MECHANISMS OF PERIPHERAL TOLERANCE IN TRANSPLANTATION

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THIS THESIS IS DEDICATED TO MY PARENTS

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ABSTRACT

MECHANISMS OF PERIPHERAL TRANSPLANTATION TOLERANCE

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A short-term treatment leading to long-term acceptance of transplanted tissues has been one of the major objectives in transplantation immunology. Non-depleting monoclonal antibodies, such as those targeting CD4, CD8 and CD154, have been shown effective in inducing transplantation tolerance. The cellular and molecular mechanisms that allow tolerance induction and maintenance are still largely unknown. A more precise identification of such mechanisms would allow the development of more robust tolerogenic strategies, and the generation of new diagnostic tools.

This thesis demonstrates that transplantation tolerance induced by co-stimulation blockade leads to a dominant and infectious form of tolerance maintained by $CD4^+$ T cells. Co-stimulation blockade, when combined with co-receptor blockade, led to robust tolerance of fully mismatched skin allografts. Such tolerance was also dominant, manifest by linked-suppression and a dependence on regulatory $CD4^+$ T cells.

I examined the phenotype of T cells maintaining dominant tolerance, and concluded that these could be found within both the $CD4^+CD25^+$ and $CD4^+CD25^-$ populations of tolerised mice, yet only among the $CD4^+CD25^+$ T cells of naïve animals. Such regulatory cells were found not only in the spleen of tolerised mice, but also in the tolerated tissue. Finally, I describe a strategy to eliminate the immunogenicity of “therapeutic” mAbs by temporarily interfering with their capacity to bind to cells. Further elucidation of mechanisms of transplantation tolerance, namely the identification of specific markers for regulatory T cells, may lead to significant advances on our understanding of T cell suppression and may greatly facilitate the clinical application of tolerogenic strategies.

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ABBREVIATIONS

Ab	Antibody
ADCC	Antibody-dependent cell cytotoxicity
Ag	Antigen
AH	Armenian hamster
AICD	Activation-induced cell death
APC	Antigen presenting cell
BSA	Bovine serum albumin
CDR	Complementarity determining regions
Con A	Concavalin A
CsA	Cyclosporin A
CTL	Cytotoxic T lymphocyte
CyCr	Cy-Chrome
DC	Dendritic cell
DMSO	Dimethylsulphoxide
EAE	Experimental allergic encephalitis
ELISA	Enzyme-linked immunosorbant assay
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FDA	Food and Drug Administration, USA
FITC	Fluorescin isothiocyanate
HA	Hemaglutinin
HINRS	Heat inactivated normal rabbit serum
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MST	Median survival time
NK	Natural killer
NMS	Normal mouse serum

NOS	Nitric oxide synthase
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin chlorophylla protein
QR	Quantum red
RA	Rheumatoid arthritis
RT-PCR	Reverse transcription PCR
SAGE	Serial analysis of gene expression
SEB	Staphylococcal enterotoxin B
SPF	Specific pathogen free
ST	Streptavidin
ST-APC	Streptavidin-allophycocyanin
TCR	T cell receptor
T_{CM}	Central-memory T cell
TE	Thymic epithelium
T_{EM}	Effector-memory T cell
TGF	Transforming growth factor
Th	T-helper
TNF	Tumour necrosis factor
Tr	T-regulatory
UV	Ultra-violet

BRIEF TABLE OF CONTENTS

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Immunological tolerance to a foreign antigen or tissue can be defined as the state of unresponsiveness to the antigen, following prior contact with that antigen, where the host remains competent to mount an effective aggressive immune response against third-party antigens. The mammalian immune system is usually tolerant to the host's own cells and molecules (self-tolerance), except in pathological conditions designated as autoimmunity. It is then the normal function of the immune system to be able to generate aggressive immune responses against non-self constituents entering the body, particularly microbes and their products, whilst remaining harmless to the autologous components.

The attainment of therapeutic tolerance has been considered the Holy Grail of immunology ever since the pioneering work of Medawar and colleagues (Billingham *et al*., 1953). This goal is not only important to enable successful allogeneic transplantation, but also to overcome unwanted immune responses to therapeutic proteins and gene products. This is the case for the majority of therapeutic monoclonal antibodies (mAbs) and correction of genetic defects by enzyme replacement and gene therapy. Immune responses against such therapeutic products shorten their half-life and utility.

The main objective of this thesis' work was to investigate how the immune system may become tolerised to therapeutic proteins and transplanted cells and organs. An understanding of the mechanisms involved, could enable us to manipulate the system more effectively.

1. 1 Self-tolerance

The immune system has evolved as a mechanism to protect the body against foreign infectious pathogens. However, in spite of being capable of detecting and destroying foreign microbes without prior experience, the immune system has to remain unresponsive against self-antigens. This unresponsiveness towards “self” is usually referred to as “self-tolerance”. There is therefore a balance between processes leading to aggression towards non-self and those enabling self-tolerance. Occasionally this balance is disturbed leading to immunodeficiency and susceptibility to infection on the one hand, and autoimmunity on the other. As a consequence, the capacity to maintain self-tolerance without compromising protective immunity must have been a major issue in natural selection. Thus, it is not surprising that several immune mechanisms have co-evolved so as to maintain both self-tolerance and immunity. The mammalian immune system of today shows a remarkably robust capacity for self-tolerance, in spite of its equally efficient performance in attacking foreign microbes – the latter creating a major hurdle for transplantation and the therapeutic administration of “foreign” proteins and genes.

1.1.1 Central tolerance

In order for the immune system to be capable of mounting an efficient immune response to any foreign pathogen, always evolving and consequently changing its antigenic content, antigen-specific receptors are generated by somatic rearrangement of germ-line gene segments (Tonegawa *et al*., 1974). In this way, a sufficient diversity of receptors is generated to allow the recognition of virtually any antigen generated by infectious agents. Naturally, many of those generated antigen receptors of T

cells will bind to self. Without a censoring mechanism these autoreactive cells might then lead to autoimmune disease.

The thymus is the organ where developing thymocytes, precursors of mature T cells, are generated and selected for their capacity to interact with self-MHC and associated antigens. During thymic “education” thymocytes interact with thymic stroma, first in the outer cortex, and finally in the medulla. The thymic stroma provides survival factors to thymocytes expressing a T cell receptor (TCR) capable of binding self-MHC loaded with antigen. Thymocytes failing to engage in such interactions are committed to apoptosis. This process, known as positive selection, leads to a T cell repertoire where the TCRs are restricted to self MHC molecules (Zinkernagel and Doherty, 1974; Zinkernagel, 1974; Zinkernagel *et al*., 1978). However, due to similarities between different MHC alleles, and to the selection of T cells whose TCRs weakly interact with a MHC molecule, T cells can be activated by cells expressing non-self MHC. This phenomenon is one of the major factors determining transplant rejection of MHC mismatched tissues, being known as “direct presentation” (described below).

“Positive selection” would lead by itself to the generation of many autoreactive T cells. It has been suggested that the fate of developing thymocytes depends on the avidity of the interaction they establish with the thymic stroma (Jameson *et al*., 1994; Sebzda *et al*., 1994; Alam *et al*., 1996). When a TCR interaction with peptide-loaded MHC results is of low or intermediate avidity, the cells are rescued from death by neglect due to receiving a survival factor. However, a high avidity interaction results in the delivery of an apoptotic signal leading to cell death. Such is the basis of thymic “negative selection”, a process evolved to purge the T cell repertoire of cells expressing TCRs which bind avidly to self-antigens presented by

thymic antigen presenting cells (APCs) (Kappler *et al*., 1987; MacDonald *et al*., 1988; von Boehmer *et al*., 1989).

Thymic negative selection gave support to the view that self-tolerance could be explained by the deletion of autoreactive T cell clones, an idea originally introduced by Burnet to explain aspects of antibody (Ab) production (Burnet, 1957). Clonal deletion, or central tolerance, was based on the assumption that negative selection in the thymus could purge the immune system of any autoreactive T cells. Indeed, thymic selection has a major impact in shaping the repertoire of peripheral T cells, with more than two thirds of positively selected thymocytes being subsequently deleted by exposure to self peptides in the thymic medulla (Murphy *et al*., 1990). However, despite this, T cells with autoreactive TCRs are exported from the thymus and can be demonstrated to be present in the periphery (Ramsdell *et al*., 1989; Schonrich *et al*., 1992).

1.1.2 Peripheral tolerance

Not all autoreactive T cells are deleted or anergised in the thymus. It is possible to identify peripheral T cells with capacity to cause damage to self-tissues from normal animals (Powrie and Mason, 1990; Fowell and Mason, 1993). It is likely that the majority of such autoreactive T cells are specific for antigens that are not expressed in the thymus, or that are expressed at low levels. Consequently, there may be a number of fail-safe mechanisms operating to prevent autoimmune disease. Peripheral mechanisms such as deletion, anergy, ignorance and regulation are thought to offer a further level of control over autoreactive T cells in the periphery. Furthermore, there is evidence that the thymus can export regulatory T cells important for the maintenance of immune tolerance (Seddon and Mason, 2000).

1.1.2.1 Peripheral deletion

Clonal deletion of auto-reactive T cell clones is not exclusive to the thymus. Several experiments show that deletion can also occur in the periphery. In one such experiment, *in vivo* injection of the superantigen Staphylococcal enterotoxin B (SEB) led to transient proliferation of SEB reactive $V\beta8^+$ T cells, followed by their deletion to numbers below those prior to treatment (Kawabe and Ochi, 1991; MacDonald *et al*., 1991). Similar results were obtained following administration of the superantigen Mls-1a (Webb *et al*., 1990; Jones *et al*., 1990), or in T cell transgenic mice specific for a male H-Y peptide in the context of MHC class I molecules (Zhang *et al*., 1992).

However, peripheral T cell deletion is unlikely to account for the whole of peripheral tolerance. High doses of antigen are required for peripheral deletion, and even in such circumstances a residual population of antigen-specific cells was seen to persist (Chen *et al*., 1995). Furthermore, it has been suggested that given the high degree of cross-reactivity of TCRs, self-tolerance by deletion alone would create a major hole in the T cell repertoire (Mason, 1998).

1.1.2.2 Ignorance and indifference

Immune ignorance has been defined as the situation where T cells do not lead to immune reactivity towards their specific antigens, as such antigens are not encountered in an immunogenic form by T cells during their normal recirculation (Oldstone *et al*., 1991; Ohashi *et al*., 1991).

It has been shown that naïve T cells do not circulate through non-lymphoid tissues (Mackay *et al*., 1990), and consequently lack the capacity to inflict

damage in those tissues even when they are reactive against tissue specific antigens. In support of this hypothesis it was shown that transgenic mice expressing the MHC class I molecule H-2K^b in pancreatic islet β-cells (rat insulin promoter RIP-K^b mice) do not develop diabetes even when most of their $CD8^+$ T cells express a K^b specific TCR (Heath *et al*., 1995). However, when primed with K^b-bearing skin grafts, as the skin transplants are rejected, the pancreatic islets become infiltrated and are ultimately destroyed (Heath *et al*., 1995). More recently it was shown that T cell replete mice lacking secondary lymphoid tissue fail to reject vascularised cardiac allografts, suggesting that alloimmune responses to vascularised organ transplants are not initiated in the graft itself (Lakkis *et al*., 2000).

The danger model, proposed by Matzinger in 1994 (Matzinger, 1994), can be seen as a variation on the ignorance theme (Matzinger, 2001; Matzinger, 2002). In brief, the danger model postulates that autoreactive cells present in the periphery do not cause damage for as long as signals of cell death or distress ("danger") are absent. It differs from the immune ignorance model in that T cells remain unresponsive even when they have access to the antigen, providing there are no "danger" signals. In other words, the autoreactive T cells are indifferent rather than ignorant to the antigen. The finding of dominant regulation, discussed below, casts doubts on whether such a model is useful as a complete explanation for tolerance. Without doubt "danger" or "adjuvanticity" are important initiators of immune responses. However, there is now compelling evidence that regulatory T cells can prevent aggressive immune responses even in situations where danger is present (see below).

1.1.2.3 Anergy

T cell anergy was described as the functional state in which T cells remain viable but unable to respond to optimal stimulation through both the TCR and costimulatory molecules (Schwartz, 1996). The functional state of anergic T cells can be defined by their incapacity to proliferate or to produce interleukin-2 (IL-2) (Lamb *et al*., 1983; Schwartz, 1990).

Anergy was first described in studies with human T cell clones specific for influenza virus haemaglutinin peptides (Lamb *et al*., 1983). In such studies, incubation of T cells with high doses of the antigen resulted in T cell unresponsiveness to subsequent presentation of the peptide in normal stimulatory conditions (Lamb *et al*., 1983). Later, it was shown that anergy could be induced by antigen recognition in the absence of co-stimulation (Quill and Schwartz, 1987; Jenkins and Schwartz, 1987), the use of altered peptide ligands (Sloan-Lancaster *et al*., 1993; Sloan-Lancaster *et al*., 1994), or direct presentation by activated rat or human T cells which express MHC class II molecules (Lombardi *et al*., 1996; Taams *et al*., 1998).

T cell anergy has also been described *in vivo* in several animal models. Initially it was reported following transplantation tolerance induced by anti-CD4 mAbs (Qin *et al*., 1989; Alters *et al*., 1991). But *in vivo* T cell anergy was also decribed following injection of cells expressing a self-superantigen (Mls-1a) into mice (Rammensee *et al*., 1989), following aqueous peptide antigen administration in mice (Burstein *et al*., 1992), in double transgenic mice for a TCR and its surrogate self antigen (Schonrich *et al*., 1992; Jordan *et al*., 2000), and in oral tolerance (Chen *et al*., 1994).

Interestingly, anergic T cells were found capable to suppress proliferation of naïve T cells *in vitro* (Lombardi *et al*., 1994) and *in vivo* (Chai *et al*., 1999). However, the T cells used in both instances were rendered anergic following *in vitro* incubation with immobilised anti-CD3 mAb. There has been no report yet of suppressive function of T cells obtained following physiological *in vivo* induction of anergy by antigen stimulation. In any case, such observations support the "civil service model" proposed by Waldmann (Waldmann *et al*., 1992), that postulates that antigen specific unresponsive cells can interfere with the generation of help by co-localising with other T cells and competing for elements in the microenvironment (such as adhesion molecules or cytokines). As a consequence by preventing adequate "help" from being generated the anergic cells could suppress the proliferation and effector function of the naïve T cells.

1.1.2.4 Regulatory T cells

The existence of regulatory T cells, at that time named suppressor T cells, was first suggested in the early 70s following the observation that it was possible to transfer T cell unresponsiveness between animals (Gershon and Kondo, 1970; Droege, 1971). At that time $CD8^+$ T cells were believed to contain the population of suppressor T cells. However, interest in the phenomenon faded as many experimental systems proved hard to sustain, and the results difficult to explain. As a consequence the field was brought into disrepute and the term "suppressor T cells" became a taboo word for immunologists (Bloom *et al*., 1992; Green and Webb, 1993).

However, in recent years, several different groups have clearly established the existence of $CD4^+$ T cells with suppressive properties, now named regulatory T cells (reviewed in Maloy and Powrie, 2001; Sakaguchi, 2000;

Shevach, 2000; Waldmann and Cobbold, 2001). First, indirect evidence for the existence of T cells capable of suppressing autoimmune responses resulted from studies with mice thymectomised at day 3. Such animals developed autoimmune diseases such as oophoritis (Nishizuka and Sakakura, 1969), thyroditis (Kojima *et al*., 1976), or gastritis (Kojima *et al*., 1980), that could be prevented by adoptive transfer of thymocytes or splenocytes from normal syngeneic animals. With the development of methods allowing specific depletion or sorting of T cell subsets it became possible to further characterise the phenotype of regulatory T cells that could prevent the onset of autoimmune diseases or gut immunopathology upon adoptive transfer into susceptible animals and also in therapeutically induced tolerance. Initially Sakaguchi identified the regulatory capacity among the $CD5^+$ T cells (Sakaguchi *et al*., 1985), while in experimentally induced tolerance regulatory activity was present among the $CD4^+$ T cells (Hall *et al*., 1985). The $CD4^+$ cells have been further subdivided: first the regulatory capacity was found to be within the $CD4^+CD45RC^{low}$ compartment in the rat (Powrie and Mason, 1990; Fowell and Mason, 1993) or the $CD4^+CD45RB^{low}$ compartment in mice, and later within the $CD4^+CD25^+$ compartment (Sakaguchi *et al*., 1995; Asano *et al*., 1996). Read *et al* has shown, by comparing the regulatory capacity of $CD4^+CD25^+CD45RB^{low}$ and $CD4^+CD25^-CD45RB^{low}$ that only the first population could suppress colitis in scid mice co-transferred with $CD4^+CD45RB^{high}$ T cells (Read *et al*., 2000). It can thus be concluded that CD25 is a more useful marker than CD45RB. However, it was shown that $CD4^+CD25^-CD45RC^{low}$ T cells can prevent diabetes in lymphopenic rats, although the regulatory capacity of these cells was inferior to the potency of $CD4^+CD25^+CD45RC^{low}$ (Stephens and Mason, 2000). More recently an equivalent subset of $CD4^+CD25^+$ regulatory T cells was isolated from humans, and suppressive capacity was confirmed *in vitro*, by inhibition of proliferation of target T cells (Ng *et al*., 2001; Dieckmann *et al*., 2001;

Jonuleit *et al*., 2001; Stephens *et al*., 2001; Levings *et al*., 2001; Taams *et al*., 2001; Baecher-Allan *et al*., 2001).

Other markers, such as L-selectin (CD62L) (Herbelin *et al*., 1998) or CD38 (Read *et al*., 1998) have been suggested as possible surface markers of regulatory cells. However they were not shown to be useful markers to further subdivide the $CD4^+CD25^+$ regulatory T cell population (Read *et al*., 2000).

It is still unclear whether regulatory T cells constitute a specific lineage originating in the thymus. Recent evidence has suggested that thymocytes are more potent than splenocytes in preventing autoimmunity in lymphopenic mice (Saoudi *et al*., 1996). Furthermore, thymectomised PVG rats treated with sublethal irradiation develop autoimmune thyroiditis that can be prevented by adoptive transfer of $CD4^+CD45RC^{low}$ T cells or $CD4^+CD8^-$ thymocytes from syngeneic donors. However, when thyroids were ablated *in utero* with I^{131}, peripheral T cells, but not thymocytes, lost the capacity to prevent thyroiditis (Seddon and Mason, 1999a). Such result suggests that regulatory T cells develop within the thymus, and once exported to the periphery require contact with the antigen in order to keep their regulatory function. It was also shown that it is possible to induce allospecific tolerance by grafting allogeneic thymic epithelium (TE) into athymic (nude) mice at birth. The grafted mice reconstitute normal numbers of T cells and accept skin grafts syngeneic with the TE, being competent to reject third-party allografts (Modigliani *et al*., 1995). In addition, when $CD4^+$ T cells from the TE chimeras were transferred into immunocompetent syngeneic hosts, such mice became themselves tolerant for TE-type allografts (Modigliani *et al*., 1995). Taken together, these results led to the notion that the production of regulatory T cells may be a further property of the thymus (Seddon and

Mason, 2000). Significantly, a role for the thymus in transplantation tolerance induced with non-depleting mAbs has never been shown (see below).

1.1.2.5 Functional characteristics of regulatory T cells

The functional characteristics of natural regulatory T cells still remain to be elucidated.

Most studies have used inhibition of T cell proliferation as the readout of T cell suppression. Such studies have been used to confirm the suppressive capacity of T cells rendered anergic following incubation with immobilised anti-CD3 mAb (Lombardi *et al*., 1994), or regulatory T cell clones (Tr1) (Groux *et al*., 1997), or more recently $CD4^+CD25^+$ T cells (reviewed in (Sakaguchi, 2000; Shevach *et al*., 2001).

In the first case, anergic T cells were shown to inhibit antigen specific T cell proliferation *in vitro*, providing the antigen recognised by both the anergic T cells and these specific T cells being suppressed was present in the same APC (Lombardi *et al*., 1994). The Tr1 clones were also shown capable of inhibiting proliferation of $CD4^+$ T cells in response to antigen (Groux *et al*., 1997). $CD4^+CD25^+$ T cells required activation prior to their suppressive activity. Following such antigen specific activation, their suppressive activity was antigen non-specific (Thornton and Shevach, 2000). Inhibition mediated by $CD4^+CD25^+$ cells was not dependent on cytokines or other soluble factors, but required direct cellular contact (Takahashi *et al*., 1998; Thornton and Shevach, 1998).

Other *in vitro* studies have suggested that anergic or $CD4^+CD25^+$ regulatory T cells exerted their suppressive function by down-regulating the expression

of MHC class I and the co-stimulatory molecules CD80 and CD86 on dendritic cells (DCs) (Vendetti *et al*., 2000; Cederbom *et al*., 2000). As a consequence the DC would become "decommissioned" for T cell activation (Fairchild and Waldmann, 2000). Such concept is particularly appealing considering that DCs were shown capable of acting as a temporal bridge between helper T cells, and $CD8^+$ T cells (Ridge *et al*., 1998; Bennett *et al*., 1998; Schoenberger *et al*., 1998). It is tempting to establish a parallel and predict that DCs may also act as a temporal bridge between T cells with suppressive function and the T cells being suppressed. This could be achieved by modulation of the maturation state of the DC, as it has been reported that immature DCs have tolerogenic properties (Jonuleit *et al*., 2000; Dhodapkar *et al*., 2001; Roncarolo *et al*., 2001). The recent identification of a method to induce the differentiation of DCs from embryonic stem cells (Fairchild *et al*., 2000) may allow confirmation that by modifying the maturation state of DCs one can render them tolerogenic.

Alternatively, it was suggested that suppression mediated by $CD4^+CD25^+$ T cells require direct interaction between the suppressive T cell and the T cell being suppressed, as it could operate with fixed APCs or in the absence of APCs (Takahashi *et al*., 1998; Thornton and Shevach, 2000). It is not clear at this moment whether regulatory T cells induce suppression via DC modulation in addition to a direct suppressive effect on other T cells, or whether the two mechanisms are distinct regulatory circuits.

Following the identification that CTLA-4 is constitutively expressed on the majority of $CD4^+CD25^+$ regulatory T cells, different groups investigated whether such molecule could be an important mediator of T cell regulation. Indeed, by using anti-CTLA4 neutralising mAbs it was shown, both *in vitro* and *in vivo*, that in the presence of such mAbs suppression mediated by

$CD4^+CD25^+$ T cells was abrogated (Read *et al*., 2000; Takahashi *et al*., 2000). Such results were not confirmed in other experiments (Ng *et al*., 2001; Dieckmann *et al*., 2001; Jonuleit *et al*., 2001; Stephens *et al*., 2001; Levings *et al*., 2001; Taams *et al*., 2001; Baecher-Allan *et al*., 2001; Shevach, 2001; Graca *et al*., 2002b; Chapter 5). It is possible that some of the effects seen when anti-CTLA4 mAbs are used, result from their effect on the effector cell population rendering them more sensitive to antigen mediated signals (Hurwitz *et al*., 2002; Egen and Allison, 2002). However, recently Read *et al* found that when $CD4^+CD45RB^{high}$ T cells from CTLA-$4^{-/-}$ mice were co-transferred with wild-type $CD4^+CD25^+$ T cells, into T cell-deficient mice, these mice were protected from inflammatory bowel disease (IBD). However, when the $CD4^+CD45RB^{high}$ T cells from CTLA-$4^{-/-}$ mice were injected in the absence of $CD4^+CD25^+$ T cells, or when both populations were co-administered together with anti-CTLA4 mAb, the recipient mice developed IBD (S. Read, personal communication). Such results suggest that, in addition to possible effects of anti-CTLA4 mAb on effector cells, the same mAbs may have an effect on regulation by $CD4^+CD25^+$ T cells, at least in relation to specific situations.

Several cytokines were also suggested as being key factors mediating T cell suppression. Much of the evidence supporting the suppressive role of key cytokines derived from studies of “immune deviation” discussed below.

1.1.2.6 Immune deviation: the Th1 – Th2 (and Th3 / Tr1) paradigm

It was shown that terminally differentiated $CD4^+$ T cells could be functionally divided according to the cytokines they produce: T helper 1 (Th1) cells that produce IL-2, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α); and the Th2 cells, which produce IL-4, IL-5, IL-6, IL-10, and IL-13 (Mosmann *et*

al., 1986; Mosmann and Coffman, 1989). Naïve T cells, also named Th0, are believed to have the potential to differentiate towards any of the above mentioned fates (reviewed in O'Garra, 1998). Th0 cells are consequently non-polarised and capable of secreting low levels of Th1 or Th2 type cytokines. At the onset of an immune response the microenvironment in which the cells are found determines the direction in which they polarise, which is further reinforced by the effect of the cytokines the cells produce in driving differentiation towards that same type while inhibiting differentiation towards the opposing cell type (Gajewski and Fitch, 1988; Fiorentino *et al*., 1989; McKnight *et al*., 1994; Sad and Mosmann, 1994).

It has been suggested that autoimmunity and allograft rejection are immune responses dependent on Th1 cells, that could be suppressed by "deviating" the immune response towards the Th2 type of behaviour (Waldmann and Cobbold, 1993; Abbas *et al*., 1996). This hypothesis was supported by evidence of an absence of Th1 type cytokines, and presence of Th2 type cytokines, in animals demonstrating long-term graft survival (Takeuchi *et al*., 1992; Hancock *et al*., 1993; Mottram *et al*., 1995; Sayegh *et al*., 1995). Furthermore, in animal models of gut immunopathology and autoimmune disease, a Th1 type of response seem to correlate with the pathology, that could be abrogated by deviating the response towards Th2 (Powrie and Mason, 1990; Saoudi *et al*., 1993; Kuchroo *et al*., 1993; Powrie *et al*., 1994b; Powrie *et al*., 1994a).

However, a number of studies have suggested that tolerance could not simply be explained by immune deviation towards Th2 type responses. Although it was shown that prolongation of graft survival could be achieved by administration of Th2 type cytokines (Levy and Alexander, 1995; Takeuchi *et al*., 1997), other studies found no evidence for Th2 involvement in

promoting long-term graft survival (Krieger *et al*., 1996; Plain *et al*., 1997). Furthermore, Th2 cells were shown capable of inducing autoimmune disease (Lafaille *et al*., 1997; Pakala *et al*., 1997) and to mediate allograft rejection (Chan *et al*., 1995; VanBuskirk *et al*., 1996; Zelenika *et al*., 1998). Furthermore, in transplantation tolerance induced by non-depleting anti-CD4 and anti-CD8 mAbs, the frequency of Th1 and Th2 cells, as determined by limiting dilution analysis, was shown to be similar in tolerised and rejecting mice (Cobbold *et al*., 1996).

The role of Th2 type cytokines in immune tolerance has been extensively studied, but no consensus has yet been reached as there are several contradictory reports. Antibodies targeting IL-4 were shown to abrogate transplantation tolerance (Donckier *et al*., 1995; Davies *et al*., 1996b; Onodera *et al*., 1997), although in one of these studies the effect was only partial (Davies *et al*., 1996b). However, in other studies transplantation tolerance was shown to be IL-4 independent (Hall *et al*., 1998; Chapter 5), as was prevention of gut immunopathology by $CD4^+CD45RB^{low}$ cells (Powrie *et al*., 1996).

Several groups have used mAbs to neutralise IL-10, showing a role for this molecule in immune tolerance (Asseman *et al*., 1999; Hara *et al*., 2001; Kingsley *et al*., 2002). However, injection of anti-IL10 mAb failed to break anti-CD4 induced transplantation tolerance (Davies *et al*., 1996b; Graca *et al*., 2002b; Chapter 5).

More recently, a $CD4^+$ T cell clone producing IL-4, IL-10 but also tranforming growth factor β (TGF-β) and capable of suppressing autoimmune encephalomyelitis (EAE) in mice was described (Chen *et al*., 1994). Such clone was named Th3. Another T cell clone capable of suppressing gut

immunopathology induced following transfusion of $CD4^+CD45RB^{high}$ splenic T cells into SCID mice was defined as T regulatory 1 (Tr1) (Groux *et al*., 1997). Such cells can be generated *in vitro* following stimulation in the presence of IL-10, have a low proliferative capacity, produce high levels of IL-10, moderate levels of TGF-β, low levels of IL-2 and IL-4, and are capable of suppressing the proliferation of $CD4^+$ T cells *in vitro* (Groux *et al*., 1996). Consistent with a role for these types of cells *in vivo* was the finding that prevention of EAE, or other immunopathology triggered by $CD4^+CD45RC^{high}$ cell transfusion into lymphopenic mice, could be abrogated following administration of anti-IL10 and anti-TGFβ mAb (Doetze *et al*., 2000; Kitani *et al*., 2000; Read *et al*., 2000). The relationship between Th3 and Tr1 $CD4^+$ T cell populations, if any, is not yet clear (reviewed in Roncarolo and Levings, 2000). It is also unclear whether Th3/Tr1 cells bear any relationship to the naturally occurring $CD4^+CD25^+$ T cells, as they do not seem to share an obvious mechanism of action (Roncarolo and Levings, 2000). A direct comparison of genes expressed by Th1, Th2 and Tr1 T cell clones suggested that Th2 and Tr1 cells may be very closely related (Zelenika *et al*., 2002).

1.1.2.7 *In vitro* and *in vivo* assays of regulatory T cell function

The development of reliable *in vitro* systems have been useful in the elucidation of many immune mechanisms. However, recent findings raise doubts on whether current assays are adapted for the study of T cell regulation. The most popular of the assays used measures the capacity of putative regulatory cell population to inhibit proliferation of target T cells. Such assays have been used to assess the regulatory capacity of anergic T cells (Lombardi *et al*., 1994), Tr1 clones (Groux *et al*., 1996), or more recently $CD4^+CD25^+$ T cells (Thornton and Shevach, 1998; Takahashi *et al*., 1998; Ng

et al., 2001; Dieckmann *et al*., 2001; Jonuleit *et al*., 2001; Stephens *et al*., 2001; Levings *et al*., 2001; Taams *et al*., 2001; Baecher-Allan *et al*., 2001).

Recently it was shown in the host laboratory that *in vitro* proliferation of $CD4^+$ T cells and IFN-γ secretion can be inhibited by co-culture with antigen specific Th1, Th2 or Tr1 clones (Zelenika *et al*., 2001). Although inhibition of proliferation mediated by Th1 cells could be abrogated by addition of inhibitors of nitric oxide synthase (NOS), suppression mediated by Th2 and Tr1 clones was NOS independent (Zelenika *et al*., 2001). Interestingly, when suppressive capacity of the same T cell clones was assessed by *in vivo* capacity to prevent skin graft rejection, only the Tr1 clone was found to have regulatory properties (Zelenika *et al*., 2002).

Furthermore, Chun-Yen Lin, also in the host laboratory, conclusively demonstrated that when allo-specific $CD8^+$ T cells are injected into tolerant mice, they proliferate and survive to the same extent as in non-tolerant mice. The only apparent difference between the two cases is that when transfused into tolerant mice the allo-specific $CD8^+$ T cells fail to produce IFN-γ, generate cytotoxic T cells (CTL) and reject grafts (Lin *et al*., 2002; Lin, 2002). In other words, it appears that the *in vivo* function of regulatory T cells is not inhibition of proliferation or abrogation of T cell help, but rather they act by “disarming” the effector T cells. Preliminary results suggest that the same conclusions can be extended to suppression of $CD4^+$ T cells (Lin, 2002).

When taken together, these results question whether the currently available *in vitro* tests are adequate for the study of T cell suppression. It is possible, due to the uncertainties about the mechanism of action of regulatory T cells, that *in vitro* assays still do not include all the factors relevant for the study of T cell regulation. Until *in vitro* assays are further refined I believe the *in vivo*

experimental systems to be more reliable for the study of this complex phenomenon.

1.2 Therapeutically induced tolerance

Induction of therapeutic tolerance (Figure 1.1) has been one of the major aspirations of immunologists. This is not only important for transplantation, and the treatment of autoimmune diseases, but also to overcome immune responses that target molecules therapeutically introduced in the patient. Currently, the prevention of transplant rejection is achieved by administration of immunosuppressive drugs, with their associated side effects.

Such immunosuppressive regimens target the whole immune system. However, an elective ablation of only the alloreactive clones, if feasible, offers a way of preventing graft rejection while sparing host's immunocompetence. One possible approach to achieving this involves the establishment of mixed hematopoietic chimerism or macro-chimerism by transfer of a high dose of donor bone marrow cells (Ildstad and Sachs, 1984; Wekerle and Sykes, 1999; Wekerle *et al*., 2000). This permits *in vitro* monitoring of the tolerant state by sampling lymphocytes from the host and testing their reactivity against donor-type cells. Such "functional" assays may be impracticable, inconvenient and not always reliable, as it might prove difficult to deplete all alloreactive T cell clones, and any expansion of residual cells might give rise to delayed transplant rejection. Furthermore, the practical logistics concerning myeloablation and bone marrow transplantation prior to organ grafting, may preclude the application of this method to cadaveric transplantation.

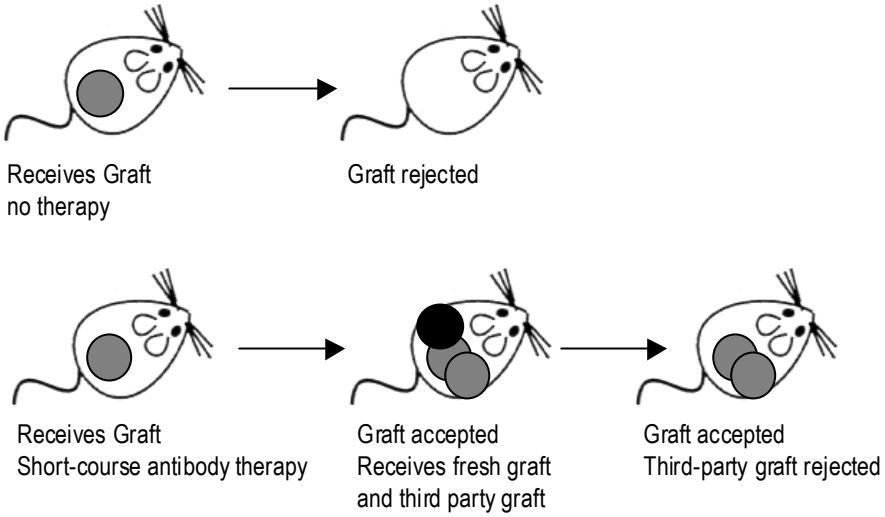

Figure 1.1 *Demonstration of tolerance in antibody treated animals.* Mice accept a second challenge with a graft of the same type, but readily reject third party grafts. Alloreactive cells, as demonstrated by proliferation assays, are present at any time point.

The complementary strategy aims to control alloreactive cells in a different way. It is based on the induction of a dominant tolerance state and its hallmark is the emergence of regulatory $CD4^+$ T cells (Waldmann and Cobbold, 1998). Unlike tolerance by deletion, alloreactive T cells may still be demonstrated, but grafts are accepted indefinitely. Furthermore, tolerance can be very robust and resists the adoptive transfer of large numbers of cells with the potential to mediate graft rejection – the reason why it is termed dominant (Figure 1.2) (Qin *et al*., 1990; Scully *et al*., 1994). The regulatory cells can even do more than just "suppress": if they are allowed to coexist with the naïve cells, they have the capacity to recruit new regulatory $CD4^+$ T cells from that naïve pool. After this recruitment, the initial regulatory T cells can be removed experimentally and one observes that the new regulators can maintain tolerance themselves (Qin *et al*., 1993). This process can be repeated experimentally for several cell transfers, and has therefore been named "infectious tolerance" (Figure 1.3) (Qin *et al*., 1993; Chen *et al*., 1996; Cobbold and Waldmann, 1998).

1.2.1 mAbs as dominant tolerance inducing agents

Short courses of therapeutic antibodies have been shown to lead to long-term acceptance of foreign grafts in several experimental systems (reviewed in Waldmann and Cobbold, 1998). The first examples of peripheral tolerance induced with monoclonal antibodies were reported in 1986 (Benjamin and Waldmann, 1986; Gutstein *et al*., 1986). In these experiments tolerance to foreign immunoglobulins was achieved after a short-term treatment with depleting anti-CD4 antibodies. It was soon demonstrated that depletion of $CD4^+$ cells was not required for tolerance induction, as similar results were found using F(ab')2 fragments (Benjamin *et al*., 1988; Carteron *et al*., 1988; Carteron *et al*., 1989), non-depleting isotypes (Qin *et al*., 1990) or non-

depleting doses of synergistic pairs of anti-CD4 antibodies (Qin *et al*., 1987). Treatment with anti-CD4 antibodies was also shown to lead to long-term acceptance of skin grafts differing by "multiple-minor" antigens (Qin *et al*., 1990), even in pre-sensitised recipients (Marshall *et al*., 1996). The same results were also demonstrated for heart grafts across MHC barriers (Chen *et al*., 1992; Onodera *et al*., 1996) or concordant xenografts (Chen *et al*., 1992).

Further demonstrations of transplantation tolerance were later reported with anti-LFA-1 antibodies, alone (Benjamin *et al*., 1988) or in combination with anti-ICAM-1 (Isobe *et al*., 1992), and also with anti-CD2 and anti-CD3 antibodies (Chavin *et al*., 1993).

More recently, co-stimulation blockade of CD28 (Lenschow *et al*., 1992), CD40L (CD154) (Parker *et al*., 1995) or both in combination (Larsen *et al*., 1996b) has been shown effective. These findings have recently been extended to non-human primates. In one study, long-term survival of renal allografts was achieved following blockade of CD40L alone (Kirk *et al*., 1999). Another group achieved prolonged islet allograft acceptance after a similar treatment (Kenyon *et al*., 1999). Interestingly, the association of tacrolimus or steroids to the therapeutic regime abrogated tolerance (Kirk *et al*., 1999).

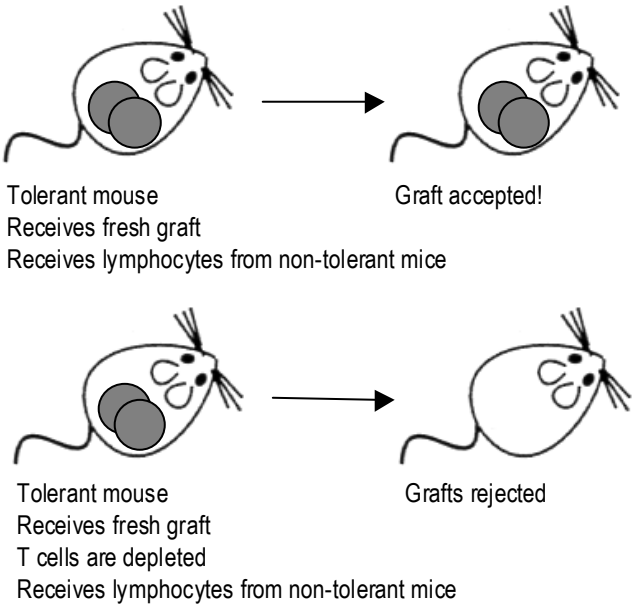

Figure 1.2 *Demonstration of dominant tolerance*. This requires the demonstration of tolerance being imposed on cells with the ability to reject a graft in the absence of regulatory cells.

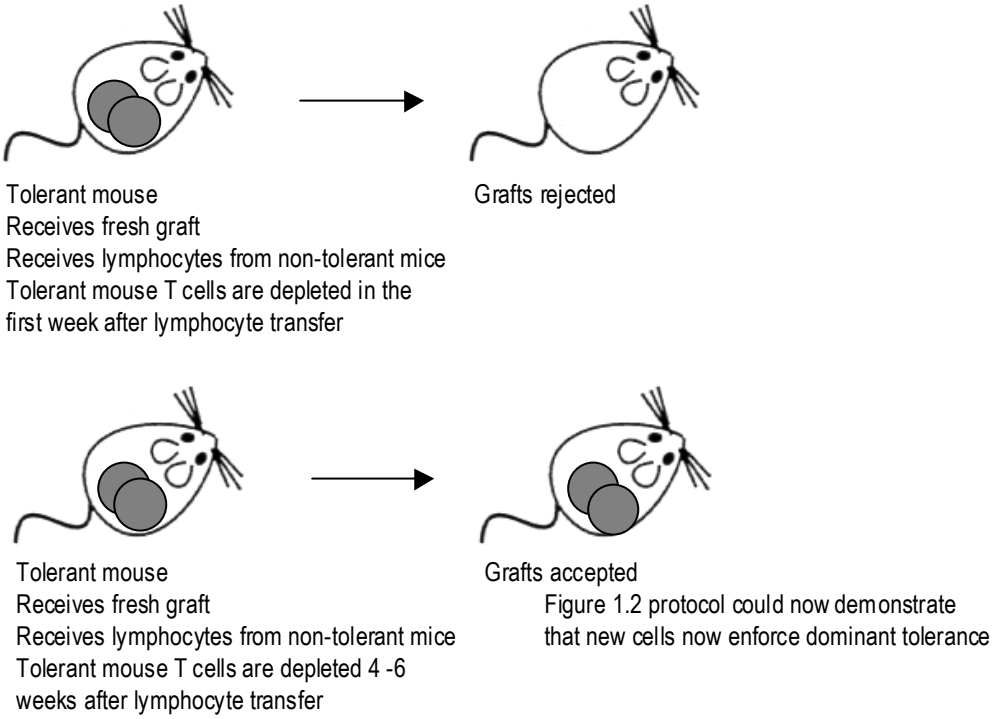

Figure 1.3 *Demonstration of infectious tolerance*. This requires the demonstration that cells with the ability to reject are converted into the regulatory type after coexistence with cells from a tolerant animal.

