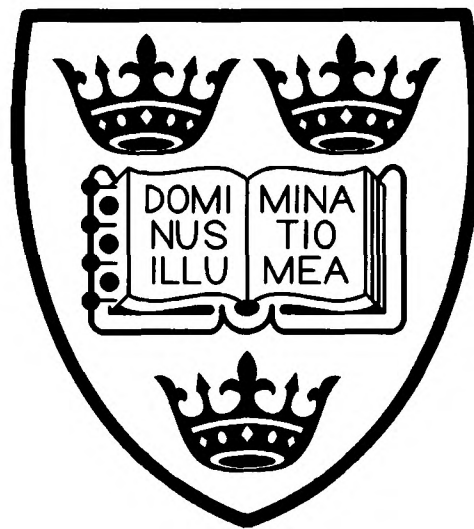


The Role of CD4⁺CD25⁺ Regulatory T Cells in a Mouse Transplantation Tolerance Model

**Thesis submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy**

Mahzuz Karim



**Brasenose College, Oxford
and
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Michaelmas Term 2003



This thesis is dedicated to my parents

ABSTRACT

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Clinical transplantation continues to rely on the use of non-specific immunosuppressive therapy, which reduces the incidence of graft rejection but also carries with it undesirable side effects such as infection and malignancy. A preferable option would be to induce operational graft tolerance without the need for such non-specific therapy, for example by harnessing natural mechanisms. In recent years there has been much progress in the characterisation of CD4⁺ cells that possess suppressive or regulatory properties in experimental systems; particular attention has been focussed upon CD4⁺ cells expressing CD25, the α subunit of the IL-2 receptor, which have been shown to possess regulatory capacity both *in vitro* and *in vivo* in autoimmune disease and transplantation models. The aim of this study was to examine the potential role of CD4⁺CD25⁺ regulatory T cells (Treg) in the induction phase of tolerance in a transplantation model.

Pre-treatment of mice with fully allogeneic blood administered under the cover of anti-CD4 antibody is shown to lead to the generation of CD4⁺CD25⁺ cells capable of preventing the rejection of donor type, but not third party, skin allografts mediated by CD4⁺CD45RB^{high} cells in secondary recipients. In addition to their suppressive properties *in vivo*, these CD4⁺CD25⁺ cells also display the ability to regulate the proliferation of target T cell populations *in vitro*.

Generation of CD4⁺CD25⁺ Treg by the pre-treatment protocol is not reliant upon an intrathymic selection process nor upon the expansion of a pre-existing CD4⁺CD25⁺ Treg population, but can occur through the conversion of peripheral CD4⁺CD25⁻ cells to a regulatory phenotype.

Although the regulatory function of the CD4⁺CD25⁺ cells generated by pre-treatment is donor strain-specific *in vivo*, this specificity can be overcome by activating the cells before their regulatory capacity is tested. Moreover, CD4⁺CD25⁺ cells generated by pre-treatment with a non-cellular protein antigen completely unrelated to the graft can also regulate skin allograft rejection provided that these Treg are first activated.

It is hoped that the principles defined by these findings identify a strategy that may be applicable in clinical transplantation and in the therapy of autoimmune disease.

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ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
AIRE	Autoimmune regulator
AITR	Activation-inducible tumour necrosis factor receptor family-related gene
APC	Allophycocyanin
APC	Antigen presenting cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	5-(and 6-)-carboxyfluorescein diacetate, succinimidyl ester
CPM	Counts per minute
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CyC	Cy-chrome
DST	Donor-specific transfusion
ELISA	Enzyme-linked immunosorbent assay
FACS [®]	Fluorescence-activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FKBP	FK binding protein
Foxp3	Forkhead box P3
GITR	Glucocorticoid-induced tumour necrosis factor receptor family-related gene
H2	Mouse histocompatibility system 2
HA	Haemagglutinin
HBSS	Hanks' balanced salt solution
H&E	Haematoxylin and eosin

HGG	Human gamma globulin
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILT	Immunoglobulin-like transcript
IPEX	Immune dysregulation polyendocrinopathy, enteropathy, X-linked syndrome
mAb	Monoclonal antibody
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte response
MSH	Melanocyte stimulating hormone
MST	Median survival time
NDS	Nuffield Department of Surgery
NFAT	Nuclear factor for activated T cells
NOD	Non-obese diabetic
OD	Optical density
PBS	Phosphate-buffered saline
PCC	Pigeon cytochrome C
PE	R-phycoerythrin
PerCP	Peridinin chlorophyll protein
PMA	Phorbol myristate acetate
Rag	Recombination activating gene

RPMI	Roswell Park Memorial Institute
SA	Streptavidin
SAGE	Serial analysis of gene expression
SD	Standard deviation
TCR	T cell receptor
TGF	Transforming growth factor
TNF	Tumour necrosis factor
Treg	Regulatory T cells
TOR	Target of rapamycin

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CHAPTER 1

INTRODUCTION

1.1 Introduction

In the last forty years, transplantation has become a mainstay of therapy for end stage organ failure. This has occurred partly as a result of advances in areas such as surgery, anaesthesia and other technology, but also largely due to the introduction of effective immunosuppressive therapy. For organs such as the heart or liver, transplantation may provide the only realistic option for prolonging life; even in the case of the kidney, where artificial support is available in the form of dialysis, transplantation provides an improvement in quality of life and a significant survival advantage in many cases [1]. However, two major obstacles remain that hamper the success of clinical transplant programmes. The first is the relative paucity of donor organs available compared to the number of patients who could potentially benefit from transplantation (Table 1.1). The second is that grafts have a limited lifespan, partly as a result of immunological processes that can lead to graft rejection. In order to avoid this, all patients receiving solid organ transplants (with the exception of grafts from identical twins) require

	Patients on waiting list (at 31 st December 2001)	Transplants performed
Kidney and / or pancreas	6252	1749
Heart	113	171
Heart / lung	74	33
Lung	243	93
Liver	160	684
TOTAL	6842	2717†

Table 1.1. United Kingdom transplant activity for the year 2001

†Includes 13 multi-organ transplants
Source: UK Transplant Annual Report 2001

treatment with immunosuppressive therapy for the life of the graft. At present this immunosuppressive therapy is non-specific in nature, and as a result carries with it the risk of infections (with “opportunistic” organisms that do not normally cause disease in immunocompetent hosts, as well as with conventional pathogens) [2] and malignancy (in particular skin tumours and lymphoproliferative disorders) [3-7]. In addition, however, the individual drugs used also have their own specific side effects. An ironic example of this is that the calcineurin inhibitors (such as cyclosporin), which have had a major beneficial effect on the success of clinical transplantation, are also nephrotoxic. This can result in damage to renal allografts, but may also cause renal dysfunction in recipients of other organs such as hearts, where up to ten per cent of recipients may develop end stage renal failure (requiring dialysis or transplantation) as a consequence of immunosuppressive therapy [8]. Thus, immunosuppressive therapy not only carries specific risks, but in some settings may also contribute to graft failure in the long term, compounding the problem of lack of donor organ availability. For these reasons, the pursuit of strategies that would obviate the need for long term non-specific immunosuppression remains a major goal of transplantation research.

1.2 A brief history of solid organ transplantation

The concept of transplantation of tissues or organs from one human being to another has been around for many years. For example, the legend of Cosmos and Damian, the patron saints of medicine, describes the transplantation of a leg from a recently deceased man onto an amputee around the fourth century AD [9]. However, the main advances that have made transplantation a clinical reality have occurred during the

twentieth century. The development of techniques for overcoming immunologically-mediated graft rejection will be described later in this chapter; in this section some of the more general aspects will be discussed.

The fundamental requirement for successful solid organ transplantation is the ability to perform the requisite surgery, and the most important aspect of this is to construct vascular anastomoses to provide the organ with a blood supply. In the early part of the 20th century the French surgeon Alexis Carrel, working initially in Lyon and then in Chicago, developed techniques allowing the successful anastomosis of blood vessels [10]. His methods still form the basis of those used in both transplant surgery and in vascular surgery to this day. He went on to examine aspects of experimental transplantation, and was awarded the Nobel Prize for Medicine in 1912 *“in recognition of his work on vascular suture and the transplantation of organs”*.

At around the same time that Carrel was carrying out his work, the Austrian pathologist Karl Landsteiner was investigating use of blood transfusion in humans. He demonstrated that transfusion of blood from one individual to another often led to catastrophic haemolytic reactions, and went on to describe the ABO blood group system in man [11]. He was awarded the Nobel Prize for Medicine in 1930 *“for his discovery of human blood groups”*. This work paved the way for clinical blood transfusion (the first widely practised form of allogeneic transplantation), a supportive technique that has been essential to enable the support of sick patients and to allow complex surgical procedures to be undertaken. Furthermore, the discovery of blood groups also had a more direct impact on transplantation specifically, since the molecules involved are important histocompatibility antigens that can play a major role in initiating graft rejection (discussed further later).

In a similar way to blood transfusion, the development of other techniques has not only been necessary to allow the surgical procedure of transplantation to be carried out, but also to support patients for long enough to undergo the procedure. The most obvious example is the invention by Willem Kolff in the 1940s of the artificial kidney [12], which was able to keep alive patients who had developed renal failure. Similarly, the development of cardiopulmonary bypass, first used successfully in man by John Gibbon in the United States, enabled complex cardiac surgical procedures (including transplantation) to be undertaken. Many advances in techniques of anaesthesia, surgery, medicine, and intensive care have similarly contributed to the success of transplantation, as have methods allowing organ preservation prior to transplantation.

The first demonstration in man that, in the absence of the immunological barrier of rejection, transplantation could be successfully carried out was provided in 1954. A group in Boston, led by Joseph Murray, transplanted a kidney from a healthy man to his identical twin brother who was suffering from chronic renal failure [13]. The procedure was a success, and the recipient survived for eight years. Murray went on to make many other contributions in the field of transplantation, both in research and in clinical practice, and in 1990 was awarded the Nobel Prize for Medicine together with E. Donnall Thomas (a pioneer in the field of bone marrow transplantation) *“for their discoveries concerning organ and cell transplantation in the treatment of human disease”*. The development of effective immunosuppressive therapy allowed the transplantation of kidneys between genetically non-identical individuals [14], and enabled the transplantation of other solid organs such as the liver (first performed by Thomas Starzl in Denver in 1963 [15]), heart (by Christiaan

Barnard in Capetown in 1967 [16]), pancreas (by Richard Lillehei in Minnesota in 1967 [17]), lung [18], and small bowel [19].

In addition to the many practical techniques that have enabled transplantation to become a successful clinical procedure, there have also been many ethical and political issues involved. These have mainly centred around the source of the organs to be transplanted. For example, in the context of cadaveric transplantation there was debate in France over whether organs from executed prisoners should be used for transplantation. An even more fundamental concept was the introduction of criteria for the diagnosis of brain death, which led to redefinition of the concept of death itself, an issue that caused much controversy. More recently the development of live donor transplantation has led to fears over the potential abuse of donors. Finally, attempts to use solid organ xenografts (for example from pigs) in clinical practice are currently under a moratorium, partly due to concerns over the potential for the transmission of infectious agents such as porcine endogenous retroviruses.

1.3 Allograft rejection

By the early 20th century it was already well recognised that allografts transplanted between non-genetically identical individuals undergo rejection. However, it was in the 1940s that work by Medawar and colleagues demonstrated that this is an immunologically-mediated phenomenon, both in animal models [20] and in humans [21]. This finding led to many efforts to attempt to characterise the processes responsible.

One major focus of research was to identify the antigenic stimuli that precipitate graft rejection. In the 1930s Gorer demonstrated that erythrocytes from

mice of different strains could be distinguished by serological reactions [22, 23], and that the antigens responsible also appeared to play a role in initiating the rejection of allogeneic tumours [24, 25]. This work eventually led to the description of the H2 antigen system, the mouse form of the major histocompatibility complex (MHC). The human form of MHC, the human leukocyte antigen (HLA) system, was first described by Dausset in 1952 [26] and, as the name suggests, it soon became apparent that these antigens play a major role in allograft rejection.

As understanding of the immune system grew, the reasons that MHC antigens act as such a powerful stimulus to allograft rejection became more clear. Figure 1.1 summarises the main events that occur during T cell activation (reviewed in [27, 28]). T cells recognise antigen through the T cell receptor (TCR). However, unlike antigen receptors on B cells, TCR do not ligate intact protein antigen but instead bind to peptide fragments of antigen lying within a binding groove of MHC molecules on the surface of antigen presenting cells (APC) [29]. The specificity of this TCR interaction is not dependent solely upon the peptide antigen fragment, but rather is determined by the structure of the peptide-MHC complex as a whole. During immune responses to pathogens, peptide is presented in the context of self MHC molecules; however, following allogeneic transplantation donor MHC can be recognised in two ways, the so-called “direct” and “indirect” pathways (Figure 1.2). In indirect allorecognition, donor MHC molecules are processed by recipient APC and then fragments are presented to T cells in the context of recipient MHC molecules, a process analogous to the presentation of viral or bacterial antigens. In contrast, in direct allorecognition, recipient T cells bind directly to intact donor MHC molecules on the surface of donor cells. The process of direct allorecognition is not one that has developed specifically during evolution, since encounter of intact foreign MHC molecules is not a natural

occurrence. Rather, direct allorecognition appears to be a by-product of cross-reactivity in binding of TCR to their cognate ligands. Surprisingly it has been shown that a high proportion of T cells are able to recognise allogeneic MHC molecules via the direct pathway [30-38]. Two potential explanations for this have been proposed [39]. In the “binary complex” model [36] it is suggested that intact donor MHC molecules present a range of peptides; some of these peptide-MHC combinations are capable of stimulating recipient T cells (through cross-reactivity with antigen restricted by recipient MHC molecules). Alternatively, in the “high determinant density” model [40], recognition by T cells is primarily focussed upon the donor MHC molecule itself, with the peptide carrying less importance. Although the individual interactions between donor MHC molecules and recipient T cell receptors may be of relatively low affinity, the high determinant density provided by all the MHC molecules on the donor cells provides an overall avidity high enough to lead to T cell activation.

Binding of TCR to their cognate peptide-MHC ligand is not on its own sufficient to activate T cells: there is also a requirement for one or more additional “costimulatory” signal(s) [41] (Figure 1.1). The best known such costimulatory signal is provided by the binding of the CD80 (B7-1) or CD86 (B7-2) molecules on APC to CD28 expressed on T cells [42, 43]. T cell stimulation in the absence of such a signal can lead to anergy [44-46] or even cell death [47] rather than activation. Another costimulatory signal that has been identified is the binding of CD40 on APC to CD40 ligand (CD40L, CD154) on T cells [48]. Many other similar costimulatory pathways have now been identified [49], and this list is likely to continue to grow.

Antigen binding to the TCR in the presence of appropriate costimulation leads to a signalling cascade resulting in several effects including a rise in intracellular calcium within the T cell. This activates the calcium-dependent enzyme calcineurin,

leading to the dephosphorylation of the transcriptional regulatory protein nuclear factor for activated T cells (NFAT). NFAT then migrates to the cell nucleus where it induces the transcription of genes including IL-2 and other cytokines. Translation and secretion of the protein products of these genes then occurs. While the secreted cytokines have effects on other cells such as B cells and cytotoxic T cells, IL-2 can also bind to IL-2 receptors on T cells in an autocrine or paracrine fashion. This IL-2 binding leads to transduction of further signals to the cell nucleus. The combination of all these signals leads to T cell proliferation and thus clonal expansion of antigen-specific T cell populations. In addition, “help” provided by CD4⁺ T cells, partially mediated through the secretion of soluble factors, leads to the activation of effector cell populations which, in the setting of transplantation, results in graft rejection [50] (Figure 1.3).

The rejection of allografts is traditionally classified according to three categories, namely hyperacute, acute, and chronic [51]:

Hyperacute rejection is normally mediated by pre-formed antibodies within the recipient that bind to the donor organ, and can lead to graft loss within minutes. These antibodies may occur naturally, as in the case of antibodies against ABO blood group antigens. Alternatively they may be produced in response to previous sensitisation with alloantigen, for example by pregnancy, blood transfusion, or previous transplantation. Antibody-mediated hyperacute rejection is preventable by performing a cross-match test prior to transplantation. Another type of hyperacute rejection occurs following xenotransplantation, where the graft can activate the recipient complement cascade directly [52, 53].

Acute rejection follows the scheme depicted in Figure 1.3, and is predominantly cell-mediated. CD4⁺ T cells play a central role in orchestrating this

process, providing help to other effector cell populations which then mediate graft rejection over a period of days, weeks, or months. Current immunosuppressive therapy mainly targets the acute rejection process and, as will be discussed later, the majority of agents exert their action through inhibition of T cell activation.

