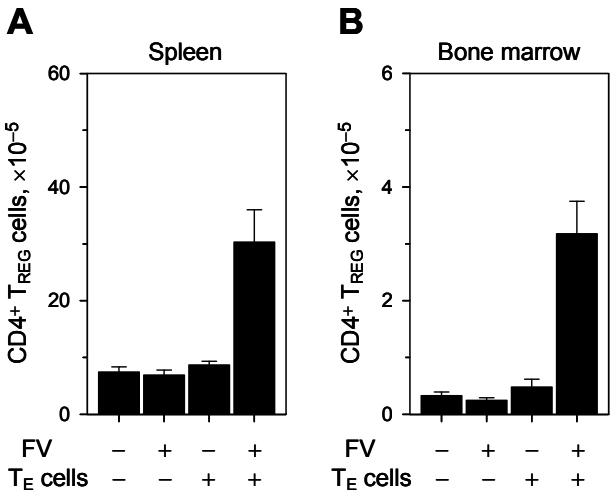

Figure 29. Enhanced proliferation of Treg cells in the presence of both virus infection and EF4.1 TCRβ-transgenic CD4$^+$ T cells.
Absolute number of CD45.2$^+$ Treg cells recovered from the spleen (**A**) or the bone marrow (**B**) of uninfected (–FV) or FV-infected (+ FV) *Rag1*$^{-/-}$ recipients, which received CD45.2$^+$ wild-type CD25$^+$ CD4$^+$ Treg cells alone (–T_E cells) or together with CD45.1$^+$ EF4.1 TCRβ-transgenic total CD4$^+$ T cells at 1:1 ratio (+ T_E cells). Values represent the mean (±SEM) of 3-6 mice per group analyzed on day 21.

Chapter 4

Discussion

Uncontrolled viral replication may result in chronic stimulation and activation of T cells, which may cause immune pathology. Several control mechanisms, including suppressive Treg cells, have arisen to regulate immune responses and limit the damage they may cause. This study demonstrated that unresolving FV infection in immunodeficient mice causes the activation and expansion of virus-specific $CD4^+$ T cells. FV infection of bone marrow cells attracts IFN-γ producing virus-specific $CD4^+$ T cells, leading to bone marrow pathology, which manifests as anaemia. Treg cells have a protective role in the development of disease, the severity of which depends on the balance between FV-specific $CD4^+$ T cells and Treg cells. Analyses of TCR specificity of the Treg cell population from naïve mice did not reveal any virus-specific clones. Furthermore, virus-specific Treg cells were also absent from FV-infected mice and no conversion of virus-specific pathogenic $CD4^+$ T cells into FoxP3-expressing Treg cells is detected during FV infection. Moreover, wild-type polyclonal Treg cells efficiently suppress bone marrow pathology, suggesting that direct viral recognition is not necessary for Treg cell function.

4.1 Role of T helper response in bone marrow pathology

Bone marrow pathology is commonly associated with persistent human viral infections such as Epstein-Barr virus, cytomegalovirus and HIV. Bone marrow dysfunction is responsible for anaemia development in HIV infection, occurring in approximately 60% and 90% of acute and chronic HIV-1 infection, respectively, and represents a significant contributor to other cytopenias, including reduced lymphocyte production (Moses et al., 1998; Kulkosky et al., 1999; Redd et al., 2007; Mlisana et al., 2008). The causes of these cytopenias are heterogeneous. They can be attributed to the hematopoietic suppressive effects of certain types of intercurrent infections or to drugs commonly used for treatment of AIDS patients (Moses et al., 1998). Nevertheless, direct involvement of HIV-1 infection may be important at various levels, leading to hematopoietic stem cell failure and bone marrow dysfunction (Moses et al., 1998). Most studies suggest that hematopoietic stem cells are refractory to HIV-1 infection and must undergo some differentiation to express critical surface receptors to permit virus entry and replication (Kulkosky et al., 1999). There is strong evidence suggesting that HIV-1 infects bone marrow stromal cells, resulting in impaired stromal function and alteration of the hematopoietic growth factor network, leading to bone marrow dysfunction (Kulkosky et al., 1999). However, some data also point to direct cytopathic effects of the virus in bone marrow pathology. Different clades of HIV-1 seem to have different capacities to infect bone marrow precursors. HIV-1C has been shown to be able to infect progenitor cell

populations both *in vitro* and *in vivo* and this seems to be associated to the higher rate of anaemia in HIV-1C-infected patients (Redd et al., 2007).