1.2.2 Infectious tolerance

Models of transplantation tolerance induced with anti-CD4 or anti-CD40L antibodies have shown that tolerant mice do not reject the grafts even after the adoptive transfer of fresh lymphocytes from a non-tolerant animal (Qin *et al*., 1990; Scully *et al*., 1994; Graca *et al*., 2000). Spleen cells from animals made tolerant to skin and heart grafts using anti-CD4 or anti-CD40L antibodies could regulate naïve T cells, and in so doing, rendering them regulatory in their own right (Qin *et al*., 1993; Chen *et al*., 1996; Graca *et al*., 2000). Using transgenic mouse strains carrying specific cell surface markers in their lymphocytes, it was possible to selectively eliminate the host-type T cells from the tolerant animal (Qin *et al*., 1993; Graca *et al*., 2000). If this cell-depletion was performed immediately after cell transfer, the tolerant state was broken and indicator grafts were readily rejected by the transferred non-tolerant lymphocytes (Qin *et al*., 1993; Graca *et al*., 2000). If, however, the host cells were allowed to coexist with the adoptively transferred set for 4 – 6 weeks, then tolerance was maintained even after the depletion of the host cells (Qin *et al*., 1993; Graca *et al*., 2000). The remaining cells were nevertheless fully competent to reject an unrelated graft. Not only were they unable to reject a graft from a similar donor, but they could now regulate another population of spleen cells from a non-tolerant animal in a similar transfer experiment (Qin *et al*., 1993). This effect, named “infectious tolerance”, provides compelling evidence for the existence of regulatory T cells: the regulatory cells from a tolerant animal can suppress the aggressive action of graft-reactive T cells and induce members of that population to become regulatory as well.

A further important finding underlining the significance of infectious tolerance comes from the demonstration of a phenomenon named “linked suppression”

(Figure 1.4). In the original experiment (Davies *et al*., 1996a) CBA/Ca mice were rendered tolerant to B10.BR skin grafts (different in multiple minor transplantation antigens) with non-depleting anti-CD4 and anti-CD8 treatment. These mice readily rejected a third-party CBK skin graft (CBA/Ca mice transgenic for the MHC class I antigen K^b) even if simultaneously grafted with a fresh B10.BR skin into the same graft bed. However, when tolerised mice were transplanted with (CBK x B10.BR)F_1 skin grafts, harbouring simultaneously the tolerated and the third-party antigens, rejection was delayed or absent. Furthermore, mice that had accepted the (CBK x B10.BR)F_1 skin grafts, accepted CBK skin transplants at a later time point. The same phenomenon was recently demonstrated for anti-CD40L antibody induced tolerance (Honey *et al*., 1999), and tolerance induced following donor bone marrow infusion (Bemelman *et al*., 1998).

1.2.3 T cell regulation

Evidence for the existence of regulatory T cells does not come exclusively from studies of transplantation tolerance. Regulatory T cells have been found in several autoimmunity models (reviewed earlier and in Mason and Powrie, 1998; Roncarolo and Levings, 2000). Even among the T cell population of normal individuals, T cells with the capacity of causing autoimmune disease have been identified, as well as regulatory cells that prevent this pathological auto-aggression (Powrie and Mason, 1990; Fowell and Mason, 1993). It is therefore likely that, in addition to thymic tolerance, peripheral tolerance mechanisms operate to safeguard tolerance to extra-thymic antigens.

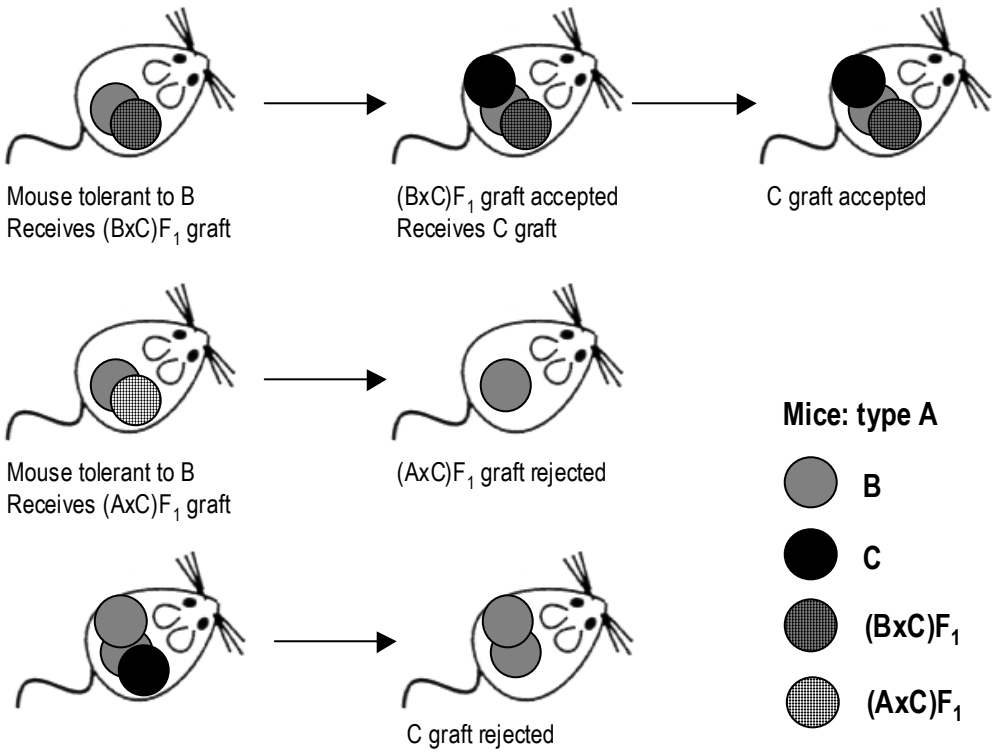

Figure 1.4 *Demonstration of linked suppression*. This requires the demonstration that tolerant animals accept grafts where a third party antigen is present in cells that also have the tolerated antigens $(BxC)F_1$, but reject third party grafts (C) if the tolerated antigen absent from the graft cells (even if a concomitant tolerated-type graft (B) is given). The animals that accept the grafts with the "linked" third party antigen $(BxC)F_1$ should accept later grafts of the third party (C).

The phenotype of these regulatory cells, and their proposed mechanisms of action is not yet totally clear. Although it is possible to induce transplantation tolerance with mAbs in thymectomised mice (Qin *et al*., 1987; Honey *et al*., 1999), there is evidence suggesting that regulatory cells in some autoimmunity models are a defined lineage originating in the thymus (reviewed by Seddon and Mason, 2000). This lineage was shown to have some distinctive surface markers: they are included in the $CD45RC^{low}$ population of $CD4^+$ cells in the rat (Fowell and Mason, 1993), or in the $CD45RB^{low}$ in the mice (Powrie *et al*., 1996). It also seems that expression of the IL-2 receptor α-chain (CD25) is a good indicator of the presence of a putative regulatory $CD4^+$ cell that further subdivide the $CD45RB^{low}$ population (Sakaguchi *et al*., 1995; Read *et al*., 2000). Given that CD25 seems to be a marker of suppressor cells it may seem paradoxical that an antibody targeting CD25 is licensed for use as immunosuppressive agent in clinical transplantation (reviewed in Waldmann and O'Shea, 1998). A theoretical risk for a therapy that targets CD25 expressing cells might be the loss of potential to induce tolerance to the graft, as well as a possible disruption of normal regulatory mechanisms that prevent autoimmunity. It may be relevant to note that mice treated with the anti-CD25 mAb PC61 were more effective in rejecting tumour cells (Shimizu *et al*., 1999).

It is hoped that purification and cloning of the elusive regulatory T cells will allow a better understanding of their biology.

1.2.4 Tolerance and cell death

During immune responses, large numbers of activated T cells are generated. The majority of them are eliminated by apoptosis (Murali-Krishna *et al*., 1998). Two distinct forms of apoptotic cell death associated with the

termination of T cell responses have been described (reviewed in Lenardo *et al*., 1999). Passive cell death occurs at the later stages of immune responses, when activated T cells are deprived of T cell growth cytokines. As a consequence of apoptosis by cytokine withdrawal, the pool of reactive T cells shrinks following an immune response (Jones *et al*., 1990; Kuroda *et al*., 1996). The second form of apoptosis was named activation induced cell death (AICD), and occurs during active proliferation of T cells, which have undergone one or more rounds of cell division and during G1 or S phase of the cell cycle (Lenardo, 1991; Boehme and Lenardo, 1993; Lissy *et al*., 1998). AICD was shown to be IL-2 dependent (Lenardo, 1991), with IL-$2^{-/-}$ mice developing a lymphoproliferative disease with accumulation of lymphocytes in secondary lymphoid tissue, infiltration of several organs and overproduction of cytokines and autoantibodies (Schorle *et al*., 1991; Sadlack *et al*., 1995). A similar phenotype was also described for mice deficient in the IL-2 receptor α chain (CD25) (Willerford *et al*., 1995). In addition, apoptosis also seems to be mediated by members of the tumour necrosis factor (TNF)-receptor family, such as Fas (CD95) (Zheng *et al*., 1995; Dhein *et al*., 1995).

There are now many examples where evidence is found for alloreactive T cell death in response to tolerance induction to transplanted tissue without the need for intended chimerism. For example, two interesting recent papers demonstrate that tolerance induction with therapeutic anti-CD40L mAbs requires cell death (Li *et al*., 1999; Wells *et al*., 1999). In fact, blockade of activation induced cell death (AICD) either by using transgenic mice resistant to apoptosis (Wells *et al*., 1999), or by using Cyclosporin-A (CsA) (Li *et al*., 1999) resulted in graft rejection in animals subjected to antibody blockade of CD28 and CD40L.

In spite of the importance of AICD in anti-CD40L transplantation tolerance, regulatory cells also play a role in its maintenance. In fact, tolerance induction with therapeutic anti-CD40L results in linked-suppression (Honey *et al*., 1999) and infectious tolerance (Chapter 3 and Graca *et al*., 2000). Thus, regulatory $CD4^+$ T cells emerge, following tolerance induction, and actively enforce a dominant tolerance state.

One can safely speculate that amplification of regulatory cells and induction of AICD are probably general mechanisms exploited in the different tolerance-inducing strategies. It is likely, although not yet demonstrated, that anti-CD4 therapeutic mAbs also require some cell death for the induction of transplantation tolerance. Supporting this hypothesis, it was recently shown that treatment of diabetic NOD mice with non-depleting anti-CD4 mAb can lead to apoptosis of diabetogenic T cells (Phillips *et al*., 2000). In these experiments, besides physical elimination of aggressive clones the tolerogenic treatment leads to the development of protective regulatory T cells. With regard to the role of apoptosis in transplantation tolerance induced with non-depleting anti-CD4 mAbs, it was recently shown that tolerance so achieved is independent of the Fas (CD95), so ruling out that pathway (Honey *et al*., 2000b). When all the above experimental results are taken together, it seems likely that in all tolerance inducing strategies some cell clones may remain fully committed towards an aggressive phenotype and their physical (AICD) or functional (anergy) deletion is required if tolerance induction is to be successful.

Information is lacking on whether therapeutic protocols targeting deletion of alloreactive clones, such as those based on macrochimerism (Wekerle *et al*., 2000), also encourage the emergence of regulatory cells. In this respect, it is important to note that when a small dose of T cell depleted bone marrow (<4

x 10^5 cells) was administered to mice, under the cover of non-depleting anti-CD4 mAbs, it led to dominant tolerance, mediated by $CD4^+$ T cells (Bemelman *et al*., 1998). However, when the dose of bone marrow is increased (>4 x 10^5 cells), the outcome is tolerance by deletion of alloreactive clones (Bemelman *et al*., 1998). High dose bone marrow mediated deletion of alloreactive clones was shown to be Fas independent (Honey *et al*., 2000a). Even a high dose of bone marrow should be equivalent to a low dose for sparse antigens. I would predict a low frequency of regulatory $CD4^+$ T cells to these residual antigens. In another study, the role of Fas/FasL in transplantation tolerance induced by co-stimulation blockade (using a combination of anti-CD40L mAb and human-CTLA4-Ig fusion protein), a similar result was observed (Trambley *et al*., 2001). It was shown that tolerance is independent of Fas/FasL signalling, as graft survival in tolerised *lpr* mice (deficient in Fas) or *gld* mice (deficient in FasL) is not compromised.

1.2.4 Tolerance and T cell homeostasis

The number of T cells in a normal adult immune system is largely stable, in spite of constant input of newly formed cells from the thymus, clonal expansion of peripheral cells, and cell death. The control of cell numbers in the immune system is one of the least known aspects of the whole immunology (reviewed in Freitas and Rocha, 2000).

It has been suggested that homeostatic control of T cell numbers is a consequence of competition for limited resources as yet to be identified (Freitas and Rocha, 2000). Supporting this hypothesis it was shown that different populations of $CD8^+$ TCR transgenic T cells compete with each other and with non-transgenic $CD8^+$ T cells in bone marrow chimeras, parabiosis, and adoptive transfers into T cell deficient mice (Freitas *et al*.,

1996). In these experiments the steady state size of each population was dependent on the presence of other populations and on the time the different populations started to co-exist. A similar competition effect had been previously reported for different transgenic B cell populations (Freitas *et al*., 1995). Interestingly, different types of lymphocytes have been shown to behave independently, i.e. without competing with each other, suggesting the existence of different types of "niches" that are occupied by different types of lymphocytes. This is the case of B and T cells: B cell deficient mice have normal T cell numbers (Kitamura *et al*., 1991), and mice without T cells have a normal number of B lymphocytes (Mombaerts *et al*., 1992). It is therefore likely that B and T cells exploit different resources. Also $\alpha\beta$ and $\gamma\delta$ T cells seem to be independently regulated: in the absence of one type of these populations the other is not expanded (Mombaerts *et al*., 1991). However, $CD4^+$ and $CD8^+$ T cells are likely to exploit the same resources of common "niches" being co-regulated. In the absence of any of the two T cell subsets, the other expand leading to a normal number of $\alpha\beta$ T cells (Rocha *et al*., 1989; Zijlstra *et al*., 1990; Rahemtulla *et al*., 1991; Cosgrove *et al*., 1991).

Interestingly naïve and memory/activated T cells seem to have independent homeostatic control (Tanchot and Rocha, 1995; Tanchot *et al*., 1997). Mice manipulated in order to contain only naïve $CD8^+$ T cells, in spite of the available space, only have half of the normal number of $CD8^+$ T cells, and vice-versa (Tanchot and Rocha, 1995). In addition, the continuous thymus output of naïve T cells do not replace resident memory T cells (Tanchot *et al*., 1997).

It has been known that when T cells are adoptively transferred into lymphopenic hosts they undergo homeostatic driven proliferation (Bell *et al*., 1987; Rocha *et al*., 1989). Such proliferation is largely antigen non-specific,

although some low-affinity TCR interactions may be required as suggested by dependence on MHC expression by the host. In fact, the peripheral T cell survival and homeostatic proliferation can be driven by the same peptide ligands that mediate positive selection in the thymus (Ernst *et al*., 1999; Goldrath and Bevan, 1999). Given the dependency of homeostatic expansion on self-antigens it is perhaps not surprising that many animal models of autoimmune diseases are lymphopenic. It may be that homeostatic proliferation is a major factor determining susceptibility to autoimmunity (Theofilopoulos *et al*., 2001).

Recent studies have suggested a role for $CD4^+$ regulatory T cells in controlling T cell homeostasis (Annacker *et al*., 2000; Annacker *et al*., 2001b). It has been shown that both $CD4^+CD25^-$ and a fraction of the $CD4^+CD25^+$ T cells do expand when injected into $RAG2^{-/-}$ mice (Annacker *et al*., 2001b). Such *in vivo* results contrast with the poor proliferative capacity of $CD4^+CD25^+$ cells *in vitro* (Takahashi *et al*., 1998; Thornton and Shevach, 1998). But in *in vivo* experiments only ~10% of the injected $CD4^+CD25^+$ T cells underwent proliferation, involving 10 – 11 rounds of division, such that the progeny contributed to more than 99% of the cell pool once a steady state was reached (Annacker *et al*., 2001b). Nonetheless, in co-transfer experiments $CD4^+CD25^+$ T cells were shown to be capable of controlling the expansion of $CD4^+CD45RB^{high}$ T cells (Annacker *et al*., 2001b).

Most *in vivo* studies of T cell suppression in autoimmunity use lymphopenic mice where T cell populations (such as the $CD4^+CD45RB^{high}$ T cells) cause disease upon transfer. Preliminary evidence suggests that in some of these experimental systems disease is prevented by abrogating homeostatic expansion of the putative aggressive $CD4^+CD45RB^{high}$ T cells, for example by adoptive transfer of a larger number of such cells (Barthlott *et al*., 2002). It

will therefore be important to develop *in vivo* assays for regulatory T cell function in the absence of homeostatic proliferation. In the case of transplantation tolerance it has been demonstrated conclusively that regulation of alloreactive cells is independent of their proliferation. Lin *et al*, in the host laboratory, have shown that TCR transgenic $CD8^+$ T cells when injected into tolerised or naïve mice proliferate to the same extent (Lin *et al*., 2002). However, only the cells injected into the tolerised mice are "disarmed", failing to produce IFN-γ, generate CTL and reject grafts (Lin *et al*., 2002).

1.3 Experimental models of transplantation

In the work described in this thesis, I focus on mechanisms of transplantation tolerance in mice transplanted with skin grafts.

Studies of transplantation offer important advantages when compared with other experimental systems of adult immune tolerance, such as animal models of autoimmune diseases. One of the main advantages is the capacity to control the time when the immune system encounters the antigen, as well as the type of antigen provided. Furthermore, the identity of the antigens involved in transplant rejection is generally better characterised than the identity of antigens that initiate a destructive autoimmune process. It is thus likely that experimental transplantation tolerance offers a better opportunity to study antigen specificity of the cells inducing and maintaining the tolerant state.

Transplant rejection or tolerance does not depend exclusively on the degree of mismatch between the host and the donor. Different strains of mice have a distinct behaviour concerning the capacity to reject or become tolerant to

transplants (Davies *et al*., 1997; Trambley *et al*., 2000). The genetic basis of strain variability is still unknown.

The capacity to accept a transplant also depends on the graft itself. Vascularised grafts are, in general, more easily accepted than non-vascularised ones such as skin. Also, different organs have been shown to have different requirements for being tolerised (Zhang *et al*., 1996; Jones *et al*., 2001; Trambley *et al*., 2000). Liver acceptance is relatively easy to achieve, with many liver allografts being spontaneously accepted without any treatment in permissive strains (Qian *et al*., 1994). Kidney allografts are also occasionally spontaneously accepted in rodents, although not as consistently as liver (Zhang *et al*., 1996). In contrast, pancreatic islets and heart allografts are usually rejected in the absence of therapeutic intervention. However, skin grafts are even more difficult to tolerise as some treatments capable of preventing rejection of heart allografts are ineffective in inducing long term survival of skin allografts (Pearson *et al*., 1993; Pearson *et al*., 1994; Cavazzana-Calvo *et al*., 1995; Isobe *et al*., 1996).

In the experiments described in this thesis I have used skin transplants with different mismatches as they allow regular observation for rejection, and being the most difficult organ to tolerise constitute a stringent experimental system.

1.4 Mechanisms of transplant rejection

Transplant rejection is absolutely dependent on T cells. This can be shown experimentally as nude mice do not reject skin allografts until they are reconstituted with immunocompetent T cells (Rosenberg *et al*., 1987). The

rejection process is initiated by T cell activation by non-self transplantation antigens (Rosenberg and Singer, 1992).

Transplantation antigens have been classically divided in two groups: the major and the minor alloantigens. The major transplantation antigens consist of MHC class I and class II molecules, while minor antigens are all other allo-peptides (Sherman and Chattopadhyay, 1993). Host T cells can recognise alloantigens through two different pathways: the direct pathway, consisting of direct interaction of host T cells with allo-MHC molecules on the surface of donor cells; and the indirect pathway, that involves the recognition of peptides derived from donor major and minor transplantation antigens processed and presented on host MHC class II molecules on the surface of host APCs (Gould and Auchincloss, 1999).

In transplantation, as in other immune responses, activation of naïve T cells require presentation by DCs that are capable of providing costimulatory signals (Fairchild and Waldmann, 2000). The role of DCs in promoting graft rejection was initially established concerning direct presentation, as thyroid allografts depleted of APCs were accepted permanently (Lafferty *et al*., 1975; Lafferty *et al*., 1976). Similar results were obtained following depletion of APCs from pancreatic islet allografts (Faustman *et al*., 1984). Furthermore, when the number of DCs in cardiac grafts was increased by prior treatment with Flt3-ligand, graft rejection was accelerated (Steptoe *et al*., 1997).

Given the evidence for a crucial role of the direct pathway in transplant rejection, $CD8^+$ T cells were long considered to be the critical effector cells, often requiring $CD4^+$ T cell help (Rosenberg and Singer, 1992). However, *in vivo* use of depleting anti-CD4 and anti-CD8 mAbs demonstrated that both $CD4^+$ and $CD8^+$ T cells could contribute to graft rejection, and reject grafts

independently (Cobbold *et al*., 1986). This finding suggested that any protocol for inducing transplantation tolerance would need to target both major T cell populations.

It was also assumed that while $CD4^+$ T cells participated in the rejection of MHC class II mismatched grafts, $CD8^+$ cells were the main contributors for rejection of MHC class I mismatched allografts. Such a conclusion was derived from studies of mice mutated for expression of MHC class I or II molecules. Using these mutants as bone marrow donors, it was observed that the injected cells were rejected by $CD4^+$ or $CD8^+$ cells depending on whether they were respectively expressing MHC class II or class I (Korngold and Sprent, 1985). However, subsequent studies have proven that in some cases rejection of MHC class I mismatched allografts was dependent on $CD4^+$ T cells (Morton *et al*., 1993; Wise *et al*., 1999).

As described previously, during the course of an immune response, $CD4^+$ T cells can differentiate along, at least, two different pathways: the Th1 and Th2. Th1 cells express IFN-γ and IL-2 that, in the context of graft rejection, can lead to clonal expansion and activation of $CD8^+$ T cells, but also to recruitment and activation of macrophages driving a delayed type hypersensitivity allo-reaction (Valujskikh *et al*., 1998). Th1 cells may also become directly cytotoxic by the expression of FasL on their surface (Rosenberg and Singer, 1992; Kagi *et al*., 1996; Le Moine *et al*., 2002).

Th2 cells were also shown to be capable of mediating graft rejection (Zelenika *et al*., 1998). Although Th2 cells do not become directly cytotoxic by expressing FasL (Matesic *et al*., 1998), they can recruit and activate eosinophils in a process mediated by IL-5, which can lead to graft rejection (Le Moine *et al*., 1999a; Le Moine *et al*., 1999b; Goldman *et al*., 2001).

Another pathway through which $CD4^+$ T cells can lead to graft rejection is by providing help to allo-specific B cell clones, driving the production of alloantibodies (Baldwin *et al*., 2001). This type of immune response is dependent on the indirect presentation pathway (Pettigrew *et al*., 1998). When alloantibodies bind to transplanted cells they can lead to cell killing through different mechanisms, such as activation of the complement cascade, antibody-dependent cell cytotoxicity (ADCC) mediated by natural killer (NK) cells, and activation mediated by cross-linking of Fc receptors on graft infiltrating macrophages, neutrophils and eosinophils (reviewed in (Baldwin *et al*., 2001). The role of alloantibodies can be demonstrated by graft rejection in nude rats injected with allospecific hyperimmune serum (Gracie *et al*., 1990). It has also been shown that a prolongation of cardiac allograft survival can be achieved when specific B cell tolerance is induced following CsA plus donor-specific blood transfusion pretreatment (Yang *et al*., 2000).

In summary, due to the many redundant mechanisms that lead to transplant rejection, a protocol for the therapeutic induction of transplantation tolerance must be aimed at an upstream target (or targets) on which the other components are dependent. In this respect, tolerance induction to fully mismatched skin grafts (described in Chapter 4) is particularly relevant, as an effective control of all rejection pathways is required.

1.5 Therapeutic induction of transplantation tolerance: How can the present knowledge translate to the clinic?

Current immunosuppressive agents, although the best option available, are far from being ideal drugs. However, their known efficacy in preventing acute allograft rejection makes it ethically difficult to displace them in clinical trials of

potential tolerogenic drugs. CsA is known to hinder tolerance induction with therapeutic mAbs (Li *et al*., 1999). Is it wise though to give transplanted patients an experimental therapeutic regime in the absence of CsA?

One reason why CsA exerts a tolerance-blocking effect is due to its capacity to interfere with AICD (Li *et al*., 1999). In fact, both CsA and sirolimus (FK506) are calcineurin inhibitors that block transcriptional activation of the IL-2 gene in response to antigen stimulation. As lymphocytes are prevented from being activated, AICD does not occur. In that respect the new immunosuppressive drug rapamycin might be a good alternative. It does not interfere with activation and AICD. It rather functions by arresting the cell cycle, rendering lymphocytes insensitive to proliferative signals. Therefore, although CsA prevents tolerance induction with anti-CD40L antibodies, rapamycin does not affect, and can even facilitate, tolerance induction in this experimental system (Li *et al*., 1999). One can predict that also anti-CD4 tolerance induction might be achieved in spite of concomitant administration of rapamycin, if given at the appropriate dose and time.

In any case, development of diagnostic tests for tolerance is required to translate experimental results into clinical practice. Currently, abrogation of tolerance is not diagnosed until the tissue is damaged, both in autoimmunity and transplantation. Safe clinical trials of experimental tolerogenic regimens will be greatly facilitated when tolerance can be monitored *in vitro*, allowing the use of conventional immunosuppressive drugs as soon as there is evidence of tolerance failure. In this respect, the study of cellular and molecular characteristics of therapeutic induced tolerance might identify cell populations, or gene transcripts, whose presence or absence correlates with the maintenance of tolerance.

1.6 Aims of the thesis

The present thesis describes studies of therapeutic transplantation tolerance induced with mAbs. As immunogenicity of therapeutic monoclonal antibodies, such as some of the ones used experimentally, may induce artefacts, a strategy to overcome this problem was also studied.

1. Anti-CD154 (CD40 ligand) therapeutic mAbs have been shown to be effective in the induction of transplantation tolerance. Chapter 3 describes a study to confirm that $CD4^+$ regulatory T cells are induced following anti-CD154 tolerance induction, and lead to a state of dominant tolerance where infectious tolerance can be demonstrated.

2. As a consequence of the findings implicating $CD4^+$ regulatory T cells in transplantation tolerance induced with non-depleting mAbs targeting co-receptors (such as CD4 and CD8) or co-stimulatory molecules (as CD154), it was investigated whether a combination of both types of mAbs leads to more robust transplantation tolerance. The results are described in Chapter 4.

3. $CD4^+CD25^+$ T cells have been implicated as the main regulatory T cells involved in the maintenance of self-tolerance. Chapter 5 describes experiments establishing that regulatory T cells mediating dominant transplantation tolerance are contained in both the $CD4^+CD25^+$ and $CD4^+CD25^-$ populations, although the two populations are distinct in terms of gene expression. It is also described that $CD4^+CD25^+$ T cells with the capacity to prevent

allograft rejection by non-tolerant cells can be isolated from naïve mice.

4. It has been known, and confirmed in experiments described in Chapter 5, that regulatory T cells that mediate transplantation tolerance can be isolated from the spleen of tolerised animals. In Chapter 6 a group of experiments demonstrates that regulatory T cells with the capacity to maintain dominant transplantation tolerance are present in tolerated skin allografts.

5. Therapeutic mAbs, such as the CAMPATH-1H, are commonly used in the treatment of human pathology. As foreign proteins with the ability to bind to cells of the body, mAbs elicit immune responses leading to a reduction of their efficiency unless immunosuppressive drugs are co-administered. Such antiglobulin responses may also limit the usefulness of mAb in some experimental systems where a long-term effect or repeated administration of the mAb is desired. It has been shown that mAbs with mutations that prevent their cell binding activity induce tolerance to their cell binding form (Gilliland *et al*., 1999). Chapter 7 describes the characteristics of a mAb that was constructed with its binding site occupied by an epitope-like peptide (mimotope). Such mAb has the capacity to induce tolerance to itself as well as to the wild type mAb. Furthermore, it has the capacity to spontaneously acquire cell-binding capacity and mediate its biological effect, without hindrance to the development of tolerance. Such mAb constructs, by having a biological effect without eliciting anti-globulin responses are useful for animal studies, such as some experiments described in Chapter 5.

CHAPTER 2

MATERIALS AND METHODS

2.1 Experimental animals

All mice were bred in a specific pathogen free (SPF) facility at the Sir William Dunn School of Pathology, Oxford, U.K. Age and sex matched groups of animals were used in experiments, unless otherwise stated. Animals from strains listed in Table 2.1 were used.

Mouse Strain	Haplotype	References
CBA/Ca	H-2^k + CBA "minors"	
BALB/c	H-2^d + BALB "minors"	
C57/BL10	H-2^b + Black 10 "minors"	
B10.BR	H-2^k + Black 10 "minors"	
CP1-CBA/Ca	H-2^k + CBA "minors" Transgenic for human-CD52 expressed on T cells	(Gilliland *et al*., 1999)
RAG1 $^{-/-}$-CBA/Ca	H-2^k + CBA "minors" + RAG1$^{-/-}$	(Zelenika *et al*., 1998)

Table 2.1 Strains of mice used in experiments described in the thesis

2.2 *In vivo* Techniques

All procedures were carried out in accordance with the UK. Home Office Animals (Scientific Procedures) Act of 1986.

2.2.1 General anaesthesia

Mice were anaesthetised using a cocktail of 10 mg/ml metomidate (Hypnodil, Janssen Pharmaceutical, Tilburg, The Netherlands) and 2 μg/ml fentanyl (Sublimaze, Janssen Pharmaceutical, Tilburg, The Netherlands), diluted in sterile water. Anaesthetic was injected i.p., 0.12 ml per 20 g body weight.

2.2.2 Skin grafting

Skin grafts were performed according to a modified technique of Billingham *et al* (Billingham *et al*., 1953). Briefly, full thickness tail skin (1 cm x 1 cm) was transplanted onto a vascularised graft bed on the lateral thoracic flank. Grafts were placed with the direction of hair growth opposite to that of the recipient. The grafts were covered with paraffin gauze (Smith and Nephrew, UK), which was kept in place by wrapping the mice in cotton gauze (Johnson and Johnson, USA) and then plaster of Paris (Gypsona, Smith and Nephrew, UK.). The casts were secured to the scruff of the neck with autoclips (Becton Dickinson,UK.). Casts were removed 8 days after skin grafting. Skin grafts were considered rejected when no viable donor tissue was visible.

2.2.3 Transfer of tolerated skin grafts

Tolerated skin allografts were collected 100 to 120 days following transplant and tolerisation protocol. They were washed in phosphate sulphate buffered saline (PBS; Oxoid Ltd., U.K. Cat. No.: BR14) and grafted, as described above, on the lateral flank of new hosts.

2.2.3 Thymectomy

Mice were thymectomised between 4 and 6 weeks of age according to a modified technique of Monaco *et al* (Monaco *et al*., 1966). Briefly, a longitudinal incision was made in the ventral surface of the neck and the pre-tracheal fascia exposed. A glass tube was inserted through the pre-tracheal fascia into the anterior mediastinum. The thymus was removed as two intact lobes by the application of negative pressure through the glass tip. The incision was closed using VetBond^{TM} (3M, Minneapolis, USA).

2.2.4 Lymphocyte depletion *in vivo*

In vivo $CD8^+$ lymphocyte depletion was achieved by intraperitoneal co-injection of 1 mg of each of the lytic anti-CD8 mAbs, YTS 156.7 and YTS 169.4 (see Table 2.2). $CD4^+$ lymphocyte depletion was achieved by intraperitoneal co-injection of 1mg of each of the lytic anti-CD4 mAbs, YTA 3.1.2 and YTS 191.2 (see Table 2.2). Depletion of the total lymphocyte population was achieved by intraperitoneal injection of a cocktail of 1 mg of each of the lytic anti-CD8 mAbs, plus the anti-CD4 mAbs, as above. Injections were carried out 3 and 2 days prior to using the mice in the indicated experiments. T cells from CP1-CBA transgenic mice were depleted using an intraperitoneal injection of 0.25 mg of anti-human CD52 mAb CAMPATH-1H.

2.2.5 Induction of transplantation tolerance

Tolerance was induced in CBA/Ca or CP1-CBA/Ca mice by treatment with 1 mg YTS177.9 and 1 mg YTS105.18 at days 0, 2, and 4 after B10.BR skin transplantation (Chapters 5 and 6). Alternatively with 1 mg MR1 at days 0, 2,

and 4 following B10.BR skin transplantation (Chapter 3). Or with a combination of the three mAbs administered at the same days following C57/B10 skin transplantation (Chapter 4).

2.2.6 Adoptive transfer of splenocytes

Spleens were dissected from donor mice and macerated through a 70 μm filter (Becton Dickinson,UK. Cat. No.: 352350) in R10 medium (see section 2.4.1). Cells were centrifuged at 1300 rpm (Sorvall RT 6000D) for 10 minutes at room temperature; these conditions were standard when washing cells. Erythrocytes were lysed by 5 seconds incubation with 0.9 ml sterile water followed by addition of 100 μl PBS 10x concentrated, then washed in PBS, 1% w/v BSA and passed through another 70 μm filter into a new tube to remove erythrocyte ghosts. Cells were counted in Trypan Blue (Sigma) using a haemocytometer. Cells were re-suspended in PBS at the desired cell density and 0.2 ml cell suspension was injected into the lateral tail vein of mice heated using an infra-red lamp (IMS, UK) to allow vasodilatation.

2.2.7 Statistics

Significant differences in skin graft survival were analysed using the non-parametric Mann-Whitney U test and the log rank method by computer software Prism version 3.02 (GraphPad Software Inc.).

2.3 Monoclonal Antibodies

The mAbs used in this thesis are listed in Table 2.2. Constructs based on CAMPATH-1H linked to its mimotope are described in detail in Chapter 7.

Name	Isotype	Specificity	Reference
YTS 177.9.6.1	Rat IgG2a	Mouse CD4	(Qin *et al*., 1990)
YTS 105.18.10	Rat IgG2a	Mouse CD8	(Qin *et al*., 1990)
YTA 3.1.2	Rat IgG2b	Mouse CD4	(Qin *et al*., 1987)
YTS 191.2	Rat IgG2b	Mouse CD4	(Cobbold *et al*., 1984)
YTS 156.7.7	Rat IgG2b	Mouse CD8	(Cobbold *et al*., 1984)
YTS 169.4.2	Rat IgG2b	Mouse CD8	(Cobbold *et al*., 1986)
11B11	Rat IgG1	Mouse IL-4	(Ohara and Paul, 1985)
JES5.2A5	Rat IgG1	Mouse IL-10	(Sander *et al*., 1993)
PC61	Rat IgG1	Mouse CD25	(Lowenthal *et al*., 1985)
CAMPATH-1H	Human IgG1	Human CD52	(Riechmann *et al*., 1988)
CAMPATH-1G	Rat IgG2b	Human CD52	(Dyer *et al*., 1989)
187.1	Rat IgG1	Mouse Ig kappa	(Yelton *et al*., 1981)
M5/114	Rat IgG2b	Mouse MHC II ($I\text{-}A^{b,d,q}$,$I\text{-}E^{d,k}$)	(Bhattacharya *et al*., 1981)
MR1	AH IgG3	Mouse CD154	(Noelle *et al*., 1992)
YCATE55	Rat IgG1	Canine CD8	(Cobbold and Metcalfe, 1994)
KT3	Rat IgG2a	Mouse CD3	(Tomonari, 1988)
4F10	AH IgG1	Mouse CTLA-4	(Walunas *et al*., 1994)
YNB46-HG1	Human IgG1	Human CD4	(Isaacs *et al*., 1997)

Table 2.2 mAbs used in this thesis. AH, Armenian hamster.

2.3.1 Preparation of monoclonal antibodies

mAbs were produced by culture in hollow-fibre bioreactors designed for generating high yields of concentrated antibodies (see website: http://www.molbiol.ox.ac.uk/pathology/tig/mprod.html). Briefly, cells were grown on semi-permeable hollow-fibres to allow free exchange of metabolites, but not proteins, with the medium being pumped through the fibres. The mAbs containing supernatants was harvested and further processed as described in Sections 2.3.1.1 and 2.3.1.2.

2.3.1.1 Purification of monoclonal antibodies by ammonium sulphate precipitation

Ammonium sulphate precipitation was carried out according to a standard protocol for mAbs purification (Cooling *et al*., 1994). Ammonium sulphate was added to the culture supernatant to make a 50% w/v saturated solution. This was stirred continuously overnight at 4 ºC, centrifuged, and the supernatant discarded. The pellet was re-suspended in a minimal amount of distilled water (~ 10% starting volume) and dialysed against PBS. The protein concentration was determined by measuring the optical density at 280 nm. The purity of the preparation was assessed by native gel eletrophoresis (PhastGel, Pharmacia, St. Albans, U.K. Cat. No.: 17-0517-01) according to the manufacturer's instructions. The mAb preparations were aliquoted and stored at –30 ºC.

2.3.1.2 Ion Exchange Purification

2.3.1.2-A Reagents

10x stock of buffer A:

Malonic Acid	104 g
NaOH	60 g
Betaine	20 g

Make up to 2 litres with endotoxin free water.

pH 5.2 - pH 5.4 at 25 ºC

Sterile filter to 0.2 μm and store at 4 ºC

Buffer B (1x):

Make 1x buffer A, but containing 0.5M NaCl

2.3.1.2-B Procedure

Antibody preparation was dialyzed into 1x Malonate buffer (buffer A). Any precipitate was spun out and discarded. Supernatant was measured for OD_{280} and adjusted to between 1 and 20 mg/ml buffer A. Fast Flow SP-Sepharose (Pharmacia Biotech, Sweden, Cat. No.: 17-0729-01) was washed three times (by centrifugation for 2 mins at 2000 rpm) in buffer A. The antibody previously dialysed into buffer A was incubated with the correct volume of SP-Sepharose (1 ml gel for 20 mg antibody) for 1 hour at room temperature, with gentle rotation. The supernatant was then removed (and checked to establish whether the antibody had been adsorbed by running analytical gel). The gel was washed three times with buffer A (each wash at least 5 gel volumes). The antibody was eluted by adding 1 gel volume of buffer B, rotated at room temperature for 5 minutes, centrifuged, and the supernatant collected. The process was repeated and the eluted volumes pooled. The protein concentration and purity was checked as stated above (section 2.3.1.1).

2.3.1.3 Biotinylation of monoclonal antibodies

mAb purified using ammonium sulphate precipitation (Section 2.3.1.1) or ion exchange purification (Section 2.3.1.2) was dialysed against sodium bicarbonate buffer, pH 9.3 (8.65 g anhydrous $NaHCO_3$ + 8.6 g Na_2CO_3 per litre of distilled water). Fresh Sulfo-NHS-LC-Biotin (Pierce, USA, Cat. No.: 21335), 1 mg/ml in water, was added to a final concentration of 37 μg per mg of mAb and incubated on ice for 2 hours. The biotinylated mAb was dialysed against PBS overnight to remove excess biotin. Bovine serum albumin (BSA; Sigma, UK; Cat. No.: A-9647) was added to 1% w/v, and sodium azide to 0.1% w/v. Aliquots were stored at 4 ºC and –80 ºC.

2.3.1.4 Fluorescin isothiocyanate conjugation of monoclonal antibodies

mAb purified using ammonium sulphate precipitation (Section 2.3.1.1) and ion exchange purification (Section 2.3.1.2) was dialysed into 0.1M sodium bicarbonate buffer and the concentration adjusted to 5 mg/ml. Fresh fluorescein isothiocyanate (FITC; Sigma, UK; Cat. No.: F7250), 1 mg/ml in dimethylsulphoxide (DMSO; Sigma, UK; Cat. No.: D5879), was added to a final concentration of 40 μg per mg of mAb and incubated at room temperature for 2 hours with occasional mixing. The FITC-conjugate was dialysed against PBS overnight. The protein concentration in mg/ml was determined using: $[OD_{280}nm - (0.36 \times OD_{493}nm)]/1.4$. The molar FITC/protein ratio determined using a nomogram (Wells *et al.*, 1966) with 3-5 being preferred. BSA was added to a final concentration of 1% w/v and sodium azide to 0.1% w/v. Aliquots were stored at 4 ºC and –80 ºC.

2.4 Tissue Culture

2.4.1 Media and buffers

R10 RPMI 1640 (Life Technologies, UK. Cat. No.: 21875-034)
10% v/v Foetal Calf Serum (FCS) (Life Technologies, UK. Cat. No.: 192-1005PJ)
50 μg/ml Penicillin/Streptomycin (Life Technologies, UK. Cat. No.: 15070-063)
0.01M Hepes Buffer (Life Technologies, UK. Cat. No.: 15630-056)
5×10^{-5}M 2-Mercaptoethanol (BDH, UK. Cat. No.: 436022A)

IMDM Hepes-free Iscove's Modified DMEM (Life Technologies, U.K. Cat. No.: 041-91344P)

7 mg/L adenosine

7 mg/L cytidine

7 mg/L guanidine

7 mg/L uridine

2.4 mg/L thymidine

357 mg/L L-glutamic acid

325 mg/L L-asparagine (free base)

5660 mg/L NaCl

IMDM 5% IMDM

2mM L-glutamine (Life Technologies, U.K. Cat. No.:25030-024)

5% v/v FCS (Life Technologies, U.K. Cat. No.:192-1005PJ)

50 μg/ml Penicillin/Streptomycin (Life Technologies, UK. Cat. No.:15070-063)

0.01M Hepes Buffer (Life Technologies, UK. Cat. No.: 15630-056)

5 x 10^{-5}M 2-Mercaptoethanol (BDH, UK. Cat. No.:436022A)

2.4.2 Preparation of mouse T cell blasts by concavalin A activation

A single cell suspension of murine splenocytes was prepared by macerating a spleen through a 70 μm filter. The cells were washed in R10, re-suspended in 20 ml R10 containing 2.5 μg/ml concavalin A (Con A) and incubated for 48 hours at 37 ºC and 5% v/v CO_2 prior to harvesting.

2.4.3 T cell activation with immobilized anti-CD3

24-well plates (Corning, New York, USA) were pre-coated with 1 ml per well of 1 μg/ml KT3 in PBS for 1 hour at 37 ºC. A single cell suspension of T cells was counted in trypan blue, and 10^6 cells were transferred into each well in 1 ml R10. The cells were incubated with the immobilized KT3 overnight at 37 ºC (typically 16 hours).