Chronic rejection is not a single specific process, but, as its name suggests, is much more insidious, with graft damage occurring over a period of months or years. The mechanisms are less well understood than those of hyperacute or acute rejection [54, 55]. Immunological factors are thought to contribute, since isografts are less susceptible than allografts [56], and better HLA matching of solid organ allografts reduces the incidence of chronic rejection [57]; however, non-immunological mechanisms also play a role. The latter include non-specific responses to graft injury (for example due to the ischaemia-reperfusion process, or incurred during acute rejection episodes), and, in the case of renal allografts, side effects of therapy with agents such as cyclosporin. Therefore, chronic rejection shows a limited response to immunosuppressive therapy and, although modern immunosuppressive regimens have been very effective at reducing rates of acute rejection, they have had less influence on chronic rejection and thus long term graft survival.

1.4 Immunosuppressive therapy

Medawar's observation in the 1940s that the rejection of allografts is immunologically-mediated [20, 21] led to attempts to prolong graft survival by suppressing the activity of the recipient immune system. One early approach used was recipient irradiation. This was shown to enhance the survival of skin allografts in animals [58], but its use in humans proved to carry unacceptable morbidity and

mortality [59]. At around the same time it was noted that the administration of glucocorticoids could prolong the survival of allografts in animal models [60-62]. However, the major breakthrough in immunosuppressive therapy came with the discovery of the compound 6-mercaptopurine and its imidazole derivative azathioprine. 6-mercaptopurine was first identified by Gertrude Elion and George Hitchings (who were subsequently awarded the Nobel Prize for Medicine in 1988 “*for their discoveries of important principles for drug treatment*”) as part of a screening programme for anti-mitotic agents that may be useful for cancer treatment [63], but was subsequently found to have immunosuppressive properties in submyelotoxic doses [64]. This led to its use in transplantation models where it was shown to suppress the rejection of skin allografts in rabbits [65, 66] and renal allografts in dogs [67, 68]. These results were so promising that, in the early 1960s in Boston, combination therapy with steroids and azathioprine was tried in human renal allograft recipients [14]. This proved to be extremely successful in preventing graft rejection without carrying unacceptable toxicity and represented the first effective clinical immunosuppressive regimen, heralding a new era in clinical transplantation and paving the way for the transplantation of other solid organs.

The next significant development in clinical immunosuppression came with the introduction of antibody therapy to target T cells. Polyclonal anti-lymphocyte globulin (made by immunising animals with human lymphocytes) was shown to be effective in preventing the rejection of human renal allografts [69], and polyclonal anti-thymocyte globulin (active against T cells) remains in widespread clinical use. OKT3, a monoclonal antibody directed against the CD3 complex on T cells, underwent trial for the treatment of acute renal allograft rejection in the early 1980s

[70, 71], and has the distinction of being the first monoclonal antibody introduced into clinical practice.

A further seminal advance in immunosuppressive therapy occurred with the introduction of cyclosporin. This agent was first extracted from the fungus *Trichoderma polysporum* and was shown by Jean Borel at the pharmaceutical firm Sandoz in Basle to have immunosuppressive properties [72-74]. This eventually led to clinical trials of cyclosporin for the prevention of renal allograft rejection by Calne and colleagues in Cambridge [75, 76], and for treatment and prophylaxis of graft versus host disease in bone marrow transplant recipients by Powles *et al.* at the Royal Marsden Hospital in London [77, 78]. The use of cyclosporin had a dramatic effect on the success of cadaveric renal transplantation, improving the one year graft survival rate from around 50% to approximately 80%.

“Triple therapy” with steroids, azathioprine, and cyclosporin remained the cornerstone of immunosuppressive therapy in clinical transplantation for many years, but more recently other drugs have been introduced. The main agents in current use in the United Kingdom are listed below, and their mechanisms of action are summarised in Figure 1.4. However, current immunosuppressive therapy remains non-specific in its action and thus, in addition to reducing the immune response against the graft, also increases susceptibility to infection [2] and malignancy [3-7].

Anti-thymocyte globulin and **OKT3**, as discussed above, are respectively polyclonal and monoclonal antibodies directed against T cells. They exert their activity by deleting T cells and / or modulating their function, thus inhibiting graft rejection [79].

Cyclosporin is a cyclic endecapeptide, and acts to block signal transduction via the calcineurin pathway. It binds to the intracytoplasmic molecule cyclophilin, and

the cyclosporin-cyclophilin complex thus formed then inhibits the activity of calcineurin, preventing the dephosphorylation of NFAT [80-83]. Ironically, although (as discussed above) the use of cyclosporin led to a dramatic improvement in outcome following cadaveric renal transplantation, it is toxic to the kidney and so can contribute to the loss of renal allografts and cause renal damage in recipients of other solid organ allografts [8]; this nephrotoxicity may, at least in part, be due to increased production of the pro-fibrotic cytokine TGF- β [84].

Tacrolimus (FK506) is a cyclic macrolide that binds to its intracellular target, FK binding protein 12 (FKBP12) and then, like the cyclosporin-cyclophilin complex, inhibits the activity of calcineurin [83, 85-89]. As a calcineurin inhibitor, tacrolimus shares the nephrotoxicity of cyclosporin.

Glucocorticoids such as prednisolone have complex effects. They bind to intracytoplasmic receptors that then migrate to the cell nucleus and influence gene transcription [90, 91]. They have specific actions related to IL-2, inhibiting both IL-2 gene transcription [92] and signalling via the IL-2 receptor [93]. In addition they suppress the activity of the enzymes involved in prostaglandin metabolism, thus also exerting a direct anti-inflammatory effect [94, 95].

Azathioprine acts to inhibit nucleic acid synthesis [96], and thus targets proliferating cells in a relatively non-specific manner. However, rapidly-dividing cells such as proliferating lymphocytes are particularly susceptible to this inhibition. For the same reason, the main toxicity of azathioprine is bone marrow suppression.

Mycophenolate mofetil also acts to inhibit nucleic acid synthesis but is more lymphocyte-specific in its action than azathioprine. It is converted to mycophenolic acid, which inhibits inosine monophosphate dehydrogenase, an enzyme required for the synthesis of purines via the *de novo* pathway [97-100]. While most other cell types

are able to synthesise purines via both the *de novo* and salvage pathways, lymphocytes are entirely dependent upon the *de novo* pathway and are thus particularly susceptible to the effects of mycophenolate mofetil.

Basiliximab and **daclizumab** are monoclonal antibodies directed against the CD25 molecule, the α subunit of the IL-2 receptor [101-112]. CD25 is not generally expressed at significant levels on resting T cells, but is upregulated following cell activation [113-115]. These antibodies therefore theoretically target activated T cells and spare resting cells that have not been stimulated by the graft. However, as will become clear in this thesis, CD25 may also be expressed by regulatory cells that have the potential to confer graft protection. The use of these agents will be discussed further in Chapter 7.

Sirolimus (rapamycin) and its more recently developed analogue **everolimus** are macrolide antibiotics that also have immunosuppressive properties. They bind to the molecule “target of rapamycin” (TOR) [116, 117] and inhibit transduction of the signal generated by the extracellular binding of IL-2 to its cell surface receptor [89, 118].

1.5 The blood transfusion effect

Following the introduction of successful immunosuppressive regimens into clinical practice in the 1960s, transplant programmes were established, with cadaveric renal transplantation leading the way. Unsurprisingly, there was much interest in analysing the results of these new programmes, with particular emphasis being placed on identifying factors that may have an influence on graft outcome. In 1967 Dossetor and colleagues published a retrospective analysis of the results of their cadaveric renal

transplant programme [119]. One of their findings was that patients who had received blood transfusions (for the treatment of anaemia secondary to chronic renal failure) while awaiting transplantation showed superior graft survival compared to those who had not. This was a surprising observation since it was assumed that pre-operative blood transfusion would lead to recipient sensitisation and thus predispose to graft rejection. However, a similar retrospective study published the following year by Morris and colleagues also showed that blood transfusion conferred a beneficial effect on graft outcome [120]. These observations prompted more detailed investigation of this phenomenon, and thus larger studies were performed, predominantly led by Opelz and Terasaki [121-124]. The data obtained were striking, and the degree of benefit was found to increase with the number of transfusions given. Patients who received more than 20 transfusions had a one year graft survival of 75% compared to 59% in recipients of a single transfusion and 41% in non-transfused patients [123]. This “blood transfusion effect” led the authors to advocate routine blood transfusion for patients awaiting renal transplantation, and fitted well with the need for transfusion to treat the anaemia resulting from chronic renal failure. However, the introduction of erythropoietin therapy in the 1980s meant that anaemia in dialysis patients could be treated without the need for blood transfusion [125-127], and in addition it was suggested that, following the introduction of cyclosporin, pre-operative transfusion no longer had such a significant effect on graft outcome [128]; these factors, together with concerns over the potential for the transmission of infection, led to a wane in popularity of the practice of routine blood transfusion prior to transplantation. However, a more recent randomised prospective study in cadaveric renal transplant recipients has shown that, even in the cyclosporin era, transfusion still carries benefit, with one year graft survival in non-transfused recipients of 82% compared to 90% in

patients who had received one or more transfusions, and five year graft survival of 70% and 79% respectively [129].

Blood administered to patients awaiting transplantation is, of necessity, random in terms of matching to the eventual graft donor. With the advent of live donor transplantation, where the identity of the donor is known well in advance of the procedure, there was speculation that pre-operative administration of blood from the graft donor may have an even more beneficial effect than transfusion of random blood. Several clinical trials have been performed in which donor-specific transfusion (DST) has been administered prior to live donor renal transplantation [130-143]. The results suggest that DST does indeed have a beneficial effect on graft survival, although follow-up has been relatively short and the effects on long term graft outcome have not been well studied. A caveat from these trials is that a significant proportion of recipients became sensitised to the donor following transfusion, precluding subsequent transplantation; however, the rate of sensitisation was reduced by the administration of immunosuppressive therapy at the time of transfusion.

In order to investigate the mechanisms underlying the blood transfusion effect, various experimental models have been developed. Such models have shown that pre-treatment of allograft recipients with donor antigen (for example in the form of transfused blood) can lead to prolonged (and in some cases indefinite) allograft survival in situations such as rat renal [144-147] and cardiac [148-151], and mouse skin [152] and cardiac [153-158] transplantation. In our laboratory it has been demonstrated that pre-treatment of mice with donor-specific blood transfusion under the cover of anti-CD4 antibody therapy leads to the long term survival of donor strain, but not third party, cardiac allografts [155-158]; this pre-treatment protocol will be a major focus of this thesis and will be discussed further in Chapter 3.

1.6 Mechanisms of tolerance

During the Second World War, following the crash of an aircraft near his home, the young zoologist Peter Medawar became aware that airmen who suffered widespread burns often died as a direct result of skin loss. This fortuitous event led him to become interested in the question of why skin transplanted from one person to another undergoes rejection, and provided the foundation for the focus of the rest of his career [159]. As discussed earlier, Medawar showed that the rejection of allografts is an immunologically-mediated process [20, 21]. This observation prompted him to investigate a phenomenon that has long been of interest to immunologists, namely “self-tolerance”, the term applied to mechanisms by which the immune system is able to avoid responses directed against the host. The dogma at the time was that all tissue or organ grafts performed between non-genetically identical individuals would undergo rejection. However, Medawar and his colleagues made the surprising observation that there was an exception to this rule: dizygotic cattle twins are able to accept grafts from one another without any evidence of rejection, but show normal ability to reject grafts from other donors [160]. The geneticist Ray D. Owen, working at the University of Wisconsin, had observed that dizygotic cattle twins show microchimerism, containing a mixture of circulating cells derived from their twin as well as themselves, and demonstrated that this was due to the sharing of a single placenta [161]. This led Medawar to speculate that exposure of the developing immune system to foreign antigen could lead to antigen-specific tolerance. Together with his colleagues Rupert Billingham and Leslie Brent, Medawar tested this hypothesis by inoculating recipient CBA mice *in utero* with cells from mice of the fully allogeneic donor strain A. In a seminal paper published in 1953 [162], they showed that, as adults, these CBA mice accepted A strain skin grafts long term,

demonstrating that tolerance can be an actively acquired phenomenon. Medawar subsequently received the 1960 Nobel Prize for Medicine “*for the discovery of acquired immunological tolerance*”. Since that time, many groups have worked to determine the means by which tolerance (either to self or exogenous antigens) can be achieved; the main mechanisms identified to date are summarised below. It is hoped that it may be possible to achieve operational tolerance to allografts by harnessing these mechanisms, thus obviating the need for non-specific immunosuppressive therapy.

1.6.1 Central (deletional) tolerance

The development of T cells includes a period of maturation within the thymus, during which they are subjected to positive and negative selection. These selection processes will be discussed in greater detail in Chapter 5, and are summarised in Figure 1.5a,b. T cells that possess receptors with low affinity for self peptide-MHC complexes die through neglect as they are not rescued from apoptosis during the process of positive selection [163, 164], whereas those with high affinity are deleted during negative selection [165-169]. The former process ensures that mature T cells are capable of activation by peptide antigen presented in the context of self MHC molecules, whereas the latter deletes T cells that could be reactive against self antigens and that may therefore have the capacity to cause autoimmune disease. This negative selection process is referred to as central tolerance, and is regarded as a major mechanism by which self tolerance is achieved [170, 171]. However, it is not the sole mechanism, partly because not all self antigens are expressed within the thymus.

1.6.2 Peripheral deletion

Under certain circumstances mature T cells may undergo deletion in the periphery following antigen encounter [172-177], a process that may contribute to tolerance. One example of this is through the action of so-called “veto cells”, APC with the capacity to delete target T cell populations [178, 179]. A further example is the deletion of donor-reactive T cells that can occur following hepatic transplantation [180, 181], contributing to the “liver effect” whereby liver allografts are relatively resistant to rejection [182-185] and can confer protection upon other grafts transplanted from the same donor [186-189].

1.6.3 Anergy

A third potential outcome (other than activation or deletion) following antigen encounter by T cells is that they become “anergic” (functionally inactive): although the T cell populations in question remain present, they are unable to mediate an immune response [45, 190-192]. As discussed in Section 1.3, one situation where anergy may result is when T cells are presented with antigen in the absence of a suitable costimulatory signal [44-46].

1.6.4 Ignorance

In order for an antigen to stimulate a response it must be “seen” by the immune system. If it is expressed at a site at which it is not normally accessible or it is not processed and presented appropriately, then immunological ignorance (and thus non-responsiveness) can result [193-196]. The importance of intact antigen presentation pathways for the rejection of allografts is demonstrated by the fact that rejection does not occur in the absence of afferent lymphatic drainage [197] or of

secondary lymphoid organs [198]. The new immunosuppressive drug FTY720 exploits this phenomenon by altering lymphocyte trafficking, leading to sequestration within secondary lymphoid organs and thus potentially maintaining immunological ignorance by preventing lymphocyte circulation through the graft or tissue [199-201].

1.6.5 Regulation

Immunological “suppression” or “regulation” is a phenomenon whereby cell populations are able to suppress immune responses in an active manner. Such regulation will form the major subject of this thesis, and is therefore discussed in greater detail below. It is possible that the generation of regulatory cells is, at least in part, responsible for the blood transfusion effect [156, 202-204].

1.7 Regulatory T cells

1.7.1 Introduction

The existence of lymphocyte populations able to suppress antigen-specific immune responses was first described over thirty years ago by Gershon and Kondo [205]. However, in recent years there has been a resurgence of interest in this area, and evidence supporting the importance of active immunoregulation has continued to accumulate.

Various experimental systems have demonstrated the ability of cells from tolerant animals to suppress responses by pathogenic cells both in models of autoimmune disease [206-227] and transplantation [175, 202, 228-241]. Similarly, in a clinical study, patients with long term surviving kidney and liver allografts but who

were off maintenance immunosuppression were shown to contain cells that were able to suppress donor-specific immune responses *in vitro* [242].

Further evidence for the existence of active regulatory mechanisms is provided by the phenomenon of infectious tolerance, whereby regulatory cells are not only able to suppress the activation of other cell populations but can actually convert them to a regulatory phenotype [175, 231, 232], and by the observation of linked epitope suppression, where regulatory cells can suppress responses not only to their specific antigens, but also to other antigens expressed on the same target [154, 175, 243-247].

The discovery of the existence of regulatory cells has led to many studies attempting to identify T cell populations that have such properties. Although the majority of the regulatory T cell (Treg) populations so far identified are contained within the CD4⁺ fraction, other T cell subsets such as CD8⁺ [226, 238, 248, 249], CD8⁺CD28⁻ [250-253], and TCR⁺CD4⁻CD8⁻ (“double negative”) [235, 254-256] have been shown to contain cells with suppressive properties.