In mice, LCMV infection of perforin-deficient mice causes anaemia resulting from bone marrow pathology (Binder et al., 1998). These mice exhibit a vigorous T cell response, but are unable to eliminate the virus. The anaemia seems to be the consequence of an unbalanced antiviral immune response caused by a conflict between persisting viral antigen and continuously expanding $CD8^+$ T cells in the bone marrow. The pathogenic effect of $CD8^+$ T cells is, at least in part, IFN-γ-mediated. Depletion of $CD4^+$ T cells does not affect the development of the disease, suggesting that in this model they do not play a major role in the immune pathology (Binder et al., 1998).

Anaemia can also develop in the absence of infection as in the case of chronic graft-versus-host (GVH) reaction driven by histocompatibility disparities (Chen et al., 2004; Bloom et al., 2004). Alloantigen-specific $CD4^+$ T cells can contribute to GvH-driven anaemia either directly, by recognition of major histocompatibility alloantigens on bone marrow precursors (Sprent et al., 1994) or indirectly, by provision of help to minor histocompatibility alloantigen-specific $CD8^+$ T cells (Chen et al., 2004). Again, IFN-γ seems to be involved in the development of the bone marrow pathology, since IFN-γ blockage rescued mice from disease (Bloom et al., 2004).

Similarly, the present study revealed that FV infects a large number of distinct bone marrow precursors. This infection is non cytopathic and has no apparent consequences

for bone marrow function in the absence of an adaptive virus-specific immune response. The effect of FV infection of the bone marrow on immune pathology is in this case due to the migration and expansion of virus-specific $CD4^+$ T cell response into the bone marrow and these cells seem to be directly responsible for bone marrow pathology development in an IFN-γR-dependent manner. Together, these studies reveal the pathogenic potential of T cell immune responses, which may cause the development of bone marrow pathology in chronic viral infection, which would otherwise not develop due to the non cytopathic nature of the infection.

4.2 Treg cells mediate protection from bone marrow pathology

Viral infections may cause pathology through different mechanisms. If the viral infection causes death of the infected cells, the pathology cannot be controlled by the immune system. Viral infection may also cause pathology indirectly through the activation of the immune system, as in the case of FV infection. In contrast to the cytopathic effects of viral infection, immune-mediated pathology can be regulated by the immune system and distinct cell types can have suppressive functions in such cases.

In the model of FV-induced immune pathology, the absence of $CD8^+$ T cells and B cells in the recipient mice allows the propagation of the virus, resulting in systemic infection which also affects the bone marrow. The virus specific $CD4^+$ T cells are attracted to the bone marrow, causing depletion of bone marrow precursors through secretion of IFN-γ. An important role for Treg cells was described in this setting. Transfer of $CD4^+$ T cells from wild-type donor mice causes very mild anaemia. This is due to the very low frequency of FV-specific $CD4^+$ T cells in wild-type mice and the presence of Treg cells in the transferred population, which mediate almost complete protection from the development of bone marrow pathology. However, depletion of the Treg cell population from the wild-type $CD4^+$ T cells leads to aggravation of disease, unmasking the pathogenic potential of the few FV-specific $CD4^+$ T cells in the wild-type population. This suggests that the balance between the frequency of effector T cells and suppressive Treg cells is crucial for maintaining the equilibrium of immune responses. In EF4.1