2.5 Cell separation by magnetic sorting (MACS™)

2.5.1 Solutions for magnetic sorting

MACS buffer: PBS supplemented with 0.5 % w/v BSA and 2mM EDTA. pH 7.2; Rinsing solution: PBS, 2mM EDTA.

2.5.2 $CD4^+$ T cell sorting

Cells were obtained from spleens of adult CBA/Ca mice. A single-cell suspension was obtained by passing the splenocytes through a 70 μm cell strainer (BD Biosciences, Oxford, U.K) and the erythrocytes were depleted by water lysis. Cells were pelleted a further time and re-suspended at $1x10^7$ cells per 90 μl in MACS buffer and 10 μl of MACS™ CD4 (L3T4) Microbeads (Miltenyi Biotec, Germany; Cat. No.:492-01). The cells were incubated with the microbeads for 15 minutes on ice. Cells were then washed once with 10 ml MACS buffer and re-suspended at 10^8 cells/ml. Magnetic sorting by autoMACS™ (Miltenyi Biotec, Germany; Cat. No.:201-01) was performed according to the manufacturer's manual using POSSEL program.

2.5.3 $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell separation

Single cell suspension from adult CBA/Ca spleens were first enriched for $CD4^+$ cells by negative selection of $CD8^+$ cells, MHC class II^+ cells, and B cells by incubation with the mAbs M5/114, 187.1, and YTS 156.7, and subsequent incubation with 1ml goat-anti-rat IgG Dynabeads (Dynal Biotech, Oslo, Norway; Cat. No.: M450) for $3x10^8$ cells and magnetic removal. For separation of $CD4^+CD25^+$ T cells, the CD8, class II, and B cell-depleted single-cell suspension was incubated 45 min at 4°C with anti-CD25 (7D4) biotin 1:100 in PBS, 1% w/v BSA, 5% v/v HINRS, and 0.1% w/v sodium azide. Following washing, the cells were incubated 15 min at 4°C with 2 μl streptavidin-microbeads (Miltenyi Biotech) per 10^7 cells, and positively selected over two columns using autoMACS POSSELD program. $CD4^+CD25^-$ cells were sorted from the negative fraction obtained following $CD4^+CD25^+$ cell separation, by incubation with 100 μl streptavidin-microbeads per 10^7 cells, and subsequent negative selection of any remaining $CD25^+$ cells using autoMACS POSSELS program. Finally, $CD4^+CD25^-$ were obtained following a positive selection step with anti-CD4 microbeads as described above. All sorted fractions were labeled with CD8 (YTS156.7)-FITC, CD25 (PC61)-PE, and CD4 (H129.19)-CyCr; and assessed in a flow cytometer. Typical purity of $CD4^+$ and $CD4^+CD25^-$ T cells was >98%, and purity of $CD4^+CD25^+$ T cells >90%.

2.6 Flow cytometric analysis (FACS)

2.6.1 Conjugated antibodies used in this thesis

All commercial conjugated antibodies used were from BD Pharmingen, San Diego, CA, USA, except for CAMPATH-1H phycoerythrin (PE) conjugated

(from Serotec, Oxford, UK). They are represented in Table 2.3. In addition to these mAbs, home grown mAbs conjugated with biotin or FITC were used (see Table 2.2). Streptavidin-PE (Sigma, UK, Cat. No. S3402) and streptavidin-allophycocyanin (ST-APC; BD Pharmingen, Cat. No. 13049A) were used as secondary reagents.

Antigen	Fluorochrome	Clone	Isotype	Cat. No.
CD3ε	APC	2C11	AH IgG1	01089A
CD4	PE	H129.19	Rat IgG2a	01065A
CD4	CyCr	H129.19	Rat IgG2a	01068A
CD4	PerCP	H129.19	Rat IgG2a	0106PA
CD8	PE	53-6.7	Rat IgG2a	01045A
CD8	CyCr	53-6.7	Rat IgG2a	01048A
CD8	PerCP	53-6.7	Rat IgG2a	0104PA
CD25	PE	PC61	Rat IgG1	01105A
CD25	Biotin	7D4	Rat IgM	01092D
CD44	PE	IM7	Rat IgG2b	01225A
CD45RB	PE	16A	Rat IgG2a	01145A
hCD52	PE	CAMPATH-1	Rat IgG2b	MCA1642PE
CD152	PE	4F10	AH IgG1	09385A

Table 2.3 Commercially available conjugated antibodies used in this thesis. AH, Armenian hamster; CyCr, Cy-Chrome; PerCP, Peridinin chlorophyll-a protein.

2.6.2 Four-colour cytometry (surface staining)

Cells stained during this thesis were splenocytes and peripheral blood lymphocytes (PBL). Red blood cells were removed from splenocytes and PBL samples by water lysis (900 μl of water followed, 5 seconds later by 100 μl 10x PBS) at room temperature prior to staining. Samples were then washed at 4 ºC in 200 μl washing buffer (PBS, 0.1% w/v BSA, 0.1% w/v sodium azide). Cells were re-suspended, using a plate shaker, in 50 μl mAb

diluted appropriately in blocking buffer (PBS, 5% heat-inactivated normal rabbit serum (HINRS), 1% w/v BSA, 0.1%w/v sodium azide). The mAbs used were directly conjugated with fluorescent markers or with biotin. Cells were incubated in the dark at 4 ºC for 45 minutes and then washed twice in 200 μl wash buffer. If a biotinylated primary reagent was used cells were re-suspended, using a plate shaker, in 50 μl secondary reagent (ST-APC or ST-PE, see 2.6.1) diluted appropriately (usually 1:100) in wash buffer and incubated in the dark at 4^0C for a further 45 minutes. Cells stained with a biotin conjugated and a secondary reagent were washed once in 200 μl IMDM, 0.1% w/v BSA, 0.1%w/v sodium azide to wash multivalent streptavidin conjugates and once in 200 μl wash buffer. The cells were mixed in 100 μl PBS, 1% w/v BSA, 0.1% w/v sodium azide + 4% v/v formaldehyde. Fixed samples could be stored 4 ºC in the dark for a number of days. Samples were analysed using a FACScan (Becton Dickinson, UK) with dual laser (488nm and 633nm) excitation in combination with data acquisition and cross beam colour compensation using CellQuest 3.1 version software (Becton Dickinson, UK).

2.6.3 Four colour cytometry (intra-cytoplasmic staining)

Cells were stimulated for 5 hours with 50ng/ml Phorbol 12-myristate 13-acetate (PMA; Sigma, U.K.; Cat. No.: P8139) plus 500ng/ml ionomycin (Sigma, U.K.; Cat. No.: I-0634) in phenol red free-R10 at 37 ºC, with the addition of 10 μg/ml Brefeldine A (Sigma, U.K.; Cat. No.: B-7651) in the last three hours. After washing with washing buffer twice, cells were re-suspended, using a plate shaker, in 100 μl mAb diluted appropriately in blocking buffer. The mAbs used were directly conjugated with flourescent markers or with biotin (section 2.3.1). Cells were incubated in the dark at 4^0C for 30 minutes and then washed twice in 200 μl wash buffer. If a biotinylated

primary reagent was used cells were re-suspended, using a plate shaker, in 100 μl secondary reagent (ST-APC) diluted appropriately in wash buffer and incubated in the dark at 4 ºC for a further hour. Cells stained with a biotin conjugated and a secondary reagent were washed once in 200 μl IMDM, 0.1% w/v BSA, 0.1%w/v sodium azide to quench multivalent streptavidin conjugates and once in 200 μl wash buffer. The cells were mixed in 50 μl PBS, 1% w/v BSA, 0.1% w/v sodium azide and 50 μl 4% v/v formaldehyde for 15 minutes. Cells were then washed and permeabilized with PBS plus 0.5% saponin (Sigma, U.K.; Cat. No.: S-2149) for 20 minutes. Cells were pelleted and re-suspended in 100 μl saponin buffer with intracellular mAbs (section 2.7.3.1) and incubated for 30 minutes at 4 ºC. The cells were extensively washed with saponin buffer twice and followed by washing buffer once. The cells were mixed in 50 μl PBS, 1% w/v BSA, 0.1% w/v sodium azide + 50 μl 4% v/v formaldehyde. Samples were analysed by flow cytometry as stated above.

2.6.4 Detection of PC61 mAb in mouse serum

Serum PC61 was determined by inhibition of binding of anti-CD25 (PC61) PE conjugated detected by flow cytometry. $CD25^+$ Con A blasts were incubated with the experimental serum diluted 1/5 in PBS, 1% w/v BSA for 30 min at 4°C, followed by addition of anti-CD25 (PC61) PE-conjugated, as well as anti-CD4 CyCr. The experimental serum samples were compared with serum from untreated animals, and serum spiked with mAb to known concentrations. Three-colour FACSCaliber analysis was performed using CellQuest software.

2.7 Enzyme-Linked ImmunoSorbant Assays (ELISAs)

2.7.1 Analysis of CAMPATH-1H antiglobulin titres in mouse serum

For detection of anti-CAMPATH-1H antibodies, flat bottom 96 well microtitre plates were coated with CAMPATH-1H, diluted to 10 μg/ml in coating buffer (0.1M $NaHCO_3$ pH 8.6), by incubation for 1 hour at room temperature with 100 μl per well. The coated plates were then blocked with 200 μl per well blocking buffer (PBS, 1% w/v BSA, 0.1% w/v sodium azide) for 1 hour at room temperature or overnight at 4 ºC. Following removal of the blocking buffer, serum samples were added in duplicate, diluted 1:20 in wash buffer (PBS, 0.1% w/v BSA, 0.1% w/v sodium azide). In each plate a positive control (hyper-immune serum) and a negative control (normal mouse serum - NMS) were included, also in duplicate. A two fold serial dilution of each of these samples were performed. The plates were incubated for 1 hour at room temperature, with a final volume of 50 μl per well. Bound antiglobulins were detected with 50 μl per well peroxidase conjugated anti-mouse IgG (Sigma, UK, Cat. No. A-9309), diluted 1:1,000 in wash buffer. Plates were incubated for 1 hour at room temperature. Plates were washed with PBS, 0.05% v/v Tween between each step. Following the final wash, the assay was developed using 100 μl substrate buffer (1 mg/ml o-phenylenediamine dihydrochloride in 0.025M citrate, 0.05M Na_2HPO_4, 0.04% H_2O_2) per well. This reaction was stopped at an appropriate time by adding 50 μl 1M H_2SO_4 per well. The absorbance at 492 nm was read using a microplate reader (Labsystems Multiskan Plus, Labsystems, Finland) with the negative control as a blank.

2.7.2 Detection of human mAb in mouse serum

96-well plates were coated with 10 μg/ml affinity-purified goat anti-human IgG, Fcγ-specific (Jackson Laboratories, USA, Cat. No. 109-005-098) in 0.1M $NaHCO_3$, and blocked with 50 μl PBS, 1% w/v BSA, as described above. Following removal of the blocking buffer, serum samples were added in duplicate, diluted 1:20 in wash buffer, together with standards with known concentrations of human IgGγ1 and NMS as a negative control. A two fold serial dilution of each of these samples was performed. The plates were incubated for 1 hour at room temperature, with a final volume of 50 μl per well. Bound human antibodies were detected by 1-hour incubation at room temperature with 50 μl per well biotin conjugated affinity-purified goat anti-human IgG, Fcγ-specific (Jackson Laboratories, USA, Cat. No. 109-065-098), diluted 1:10,000 in wash buffer, followed by 30 minutes incubation with extravidin-peroxidase (Sigma, UK, Cat. No.: E-2886) diluted 1:1,000 in wash buffer. The assay was developed and analysed as described above.

2.7.3 *In vitro* binding to CAMPATH-1 antigen

96-well plates were coated with 10 μg/ml BHK.CAM17.5, a fusion protein with the CAMPATH-1 antigen (Gilliland *et al*., 1999), in 0.1M $NaHCO_3$, and blocked with 50 μl PBS, 1% w/v BSA, as described above. Following removal of the blocking buffer, mAb samples (CAMPATH-1H, MIM-IgG1, AG-MIM-IgG1, p61-IgG1, and anti human-CD4: negative control) were added in duplicate diluted to 50 μg/ml in wash buffer. A two fold serial dilution of each of these samples was performed. The plates were incubated for 1 hour at room temperature, with a final volume of 50 μl per well. Bound human antibodies were detected by 1-hour incubation at room temperature with 50 μl per well peroxidase conjugated affinity-purified goat anti-human IgG, Fcγ-

specific (Jackson Laboratories), diluted 1:10,000 in wash buffer, followed by 30 minutes incubation with extravidin-peroxidase (Sigma, UK) diluted 1:1,000 in wash buffer. The assay was developed and analysed as described above.

2.7.4 Detection of JES5 and 11B11 mAbs in mouse serum

96-well plates were coated with 10 μg/ml JES5 or 11B11 in 0.1M $NaHCO_3$, and blocked with 50 μl PBS, 1% w/v BSA, as described above. Serum samples from the JES5 and 11B11 treated mice, as well as normal mouse serum (negative control) and serum spiked to known concentrations of the mAbs (positive control), were diluted 1/20 in PBS, 1% w/v BSA containing either recombinant IL-10 (10 ng/ml) or recombinant IL-4 (2 ng/ml), and pre-incubated for 60 min at room temperature before being transferred into the JES5- or 11B11-coated plates, where the samples were incubated for a further 60 min. After washing with PBS, 0.05% Tween, the plates were incubated 60 min with anti-IL10 (SXC-1) biotin or anti-IL-4 (BVD6-24G2) biotin (both from BD Biosciences, UK). The plates were then incubated with extravidin-peroxidase (Sigma-Aldrich, U.K.) for 30 min, and developed with substrate buffer and absorbance at 492 nm analysed with a microplate reader (Labsystems, Finland).

2.7.5 Detection of 4F10 mAb in mouse serum

96-well plates were coated anti-hamster IgG (HIG-29) (BD Biosciences) and blocked as described above. Serum from 4F10 treated mice, as well as normal mouse serum (negative control) and serum spiked to known mAb concentrations (positive control), was diluted 1/20 in PBS 1% w/v BSA and incubated for 1 hour at room temperature in the coated plates. After washing with PBS 0.05% v/v Tween, any bound hamster mAb was detected with

biotin-conjugated HIG-29 (BD Biosciences). The plates were then incubated with extravidin-peroxidase, developed and analysed as described above.

2.8 Detection of antibody binding to CAMPATH-1 Ag by surface plasmon resonance (BIAcore™)

The experiments were performed at 25 ºC, using a BIAcore 2000 apparatus (Biacore AB, UK), and a flow rate of 10 μl/min unless stated otherwise. Proteins were covalently coupled to the carboxymethylated dextran matrix on CM5 sensor chip (Biacore AB, UK, Cat. No.: 0371) using amine coupling as suggested by the manufacturer. Before activating the surface of the sensor chip, the best pH to bind the coating Ab, at which > 10,000 response units of protein bind electrostatically, was assessed injecting 25 μg/ml goat anti-human IgG, Fcγ-specific (Jackson Laboratories) in 10mM sodium acetate at different pH (4.0, 4.5, 5.0, 5.5) over the flow cell. The best pH was found to be 5.0. The chip surface was activated by injecting 70 μl 0.05M N-ethyl-N-(dimetylaminopropyl) carbomide (EDC) and 0.2M N-hydroxisuccinimide (NHS). Then, 70 μl of the coating Ab in 10mM sodium acetate pH 5.0, was injected at the same rate. Any remaining active carboxyl groups were subsequently blocked with a 70 μl injection of 1M ethanolamine hydrochloride, pH 8.5. The coated chip was regenerated by sequential 3 minute injections of 5mM NaOH and 0.1M glycin-HCL, pH 2.5. 70 μl of BHK.CAM17.5 (the fusion protein containing CAMPATH-1 Ag) at 10 μg/ml in HBS-EP (10mM HEPES pH 7.4, 150 mM NaCl, 3mM EDTA and 0.005% v/v surfactant P20) was then injected at 5 μl/minute to confirm no background binding to the coated plate. The four flow-cells of the sensor chip were coated with 70 μl of CAMPATH-1H, MIM-IgG1, P61-IgG1 and the control anti-human CD4 mAb YNB46-HG1 (all at 25 μg/ml in HBS-EP) injected at 5 μl/minute. Subsequently, 70 μl of the ligand, BHK.CAM17.5, was injected

simultaneously in all flow cells and binding was monitored. Different concentrations of BHK.CAM17.5 (from 1 μg/ml to 800 μg/ml were tested). Between experiments the sensor chip was regenerated as described above. Following the Ab stripping, the flow cells were re-coated with the experimental Abs as described. Data was collected using BIAcore 2000 control software and analysed using BIAevaluation 3.0 (BIAcore AB).

2.9 SAGE libraries and differential analysis of gene expression

The SAGE libraries were made by Sara Thompson. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were sorted from spleens of naive CBA/Ca mice and activated with overnight incubation with solid-phase anti-CD3 mAb (KT3), as described above. T cell populations were pelleted and re-suspended in Promega SV total RNA isolation system (Promega Z3100; Promega, Madison, WI) lysis buffer (175 μl / 2 x 10^6 cells), and total RNA was isolated according to the manufacturer's instructions. First strand cDNAs were prepared from 1 μg of total RNA from each cell fraction using Superscript II (Life Technologies, Gaithersburg, MD). Reverse transcription was initiated using the anchoring primer 5'-GACTCGAGTTGACATCGAGG$(T)_{20}$V-3' with incorporation of the SMARTII oligonucleotide (Clontech, Palo Alto, CA) 5'-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3' at the 5' end. The cDNAs were pre-amplified with the forward 5'-AGTGGTAACAACGCAGAGTAC-3' and reverse 5'-GACTCGAGTTGACATCGAG-3' primers using the Advantage-GC cDNA PCR kit (Clontech) with 1M of the GC-Melt, following the manufacturer's protocol. cDNAs were subjected to 16 cycles of pre-amplification, 94°C for 30 s, 68°C for 7 min. The pre-amplification steps were monitored by PCR using various house-keeping and cytokine primers as tests. SAGE was performed using *Nla*III as the anchoring enzyme, *BsmF1* as the tagging enzyme, and *Sph*I as the cloning enzyme, as described

(Velculescu *et al*., 1995). DNA sequencing was performed using the Megabase 1000 (Molecular Dynamics, Sunnyvale, CA). Sequence analysis software SAGE 3.04 β was provided by K. W. Kinzler (Johns Hopkins Oncology Center, Baltimore, MD). A conservative estimate of the differential up-regulation of each gene within the given library, compared with a pool of other libraries, was calculated using a Bayesian statistics model (Zelenika *et al*., 2001).

CHAPTER 3

ANTI-CD40 LIGAND THERAPEUTIC ANTIBODIES INDUCE INFECTIOUS TRANSPLANTATION TOLERANCE

Non-depleting anti-CD154 (CD40 ligand) monoclonal antibodies have proven effective in inducing transplantation tolerance in rodents and primates. In the induction phase anti-CD154 antibody therapy is known to enhance apoptosis of antigen reactive T cells. This may not, however, be the sole explanation for tolerance induced in this way. I show that tolerance is maintained through a dominant regulatory mechanism which, like that elicited with anti-CD4 antibodies, manifests as "infectious tolerance". Tolerance induced with antibodies that target co-stimulatory molecules, like CD154, therefore involves not only the deletion of potentially aggressive T cells, but also a contagious spread of tolerance to new cohorts of graft-reactive T cells as they arise[1].

3.1 Introduction

The main goal of transplantation research has been to induce tolerance by a short pulse of therapy. Long-term graft survival has been achieved in rodents through the use of non-depleting monoclonal antibodies such as anti-CD4, and anti-CD154 (Qin *et al*., 1990; Parker *et al*., 1995; Larsen *et al*., 1996b; Markees *et al*., 1998; Waldmann and Cobbold, 1998; Waldmann, 1999). The potential of anti-CD154 therapy to produce prolonged graft survival even across a full MHC mismatch in non-human primates (Kirk *et al*., 1999; Kenyon *et al*., 1999) has prompted an analysis of the mechanisms involved.

[1] Most of the results presented in this Chapter were published in (Graca *et al*., 2000).

Treatment of mice with non-depleting anti-CD154 has been shown to be capable of prolonging graft survival of allogeneic hearts, but did not lead to indefinite graft survival (Larsen *et al*., 1996a). Such co-stimulation blockade resistant allograft rejection appears to be dependent on $CD8^+$ T cells, apparently not tolerised following anti-CD154 treatment (Honey *et al*., 1999), CTLA4-Ig treatment (Newell *et al*., 1999), or both in combination (Trambley *et al*., 1999). The different susceptibility to tolerance induced co-stimulation blockade shown by $CD4^+$ and $CD8^+$ T cells may simply reflect their differential expression of co-stimulatory molecules.

An additional concern with regard to the use of anti-CD154 in clinical trials has been raised following the report of thromboembolic complications following the use of one such mAb (5C8.33) in non-human primates (Kawai *et al*., 2000). Such complications were not observed in similar studies by another group (Kirk and Harlan, 2000b). CD154 has been shown to be expressed on activated platelets *in vitro*, and *in vivo* on platelets participating in thrombus formation (Henn *et al*., 1998), having a role on the stabilisation of arterial thrombi (Andre *et al*., 2002). It may be necessary to develop strategies allowing the tolerogenic action of anti-CD154 mAb without thromboembolic complications in humans.

It has been a long held assumption that activation of T cells requires two signals (Bretscher and Cohn, 1970). Signal 1 is delivered by the CD3 complex as a peptide – MHC complex is engaged by a specific TCR, in an interaction stabilised by the co-receptor molecules CD4 or CD8. Signal 2, also known as co-stimulation, is provided by the APC through CD80 and CD86 (ligands of CD28 on T cells) and CD40 (ligand of CD154). It has been postulated that both signals are required to induce T cell activation, while the delivery of signal 1 in the absence of signal 2 would result in cell death or its

functional inactivation (Bretscher and Cohn, 1970; Lafferty and Cunningham, 1975). In spite of several reports that cannot easily be explained by the two signal model of T cell activation it still remains an integral part of current immunological thought, with some authors postulating additional signals to reconcile the model with experimental results (Bretscher, 1999; Matzinger, 1999; Matzinger, 2001).

Two reports showed that activation induced cell death (AICD) of potentially aggressive T cells is an important feature of the induction phase of the prolonged graft survival with CD154 antibodies (Li *et al*., 1999; Wells *et al*., 1999). These results may be interpreted in the light of the two signal model described above, as a consequence of blocking signal 2 when signal 1 is provided, giving rise to AICD and tolerance by purging the T cell repertoire of alloreactive cells. When apoptosis was impaired, in those studies, alloreactive clones were not deleted and tolerance could not be achieved.

Previous work in our group has established that the $CD4^+$ T cell population in mice could indeed be tolerised by CD154 antibodies, in circumstances where the $CD8^+$ population had been removed by prior antibody ablation (Honey *et al*., 1999), as such treatment cannot efficiently control the behaviour of $CD8^+$ cells that express low levels of CD154 (Trambley *et al*., 1999; Honey *et al*., 1999). In such circumstances it was found that tolerised mice demonstrate "linked suppression" to third party antigens (Honey *et al*., 1999). This result cannot easily be explained without considering the emergence of $CD4^+$ regulatory T cells following the tolerising mAb treatment.

In a similar experimental system I further analysed the maintenance phase of tolerance, and uncovered a role for a contagious process of tolerance (*infectious tolerance)*. Infectious tolerance has been observed following

tolerance induction with non-depleting CD4 mAb in skin and marrow transplants over multiple-minor histocompatibility barriers, and heart transplants across complete MHC histocompatibility barriers (Waldmann and Cobbold, 1998). In this Chapter I describe how tolerance, once it has been induced by CD154 antibodies, cannot be broken by the adoptive transfer of large numbers of naïve non-tolerant T cells. When these naïve cells are allowed to coexist with the "regulatory" population for 6 weeks, they become tolerant themselves, so exhibiting "infectious tolerance". These results lead me to conclude that tolerance induced with CD154 antibodies involves not just the deletion of alloreactive T cells but also maintenance through a contagious spread of tolerance to new graft-reactive T cells as they arise.

3.2 Results

3.2.1 The experimental setting

I used CD8-depleted mice treated with CD154 antibodies to produce tolerance to B10.BR skin grafts which differ over multiple minor histocompatibility antigens. This model has proven useful in previous descriptions of infectious tolerance with CD4 antibodies, and many parameters of cell dose and potency in adoptive transfers studies have been well characterised (Davies *et al*., 1996b). Previous studies have demonstrated that regulatory cells involved in CD4-antibody induced transplantation tolerance are themselves $CD4^+$ (Qin *et al*., 1993). I wished to be able to distinguish any such $CD4^+$ regulatory T cells from naïve CD4 T cells that could reject grafts. I therefore used CBA/Ca mice transgenic for the human CD52 gene, expressed under the control of the CD2 promoter, as the tolerised host, and normal CBA/Ca mice as a source of naïve non-tolerant T cells for adoptive transfer. It was thus possible to identify and specifically

deplete host T cells using the CD52 specific CAMPATH-1H mAb (Gilliland *et al*., 1999). These transgenic mice, named CP1-CBA, are histocompatible with CBA/Ca mice, as was confirmed by acceptance of reciprocal skin grafts (Figure 3.1). Furthermore, when grafted with B10.BR skin (differing by multiple minor histocompatibility antigens), both transgenic and CBA/Ca strains rejected at a comparable rate (Figure 3.1). In order to study infectious tolerance I needed to be sure that once host T cells had been ablated by the CAMPATH-1H antibody, they would not replenish from the thymus. For that reason I used adult-thymectomised (ATx) CP1-CBA mice. Such ATx mice depleted of T cells with 0.1 mg of CAMPATH-1H mAb accept B10.BR skin grafts indefinitely (Figure 3.2A). Analysis of PBLs from CAMPATH-1H treated mice by flow cytometry confirms that T cells are depleted to less than 1% (Figure 3.2B and 3.2C). This enabled me to use the CAMPATH-1H mAb to ablate T cells of the tolerant transgenic host whenever I wished, allowing me to determine the impact of their prolonged co-existence with naïve CBA/Ca T cells.

In this study, ATx, $CD8^+$ cell depleted CP1-CBA were tolerised to B10.BR skin under the cover of three doses of the non-depleting anti-CD154 mAb MR1, administered on days 0, 2 and 4 with respect to time of transplantation as previously described (Honey *et al*., 1999).

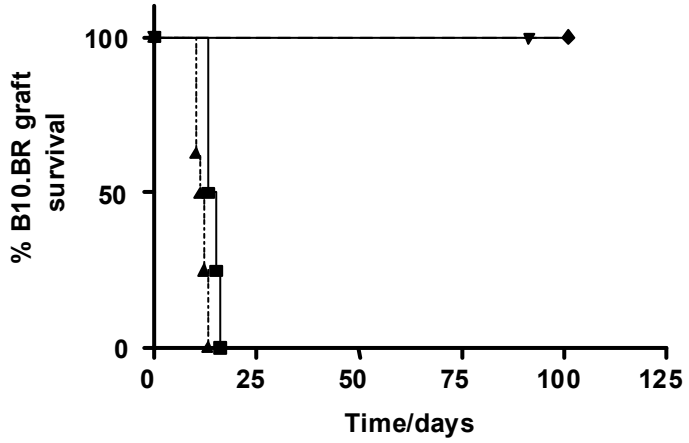

Figure 3.1 *CP1-CBA and CBA/Ca mice are histocompatible*. Adult CP1-CBA mice reject B10.BR skin grafts readily (■, n=4), but do not reject CBA/Ca skin transplants (▼, n=6). Similarly, CBA/Ca are able to reject B10.BR skin grafts (▲, n=8), while accepting CP1-CBA skin transplants (▼, n=6).

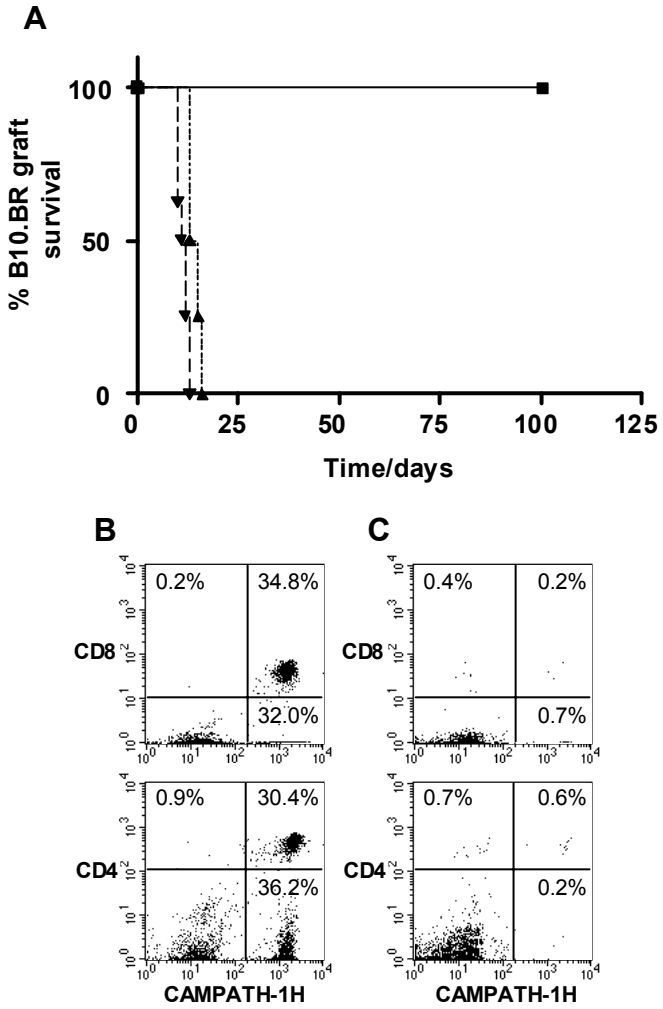

Figure 3.2 *CP1-CBA mice reject B10.BR skin grafts at a similar rate to CBA/Ca, but not after T cell depletion with CAMPATH-1H mAb*. **A,** Adult CP1-CBA (▲, n=4) mice reject B10.BR skin grafts at a rate comparable with CBA/Ca (▼, n=8) recipients. Adult ATx CP1-CBA donors treated with 0.1 mg of CAMPATH-1H mAb prior to B10.BR skin grafting permanently accept these grafts (■, n=5, median survival time (MST) >100 days). **B,** PBLs were analysed by flow cytometry following staining with CAMPATH-1H-FITC, CD8-PE and CD4-CyCr. CP1-CBA mice T cells are double positive for either CD4 or CD8 and CAMPATH-1H. **C,** Euthymic CP1-CBA mice treated with 0.1 mg of CAMPATH-1H show depletion of both $CD4^+$ and $CD8^+$ T cells 7 days after treatment.

3.2.2 Anti-CD154 mAb induces dominant transplantation tolerance

I investigated whether anti-CD154 induced tolerance was dominant by testing if the CP1-CBA mice tolerised to B10.BR skin as described above could resist the adoptive transfer of spleen cells from naïve CBA/Ca mice (Scully *et al*., 1994). 90 days after tolerance induction, CP1-CBA tolerant mice were injected intravenously with $50x10^6$ spleen cells from naïve CBA/Ca mice, and received a fresh B10.BR skin graft the following day. The naïve cells did not "break" tolerance, as both the new and old skin grafts were accepted indefinitely (Figure 3.3A). However, in control groups in which mice received MR1 treatment in the absence of a first skin graft, or where, at the time of cell-transfer, the host T cells had been depleted with CAMPATH-1H antibody, B10.BR skin grafts were readily rejected (see Figure 3.3A). The levels of donor T cell chimerism, and of host T cell depletion, were analysed by flow cytometry of PBLs (Figure 3.3B). These observations indicate that although naïve T cells did engraft, they were prevented from rejecting transplanted B10.BR skin by tolerised host T cells.

3.2.3 Anti-CD154 mAb induces infectious transplantation tolerance

As dominant-tolerance induced with CD4 antibodies has been shown to involve "infectiousness" – tolerant cells imposing tolerance on naïve cells (Qin *et al*., 1993; Cobbold and Waldmann, 1998; Waldmann, 2001) – I investigated whether infectious tolerance had been induced by anti-CD154 therapy. "Indicator" CP1-CBA mice which had been adult thymectomised, and depleted of $CD8^+$ T cells were tolerised to B10.BR skin grafts as above. After tolerance had been confirmed by graft maintenance for 90 days, $50x10^6$ spleen cells from naïve CBA/Ca mice were transferred intravenously into these tolerant mice, which then received a second B10.BR skin graft the

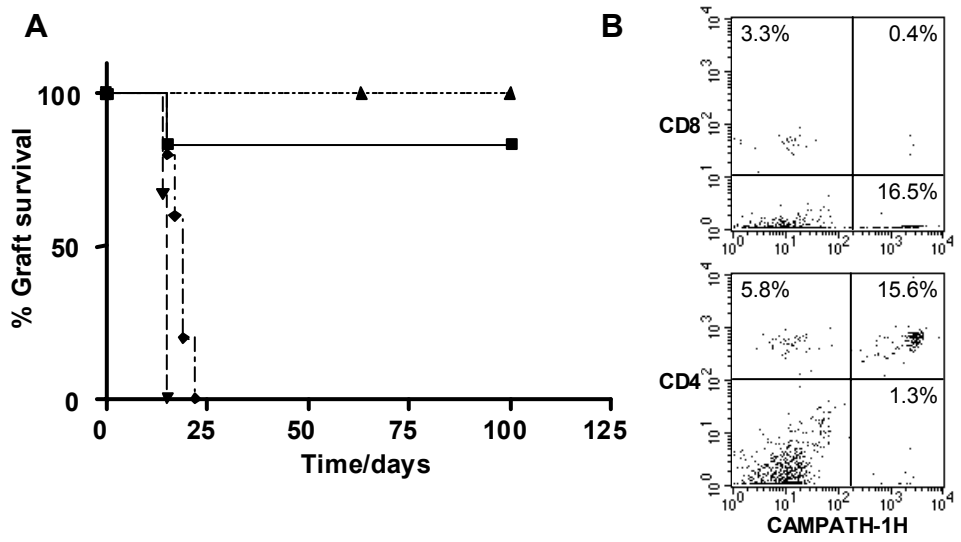

Figure 3.3 *Anti-CD154 treatment of CD8 depleted mice induces dominant tolerance that is not broken by adoptive transfer of naïve spleen cells*. **A,** Adult ATx and CD8 cell depleted CP1-CBA transgenic mice received a B10.BR skin graft (multiple minor histocompatibility differences) at day -90, with three doses of 0.67 mg of the non-depleting anti-CD154 mAb MR1 i.p. at days -90, -88 and -86 to induce tolerance. At day 0 mice from groups designated ▲, ▼ and ◆ received $50x10^6$ spleen cells i.v. from naïve CBA donors. All mice received a fresh B10.BR skin graft the following day and were monitored for graft rejection. Tolerant mice that received naïve spleen cells accepted the grafts indefinitely (▲, n=7, MST>100), as did tolerant mice receiving no naïve cells (■, n=6, MST>100 days, $p<0.28$). The absence of rejection was not due to the persistence of MR1 mAb as all animals in the control group that received the tolerising MR1 treatment in the absence of an initial skin graft rejected the graft (▼, n=6, MST=15 days). The transferred cells were competent to reject the B10.BR skin, as mice depleted of their own T cells with 0.1 mg of CAMPATH-1H i.p. prior to cell transfer and skin grafting also rejected (◆, n=5, MST=19 days). **B,** PBLs were analysed by flow cytometry following staining with CAMPATH-1H-FITC, CD8-PE and CD4-CyCr. Host lymphocytes, from CP1-CBA transgenic mice, are CAMPATH-$1H^+$. The CBA/Ca T cells that were adoptively transferred were CAMPATH-1H-ve. It was therefore possible to monitor the efficiency of cell transfer by flow cytometry of the CD4 and CD8 populations of CAMPATH-1H-ve cells. An example of a mouse from group ▲ is shown where ~25% of $CD4^+$ and almost all of $CD8^+$ T cells are from the donor (the host had been CD8 depleted).

following day. This dose of spleen cells is well in excess of the dose needed to get rapid graft rejection in this model (Davies *et al*., 1996a). When host cells were depleted with the CAMPATH-1H mAb on the day following the adoptive transfer, the naïve CBA/Ca cells were fully competent to reject the new skin graft, as well as the original one (Figure 3.4A). However, if the naive CBA/Ca cells were allowed to co-exist with the tolerant cells for 6 weeks, before depletion of host cells with CAMPATH-1H mAb, and challenged with a third B10.BR skin graft on the day following depletion, all three B10.BR skin grafts were accepted indefinitely (Figure 3.4A). Flow cytometry confirmed donor T cell chimerism, as well as effective host T cell depletion (Figure 3.4B).

3.3 Discussion

Taken together with previous findings of linked suppression in this model of transplantation tolerance (Honey *et al*., 1999), these results indicate that therapy with anti-CD154 in this context has a more profound impact than can be explained just by deletion of potentially aggressive T cells by AICD (Wells *et al*., 1999; Li *et al*., 1999). The notion of the need for AICD arose from transplants across MHC barriers, and it is conceivable that AICD may not be essential in tolerance across multiple minor differences (Li *et al*., 1998), although this remains to be established. Equally, although infectious tolerance can be shown to operate across MHC barriers when tolerance is induced with CD4 antibodies, it has not been formally demonstrated with CD40L antibodies. Whether or not AICD is operational in tolerance achieved across multiple minor differences, I must conclude that a population of $CD4^+$ regulatory T cells emerges from amongst the antigen-reactive T cells, and that these are responsible for the maintenance phase of tolerance. They do

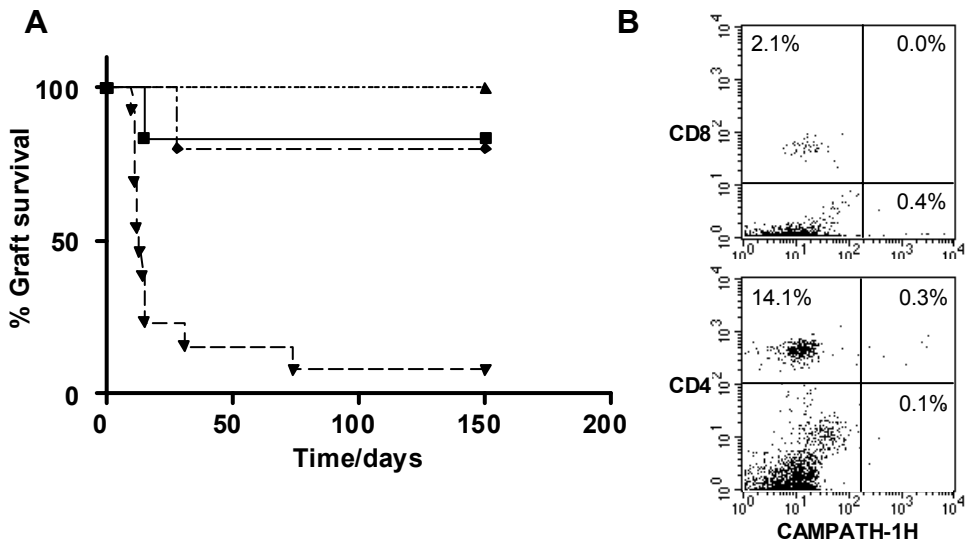

Figure 3.4 *Anti-CD154 treatment induces infectious transplantation tolerance.* **A,** Transplantation tolerance to B10.BR skin was induced in ATx CP1-CBA transgenic mice with MR1 mAb as described before. Mice from the group designated ■ (n=6) did not receive naïve cells. $50x10^6$ spleen cells from naïve CBA donors were adoptively transferred into the animals of all other groups at day 0 (90 days after tolerance induction), followed by a fresh B10.BR skin graft at day 1. In group ▼ (n=13), where host transgenic T cells were depleted at day 1 with 0.1 mg of CAMPATH-1H mAb i.p., both fresh and old skin grafts were rejected (MST=13 days). In group ◆ (n=5), the CBA lymphocytes were allowed to co-exist with the CP1-CBA cells for 6 weeks, after which host-T cells cells were depleted with CAMPATH-1H and a further B10.BR skin grafted (MST>150). There was no statistically significant difference in graft survival for this group when compared with the groups that did not receive naïve CBA spleen cells (■, n=6), or in which the CP1-CBA cells were not depleted (▲, n=7). There is a significant difference between the groups depleted at day 1 (▼) and day 45 (◆) after transfer ($p<0.006$). In animals receiving multiple grafts the most recent one was always the first to be lost and the one considered in the analysis of data. **B,** PBLs were stained with CAMPATH-1H-FITC, CD8-PE and CD4-CyCr and analysed by flow cytometry, as before, to confirm the efficiency of depletion and cell transfers. An example of a mouse from group ◆, 45 days after CAMPATH-1H depletion is shown, where the presence of CAMPATH –1H-ve T cells of donor origin can be seen while the host T cells are less than 1%.

so not just by actively suppressing rejection, but also by imposing tolerance on naïve cells through infectious tolerance.

There are two possible mechanisms by which these regulatory cells may arise. One (two-population model) is that these regulatory cells are already present in the T cell pool as a distinct sub-population. If they were less susceptible to AICD than potentially aggressive cells they would persist after anti-CD154 therapy and thus the ratio between regulators and aggressors would be altered to favour tolerance. A variant of this first model might require that AICD of the potentially aggressive T cells permits the graft to survive long enough for a regulatory $CD4^+$ cell population to expand and then dominate. The other possible mechanism (single-population model) is that potentially aggressive T cells which failed to die from AICD might have changed function to become regulators as a consequence of the antigen recognition in a tolerogenic environment. I cannot, at present, distinguish between these two possibilities.

To exert infectious tolerance such regulatory T cells would need to influence naïve T cells. This could occur either by influencing the local microenvironment of antigen presentation where both types of cells (regulatory and naïve) were in close proximity, or alternatively by decommissioning APCs so that they present to naïve T cells for tolerance rather than immunity.