The presence of Treg within the CD4⁺ population has been demonstrated by numerous studies. Adoptive transfer of whole CD4⁺ populations from tolerant animals to naïve recipients has been shown to confer protection against disease in rat autoimmunity [211], rat cardiac [229] and renal [202], and mouse skin [175, 231, 232], and cardiac [175] allograft models. Similarly, depletion of CD4⁺ populations from tolerant animals can result in rejection of mouse skin [257] or cardiac [156] allografts. Within the CD4⁺ population various fractions have been identified that possess regulatory properties.

1.7.2 *Tr1 and Th3 cells*

Culture of CD4⁺ cells in the presence of antigen and IL-10 leads to the generation of IL-10-secreting cells, designated Tr1, that are able to suppress antigen-specific T cell responses *in vitro* and the development of autoimmune colitis *in vivo* [258, 259]. Generation of Tr1 cells has been shown to be enhanced by the addition of IFN- α , but not of TGF- β , to cultures [260] and by CD2 costimulation [261]. Cells with a similar suppressive phenotype have been isolated following culture of CD4⁺ T cells and antigen in the presence of vitamin D and dexamethasone [262].

A further subset of regulatory CD4⁺ cells, designated Th3, has been identified in mouse models, where they are characteristically produced following oral antigen administration and predominantly secrete IL-4, IL-10, and TGF- β [212, 263], while IL-4 may play a role in their generation [264]. Like their Tr1 counterparts, Th3 cells are able to ameliorate autoimmune disease *in vivo* [212, 263, 264] and suppress antigen-specific T cell proliferation *in vitro* [263]. Similar cells have been identified in humans, either as naturally-occurring autoreactive cells [265], or following oral myelin administration to multiple sclerosis patients [266]. In both the mouse and human studies, regulation *in vitro* was abrogated by blockade of TGF- β and, in the case of the human cells, IL-10 [263, 265].

1.7.3 *CD4⁺CD45RB^{low} and CD4⁺CD45RC^{low} Treg*

Although the ability to distinguish Treg on the basis of functional characteristics is useful, identification of phenotypic markers such as cell surface molecules that identify, or at least enrich for, regulatory populations would provide an immensely useful tool for experimental study and, potentially, clinical application.

One of the first cell surface molecules identified that enriches for Treg was CD45. This is the leukocyte common antigen and is expressed on the surface of all leukocytes. However, the gene contains several exons that are differentially utilised, giving rise to several splice variants and thus different final protein products [267-270]. It is one of these proteins, CD45RB in the mouse and CD45RC in the rat, which helps to distinguish Treg. CD45RB is expressed at high levels on naïve T cells (“CD45RB^{high}”), but is downregulated following antigen exposure (“CD45RB^{low}”) [271]. It is the latter CD4⁺CD45RB^{low} (CD45RC^{low} in the rat) population that contains cells with regulatory properties. In the rat, CD4⁺CD45RC^{low} cells can prevent the development of autoimmune thyroiditis and diabetes induced by thymectomy and irradiation [215]. In the mouse, CD4⁺CD45RB^{low} cells have been shown to prevent CD4⁺CD45RB^{high}-induced autoimmune wasting disease [207] and colitis [208, 214]. In the transplantation setting, CD4⁺CD45RB^{low} cells from mice with long term surviving cardiac allografts are able to prevent CD4⁺CD45RB^{high} cell-mediated rejection of donor-specific skin allografts when adoptively transferred into T cell-deficient recipients [236], and, similarly, CD4⁺CD45RB^{low} cells can suppress rejection of pancreatic islet allografts [234].

In addition to their regulatory properties *in vivo*, CD4⁺CD45RB^{low} cells have also been shown to exert suppressive activity *in vitro*. One study has suggested that it is the CD38⁺ subfraction that possesses regulatory properties *in vitro* [272], and in the model using CD4⁺CD45RB^{low} cells from mice with long term surviving cardiac allografts, regulation *in vitro* appears to require alloantigen presentation via the indirect pathway [236].

Although the pattern of expression of CD45 isoforms may be helpful in identifying Treg, it does not provide an absolute marker. For example, a study by Zhai

et al. demonstrated that, in rats with long term surviving cardiac allografts, regulatory cells existed within the $CD4^+CD45RC^+$, but not the $CD4^+CD45RC^-$, subset. The authors postulated that this population may represent memory cells that had reverted from a $CD45RC^-$ to a $CD45RC^+$ phenotype [239].

1.7.4 $CD4^+CD25^+$ Treg

A further cell surface marker that enriches for $CD4^+$ cells with regulatory activity is the CD25 molecule, a component of the IL-2 receptor. The high affinity receptor for IL-2 consists of three subunits [273]. The α subunit, CD25, is unique to the IL-2 receptor; it is not in general expressed at significant levels on resting $CD4^+$ cells, but is upregulated following antigen experience [115]. The β subunit, CD122, is utilised only by the receptors for IL-2 and IL-15. The third (γ) chain, CD132, is responsible for signalling activity [274], and is also known as the common γ chain as it forms part of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Cells within the $CD4^+CD25^+$ population possess regulatory properties. In 1995, Sakaguchi and colleagues published the seminal observation that this population is able to prevent the development of autoimmune disease that otherwise occurs in nude mice reconstituted with the reciprocal $CD4^+CD25^-$ subset [209]. Since then there have been numerous further examples of the ability of $CD4^+CD25^+$ cells to regulate autoimmune disease *in vivo* in mouse [210, 213, 216-218, 222-224, 227, 275-281] and rat [220, 221] models. Studies *in vitro* have revealed that $CD4^+CD25^+$ cells do not proliferate in response to stimulation, a state reversed by exogenous IL-2 [276, 277, 282-287], but they are able to regulate the proliferation of other T cell populations, both $CD4^+$ [217, 240, 276, 277, 282, 285, 286, 288, 289] and $CD8^+$ [240, 285, 290-292]. This suppressive effect requires TCR stimulation of the Treg and also requires direct cell contact with the

population being regulated but, interestingly, is not antigen-specific: the Treg do not need to be activated by the same antigen, or indeed the same APC, as the population being regulated [276, 289, 291, 293].

In addition to their ability to prevent autoimmune disease, CD4⁺CD25⁺ cells can also play a role in areas such as tumour immunity [294-297] and transplantation. In the case of the latter, such cells can inhibit the development of graft versus host disease [298-300] or, more importantly in the context of this thesis, may facilitate allograft survival. The first example of this was provided by the observation by Hall *et al.* who showed, as early as 1990, that tolerance could be adoptively transferred by CD4⁺CD25⁺ cells taken from rats with long term surviving cardiac allografts [229]. The accumulating data from autoimmune disease models have prompted a more thorough examination of the role of such cells in transplantation tolerance, and donor-specific CD4⁺CD25⁺ Treg capable of preventing graft rejection have now been identified in mice with long term surviving cardiac [236] and pancreatic islet [237, 240] allografts; indeed, as previously mentioned, it is possible that the generation of such Treg may contribute to the clinical blood transfusion effect described earlier [156, 202-204].

CD4⁺CD25⁺ Treg are not confined to rodents. In humans it has been demonstrated that CD4⁺CD25⁺ cells in the peripheral blood are able to suppress T cell proliferation in response to both polyclonal and alloantigen stimulation [283-287, 301-303]. Human CD4⁺CD25⁺ cells with regulatory properties have also been isolated from other tissues such as the thymus [304] and the tonsils [305].

As mentioned above, CD25 is not expressed on resting CD4⁺ cells but is upregulated following cell activation. In an attempt to distinguish regulatory from activated cells within the CD4⁺CD25⁺ population, Sakaguchi's group has shown that,

in the mouse, cells with regulatory properties express high and persistent levels of CD25, whereas activation of naïve cells leads to expression of CD25 at intermediate levels which is only transient [217]. Similar data from humans support the suggestion that the “CD25-intermediate” population does not possess regulatory properties [283].

In view of the fact that CD25 expression does not serve to identify Treg populations absolutely, other phenotypic markers have been sought that may define subfractions of the CD4⁺CD25⁺ population that possess regulatory activity. Some of the markers that have been proposed include CD122 [283, 285, 286, 301, 306, 307], CTLA-4 (cytotoxic T lymphocyte-associated antigen 4, CD152) [216, 218, 237, 284-288, 301, 302, 304, 306-310], TGF- β [279, 304, 311], GITR (glucocorticoid-induced tumour necrosis factor receptor family-related gene) [308, 312-314], the integrin $\alpha_E\beta_7$ (CD103) [225, 308, 313], and the transcription factor Foxp3 (forkhead box P3, also known as scurfin) [249, 315, 316]; these markers will be discussed in greater detail in Chapter 3.

Although at present CD25 appears to be the most convenient marker to identify CD4⁺ Treg with the greatest overall sensitivity and specificity, cells with regulatory properties have been identified within the CD4⁺CD25⁻ population [220, 223-225, 241, 279, 317-319]; this also provides further impetus for the quest for methods allowing more precise identification of Treg.

1.7.5 Generation of CD4⁺CD25⁺ Treg

Current data suggest that naturally-occurring CD4⁺CD25⁺ Treg are generated through a positive selection process within the thymus [307, 317, 320-328], which will be discussed in greater detail in Chapter 5. These cells possess receptors with relatively

high affinity for self peptide-MHC complexes, and leave the thymus already committed as CD4⁺CD25⁺ Treg (Figure 1.5c).

Intact costimulatory pathways appear to be required for the generation and maintenance of CD4⁺CD25⁺ Treg. CD40-deficient mice show a reduction in the size of the CD4⁺CD25⁺ population and develop autoimmune disease, and CD40-deficient APC are unable to induce Treg development *in vitro* [329]. Non-obese diabetic (NOD) mice that are deficient in B7 or CD28 have reduced numbers of CD4⁺CD25⁺ cells and show exacerbated autoimmune diabetes mellitus, and B7-CD28 blockade in wild type NOD mice leads to a five-fold reduction in the size of the CD4⁺CD25⁺ population [216].

As well as developing naturally, CD4⁺CD25⁺ Treg can be generated by exposure to exogenous antigen. As discussed earlier, CD4⁺CD25⁺ cells capable of regulating anti-donor responses may be found in animals with long term surviving allografts in some transplantation models [236, 237, 240]. It has also been shown that oral antigen administration can lead to the development of CD4⁺CD25⁺ Treg [309, 330-332]. Furthermore, such cells may be generated by exposure to antigen *in vitro*. CD4⁺CD25⁺ cells capable of preventing the development of uveoretinitis *in vivo* have been successfully isolated following culture of mouse CD4⁺ cells in the presence of α -MSH (melanocyte stimulating hormone) [333]. Similarly, culture of human CD4⁺ cells under appropriate conditions can generate CD4⁺CD25⁺ cells that are able to regulate T cell responses *in vitro* [291, 334].

1.7.6 The role of cytokines in Treg function

A further area of intense investigation has been to attempt to elucidate the mechanisms by which Treg may exert their suppressive function. One possibility that

has been suggested is the secretion of cytokines; however, this has proved controversial. Peripheral $CD4^+CD25^+$ cells from normal unmanipulated mice transcribe IL-4, IL-10, and TGF- β mRNA more actively than does the reciprocal $CD4^+CD25^-$ population [210], and, similarly, $CD4^+CD25^+$ cells generated following oral antigen administration secrete IL-4, IL-10, and TGF- β [331]. However, although production of these cytokines by normal *ex vivo* $CD4^+CD25^+$ cells when stimulated *in vitro* has been demonstrated in some studies [285, 286, 331], this has not been the case in others [276], and furthermore a number of groups have suggested that they play no role in regulation *in vitro* [276, 285, 286, 291, 335], whereas other authors have demonstrated abrogation of regulation *in vitro* by blockade of IL-10 or TGF- β [309].

In vivo experiments have been rather more persuasive in demonstrating a role for cytokines in Treg function. Pontoux and colleagues have shown that $CD4^+CD25^+$ cells from IL-10 knockout mice do not exert regulatory activity *in vivo* [336], and, in an autoimmune disease model, it has been demonstrated that regulation is dependent upon IL-10 and TGF- β , but not IL-4 [214, 218, 223, 281, 337]. Similarly, in transplantation, blockade of IL-10, but not of IL-4, has been shown in one model to abrogate regulation (by cells taken from mice with long term surviving cardiac allografts) of skin allograft rejection [236]; however, this is not the case in all models, since in another transplantation study blockade of neither IL-4 nor IL-10 was able to abrogate regulation of skin allograft rejection [241]. A potential explanation for the apparent discrepancy in the role of IL-10 in these two studies is that in the former, IL-10 blockade was achieved by administration of an antibody directed against the IL-10 receptor, whereas in the latter, an anti-IL-10 antibody (which may provide less complete blockade) was used.

Although the mechanisms by which IL-10 and TGF- β contribute to regulation have not yet been clearly elucidated, both cytokines are known to have immunomodulatory effects. IL-10 can inhibit secretion of pro-inflammatory cytokines [338] and expression of both B7 costimulatory molecules [339] and MHC class II [340] by APC. In addition, IL-10 inhibits cytokine secretion [341] and promotes upregulation of TGF- β receptor expression [342] by T cells. TGF- β itself acts on macrophages to increase IL-10 secretion [343], and on leukocytes to downregulate adhesion molecule expression thus inhibiting endothelial adhesion [344]. However, despite these potentially beneficial effects of IL-10 and TGF- β , it has been shown that they can also have adverse effects in experimental systems of both autoimmunity [345] and transplantation [346-348] by exacerbating disease and rejection respectively.

1.7.7 The role of CTLA-4

As alluded to earlier, published data suggest that both mouse and human CD4⁺CD25⁺ Treg express the B7 ligand CTLA-4 [216, 218, 237, 284-288, 301, 302, 304, 306-308, 310]. Furthermore, some groups have found that an intact CTLA-4 pathway is required for regulation [218, 222, 240, 288, 304, 309], although other studies have suggested the opposite [241, 282, 283, 286, 293, 349]. This controversial subject will be addressed in greater detail in Chapters 3 and 4.

1.7.8 Outcomes of Treg activity

Just as there are a number of mechanisms through which Treg may act, there are also several possible consequences of these actions (Figure 1.6). APC may be induced to downregulate B7 costimulatory molecules [253, 339] or to secrete IL-10 [343] as a

result of IL-10 and TGF- β activity respectively, or skewed towards a tolerogenic phenotype by induction of the inhibitory receptors ILT3 (immunoglobulin-like transcript 3) and ILT4 [253]. Treg may also exert effects on other (“target”) T cells, such as inhibition of cytokine secretion [341], downregulation of adhesion molecule expression [344], inhibition of cell expansion [207, 219], induction of anergy [290] or death [235], or even conversion to a Treg phenotype in the process of infectious tolerance [175, 231, 232].

1.8 Aims of this thesis

The overall goal of this thesis is to investigate the role of CD4⁺CD25⁺ Treg in a mouse transplantation model, with particular emphasis on the induction phase of tolerance. The aims of Chapter 3 will be to examine the phenotypic characteristics of CD4⁺CD25⁺ cells and to develop a protocol allowing the generation of CD4⁺CD25⁺ cells capable of preventing allograft rejection *in vivo*. Chapter 4 will describe attempts to design *in vitro* assays mirroring the regulatory activity of CD4⁺CD25⁺ cells *in vivo*. In Chapter 5 the pathways by which Treg are generated will be studied, and the focus of Chapter 6 will be to examine aspects of Treg function *in vivo*, in particular their antigen specificity requirements. It is hoped that the data obtained in this thesis might help to elucidate principles facilitating the development of protocols that may eventually be useful clinically in transplantation or in the therapy of autoimmune disease.