TCRβ transgenic mice, the balance between Treg cells and FV-specific $CD4^+$ T cells is significantly affected, with an increased frequency of FV-specific conventional $CD4^+$ T cell clones, while the numbers of Treg cells remain the same as in wild-type controls. This change in the equilibrium favouring the virus-specific $CD4^+$ T cells leads to the development of bone marrow pathology despite the presence of Treg cells. Nevertheless, Treg cell removal from the EF4.1 TCRβ-transgenic $CD4^+$ T cell population also exacerbates bone marrow pathology, indicating that although incomplete, the Treg cells contained in this population mediate significant protection. Furthermore, enrichment of the EF4.1 TCRβ-transgenic $CD4^+$ T cell population with additional Treg cells confers protection against bone marrow pathology. Therefore, these results demonstrate that, at least in unresolving FV infection, $CD4^+$ T cell-mediated bone marrow pathology is triggered by Treg cell insufficiency. The involvement of Treg cells in suppressing the immune responses of other T cells during FV infection is supported by studies showing that during FV infection there is expansion of a Treg cell population with the capacity to suppress the FV-specific $CD8^+$ T cell response (Dittmer et al., 2004). Together, these studies confirm the versatility of Treg cell function on the adaptive immune system, where in the same setting they can suppress different cell types.

The results presented here revealing the importance of Treg cell numbers in controlling immune responses against retroviruses suggest that similar mechanisms may occur during HIV-1 infection. The role and fate of Treg cells in HIV-1 infection is controversial. It has been clearly shown that HIV-specific $CD4^+$ T cell responses can be suppressed by Treg

cells (Aandahl et al., 2004) and that Treg cells migrate and are expanded in tissue sites of HIV replication (Epple et al., 2006), where they suppress HIV-specific responses and allow for viral replication (Kinter et al., 2007). However, Treg cells express the HIV-coreceptor CCR5 and are highly susceptible to HIV infection and replication (Oswald-Richter et al., 2004). Some studies have suggested that targeting of Treg cells by HIV leads to their depletion and may be responsible for the hyperactivation of conventional T cells, a characteristic of HIV disease progression (Oswald-Richter et al., 2004; Nixon et al., 2005).

In chronic graft-versus-host disease (GVHD), which can result in bone marrow cell depletion and anaemia, it has been shown that patients with active chronic GVHD have reduced frequencies of Treg cells, although their function remains normal (Zorn et al., 2005). High $FoxP3^+$ Treg cell content in the cell population transplanted into the patients is associated with a reduced risk of GVHD (Rezvani et al., 2006), showing Treg cells can suppress the immune responses of effector T cells and control development of GVHD. Moreover, relative Treg cell deficiency is found in the majority of patients with acquired aplastic anaemia caused by autoimmune reactions (Solomou et al., 2007). In a mouse model of aplastic anaemia caused by bone marrow failure due to minor histocompatibility differences it has been shown that adoptive transfer of Treg cells can prevent T cell expansion and bone marrow destruction (Chen et al., 2007b). Therefore, Treg cells seem to have fundamental roles in protecting the bone marrow from a diverse spectrum of immune pathologies.

4.2.1 Targets of Treg cell-mediated suppression

Treg cells are known to suppress distinct steps in effector T cell activation, from APC-stimulation to cytokine production. The stage at which Treg cells suppress immune responses may depend on the particular setting and immune cell type. In this study it was revealed that the protective Treg cells have no effect on the expansion of the FV-specific $CD4^+$ T cell clones in the spleen or bone marrow, in agreement with previous studies using diverse models where no effect on expansion of antigen-specific effector cells by Treg cells was found (DiPaolo et al., 2005; Chen et al., 2005; Sarween et al., 2004; Xu et al., 2003; Mempel et al., 2006; Dittmer et al., 2004). Here it is reported that Treg cells protect from FV-induced immunopathology by inhibiting the local IFN-γ production by pathogenic $CD4^+$ T cells, which would otherwise adversely affect bone marrow function. The reduction observed in the IFNγ-producers is of around 45% in the bone marrow and the mean fluorescence intensity (MFI) is reduced by around 34%. Thus, the total reduction in IFN-γ production (frequency x intensity) in the bone marrow is of 63%, demonstrating Treg cells do not completely abolish cytokine production by effector T cells. This partial suppression was reflected in their incomplete protection from bone marrow pathology. The immune system needs to keep a balance between strong and efficient immune responses and development of immune pathology. In the FV-induced immunopathology model the $CD4^+$ T cell responses are not able to control viral replication mainly due to the lack of $CD8^+$ T cells and end up being detrimental to the host. Treg cells seem to play an important role in achieving equilibrium of the virus-

specific $CD4^+$ T cells, but their action may also need to be limited as to not completely prevent induction of the immune responses.