These results show that common characteristics can be found following tolerance induction with mAb targeting co-stimulatory molecules (such as CD154) or mAb targeting the co-receptor molecules CD4 and CD8 (reviewed in Waldmann and Cobbold, 1998). It is even likely that other tolerance inducing regimens like vitamin D3 and mycophenolate mofetil, although not

yet studied with the same detail, will also share the same characteristics of dominant tolerance, infectious tolerance and linked suppression as they give rise to $CD4^+$ regulatory T cells (Gregori *et al*., 2001). As a consequence, it may be possible to explore synergistic effects of such tolerance inducing agents, as described in the next chapter, in order to achieve an even more robust tolerance.

One can speculate that any therapeutic intervention allowing peaceful coexistence between foreign antigen, some of it presented indirectly by host APCs, and $CD4^+$ T cells can result in the expansion of regulatory T cells and dominant transplantation tolerance. It may even be that in the absence of inflammation or “danger”, where effector mechanisms are not deployed, the outcome is the expansion of regulatory T cells. This hypothesis is consistent with the finding described in Chapter 7 concerning the immunogenic and tolerogenic properties of mAb variants. There I show that two mAbs with the same sequence except for a single amino-acid mutation that renders one of the mAbs non-lytic have different properties. The mAb that do not kill cells is not immunogenic, being remarkably tolerogenic. In other words, absence of danger may drive tolerance. This would not be tolerance by indifference as suggested in Matzinger’s danger theory (Matzinger, 1994), but dominant tolerance enforced by regulatory T cells and capable of resisting subsequent “dangerous” encounters with the tolerated antigens, and even third-party “linked” antigens.

CHAPTER 4

INDUCTION OF DOMINANT TRANSPLANTATION TOLERANCE TO FULLY MISMATCHED SKIN GRAFTS WITH NON-DEPLETING ANTIBODIES

Non-depleting therapeutic antibodies, such as those targeting CD4, CD8 and CD154 (CD40 ligand) have been shown effective in inducing tolerance to fully mismatched kidneys, heart and islet grafts, or to multiple minor antigens disparate skin grafts. A non-depleting antibody regimen capable of inducing tolerance to fully mismatched skin grafts has remained elusive. This Chapter reports that a combination of non-depleting antibodies to CD4, CD8 and CD154 is capable of inducing tolerance to fully mismatched skin transplants in euthymic mice. Tolerised animals accept subsequent skin grafts of the tolerated type while remaining fully competent to reject skin grafts of unrelated donors. Tolerance so induced appears to be dominant and mediated by $CD4^+$ T cells, reflected as a T cell dependent resistance of rejection mediated by transfused naïve lymphocytes and as "linked suppression" toward third party antigens associated with the tolerated set. This state of dominant tolerance is remarkably powerful, leading to tolerance of any third party antigens genetically associated with the tolerated set.

4.1 Introduction

The Holy Grail of transplantation research has been the achievement of life-long tolerance, following a short treatment, and without significant long-term immunosuppression. Therapeutic non-depleting mAbs have been shown effective in inducing tolerance to fully mismatched kidneys, hearts and pancreatic islets (Chen *et al*., 1992; Lenschow *et al*., 1992; Isobe *et al*., 1992; Nicolls *et al*., 1993; Yin and Fathman, 1995b; Onodera *et al*., 1996; Kirk *et al*., 1999; Kenyon *et al*., 1999) and to skin grafts mismatched for multiple minor histocompatibility antigens (Qin *et al*., 1990). However, complete tolerance to the most challenging of allografts, the fully mismatched skin grafts, and defined by acceptance of repeat transplants, has remained elusive and considered a most stringent test for any tolerance inducing regimen. The two experimental protocols that have come closest to inducing tolerance to fully mismatched skin grafts have been either the combination of non-depleting anti-CD4 and anti-CD8 mAbs, or the combination of anti-CD154 and CTLA4-Ig with indefinite survival of fully mismatched skin grafts being obtained in both cases (Larsen *et al*., 1996b; and S.P. Cobbold, unpublished). Both tolerance inducing regimens elicit the emergence of $CD4^+$ regulatory T cells capable of suppressing rejection by transfused non-tolerant T cells (dominant tolerance) and inducing the non-tolerant T cells to become themselves tolerant (infectious tolerance) (Qin *et al*., 1993; Graca *et al*., 2000). Operational tolerance of fully mismatched skin grafts has been obtained with the use of depleting mAbs (Cobbold *et al*., 1990), induction of macrochimerism (Wekerle and Sykes, 2001), or a combination of non-depleting mAbs with immunossupressive drugs (Li *et al*., 1999).

Based on evidence presented in the previous chapter, that either mAbs targeting co-receptor or mAbs specific for co-stimulatory molecules can

induce dominant tolerance mediated by $CD4^+$ regulatory T cells, I decided to investigate whether the combination of those mAbs would synergise leading to a more robust tolerant state. Indeed, I found that a combination of non-depleting mAbs targeting CD4, CD8 and CD154 synergise to enable dominant tolerance to be achieved for fully mismatched skin grafts. Tolerised mice retain the capacity to reject unrelated skin grafts, while being disabled from rejecting repeat grafts of the tolerated type. Tolerance so induced appears to be dominant and mediated by $CD4^+$ regulatory T cells, as expected from the known characteristics of tolerance induced by co-receptor or co-stimulatory blockade (Qin *et al*., 1993; Graca *et al*., 2000; Waldmann and Cobbold, 2001). A remarkably powerful form of linked suppression is readily demonstrated in tolerant mice, which is able to spread to third party antigens if these are genetically associated with the tolerated antigen set, and most surprisingly with the host antigens. This suggests that the repertoire of regulatory T cells which recognise "donor" antigens processed by host APCs, cross reacts significantly with that recognising third-party antigens (full mismatches) presented in association with host-type MHC.

4.2 Results

4.2.1 Non-depleting antibodies to CD4, CD8 and CD154 synergise to induce tolerance to fully mismatched skin grafts

A short treatment with a combination of non-depleting CD4, CD8 and CD154, but not a combination of any two of the mAbs, is capable of inducing tolerance to fully mismatched skin grafts. Adult euthymic CBA/Ca mice were transplanted with C57/B10 skin and treated with a short course of non-depleting CD4 (YTS 177), CD8 (YTS 105) and CD154 (MR1) mAbs. Mice treated with a combination of the three mAbs accepted the tolerated skin

grafts indefinitely, as did most of the animals treated with anti-CD4 + anti-CD8. All mice treated with other mAb combinations readily rejected the skin grafts (Figure 4.1A).

At day 100 following tolerance induction, mice with surviving grafts received a fresh C57/B10 skin graft, as did animals treated with all three mAbs in the absence of the initial skin grafts (Figure 4.1B). No rejection was observed in the group rendered tolerant to C57/B10 skin by treatment with the three mAbs, a result which cannot be consequence of persistence of the mAbs in circulation, as the control group injected with the same mAbs, but in the absence of an initial graft, readily rejected the transplanted skin. Mice pre-treated with just CD4 and CD8 mAbs, in spite of prolonged acceptance of initial allografts, rejected both fresh and old grafts following the secondary graft challenge. Mice tolerised with the three mAbs remained fully competent to reject third-party BALB/c skin transplants, at a similar rate to non-tolerant animals.

4.2.2 Non-depleting antibodies to CD4, CD8 and CD154 induce dominant tolerance to fully mismatched skin grafts

Treatment with either non-depleting CD4 and CD8 mAbs, or with CD154 and CD8 mAb can lead to dominant transplantation tolerance to skin grafts mismatched for multiple minor transplantation antigens (Qin *et al*., 1993; Graca *et al*., 2000). Such dominant tolerance is easily identified by the demonstration that transfusions of naïve syngeneic lymphocytes will not break the tolerant state. This was indeed seen to be the case when tolerance had been induced to fully mismatched skin, as tolerised animals transfused with a large number of naïve lymphocytes resisted graft rejection.

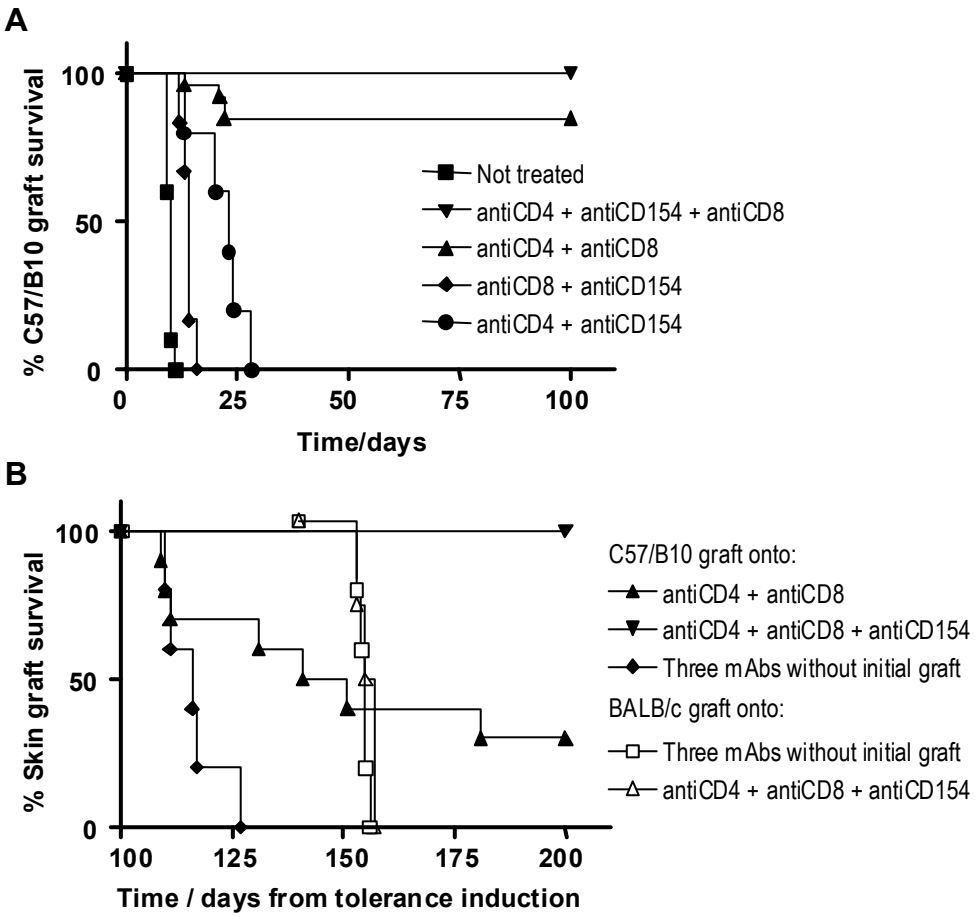

Figure 4.1 *Tolerance induction to fully mismatched skin grafts.* **A**, CBA/Ca mice were treated with 3 doses of 1 mg of the anti-CD4, anti-CD8 and anti-CD154 non-depleting mAbs over 1 week following C57/B10 skin transplantation at day 0. Only mice treated with the three mAbs (▼, *n*=6, MST>100d) and anti-CD4 + anti-CD8 (▲, *n*=26, MST>100d) showed indefinite graft survival ($P<0.05$ to any other group). All grafts from mice treated with anti-CD4 + anti-CD154 (●, *n*=6, MST=21.5d), anti-CD8 + anti-CD154 (◆, *n*=6, MST=14d) and untreated controls (■, *n*=10, MST=10d) were rejected. **B**, After 100 days, mice with surviving skin allografts received fresh C57/B10 skin transplants. In animals treated with the three mAbs no rejections were observed (▼, *n*=6, MST>100d). Mice treated with only anti-CD4 and anti-CD8 rejected the skin allografts (▲, *n*=10, MST=46d, $P=0.011$ vs. ▼). The control group treated with the same three mAbs (as ▼) in the absence of initial transplant rejected the skin grafts (◆, *n*=5, MST=16d). BALB/c skin was transplanted at day 140 onto mice tolerised with the three mAbs (□, *n*=6, MST=16d) and the control mice treated with the mAbs in the absence of an initial graft (△, *n*=5, MST=15d).

Adult thymectomised CP1-CBA mice were tolerised to C57/B10 skin grafts, by treatment with the three mAbs over the first week following transplantation. At 100 days following tolerance induction, the CP1-CBA mice received a transfusion of $20x10^6$ splenocytes from naïve CBA/Ca mice, as well as a fresh C57/B10 skin graft. Figure 4.2A shows that where $20x10^6$ non-tolerant splenocytes were transfused into T cell depleted hosts the grafts were readily rejected. Rejection at a similar rate was observed when host T cells were depleted from tolerised CP1-CBA mice one day following the transfusion with naive splenocytes. However, when host T cells were allowed to co-exist with the transfused splenocytes, graft rejection was significantly delayed. The extent of the delay was not due to the persistence of the therapeutic mAbs as control animals treated with the same mAb combination, but in the absence of an initial skin graft, readily rejected C57/B10 skin grafts even without being transfused with naive CBA/Ca splenocytes. Figure 4.2B shows that when non-tolerant splenocytes co-exist with tolerised T cells for 45 days, their ability to reject fresh C57/B10 skin grafts was significantly reduced by comparison to those which have co-existed for just one day (MST=11d vs. 18.5d; P=0.018).

The delay in graft rejection following the adoptive transfer of $20x10^6$ splenocytes from naïve mice is significant as I determined that as few as $5x10^4$ splenocytes from naïve CBA/Ca are sufficient to mediate C57/B10 skin graft rejection upon transfer into $RAG1^{-/-}$ mice (n=4, MST=23.5d; Figure 4.2C).

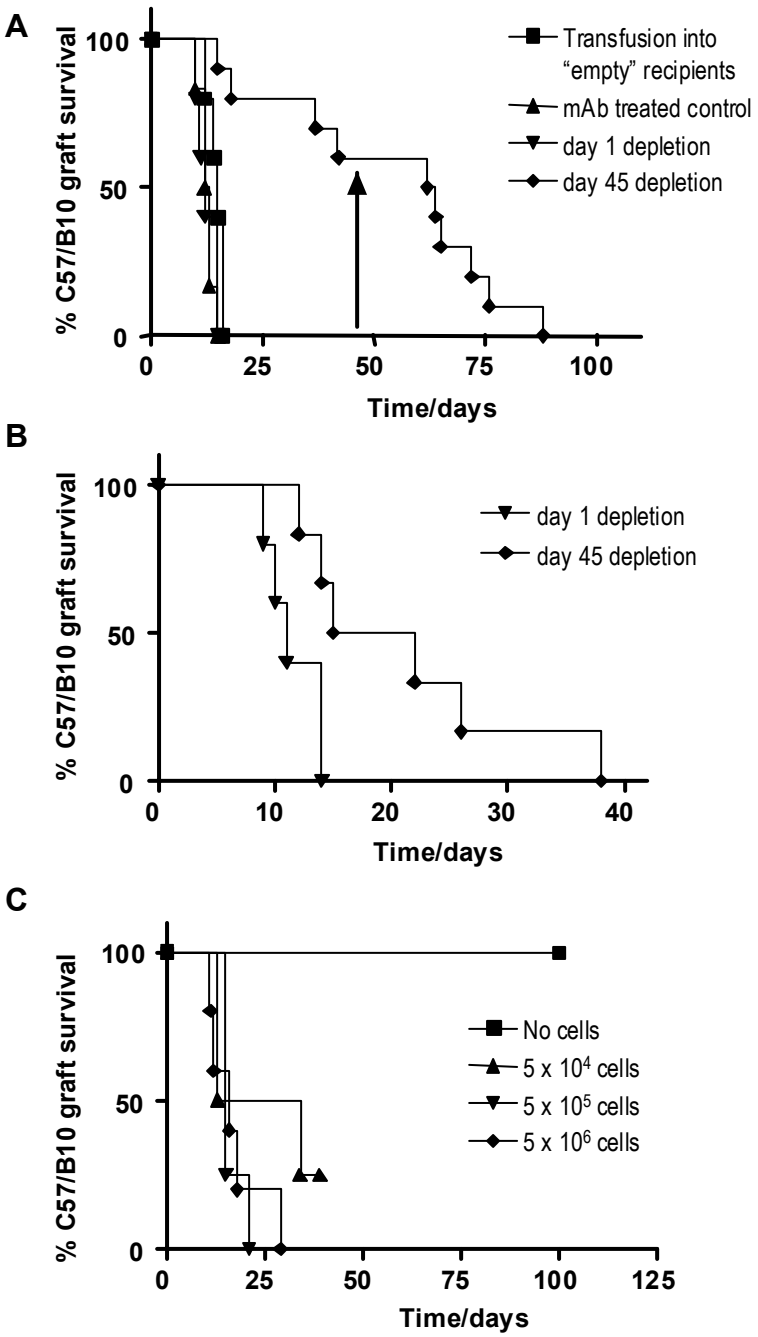

Figure 4.2 *Tolerised mice resist transfusion of non-tolerant cells.* Adult thymectomised CP1-CBA mice were tolerised to C57/B10 skin grafts as described. **A**, 100 days after initial transplantation (day -1) the animals from two groups (▼ and ◆) were transfused with $20x10^6$ splenocytes from naïve CBA/Ca mice. The following day all mice were grafted with C57/B10 skin, and one group depleted of host T cells with CAMPATH-1H (▼). Animals from the depleted group readily rejected skin grafts (▼, n=5, MST=12d) at rate comparable with non-tolerant T cell depleted animals transfused with the same number of splenocytes (■, n=5, MST=12.5d), or animals treated with tolerogenic mAbs in the absence of initial skin graft, and not transfused (▲). The animals whose T cells had not been depleted resisted transfusion

showing delayed graft rejection (◆). When their host T cells were depleted 45 days after transfusion, and another C57/B10 skin transplanted, grafts were rejected. **B**, Direct comparison of rejection rate following depletion of host T cells from tolerised mice, as described in (A). In mice from group ▼ the transfused non-tolerant splenocytes were allowed to coexist with tolerised T cells for only 1 day (n=5, MST=11d). In mice from group ◆ the coexistence was extended to 45 days (n=6, MST=18.5d, P=0.018). **C**, Number of splenocytes from naïve CBA/Ca capable to mediate C57/B10 skin graft rejection upon adoptive transfer into $RAG1^{-/-}$ mice. As few as $5x10^4$ splenocytes can lead to allograft rejection.

4.2.3 Tolerance is maintained by $CD4^+$ regulatory T cells

Dominant tolerance induced with either non-depleting CD4 and CD8 mAbs, or with CD154 mAb is maintained by $CD4^+$ regulatory T cells (Qin *et al*., 1993; Graca *et al*., 2000). We also confirmed that dominant tolerance induced with a combination of the three mAbs to fully mismatched skin grafts is mediated by $CD4^+$ T cells.

$RAG1^{-/-}$ mice were grafted with C57/B10 skin and transfused with 10^7 splenocytes from CBA/Ca mice tolerised to C57/B10 skin 100 days earlier (Figure 4.3). Different groups of mice were depleted of $CD4^+$ or $CD8^+$ T cells with mAbs. All recipients of splenocytes from naïve donors rejected skin grafts regardless of depletion of either $CD4^+$ or $CD8^+$ cells, but rejection was significantly impaired when both populations of T cells were simultaneously depleted. However, where $RAG1^{-/-}$ mice were transfused with splenocytes from tolerised donors, rejection was only observed following depletion of $CD4^+$ T cells, and at a delayed rate. The differences observed between the groups where "tolerant" splenocytes were depleted of $CD4^+$ or $CD8^+$ T cells is statistically significant ($P<0.05$).

4.2.4 Non-depleting antibodies to CD4, CD8 and CD154 induce linked suppression to fully mismatched skin grafts

Linked suppression has been a constant feature of dominant transplantation tolerance induced by treatment with non-depleting CD4 or CD154 mAbs (Davies *et al*., 1996a; Chen *et al*., 1996; Wong *et al*., 1997; Honey *et al*., 1999). I found that the combination of the three mAbs induces a very powerful form of linked suppression across fully mismatched skin allografts.

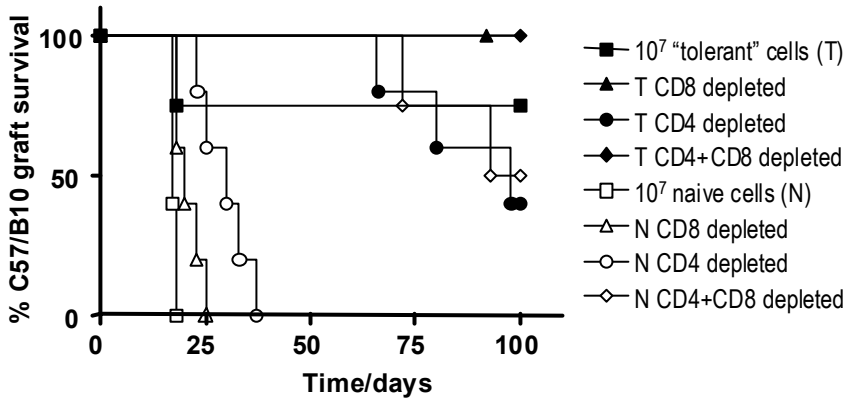

Figure 4.3 *Dominant transplantation tolerance is mediated by CD4$^+$ T cells.* RAG1$^{-/-}$mice were transfused with 10^7 splenocytes from tolerant (filled symbols) or naïve donors (open symbols). CD4$^+$ cells (circles), CD8$^+$ cells (triangles), or both (diamonds), were depleted in different groups of mice. The difference in graft survival between groups where "tolerant" splenocytes were depleted of CD4$^+$ or CD8$^+$ T cells is statistically significant (● and ▲, n=5, P<0.05).

CBA/Ca mice were tolerised to C57/B10 skin grafts as described. Figure 4.4A shows graft survival for skin transplanted at 100 days. Mice grafted with (C57/B10 x BALB/c)F_1 skin held their grafts indefinitely, while non-tolerant mice rejected their grafts. When mice were transplanted with both C57/B10 and BALB/c skin in the same graft bed, the BALB/c graft was rejected, with two mice from this group subsequently rejecting the C57/B10 grafts (on days 22 and 27, not represented in the figure). In a repeat experiment BALB/c rejection did not lead to C57/B10 rejection in any animal (*n*=5), and in all experiments where BALB/c and C57/B10 skin grafts were transplanted sequentially only BALB/c grafts were rejected (see Figure 4.1B). It is possible that in the two mice that had rejected the tolerated C57/B10 grafts, cross-reactivity of T cells recognising antigens presented by donor APCs (direct presentation) overcame the effect of regulatory cells that were maintaining C57/B10 grafts.

Surprisingly, tolerant mice grafted with (CBA/Ca x BALB/c)F_1 skins rejected the grafts at a slower rate, with 3/6 of the mice accepting the grafts indefinitely (Figure 4.4A). Naïve CBA/Ca mice readily rejected (CBA/Ca x BALB/c)F_1 or (CBA/Ca x C57/B10)F_1 skin grafts.

One of the potential therapeutic benefits of linked suppression is to build up tolerance to transplants of a third party type, by regrafting hosts tolerant to the second party with tissues exhibiting both the tolerated and the third-party antigens(Davies *et al*., 1996a; Chen *et al*., 1996; Wong *et al*., 1997). We confirmed that this was possible by transplanting BALB/c skin onto those CBA/Ca mice, pre-tolerised to C57/B10 skin, where (C57/B10 x BALB/c)F_1 skin had survived 50 days (Figure 4.4B). All animals accepted the BALB/c skin. These results show how tolerance induced to fully mismatched skin grafts can be extended to third-party antigens. Most interestingly, grafts from

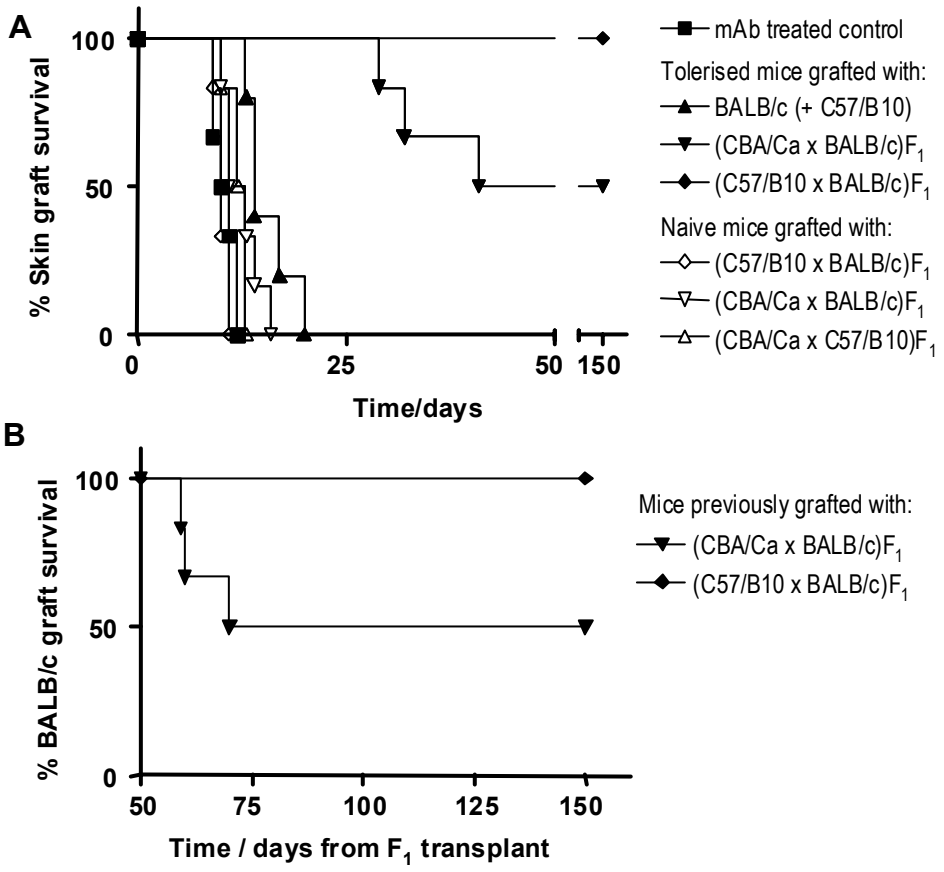

Figure 4.4 *Linked suppression across fully mismatched skin transplants.* CBA/Ca mice were tolerised to C57/B10 skin grafts as described. **A**, 100 days after tolerance induction (day 0 in the graph) mice were transplanted with (CBA/Ca x BALB/c)F_1 (▼, *n*=6, MST=80.5d), (C57/B10 x BALB/c)F_1 (◆, *n*=6, MST>150d) skin grafts, or both BALB/c and C57/B10 skin grafts onto the same graft bed (only BALB/c graft survival represented: ▲, *n*=5, MST=14d). Antibody treated mice not transplanted with tolerising skin were now grafted with C57/B10 skin (■, *n*=6, MST=10.5d). Naive mice were transplanted with (C57/B10 x BALB/c)F_1 (◇, *n*= 6, MST=10d), (CBA/Ca x BALB/c)F_1 (▽, *n*=6, MST=12d), or (CBA/Ca x C57/B10)F_1 (△, *n*=6, MST=12.5d) skin grafts. All tolerised mice grafted with (C57/B10 x BALB/c)F_1 skin accepted the grafts (◆, $P<0.001$ to any other group except ▼: non-significant), while mice grafted with (CBA/Ca x BALB/c)F_1 skin showed a delayed rejection with half the mice accepting the grafts indefinitely (▼, $P<0.001$). **B**, All mice from (A) that failed to reject either (C57/B10 x BALB/c)F_1 (◆) or (CBA/Ca x BALB/c)F_1 (▼) skin grafts, accepted subsequent BALB/c skin grafts transplanted at day 150. The same mice that rejected (CBA/Ca x BALB/c)F_1 (▼) skin grafts, rejected subsequent BALB/c skin transplants.

(CBA/Ca x BALB/c)F_1 hybrid mice, where the host-type parent (CBA/Ca) had been crossed with the third-party (BALB/c), were also able to elicit a similar, albeit less powerful, effect than (C57/B10 x BALB/c)F_1 grafts.

4.3 Discussion

It can therefore be concluded that a combination of non-depleting CD4, CD8 and CD154 mAbs, but not a combination of any two of the mAbs, induce transplantation tolerance specific to the antigens present in the skin allograft at the time of treatment, without impairing immune responses to unrelated antigens (Figure 4.1). The treated mice are therefore tolerant and not immunosuppressed. Furthermore, mAbs targeting co-receptor molecules apparently synergise with mAbs targeting co-stimulatory molecules, and that CD4 blockade is more efficient that CD154 blockade in inducing tolerance, contrary to what would be predicted by a strict interpretation of the two signal model of T cell activation (Matzinger, 1999).

The finding that non-depleting CD4 and CD8 mAb treatment leads to long-term allograft survival, but cannot prevent rejection following a transplant rechallenge is intriguing. During the induction phase of tolerance it is possible that the T cell repertoire specific for directly presented antigens (on donor type APCs) is not efficiently deleted. It is known that dominant tolerance requires the persistence of the tolerated antigens in order to be maintained (Scully *et al*., 1994), and It is most unlikely that donor type APCs persist for a long period of time (Lechler and Batchelor, 1982). As a consequence, at the time of secondary skin transplantation, dominant tolerance towards directly presented antigens is probably absent, while tolerance to indirectly presented antigens persists. This effect cannot be seen in the context of minor antigens where there is no difference between direct and indirect presentation. It is

possible (although speculative) that addition of anti-CD154 to the tolerizing protocol could allow a more complete depletion of the T cell repertoire specific for antigens presented on donor APCs, most likely by AICD (Wells *et al*., 1999; Li *et al*., 1999). According to this hypothesis, one would predict that a persistent source of donor type APCs, such as bone marrow transplantation or repeated donor type transfusions, should allow the maintenance of regulatory T cells capable of suppressing immune responses to repeat transplantation in mice treated with only anti-CD4 and anti-CD8. Another prediction is that if rejection of secondary grafts is prevented by conventional immunosuppressive drugs for the short time donor APCs are present, then tolerance will not be broken. It is important to note that this hypothesis is different from Matzinger's danger theory (Matzinger, 1994). Although inflammatory stimuli may skew the balance between suppression / aggression towards the latter, the key issue is that direct presentation does not elicit regulatory cells unless there is a persistent source of donor APCs. When regulatory T cells are present, for example in the context of indirect presentation, usually "danger" cannot break tolerance.

The results also suggest that in tolerant mice $CD8^+$ T cells capable of rejecting the tolerated skin allografts persist, but are controlled by $CD4^+$ regulatory T cells (Figure 4.3). Delayed rejection mediated by tolerated $CD8^+$ T cells, when compared with rejection by naïve $CD8^+$ cells, can be due to either the presence of some $CD4^-$ regulatory cells (not powerful enough to completely control rejection), or to deletion of a significant pool of alloreactive $CD8^+$ T cells. I favour this last hypothesis, as activation induced cell death has been shown to contribute to mAb-induced transplantation tolerance (Wells *et al*., 1999; Li *et al*., 1999).

The demonstration of dominant tolerance is compatible with a role for regulatory T cells, as it has been demonstrated for tolerance induced with non-depleting CD4 and CD8 mAbs (Qin *et al*., 1993), and with anti-CD154 and CD8 mAbs (Graca *et al*., 2000).

Furthermore, as tolerance can be induced in either euthymic (Figure 4.1), or adult thymectomised mice (Figure 4.2), I confirmed that the potential for this form of tolerance does resides in the periphery, and that any alloreactive T cells exported from the thymus are not able to break the tolerant state.

Finally, it was shown that tolerance induced to fully mismatched skin grafts can be extended to skin of a third-type, by exposure of the immune system to a graft carrying both sets of antigens in their cells. Most interestingly, (CBA/Ca x BALB/c)F_1 skin grafts, from syngeneic host-type mice crossed with third-party mice (whose cells harbour simultaneously autologous and third-party antigens), could also elicit a similar albeit less powerful outcome than (C57/B10 x BALB/c)F_1 skin grafts (whose cells harbour simultaneously antigens of the tolerated type and third-party antigens). These observations contrast with findings from some studies where grafts mismatched for minor antigens were used (Davies *et al*., 1996a; Honey *et al*., 1999). In such experiments, syngeneic by third party F_1 skin grafts were shown to be consistently rejected.

Our results can be understood if we recognise that many donor proteins are presented on host APCs (indirect presentation) for priming of regulatory T cells (Wise *et al*., 1998; Yamada *et al*., 2001). It is possible that the two fully mismatched donor skin grafts (BALB/c and C57/B10) share sufficient polymorphic proteins so as to engage the abundant pool of regulatory T cells established following initial tolerisation of C57/B10 skin graft, when presented

on host-type MHC. (CBA/Ca x BALB/c)F_1 skin grafts would present many of these shared antigens through indirect presentation by H-2^k on F_1 APC. In contrast, BALB/c grafts would not be able to present “directly” to regulatory T cells, and so rejection would be advanced before regulation through indirect presentation had been harnessed. When tolerance is induced to some minor antigens, there may not be a sufficient overlap between the tolerised minor antigens, and the third-party antigens tested. In such conditions the balance would favour rejection (Davies *et al*., 1996a; Honey *et al*., 1999). It is, however, important to stress that skin grafts different in minor antigens are often not rejected, as it is the case of BALB/k skin grafts transplanted onto CBA/Ca (Davies *et al*., 1996a). Such phenomenon may also be due to “linked suppression” mediated by regulatory T cells maintaining peripheral self-tolerance.

The results presented in this chapter show that a combination of non-depleting mAbs aiming for simultaneous co-receptor and co-stimulation blockade achieve tolerance in the most stringent of the mouse transplantation models. The observation of linked suppression offers the prospect of significant clinical application: it may be possible to identify sets of common transplantation antigens to which tolerance once induced would be permissive for acceptance, through linked suppression, of organs from diverse donors. Patients receiving such “prophylactic vaccines” prior to a transplant might tolerate a broader degree of genetic mismatch. A concern for clinical trials of mAb to induce tolerance to a transplanted organ is the risk of rejection where current drug immunosuppression is considered effective in the short-term. The concept of prophylactic tolerance induction to a limited set of common (shared) transplantation antigens could thus lessen the risk of rejection in experimental tolerance protocols.

CHAPTER 5

BOTH CD4+CD25+ AND CD4+CD25- REGULATORY T CELLS MEDIATE DOMINANT TRANSPLANTATION TOLERANCE

$CD4^+CD25^+$ T cells have been proposed as the principal regulators of both self-tolerance and transplantation tolerance. Although $CD4^+CD25^+$ T cells do have a suppressive role in transplantation tolerance, so do $CD4^+CD25^-$ T cells although ten-fold less potent. Antibodies to CTLA-4, CD25, IL-10 and IL-4 were unable to abrogate suppression mediated by tolerant spleen cells, so excluding any of these molecules as critical agents of suppression. $CD4^+CD25^+$ T cells from naïve mice can also prevent rejection despite the lack of any previous experience of donor alloantigens. However, this requires many more naive than tolerised cells to provide the same degree of suppression. This suggests that a capacity to regulate transplant rejection pre-exists in naïve mice, and may be amplified in "tolerised" mice. Serial Analysis of Gene Expression (SAGE) confirmed that cells sorted into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations were distinct in that they responded to TCR ligation with very different programs of gene expression. Further characterization of the differentially expressed genes may lead to the development of diagnostic tests to monitor the tolerant state[2].

[2] Some of the results presented in this Chapter were published in (Graca *et al*., 2002b).

5.1 Introduction

Ever since the description of classical transplantation tolerance by Medawar and his colleagues (Billingham *et al*., 1953), the attainment of clinical transplantation tolerance has been considered the Holy Grail of immunology. In rodents, the therapeutic administration of non-depleting monoclonal antibodies (mAb), such as the combination of anti-CD4 and anti-CD8, at the time of transplantation can lead to a robust form of peripheral tolerance (Qin *et al*., 1990; Qin *et al*., 1993; Davies *et al*., 1996a; Chen *et al*., 1996; Waldmann and Cobbold, 1998). The tolerant state so achieved, creates an environment in the host which can disarm non-tolerant naïve cells from rejecting the transplant (dominant tolerance), as well as enabling the emergence of novel regulatory cells from the naïve lymphocyte population (infectious tolerance). This has been observed not only with tolerance induced following co-receptor blockade of CD4 and CD8 (Qin *et al*., 1993), but also with tolerance resulting from co-stimulation blockade using non-depleting anti-CD40L (CD154) mAb (Graca *et al*., 2000). Thus far, the regulatory cells mediating dominant tolerance have been identified as $CD4^+$ T cells in all the models studied (Hall *et al*., 1985; Qin *et al*., 1993; Yin and Fathman, 1995a; Zhai and Kupiec-Weglinski, 1999; Waldmann and Cobbold, 2001).

In parallel studies, animal models of autoimmune disease and inflammatory bowel disease have provided compelling evidence of $CD4^+$ regulatory cells that prevent immunopathology (Sakaguchi *et al*., 1985; Powrie and Mason, 1990; Fowell and Mason, 1993; Mason and Powrie, 1998). The phenotype of these regulatory T cells has been further refined, so that $CD4^+CD25^+CD45RB^{low}$ T cells are now considered the principal exponents (Sakaguchi, 2000; Shevach, 2000; Maloy and Powrie, 2001). These cells

have been shown capable of regulating $CD4^+CD25^-$ or $CD4^+CD45RB^{high}$ non-tolerant cells both *in vitro* and *in vivo*, preventing the onset of autoimmunity and gut immunopathology (Sakaguchi *et al*., 1995; Asano *et al*., 1996; Thornton and Shevach, 1998; Takahashi *et al*., 1998). They have also been shown capable of suppressing *in vitro* proliferation and IFN-γ secretion by $CD8^+$ T cells (Piccirillo and Shevach, 2001).

In order to establish the relationship, if any, between the T cells that regulate transplant rejection and those that regulate self-immunopathology we compared the suppressive ability of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from mice rendered tolerant to skin transplants, as well as from naïve mice with no previous experience of those particular transplantation alloantigens. We found that both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from tolerant mice could mediate suppression, although the $CD4^+CD25^-$ cells were required in larger numbers. However, as mice have ten times more $CD4^+CD25^-$ than $CD4^+CD25^+$ T cells, we are led to conclude that regulatory cells within both populations are involved in suppression, perhaps acting in concert. In contrast, it was only the $CD4^+CD25^+$, but not the $CD4^+CD25^-$, cells from naïve mice that could prevent naïve splenocytes cells from rejecting a skin graft, although at least five-fold more cells were required than from tolerant donors. This could mean that tolerance inducing protocols either drive an expansion of regulatory T cells (both $CD4^+CD25^+$ and $CD4^+CD25^-$) or that they bring about selective deletion of non-tolerant cells, or indeed both – the outcome being a tolerance-permissive regulator to immune-effector ratio. These results appear to differ from previously published work (Hara *et al*., 2001; Gregori *et al*., 2001) where only the $CD4^+CD25^+$ cells from tolerant animals have been shown to be regulatory. These differences may be apparent rather than real, simply reflecting minutiae of the protocols involved in the previous studies.

Transcriptional profiling by SAGE (Velculescu *et al*., 1995) of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from naïve mice was used to establish that the two populations have very distinct gene profiles. These may reflect the differing functions of such populations, and, with further characterization, may provide diagnostics to allow monitoring of the contributions of each $CD4^+$ subpopulation in circumstances where therapeutic tolerance is desirable.

5.2 Results

5.2.1 Spleen cells from "tolerized" mice abrogate skin graft rejection by spleen cells from naïve mice

Regulatory cells that suppress graft rejection by naïve spleen cells can be found in the spleens of mice made tolerant to an allograft with therapeutic mAbs (Qin *et al*., 1993). We confirmed that 10^7 spleen cells from naïve CBA/Ca mice were, upon adoptive transfer, sufficient to reject B10.BR skin grafts in T cell depleted hosts (Davies *et al*., 1996b). An equal number of spleen cells from mice tolerant to B10.BR skin grafts could prevent graft rejection when co-administered with the naïve cells.

Tolerance was induced in CBA/Ca mice by treatment with 3 mg of the combination of non-depleting CD4 and CD8 mAbs administered over one week following the transplantation of B10.BR skin grafts. I used CP1-CBA mice as T cell depleted hosts for cell transfusion (Gilliland *et al*., 1999). CP1-CBA mice were thymectomised at 4 weeks of age, and depleted of T cells with CAMPATH-1H one week prior to cell transfer (designated as "empty" mice). The "empty" mice were transfused with 10^7 spleen cells from tolerant mice; 10^7 spleen cells from naïve CBA/Ca; or an equal number (10^7) spleen cells from both tolerant and naïve mice. All animals were grafted with B10.BR

skin the following day. Rejection was only observed in those mice transfused with cells from naïve CBA/Ca (Figure 5.1). Spleen cells from tolerant mice not only failed to reject the skin grafts, but also abrogated rejection mediated by naïve T cells: so demonstrating dominant tolerance. Similar results were obtained in experiments where $2x10^7$ and $4x10^7$ spleen cells from both tolerant and naïve mice were transfused. I decided to use 10^7 spleen cells from naïve CBA/Ca as the target population to assess the number and phenotype of regulatory cells able to prevent rejection.

5.2.2 $CD4^+CD25^+$ cells from naïve mice prevent graft rejection upon adoptive transfer

I investigated whether the capacity to suppress transplant rejection pre-existed in naïve mice. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from the spleens of naïve CBA/Ca mice. “Empty” CP1-CBA mice were injected with 10^7 unsorted spleen cells from naïve CBA/Ca, together with 10^6 of either $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells, also from naïve CBA/Ca. All mice were transplanted with B10.BR skin on the following day. Delayed graft rejection was observed in the group transferred with the $CD4^+CD25^+$ cells, with 6/10 of the mice showing indefinite graft survival (Figure 5.2). The animals injected with unsorted spleen cells alone rejected the skin grafts at a rate similar to the group receiving $CD4^+CD25^-$ T cells. To rule out an artefact of the sorting procedure, a control experiment was performed where spleen cells from naïve CBA/Ca were sorted and subsequently remixed to the exact starting proportions. These cells failed to prevent skin graft rejection upon adoptive transfer (Figure 5.3).