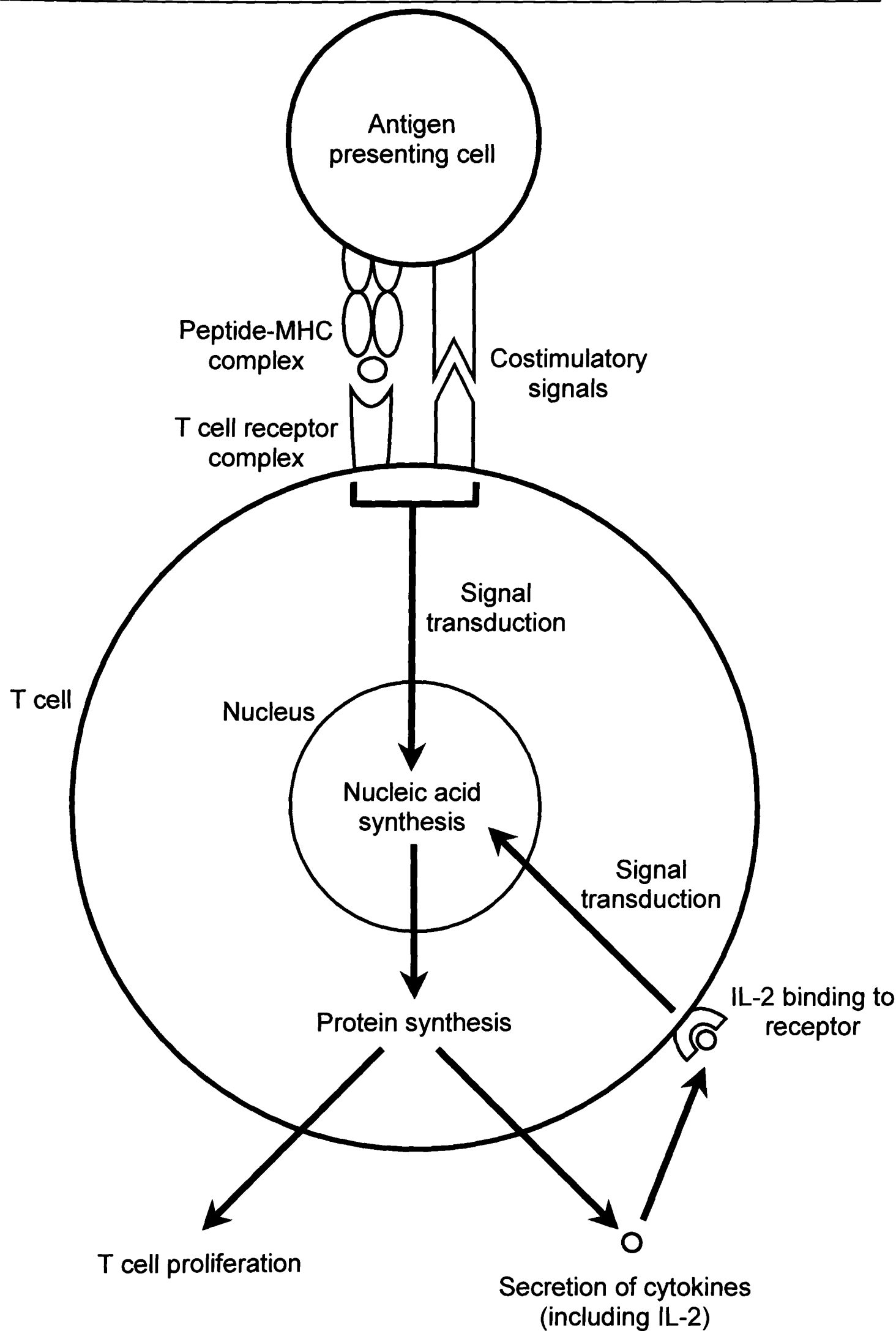


Figure 1.1. Overview of the main events during T cell activation

The T cell receptor complex binds to a peptide-MHC complex on the antigen presenting cell; this interaction is stabilised by adhesion molecules and coreceptors. The presence of costimulatory signal(s) results in signal transduction to the nucleus, mediated via the calcineurin pathway. Within the nucleus DNA replication and RNA synthesis occur. Proteins are synthesised within the cytoplasm, including cytokines (such as IL-2) that are secreted. IL-2 binds to the IL-2 receptor providing an autocrine / paracrine signal which is transduced back to the nucleus. Together these events result in T cell activation and proliferation.

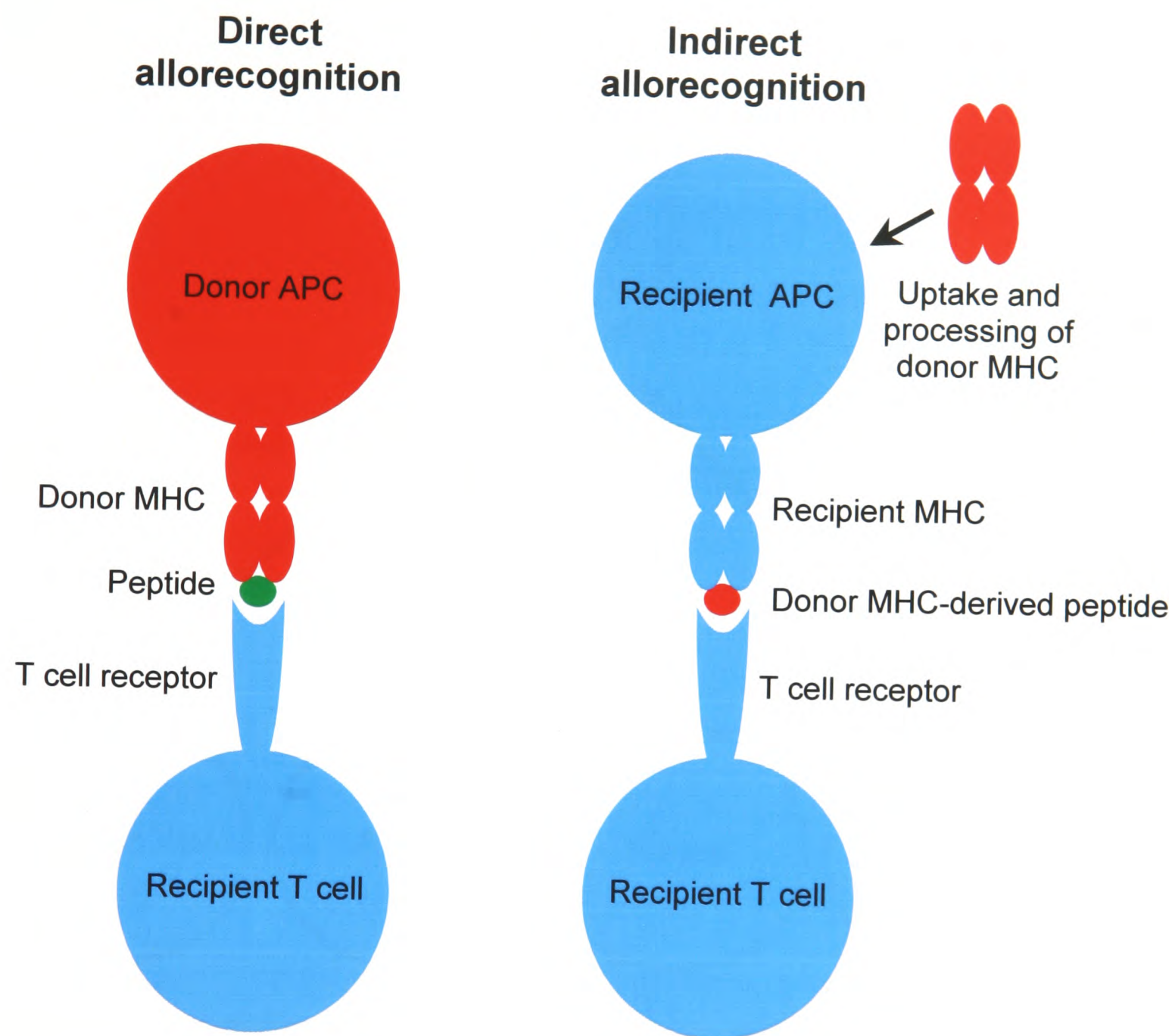


Figure 1.2. Direct and indirect pathways of alloantigen recognition by T cells
In the process of direct allorecognition recipient T cell receptors bind to intact donor MHC molecules expressed on donor antigen presenting cells. In indirect allorecognition recipient T cell receptors bind to recipient MHC molecules presenting donor MHC-derived peptide expressed on recipient antigen presenting cells.

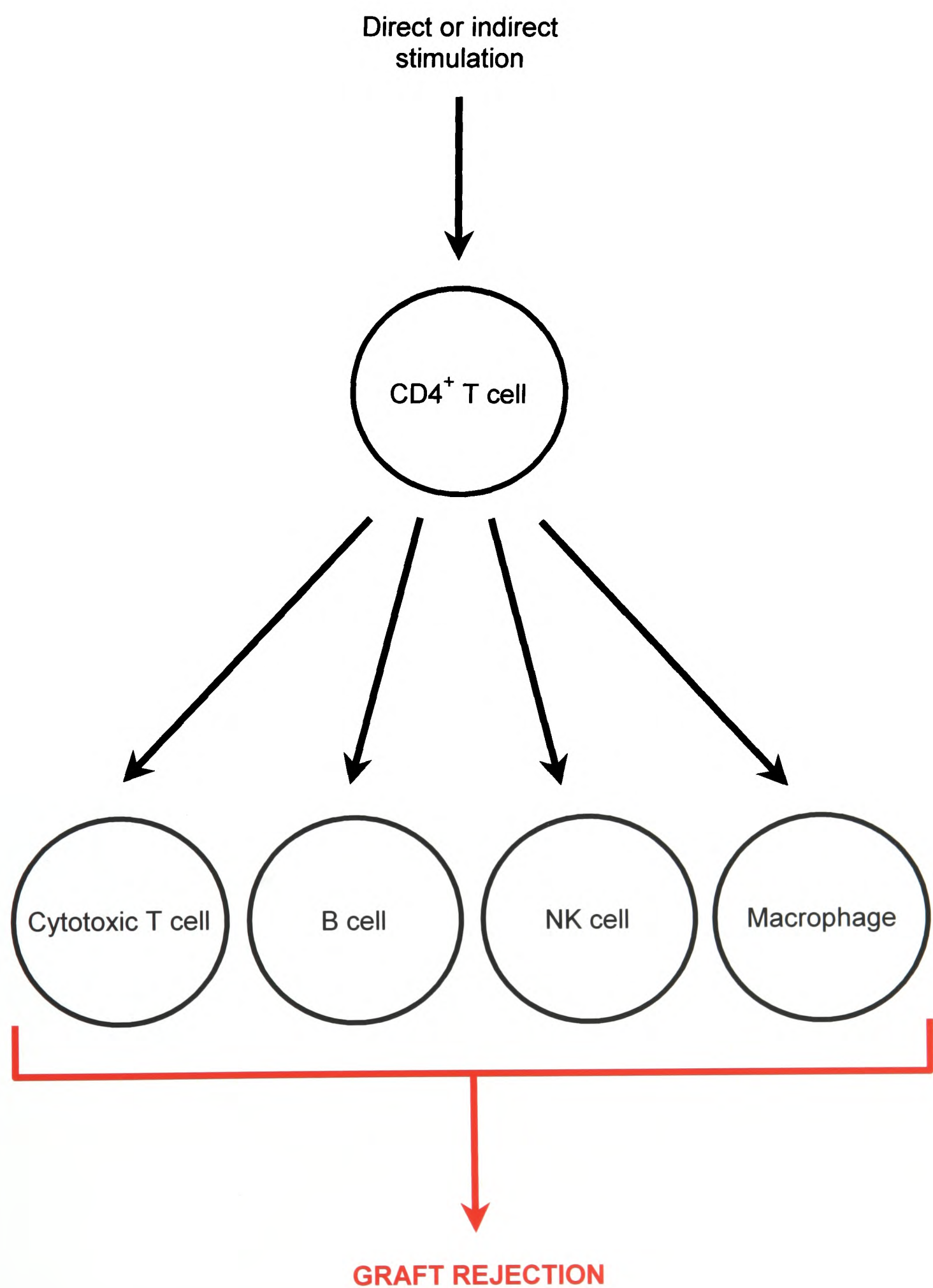


Figure 1.3. The role of CD4⁺ T cells in allograft rejection
Stimulation of CD4⁺ T cells by donor alloantigen through the direct or indirect pathways of alloantigen presentation results in the activation of effector cell populations partially through the secretion of soluble mediators. These effector cells then mediate allograft rejection by mechanisms such as direct cell killing (by cytotoxic T cells and NK cells), antibody production (B cells), or delayed type hypersensitivity reactions (macrophages).

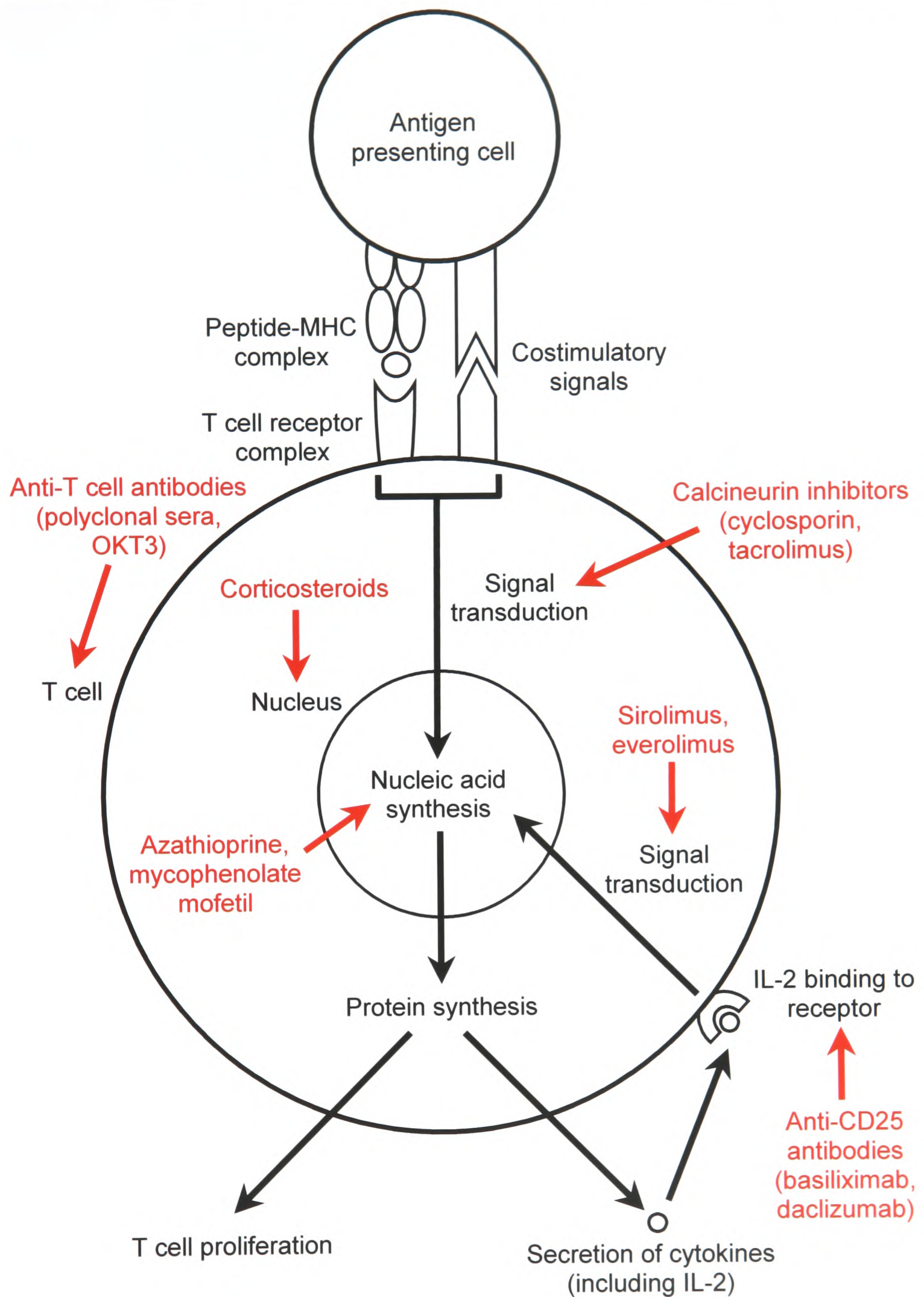


Figure 1.4. Mechanisms of action of the main clinical immunosuppressive agents Anti-T cell antibodies including polyclonal sera (such as thymoglobulin) and monoclonal antibodies (such as OKT3) lead to T cell depletion or modulation of cell surface molecules. Calcineurin inhibitors (such as cyclosporin and tacrolimus) inhibit signal transduction from the T cell receptor. Corticosteroids affect gene transcription within the nucleus. Azathioprine and mycophenolate mofetil inhibit nucleic acid synthesis. Anti-CD25 antibodies (such as basiliximab and daclizumab) target the IL-2 receptor complex expressed on activated T cell. Sirolimus and everolimus inhibit transduction of the signal from the IL-2 receptor.