Treg cells have a clear effect on IFNγ-producing pathogenic $CD4^+$ T cells which is also associated with protection from immune pathology. This result did not exclude, however, that Treg cells may be acting on cells of the innate immune system which could also contribute to bone marrow pathology. The effect of Treg cells on the innate immune system has been described in the colitis mouse model, where they have a protective effect against disease development (Maloy et al., 2005). Moreover, Treg cells could have a direct or indirect effect on $CD4^+$ T cells. Several studies have shown a direct *in vitro* effect of Treg cells in T and B cells (Dittmer et al., 2004; Lim et al., 2005). However, Treg cells can also act indirectly on the adaptive immune system through innate immune cells (Maloy et al., 2005), specifically on dendritic cells, where they downregulate the expression of costimulatory molecules, thus reducing their stimulatory capacity (Tang et al., 2006).

4.2.2 Mechanisms of Treg cell-mediated suppression

The mechanisms through which Treg cells achieve their suppressive functions remain controversial. *In vitro* studies suggest Treg cells act on a cell-to-cell contact-dependent, cytokine-independent way, while many *in vivo* studies have pointed to a major role of some cytokines such as IL-10 and TGF-β. Although the precise mechanisms underlying Treg cells-mediated protection against bone marrow pathology are currently unclear, the role of IL-10 and TGF-β, the main cytokines associated with Treg cell function *in vivo*, was tested. No major role for IL-10 could be demonstrated, as anaemia development is comparably suppressed by either wild-type or IL-10-deficient Treg cells (data not shown). The importance of IL-10 for Treg cell function has been shown in the colitis mouse model (Asseman et al., 1999) and in Treg cell-controlled inflammation and homeostatic expansion (Belkaid et al., 2006; Asseman et al., 1999). However, a recent study where IL-10 was depleted exclusively in the Treg cell subset has shown that IL-10 is needed for Treg cell function only in particular settings, namely at the interfaces with the environment (Rubtsov et al., 2008), suggesting IL-10 may be used by Treg cells in particular settings. Similarly, no major role for TGF-β was found, as treatment with TGF-β blocking antibodies has no effect on Treg cell-mediated suppression of bone marrow pathology. However, TGF-β is a pleiotropic cytokine which can also potently suppress bone marrow function on its own (Dybedal and Jacobsen, 1995). Thus, although mice treated with anti-TGF-β antibodies were still protected from anaemia development by the transfer of Treg cells, given the complex role of TGF-β, specifically in bone marrow

function, delineation of the precise cellular sources or cellular targets of TGF-β will require much more sophisticated tools. IL-35 is a cytokine recently described which, at least in mice, seems to be important for Treg cell function both *in vitro* and *in vivo* (Collison et al., 2007) and could play a role in Treg cell function during FV infection. Together these findings suggest that *in vivo* Treg cells may use different cytokines in different inflammatory settings.

Different modes of Treg cell function *in vivo* independent of cytokine secretion have been described that could play a role in Treg cell function during FV infection. These mechanisms can be cell-to-cell contact dependent, such as granzyme B and perforin, cAMP which was shown to suppress cytokine production in the target cells, and CTLA-4 which was shown to be important for Treg cell suppression of dendritic cells. Treg cells can also suppress other cells of the immune system by competing for growth factors, a common mechanism for homeostatic control of the immune system. The IL-2 receptor is constitutively expressed in Treg cells and is expressed upon activation of T cells and Treg cells may suppress effector function of other T cells by IL-2 consumption. The variety of distinct and nonoverlapping mechanisms used by Treg cells to suppress the immune system suggests they may be used independently of each other in specific inflammatory settings.

4.3 Antigenic specificity of Treg cells

To be able to manipulate the immune responses to foreign antigens, a detailed understanding of the mechanisms involved in Treg cell function is needed, namely how they are activated during immune responses to pathogens.