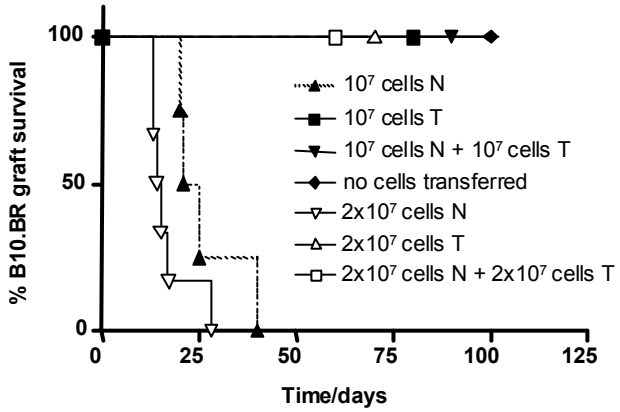

Figure 5.1 *Spleen cells from tolerant mice prevent rejection mediated by non-tolerant cells*. CP1-CBA mice were thymectomised at 4 weeks of age, and depleted of T cells with 0.1mg CAMPATH-1H at days –7 and –8. (A) At day –1, these mice received an i.v. injection of 10^7 (▲) or $2x10^7$ (▽) spleen spleen cells from naïve CBA/Ca;10^7 (■) or $2x10^7$ (△) spleen cells from CBA/Ca mice tolerant of B10.BR skin grafts;10^7 or $2x10^7$ spleen cells from the same naïve CBA/Ca donors, together with an equal number of spleen cells tolerant donors (▼ and □); and a final group was not injected (◆). All animals received a B10.BR skin graft on the following day (day 0). Only mice transfused with cells from naïve donors rejected the skin grafts, the rejection being slightly faster when more cells were transfused. The spleen cells from tolerant donors not only allowed indefinite graft survival, but were also able to suppress rejection mediated by the naïve cells injected at the same time.

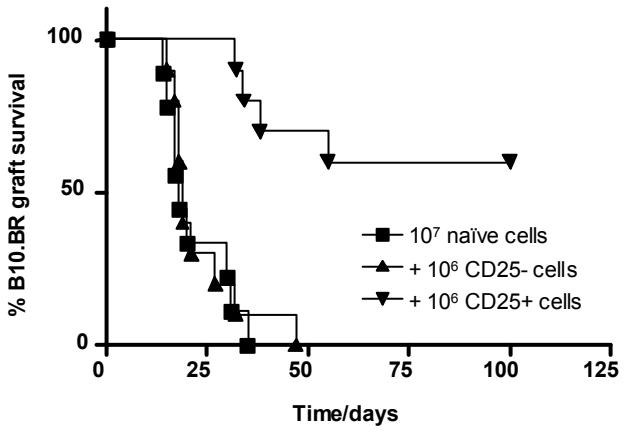

Figure 5.2 *$CD4^+CD25^+$ cells from naïve mice prevent graft rejection upon adoptive transfer*. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were sorted from spleens of CBA/Ca mice. "Empty" CP1-CBA mice were injected i.v. with 10^6 of either $CD4^+CD25^+$ or $CD4^+CD25^-$ cells together with 10^7 unsorted spleen cells. All mice were transplanted with B10.BR skin the following day. The group injected with $CD4^+CD25^-$ cells (▲, n=10, MST=19d) rejected the skin grafts at a similar rate as the control group where only 10^7 unsorted spleen cells were transferred (■, n=9, MST=18d). However, when the $CD4^+CD25^+$ cells were co-injected with the unsorted cells, graft rejection was significantly delayed, and several mice accepted the transplanted skin indefinitely (▼, n=10, MST>100, P<0.0001). This Figure represents pooled results from two different experiments.

I confirmed that the $CD4^+CD25^+$ cells used in the present study have the principal phenotypic characteristics of the $CD4^+CD25^+$ regulatory cells described in the literature (reviewed in Shevach, 2000). By performing FACS staining we confirmed that approximately 10% of the splenic $CD4^+$ T cells express CD25, and that the majority of the $CD4^+CD25^+$ cells constitutively express CTLA-4 and CD44, and are contained among the $CD45RB^{low}$ T cells (Figure 5.4). The $CD4^+CD25^-$ cells are predominantly CTLA-4 negative, and contained within the $CD44^-$ $CD45RB^{high}$ cell fraction, although ~20% are $CD44^+$ $CD45RB^{low}$.

5.2.3 $CD4^+CD25^+$ T cells from "tolerized" mice are more efficient than $CD4^+CD25^-$ cells as mediators of dominant transplantation tolerance

Having established that $CD4^+CD25^+$ T cells from naïve animals suppress graft rejection, I compared the potency of both $CD4^+CD25^+$ and $CD4^+CD25^-$ populations from "tolerized" mice in preventing rejection. The $CD4^+CD25^+$ and $CD4^+CD25^-$ populations were purified from the spleens of CBA/Ca mice made tolerant to B10.BR skin transplants 100 to 120 days earlier (Figure 5.5A). Different numbers of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were transferred with the fixed number 10^7 of naïve spleen cells into "empty" CP1-CBA recipients. All recipients received B10.BR skin grafts on the following day. When 10^5 $CD4^+CD25^+$ cells were transferred together with 10^7 spleen cells from naïve CBA/Ca a delay in graft rejection was observed, when compared with the groups transferred with the same number of $CD4^+CD25^-$ cells, or with controls which had received naive spleen cells only (Figure 5.5B). However, when the number of $CD4^+CD25^-$ T cells was increased ten-fold to 10^6, graft rejection was delayed to an extent comparable to the 10^5 $CD4^+CD25^+$ group. No skin graft rejection by the naïve cells was observed in

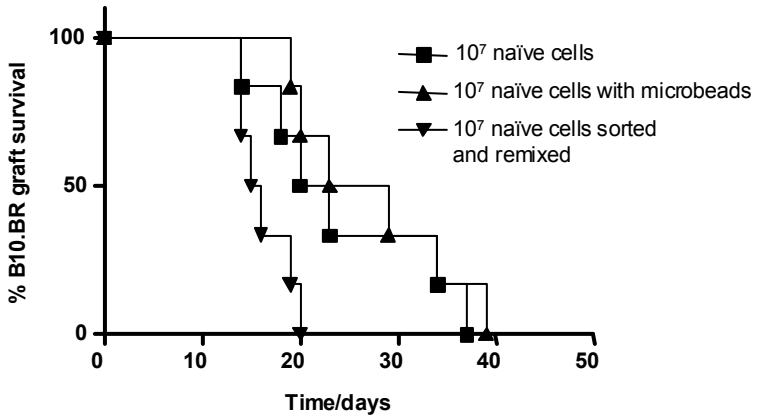

Figure 5.3 *Cell sorting procedure does not affect rejection capacity of cells*. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were sorted from naive CBA/Ca spleens, mixed together into the original proportion, and 10^7 injected iv into CP1-CBA mice, transplanted with B10.BR skin on the following day. All skin grafts were rejected (▼, n=6, MST=15.5d). Other empty CP1-CBA mice were transfused with the same number of cells, from the same naive CBA/Ca donors, either labeled with mAb and streptavidin-microbeads (▲, n=6, MST=26d) or in the absence of any manipulation (■, n=6, MST=21.5d). Differences in graft survival between the groups were not significant.

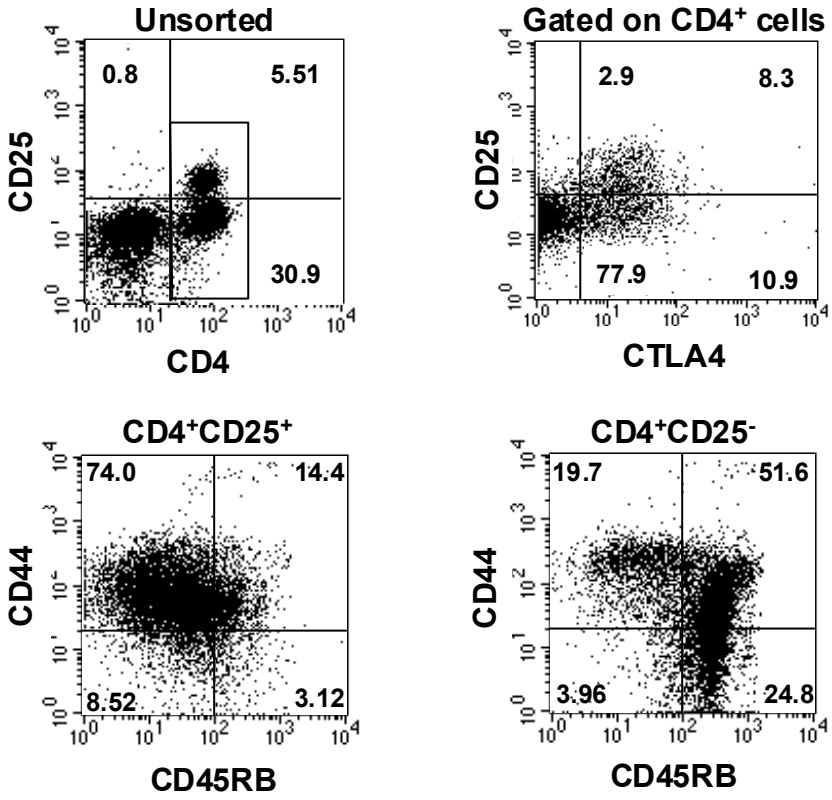

Figure 5.4 *$CD4^+CD25^+$ T cells express CTLA-4, CD44 and low levels of CD45RB*. Spleen cells from naïve CBA/Ca mice were stained *ex vivo*. Approximately 10% of the CD4+ population express CD25. The majority of the $CD4^+CD25^+$ cells constitutively express CTLA-4 and CD44, and are contained among the $CD45RB^{low}$ T cells. The $CD4^+CD25^-$ cells are predominantly CTLA-4 negative, and contained among the $CD44^-CD45RB^{high}$ cell fraction, although ~20% of these cells are $CD44^+CD45RB^{low}$.

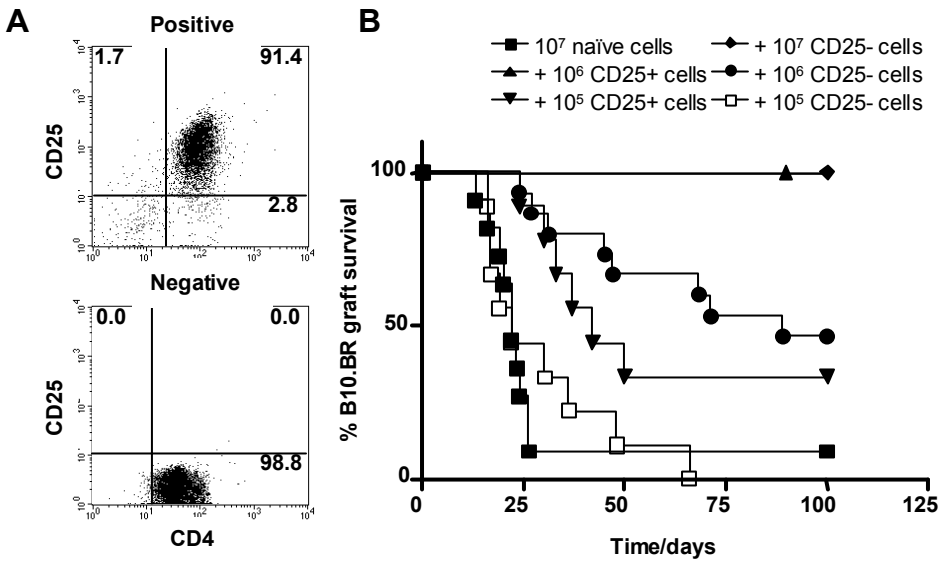

Figure 5.5 *$CD4^+CD25^+$ T cells from tolerant mice are more efficient than $CD4^+CD25^-$ cells as mediators transplantation tolerance*. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were sorted from the spleens of CBA/Ca mice tolerant to B10.BR skin transplants as described in Chapter 2. Different numbers of these cells were co-transferred i.v. with 10^7 unsorted spleen cells from naïve CBA/Ca into "empty" CP1-CBA mice. **A**, FACS profiles of the transferred $CD4^+CD25^+$ and $CD4^+CD25^-$ cells. **B**, All mice were transplanted with B10.BR skin the day following cell transfer. When unsorted spleen cells were transferred in the absence of cells from tolerant mice, the grafts were readily rejected (■, n=11, MST=22d). A similar rate of rejection was observed when 10^5 $CD4^+CD25^-$ cells were added (□, n=9, MST=22d). When the number of $CD4^+CD25^-$ cells was increased to 10^6 (●, n=15, MST=89d), or when 10^5 $CD4^+CD25^+$ cells were transferred (▼, n=9, MST=42d), rejection was significantly delayed and several animals accepted the grafts indefinitely. No rejection was observed in the groups injected with 10^6 $CD4^+CD25^+$ cells (▲, n=13, MST>100) or 10^7 $CD4^+CD25^-$ cells (◆, n=4, MST>100). This figure represents pooled results from three different experiments.

the groups transferred with 10^6 $CD4^+CD25^+$ or 10^7 $CD4^+CD25^-$ T cells. These results suggest that both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells can mediate transplantation tolerance, the $CD4^+CD25^+$ T cells being ten times more potent than $CD4^+CD25^-$ cells. However, as the number of $CD4^+CD25^-$ T cells in tolerant mice is approximately ten times higher than that of $CD4^+CD25^+$ cells, it is likely that both populations have a significant role in maintaining transplantation tolerance.

5.2.4 Regulatory potency of unsorted $CD4^+$ cells suggests that $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells are both responsible for suppression of graft rejection

I sought to study whether both $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells contribute to graft acceptance, by determining the minimum number of unseparated tolerised $CD4^+$ cells (containing both $CD4^+CD25^+$ and $CD4^+CD25^-$ subpopulations) capable of preventing graft rejection mediated by 10^7 splenocytes from naïve CBA/Ca mice. The $CD4^+$ cells were sorted from spleens of CBA/Ca mice tolerant to B10.BR skin grafts using magnetic microbeads, and different numbers of these cells were injected together with the fixed number of 10^7 splenocytes from naïve CBA/Ca mice into “empty” CP1-CBA recipients (Figure 5.6A). All animals received a B10.BR skin graft on the following day. When 10^5, or less, $CD4^+$ spleen cells were transfused the outcome was rejection (Figure 5.6B). However, adoptive transfer of $5x10^5$, or more, $CD4^+$ spleen cells from tolerant mice resulted in graft acceptance. In the CBA/Ca mouse strain ~10% of $CD4^+$ cells co-express CD25, in both naïve and tolerant animals. On the basis of these figures we can calculate that it takes approximately $5x10^4$ $CD4^+CD25^+$ cells combined with $4.5x10^5$ $CD4^+CD25^-$ cells from tolerant mice to suppress 10^7 naive spleen cells. However, we found that neither 10^5 $CD4^+CD25^+$ nor 10^6

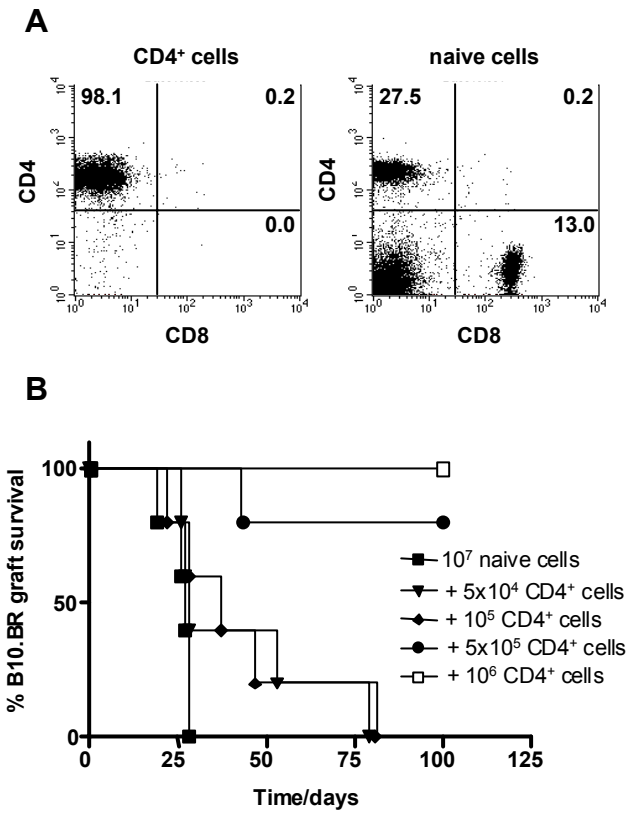

Figure 5.6 *$CD4^+$ T cells from tolerant mice prevent rejection by spleen cells from naïve mice in a dose dependent way*. Splenic $CD4^+$ cells were sorted from CBA/Ca mice tolerant to B10.BR skin grafts, as described in Chapter 2, and injected i.v. with 10^7 spleen cells from naïve CBA/Ca mice into "empty" CP1-CBA mice. **A**, FACS profiles of the sorted $CD4^+$ cells and the unsorted CBA/Ca spleen cells. **B**, All mice were grafted with B10.BR skin the day following adoptive cell transfer. The group injected with spleen cells in the absence of $CD4^+$ cells from tolerant mice, readily rejected the skin grafts (■, n=5, MST=26d). The rejection rate was not significantly delayed in the groups where $5x10^4$ (▼, n=5, MST=28d) or 10^5 (◆, n=5, MST=37d) $CD4^+$ cells were added to the 10^7 spleen cells from naïve CBA/Ca mice. However, when $5x10^5$ (●, n=5, MST>100, P=0.0027) and 10^6 (□, n=5, MST>100, P=0.0027) $CD4^+$ cells were added, the grafts were accepted indefinitely, these results being statistically significant when compared with the control group ■.

$CD4^+CD25^-$ cells alone could provide this degree of suppression (see Figure 5.5). This suggests that the "unseparated" $CD4^+$ cell population shows greater potency than the equivalent numbers of sorted $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells. Such a result could possibly reflect impairment of regulatory function from the cell-separation manipulations, or perhaps, the enhanced regulation from $CD4^+CD25^+$ and $CD4^+CD25^-$ regulatory cells operating together. Further experiments are needed to clarify this.

5.2.5 $CD4^+CD25^+$ regulatory T cells mediate infectious transplantation tolerance

Infectious transplantation tolerance has been described as the capacity of tolerant T cells to induce regulatory function on a population of non-tolerant T cells upon coexistence in a tolerised animal (Qin *et al*., 1993; Cobbold and Waldmann, 1998). I decided to investigate whether tolerant T cells "infectiously" induce regulatory capacity within the $CD4^+CD25^+$ or $CD4^+CD25^-$ cell population of non-tolerant T cells that are allowed to coexist for 6 weeks. The preliminary results suggest that regulatory function can be induced among the $CD4^+CD25^+$ T cells.

CP1-CBA mice were tolerised to B10.BR skin grafts as described. At day 100 all mice were transfused with $3.5x10^7$ spleen cells from naïve CBA/Ca mice. Three weeks following adoptive cell transfer all mice were injected with 0.5 mg of AG-MIM-IgG1 mAb. The objective of such treatment was to prevent antiglobulin response following subsequent administration of CAMPATH-1H, that was performed 21 days later (see Chapter 7). With such CAMPATH-1H treatment I was able to deplete the first cohort of tolerant T cells, with pre-treatment with AG-MIM-IgG1 and using these mAb doses depletion of T cells can be extended for at least 60 days in euthymic mice (see Chapter 7, Figure

7.10). On the same day of CAMPATH-1H depleting treatment all mice received a second B10.BR skin graft. Spleens from these mice were collected 4 weeks later and $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were sorted as described, and adoptively transferred together with 10^7 unsorted spleen cells from naïve CBA/Ca into "empty" CP1-CBA hosts. Such test mice were grafted with B10.BR skin on the following day and monitored for transplant rejection.

Mice transfused with 10^7 unsorted spleen cells alone rejected the skin grafts, as did mice where $2x10^6$ $CD4^+CD25^-$ T cells were added (Figure 5.7). However no skin graft rejection was observed in the group where $3x10^5$ $CD4^+CD25^+$ T cells were administered together with the unsorted spleen cells. Although this result suggests that infectious tolerance operates via induction of $CD4^+CD25^+$ regulatory T cells, the data lacks statistically significance due to the reduced number of animals per group. As a consequence, this observation requires confirmation in repeat experiments.

5.2.6 $CD4^+CD25^+$ and $CD4^+CD25^-$ cells differ in gene expression

In order to validate the separation procedures adopted to isolate $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells, we examined the nature of genes expressed in each population, either resting or activated with solid-phase CD3 mAbs, using SAGE[3]. A differential analysis of the four SAGE libraries is displayed in Figure 5.8 as scatter plots comparing $CD4^+CD25^-$ spleen cells with $CD4^+CD25^+$ spleen cells before and after stimulation. A number of transcripts that do not differ between the two populations before activation are highlighted in Figure 5.8A. These include the housekeeping genes EF-1α and GAPDH, the T cell specific genes CD3δ and Ly116 (a Th1 marker), the

[3] The SAGE libraries were made by Sara Thompson.

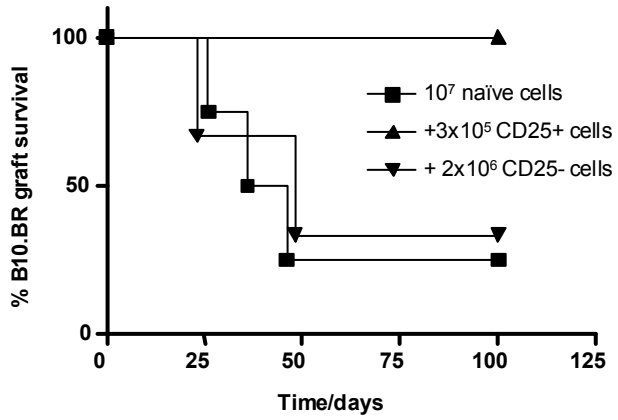

Figure 5.7 *$CD4^+CD25^+$ T cells mediate infectious transplantation tolerance*. $3.5x10^7$ spleen cells from naïve CBA/Ca mice were transfused into tolerised CP1-CBA recipients, and allowed to co-exist with the T cells of the tolerised mice for 6 weeks. After that time the CAMPATH-$1H^+$ cells were depleted and the mice received an additional B10.BR skin graft. The transfused cells were allowed to expand for another 4 weeks, before spleens were removed and their cells separated into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. $3x10^5$ $CD4^+CD25^+$ T cells prevented B10.BR graft rejection when transfused into empty CP1-CBA mice together with 10^7 unsorted spleen cells from naïve CBA/Ca mice (▲, n=4, MST>100). Most of the skin grafts were rejected when the empty CP1-CBA mice were transfused with 10^7 unsorted spleen cells alone (■, n=4, MST=41d), or in combination with $2x10^6$ $CD4^+CD25^-$ T cells (▼, n=3, MST=48d). The difference in graft survival is statistically significant between the groups where naïve cells were transfused on their own or in combination with $CD4^+CD25^+$ T cells (P=0.0401), but not between any other groups.

activation marker OX40, together with β2 microglobulin and MHC-I (K, D and L, although this latter tag is slightly higher in $CD4^+CD25^-$ cells). Very few tags appear to be specific to either one of the populations, but 28 tags are significantly up-regulated in $CD4^+CD25^+$ cells, most of which we have, as yet, been unable to assign to known genes, and 97 tags significantly up-regulated in $CD4^+CD25^-$ cells. The majority of the latter tags (at least 58) map to transcripts normally considered housekeeping genes (shown in grey, and defined as being non-differential (ie. SD<Mean) across 16 other SAGE libraries, including ribosomal proteins and essential metabolic enzymes (Zelenika *et al*., 2001; Zelenika *et al*., 2002). This relative loss of housekeeping transcripts is further exemplified after CD3 stimulation of the two populations (Figure 5.8B), and includes GAPDH, EF-1α and also β2 microglobulin (while CD3δ, MHC-I and OX40 change little). This apparent loss of housekeeping gene expression may be explained by the different capacities of the two populations to proceed through the cell cycle: it may be that $CD4^+CD25^+$ cells, which do not proliferate in response to TCR ligation, do not require many of the synthetic and metabolic enzymes, but express a set of new functional proteins without any cell division. It is clear that $CD4^+CD25^+$ cells have indeed expressed at least 103 new transcripts as a result of their activation (Figure 5.8B) and are therefore behaving in a manner that is quite distinct from $CD4^+CD25^-$ cells. Most of these tags are unique amongst the SAGE libraries that have been constructed so far (Zelenika *et al*., 2001; Zelenika *et al*., 2002), and have not yet been assigned to known genes. Although, I used SAGE results exclusively to confirm the distinctiveness of the $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, I have thus far identified 4 novel candidate genes from the Celera Discovery System mouse gene database (marked as transcripts mCT5392, mCT2519, mCT6469 and mCT4200), whose roles are currently under investigation.

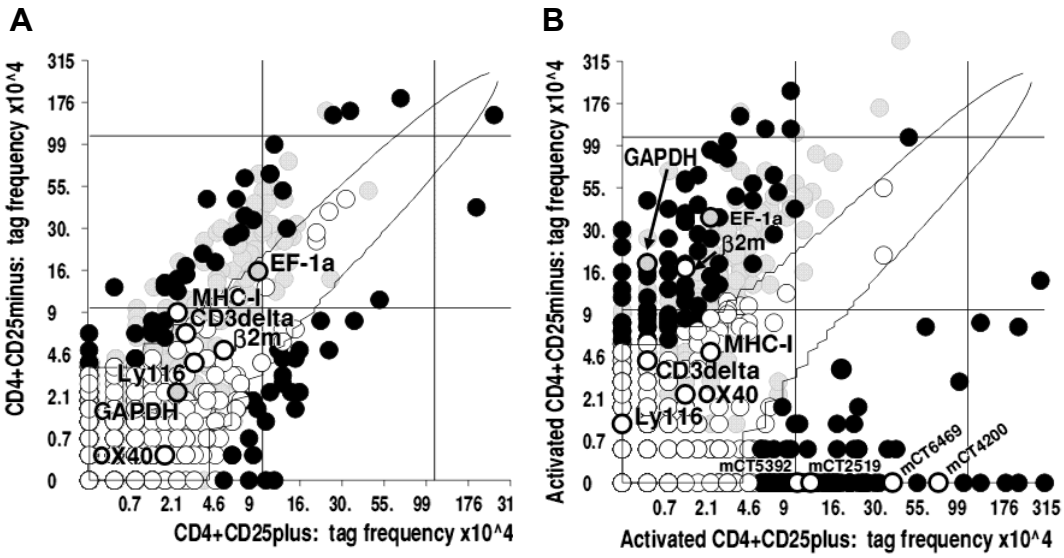

Figure 5.8 *$CD4^+CD25^+$ and $CD4^+CD25^-$ cells express different genes*. **A**, Comparison of gene expression profile of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells sorted from naïve CBA/Ca mice. **B**, Comparison of gene expression profile of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells following activation with solid-phase anti-CD3 mAb. SAGE libraries were compared using scatter plots where each SAGE gene tag is represented by a point plotted at the coordinates corresponding to the tag frequency per 10,000 tags (note logarithmic scale). Tags corresponding to genes whose expression is not differential are represented inside the diagonal area shown. Tags with a statistically differential expression (95% confidence of >1.2 fold upregulation) are those plotted outside the diagonal area. House keeping genes are defined as tags that are non-differentially expressed across a group of 20 SAGE libraries from different cell types (defined by SDev <= Mean) and are depicted in grey. For clarity, most of differentially expressed tags are represented in black, and the non-differential in white. The following gene transcripts were identified by their SAGE tags as follows: β2 microglobulin, TTTTCAAAAA; CD3δ, AGACCGGAAG; EF-1α, AGGCAGACAG; GAPDH, GCCTCCAAGG; Ly116, GCAGTGGTTC; MHC-I (K; D; L), GATTGAGAAT; OX40, CTAGCAGCTG; mCT5392, CCCAGCATCC; mCT2519, AAGGCTATGT; mCT6469, CTTCTACCAA; mCT4200, GTGGCAGGAG.

5.2.7 Dominant tolerance is not compromised by administration of mAbs targeting IL-10, IL-4, CTLA-4 and CD25

There are several conflicting reports in the literature implicating particular cytokines and cell surface molecules in dominant tolerance. In some *in vivo* and *in vitro* studies, antibodies to CTLA-4 (4F10) and CD25 (PC61) have interfered with suppression (Takahashi *et al*., 2000; Read *et al*., 2000), although others did not find a role for CTLA-4 *in vitro* (Shevach *et al*., 2001). I could not implicate CTLA-4 in our readout of suppression (see below). Other studies have also described a role for IL-10, IL-4 and TGF-β in suppression by regulatory T cells, where high-doses of mAbs were used in an attempt to neutralize the effect of the target cytokines (Powrie *et al*., 1996; Davies *et al*., 1996b; Asseman *et al*., 1999; Seddon and Mason, 1999b; Hara *et al*., 2001). I could not implicate IL-10 and IL-4 in my own studies.

$20x10^6$ spleen cells from CBA/Ca mice tolerant to B10.BR skin were transfused into "empty" CP1-CBA mice, together with the same number of spleen cells from naïve CBA/Ca animals (as described in Figure 5.1). Separate groups of these animals were treated with high doses of anti-IL4 mAb (11B11), anti-IL10 mAb (JES5) or both in combination; anti-CD25 mAb (PC61), anti-CTLA4 (4F10) or both in combination. One control group was treated with anti-canine CD8 (YCATE55), and another control group received naïve spleen cells in the absence of spleen cells from tolerant mice. The mAbs were administered in doses of 2mg at days –4, –2, 0, 5, and then weekly until rejection. The adoptive cell transfer was performed at day –1, and B10.BR skin transplants at day 0. Blood samples from all mice were collected at days 20 and 60 to determine the level of the injected mAbs in the sera. The serum concentration of the injected Abs, as determined by binding inhibition, was over 100 μg/ml in all PC61 treated mice (two mice injected

with PC61 + 4F10 had serum levels between 1 μg/ml and 10 μg/ml at day 60); all JES5 treated mice had serum concentrations of the mAb between 10 μg/ml and 100 μg/ml; all 11B11 treated mice had serum concentrations of the mAb over 100 μg/ml (except 4 mice also injected with JES5 where the mAb concentrations were between 10 μg/ml and 100 μg/ml at day 20 and 60). The serum concentration of 4F10 was determined through an anti-hamster IgG ELISA. All mice had a serum concentration of hamster Ab between 1 μg/ml and 10 μg/ml at day 20, dropping to below 1 μg/ml at day 60.

The group transferred with cells from naïve mice readily rejected the test skin grafts (Figure 5.9A). However, indefinite graft survival was observed in all other groups, suggesting that those particular targeted molecules do not play a critical role in dominant transplantation tolerance in this model. A similar experiment focussed on the naïve $CD4^+CD25^+$ population. I transferred 10^6 sorted $CD4^+CD25^+$ spleen cells from naïve CBA/Ca mice, together with 10^7 splenocytes from naïve CBA/Ca mice, as described. Some of the mice were treated with a combination of anti-CTLA4 and anti-IL10 mAbs in the doses mentioned above. Treatment with these mAbs did not result in any significant difference in tolerance induced by $CD4^+CD25^+$ cells (Figure 5.9B). A similar experiment with sorted $CD4^+CD25^+$ spleen cells from tolerised mice had a comparable result, with none of the mice treated with the mAbs rejecting their grafts.

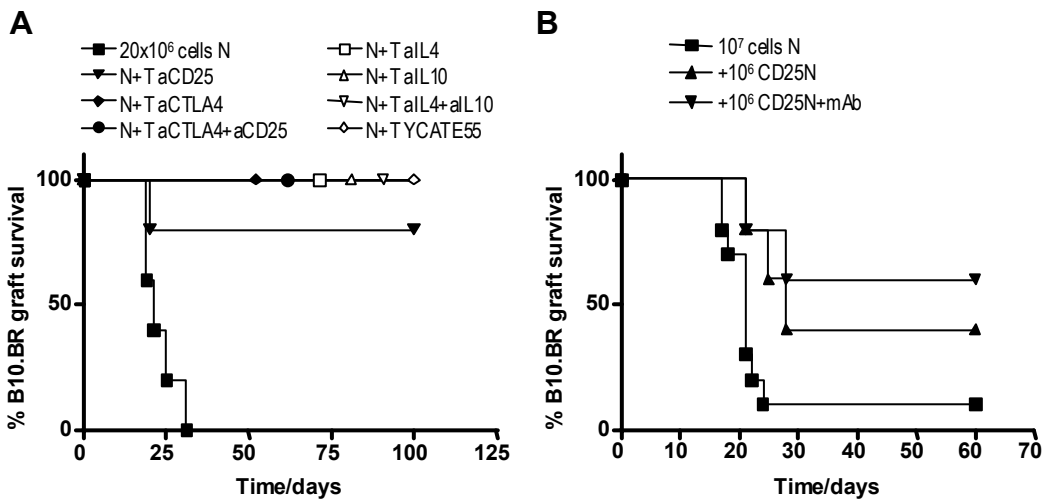

Figure 5.9 *Dominant tolerance operates in spite of treatment with high-dose mAbs targeting IL-10, IL-4, CTLA-4 and CD25*. **A**, 20x10^6 spleen cells from naïve CBA/Ca were injected into "empty" CP1-CBA mice i.v., either alone (N) or together with the same number of spleen cells from CBA/Ca tolerant to B10.BR skin (N+T). Mice were injected i.p. with 2 mg of mAbs targeting CD25, CTLA-4, IL-4, IL-10 and canine CD8 (control) at days –4, –2, 0, 2, 5 and then weekly until rejection. Cells were injected i.v. at day –1, and all animals received B10.BR skin transplants at day 0. Animals transferred with 20x10^6 spleen cells from naïve CBA/Ca in the absence of mAb treatment, readily rejected the grafts (■, n=5, MST=21d). However, in all other groups grafts were accepted indefinitely (n=5, MST>100, P=0.0198 for ▼, P=0.0017 for any other group). Blood samples were collected at days 20 and 60 to confirm the presence of the injected mAb in the sera. **B**, "Empty" CP1-CBA mice were transfused with 10^7 spleen cells from naïve CBA/Ca mice, alone (■, n=10, MST=21d) or in combination with 10^6 CD4$^+$CD25$^+$ spleen cells also from naïve CBA/Ca donors (▼ and ▲, n=5 in each group). All mice were transplanted with B10.BR skin on the following day. Some mice transfused with CD4$^+$CD25$^+$ T cells were treated with 2 mg of each of anti-IL10 and anti-CTLA4 as described above (▼). There was no significant difference in graft survival between the mAb treated and untreated groups.

5. 3 Discussion

One outstanding issue concerning *in vivo* suppression by $CD4^+CD25^+$ regulatory T cells concerns their antigenic specificity. Previous studies have established that dominant transplantation tolerance induced with non-depleting mAb is antigen specific, as mice tolerised to one type of skin graft reject subsequent transplants of different types (Qin *et al*., 1990; Chen *et al*., 1996; Chapter 4). Experimental transplantation offers the opportunity to assess the suppressive capacity of $CD4^+CD25^+$ regulatory T cells isolated from animals that had never experienced the transplantation antigens. When such experiments were performed I uncovered the capacity of $CD4^+CD25^+$ T cells from non-transplanted animals to prevent graft rejection mediated by unsorted splenocytes from the same donors (Figure 5.2). One interpretation for these results is that aggression and tolerance are the outcome of situations dictated by the numerical balance between regulatory and effector cells, and that by changing such balance one may alter the outcome of an immune response.

This result differs from previously published data (Gregori *et al*., 2001; Hara *et al*., 2001; Taylor *et al*., 2001), however, in my experiments the number of $CD4^+CD25^+$ cells injected into each recipient was much higher than the numbers used in those studies. I suggest that the optimal regulator-effector ratio can only be reached when such high numbers of regulatory cells are transferred. In fact, one previous study demonstrated that purified $CD4^+CD25^+$ from naïve animals were incapable of making a graft versus host allogeneic response upon transfer into a mismatched immunodefficient recipient (Taylor *et al*., 2001). In another study, analyzing islet graft rejection, $CD4^+CD45RB^{low}$ cells from naïve donors could, when co-administered at an appropriate ratio, prevent graft rejection by $CD4^+CD45RB^{high}$ cells (Davies *et*

al., 1999). This is also consistent with the observation of Sakaguchi *et al*, that depletion of the $CD25^+$ T cells increases the speed of first-set allograft rejection (Sakaguchi *et al*., 1995).

The antigen specificity of the $CD4^+CD25^+$ T cells from naïve animals that suppress transplant rejection is currently unknown. $CD4^+CD25^+$ cells have been shown to inhibit the proliferation of $CD4^+CD25^-$ cells to different alloantigens *in vitro*, as long as the $CD4^+CD25^+$ cells are themselves pre-activated (Thornton and Shevach, 2000). It may be that their repertoire contains receptors directed towards self-antigens (Seddon and Mason, 1999b). If so, then there is the possibility that self-reactive regulators mediate graft acceptance through "linked suppression" where they are brought into the local microenvironment of the alloreactive cell. Alternatively, the receptor repertoire of $CD4^+CD25^+$ T cells may show cross-reactivity to alloantigens present in the graft. Given the finding that suppression involves indirect presentation of antigen (Wise *et al*., 1998), the "alloantigens" in question are likely to be donor-type peptides presented in conjunction with host-type MHC. At this moment all the above hypotheses are possible, and the specificity of regulatory T cells remains a fundamental issue to resolve in the field of immune tolerance.

By studying the regulatory potency of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from tolerised mice I established that both populations can mediate transplantation tolerance, the $CD4^+CD25^+$ T cells being ten times more potent than $CD4^+CD25^-$ cells from tolerised animals or $CD4^+CD25^+$ cells from naïve mice (Figure 5.5).

These findings also contrast with previous reports describing a lack of regulatory capacity within the $CD4^+CD25^-$ population in transplantation

tolerance (Gregori *et al*., 2001; Hara *et al*., 2001). Although our experimental system is different to those used by Hara *et al* and Gregori *et al* in many respects, they may not have reached the appropriate cell doses of $CD4^+CD25^-$ cells required for suppression. Experiments in animal models of autoimmunity have also described a regulatory role for $CD4^+CD25^-$ peripheral T cells (Fowell and Mason, 1993; Stephens and Mason, 2000; Olivares-Villagomez *et al*., 2000; Shevach, 2001). In one of these reports the difference in cell numbers between $CD4^+CD25^+$ and $CD4^+CD25^-$ cells required to achieve equivalent suppression is comparable to our findings (Stephens and Mason, 2000).

The observation that the potency of the $CD4^+CD25^+$ population seems to increase following induction of transplantation tolerance is intriguing. It is not clear at this time whether this is due to an expansion of the regulatory cells from pre-existing regulators, whether it results from *de novo* formation of regulatory cells, or whether this reflects selective inactivation or death of non-tolerant cells, so shifting the functional bias of the population towards regulation. It is equally interesting that the $CD4^+CD25^-$ population is only seen to regulate if derived from tolerant, but not naïve populations. It may be that some "tolerant" $CD25^+$ regulatory cells lose the expression of CD25 and endow the $CD4^+CD25^-$ population with new regulatory powers, as it has been suggested following homeostatic expansion of $CD4^+CD25^+$ T cells (Gavin *et al*., 2002). Alternatively, AICD previously reported to occur in the induction of transplantation tolerance (Wells *et al*., 1999; Li *et al*., 1999), may selectively remove effector cells from the $CD25^-$ population so unmasking residual regulatory cell activity.

It is remarkable that the difference in regulatory potency between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from tolerised mice is about 10-fold, as

the number of $CD4^+CD25^-$ T cells in tolerant mice is approximately ten times higher than that of $CD4^+CD25^+$ cells. Taken these figures in consideration, it is likely that both populations have a significant role in maintaining transplantation tolerance. Such an hypothesis is further reinforced by the demonstration that the regulatory potency of unsorted $CD4^+$ cells is greater than the equivalent number of each $CD4^+CD25^+$ and $CD4^+CD25^-$ subpopulations (Figure 5.6).

The cellular mechanisms underlying the well established phenomenon of infectious tolerance still remain to be elucidated. I investigated whether coexistence of spleen cells from naïve donors with regulatory T cells within tolerised mice could increase the suppressive potency of the $CD4^+CD25^+$ or $CD4^+CD25^-$ cells, as seen following mAb induced tolerance. Although the described results still require confirmation, they suggest that by means of infectious tolerance the $CD4^+CD25^+$ T cells acquire a regulatory capacity superior to the one demonstrated by $CD4^+CD25^+$ cells from naïve mice, and comparable to the potency of cells with this phenotype from tolerised mice (Figure 5.7). The reciprocal population of $CD4^+CD25^-$ T cells did not seem to acquire regulatory capacity as seen following mAb tolerisation. If confirmed, such results may suggest that tolerisation of the $CD4^+CD25^+$ and $CD4^+CD25^-$ compartments may have different requirements. One can speculate that while the increased potency of $CD4^+CD25^+$ cells may be due to expansion of specific regulatory cells, the regulatory capacity found within the $CD4^+CD25^-$ cells may be determined by another mechanism, such as deletion of aggressive cells. Infectious tolerance and mAb induced tolerance may explore diverse mechanisms for tolerance induction.

When neutralizing mAb to IL-10, IL-4, CTLA-4 or CD25 were used, I failed to demonstrate a role for any of these molecules as being essential for

regulatory function (Figure 5.9). Previous demonstrations of roles for these molecules in other experimental readouts suggest: (a) that regulation may involve diverse molecular mediators depending on the precise microenvironment where it operates; or (b) that dominant tolerance exploits multiple redundant suppressive pathways where blockade of any one would not impact the outcome; or (c) that some of the effects seen are on the effector population in rendering them more sensitive to antigen mediated signals – this may be a plausible explanation for the effects of CTLA-4 mAbs, which should in principle enhance signalling (Hurwitz *et al*., 2002; Egen and Allison, 2002); or (d) that regulation takes place within a compartment which the injected mAb cannot easily access, such as the transplanted skin graft itself (see Chapter 6).