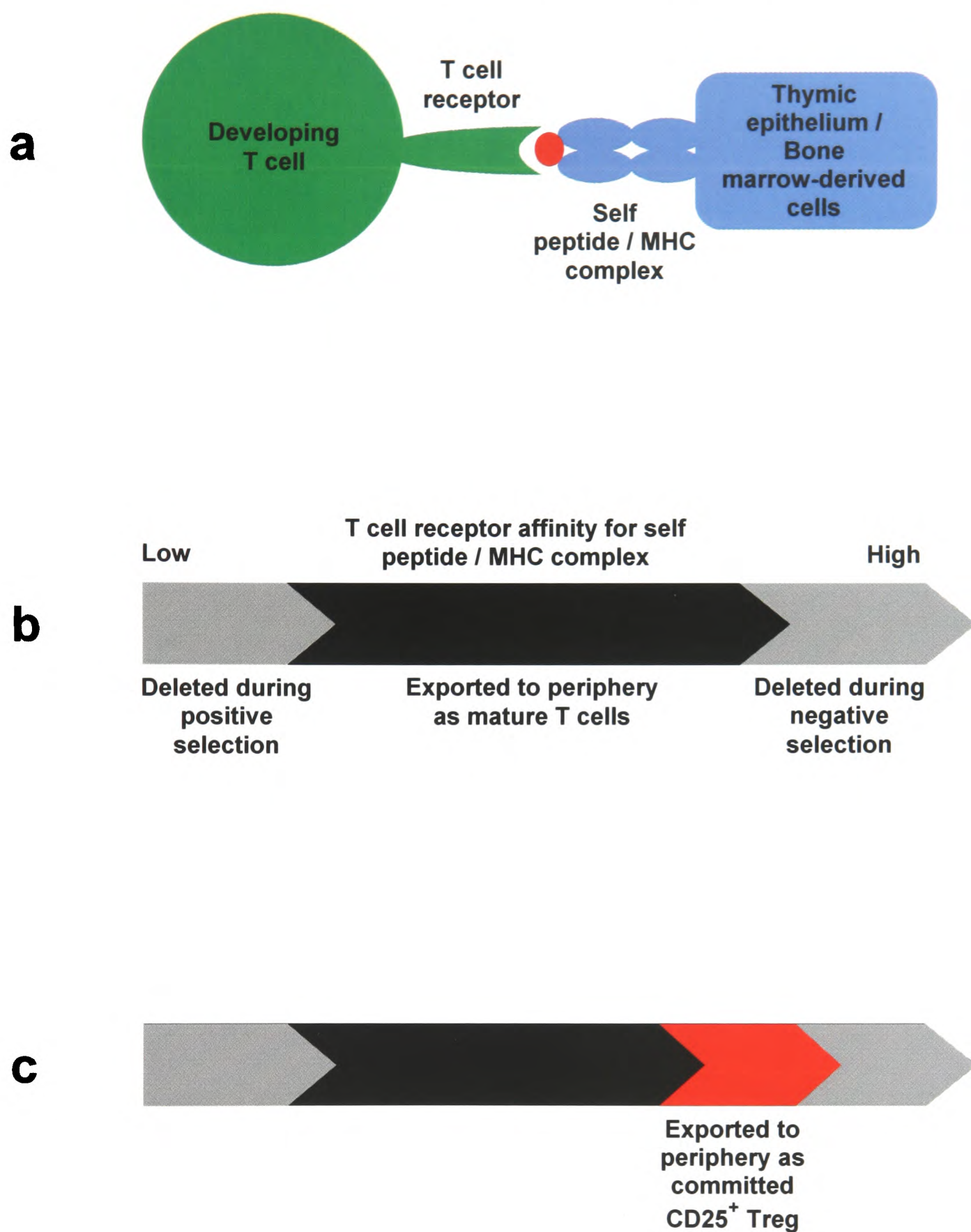


Figure 1.5. Intrathymic development of normal and regulatory T cell populations

(a) During intrathymic maturation developing T cells interact via their T cell receptors with self peptide-MHC complexes expressed by thymic epithelial or bone marrow-derived cells. (b) T cells with receptors for low affinity for self peptide-MHC complexes are deleted during the process of positive selection and those with high affinity during the process of negative selection. The remaining cells with intermediate affinity are exported to the periphery as mature T cells. (c) Treg undergo a process of positive intrathymic selection. They express receptors with relatively high affinity for self peptide-MHC and are exported to the periphery as committed CD25⁺ regulatory cells.

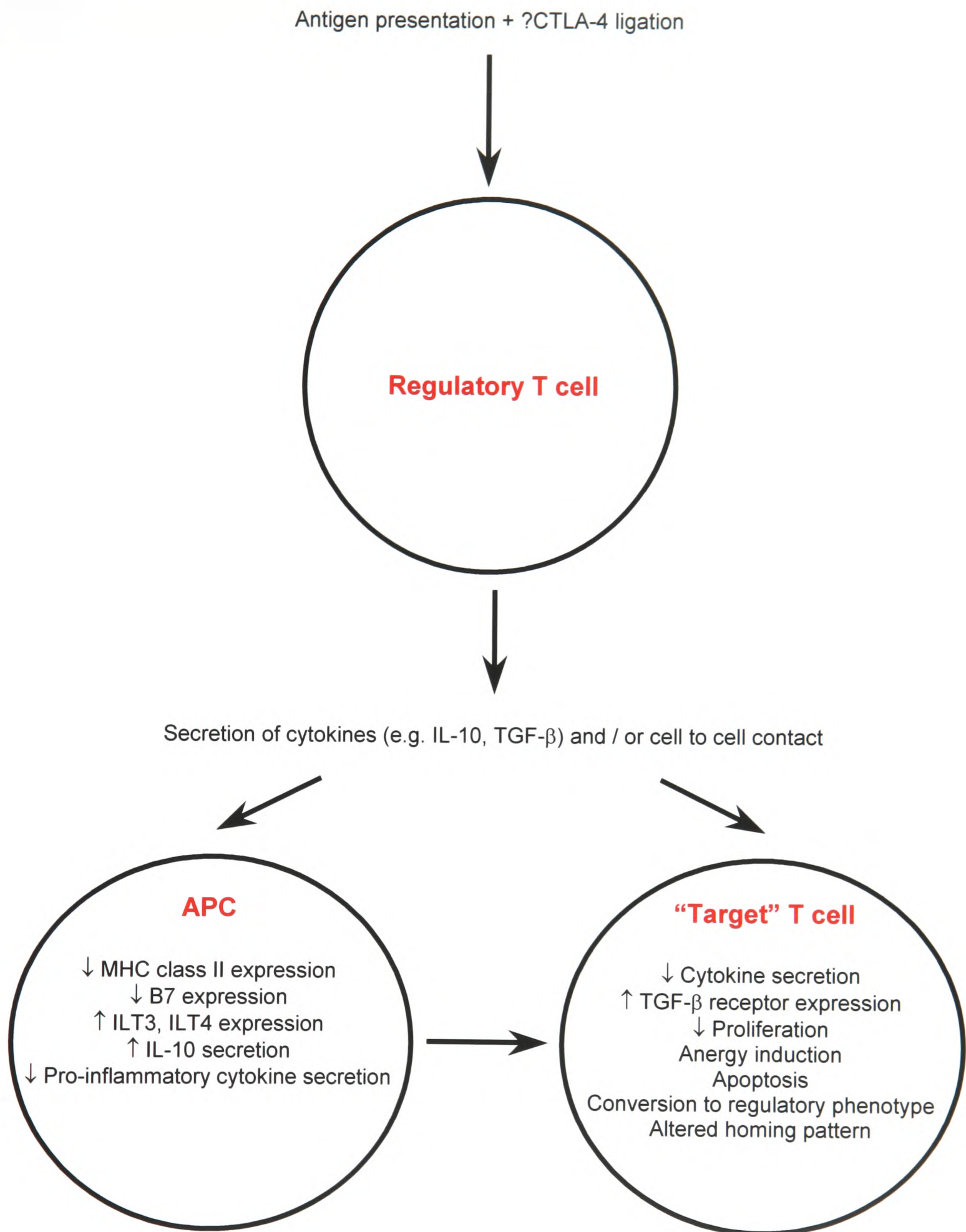


Figure 1.6. Potential mechanisms of action of regulatory T cells

Activation of regulatory T cells by antigen, possibly in combination with CTLA-4 ligation, may inhibit activity of "target" T cell populations directly or via effects on APCs; these actions may require cell to cell contact or involve secretion of soluble factors.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Animals were bred and housed in the conventional facilities of the Biomedical Services Unit (BMSU), John Radcliffe Hospital, Oxford, UK. All housing and procedures were in accordance with the Animals (Scientific Procedures) Act 1986. The CBK mice (H2^k also expressing K^b as a transgene) [350] and DES mice (bearing a transgenic TCR specific for K^b) [351] were a generous gift from Dr A Mellor, Medical College, Augusta, GA, USA. The CBA-Rag 1 knockout mice (CBA mice deficient in the recombination activating gene 1) [352] were a generous gift from Dr D Kioussis, Division of Molecular Immunology, National Institute for Medical Research, Mill Hill, London, UK. The mouse strains used are detailed in Table 2.1. All mice were aged 6-12 weeks at the time of first experimental procedure.

Strain	Abbreviation	H2 haplotypes					Notes
		K	D	L	A	E	
CBA/Ca	CBA	k	k	k	k	k	Rag 1-deficient K ^b transgenic
CBA-Rag 1 ^{-/-}	CBA-Rag	k	k	k	k	k	
CBK	CBK	k,b	k	k	k	k	
C57BL/10	B10	b	b	b	b	b	
(CBA × C57BL/6)F ₁	(CBA×B6)F ₁	k,b	k,b	k,b	k,b	k,b	
BALB/c	BALB	d	d	d	d	d	TCR transgenic
DES	DES	k	k	k	k	k	

Table 2.1. Mouse strains used in this thesis

2.2 Monoclonal antibodies and flow cytometry reagents

The purified monoclonal antibodies used for cell purification, *in vitro* cell culture, and *in vivo* administration are listed in Table 2.2. The hybridomas for YTS169.4.2 (abbreviated as YTS169) and YTS177.9 (YTS177) were a generous gift from Prof H Waldmann, Sir William Dunn School of Pathology, Oxford, UK. 4F10 was a generous gift from Dr J Bluestone, Diabetes Center, University of California, San Francisco, CA, USA. DTA-1 was a generous gift from Dr S Sakaguchi, Institute for Frontier Medical Sciences, Kyoto, Japan. GL113, a rat IgG₁ isotype control antibody reactive with β -galactosidase, was used as a control for the anti-IL-10R antibody 1B1.2. Purified hamster IgG, used as a control for 4F10, was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Monoclonal antibodies used for flow cytometry were conjugated to fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), Cy-chrome (CyC), peridinin chlorophyll protein (PerCP), allophycocyanin (APC), or biotin. Antibodies purchased from Pharmingen (San Diego, CA, USA) are listed in Table 2.3. Fluorochrome-conjugated streptavidin (SA) was also purchased from Pharmingen.

Other reagents used for flow cytometry were as follows. Biotinylated anti-TGF- β_1 , a chicken IgY antibody, was purchased from R&D Systems Inc (Minneapolis, MN, USA). Biotinylated chicken IgY whole molecule, used as a negative control, and FITC-conjugated mouse anti-rat IgG (H+L) were purchased from Jackson. For staining of cell surface IL-15R α chain expression, we used a biotinylated mutant IL-15-Fc γ 2a fusion protein kindly provided by Drs X Zheng and T Strom, Department of Medicine, Harvard Medical School, Boston, USA. This fusion protein consists of mutant human IL-15 coupled to mouse Fc γ 2a and has been shown to bind with high affinity to the α chain of the mouse IL-15 receptor [353].

Specificity	Clone	Species	Isotype	Source	Ref
CD3	145-2C11	Hamster	IgG group 1, κ	NDS	[354]
CD4	YTS177.9	Rat	IgG _{2a}	HW	[355]
CD8	YTS169.4.2	Rat	IgG _{2b}	HW	[356]
CD25	PC61	Rat	IgG ₁	NDS	[357]
CD28	37.51	Hamster	IgG group 2	NDS	[358]
CD45	M1/9.3.4.HL.2	Rat	IgG _{2a}	NDS	[359]
CTLA-4	4F10	Hamster	IgG group 1, κ	JB	[360]
Hamster IgG	Polyclonal	Hamster	IgG	Jackson	
GITR	DTA-1	Rat	IgG _{2a}	SS	[312]
MHC class II	M5/114.15.2	Rat	IgG _{2b} , κ	NDS	[361]
IL-10R	1B1.2	Rat	IgG ₁	NDS	[362]
Isotype control	GL113	Rat	IgG ₁	NDS	[363]

Table 2.2. Antibodies used *in vitro* and *in vivo* in this thesis
NDS: Nuffield Department of Surgery
HW: Prof H Waldmann
JB: Dr J Bluestone
SS: Dr S Sakaguchi

Specificity	Clone	Species	Isotype
CD3	145-2C11	Hamster	IgG group 1, κ
CD4	RM5-5	Rat	IgG _{2a} , κ
CD8	53-6.7	Rat	IgG _{2a} , κ
CD25	7D4	Rat	IgM, κ
CD44	IM7	Rat	IgG _{2b} , κ
CD45RB	16A	Rat	IgG _{2a} , κ
CD62L	MEL-14	Rat	IgG _{2a} , κ
CD69	H1.2F3	Hamster	IgG group 1, λ
CD122	TM- β 1	Rat	IgG _{2b} , κ
CD132	4G3	Rat	IgG _{2a} , κ
CTLA-4	UC10-4F10-11	Hamster	IgG group 1, κ
Isotype control	Polyclonal	Hamster	IgG group 1, κ

Table 2.3. Pharmingen antibodies used for flow cytometry in this thesis

2.3 Other reagents

Recombinant mouse IL-2 was obtained from culture supernatant from an IL-2-secreting X63 myeloma cell line kindly provided by Dr. Hajime Karasuyama, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan [364].

Human gamma globulin (HGG) was purchased from Sigma-Aldrich Co Ltd (Poole, Dorset, UK), and was heat-aggregated at 63°C for 25 minutes and then incubated overnight on ice prior to use, according to the protocol previously described by Benjamin and Waldmann [365].

2.4 Flow cytometric analysis

All incubation steps were carried out for 20 minutes at 4°C. Single cell splenocyte suspensions were prepared in FACS buffer consisting of PBS (Oxoid Ltd, Basingstoke, UK, containing 2.6 mM KH_2PO_4 , 26 mM Na_2HPO_4 , 145 mM NaCl, final pH 7.2) supplemented with 2% fetal calf serum (FCS, PAA Laboratories GmbH, Linz, Austria) and 0.02% sodium azide (Sigma). Erythrocytes were removed by hypotonic lysis using distilled water and the cells re-suspended in FACS buffer at a concentration of 10^7 cells/ml. 50 μl of cell suspension were then incubated with 50 μl of FACS buffer containing the appropriate mAbs for staining of cell surface markers. Following this incubation the cells were washed in FACS buffer, and if necessary incubated with the appropriate second stage reagents before being washed again. Finally the cells were re-suspended in 200 μl of FACS buffer containing 1% v/v formaldehyde (Sigma) and stored at 4°C until acquisition.

For intracellular staining, cell surface markers were stained as described above. The cells were then fixed by incubating in PBS containing 4% v/v formaldehyde, washed, and incubated in permeabilisation buffer consisting of FACS buffer supplemented with 0.5% saponin (Sigma). They were then washed again in permeabilisation buffer prior to incubation with the appropriate mAbs for intracellular staining. They underwent a final wash in permeabilisation buffer and then in FACS buffer following which they were re-suspended in FACS buffer containing 1% v/v formaldehyde and stored at 4°C until acquisition.

Data were acquired using a FACSort (Becton Dickinson, San Jose, CA, USA) and analysed using the CellQuest software package (Becton Dickinson). Results are presented as mean $\pm$ standard deviation, and statistical comparison between groups was performed using the 2 tailed Student's t test.

2.5 177/DST *in vivo* pre-treatment protocol

For the basic “177/DST” *in vivo* pre-treatment protocol adult CBA mice received 200 µg of the anti-CD4 mAb YTS177 intravenously on days –28 and –27, and on day –27 they also received a DST (donor-specific transfusion) of 250 µl of B10 blood intravenously. The spleens were then harvested on day 0 for cell isolation (Figure 2.1).

In some experiments further doses of DST were administered as indicated, or B10 blood was replaced with 250 µl of blood from other mouse strains or with 500 µg of heat-aggregated HGG prepared as described above.