One central question that must be addressed is whether the regulatory function of Treg cells is associated with their capacity to recognize foreign antigens or if it is the consequence of bystander activation through self-antigen recognition. There are different ways by which Treg cells could be activated during viral infection: the first is that viruses activate and expand Treg cells that are antigen-specific via their TCR. Another possibility is that viruses can mimic self antigens and the cross-reactive Treg cells would be activated and expand during infection. Treg cells could also be specific for endogenous proviral sequences expressed during viral infection. However, the possibilities that viruses could activate Treg cells non-specifically via their TLRs (either by expression of TLR ligands or by induction of host-derived TLR ligands) or, alternatively, that Treg cells could be activated by indirect mechanisms such as induction of cytokines or inflammatory mediators also need to be taken into consideration.

4.3.1 TCR-dependent Treg cell activation

The crucial role of Treg cells in regulation of immune responses has been shown in many different models. However, few have addressed their antigenic specificity, mainly due to difficulties in the analysis of their highly variable TCR repertoire. Studies addressing thymic development of Treg cells suggest that thymocytes surviving negative selection with the highest avidity for self-peptide/MHC II complexes are diverted into the Treg cells lineage (Picca et al., 2006). Studies that have compared the TCR repertoires of conventional and Treg cells used TCRβ transgenic mice, in which the repertoire is restricted but still diverse enough to allow detection of TCR usage differences between the T cell populations. These studies have found that the TCR repertoires are similarly diverse with a variably small degree of overlap (Hsieh et al., 2004; Pacholczyk et al., 2006), suggesting conventional and Treg cells may not recognize the same ligands. Despite these results, the model of T cell thymic selection implies that a certain degree of self-peptide/MHC II recognition is needed for all thymocytes to be selected by the process of positive selection. Since conventional T cells are able to recognize foreign peptides, the previous studies could not exclude the possibility of recognition of foreign antigens by Treg cell clones despite their highest avidity for self ligands.

This study was aimed at analyzing the function of $CD4^+$ T cells during chronic FV infection. To this end a TCRβ transgenic mouse strain with an increased frequency of FV-specific $CD4^+$ T cells was used. The use of TCRβ transgenic mice allows the expression of endogenous TCRα chains, which was fundamental for the studies of the

TCR repertoire of Treg cells. In contrast to TCRβ transgenic mice, TCRα/β transgenic mice are not suitable for the study of the TCR repertoire of Treg cells because in these mice Treg cells only develop by the expression of a second endogenous TCR that allows for their thymic selection. Another problem with TCRα/β transgenic mice is that the same transgenic TCR is forced to be expressed on both conventional and Treg cells and this condition is not likely to occur in normal mice, because the two populations have largely distinct TCR repertoires (Hsieh et al., 2004; Pacholczyk et al., 2006). Also, in TCRα/β transgenic mice there is an inflated percentage of antigen-specific cells, both conventional and Treg cells, and this stoichiometry does not replicate what happens in a normal polyclonal response. Studies with TCRβ transgenic mice have shown normal development of $CD4^+$ T cells, including Treg cells (Hsieh et al., 2004; Pacholczyk et al., 2006). Therefore, the use of TCRβ transgenic mice seems to be a more valuable approach for the project described here.

The EF4.1 TCRβ transgenic mice showed an enrichment of FV-specific $CD4^+$ T cells of up to 4%. However, the *in vitro* and *in vivo* analyses, using both naïve and FV-infected mice, demonstrated that the Treg cell repertoire contains very few, if any, FV $env_{122\text{-}141}$-reactive clones. Recent work has been published supporting this data. A study analyzing the requirements for Treg cell development has shown that in wild-type B6 mice, binding to the MHC class II $E\alpha_{52\text{-}68}$ tetramer, which is a foreign peptide for B6 mice, is restricted to $CD4^+CD25^-FoxP3^-$ T cell clones, demonstrating that this TCR specificity is underrepresented in the Treg cell pool (Burchill et al., 2008). Another study analysed the

effects of Treg cells in HIV pathogenesis using samples from HIV-infected patients and obtained similar results. It was found that the majority of Gag-specific $CD4^+$ T cells had low FoxP3 expression and staining with HLA class II tetramers did not identify subsets of HIV-specific Treg cells, suggesting the effects of Treg cells on HIV pathogenesis are achieved by HIV nonspecific Treg cell clones (Kaufmann et al., 2007). These reports together with the results presented here support the hypothesis that the TCR repertoire of naturally-occurring Treg cells is not normally equipped to detect foreign antigens.