The present results confirm that where transplantation tolerance was induced with therapeutic mAbs the subpopulation most potent in maintaining tolerance are the $CD4^+CD25^+$ T cells. However, regulatory activity can also be demonstrated within the $CD4^+CD25^-$ population. It is not clear whether this is a result of redundant tolerogenic strategies mediated by different cell populations, or whether it reflects the fact that a proportion of the regulatory T cell population does not express CD25 constitutively. Either way, the characterization of genes differentially expressed by $CD4^+CD25^+$ and $CD4^+CD25^-$ cells may certainly contribute to a better understanding of their physiology, may provide new markers that better define regulatory T cells, and may lead to the development of diagnostic tests for monitoring T cell population changes in autoimmunity or therapeutic tolerance.

CHAPTER 6

IDENTIFICATION OF REGULATORY T CELLS IN TOLERATED ALLOGRAFTS

Induction of transplantation tolerance with certain therapeutic non-depleting monoclonal antibodies can lead to a robust state of peripheral "dominant" tolerance. Regulatory $CD4^+$ T cells, which mediate this form of "dominant" tolerance can be isolated from the spleen of tolerant animals. In order to determine whether there were any extra-lymphoid sites that might harbour regulatory T cells their presence was sought in tolerated skin allografts and in normal skin. When tolerated skin grafts are re-transplanted onto T cell depleted hosts, graft infiltrating T cells exit the graft and recolonise the new host. These colonising T cells can be shown to contain members with regulatory function, as they can prevent non-tolerant lymphocytes from rejecting fresh skin allografts, without hindrance of rejection of third party skin. Such results suggest that T cell suppression of graft rejection is an active process that operates beyond secondary lymphoid tissue, and involves the persistent presence of regulatory T cells at the site of the tolerated transplant[4].

[4] Most of the results presented in this Chapter were published in (Graca *et al*., 2002a).

6.1 Introduction

In recent years significant advances have been made in enabling the therapeutic induction of transplantation tolerance (Waldmann, 1999; Wekerle and Sykes, 2001; Knechtle, 2000; Kirk and Harlan, 2000a; Li *et al*., 2001; Waldmann, 2001). In rodents it is possible to induce a robust form of peripheral tolerance by treatment with non-depleting mAbs, such as the combination of anti-CD4 and anti-CD8, at the time of transplantation (Qin *et al*., 1990; Qin *et al*., 1993; Davies *et al*., 1996a; Chen *et al*., 1996; Waldmann and Cobbold, 1998; Waldmann and Cobbold, 2001). Tolerance so achieved is dependent on regulatory T cells that disarm non-tolerant naïve cells (dominant tolerance) and facilitate the emergence of novel regulatory cells from the naïve lymphocyte population (infectious tolerance) (Qin *et al*., 1993; Chen *et al*., 1996; Graca *et al*., 2000); Chapters 3 and 4). The regulatory cells which fulfill this role are known to be $CD4^+$ (Qin *et al*., 1993), and contained in both the $CD4^+CD25^+$ and $CD4^+CD25^-$ populations (Graca *et al*., 2002b; Chapter 5).

Dominant transplantation tolerance has been shown capable to be extended to third-party antigens provided they are genetically linked to the tolerated ones in the same tissue (Davies *et al*., 1996a; Chen *et al*., 1996; Wong *et al*., 1997; Honey *et al*., 1999; Chapter 4). This phenomenon, known as “linked suppression”, does not occur when the tolerated and third party antigens are provided in two separate skin grafts transplanted at the same time onto the same graft bed (Davies *et al*., 1996a; Honey *et al*., 1999; Chapter 4). It may be that regulatory T cells acting at the level of the graft itself mediate linked suppression.

It has been repeatedly demonstrated that such regulatory T cells can be isolated from the spleens of tolerant mice (Qin *et al*., 1993; Zhai and Kupiec-Weglinski, 1999; Waldmann and Cobbold, 2001; Chapter 5). Recent work has suggested that in tolerant rats T cells infiltrating tolerated kidneys are enriched for regulatory cells when compared with the splenic T cells (Sawitzki *et al*., 2001). The work described in this chapter shows that regulatory T cells which can mediate dominant transplantation tolerance are present within tolerated skin allografts. The presence of regulatory T cells in the tolerated transplanted tissue may indicate that they have a protective role within that tissue.

6.2 Results

6.2.1 The experimental system

As the number of T cells that can be isolated from tolerated skin grafts is very low, it was necessary to develop an experimental system allowing expansion of rare resident T cells for analysis of their suppressive capacity *in vivo* (Figure 6.1).

Tolerised CBA/Ca or CP1-CBA mice were used as donors of tolerated skin, 100 to 120 days following tolerance induction with three doses of 1 mg of each of non-depleting CD4 and CD8 mAbs over one week. The experimental skin allografts were removed from the initial hosts and regrafted onto recipients without T cells ("empty" mice). These "empty" mice were either RAG1$^{-/-}$ mice, or adult thymectomised CP1-CBA mice T cell depleted with CAMPATH-1H mAb.

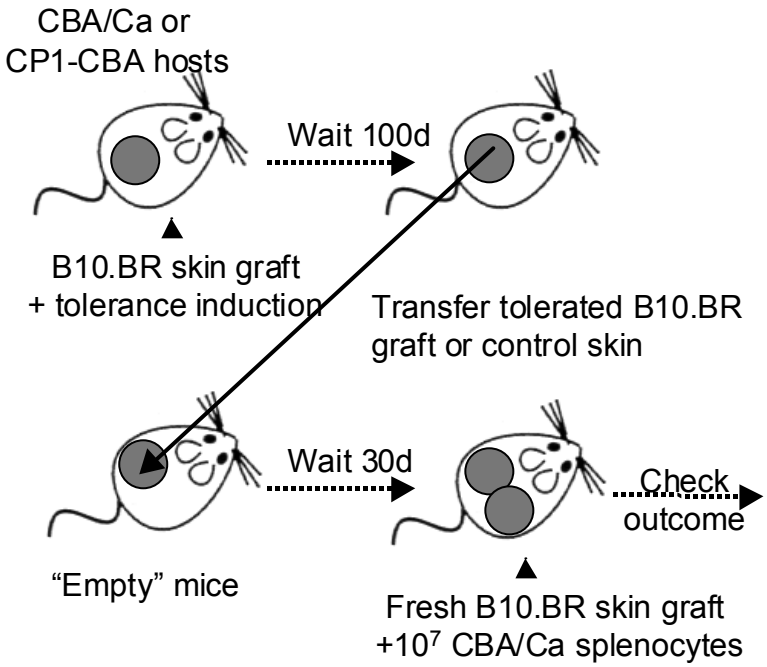

Figure 6.1 *The experimental system.* CBA/Ca or CP1-CBA were made tolerant to B10.BR skin grafts by treatment with non-depleting CD4 and CD8 mAbs. 100 days following tolerance induction the tolerated skin grafts, or autologous control skin, were removed and transplanted onto "empty" mice (either adult thymectomised and T cell depleted CBA-CP1 mice, or RAG1$^{-/-}$-CBA mice). Following 30 days the mice were transfused with 10^7 splenocytes from naive CBA/Ca mice, together with a fresh B10.BR skin graft. The possible outcomes are: rejection, when a non-tolerant pre-existing state permits the transfused cells to mediate graft rejection; or acceptance of the skin grafts, when tolerated grafts lead to a tolerance state that is non-permissive for graft rejection by the transfused splenocytes.

Cells were allowed to expand from the allograft for 30 days. At that time the mice were challenged with a fresh B10.BR skin graft, together with a transfusion of 10^7 splenocytes from naive CBA/Ca donors. The suppressive capacity of any regulatory T cells which may have emerged from tolerated allografts was assessed by their capacity to prevent skin graft rejection mediated by the transfused naïve splenocytes.

6.2.2 Tolerated skin grafts can transfer dominant tolerance when re-grafted onto new recipients

I investigated whether tolerated skin from animals exhibiting dominant tolerance plays host to regulatory T cells. Here, I show that the re-grafting of tolerated B10.BR skin transplants into T cell depleted hosts leads to a dominant tolerant state, such that adoptively transferred splenocytes from naïve donors are prevented from rejecting fresh allografts (Figure 6.2).

Tolerated B10.BR skin grafts, as well as control CBA/Ca skin, were re-grafted onto “empty” CP1-CBA mice. 30 days following the grafting of these empty CP1-CBA mice with tolerated B10.BR or control CBA/Ca skin grafts, all mice were transfused with 10^7 splenocytes from naïve CBA/Ca donors and challenged with a fresh B10.BR skin graft. Figure 6.2A shows that the group of mice transplanted with tolerated B10.BR skin grafts, was able to resist the rejection by naïve cells. However, groups transplanted with CBA/Ca skin from the same tolerant donors remained permissive for rejection, with a rate similar to the animals grafted with CBA/Ca skin from naïve donors, and to the control recipients that had not received any preparatory skin graft.

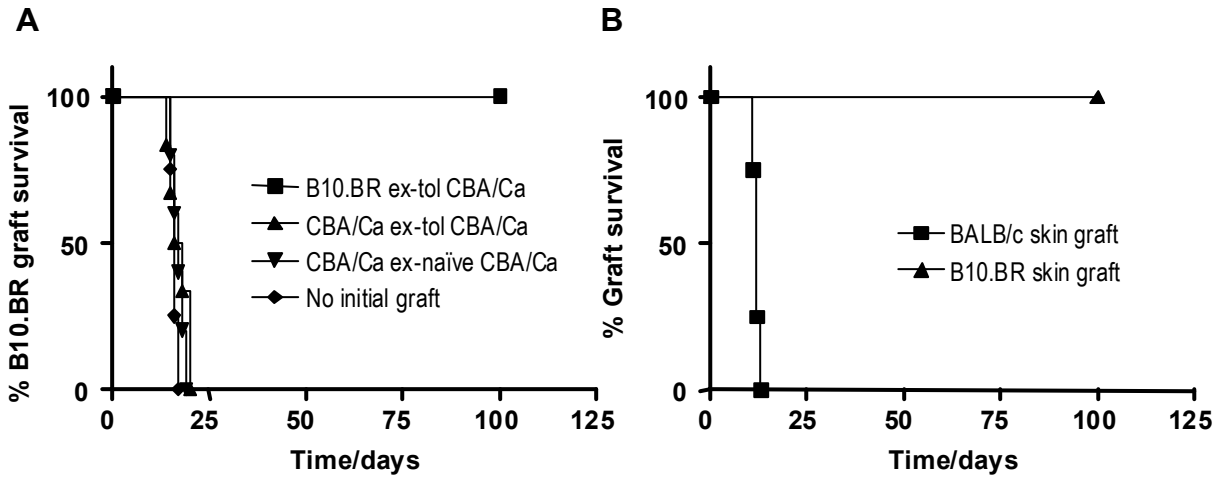

Figure 6.2 *Tolerated skin grafts can transfer the tolerant state upon regraft*. CP1-CBA mice were thymectomised at 4 weeks of age, and depleted of T cells with 0.25 mg CAMPATH-1H. **A**, At day –30, these mice were transplanted with tolerated B10.BR skin grafts from tolerant CBA/Ca (■), CBA/Ca skin from the CBA/Ca tolerant to B10.BR skin grafts (▲), or CBA/Ca skin from naïve donors (▼). A control group of mice did not receive any initial skin graft (◆). All mice were transfused with 10^7 spleen cells from naïve CBA/Ca at day –1, and transplanted with a fresh B10.BR skin on the following day. Only mice with tolerated skin grafts resisted the challenge transfusion of non-tolerant splenocytes and accepted the B10.BR skin grafts indefinitely (■, n=5, median survival time (MST)>100d, $P<0.002$ to other groups). In all other groups the B10.BR skin grafts were rejected at a similar rate. **B**, Tolerant mice were grafted with both BALB/c (■) and B10.BR (▲) skin grafts in the same graft bed, 60 days following challenge with naïve CBA/Ca splenocytes and a fresh B10.BR skin. Only BALB/c skin grafts were rejected ($P<0.007$).

To confirm that tolerant animals were not globally immunosuppressed, I showed that the same recipient test animals remained permissive for rejection of third-party skin. BALB/c and fresh B10.BR skin were transplanted in the same graft bed of mice of the non-permissive group. Figure 6.2B shows that the third-party BALB/c skin grafts were promptly rejected while the B10.BR skin grafts were accepted indefinitely.

Taken together, these results confirm that only the tolerated skin grafts, but not autologous skin from tolerant animals, had the capacity to transfer dominant tolerance.

6.2.3 Tolerance is not due to microchimerism

There is evidence implicating donor microchimerism as a mechanism capable of enhancing graft acceptance (Ko *et al*., 1999; Anderson and Matzinger, 2001). To investigate whether microchimerism was the explanation for tolerance induced by transfer of tolerated skin grafts, the experiment was repeated but this time grafting CBA/Ca mice with skin from (B10.BR x CBA/Ca)F_1. Such skin grafts could contribute to the generation of donor-type microchimerism, with cells simultaneously carrying CBA/Ca and B10.BR antigens and being naturally tolerant, by deletion, to both sets of antigens (without B10.BR specific regulatory T cells). “Empty” CP1-CBA mice were transplanted with tolerated B10.BR skin grafts from tolerant CBA/Ca, another group with (B10.BR x CBA/Ca)F_1 skin grafts previously transplanted onto syngeneic F_1 mice, and yet another group with fresh (B10.BR x CBA/Ca)F_1 skin. In one control group, the empty CP1-CBA mice received no grafts. A i.v. infusion of 10^7 spleen cells from naïve CBA/Ca mice was administered to all CP1-CBA mice 30 days following grafting. All mice received a fresh B10.BR skin graft on the following day. Figure 6.3 shows

that only the animals grafted with tolerated B10.BR skin from tolerant CBA/Ca were non-permissive for naïve cells to reject the B10.BR skin grafts. The empty mice, which had been grafted with (B10.BR x CBA/Ca)F_1 skin, remained permissive and skin was rejected at rate similar to controls.

6.2.4 Tolerance is due to regulatory T cells present in the skin graft

To establish the role of putative regulatory T cells infiltrating the skin graft CP1-CBA mice, tolerised to B10.BR skin grafts, were used as donors of tolerated B10.BR skin. This enabled the use CAMPATH-1H mAb to deplete donor T cells present in the tolerated skin, once it had been re-transplanted. Figure 6.4 shows that when tolerated skin was obtained from tolerant CBA/Ca donors, hosts became non-permissive for the rejection of fresh B10.BR skin graft after transfusion of 10^7 non-tolerant spleen cells. However, when the tolerated B10.BR skin was derived from tolerant CP1-CBA donors, and the hosts depleted of all donor derived and recipient T cells by using 0.25 mg CAMPATH-1H at the time of re-graft, grafts were rejected after the transfusion of naïve CBA/Ca splenocytes.

6.2.5 T cells can expand from the tolerated B10.BR skin grafts

We used RAG1$^{-/-}$ mice as hosts completely deficient in T cells to determine whether T cells infiltrating tolerated grafts could expand from the skin. These mice were grafted with tolerated B10.BR skin from either tolerant CBA/Ca or tolerant CP1-CBA, or autologous CBA/Ca skin from CBA/Ca mice tolerant to B10.BR skin grafts. A sample of peripheral blood was collected 30 days

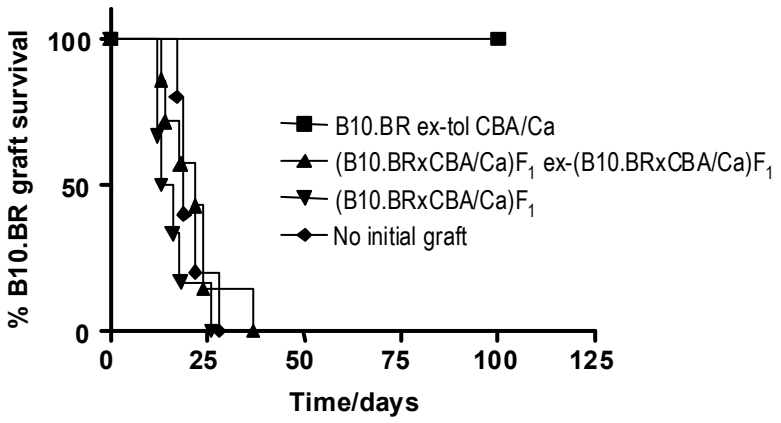

Figure 6.3 *Tolerance is not due to microchimerism.* Empty CP1-CBA mice were transplanted at day –30 with tolerated B10.BR skin grafts from tolerant CBA/Ca (■), $(B10.BRxCBA/Ca)F_1$ skin grafts (▼), or $(B10.BRxCBA/Ca)F_1$ skin grafts transplanted 30 days before into syngeneic hosts (▲). A control group of mice did not receive any initial skin graft (◆). All mice were transfused with 10^7 spleen cells from naïve CBA/Ca at day –1, and transplanted with a fresh B10.BR skin on the following day. Only recipients of tolerated skin grafts resisted the transfusion of non-tolerant splenocytes and accepted the B10.BR skin grafts indefinitely (■, n=5, MST>100d, P<0.002). In all other groups the B10.BR skin grafts were rejected at a similar rate.

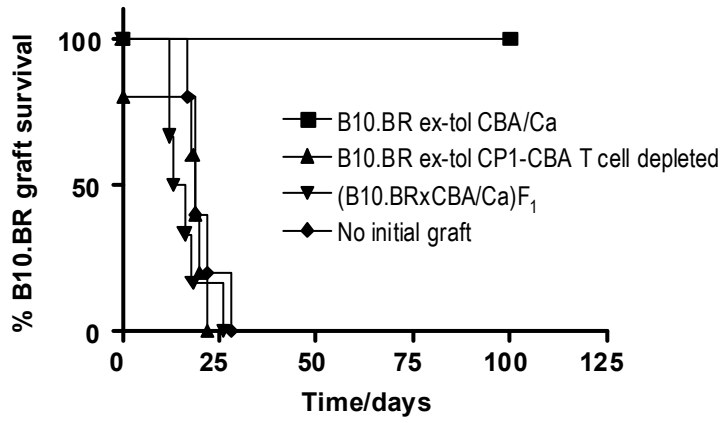

Figure 6.4 *Tolerance is due to regulatory T cells present in the skin graft*. Empty CP1-CBA mice were transplanted at day –30 with tolerated B10.BR skin grafts from tolerant CBA/Ca (■), tolerated B10.BR skin grafts from tolerant CP1-CBA (▲), or (B10.BRxCBA/Ca)F_1 skin (▼). A control group of mice did not receive any initial skin graft (◆). All mice were transfused with 10^7 spleen cells from naïve CBA/Ca at day –1, and transplanted with a fresh B10.BR skin on the following day. Mice transplanted with tolerated B10.BR skin grafts from tolerant CP1-CBA (▲) were depleted of infiltrating T cells by treatment with 0.25 mg CAMPATH-1H at days –30 and –1. Recipients of tolerated B10.BR skin grafts from tolerant CBA/Ca were also treated with CAMPATH-1H as described. Only recipients of tolerated skin grafts whose T cells had not been ablated resisted the transfusion of non-tolerant splenocytes and accepted B10.BR skin grafts indefinitely (■, n=5, MST>100d, P<0.002). In all other groups the B10.BR skin grafts were rejected at a similar rate. Note that one animal in the tolerated skin, T cell depleted group (▲) rejected the initial B10.BR graft before transfusion with CBA/Ca splenocytes.

following transplantation, stained and analyzed by flow cytometry. Figure 6.5A shows that $CD4^+$ T cells can be detected in the peripheral blood of transplanted $RAG1^{-/-}$ mice 30 days following tolerated skin transplantation. Remarkably, the $CD4^+$ T cell frequency was significantly increased in recipients of tolerated skin grafts when compared with recipients of autologous skin from tolerant mice. In all mice the majority of $CD4^+$ cells that had expanded from the graft were $CD4^+CD25^-$, but a minority of $CD4^+CD25^+$ cells could also be detected (Figure 6.5B). The frequency of $CD4^+CD25^+$ T cells within the $CD4^+$ T cell population derived from tolerated skins was not significantly different from the usual frequency in naïve CBA/Ca mice.

One week after the blood sampling, all animals were transfused with 10^7 spleen cells from naïve CBA/Ca mice, and challenged with a fresh B10.BR skin graft on the following day. In one group of mice transplanted with tolerated B10.BR skin from tolerant CP1-CBA donors, donor T cells were depleted with 0.25 mg CAMPATH-1H at the time of CBA/Ca cell transfusion. These mice became permissive for rejection by naïve CBA/Ca cells, with a rejection rate comparable to the group initially grafted with CBA/Ca skin from tolerant CBA/Ca mice (Figure 6.5C). In contrast, when the $RAG1^{-/-}$ mice were initially transplanted with tolerated B10.BR skin grafts, in the absence of T cell depletion, all mice became non-permissive for rejection, and consequently all B10.BR grafts were held indefinitely.

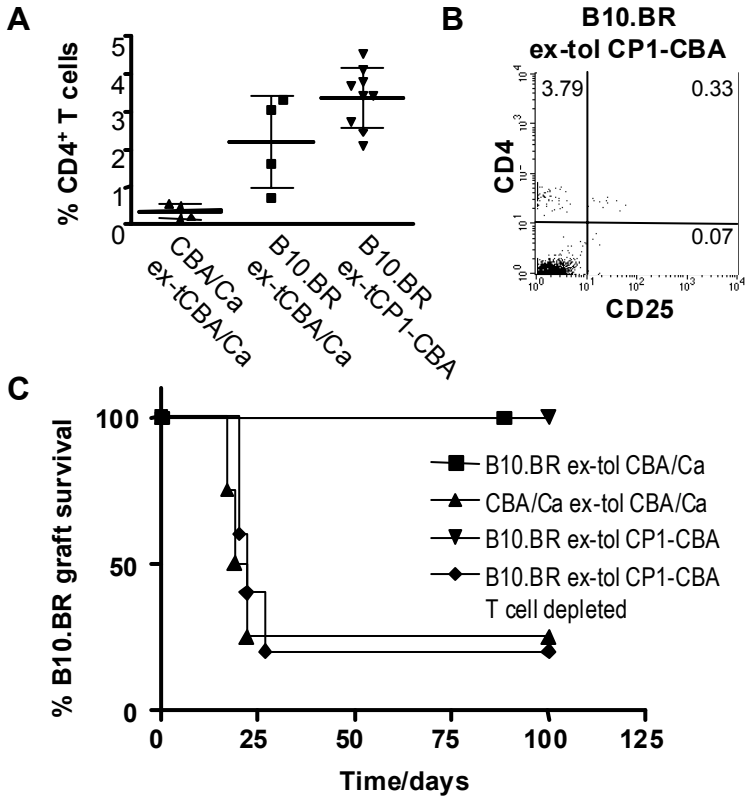

Figure 6.5 *T cells expand from the tolerated B10.BR skin grafts.* RAG1$^{-/-}$-CBA mice were grafted with tolerated B10.BR skin from tolerant CBA/Ca mice (■), CBA/Ca skin from CBA/Ca mice tolerant to B10.BR skin grafts (▲), and tolerated B10.BR skin grafts from tolerant CP1-CBA mice (▼ and ◆). Tolerated B10.BR skin grafts in group ◆ were depleted of putative infiltrating T cells with 0.25 mg CAMPATH-1H at day –1. **A**, Blood samples were collected 30 days after skin grafting and analysed by FACS. The graph represents the percentage of CD4$^+$ T cells within blood mononuclear cells. The percentage of CD4$^+$ T cells that expanded from tolerated skin grafts is significantly higher than in the animals grafted with CBA/Ca skin from tolerant syngeneic donors ($P<0.05$, unpaired t test). **B**, FACS staining from a mouse of the tolerated skin group (▼), showing that expanded T cells are mainly CD4$^+$CD25$^-$. **C**, All mice were transfused with 10^7 spleen cells from naïve CBA/Ca one week following blood tests, and transplanted with a fresh B10.BR skin on the following day (day 0). Recipients of tolerated B10.BR skin grafts whose putative regulatory T cells had not been depleted resisted the challenge with transfused CBA/Ca splenocytes and accepted the B10.BR skin grafts indefinitely (■ and ▼, MST>100d, $P<0.05$). Mice that were recipients of tolerated B10.BR skin grafts depleted of T cells rejected the grafts shortly after transfusion of CBA/Ca splenocytes (▲, MST=22d). Recipients of CBA/Ca skin from CBA/Ca mice tolerated to B10.BR skin grafts also rejected B10.BR skin grafts (◆, MST=20.5d).

6.2.6 T cells from non-tolerated B10.BR skin allografts can also expand, but this does not lead to tolerance

I wanted to exclude the possibility that the process of transplanting donor skin to $RAG^{-/-}$ recipients was not itself conducive to the development of dominant tolerance. I found that transfer of non-tolerated skin allografts indeed led to T cell expansion, but did not modify the rejection capacity of transfused splenocytes from naïve syngeneic mice. CBA/Ca mice were transplanted with B10.BR in the absence of antibody treatment. At day 8 following transplantation, when the skin grafts still appear healthy (rejection usually occurs at days 11 – 15), the skin grafts were removed from the initial hosts and re-transplanted onto $RAG1^{-/-}$ mice. These grafts were all rejected (n=6, MST=9d from the time of regraft). At day 30 following transplantation, blood samples were collected to confirm $CD4^+$ T cell expansion (4.92% $\pm$ 0.37 $CD4^+$ T cells in peripheral blood). One week after the blood sample, all mice were transfused with 10^7 splenocytes from naïve CBA/Ca donors and transplanted with a second B10.BR skin on the following day. All skin grafts were readily rejected (n=6, MST=17d) confirming that the T cell expansion from non-tolerated allografts did not alter the rejection permissive state. This result reinforces the conclusion that the regulatory T cells pre-existed in tolerated skin prior to retransplantation onto $RAG1^{-/-}$ recipients.

6.3 Discussion

One outstanding issue concerning immune regulation concerns the location where regulatory T cells operate. It has been shown in different experimental systems that regulatory T cells can be isolated from the spleen of tolerant animals (Qin *et al*., 1993; Chen *et al*., 1996; Zhai and Kupiec-Weglinski, 1999; Sawitzki *et al*., 2001; Waldmann and Cobbold, 2001). The results

described in this chapter conclusively demonstrate that regulatory T cells can also be identified within the tolerated tissue itself.

The experiments with (B10.BR x CBA/Ca)F_1 skin grafts exclude microchimerism as being sufficient to drive tolerance achieved by transferring tolerated skin grafts. In addition, they also exclude any requirement for the thymus in the maintenance of the tolerant state, as adult thymectomised recipient mice can be rendered tolerant following transplantation of a tolerated skin allograft.

Studies concerning neonatal tolerance had implicated pro-tolerogenic properties of neonatal skin in achieving tolerance to skin antigens (Alferink *et al*., 1998; Alferink *et al*., 1999). The present findings exclude pro-tolerogenic properties of the tolerated skin allografts. In fact, when T cells (defined as transgenic cells that express hCD52) carried over with tolerated skin grafts are depleted, then tolerance is not imposed on the recipient. As a corollary, when recipients of tolerated skin allografts resist rejection mediated by transfused T cells, such a non-permissive state must be due to regulatory T cells which have infiltrated the tolerated skin grafts, and not to cells of a different type.

I also established that tolerance cannot be achieved by transplantation of autologous skin from tolerised mice. The study of lymphocyte expansion from transplanted skins suggests that very few T cells infiltrate autologous skin of tolerised mice, when compared with tolerated allografts. In Chapter 5, I showed that B10.BR skin graft rejection mediated by 10^7 splenocytes transfused from naïve CBA/Ca into empty CP1-CBA mice, could be prevented by co-transfer of regulatory T cells. By titrating the number of transfused regulatory cells I concluded that abrogation of rejection requires

co-transfer of 10^6 $CD4^+CD25^+$ cells or 10^7 $CD4^+CD25^-$ cells from CBA/Ca tolerised to B10.BR skin grafts (Graca *et al*., 2002b; Chapter 5). Such observations, taken together with the present results, suggest that at the time 10^7 splenocytes from naïve CBA/Ca mice were transfused, regulatory cells from tolerated allografts had expanded to evoke a regulatory function equivalent to 10^6 $CD4^+CD25^+$ cells from a tolerised spleen.

The observations described in this chapter may in part explain the phenomenon of linked suppression (Davies *et al*., 1996a; Chen *et al*., 1996; Wong *et al*., 1997; Honey *et al*., 1999; Chapter 4). When dominant transplantation tolerance is achieved, the tolerised animals accept subsequent grafts expressing third-party antigens when such tissues also express tolerated antigens. But third-party skin grafts are rejected when transplanted simultaneously with a skin graft of the tolerated type onto the same graft bed. The present study was designed as a way to investigate whether regulatory T cells capable of maintaining dominant tolerance could be demonstrated within the tolerated tissue. It is possible that the reason for the graft acceptance when tolerated and third-party antigens are linked within the same tissue, may be due to local effects of regulatory T cells. When the tolerated and third-party antigens are present in two different grafts that mainly drain into the same lymph nodes, the absence of tissue infiltrating regulatory T cells may result in graft rejection.

The identification of regulatory T cells within tolerated allografts may relate to the recent identification of memory T cells that persist in non-lymphoid tissue (Sallusto *et al*., 1999; Lanzavecchia and Sallusto, 2000; Masopust *et al*., 2001; Reinhardt *et al*., 2001; Tuma and Pamer, 2002; Sprent and Surh, 2002). The memory T cells can be divided into two different populations based on their surface phenotype, localization pattern and effector function:

the central-memory T cells (T_{CM}) and effector-memory T cells (T_{EM}). The T_{CM} cells express the chemokine CCR7, are residents in secondary lymphoid tissue, and following subsequent interaction with antigen undergo proliferation and further differentiation acquiring effector function, some of them becoming T_{EM} cells (Sallusto *et al*., 1999). The T_{EM} are believed to be terminally differentiated effector T cells, with the capacity to deliver effector molecules (cytokines or lytic mediators) upon rechallenge with antigen. They also express CCR7, and recirculate through peripheral tissues. It is not clear why these T_{EM} cells survive for long periods of time, but it has been suggested that they upregulate anti-apoptotic genes (Lanzavecchia and Sallusto, 2000). The best characterized regulatory T cells – the $CD4^+CD25^+$ cells – share many phenotypic characteristics with memory cells (for instance they are $CD44^+CD45RB^{low}$ – see Chapter 5). It may be that following initial activation of T cells, in the same way some $CD4^+$ differentiate into T_{EM} cells in the context of protective immune responses, some other $CD4^+$ T cells can differentiate into regulatory T_{EM} cells. It has been postulated that the functional relevance for T_{EM} is a rapid effector function following antigen encounter in the peripheral tissues, while T_{CM} would allow a rapid expansion and recruitment of new effector T cells following interaction with the antigen in the lymphoid tissue (Lanzavecchia and Sallusto, 2000). It is possible that regulatory T cells in tolerated allografts are a particular population of effector-memory T cells, that unlike the T_{EM} require the persistence of antigen. It is not clear at this time what makes regulatory T cells to accumulate preferentially within tolerated allografts, when compared with syngeneic skin from the same tolerised animal.

Interestingly, a reverse transcription PCR (RT-PCR) analysis of genes expressed in tolerated and rejecting tissues showed that expression of genes associated with regulatory T cells were found to be differential (Zelenika *et*

al., 2002). This was not, however, the case, when draining lymph nodes or spleens from the same animals were compared, suggesting that regulatory activity is concentrated in the graft (Zelenika *et al*., 2002). Surprisingly, the genes associated with regulatory T cells were also found to be overexpressed in syngeneic skin when compared with rejecting tissue (Zelenika *et al*., 2002). It is possible that regulatory T cells circulate through syngeneic skin although their number is below the threshold that we could identify with the described experimental system.

It is intriguing that, on a functional basis, regulatory cells with the capacity to prevent graft rejection can be demonstrated in both the spleen and tolerated skin grafts. It is not clear at this time, given the RT-PCR data, whether graft infiltrating regulatory cells constitute a special resident population different from splenic regulatory cells. The observation that T cells expand from graft infiltrating regulatory cells may imply that regulatory T cells in grafts result from a steady-state recirculation. Perhaps, regulatory cells recirculate through the body and accumulate preferentially at the sites where their target antigens are present. As a consequence it is possible they exert their regulatory activity on peripheral tissues by default, until inflammatory signals or other as yet unknown ligands turn off their suppressive function, so permitting a “normal” protective immune response to occur. Consistent with this hypothesis, it was recently reported that $CD4^+CD25^+$ regulatory T cells express glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR, also known as TNFRSF18) (Shimizu *et al*., 2002; McHugh *et al*., 2002), and ligation of this molecule by agonistic Abs abrogates the suppressive capacity of the cells (Shimizu *et al*., 2002). In any case, the present observations strongly support the view that at least some of the suppressive activity of regulatory T cells occurs beyond secondary lymphoid tissues at the sites where their target antigens are present.

CHAPTER 7

"STEALTH ANTIBODIES" AS A STRATEGY TO ABOLISH IMMUNOGENICITY OF THERAPEUTIC ANTIBODIES

Monoclonal antibodies have proven useful in the treatment of several human diseases. However, 25 years after their discovery, the clinical use of mAbs is still hampered by immune responses directed against them. Humanisation of mAbs reduced but did not abolish their immunogenicity. It has been reported that non cell-binding mAbs are not immunogenic, but instead can induce tolerance to the cell-binding form. When depleting T cells with CAMPATH-1H, as described in previous Chapters, it would be ideal to use a non-immunogenic mAb treatment. Based on the classical principles of Chiller and Weigle, we decided to test the biological effect of mAbs with their binding site occupied by an epitope-like peptide (mimotope). Such mAbs were found to be less immunogenic, and could induce partial to complete tolerance to their wild-type form. Surprisingly, the mimotope-bound mAbs retained biological effect suggesting that some antibody must have bound cells *in vivo*. As a consequence we called these mAbs "stealth antibodies". We anticipate it may be possible to create "stealth" variations of therapeutic mAbs, that once administered can mediate the therapeutic effect (although with a delayed action) without eliciting an immune response. In addition to the possible clinical applications, such mAbs may prove more useful reagents to achieve long term depletion in experimental transplantation, as described in previous Chapters, without incurring the risk of any artefacts due to immunogenicity.

7.1 Introduction

The efficacy of mAbs to treat several human diseases is now well established. Shortly after the discovery of mAbs in 1975 (Kohler and Milstein, 1975), they were adopted for biomedical research and clinical diagnosis. However, their therapeutic use has been slower to be established. In spite of the initial enthusiasm following the first report on the therapeutic use of mAb to treat a patient with lymphoma in 1982 (Miller *et al*., 1982), progress in their therapeutic application has been slow.

A major limitation for clinical use of traditional mAbs was that they derive from rodents, with amino-acid sequences significantly different from human antibodies (Ab). As a result, murine therapeutic mAb often evoked the production of neutralising antiglobulins (Schroff *et al*., 1985; Shawler *et al*., 1985). The turning point for clinical use of mAbs was the application of genetic engineering to mAb production. First, the development of chimeric mAbs, whose constant regions were human while the variable regions were rodent (Figure 7.1), offered the provision of equivalent “human” effector function and reduced the Ab immunogenicity (Boulianne *et al*., 1984; Morrison *et al*., 1984). The heavy-chain constant region (in particular the CH2 domain) contains sites recognised by innate effector systems of the body. Furthermore, humans are, of course, largely tolerant to human constant regions derived from other individuals. It has been formally shown (as might have been predicted) that immunogenicity of mAbs is greater when the sequence of the injected mAb is more dissimilar to host antibodies’ sequences (Bruggemann *et al*., 1989). Humanisation allowed the production of mAbs with an entirely human sequence except for the complementarity determining regions (CDR, Figure 7.1) (Jones *et al*., 1986). Humanised mAbs significantly reduced the immunogenicity of therapeutic mAbs, but were

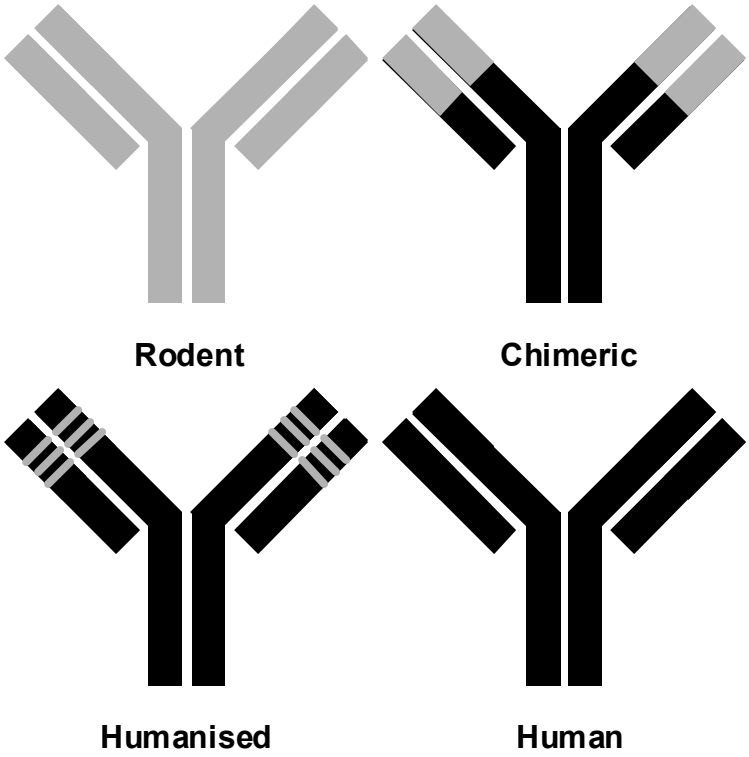

Figure 7.1 Humanization of therapeutic mAbs. Murine sequences are represented in grey and human sequences in black. In chimeric mAbs the constant region of both light and heavy chains are human, while the variable regions are murine. In the humanised mAbs only the CDRs are murine.

shown unlikely to abolish it completely (Isaacs *et al*., 1992; Weinblatt *et al*., 1995). In the early 90s an approach was developed for the production of human mAbs in mice by construction of mice transgenic for human immunoglobulin (Ig) genes (Bruggemann *et al*., 1991) as well as through phage display technology (Clackson *et al*., 1991; Winter *et al*., 1994). These advances, although offering alternatives to engineering, are also unlikely to overcome immunogenicity based on the CDRs of the mAbs. In fact, even the Abs naturally produced in the human body frequently lead to the production of anti-idiotypic Abs (i.e. directed to the CDR of the target Ab) (Eichmann, 1975; Trenkner and Riblet, 1975; Forni *et al*., 1980). Some authors even suggest that the emergence of such a network of interactions between natural Abs can be an important homeostatic mechanism governing the reactivity of the immune system (Jerne, 1974; Coutinho, 1989). As a consequence, a human mAb is probably not too different from a humanised one in terms of potential for immunogenicity.

In chronic diseases such as rheumatoid arthritis (RA) where therapeutic mAbs are repeatedly administered, the emergence of neutralising Abs significantly affects the efficacy of therapeutic mAbs by reducing their half-life and their capacity to bind target antigen. As an example, in one of the first clinical trials of humanised mAbs, CAMPATH-1H was used to treat patients with RA, and 63% of the treated patients developed neutralising Abs which may have limited the clinical benefit (Weinblatt *et al*., 1995). Of the mAbs used currently to treat RA, the chimeric mAb Infliximab^{TM} (anti – Tumour Necrosis Factor α (TNF-α)) is perhaps the most studied example, and requires the addition of immunosuppressive drugs to prevent the production of neutralising Abs. In fact, in a recent review, Feldmann and Maini discussed the immunogenicity of Infliximab^{TM}, as well as of other therapeutic mAbs, and offered no solution other than co-administration of immunosuppressive drugs

(Feldmann and Maini, 2001). Furthermore, formation of immune complexes and subsequent deposition in the tissues can lead to adverse consequences. Such formation of immune complexes is a clinical problem as it may generate a range of side effects as severe as serum sickness, and reduces efficacy of the therapeutic mAb. Similarly, artefacts associated with immunogenicity might also lead to misinterpretations of animal experiments of the type in this thesis which use mAb to manipulate cell populations.

The amino-acid sequence of the mAbs, however, is only one of the several factors determining their immunogenicity (Table 7.1). From the early 70s it has been known that the same immunoglobulins can lead to the formation of antiglobulins or to tolerance depending on the way they are administered (Weigle, 1973). Chiller and Weigle reported that aggregated human Ig were very immunogenic in mice resulting in the production of antiglobulins, however, the same human Ig administered in the monomeric form not only fail to induce an immune response, but was tolerogenic preventing antiglobulin production following subsequent immunisations with aggregated human Ig (Chiller *et al*., 1970). More recently, it was shown that mAbs binding to cellular antigens tend to be immunogenic, while non-cell binding mAbs tend to be tolerogenic (Benjamin *et al*., 1986). Immunogenicity of Abs, as well as other foreign proteins (like therapeutic administration of factors VIII and IX in haemophilia), is a consequence of an immune response dependent on $CD4^+$ T cells (Isaacs and Waldmann, 1994). This may be the explanation for the special characteristics of anti-CD4 mAbs as tolerogenic agents: although cell-binding, these mAbs induce tolerance to themselves and to other foreign antigens administered at the same time (Benjamin *et al*., 1986; Benjamin and Waldmann, 1986). This property makes CD4 mAbs very interesting for therapeutic induction of tolerance, namely to transplants (Waldmann and Cobbold, 1998; Graca and Waldmann, 2001).

mAb Structure	Humanized fraction Glycosylation
mAb Specificity	Soluble versus cellular antigen Binding affinity Multivalency of Ab and antigen
mAb Function	Binding to Fc receptors Complement activation Cell lysis Cytokine release Inflammation
mAb administration	Dose Frequency of administration Route of administration
Patient	Characteristics of the disease Immunosuppression

Table 7.1 Factors determining the immunogenicity of therapeutic mAbs.