2.6 Cell purification

CD4⁺CD45RB^{high} cells were purified from pooled spleens and lymph nodes of naïve CBA mice, and CD4⁺CD25⁺ and CD4⁺CD25[–] populations from spleens of naïve or pre-treated CBA mice. Tissues were harvested into Hanks’ balanced salt solution (HBSS, PAA Laboratories) containing 0.1% bovine serum albumin (BSA) fraction V (Sigma) and single cell suspensions prepared. Erythrocytes were removed by hypotonic lysis using distilled water. The cells were then washed and re-suspended at a concentration of 2×10^8 cells/ml in PBS / 0.1% BSA. In order to enrich for CD4⁺ cells, antibodies directed against CD8 (YTS169) and MHC class II (M5/114.15.2) were added, each to a final concentration of 100 µg/ml. The cell / antibody mixture was then incubated for 20 minutes at 4°C before being washed, re-suspended at a concentration of 10^8 cells/ml in PBS, and added to sheep anti-rat Ig-coated Dynabeads (DynaL AS, Oslo, Norway) at a ratio of one bead per cell. After incubation of the cell / bead mixture on a rotating wheel for 20 minutes at 4°C, unlabelled cells were isolated by negative separation using a Dynal magnet. The resulting CD4⁺-enriched cell

population was washed and re-suspended at 10^7 cells/ml in PBS / BSA and stained with fluorochrome-conjugated mAbs specific for CD4, CD45RB, CD25, and CD122 as appropriate. Cells were then fractionated into the desired populations by flow cytometry using a FACSVantage SE (Becton Dickinson).

2.7 *In vivo* cell adoptive transfer protocol

For *in vivo* experiments the appropriate purified cell populations were adoptively transferred into CBA-Rag recipients via the intravenous route, and the following day these mice received skin allografts (Figure 2.1). For some experiments the recipients were also treated with the anti-IL-10R antibody 1B1.2 or the isotype control antibody GL113. These were administered via the intraperitoneal route at a dose of 1 mg at the time of cell adoptive transfer and then 0.5 mg per week until graft rejection occurred or for a total of six weeks, whichever period was the shorter.

2.8 CFSE-labelling

The fluorescent dye CFSE (5-(and 6-)-carboxyfluorescein diacetate, succinimidyl ester) was purchased from Molecular Probes Inc (Leiden, the Netherlands). The cell population to be stained was washed in plain RPMI 1640 (PAA Laboratories) and then re-suspended in RPMI that had been warmed to 37°C. CFSE was then added to a final concentration of 5 μ M (0.1 μ M for cell conjugate assays where analysis was performed on the same day). This suspension was then incubated in a water bath at 37°C for 10 minutes, during which time it was agitated regularly. At the end of the incubation period the reaction was stopped by the addition of ice-cold RPMI. The

cells were mixed with a pipette to break up any clumps and then washed twice in RPMI. For *in vitro* cultures the cells were then washed and re-suspended in tissue culture medium, while for *in vivo* administration PBS was used.

2.9 PKH26-labelling

Cell membrane labelling with the red fluorescent label PKH26 was performed using a PKH26 red fluorescent cell linker kit purchased from Sigma. Cells were suspended at a concentration of 2×10^7 cells/ml in diluent C provided by the manufacturer, and an equal volume of PKH26, diluted to a concentration of 4 μ M in diluent C, was added. The sample was incubated for 5 minutes at room temperature, during which time it was agitated frequently. At the end of the incubation period the staining reaction was stopped by the addition of an equal volume of HBSS containing 1% FCS for 1 minute.

2.10 *In vitro* proliferation assays

All *in vitro* assays were performed using complete culture medium consisting of RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine (Sigma), 0.5 mM 2-mercaptoethanol (Sigma), and 100 units/ml each of penicillin and streptomycin (Sigma). Purified responder cells (labelled with CFSE where appropriate) were cultured at 2×10^5 cells per well in round-bottomed 96 well plates (Corning Inc, New York, USA) with or without various doses of putative regulatory T cell populations. For anti-CD3-stimulated cultures, the anti-CD3 mAb 145-2C11 was added at a final concentration of 2 μ g/ml, together with 5×10^5 irradiated (3600 rad) syngeneic spleen cells as antigen presenting cells. For allostimulated cultures, 5×10^5 irradiated

(3600 rad) allogeneic spleen cells were added. Cell cultures were maintained at 37°C in 5% CO₂. Cell proliferation was assessed either by incorporation of ³H-thymidine or by analysis of CFSE expression.

For measurement of ³H-thymidine incorporation anti-CD3-stimulated cultures were harvested after 4 days, and allostimulated cultures after 5 days based on pilot assays to identify optimal harvest times. In both cases cultures were pulsed with 0.5 µCi ³H-thymidine (Amersham International, Amersham, UK) per well for the final 18 hours of culture. Cells were then harvested using a Skatron Micro Cell Harvester (Skatron, Lier, Norway) onto glass fibre filter mats (Wallac, Turku, Finland). Betaplate Scint scintillation fluid (Wallac) was added, and ³H-thymidine incorporation measured using a Betaplate counter (Wallac). Assays were performed in triplicate wells and the results are presented as mean ± standard deviation.

Cultures analysed for CFSE expression were harvested after 4 days for anti-CD3-stimulated cultures and after 7 days for allostimulated cultures (based on time course experiments described in Chapter 4). Cells were harvested from the wells and washed in FACS buffer. They were then re-suspended in FACS buffer prior to data acquisition using the FACSsort.

2.11 PMA and ionomycin stimulation

Cells were cultured in complete culture medium for 4 days in the presence of phorbol myristate acetate (PMA, Sigma) at a concentration of 50 ng/ml and ionomycin (Sigma) 500 ng/ml.

2.12 IL-2 ELISA

IL-2 concentration in tissue culture supernatant samples was measured by ELISA (enzyme-linked immunosorbent assay). The anti-IL-2 capture antibody JES6-1A12 (rat IgG_{2a}, Pharmingen) was diluted to a concentration of 2 µg/ml in ELISA coating buffer consisting of 0.1 M NaOH, pH 8.2. 50 µl of antibody solution were added to the wells of Nunc-Immuno 96 MicroWell Plates (Nalge Nunc International, Rochester, NY, USA) and the plates covered and incubated overnight at 4°C. The following day unbound antibody was flicked out of the wells and the plates washed twice with PBS / Tween solution containing 0.5 ml Tween-20 (Sigma) per litre of PBS. Unoccupied protein binding sites were then blocked by the addition of 200 µl of 10% FCS in PBS to the wells and incubation for 2 hours at room temperature. The plates were then washed twice with PBS / Tween. 50 µl of sample were added to the appropriate wells, and standard wells were also prepared containing 50 µl of doubling dilutions (prepared in PBS / 10% FCS) of recombinant mouse IL-2 (Pharmingen) starting at a concentration of 4000 pg/ml. The plates were incubated overnight at 4°C and then washed 4 times with PBS / Tween. Biotinylated anti-IL-2 detection antibody (JES6-544, rat IgG_{2b}, Pharmingen) was diluted to 1 µg/ml in PBS / 10% FCS and 100 µl added to each well, and the plates were incubated for 45 minutes at room temperature and then washed 6 times with PBS / Tween. Streptavidin-conjugated horseradish peroxidase (HRP, Vector Laboratories Ltd, Peterborough, UK) was diluted 1:2000 in PBS / 10% FCS and 100 µl added to each well. The plates were incubated for 30 minutes at room temperature and then washed 8 times in PBS / Tween. 100 µl of ABTS substrate were then added to each well. This substrate was prepared by adding 150mg of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) to 500ml of 0.1 M citric acid in distilled water, adjusting the pH to 4.35

using NaOH, and finally adding H₂O₂ 1 µl/ml immediately prior to use. The plates were then incubated for between 10 and 80 minutes until the colourimetric reaction developed, and optical density (OD) at 405 nm was read using an ELISA Plate Reader (Molecular Devices, Sunnyvale, CA, USA) and analysed using SOFTmax software (Molecular Devices). Samples were assayed in triplicate and the results are presented as mean ± standard deviation.

2.13 Anti-HGG antibody ELISA

Serum anti-HGG antibodies were measured using a modification of the IL-2 ELISA protocol. Plates were coated with HGG diluted to a concentration of 100 µg/ml in PBS. After overnight incubation at 4°C the plates were washed twice with PBS / Tween, blocked with PBS / 10% FCS for 2 hours at room temperature, and washed twice with PBS / Tween. 50 µl of doubling dilutions (starting from 1:10) of test serum diluted in PBS / 10% FCS were then added to the wells. Following incubation overnight at 4°C the plates were washed 6 times, and HRP-conjugated rabbit anti-mouse IgG and IgM (Jackson) was diluted 1:500 in PBS / 10% FCS and 100 µl added per well. The plates were incubated at room temperature for 45 minutes and washed 8 times, and then the reaction was developed by the addition of ABTS substrate and OD at 405 nm measured. Samples were assayed in triplicate and the results are presented as mean ± standard deviation.

2.14 Cell biotinylation

For biotinylation of whole cells single cell suspensions were prepared, washed in PBS adjusted to pH 8.0 by the addition of NaOH, and re-suspended at a concentration of 25×10^6 cells/ml. EZ-Link Sulfo-NHS-LC-Biotin (Pierce Chemical Co, Rockford, IL, USA) was added to the cell suspension to a final concentration of 0.5 mg/ml and the mixture incubated at room temperature for 30 minutes. After the incubation period the cells were washed 3 times in pH 8.0 PBS.

2.15 Capture and detection of biotinylated cells

Nunc-Immuno 96 MicroWell Plates were coated with streptavidin by incubation overnight at 4°C with streptavidin (Sigma) diluted to a concentration of 10 µg/ml in ELISA coating buffer. The following day the plates were washed twice with PBS / Tween and then unoccupied protein binding sites were blocked by the addition of 200 µl of PBS / 10% FCS to the wells and incubation for 2 hours at room temperature. The plates were then washed twice in PBS / Tween and biotinylated B10 spleen cells suspended in PBS / 10% FCS were added at varying numbers in a total volume of 100 µl per well. The plates were then incubated for 30 minutes at 4°C following which they were washed 4 times with PBS / Tween. Anti-mouse CD45 antibody was diluted to a concentration of 100 µg/ml in PBS / 10% FCS and 100 µl added per well. After incubation at room temperature for 45 minutes the plates were washed 6 times in PBS / Tween; HRP-conjugated mouse anti-rat IgG (Jackson) was then diluted 1:500 in PBS / 10% FCS and 100 µl added per well. The plates were incubated at room temperature for 30 minutes, washed 8 times with PBS / Tween, ABTS substrate added, and OD at 405 nm read following development of the colourimetric reaction.

Assays were performed in triplicate wells and the results are presented as mean $\pm$ standard deviation.

For attempted detection of alloantigen-specific T cells, plates were coated with streptavidin and incubated with biotinylated B10 spleen cells as already described. The plates were then washed 4 times with PBS / Tween and then the appropriate test cell populations containing the T cells of interest were suspended in PBS / 10% FCS and added at doubling dilutions starting at 5×10^5 cells per well, in a total volume of 100 μ l. The plates were then incubated for 30 minutes at 4°C following which they were washed four times with PBS / Tween. In some cases the test cells were coated with anti-CD45 antibody by incubation for 20 minutes in PBS / 10% FCS at a concentration of 10^8 cells/ml and 100 μ g antibody/ml prior to addition to wells. In other cases 100 μ l of anti-CD45 antibody (100 μ g/ml) were added to wells after the incubation with the test cells, incubated for 30 minutes at 4°C, and washed 4 times with PBS / Tween. In both cases HRP-conjugated mouse anti-rat IgG was added to wells and the colourimetric reaction developed and read as previously described.

2.16 Cell conjugate assays

Attempted formation and detection of conjugates between T cells and antigen presenting cells was performed by modification of the protocol previously described by Schaefer *et al.* [366]. The two cell populations (containing the T cells and APC of interest respectively) were prepared and one stained with CFSE (0.1 μ M) and the other with PKH26. The cells were then re-suspended at various concentrations in RPMI containing 10% FCS, and 50 μ l of each cell fraction were added to FACS tubes. This mixture was incubated for varying periods of time at 37°C and then placed

on ice. In some experiments PerCP-conjugated anti-CD4 antibody was then added to the tubes and incubated at 4°C for 20 minutes, and in some experiments 4% formaldehyde was added to tubes and incubated at 4°C for 20 minutes. Data were acquired using the FACSort.

2.17 General anaesthesia

General anaesthesia was attained by administration of equal volumes of Hypnorm[®] (10 µg/ml fentanyl and 330 µg/ml fluanisone, Janssen Pharmaceutical Ltd, Oxford, UK) and midazolam (500 µg/ml, Roche Products Ltd, Welwyn Garden City, UK). Anaesthetic doses were adjusted according to animal age and size, varying from 0.2 ml to 0.4 ml of each agent (recovery doses), or a high dose of 1 ml of each agent for blood donors (lethal dose).

2.18 Intravenous injection

Intravenous injection was performed through the penile vein. Under general anaesthesia the animal was secured in the supine position with the tail facing the operator. The penis was sprayed with 70% alcohol and stretched to reveal the dorsal penile vein, and the injection was performed through a 30 gauge needle.

2.19 Blood collection

Blood donors received high dose general anaesthesia as described above. Heparinised whole blood (100 units heparin/ml, Leo Laboratories Ltd, Princes Risborough, UK) was harvested from the inferior vena cava and kept on ice until used.

2.20 Skin grafting

Skin transplantation was performed broadly according to the technique described by Billingham and Medawar [367]. The donor mouse was sacrificed by cervical dislocation and the tail sprayed with 70% alcohol. Incisions were made through the full thickness of the tail skin circumferentially around the proximal end and longitudinally to the distal end. The tail skin was then stripped, placed into normal saline, and cut into individual grafts approximately 12 mm in length. The recipient animal underwent general anaesthesia and the flank was shaved using electric clippers. The skin was sprayed with 70% alcohol and a graft bed prepared on the flank by dissection of the skin from the underlying fascia to match the graft size. The graft was placed on the bed and secured by applying Flexible Collodion BP methylated (William Ransom & Son, Herts, UK) around the margin. A povidone-iodine non-adherent dressing (Inadine[®], Johnson & Johnson Medical, Ascot, UK) was placed over the graft and covered with adhesive tape wrapped circumferentially around the animal. The dressing was removed under general anaesthesia 7 days later. Grafts that were necrotic at the time of unwrapping were considered to be technical failures. Grafts were inspected regularly and rejection was defined as complete necrosis of the donor skin. Allograft survival between groups was compared using the log rank test

[368] using software developed and kindly provided by Dr Stephen Cobbold, Sir William Dunn School of Pathology, Oxford, UK.

2.21 Thymectomy

Under general anaesthesia the animal was secured in the supine position with the head facing the operator. The head was stretched and immobilized with a piece of 7-0 silk holding the upper incisor teeth of the animal and taped to the operating board, and the skin was sprayed with 70% alcohol. A midline skin incision was made over the neck and upper thorax, and the salivary glands were retracted laterally. The manubrium was divided with sharp scissors and the strap muscles of the neck were separated in the midline using fine forceps and retracted laterally with 8/0 nylon stay sutures. The two lobes of the thymus were revealed by dissection of the pre-tracheal fascia. Each lobe was freed separately by blunt dissection and removed, taking care to remove each lobe intact and to avoid damage to surrounding structures. The stay sutures were removed and the skin closed with a continuous 4/0 catgut suture.

2.22 Histology

Histological sections were kindly prepared by the staff of the Department of Cellular Pathology, John Radcliffe Hospital, Oxford, UK. Skin grafts were harvested into 10% formaldehyde in PBS. 5 µm formalin-fixed paraffin-wax sections were de-waxed in xylene (BDH Laboratory Supplies, Poole, UK), rinsed in 100% ethanol and rehydrated with distilled water. Cell nuclei were stained using alum haematoxylin and the sections were rinsed in tap water and then immersed in 1% hydrochloric acid in

100% ethanol for 5 seconds in order to differentiate nuclear staining. After being washed in tap water the sections were counter-stained in 1% aqueous eosin (BDH), washed in running tap water, dehydrated in 100% ethanol, cleared in xylene (BDH), and mounted in DPX (BDH).