The majority of studies showing the presence of pathogen-specific Treg cells have been done during chronic infection, a situation where there is conversion of pathogen-specific conventional T cells into Foxp3-expressing Treg cells. These studies did not distinguished between naturally-occurring Treg cells and other types of T cells with suppressive functions (Belkaid, 2007). The only exception is a study where it was shown the existence of pathogen-specific naturally-occurring Treg cells at the site of infection in a model of chronic *Leishmania major* infection. It was shown that naturally-occurring Treg cells taken from the site of infection were able to expand when stimulated with *L. major*-infected dendritic cells (Suffia et al., 2006). These results suggest that pathogen-specific Treg cells can be found in a naïve mouse and argue against the results presented here. However, it is noteworthy that this study did not address the nature of the *L. major* antigens recognized by Treg cells. *L. major* has a 32.8Mb genome containing 8,300 protein-coding genes, meaning that during infection a large number of different antigens will be presented and some of them maybe similar to endogenous sequences. Therefore,

the existence of Treg cells capable of reacting to such a diverse antigenic repertoire might not be surprising. In contrast, the 8.3Kb genome of FV generates only 3 peptides containing one described MHC class II A^b-restricted epitope (Iwashiro et al., 1993). Given the much reduced antigenic diversity in FV infection, pathogen-specific Treg cell clones may not necessarily develop.

Here it was shown that the virus-naïve Treg cell repertoire does not contain FV-specific clones and that they do not arise from expansion of a population of preexisting FV-specific Treg cells with low precursor frequency or from conversion of FV-specific conventional $CD4^+$ T cells into $FoxP3^+$ Treg cells in response to FV infection. Moreover, Treg cells protecting from FV-induced immune pathology are also not reactive to FV-derived antigens, discarding the hypothesis that FV-derived antigens other than envelope may activate Treg cells during FV infection.

During infection, there is elevated expression of self antigens/MHC complexes, which could provide the TCR stimulation necessary for Treg cell activation and expansion observed during FV infection. This mechanism could provide an explanation for the effect of Treg cells independently of TCR stimulation by viral-unrelated antigens.

The expression of viral sequences as self antigens during thymic selection can lead to the deletion of reactive effector T cells and selection of Treg cells. This strategy seems to have been developed by retroviruses to suppress the immune responses to viral antigens

(Acha-Orbea et al., 1999). The TCR Vβ5 is one of the TCR Vβ chains conferring specificity to a superantigen from MMTV, leading to deletion of the majority of TCR Vβ5 thymocytes during thymic selection, while the remaining clones are of the Treg cell lineage (Fink et al., 1992; Fink et al., 1994). In this study it was confirmed that wild-type mice have an enriched frequency of TCR Vβ5$^+$ clones in the Treg cell compartment and it was also shown that the same is true in EF4.1 TCRβ transgenic mice, although it is less pronounced due to the expression of the transgenic TCRβ chain. Therefore, the hypothesis that Treg cells might be activated thought the TCR by endogenous proviral sequences that may be expressed upon FV infection needed to be considered. After FV infection, the frequency of TCR Vβ5$^+$ Treg cells was decreased when compared to the initial frequency in the adoptively transferred population. Furthermore, the reactivity of EF4.1 TCRβ transgenic Treg cells to the only endogenous envelope sequence that has been described in the B6 genome (Jenkins et al., 1982) was tested *in vitro* and it was found Treg cells are not reactive to the B6 endogenous sequence. Together these results suggest that Treg cells protecting from FV-induced immune pathology are not being activated neither by the envelope-related B6 endogenous sequence nor by the expression of the MMTV-related superantigen.

These results suggest that enhancement of Treg cell numbers or function, irrespective of peptide-specificity, may prove beneficial in the prevention and treatment of bone marrow immune pathology.

4.3.2 TCR-independent Treg cell activation

It is well established that TCR signalling is essential for T cell survival and expansion in the periphery (Polic et al., 2001; Seddon and Zamoyska, 2002). However, other signalling molecules, such as cytokines and TLRs also play a role in T cell activation.