The observation that an immunogenic mAb can be made tolerogenic by preventing its binding to cells and formation of aggregates, offers the opportunity to develop a general mechanism to eliminate immunogenicity of therapeutic mAbs. The principle has recently been established by Gilliland and colleagues in the host laboratory (Gilliland *et al*., 1999). They evolved a strategy based on the humanised CAMPATH-1H mAb, recently approved for clinical use by the Food and Drug Administration, USA (FDA). Antiglobulins have been shown to be induced during clinical administration of CAMPATH-1H in chronic diseases like RA and multiple sclerosis (G. Hale, personal communication), leading to a reduction of the therapeutic effect in some instances (Isaacs, 1990; Isaacs *et al*., 1992; Lockwood *et al*., 1993; Weinblatt *et al*., 1995; Isaacs *et al*., 1996). Gilliland and colleagues studied the immunogenicity of CAMPATH-1H and a series of mutants in CP1-CBA/Ca transgenic mice that express human-CD52 (hCD52), the target of

CAMPATH-1H, under the control of the CD2 promoter (Gilliland *et al*., 1999). All T cells from this mouse strain express hCD52. When these mice were injected with CAMPATH-1H they produced antiglobulins.

The mutants that were used above were identical to CAMPATH-1H, except that they possessed one or two amino-acid substitutions within the CDR2 of the heavy chain. *In vitro* tests identified some mutants with impaired binding to hCD52. Animals treated with non-binding mutants did not produce antiglobulins, not even following subsequent administration of CAMPATH-1H (Gilliland *et al*., 1999). However, with some of the mutants tolerogenicity to the wild-type was, as might be expected, incomplete with breakthrough responses specifically directed to the site selected for mutation (Gilliland *et al*., 1999). These experiments show that it is possible to eliminate immunogenicity of therapeutic mAbs by preventing their binding to cells. However, tolerisation in advance of treatment may not be logistically feasible for clinical use, and the possibility that immunogenicity can still be observed due to differences of the mutated residues could also become a problem.

Aside from the therapeutic arena, immunologists have increasingly utilised mAbs to deplete cell populations or block specific functions in experimental studies. Any neutralising antiglobulins might well create artefactual outcomes to the experimental study. It would therefore be desirable to have antibody reagents capable of inducing long lasting cell depletion in experimental animals, without eliciting the production of anti-antibodies with their associated disadvantages. The two step process involving prior administration of a mutated non-cell binding mAb, although useful for short-term studies, may still pose problems where longer-term “therapy” is anticipated, as antiglobulins can be generated against the non-mutated regions of the wild-type mAb (Gilliland *et al*., 1999).

We decided to tackle the problem by creating a non-cell binding version of the CAMPATH-1H mAb without modifying its primary amino-acid sequence. This antibody was designed to have the advantages of a tolerogen, but retain (in part) the cell-binding capacity required for its function. An epitope-like peptide (mimotope) was convalently linked (by genetic engineering) to the variable region of the light chain, so as to interfere with the mAb binding capacity to hCD52. One such construct, the mimotope-IgG1 (MIM-IgG1) had impaired binding to its ligand both *in vitro* and *in vivo*. MIM-IgG1 was shown to be less immunogenic and even partially tolerogenic when administered into CP1-CBA transgenic mice. Despite this, the mAb could still deplete target T cells *in vivo*, albeit at a slower rate than with wild-type CAMPATH-1H. These results suggest that around the time that the antibody is administered, the majority of MIM-IgG1 is unable to bind to cells and consequently can induce partial tolerance to itself. With time, increasing amounts of the MIM-IgG1 become bound to cells at sufficient levels to bring about cell depletion. We have coined such mAbs “stealth antibodies”.

To determine whether “effector” function contributed to immunogenicity we also removed the glycosylation site (asp 297) from the Fc region (AG-MIM-IgG1). This construct proved to be completely non-immunogenic and a far more effective tolerogen than MIM-IgG1. We conclude that the lack of “adjuvanticity” or “danger” associated with the loss of FcR-binding by AG-MIM-IgG1 resulted in that outcome.

7.2 Results

7.2.1 MIM-IgG1 and AG-MIM-IgG1 have impaired binding activity *in vitro*

Several constructs based on the CAMPATH-1H were made by Mark Frewin (see Table 7.2). I produced the AG-CAMPATH-1H construct.

MIM-IgG1 and AG-MIM-IgG1 have a reduced binding capacity *in vitro* when compared with CAMPATH-1H by ELISA, flow cytometry and surface plasmon resonance (BIAcore™).

Figure 7.2A shows the binding ability of different constructs to HUT cells expressing hCD52. It is apparent that CAMPATH-1H binds HUT cells with an efficiency approximately 5 times superior to p61-IgG1, 2000 times superior to MIM-IgG1 and more than 10,000 times superior to AG-MIM-IgG1 (experiment performed by Mark Frewin and provided for clarification). Comparable results were obtained by ELISA, using plates coated with the antigen (Figure 7.2B).

Results obtained with BIAcore™ were not as conclusive due to the large differences in binding capacities between the different constructs. However, such results confirmed a far superior binding capacity of CAMPATH-1H and p61-IgG1 when compared with MIM-IgG1, compatible with the results obtained with the other methods (Figure 7.2C).

CAMPATH-1H	Wild type CAMPATH-1H light chain V-region + CAMPATH-1H heavy chain with wild type human IgG1 constant region. Cloned into Wellcome expression vectors pRDN-1 and pBAN-2 for CHO produced Ab.
AG-CAMPATH-1H	Wild type CAMPATH-1H light chain V-region + CAMPATH-1H heavy chain with aglycosyl human IgG1 constant region. Cloned into Celltech expression vector PEE12 for NSO produced Ab.
MIM-IgG1	CD52 mimotope QTSSPSAD tethered to CAMPATH-1H light chain V-region by flexible Glycine4 Serine2 linker + CAMPATH-1H heavy chain with wild type human IgG1 constant region. Cloned into Celltech expression vector PEE12 for NSO produced Ab, and Wellcome pRDN-1 and pBAN-2 for CHO produced Ab.
AG-MIM-IgG1	CD52 mimotope QTSSPSAD tethered to CAMPATH-1H light chain V-region by flexible Glycine4 Serine2 linker + CAMPATH-1H heavy chain with aglycosyl human IgG1 constant region. Cloned into Celltech expression vector PEE12 for NSO produced Ab.
P61-IgG1	HLA P61 binding peptide SLLPAIVEL tethered to CAMPATH-1H light chain V-region by flexible Glycine4 Serine2 linker + CAMPATH-1H heavy chain with wild type human IgG1 constant region. Cloned into Wellcome expression vectors pRDN-1 and pBAN-2 for CHO produced Ab.
AG-P61-IgG1	HLA P61 binding peptide SLLPAIVEL tethered to CAMPATH-1H light chain V-region by flexible Glycine4 Serine2 linker + CAMPATH-1H heavy chain with aglycosyl human IgG1 constant region. Cloned into Celltech expression vector PEE12 for NSO produced Ab.

Table 7.2 mAb constructs based on CAMPATH-1H used in the present study. I only made AG-CAMPATH-1H. All other constructs were made by M. Frewin.

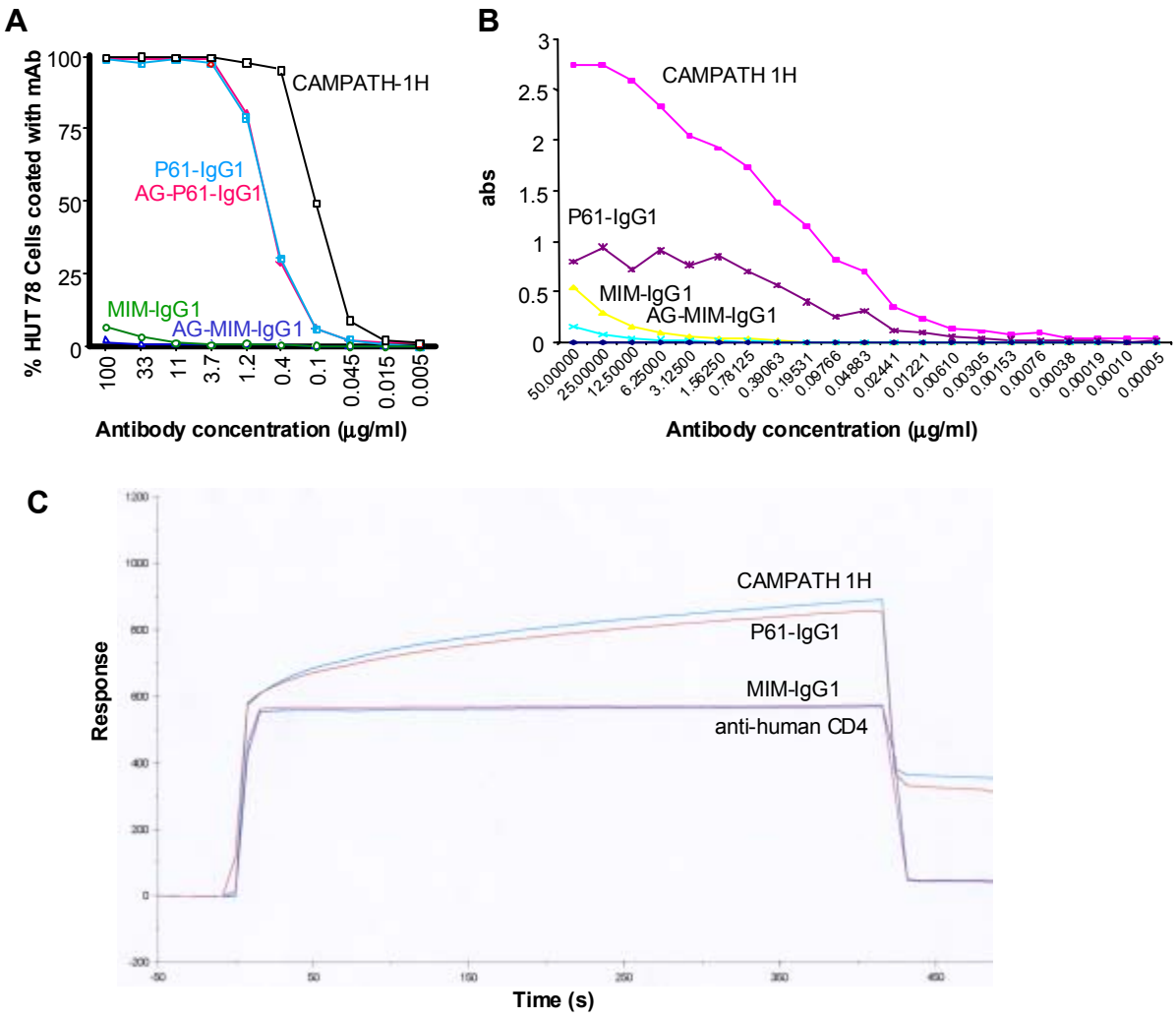

Figure 7.2 *Binding activity of different mAb constructs in vitro*. **A**, Binding capacity of different mAb constructs to HUT-78 cells expressing the hCD52 antigen, detected by flow cytometry. CAMPATH-1H is the most efficient mAb in binding to HUT cells, followed by both P61-IgG1 and AG-P61-IgG1. MIM-IgG1 and AG-MIM-IgG1 have poor binding activity (data from M. Frewin). **B**, Similar results were obtained measuring Ab binding activity to BHK21.C13 by ELISA. **C**, BIAcore™ sensorgram showing binding of different mAbs to BHK21.C13. Binding activity of CAMPATH-1H and P61-IgG1 is significantly greater than MIM-IgG1. Binding of MIM-IgG1 is not significantly different than the control mAb.

7.2.2 MIM-IgG1 and AG-MIM-IgG1 show delayed cell-binding activity *in vivo*

The inferior *in vivo* binding capacity of MIM-IgG1 and AG-MIM-IgG1 compared with CAMPATH-1H was confirmed by flow cytometric analysis of lymphocytes from CP1-CBA transgenic mice treated with the different mAbs. CP1-CBA mice express hCD52 under the control of the CD2 promoter on all T cells (Gilliland *et al*., 1999). Purified mAb was injected i.p. into CP1-CBA mice and spleen cells and peripheral blood were analysed for mAb binding to $hCD52^+$ T cells 3 hours and 8 days following mAb treatment.

Peripheral blood lymphocytes (PBL) stained three hours following mAb injection show that mice treated with CAMPATH-1H or AG-CAMPATH-1H were saturated with the mAb (mean fluorescent activity (MFI): 938 ± 237 and 1729 ± 115 respectively) (Figure 7.3A, C), and a significant proportion of the $CD3^+$ T cell population was already depleted (only 9% ± 2.9 and 32% ± 1.6 respectively of the $CD3^+$ cells were left). Both p61-IgG1 and AG-p61-IgG1 which also stained strongly (MFI 528 ± 145 and 855 ± 42 respectively), achieved significant depletion at this same dose (10.9% ± 2.3 and 20.7% ± 2.8 of $CD3^+$ PBL). MIM-IgG1 could also be detected bound to $CD3^+$ cells, although the intensity of staining was reduced by approximately two orders of magnitude (MFI = 191 ± 31), and almost no depletion could be observed (66.9% ± 4.2 of $CD3^+$ PBL were present). Finally, AG-MIM-IgG1 bound very weakly to PBL (MFI = 23.1 ± 0.8), and virtually no depletion of T cells could be observed at this stage (71.8% ± 5.1 of $CD3^+$ cells). Comparable results were obtained when splenocytes from the same animals were examined (Figure 7.3B and D). We can thus conclude that, in the first hours after treatment, both MIM-IgG1 and AG-MIM-IgG1 are significantly less efficient than all other constructs in binding to T cells *in vivo* and causing depletion.

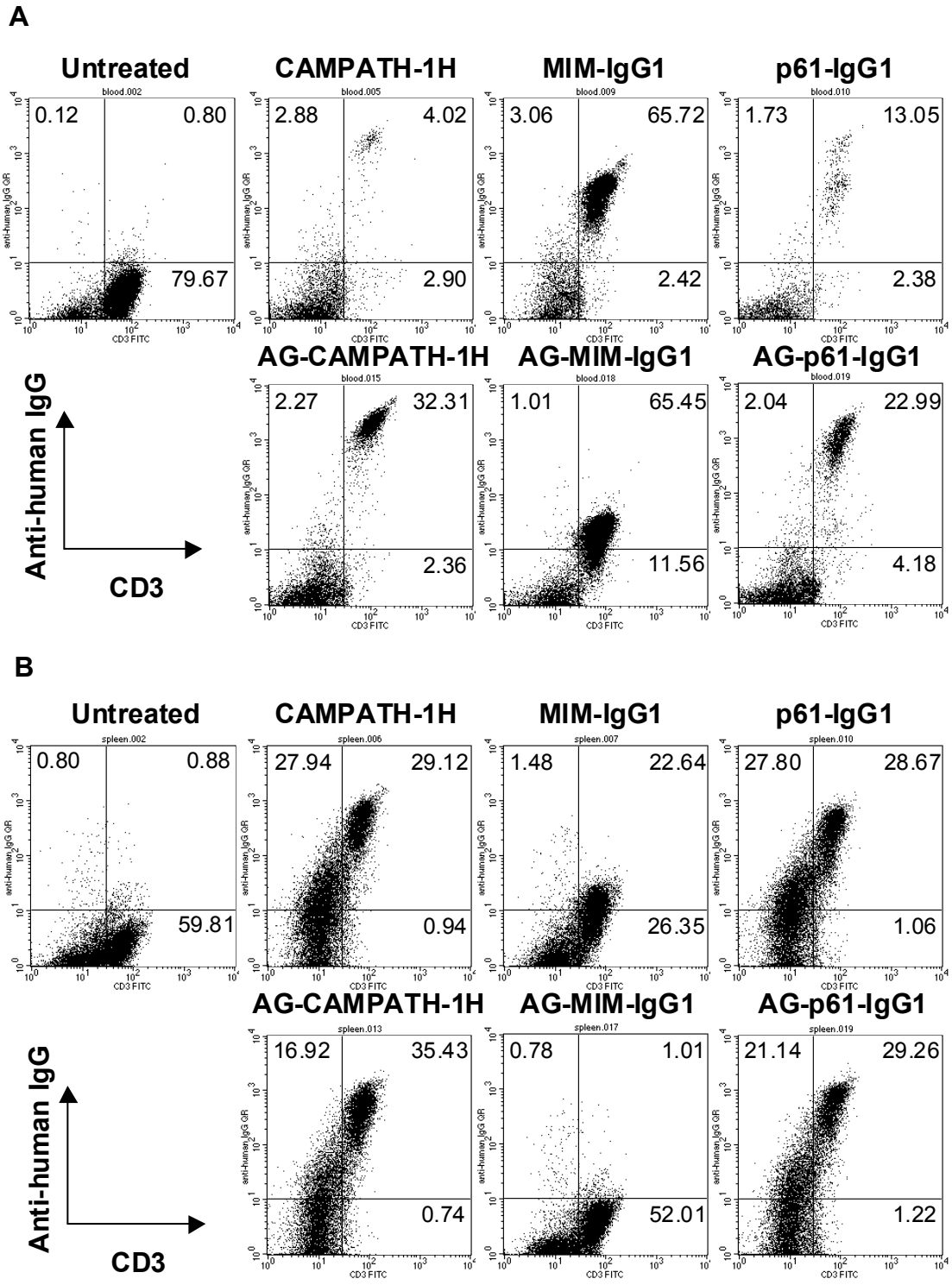

Figure 7.3 *Binding capacity of different mAb constructs to T cells in vivo*. (see legend in the next page).

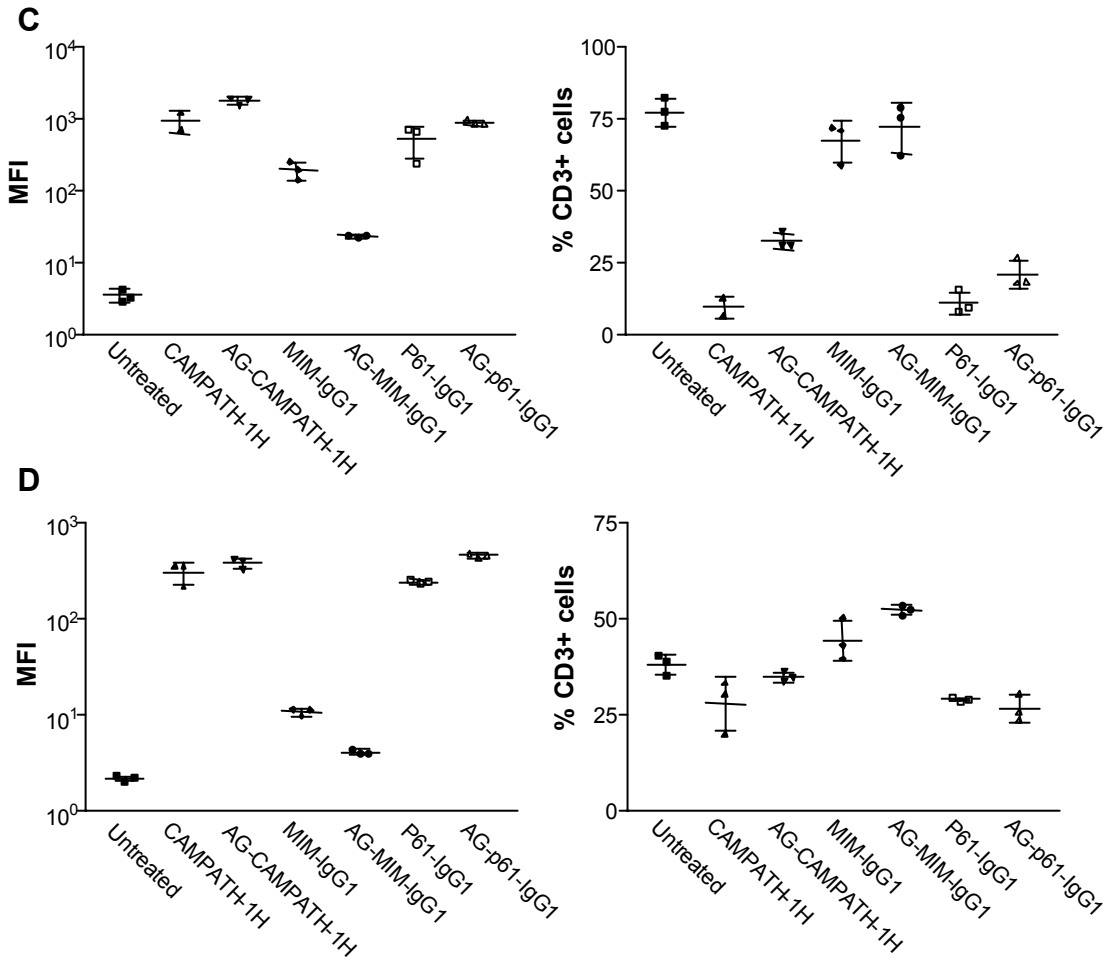

Figure 7.3 *Binding capacity of different mAb constructs to T cells in vivo*. Analysis by flow cytometry of mAb binding to T cells of CP1-CBA mice 3 hours following i.p. injection of 0.5 mg of mAb. **A**, Analysis of peripheral blood lymphocytes. $CD3^+$ T cells from mice treated with CAMPATH-1H or AG-CAMPATH-1H are coated with these mAbs as the cells are brightly stained with anti-human IgG1 mAb. Some depletion of T cells from the blood can already be seen at this time with both constructs. The P61-IgG1 and AG-P61-IgG1 also stain strongly, and achieve some depletion. MIM-IgG1 stains the blood T cells, although with lower intensity than the above constructs, and very little cell depletion is seen at this stage. Finally, AG-MIM-IgG1 binds very weakly to blood lymphocytes, and is not associated with any cell depletion at this time. **B**, Analysis of splenocytes. Results are comparable to peripheral blood. **C**, Representation of the MFI of $CD3^+$ T cells (left) and percentage of $CD3^+$ T cells among the PBL (right) amongst the mice treated with different constructs as shown in A. Mean and standard deviation are shown. **D**, Representation of MFI (left) and percentage of $CD3^+$ cells (right) amongst the splenocytes of the same mice.

When PBLs and splenocytes from mice treated with MIM-IgG1 or AG-MIM-IgG1 were analysed 8 days following mAb injection, it was apparent that there was an increase in the amount of the human mAb binding to T cells, as exemplified by AG-MIM-IgG1 when compared with that seen at 3 hours after treatment (Figure 7.4). Furthermore, depletion mediated by MIM-IgG1 was also increased. Animals treated with mAbs were observed to have near total T cell depletion at this time point.

Taken together, these results suggest that AG-MIM-IgG1 and MIM-IgG1 have the capacity to bind the cells *in vivo*, although with delayed kinetics.

7.2.3 MIM-IgG1 depletes T cells in a dose-dependent manner

Since MIM-IgG1 has the capacity to bind cells *in vivo*, I determined whether this mAb could exert its biological function by depleting cells expressing their target antigen. I found this to be the case, with MIM-IgG1 being capable of cell depletion in a dose dependent fashion.

CP1-CBA mice were treated with different doses of CAMPATH-1H or MIM-IgG1. Blood samples were collected at different time points following mAb treatment and analysed for depletion of $hCD52^+$ T cells (Figure 7.5). As expected, mice treated with CAMPATH-1H showed a marked depletion of T cells immediately following injection, while depletion in MIM-IgG1-treated mice took much longer to occur. It is also required a dose 10 to 50 times higher to achieve depletion comparable to CAMPATH-1H. Interestingly, it appears that depletion induced by MIM-IgG1 has a longer duration than that seen with CAMPATH-1H. In fact, 21 days following mAb treatment at the highest mAb doses, the percentage of $hCD52^+$ cells was higher in CAMPATH-1H treated mice than in MIM-IgG1 treated mice.

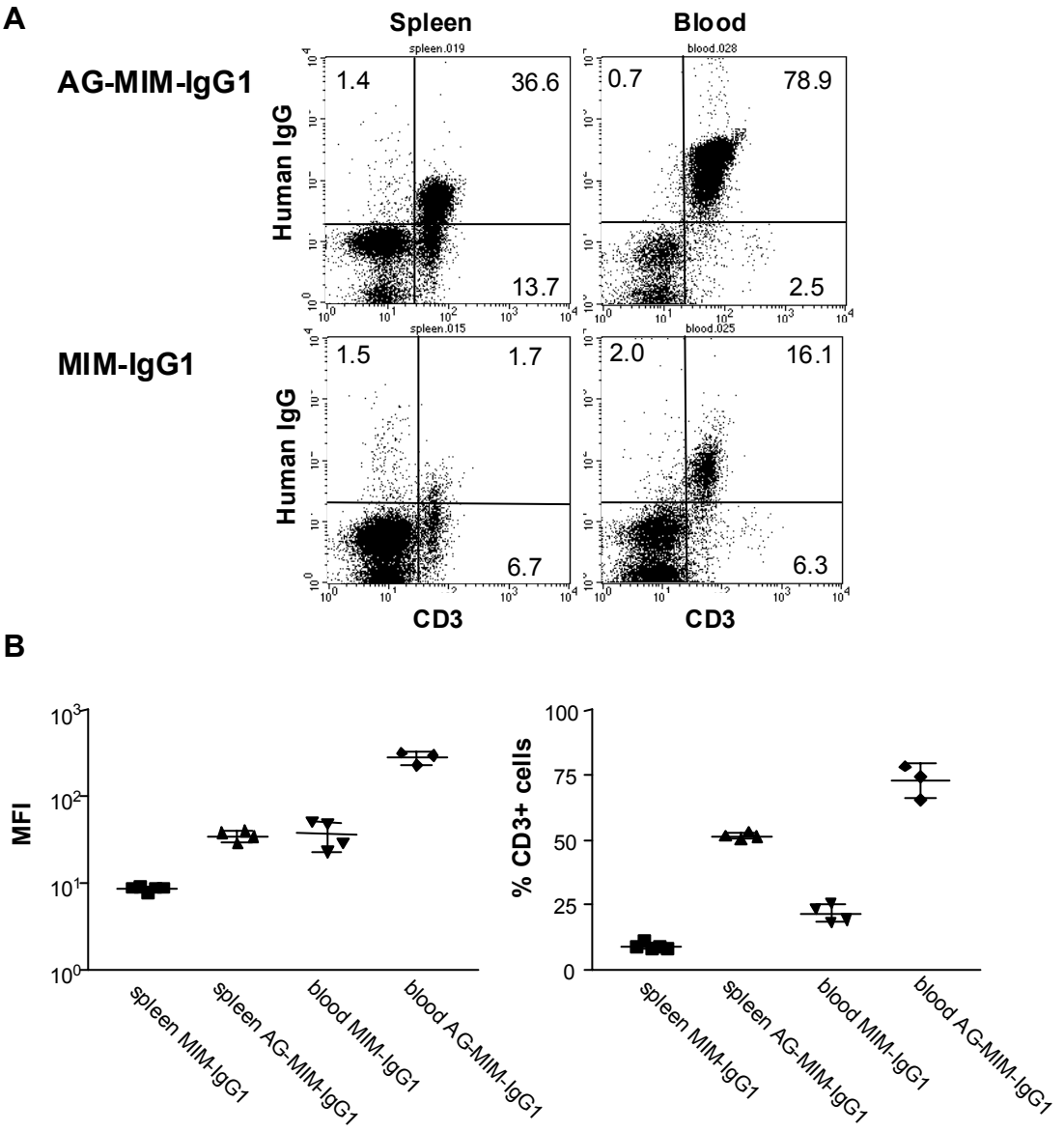

Figure 7.4 *Binding of MIM-IgG1 and AG-MIM-IgG1 to hCD52$^+$ T cells increases with time*. **A**, Analysis by flow cytometry of MIM-IgG1 and AG-MIM-IgG1 binding to T cells of CP1-CBA mice 8 days following i.p. injection of 0.5 mg of mAb. The injected AG-MIM-IgG1 mAb has bound to CD3$^+$ T cells in the spleen and peripheral blood, and that the intensity of staining is higher that in the analysis at 3 hours post-treatment (Figure 7.3). Similar results were obtained following treatment with MIM-IgG1, but in this case a significant proportion of T cells has been depleted as the percentage of CD3$^+$ cells is markedly reduced. **B**, Representation of the MFI (left) and percentage of CD3$^+$ T cells from mice treated with the different mAb constructs. Mean and standard deviation are indicated.

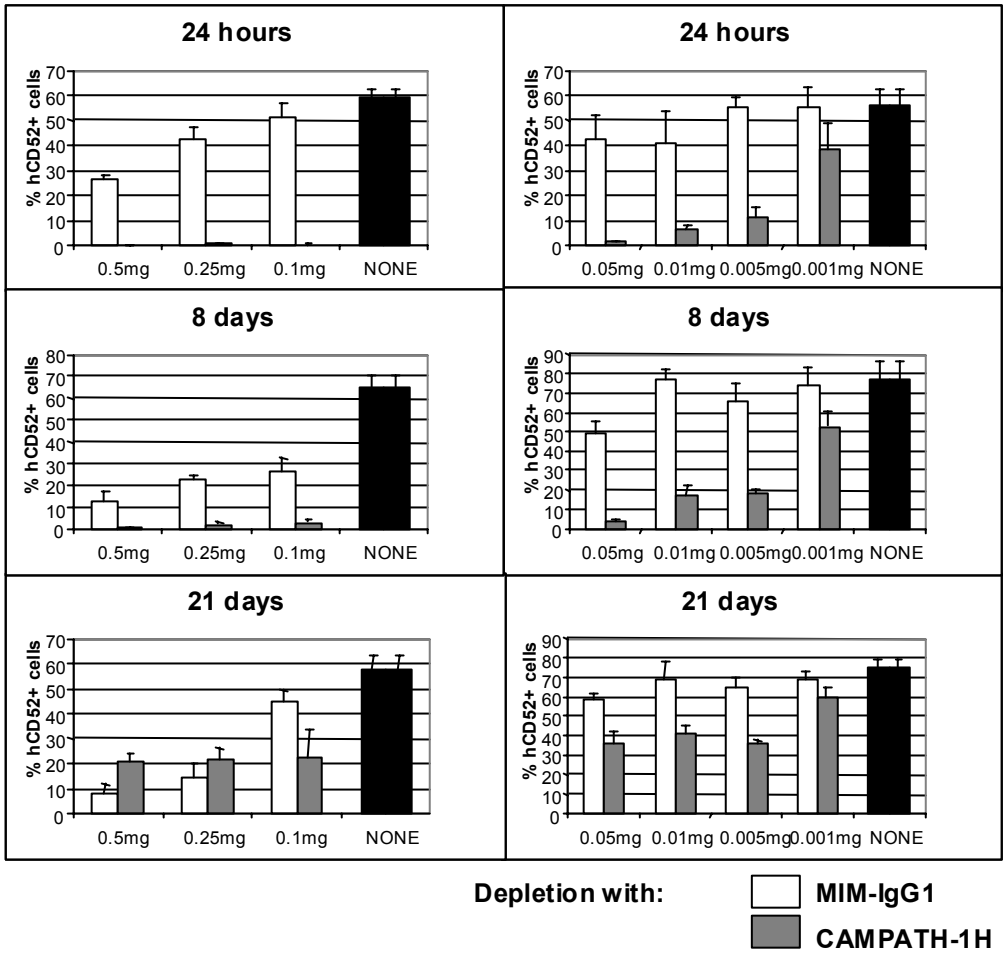

Figure 7.5 *Depletion of peripheral blood T lymphocytes by CAMPATH-1H and MIM-IgG1*. Different groups of CP1-CBA mice were injected i.p. with several doses of CAMPATH-1H or MIM-IgG1. Blood samples were collected 24 hours, 8 days and 21 days following mAb treatment, and depletion of $hCD52^+$ T cells was quantitated by flow cytometry. The left column shows the results of mice treated with 0.1 mg to 0.5 mg of mAb and the right column shows the results of a different experiment where mice were treated with 1 μg to 50 μg of mAb. CAMPATH-1H depletes host T cells within 24 hours at doses down to 5 μg/ml whereas MIM-IgG1 depletion is slower and requires doses of mAb 100 times greater. In contrast, at day 21 depletion achieved with 0.25 mg or 0.5 mg of MIM-IgG1 is similar to the one achieved with the same doses of CAMPATH-1H, where T cells are already expanding.

These results suggest that treatment with MIM-IgG1 can achieve cell depletion, when given at appropriate dose, and that this has a slower onset but a longer duration than depletion by CAMPATH-1H.

7.2.4 MIM-IgG1 and AG-MIM-IgG1 are less immunogenic than CAMPATH-1H

To assess the immunogenicity of the different mAbs, serum samples were collected 21 or 28 days following mAb treatment of CP1-CBA mice. The presence of anti-human globulins in the serum samples was quantified by ELISA as described in Chapter 2, confirming high immunogenicity of CAMPATH-1H, but not MIM-IgG1 or AG-MIM-IgG1.

CP1-CBA mice were injected with different doses of mAbs (Figure 7.6A). All doses of CAMPATH-1H tested were found to be significantly more immunogenic than any dose of MIM-IgG1 tested. Remarkably, mice treated with AG-MIM-IgG1 had no detectable anti-human globulin in the serum. The lack of immunogenicity of AG-MIM-IgG1 is not solely due to the amino-acid modification of the Fc region, as treatment with 0.5 mg AG-CAMPATH-1H or AG-p61-IgG1 leads to significant anti-human globulin titres (Figure 7.6B).

7.2.5 MIM-IgG1 and AG-MIM-IgG1 can induce tolerance to CAMPATH-1H

In order to determine the tolerogenic capacity of different Ab constructs, CP1-CBA mice were initially treated with the test mAbs, and subsequently challenged with an immunogenic dose of CAMPATH-1H as represented in Figure 7.7A. Treatment with MIM-IgG1 and AG-MIM-IgG1 severely impaired production of antiglobulins following subsequent challenge with CAMPATH-

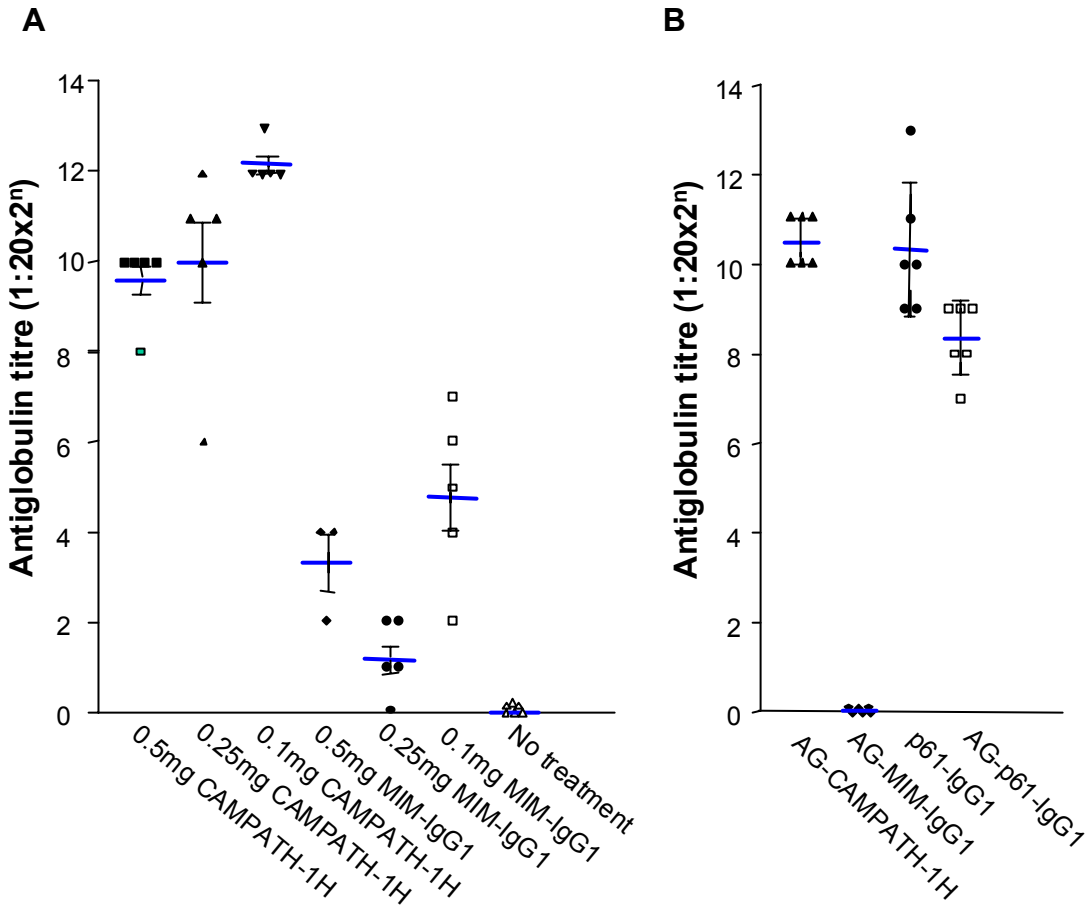

Figure 7.6 *Immunogenicity of different mAb constructs*. Sera was taken from CP1-CBA mice treated with different doses CAMPATH-1H or MIM-IgG1 mAbs on day 21 (A), or from mice treated with 0.5 mg of test mAbs on day 28 (B). The presence of anti-CAMPATH-1H Abs was assessed by ELISA. All doses of CAMPATH-1H were more immunogenic than equivalent doses of MIM-IgG1 (mean antiglobulin titres respectively $1:1.5x10^4$ to $1:10^5$ versus 1:45.9 to 1:557, corresponding to differences of approximately 100 to 400-fold for equivalent doses). Interestingly, in any of the mice treated with 0.5 mg of AG-MIM-IgG1 no antiglobulins were detected (titres <1:20). Treatment with AG-CAMPATH-1H or AG-P61-IgG1 led to the production of antiglobulins (mean titres $1:2.9x10^4$ and 1:6463). Differences between CAMPATH-1H and MIM-IgG1 treated mice, as well as AG-MIM-IgG1 treated mice compared with animals treated with AG-CAMPATH-1H or AG-p61-IgG1, are statistically significant (p<0.0005). Geometric mean and standard deviation for each group are represented in the graph.

1H. In other words, treatment with MIM-IgG1 and AG-MIM-IgG1 induced partial (MIM-IgG1) and complete (AG-MIM-IgG1) tolerance to CAMPATH-1H.

Groups of CP1-CBA mice were treated with different concentrations of mAbs. All animals were challenged with 5 daily doses of 50 μg CAMPATH-1H as described in Figure 7.7A. Such challenge with CAMPATH-1H is highly immunogenic leading to high serum levels of anti-human globulins readily detectable by ELISA 30 days following CAMPATH-1H administration (Figure 7.7B). The antiglobulin titres of mice treated initially with CAMPATH-1H were even increased four-fold following the CAMPATH-1H challenge. However, mice initially treated with different doses of MIM-IgG1 showed partial tolerance to CAMPATH-1H, as they failed to produce more antiglobulins following challenge with CAMPATH-1H, or even subsequent challenge 30 days after the first one (Figure 7.7C). Remarkably, mice initially treated with AG-MIM-IgG1 became completely tolerant to CAMPATH-1H with no detectable serum antiglobulins following any CAMPATH-1H challenge. The control group treated with p61-IgG1 showed a partial impairment in antiglobulin production following CAMPATH-1H treatment. Interestingly, one of these mice failed to produce antiglobulins following challenge with CAMPATH-1H. At the present we cannot explain this observation, which was not reproduced in a repeat experiment.

Taken together these results confirm that MIM-IgG1 and particularly AG-MIM-IgG1 have the capacity to induce degrees of tolerance to the wild-type CAMPATH-1H mAb.

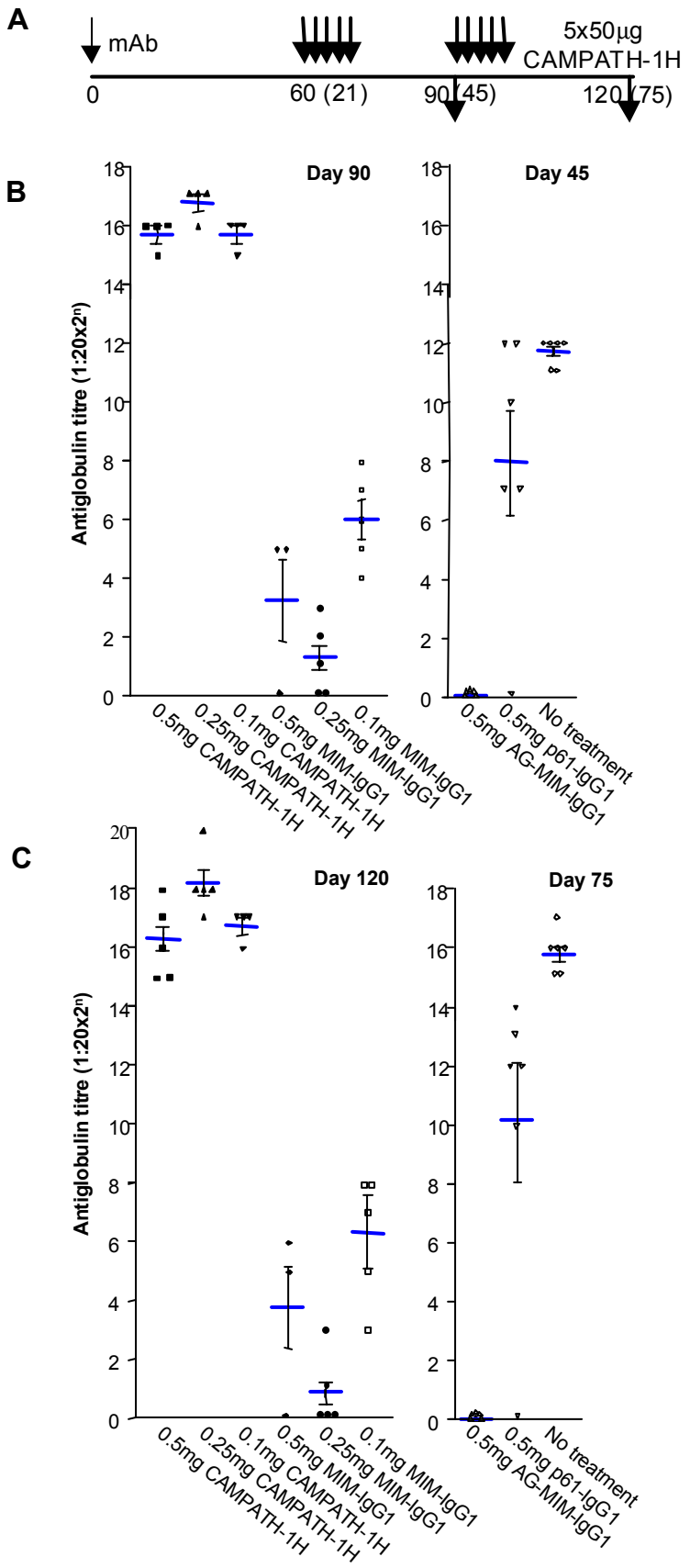

Figure 7.7 *Tolerogenicity of different mAb constructs*. (see legend in the next page).