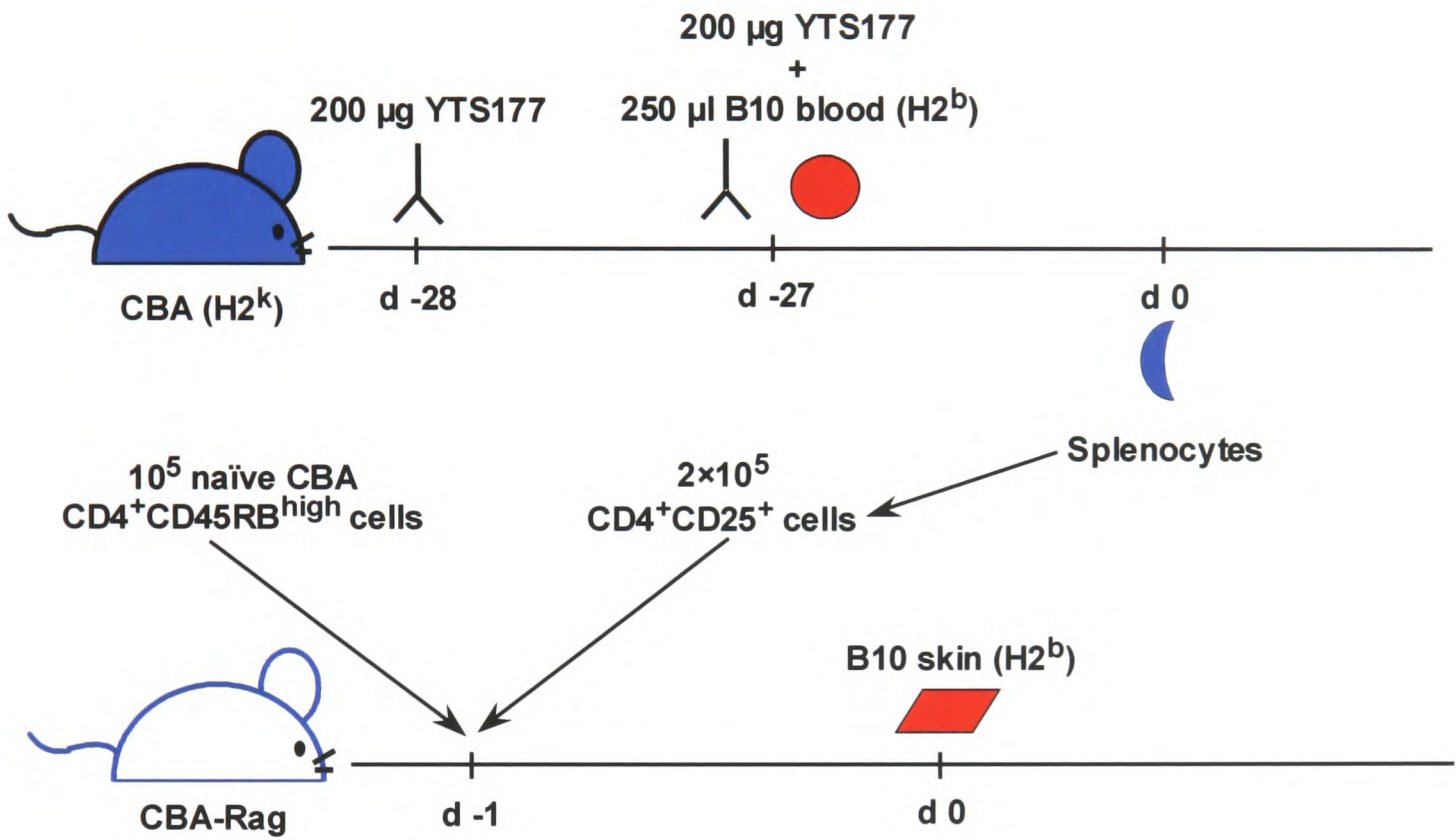


Figure 2.1. 177/DST pre-treatment and adoptive transfer protocol
CBA (H2^k) mice receive 200 µg of the anti-CD4 mAb YTS177 intravenously on days -28 and -27. On day -27 they also receive 250 µl of blood from naïve B10 (H2^b) donors intravenously. On day 0 splenocytes are isolated and the CD4⁺CD25⁺ population purified by flow cytometry. 2×10⁵ of these purified CD4⁺CD25⁺ cells are adoptively transferred into CBA-Rag recipients together with 10⁵ CD4⁺CD45RB^{high} cells purified by flow cytometry from the spleens and lymph nodes of naïve CBA mice. The day after adoptive transfer the CBA-Rag mice then receive a B10 skin allograft.

CHAPTER 3

GENERATION AND PHENOTYPE OF REGULATORY CELLS

3.1 Introduction

As discussed in Chapter 1, in recent years there has been increasing recognition that regulatory T cell populations play an important role in the maintenance of self-tolerance. As a result, much effort has been expended on attempts to define phenotypic markers that allow the identification of Treg populations. Although several potential such markers have been identified, the one that has achieved greatest prominence to date is CD25, the α subunit of the IL-2 receptor.

3.1.1 *Phenotypic characteristics of CD4⁺CD25⁺ regulatory cells*

It has now been widely demonstrated that CD4⁺CD25⁺ cells from mice [209, 210, 213, 217, 222-224, 227, 236, 237, 241, 275-282, 290, 292-300, 317, 331, 332, 336, 369-378], rats [220, 221, 229, 379, 380], and humans [283-287, 301-305, 307, 310] possess regulatory properties. Although, as discussed in Chapter 1, a few studies have suggested that cells within the CD4⁺CD25⁻ population (which represent around 90% of peripheral CD4⁺ cells) may also regulate [220, 223-225, 241, 279, 317-319], the majority of data demonstrate that regulatory activity lies predominantly within the minority CD4⁺CD25⁺ population.

3.1.1.1 “Activation” markers

Although CD4⁺ populations that express CD25 are enriched for regulatory activity, CD25 is not a marker expressed solely by Treg. Within the CD4⁺ population as a whole, CD25 is not generally expressed by naïve T cells, but is upregulated following activation [113-115]. Thus CD25 expression is not confined to cells with a Treg phenotype but also occurs on activated cells, albeit possibly at lower levels [217, 283]. However, although *ex vivo* CD4⁺CD25⁺ cells from mice exhibit regulatory properties

both *in vitro* and *in vivo*, CD4⁺CD25⁻ cells that are activated *in vitro* (and become CD25⁺) do not [217]. Other phenotypic markers have therefore been sought that might help to identify which cells within the CD4⁺CD25⁺ population possess regulatory properties. Since CD25 itself can be a cell activation marker, the expression of other markers whose levels vary according to cell activation status has therefore been examined. Such markers include: CD44, an adhesion receptor that is upregulated following cell activation [381, 382]; CD45RB, a tyrosine phosphatase that is downregulated following cell activation [271, 383]; CD62L (L-selectin, MEL-14), an adhesion molecule that mediates cell migration and is downregulated following cell activation [384-386]; and CD69, a costimulatory molecule expressed very early after cell activation [387, 388].

In their early paper characterising mouse CD4⁺CD25⁺ Treg, Sakaguchi *et al.* found that around 90% were CD44^{high}, virtually all CD45RB^{low}, 70% CD62L^{low}, and only 25% CD69^{high} [209]; however, they subsequently demonstrated that both the CD62L^{high} and CD62L^{low} fractions of the CD4⁺CD25⁺ population could regulate *in vitro* [217]. Similarly, Chai and colleagues demonstrated that CD4⁺CD25⁺ Treg expressed CD44, but also found that majority were positive for CD69 [293]. Yamashiro *et al.* found low expression of CD45RB and CD62L and high expression of CD44 [370], whereas Cohen and colleagues found that most CD4⁺CD25⁺ cells expressed high levels of CD62L [299]. Papiernik and Banz showed that CD4⁺CD25⁺ cells were CD45RB^{low}, CD44^{high}, CD69^{high}, and around half were CD62^{low} [389]. In an autoimmune diabetes model, Szanya and colleagues demonstrated that CD62L expression was required for CD4⁺CD25⁺ to suppress the development of disease *in vivo* but not to regulate *in vitro* [224]. In a human study, Ng and colleagues compared the phenotypes of *ex vivo* human CD4⁺CD25⁺ cells and

CD4⁺CD25⁻ cells that had been activated to become CD25⁺ [287]. Although they found quantitative differences in the expression of activation markers, the overall pattern was similar in both groups. Taken together these data suggest that CD4⁺CD25⁺ Treg express other markers that indicate an activated cell phenotype.

3.1.1.2 CD122

Since CD25 forms the α subunit of the IL-2 receptor, it seems probable that IL-2 binding may play a role in the activity of these cells. However, the IL-2 receptor also consists of two other chains, namely β (CD122) and γ (CD132). CD132, also known as the “common γ chain” forms part of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, and is therefore widely expressed whereas, in contrast, CD122 only forms part of the receptors for IL-2 and IL-15 [274, 390-392]. The expression of CD122 by CD4⁺CD25⁺ Treg has therefore been examined by several groups. It has been shown that CD122 is expressed by human CD4⁺CD25⁺ Treg [283, 285, 286, 301, 306, 307]; however, to date, expression by mouse CD4⁺CD25⁺ Treg has not been well studied.

3.1.1.3 CTLA-4

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a homologue of CD28 that is expressed on T cells and that binds to the B7 family molecules CD80 and CD86 expressed on antigen presenting cells [393-395] but with an affinity twentyfold higher than that of CD28 [396]. CTLA-4 is expressed at very low levels by resting T cells but is upregulated following cell activation [396-398]. In contrast to CD28 (which acts as a costimulatory molecule), ligation of CTLA-4 delivers a negative signal inhibiting IL-2 synthesis and cell activation and proliferation [399-404]; in addition, CTLA-4 ligation can lead to the synthesis and secretion of factors such as TGF- β that may

themselves have immunosuppressive effects [405, 406]. Thus CTLA-4 plays a role in the negative regulation of immune responses, and its upregulation following cell activation provides a mechanism for limiting responses and preventing damage by uncontrolled activation of the immune system. The importance of this is demonstrated by the observations that CTLA-4-deficient mice develop fatal lymphoproliferative disease [407, 408] and that CTLA-4 blockade can worsen autoimmune disease [409, 410].

In addition to its role as a negative regulator of immune responses, CTLA-4 has been specifically implicated as playing a role in the function of $CD4^+CD25^+$ Treg. It has been shown that both mouse [216, 218, 237, 288, 308] and human [284-287, 301, 302, 304, 306, 307, 310] $CD4^+CD25^+$ Treg express CTLA-4 constitutively. Furthermore, several groups have shown that CTLA-4 blockade abrogates the regulatory function of $CD4^+CD25^+$ Treg [218, 240, 288, 304], although other studies have yielded conflicting data [241, 282, 283, 286, 293, 349]. Expression of CTLA-4 appears to be required on the regulatory population rather than the target population since cells from wild type mice are able to suppress autoimmune disease in CTLA-4-deficient mice [411].

3.1.1.4 TGF- β

TGF- β is a cytokine that has immunosuppressive properties. It has been shown to increase secretion of IL-10 by macrophages [343] and to downregulate the expression of leukocyte adhesion molecules [344]. In the field of transplantation, Josien and colleagues have shown that it plays a role in the induction of tolerance in a rat cardiac allograft model, and that induction of TGF- β expression within allografts can prolong their survival [151]. TGF- β has also been suggested to play a role in the regulatory

function of CD4⁺CD25⁺ cells. Sakaguchi's group has shown that CD4⁺CD25⁺ cells from normal mice transcribe TGF- β mRNA more actively than the reciprocal CD4⁺CD25⁻ population [210], and Tsuji *et al.* found that CD4⁺CD25⁺ Treg from Peyer's patches secreted TGF- β [332]. TGF- β blockade has been demonstrated to abrogate regulation by CD4⁺CD25⁺ cells *in vivo* [218, 281]. A role for TGF- β *in vitro* has been somewhat harder to demonstrate. Although in some studies it has been shown that regulation by CD4⁺CD25⁺ cells *in vitro* is abrogated by TGF- β blockade [304, 314], in other situations this has not been found to be the case [286, 291, 302, 335]. A study by Nakamura and colleagues found that CD4⁺CD25⁺ Treg expressed TGF- β (in both active and latent forms) bound to the cell surface, and that this surface-bound TGF- β appeared to be required for regulation [311]; similarly Annunziato *et al.* also demonstrated surface TGF- β expression by human CD4⁺CD25⁺ Treg [304]. Furthermore Oida *et al.* found that CD4⁺CD25⁻ cells that expressed cell surface latent TGF- β could regulate *in vivo* [279]. However, these findings have proved controversial. Levings *et al.* examined the role of TGF- β in suppression by human CD4⁺CD25⁺ T cell clones [314]; they found that although TGF- β blockade abrogated regulation *in vitro*, no cell surface TGF- β could be detected on the Treg. Piccirillo and colleagues have also attempted to address this question. In contrast to Levings and colleagues, they found that neutralisation of TGF- β (either using anti-TGF- β antibody or sTGF- β -RII-Fc) failed to abrogate regulation by CD4⁺CD25⁺ cells *in vitro*. Furthermore they observed that CD4⁺CD25⁺ cells from TGF- β ^{-/-} mice were as suppressive as those from wild type mice, and CD4⁺CD25⁻ cells from mice that were unresponsive to TGF- β were as amenable to regulation as wild type cells [335]. Thus the role of TGF- β , and in particular cell surface-bound TGF- β , in the function of CD4⁺CD25⁺ Treg remains unclear.

3.1.1.5 GITR

GITR (glucocorticoid-induced tumour necrosis factor receptor family-related gene, also known as TNFRSF18) is a mouse cell surface molecule whose human homologue is also known as AITR (activation-inducible TNF receptor family-related gene) [412-414]. Expression of GITR by CD4⁺CD25⁺ Treg has attracted interest in recent years. Shimizu *et al.* immunised rats with mouse CD4⁺CD25⁺ cells and purified an antibody, DTA-1, reactive against GITR [312]. Using this antibody they found that GITR was expressed constitutively by CD4⁺CD25⁺ cells both within the thymus and the periphery, but they proceeded to show that binding of agonistic anti-GITR antibody to CD4⁺CD25⁺ Treg abrogated their regulatory function. Although GITR was expressed by Treg, induction of GITR expression by CD4⁺CD25⁻ cells (by means of cell activation) did not confer a regulatory phenotype.

Using a different approach, McHugh *et al.* performed DNA microarray analysis to compare gene expression by *ex vivo* CD4⁺CD25⁺ and CD4⁺CD25⁻ cells [308]. They found that GITR was one of 21 genes expressed at higher levels by the former population. In common with the study by Shimizu and colleagues, they also found that agonistic antibodies to GITR led to abrogation of regulation, and that expression of GITR following activation of CD4⁺CD25⁻ cells did not in itself confer the ability to regulate. Zelenika *et al.* have used SAGE (serial analysis of gene expression) analysis to show that GITR is expressed at higher levels by CD4⁺CD25⁺ than CD4⁺CD25⁻ cells [313]. In addition, in that study they showed that GITR expression was higher in tolerated than in rejected skin allografts. In a human study, Levings *et al.* found that human CD4⁺CD25⁺ Treg clones expressed GITR at higher levels than non-suppressive CD4⁺CD25⁺ clones [314].

At first sight, the observations that GITR is expressed by Treg but that ligation of GITR abrogates regulation seem contradictory. Although the expression pattern of the ligand for GITR, GITRL, is not well characterised, it is known to be expressed on vascular endothelial cells [413, 414], and expression levels are increased by endothelial activation [312]. It has been speculated that this GITRL upregulation, as may occur as a result of acute inflammation due to infection, leads to engagement of GITR on Treg, inhibiting their regulatory activity and thus potentiating the protective immune response [312].

3.1.1.6 Integrin $\alpha_E\beta_7$ (CD103)

The use of DNA microarray analysis has identified a further cell surface molecule whose expression is higher on $CD4^+CD25^+$ than $CD4^+CD25^-$ cells, the integrin $\alpha_E\beta_7$ (CD103) [308, 313]. Lehmann and colleagues have examined the functional significance of this expression and demonstrated that, within the $CD4^+CD25^+$ population, $CD103^+$ cells are more potent suppressors than the $CD103^-$ population [225]. Moreover, they found that $CD4^+CD25^-CD103^+$ cells also possessed regulatory properties both *in vitro* and *in vivo*. Thus, CD103 expression appears to be involved in regulation; although the precise mechanism is unknown, it is likely to influence cell migration.

3.1.1.7 Foxp3 (scurfin)

The most recent factor to have attracted interest as a marker to identify regulatory cells is the transcription factor Foxp3 (forkhead box P3), also known as scurfin. Hori and colleagues have shown that Foxp3 is transcribed predominantly by $CD4^+CD25^+$ rather than $CD4^+CD25^-$ cells both in the thymus and in the periphery [315]. In their

study stimulation of $CD4^+CD25^-$ cells did not lead to expression of Foxp3, but forced expression of Foxp3 by $CD4^+CD25^-$ cells (by means of retroviral transduction) conferred a regulatory phenotype upon these cells. These findings have been confirmed by others. Fontenot *et al.* found that targeted deletion of Foxp3 from mice led to the development of autoimmune disease [316]. $CD4^+CD25^+$ cells were absent from Foxp3-deficient mice, and disease in these animals could be prevented by adoptive transfer of $CD4^+CD25^+$ cells. They also found that forced expression of Foxp3 conferred regulatory properties on $CD4^+CD25^-$ cells. Similarly, Khattri *et al.* showed that mice in which Foxp3 was overexpressed contained increased numbers of $CD4^+CD25^+$ cells, while numbers were lower in Foxp3-deficient mice [249]. The $CD4^+CD25^+$ cells that were present in Foxp3-deficient animals did not appear to possess regulatory capacity, and $CD4^+CD25^-$ cells from Foxp3-transgenic mice were able to suppress *in vitro*. Taken together the results from these three studies suggest that Foxp3 expression is the most sensitive and specific marker of cells with regulatory activity that has been identified and validated at present. It is likely that Foxp3 expression may have similar significance in humans, since its deficiency is associated with disease, IPEX (the immune dysregulation polyendocrinopathy, enteropathy, X-linked syndrome), characterised by dysregulation of immune responses [415-417].