It has been shown that Treg cells express some members of the TLR family, namely TLR4 (Caramalho et al., 2003), which was shown to bind to the MuLV envelope (Rassa et al., 2002). These findings raised the hypothesis that Treg cells may be activated by the envelope peptide through signalling through TLR4. This hypothesis was addressed by using Treg cells form TLR4-deficient mice in the FV-induced immune pathology model. However, TLR4-deficient Treg cells could protect against development of the bone marrow pathology, showing no difference to wild-type Treg cells (data not shown) and suggesting TLR4 is not involved in Treg cell activation in this immune pathology model.

The FV-induced immune pathology model depended on the lymphopenic status of the adoptive hosts, a situation where FV replication could not be controlled and that lead to bone marrow infection and immune pathology. It was observed that lymphopenia lead to activation of Treg cells, as observed by upregulation of ICOS and CD103, and this could influence the expansion of Treg cells. However, there was a clear influence of the FV-responding $CD4^+$ T cell population in Treg cell expansion. This effect was more significant than the lymphopenic condition or the presence of $CD4^+$ T cells in uninfected mice. Therefore, it is possible that the FV-activated $CD4^+$ T cell population produces a

growth factor which favours Treg cell division. IL-2 is produced by activated T cells and is essential for Treg cell survival and expansion both *in vitro* and *in vivo*. A model has been proposed where the number of Treg cells is indexed to the number of $CD4^+$ T cells due to their dependence on IL-2 (Almeida et al., 2006) and this mechanism can be responsible for the expansion in the Treg cell population in the FV-induced immune pathology model. Furthermore, the dynamics of conventional and Treg cells *in vivo* following immunization with a foreign antigen have been analyzed (Haribhai et al., 2007). It was found that both subsets have the same cell division rate, but the frequency of Treg cells dividing is much higher. This higher rate of division could not be accounted for if only the antigen-specific Treg cells were activated, suggesting a degenerate TCR recognition and activation largely on self antigens.

Based on all these findings, a general model for Treg cell activation and expansion, which could also apply to FV infection, can be proposed. Treg cells are progressively recruited to regulate immune responses based on the level of conventional T cell activation and the local amount of IL-2. Several mechanisms may contribute to the extensive Treg activation, like their bias towards self-reactivity, allowing for the recruitment of a sufficient number of Tregs to control the magnitude of the response.

Concluding remarks and future directions

Chronic viral infection of the hematopoietic system may result in immune-mediated pathologies. Treg cells are potent suppressors of immune responses and have important roles in preventing development of disease. In this study it was revealed the crucial function of Treg cells in preventing bone marrow pathology induced by virus-specific $CD4^+$ T cells during FV infection. Treg cells seem to be protecting the host from disease by suppressing IFN-γ production by pathogenic $CD4^+$ T cells in an IL-10 independent way. It remains to be analyzed the role of other cytokines and molecules shown to be relevant for Treg cell function in other settings and which may apply to the bone marrow pathology model described here.

Importantly, it was shown in this study that, contrary to pathogenic $CD4^+$ T cells which are virus specific, Treg cells protecting from FV-induced pathology are not pathogen-specific and Treg cell activation seems to be TCR-independent. The bias of Treg cells towards self-reactivity (Hsieh et al., 2004) may be a mechanism contributing to their activation. Furthermore, in this model Treg cell expansion depends on the presence of $CD4^+$ T cells responding to FV infection, suggesting they may be responding to signals released by the activated $CD4^+$ T cells. Given the role of IL-2 in Treg cell activation and function (Papiernik et al., 1998), it is possible that in this setting Treg cells are regulated by the amount of IL-2 locally present. The activation and expansion of virus-specific $CD4^+$ T cells due to FV infection leads to increased expression of IL-2, which could be

used by Treg cells for activation and expansion. Consumption of IL-2 by Treg cells could also be the mechanisms used by these cells to suppress the $CD4^+$ T cell effector response. Analysis of the role of IL-2 in Treg cell activation and function in the FV-induced immune pathology model would be of great interest.

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