Figure 7.7 *Tolerogenicity of different mAb constructs*. CP1-CBA mice were treated with different mAb constructs at day 0. **A**, At day 60 (left panels) or 21 (right panels) the mice were challenged with 250 μg CAMPATH-1H over five days. A blood sample was collected 3 or 4 weeks following the CAMPATH-1H challenge (day 90 or 45, respectively) and analysed for the presence of antiglobulins. At this time another challenge with daily injections of 50 μg CAMPATH-1H over five days was performed. A final blood sample was collected 4 weeks following the last rechallenge (days 120 or 75). **B**, Quantitation by ELISA of serum antiglobulins after the initial challenge with CAMPATH-1H, on day 90 (left) or 45 (right). Mice initially treated with MIM-IgG1 shown a reduced capacity to mount an immune response to the injected CAMPATH-1H, suggesting some level of tolerisation. None of the mice initially treated with AG-MIM-IgG1 had detectable antiglobulins in circulation, suggesting they were completely tolerised to CAMPATH-1H. **C**, Quantitation of serum antiglobulins, from the same animals represented in (B), after secondary challenge with CAMPATH-1H, on day 120 (left) or 75 (right). Mice initially treated with CAMPATH-1H, P61-IgG1, or without any initial treatment demonstrated an even higher antiglobulin titre. That was not the case of animals initially treated with MIM-IgG1, that maintained low titres of antiglobulin, or mice initially treated with AG-MIM-IgG1, that still had no detectable antiglobulin in the circulation. Differences between CAMPATH-1H and MIM-IgG1 treated mice, as well as AG-MIM-IgG1 treated mice compared with animals treated with p61-IgG1 or without an initial mAb treatment, are statistically significant at the two studied time points (A and B) ($p<0.005$). Geometric mean and standard deviation for each group are represented in the graph.

7.2.6 Tolerance with AG-MIM-IgG1 is complete by two weeks

To assess the time that is necessary for tolerance to be complete, groups of mice treated with the tolerogenic dose of 0.5 mg AG-MIM-IgG1 were subsequently injected with an immunogenic dose of 50 μg CAMPATH-1H at different time points thereafter (Figure 7.8). Quantification of serum anti-human globulins was performed 21 days following the administration of the immunogenic mAb. Remarkably, when the time interval between tolerogenic and immunogenic treatment was greater than 24 hours, the amount of antiglobulins detected in the sera was lower, being smaller the longer the time interval from administration of tolerogen. When the immunogenic stimuli was given two weeks following the tolerogenic mAb, only 1 out of 6 mice had any detectable serum antiglobulins and this was at a very low titre (<1:40). I can therefore conclude that 1 week is sufficient to prevent the generation of high antiglobulin titres, and 2 weeks permits virtually complete tolerance.

7.2.7 An immunogenic dose of CAMPATH-1H can be overriden by tolerance processes if co-administered with an excess of AG-MIM-IgG1

I decided to establish whether an immunogenic dose of CAMPATH-1H could be overriden by tolerance if sufficient AG-MIM-IgG1 was co-administered. Such result would be important to determine whether any cell-bound antibody accumulating immediately after injection could abrogate the tolerogenic capacity of the stealth construct.

CP1-CBA mice were treated with the tolerogenic dose of 0.5 mg AG-MIM-IgG1 mixed with different immunogenic doses of CAMPATH-1H (Figure 7.9A). An immune response to CAMPATH-1H was abrogated when a ratio of

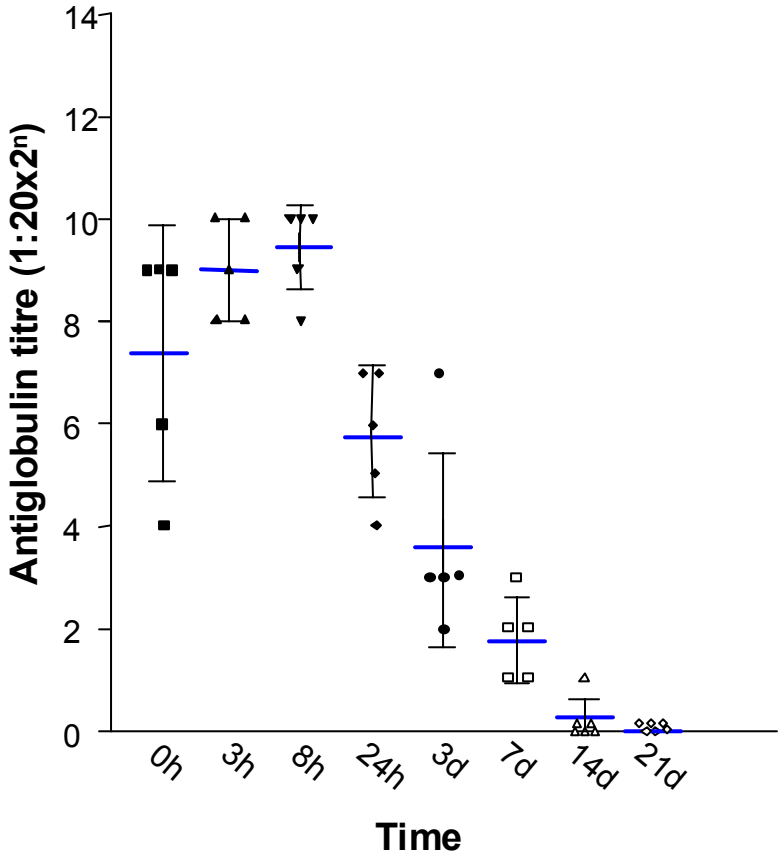

Figure 7.8 *How much time is needed for tolerance to develop*. All mice were treated with 0.5 mg AG-MIM-IgG1 at time 0. An immunogenic dose of 50 μg CAMPATH-1H was given at different time points following the Ag-MIM-IgG1 treatment. Blood samples were collected 21 days following immunogenic challenge and the presence of antiglobulins quantified by ELISA. Serum antiglobulin levels were significantly reduced when the immunogenic challenged was given three days following the tolerogenic treatment (geometric mean: 3.6 ± 0.87, p=0.0226 compared with time 0 (7.4 ± 1.03)). And even further reduced at day 7 and day 14 (1.8 ± 0.37 and 0.2 ± 0.2 respectively).

CAMPATH-1H / AG-MIM-IgG1 of 1:100 or 1:1,000 was injected (Figure 7.9B). Furthermore, when mice that failed to mount an immune response to the initial injection of CAMPATH-1H were challenged with 5 daily doses of 50 μg CAMPATH-1H they did not produce detectable levels of antiglobulins. I can therefore conclude that an appropriate mixture of the immunogenic with the tolerogenic Ab can result in tolerance or immunity depending on the injected ratios.

7.2.8 Tolerance correlates with higher serum concentration / half-life of human mAb

As antiglobulins can lead to increased clearance of their target Abs, I determined whether induction of tolerance leads to increased half-life of the injected mAbs. An inverse correlation between antiglobulin titre and serum concentration of human Abs was found.

CP1-CBA mice were treated with a mixture of AG-MIM-IgG1 and CAMPATH-1H, and challenged at day 21 with a further injection of 250 μg CAMPATH-1H over 5 days, as described above (Figure 7.9A). At day 42 the concentration of human Abs (both injected CAMPATH-1H and AG-MIM-IgG1) in the serum was determined by ELISA (Figure 7.9C). Interestingly, mice tolerant to CAMPATH-1H demonstrated higher titres of human Abs in the serum than mice that were not tolerant. In fact, there is an inverse correlation between the presence of antiglobulins and the presence of human Ab in the serum (Figure 7.9B and C). These results confirm the hypothesis that tolerance induction, by reducing the production of antiglobulins, allows a greater half-life of the injected mAbs.

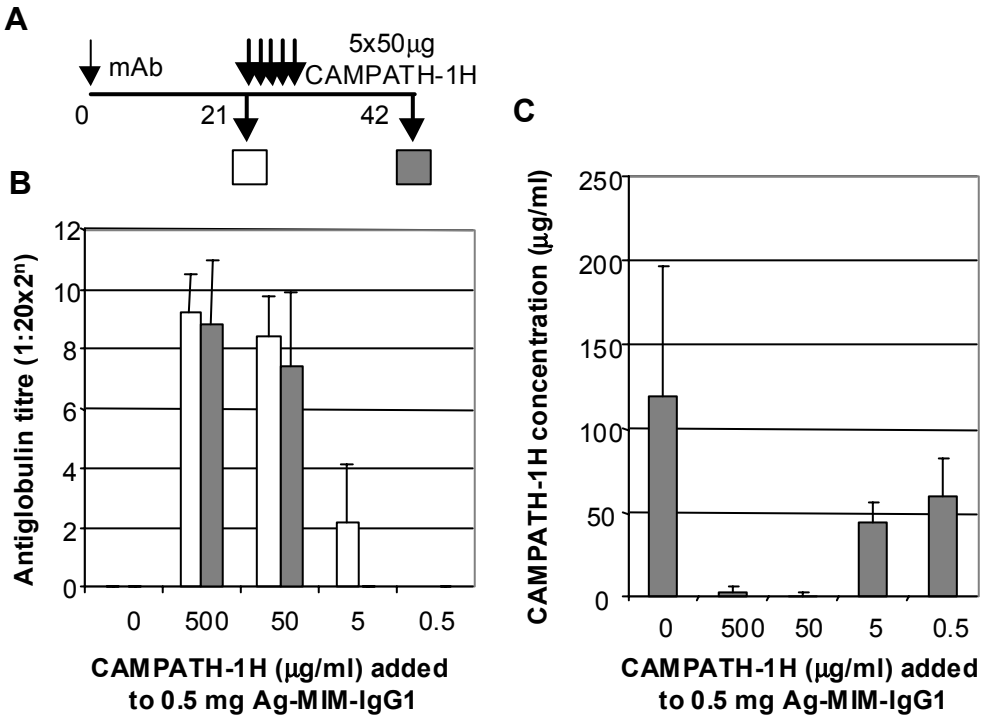

Figure 7.9 *A mixture of CAMPATH-1H and AG-MIM-IgG1 at the appropriate ratio can result in tolerance*. **A**, CP1-CBA mice were injected at day 0 with 0.5 mg AG-MIM-IgG1, alone or in combination with 0.5 mg, 50 μg, 5 μg or 0.5 μg CAMPATH-1H. All mice were bled at day 21, and antiglobulin titles measured by ELISA (white bars). All mice were then challenged with 5 doses of 50μg CAMPATH-1H between day 21 and 25. Another blood sample was collected at day 42, and antiglobulin titres determined as before (grey bars). **B**, Comparison of antiglobulin titres from mice injected with different mAb mixtures. White and grey bars correspond to samples from day 21 or 42 respectively. The titres were not significantly different between day 21 and 42 in any group except the one treated with 5 μg CAMPATH-1H ($p=0.0338$). There was a significant reduction in antiglobulin titres, both at days 21 and 42 between the groups injected with 50 μg and 500 μg CAMPATH-1H compared with any other group ($p<0.0005$). **C**, At day 42 the serum concentration of human antibody was determined in all mice by ELISA. Mice with higher antiglobulin titres had lower concentration of human mAb in the sera, being the difference between any of those two groups and any other group statistically significant ($p<0.01$).

7.2.9 Tolerance to CAMPATH-1H correlates with a longer biological effect of the mAb

Initial experiments suggested that treatment with MIM-IgG1 could result in a more prolonged T cell depletion than the use of an equal dose of CAMPATH-1H (Figure 7.5). I confirmed that a longer biological effect of this type is dependent on tolerance induction.

Different groups of CP1-CBA mice were treated with 0.5 mg of MIM-IgG1, CAMPATH-1H, or a control mAb. An additional group of mice were pre-tolerised by treatment with 0.5 mg AG-MIM-IgG1 21 days prior to CAMPATH-1H treatment (Figure 7.10). Remarkably, the two groups treated with the same dose of CAMPATH-1H showed a very different result in terms of long-term depletion. The tolerant mice maintained a very low level of T cells up to 60 days following CAMPATH-1H treatment, while in the non-tolerant group T cells started to expand in the first 3 weeks. Mice treated with MIM-IgG1 showed an intermediate effect: such treatment does not lead to near absolute cell depletion as obtained following CAMPATH-1H treatment. However, low levels of T cells are maintained for a long period of time, probably as a result of low antiglobulin levels.

7.3 Discussion

The present study was prompted by the need in animal models for non-immunogenic mAbs as reagents for *in vivo* experimental work. I used, as a starting point, the previous observation that non-cell binding mAbs can induce tolerance to the binding form (Benjamin *et al*., 1986). Gilliland and colleagues have shown that by mutating HCDR2 of CAMPATH-1H one can prevent its binding, being the mutant Ab capable of inducing tolerance to

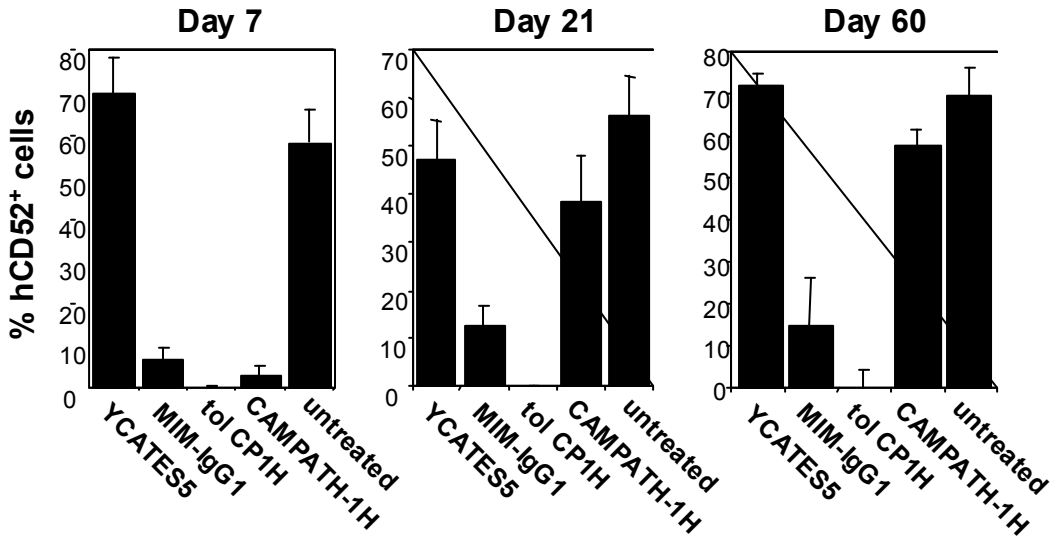

Figure 7.10 *Tolerance correlates with long-term depletion*. Mice were injected with 0.5 mg of different mAb at day 0. One group of mice treated with CAMPATH-1H at day 0, had been tolerised by treatment with 0.5 mg AG-MIM-IgG1 at day –30. Depletion of T cells among peripheral blood lymphocytes was monitored by flow cytometry. Tolerised mice treated with CAMPATH-1H showed a much longer depletion than mice injected with the same CAMPATH-1H dose in the absence of pre-tolerisation. At day 60, the percentage of $hCD52^+$ cells in the peripheral blood of mice treated with CAMPATH-1H (57.8 $\pm$ 1.60) was significantly higher than in animals treated with the same mAb but tolerised in advance with AG-MIM-IgG1 (1.80 $\pm$ 1.72; $p<0.0001$) or mice treated with MIM-IgG1 (17.3 $\pm$ 5.7; $p<0.0001$).

CAMPATH-1H (Gilliland *et al*., 1999). With the present experiments I confirmed that by constructing a mAb variant lacking cell binding activity, one can obtain an mAb capable of inducing tolerance to the cell binding form.

We decided to prevent cell-binding capacity of CAMPATH-1H using a CD52 mimotope convalently linked to the V region of its light chain. We anticipated that such a strategy would have three important advantages. First, the complete primary sequence and structure of CAMPATH-1H would be preserved in the tolerogenic mAb. Second, the process offered a new and general method for creating non-cell binding variations of different mAbs. Third, it might, in time, be possible to remove the linked mimotope once tolerance had been induced, so releasing a functional mAb.

In vitro studies confirmed that the MIM-IgG1 had a poor binding capacity when compared with CAMPATH-1H or P61-IgG1 (a control with an irrelevant peptide). Remarkably, when injected *in vivo*, MIM-IgG1 could bind to cells and even mediate cell depletion. However, MIM-IgG1 took more time to bind to cells than CAMPATH-1H, and does not seem to saturate cellular sites in the manner of CAMPATH-1H (Figures 7.3 and 7.4). It is therefore likely that a large fraction of MIM-IgG1 remained in the plasma, in spite of some cell binding activity. In the case of CAMPATH-1H treatment it is certain that the proportion of the injected mAb that remained in the circulation was lower. Such differences in the cell binding capacity offer an explanation for the distinct pattern in cell depletion observed following treatment with MIM-IgG1 or CAMPATH-1H (Figure 7.5). A larger dose of MIM-IgG1 than of CAMPATH-1H was required to achieve substantial T cell depletion, possibly due to a significant fraction of MIM-IgG1 that remained unbound. Depletion with MIM-IgG1, at the highest tested doses, had a slower onset than by CAMPATH-1H, and a “near complete” depletion, as follows CAMPATH-1H treatment, was

never observed with MIM-IgG1. Interestingly, the depletion achieved with MIM-IgG1 was maintained for a longer period than deletion by CAMPATH-1H (Figures 7.5 and 7.10).

As predicted, MIM-IgG1 was significantly less immunogenic than CAMPATH-1H and could prevent the generation of antiglobulins following subsequent treatment with CAMPATH-1H (Figures 7.6 and 7.7). Remarkably, AG-MIM-IgG1 was not immunogenic and could induce complete tolerance to CAMPATH-1H. Although the antigen binding properties of AG-MIM-IgG1 and MIM-IgG1 are slightly different (Figures 7.2 and 7.3), we believe that such differences are probably insufficient to explain the differences in immunogenicity and tolerogenicity. It is likely that a mAb capable of causing cell deletion will generate some pro-inflammatory “danger” signals. As a consequence, a similar mAb unable to activate effector function would not generate such “danger” signals and would be less immunogenic. Indeed AG-MIM-IgG1 lacked cell depleting activity altogether.

We also established that tolerance with AG-MIM-IgG1 took time to reach completion. Three days following the tolerogenic treatment, the capacity to produce antiglobulins following administration of the immunogenic wild-type mAb was clearly compromised, but not totally (Figure 7.8). It is interesting to compare this time-scale with the kinetics of cell binding *in vivo* (Figures 7.3 and 7.4). It is likely that in the initial days when tolerance is becoming established most of the MIM-IgG1 or AG-MIM-IgG1 is unbound, with progressively more Ab being deposited on T cells with time. However, depletion with MIM-IgG1 only becomes significant 8 days after treatment, at a time partial tolerance was already established. Furthermore, when a mixture of AG-MIM-IgG1 and CAMPATH-1H was injected at different ratios we confirmed that 5 μg of CAMPATH-1H could be co-administered with 0.5 mg

of AG-MIM-IgG1 without compromising tolerance induction (Figure 7.9). Such a result suggests that some cell binding mAb present at the time of tolerance induction need not prevent tolerance if an excess of unbound tolerising mAb is also present.

Taken together, these results suggest that the bulk of MIM-IgG1 and AG-MIM-IgG1, due to their slow accumulation on cells, remain mostly in the unbound form, in the circulation, during the initial days following treatment. About 1 week following mAb injection, more mAbs accumulate on T cells, resulting in greater cell depletion with MIM-IgG1. However, as tolerance is already partially established, the cell-bound mAb is less able to evoke an immune response.

It is important to stress that all *in vivo* experiments were performed using humanised mAbs in transgenic mice. As a consequence, the immunogenic challenge was much greater than that which might be expected in clinical situations where humanised mAbs are used. It may well be possible that the incomplete tolerogenic effect of MIM-IgG1 in the mouse could be sufficient to induce complete tolerance in a less stringent human – human situation. It is even likely that the partial tolerance effect obtained following MIM-IgG1 treatment could be adequate, as animals treated with MIM-IgG1 showed prolonged cell depletion (Figure 7.10), and maintained the low level of antiglobulins in spite of subsequent administrations of CAMPATH-1H (Figure 7.7).

The finding that MIM-IgG1 binds to target cells at a slower rate than CAMPATH-1H may offer important clinical benefits. One frequent problem of mAbs that target lymphocytes is the deleterious effect of cytokines released as a result, a complication known as cytokine release syndrome (Cosimi *et*

al., 1981; Group, 1985). By diminishing the speed of cell depletion in may be possible to reduce or even eliminate cytokine release syndrome when a therapeutic mAb with an obstructed binding site is used. Additionally, it is frequently difficult to remove efficiently large solid tumours with mAbs. In part this may be due to the cells at the periphery acquiring most of the available mAb, so that insufficient mAb accumulates in the central tumour cells. If so, then this might be improved with a mAb like MIM-IgG1 that binds slowly and maintains efficacy for longer than CAMPATH-1H. This way it may be possible to progressively reduce the tumour mass without “wasting” therapeutic mAb on the peripheral cells. Finally, the process of reducing immunogenicity and achieving tolerogenic capacity by interfering with cell binding with a linked peptide may prove useful for other molecules apart from therapeutic mAbs. When foreign molecules are introduced in patients, either directly, like factors VIII and IX in haemophilia and therapeutic enzymes in storage diseases, or by gene therapy, their immunogenicity can limit their effectiveness. A similar strategy to create non-cell binding tolerogenic molecules may be possible in this context.

A natural development from our experimental system will be to design “linkers” that can be cleaved at specific sites or particular time. Such “cleavable” linkers may allow a better control of the site and time for therapeutic activity, while allowing the maintenance of a large proportion of soluble mAb to induce tolerance to the cell binding form.

CHAPTER 8

DISCUSSION

There is now compelling evidence for active regulation of immune responses by T cells. The mechanisms responsible for such active regulation remain largely unknown, and it is not inconceivable that multiple redundant regulatory strategies are operating.

The suggestion that antibody-induced transplantation tolerance, as well as self-tolerance may in part be maintained by $CD4^+$ regulatory T cells (Powrie and Mason, 1990; Qin *et al*., 1993; Sakaguchi *et al*., 1995; Chen *et al*., 1996; Onodera *et al*., 1996; Asano *et al*., 1996; Hall *et al*., 1998; Thornton and Shevach, 1998) sparked interest in the characterisation of mechanisms by which T cells regulate in these model systems. When compared with other cells that have been shown capable of suppressing T cell responses (at least *in vitro*) $CD4^+CD25^+$ T cells seem to be the most potent.

It is still not known whether regulatory T cells implicated in self-tolerance have any relationship with the $CD4^+$ regulatory T cells involved in transplantation tolerance. By understanding the characteristics and mechanisms of action of regulatory T cells it may be possible to develop rational methods for the induction of tolerance both in transplantation and autoimmunity. In addition, the precise characterisation of specific markers of regulatory function may allow the biology of regulatory cells to be studied in greater detail, as well as the development of useful diagnostic tools for clinical use.

The starting point for this thesis was the study of whether tolerance induced with co-stimulation blockade lead to the development of regulatory T cells that could maintain a state of dominant tolerance and recruit new regulatory cells through infectious tolerance (Chapter 3). Indeed, long held assumptions postulated that co-stimulation blockade would lead to tolerance by inactivation or deletion of alloreactive clones (see Matzinger, 1999). I was able to demonstrate that indeed co-stimulation blockade induced the emergence of $CD4^+$ regulatory T cells (Graca *et al*., 2000; Chapter 3), adding to the body of knowledge that has also implicated reduction of alloreactive clones as part of the tolerisation process (Wells *et al*., 1999; Li *et al*., 1999; Li *et al*., 2001).

I therefore argue that the regulatory mechanisms induced following co-receptor blockade (with non-depleting anti-CD4 and anti-CD8 mAbs) are similar to the mechanisms triggered by tolerisation with co-stimulation blockade (with non-depleting anti-CD40L). In fact the properties of tolerance achieved with either one or the other strategy are remarkably similar: in both cases they involve dominant tolerance (Qin *et al*., 1990; Graca *et al*., 2000), infectious tolerance (Qin *et al*., 1993; Graca *et al*., 2000), and linked suppression (Davies *et al*., 1996a; Chen *et al*., 1996; Honey *et al*., 1999). In both cases tolerance is maintained by $CD4^+$ T cells (Qin *et al*., 1993; Graca *et al*., 2000), while AICD is probably involved during the induction phase (Li *et al*., 1999; Wells *et al*., 1999; Phillips *et al*., 2000).

Given the finding that both co-receptor or co-stimulation blockade can result in dominant tolerance, I decided to investigate whether the two strategies can be combined to induce tolerance more effectively. In Chapter 4 I show that combined co-stimulation and co-receptor blockade can enable induction of tolerance to fully mismatched skin allografts, which is not feasible with either

strategy alone. Tolerance so achieved was shown to be dominant, and dependent on $CD4^+$ T cells. Linked suppression was also observed. It is important to note that fully mismatched skin is considered to be the most stringent test for transplantation tolerance (Trambley *et al*., 2000), and that non-depleting mAbs targeting co-stimulation or co-receptors have generally failed to induce tolerance to fully mismatched skin allografts when given alone.

Given the results described above, it appears that $CD4^+$ regulatory T cells may be a final common pathway for a range of different strategies to induce transplantation tolerance. I decided therefore to study whether such $CD4^+$ regulatory T cells carried the CD25 marker which is characteristic of natural regulatory T cells in self-tolerance (Sakaguchi, 2000; Shevach, 2000; Maloy and Powrie, 2001). I have shown that naturally occurring $CD4^+CD25^+$ T cells from "naïve" mice, which had never experienced the transplantation antigens, can still prevent skin graft rejection if given in sufficient numbers together with normal splenocytes (Graca *et al*., 2002b; Chapter 5). However, $CD4^+CD25^+$ T cells from tolerised mice seemed more potent as a lower number were required to suppress allograft rejection. This result can be explained by any of: (1) a selective expansion of regulatory T cells from pre-existing precursors, (2) by *de novo* formation of $CD4^+CD25^+$ regulatory cells, or (3) by selective inactivation of putative non-tolerant cells from the $CD4^+CD25^+$ T cell population.

These observations question the antigen specificity of regulatory $CD4^+CD25^+$ T cells, one of the most important outstanding issues concerning regulatory T cell biology. Transplantation studies offer the opportunity to study antigen specificity of regulation in a more controlled way than animal models of autoimmunity. But a proper study of specificity will require a criss-cross

analysis, testing whether regulatory T cells which can prevent rejection of one type of allograft are permissive for rejection of a third-party allograft, and vice-versa. Until such experiments are performed, the question of antigen specificity of regulatory T cells remains unresolved.

In these same studies I have also observed regulatory capacity among the $CD4^+CD25^-$ population from tolerised mice, albeit 10-fold less potent than equivalent numbers of $CD4^+CD25^+$ T cells (Graca *et al*., 2002b; Chapter 5). However, as the proportion of $CD25^+$ cells among the $CD4^+$ T lymphocytes is ~10%, the overall regulatory capacity within the whole of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations is likely to be similar. Furthermore, by titrating unseparated $CD4^+$ T cells from tolerised mice, I concluded that the potency of the unseparated $CD4^+$ T cells was greater than the potency of equivalent numbers of sorted $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells. Given these results it is possible that the two populations can synergise in the maintenance of tolerance.

It is interesting that the $CD4^+CD25^-$ population is only seen to regulate if derived from tolerised, but not naïve mice. This may not be surprising as the $CD4^+CD25^-$ population in naïve animals is thought to carry the potential "aggressor cells". It may be that, in tolerance, some $CD4^+CD25^+$ regulatory cells loose the expression of CD25 and endow the $CD4^+CD25^-$ population with new regulatory powers, as has been suggested following homeostatic expansion of $CD4^+CD25^+$ T cells (Gavin *et al*., 2002). Alternatively, AICD previously reported to occur in the induction of transplantation tolerance (Wells *et al*., 1999; Li *et al*., 1999), may selectively remove effector cells from the $CD4^+CD25^-$ population so unmasking residual regulatory cell activity.

It is also an unresolved issue whether regulatory T cells (both $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells) represent a specific lineage exported from the thymus, or whether peripheral T cells can differentiate into regulatory cells in the context of a particular microenvironment.

In support of the "lineage" hypothesis, the thymus was shown to contain potent regulatory cells that are $CD4^+CD25^+CD45RC^{low}$ (Saoudi *et al*., 1996; Itoh *et al*., 1999). Furthermore, $CD4^+CD25^-$ thymocytes, unlike $CD4^+CD25^-$ peripheral T cells, seem to lack any suppressive capacity (Stephens and Mason, 2000). It has been shown that thymocytes from double transgenic mice, bearing both a high affinity TCR and its target antigen (a peptide derived from influenza hemaglutinin (HA)), can produce functional $CD4^+CD25^+$ T cells. However, double transgenic mice expressing a low affinity HA specific TCR fail to sustain the development of $CD4^+CD25^+$ T cells (Jordan *et al*., 2001). The authors suggest that thymocytes whose TCRs have high affinity for self-peptides being presented in the thymus, but not high enough to be committed towards negative selection, become $CD4^+CD25^+$ regulatory T cells.

This, however, is not conclusive evidence supporting lineage commitment. Our results have shown that dominant tolerance, mediated by $CD4^+$ T cells, can be induced in either euthymic or adult thymectomised animals (see Chapters 3 and 4). In such experiments tolerance is induced to non-self antigens. Although, such results are consistent with the hypothesis that T cells can become regulatory following peripheral "tolerisation", they are also insufficient for the conclusion that they derive *de novo*. It was recently shown that peripheral $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells, when injected into $RAG2^{-/-}$ mice proliferate yielding a mixed population where in both cases ~20% of the cells are $CD4^+CD25^+$ (Annacker *et al*., 2001b). The authors suggested that,

on the one hand, peripheral T cells are heterogeneous with regulatory cells, derived from $CD4^+CD25^+$ thymocytes, contained within both the $CD25^+$ and $CD25^-$ T cell pool (Annacker *et al*., 2001a). On the other hand, single positive thymocytes are considered to be more homogeneous for the presence of regulatory T cells, with all regulatory activity contained in the $CD4^+CD25^+$ subpopulation, while the $CD4^+CD25^-$ thymocytes are apparently devoid of any regulatory cells (Annacker *et al*., 2001a). Given the evidence suggesting that in single positive thymocytes CD25 expression defines two separate populations – regulatory and non-regulatory cells – it may be possible to further investigate the plasticity of thymic emigrants for developing into aggressive or regulatory T cells, by adoptive transfers of $CD4^+CD8^-CD25^-$ thymocytes into syngeneic thymectomised $RAG^{-/-}$ hosts. In such circumstances it may be possible to observe $CD4^+CD25^-$ T cells becoming effectors of skin graft rejection or regulatory T cells (even $CD4^+CD25^+$ regulatory T cells) if "tolerised" with non-depleting mAbs, or following co-existence with regulatory T cells (infectious tolerance). Such a result would strongly argue against a deterministic lineage of regulatory T cells, identified by the CD25 molecule, being exported from the thymus.

The effect of homeostatic-driven proliferation of transfused cells into T cell deficient hosts needs some discussion. It has been shown that both $CD4^+CD25^-$ and a fraction of the $CD4^+CD25^+$ T cells do expand when injected into $RAG2^{-/-}$ mice (Annacker *et al*., 2001b). Such *in vivo* results contrast with the poor proliferative capacity of $CD4^+CD25^+$ cells *in vitro* (Takahashi *et al*., 1998; Thornton and Shevach, 1998). However, it is important to note that in the *in vivo* experiments only ~10% of the injected $CD4^+CD25^+$ T cells underwent proliferation, involving 10 – 11 rounds of division, such that the progeny contributed to more than 99% of the cell pool once a steady state was reached (Annacker *et al*., 2001b). Nonetheless,

$CD4^+CD25^+$ T cells were shown capable of controlling the expansion of $CD4^+CD45RB^{high}$ T cells, suggesting a role in the control of homeostatic driven proliferation (Annacker *et al*., 2001b). Such findings raise the possibility that in animal models of gut immunopathology and autoimmune disease where pathology is associated with homeostatic proliferation of $CD4^+CD45RB^{high}$ T cells, the protective effect of $CD4^+CD25^+$ T cells may be due to control of homeostatic expansion rather than antigen-specific suppression.

It has been suggested that once activated, $CD4^+CD25^+$ T cells mediate suppression in an antigen non-specific way (Takahashi *et al*., 1998; Thornton and Shevach, 2000). One can speculate that under lymphopenic conditions, the $CD4^+CD25^+$ T cells have a competitive advantage over $CD25^-$ cells. Under such circumstances, the $CD4^+CD25^+$ T cells would benefit from homeostatic expansion, while simultaneously preventing the $CD25^-$ cells from expanding. As was shown by Gavin *et al*, under lymphopenic conditions homeostatic expansion is largely antigen non-specific, but MHC dependent – a situation similar to thymic positive selection (Gavin *et al*., 2002). It is likely that T cell competition for expansion in T cell replete animals is considerably tougher. In such conditions, it is improbable that simple interactions with MHC will support expansion. In a replete immune system it is therefore more likely that antigen specificity play an important role in the competition for expansion, and conversely in its inhibition. It will be important to develop *in vivo* experimental systems where the function of different cell populations can be assessed in the absence of homeostatic proliferation.

The finding that regulatory T cells can be identified in tolerated skin allografts but not, at least to the same extent, within the syngeneic skin of the same tolerised mice (Graca *et al*., 2002a; Chapter 6), bears some discussion. This

may in part explain the phenomenon of linked suppression (Davies *et al*., 1996a; Chen *et al*., 1996; Wong *et al*., 1997; Honey *et al*., 1999; Chapter 4). It is possible that the reason for graft acceptance when the tolerated and third-party antigens are linked in the same tissue, may be due to local effects of regulatory T cells. Whether the regulatory T cells present in tolerated allografts are resident peripheral regulatory cells or cells preferentially re-circulating through that tissue is presently unknown.

In some experiments, such as those where infectious tolerance is being investigated, it is desirable to use depleting mAbs to eliminate a particular T cell sub-population (host or donor). In euthymic mice, the long-term maintenance of non-detectable levels of the target lymphocytes can be difficult when anti-globulins start to neutralize the injected mAb. This problem is obviously one also encountered in the clinic when patients are treated with multiple doses of therapeutic mAb over a long period of time. In Chapter 7 I described how the immunogenicity of therapeutic mAbs can be circumvented when they are prevented from binding to cells with a mimotope that occupies their binding site. This strategy was based on previous observations that non-cell binding mAbs are not immunogenic and can induce tolerance to the binding form (Benjamin *et al*., 1986; Gilliland *et al*., 1999). Indeed I confirmed that by preventing CAMPATH-1H from binding to cells when its binding site is occupied by a mimotope, the immunogenicity is significantly reduced and at least partial tolerance to subsequent administrations of CAMPATH-1H is obtained.

It came as a surprise that such binding inhibition is not absolute, as with time MIM-IgG1 accumulates on the cell surface. Remarkably, MIM-IgG1 was found to be capable of cell depletion, although with a delayed onset when compared with wild-type CAMPATH-1H. I can therefore conclude that it is

possible to obtain two different actions from the same mAb construct: first, the MIM-IgG1 induces tolerance to itself while the majority of the mAb is unbound. Later, the mAb starts to accumulate on the cell surface leading to cell deletion once a threshold is reached. However, it no longer elicits a strong anti-globulin response as tolerance, at least partial, has already been established. As a consequence MIM-IgG1 achieves a better long-term effect, as seen by sustained cell depletion, than the wild-type CAMPATH-1H.

Such mAb variants may be useful in situations where antiglobulin responses may be undesirable. For example, when I investigated whether infectious tolerance leads to the emergence of $CD4^+CD25^+$ regulatory T cells in naive splenocytes, I used AG-MIM-IgG1 to induce tolerance to CAMPATH-1H. Subsequently, it became possible to achieve a long-term depletion of $hCD52^+$ T cells in the euthymic mice, so eliminating any experimental artefacts that might arise from anti-globulin responses (see Chapter 5).

Another finding described in Chapter 7 concerns the distinct immunogenic and tolerogenic properties of MIM-IgG1 and AG-MIM-IgG1. In spite of some minor differences in cell binding capacity between the two constructs, I believe the explanation must relate to the differences in pro-inflammatory signals that the two mAbs induce. As AG-MIM-IgG1 lacks cell depleting activity altogether, it is likely that in the absence of “danger” the outcome shifts the balance between effector and regulatory systems more towards the latter.

One can explain the results described in this thesis by considering a model where persistence of foreign antigen in the absence of danger would result in dominant tolerance mediated by regulatory $CD4^+$ T cells. Both in transplantation tolerance and tolerance to therapeutic mAbs that seems to be

the case. In tolerance induced to allografts it has been previously shown that the persistence of alloantigen is required for tolerance maintenance (Scully *et al*., 1994). Furthermore, the results described in Chapter 4 suggest that when tolerance is induced to fully mismatched skin grafts, dominant tolerance is only maintained towards alloantigens derived by the indirect pathway. As direct presentation to $CD4^+$ host T cells is necessarily transitory in the experimental system tested, regulatory T cells specific for that pathway are probably not maintained. It is possible that a major effect of tolerogenic mAbs, such as non-depleting anti-CD4, anti-CD8 and anti-CD40L, is to induce a cease-fire, therefore permitting interaction between $CD4^+$ T cells and alloantigens in the absence of "danger". Results described in Chapter 7 support this model: AG-MIM-IgG1 and MIM-IgG1 are two mAbs with essentially the same characteristics, except that the first is not lytic. I confirmed that the non-lytic mAb is a better tolerogen that the lytic one.

In the absence of "danger" it is unlikely that persistent non-self antigens (either transplantation antigens or therapeutic mAbs) would lead to presentation by fully mature DCs. It is most likely that in those circumstances tolerance is reinforced by antigen recognition in the context of immature DCs or a pro-tolerogenic set of DCs (Roncarolo *et al*., 2001; Higgins *et al*., 2002). It is not clear at this time whether $CD4^+$ regulatory T cells can drive DCs towards an alternative maturation pathway, forcing them to remain immature or "decommissioned". With time, through "infectious tolerance" the pool of regulatory $CD4^+$ T cells will increase, at the expense of naïve alloreactive cells. As a consequence, when alloantigens persist in the absence of "danger", tolerance becomes more robust with time due to the "infectious tolerance" recruitment of new regulatory cells. At a later time point dominant tolerance is robust enough to resist a challenge where "danger" is present. This has been repeatedly observed when a fresh skin graft of the tolerated

type is transplanted onto tolerised animals. But even more remarkable is the experiment described in Chapter 7, where after treatment with the tolerogenic mAb AG-MIM-IgG1, mice resist an immunogenic challenge with the lytic "danger"-inducing mAb CAMPATH-1H. Furthermore, the longer we wait until injecting the immunogenic mAb, the more robust tolerance has become (Chapter 7, Figure 7.8).

In immunology, as in human conflicts, it is likely that lasting "tolerance" does not rely on segregation, ignorance or indifference. Tolerance can only be achieved after a relatively long and peaceful coexistence, that may require active intervention from a peace-keeping force. Given enough time of peaceful coexistence tolerance may no longer be broken by "dangerous" situations.

Although many major issues in transplantation tolerance and T cell regulation remain unsolved, I anticipate that a significant advance in the field will be the development of diagnostic markers of the tolerant state *in vivo*. There are currently no reliable tests capable of distinguishing a tolerised animal from a primed one. The transfer of current knowledge of tolerogenic protocols to the clinic would be greatly facilitated if diagnostic tests for tolerance were available.

One possible strategy for the development of diagnostic tests, that is being pursued in the host laboratory, involves the characterization of genes uniquely expressed in regulatory cells. By comparing the genes expressed by different T cell sub-populations it may be possible to identify unique genes whose level of expression may correlate with tolerance (see Chapter 5). Together, with the observation that tolerated allografts harbour regulatory T cells (Chapter 6), one may predict that transplant tolerance correlates with

high tissue expression of regulatory T cell-specific genes. It may therefore be possible, although speculative, that by monitoring the level of such regulatory-specific transcripts in the patients blood or urine (in the case of kidney grafts) one may be able to monitor the tolerant status of the patients, and act accordingly.

In summary, this thesis demonstrates that transplantation tolerance induced by co-stimulation blockade leads to a dominant and infectious form of tolerance maintained by $CD4^+$ T cells (Chapter 3). It was consequently possible to achieve a synergy when co-stimulation blockade was combined with co-receptor blockade, leading to robust tolerance of fully mismatched skin allografts (Chapter 4). Such tolerance was also dominant, manifest by linked-suppression and dependent on regulatory $CD4^+$ T cells. By studying the phenotype of T cells maintaining dominant tolerance, I concluded that these could be found within the $CD4^+CD25^+$ and $CD4^+CD25^-$ populations of tolerised mice, but only among the $CD4^+CD25^+$ T cells of naïve animals (Chapter 5). Such regulatory cells were found not only in the spleen of tolerised mice, but also in the tolerated tissue (Chapter 6). Finally, I describe a strategy to eliminate the immunogenicity of therapeutic mAbs by interfering with their capacity to bind to cells by occupying the binding-site with a mimotope (Chapter 7). Further elucidation of mechanisms of transplantation tolerance, namely the identification of specific markers for regulatory T cells, may lead to significant advances on our understanding of T cell suppression and may greatly facilitate the clinical application of tolerogenic strategies.

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