3.1.2 Naturally-occurring and antigen-induced $CD4^+CD25^+$ regulatory cells

The majority of studies of $CD4^+CD25^+$ Treg have examined the properties of so-called “naturally-occurring” Treg, that is cells taken from normal, unmanipulated mice [209, 210, 213, 217, 222, 224, 227, 275-282, 290, 292, 294-300, 336, 369, 373-377] or humans [283-287, 301-307, 310]. In addition, there have been some mouse

studies characterising cells from transgenic animals [276, 289, 293]. Although these studies have yielded valuable information, further knowledge about the properties of Treg is necessary if their potential is to be harnessed in clinical practice. In the fields of transplantation and autoimmune disease, manipulation of Treg would be required in order to control deleterious immune responses. Ideally the Treg used would be antigen-specific in nature in order to avoid the problems of non-specific immunosuppression that are currently seen clinically. This would require either the expansion of a pre-existing population of antigen-specific Treg, or the *de novo* generation of such a Treg population. Recent studies have shown that this is possible. In our laboratory Hara *et al.* have shown that mice with long term surviving cardiac allografts contain Treg (purified as $CD4^+CD45RB^{low}$ or $CD4^+CD25^+$) capable of controlling anti-donor responses [236]; this model is described in more detail below. Similarly, Gregori and colleagues have identified donor-specific $CD4^+CD25^+$ Treg in mice with long term surviving pancreatic islet allografts [237], van Maurik *et al.* in mice with long term surviving cardiac allografts [371], and Graca *et al.* in mice tolerant to skin allografts [241]. In a non-transplant setting, several groups have shown that $CD4^+CD25^+$ Treg can be generated following oral antigen administration [309, 330-332]. These studies indicate that antigen-specific $CD4^+CD25^+$ Treg can indeed be induced by appropriate antigen exposure.

3.1.3 177/DST pre-treatment and adoptive transfer protocols

In our laboratory it has been shown that pre-treatment of recipient mice with donor alloantigen administered under the cover of anti-CD4 antibody allows the long term survival of donor strain cardiac allografts performed 28 days later. Using this approach long term allograft survival has been achieved in a number of strain

combinations, administering donor alloantigen in various forms, and under the cover of various anti-CD4 antibodies [155-158, 203, 233, 246, 247, 418-423]. In the study by Hara *et al.* quoted above [236], pre-treatment of CBA recipients ($H2^k$) with a donor-specific transfusion (DST) of blood from B10 mice ($H2^b$) administered on day -27 under the cover of the depleting anti-CD4 antibody YTA3.1 on days -28 and -27 (a pre-treatment protocol referred to as 3.1/DST) allowed the long term survival (over 100 days) of B10 cardiac allografts performed on day 0. The regulatory capacity of $CD4^+CD45RB^{low}$ or $CD4^+CD25^+$ cells from these mice with long term surviving grafts was then tested using an adoptive transfer system. CBA adoptive transfer recipients were rendered T cell-deficient by thymectomy followed by administration of depleting anti-CD4 and anti-CD8 antibodies. These T cell-deficient mice were unable to reject B10 skin allografts, but their ability to reject could be reconstituted by the administration of $CD4^+CD45RB^{high}$ cells from naïve syngeneic CBA donors the day prior to transplantation. However, this rejection could be prevented by the co-administration of $CD4^+CD45RB^{low}$ or $CD4^+CD25^+$ cells from mice with long term surviving cardiac allografts, demonstrating the presence of Treg during the *maintenance* phase of tolerance in this model. In this thesis, however, the aim was to examine the role of $CD4^+CD25^+$ Treg during the *induction* phase of tolerance. Therefore, for reasons discussed below, mice were pre-treated with the non-depleting anti-CD4 antibody YTS177 rather than the depleting antibody YTA3.1, since the 177/DST pre-treatment protocol has also been shown to allow the long term survival of B10 cardiac allografts transplanted into $H2^k$ recipients [158].

3.1.4 Aims of this chapter

The overall aim of this chapter was to examine the phenotypic characteristics of $CD4^+CD25^+$ Treg, initially focussing on *ex vivo* $CD4^+CD25^+$ cells from normal mice. However, in parallel we wished to develop the observations described above regarding the potential role of Treg in transplantation tolerance. In particular we wished to examine whether $CD4^+CD25^+$ Treg may be involved in the induction phase of tolerance and, if so, to investigate whether Treg in this context differ phenotypically from *ex vivo* $CD4^+CD25^+$ cells. The final part of this chapter describes attempts to enumerate antigen-specific Treg.

3.2 Results

3.2.1 Phenotypic analysis of $CD4^+CD25^+$ cells

Since it has been shown in many studies that $CD4^+CD25^+$ cells from normal unmanipulated mice possess regulatory properties demonstrable both *in vitro* [217, 224, 277, 282, 288, 292, 376] and *in vivo* [209, 210, 213, 217-219, 224, 275, 288, 294, 296, 297, 369, 376], the first aim of this chapter was to examine the phenotype of $CD4^+CD25^+$ cells taken from naïve mice. Given that $CD4^+CD45RB^{low}$ cells from normal mice have also been shown to have regulatory capacity [207, 208, 214, 234], we first decided to establish the relationship between these two populations. As shown in Figure 3.1, in one of four representative experiments 8% of $CD4^+$ splenocytes from naïve CBA mice expressed CD25, a level consistent with published reports [209, 210, 216, 282, 369], while 35% of $CD4^+$ cells expressed low levels of CD45RB. $CD4^+CD25^+$ cells lay within the $CD45RB^{low}$ population, whereas only one third of $CD45RB^{low}$ cells were $CD25^+$.

We next wished to examine the pattern of expression of other phenotypic markers by $CD4^+CD25^+$ cells in naïve mice when compared to the reciprocal $CD4^+CD25^-$ fraction (Figure 3.2). Both populations showed similar expression profiles of CD62L (Figure 3.2b) and CD69 (Figure 3.2c), and both expressed CD132, the common γ chain, constitutively (Figure 3.2e). However, the $CD25^+$ cells demonstrated significantly higher expression of both CD44 (Figure 3.2a) and CD122 (Figure 3.2d). Similarly, unlike the $CD25^-$ population, the majority of $CD25^+$ cells showed constitutive expression of GITR (Figures 3.2h and 3.3), consistent with the previous observations of Shimizu *et al.* [312]. In view of the changes observed in expression of CD122, which is the β chain of the receptor for IL-15 as well as that for IL-2, a biotinylated fusion protein [353] was used to compare expression of the α chain of the IL-15 receptor between the $CD25^+$ and $CD25^-$ populations. There was no demonstrable difference between the two populations in expression of this chain (Figure 3.2f); indeed, IL-15R α expression was relatively limited among $CD4^+$ cells, and was more conspicuous within the $CD4^-$ population (Figure 3.4). Finally, in view of recent reports suggesting that $CD4^+CD25^+$ Treg express TGF- β bound to the cell surface [279, 304, 311], cells were stained for surface expression of this cytokine; however, in contrast to these reports, no cell surface-bound TGF- β was detected (Figure 3.2g).

3.2.2 Reconstitution of skin allograft rejection in CBA-Rag recipients

In order to examine the role of $CD4^+CD25^+$ Treg in a transplantation setting *in vivo* we wished to use the adoptive transfer system previously developed in our laboratory [236] and described above (Section 3.1.3) to test the ability of putative Treg populations to prevent the rejection (mediated by $CD4^+CD45RB^{\text{high}}$ cells from naïve

syngeneic donors) of skin allografts in secondary adoptive transfer recipients. For *in vivo* experiments in this thesis, the mice used as adoptive transfer recipients were not animals that had been depleted of T cells (by thymectomy followed by treatment with depleting anti-CD4 and anti-CD8 antibodies), but rather CBA mice that were knockouts for the Rag-1 gene (CBA-Rag) and so contained no naturally-occurring T or B cells [352]. It was therefore necessary to determine whether rejection of skin allografts could be restored in the same way in these CBA-Rag mice by reconstitution with $CD4^+CD45RB^{high}$ cells from naïve syngeneic donors.

T cells were not present in normal CBA-Rag mice, as demonstrated by the absence of $CD3^+$ cells within the spleens of these animals (Figure 3.5a), and skin allografts transplanted onto these mice were not rejected (Figure 3.5b). However, as shown in Figure 3.6, adoptive transfer of 10^5 $CD4^+CD45RB^{high}$ cells from naïve CBA mice (purified by flow cytometry, as shown in Figure 3.7, typically with purity of over 99%) the day before transplantation led to acute rejection of B10 skin allografts in all cases (MST = 23 days, $n = 4$; $P = 0.008$ compared to unreconstituted animals), whereas adoptive transfer of 5×10^4 naïve $CD4^+CD45RB^{high}$ cells resulted in rejection by only 2 out of 3 recipients (MST = 23 days, $n = 3$; $P = 0.07$ compared to unreconstituted mice). 10^5 was thus deemed to be the minimum number of naïve $CD4^+CD45RB^{high}$ cells required to reconstitute skin allograft rejection reliably in CBA-Rag mice, the same number as previously determined in T cell-depleted animals [236], and this dose was therefore used for all subsequent experiments in this thesis.

3.2.3 Regulation of skin allograft rejection by CD4⁺CD25⁺ cells from 177/DST pre-treated mice

Having demonstrated that the ability of CBA-Rag mice to reject skin allografts could be restored by the administration of naïve CD4⁺CD45RB^{high} cells, it was possible to use this model to test the ability of putative Treg populations to overcome this rejection. Since a goal of this thesis was to examine the role of CD4⁺CD25⁺ Treg during the induction phase of tolerance rather than the maintenance phase, we wished to test the regulatory capacity of CD4⁺CD25⁺ cells taken not from animals in which long term survival of cardiac allografts had been induced by pre-treatment with DST under the cover of anti-CD4 antibody, but rather from animals that had received pre-treatment only. Since the putative Treg were to be harvested soon after pre-treatment, in order to increase cell yields the anti-CD4 antibody used was not the depleting antibody YTA3.1 used previously [236], but rather the non-depleting antibody YTS177. In order to determine whether 177/DST pre-treatment leads to the generation of CD4⁺CD25⁺ Treg that may play a role in the induction phase of tolerance, CBA mice were pre-treated with 177/DST and then 28 days later (the time point when transplantation would be performed in the primary cardiac allograft model) CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were isolated from the spleens of these mice by flow cytometry (Figure 3.8). These cells were then adoptively transferred into CBA-Rag mice together with CD4⁺CD45RB^{high} cells from naïve CBA donors, and a B10 skin transplant was performed the following day (Figure 3.9).

All mice reconstituted with naïve CD4⁺CD45RB^{high} cells alone all rejected their skin allografts acutely (MST = 20 days, n = 4), leading to complete graft necrosis (Figure 3.10a). However, in clear contrast, co-transfer of CD4⁺CD25⁺ cells from the pre-treated animals overcame this rejection in all cases (MST > 100 days, n = 4;

$P = 0.008$) and allowed the long term survival of these grafts for over 100 days. These long term surviving grafts showed normal appearances macroscopically (Figure 3.10b) with luxuriant hair growth, and microscopically (Figure 3.10c and d) with normal epithelium and hair follicles and no evidence of inflammatory infiltrate. $CD4^+CD25^-$ cells from the 177/DST pre-treated animals did not display regulatory properties when co-transferred at the same cell dose, since all skin allografts were rejected acutely (MST = 9.5 days, $n = 4$; $P = 0.18$ compared to group receiving naïve $CD4^+CD45RB^{high}$ cells only).

3.2.4 Effect of 177/DST pre-treatment on $CD4^+CD25^+$ population

Having established that $CD4^+CD25^+$ cells from mice pre-treated with 177/DST are able to regulate the rejection of skin allografts *in vivo*, the next objective was to determine whether any phenotypic differences in $CD4^+CD25^+$ cells taken from 177/DST pre-treated animals could be detected when compared with those from control groups. Initially we wondered whether 177/DST pre-treatment would lead to an expansion in the size of the $CD4^+CD25^+$ population. Compared to naïve controls, the absolute number of $CD4^+$ cells recovered from the spleens of mice pre-treated with YTS177 alone or in conjunction with DST was reduced from $12.0 \times 10^6 \pm 1.6 \times 10^6$ to $3.4 \times 10^6 \pm 0.76 \times 10^6$ ($P = 0.001$), and $4.3 \times 10^6 \pm 0.48 \times 10^6$ ($P = 0.001$) respectively (Figure 3.11a, $n = 3$ in each group). The absolute number of $CD4^+CD25^+$ cells recovered from these two groups was also reduced, from $1.21 \times 10^6 \pm 0.23 \times 10^6$ to $0.40 \times 10^6 \pm 0.07 \times 10^6$ for YTS177 only ($P = 0.004$), and to $0.52 \times 10^6 \pm 0.08 \times 10^6$ for 177/DST ($P = 0.008$) (Figure 3.11b). Figure 3.11c, however, shows that within the $CD4^+$ population pre-treatment did lead to a small increase in the proportion of cells expressing CD25, from $10.01\% \pm 0.52$ in naïve animals to $11.95\% \pm 0.74$ in mice

pre-treated with 177/DST ($P = 0.02$). Compared to naïve animals there was also an increase in the group pre-treated with YTS177 alone ($11.83\% \pm 0.50$, $P = 0.01$), but not in the group pre-treated with DST alone ($9.68\% \pm 1.34$, $P = 0.64$). There was no significant difference between the two groups receiving YTS177 ($P = 0.83$). In view of the depletion of $CD4^+$ cells in animals whose pre-treatment included YTS177, all further phenotypic studies were also analysed in terms of cell percentages rather than absolute cell numbers.

3.2.5 Effect of DST re-challenge after 177/DST pre-treatment

Given the finding that 177/DST pre-treatment led to only a very small increase in the proportion of $CD4^+$ cells expressing CD25, we postulated that the pre-treatment protocol leads to the generation of Treg, but that full expansion of this population may only occur when the cells re-encounter antigen in a secondary response, usually in the form of an allograft. In order to test this hypothesis, mice were given an antigen re-challenge in the form of a DST at day 0 either following no pre-treatment, following 177/DST pre-treatment, or following pre-treatment with YTS177 alone or DST alone; spleen cells were then analysed at day +2 (Figure 3.12, $n = 4$ in each group). Animals that received both YTS177 and DST components of pre-treatment followed by DST re-challenge showed an increase in the proportion of $CD4^+$ cells expressing CD25 to $15.8\% \pm 0.9$ compared to $10.0\% \pm 1.0$ in naïve animals ($P < 0.001$). The group pre-treated with YTS177 alone followed by day 0 DST also showed an increase, to $13.5\% \pm 1.6$ ($P = 0.009$); the difference between the two groups receiving YTS177 was small but significant ($P = 0.04$). However, importantly, there was no increase compared to naïve animals in the groups receiving either day 0 DST alone ($11.1\% \pm 1.1$, $P = 0.18$) or DST at days -27 and 0 ($10.1\% \pm 0.5$, $P = 0.84$),

suggesting that the increase in the groups receiving antibody was not simply a result of activation of cells by alloantigen. These findings would be consistent with the hypothesis that 177/DST pre-treatment leads to the generation of a $CD4^+CD25^+$ Treg population that undergoes further expansion following antigen re-challenge.

3.2.6 *CD122 expression by $CD4^+CD25^+$ cells*

In view of the finding that $CD4^+CD25^+$ cells from naïve mice express higher levels of CD122 than the reciprocal $CD4^+CD25^-$ population (Figure 3.2d) and previous published reports that human $CD4^+CD25^+$ Treg also express CD122 [285, 286, 301, 306], the next aim was to investigate whether pre-treatment with 177/DST followed by DST re-challenge would lead to an increase in the proportion of $CD4^+CD25^+$ cells that express CD122 (Figure 3.13, $n = 4$ in each group). While $17.1\% \pm 2.4$ of $CD4^+CD25^+$ cells from naïve animals expressed CD122, this increased to $36.4\% \pm 1.6$ for cells from mice pre-treated with 177/DST followed by day 0 DST ($P < 0.0001$). Mice pre-treated with YTS177 alone followed by day 0 DST showed a less substantial increase to $24.7\% \pm 2.8$ ($P = 0.006$ compared to naïve animals, $P = 0.0004$ compared to 177/DST group). However, there was no increase compared to naïve animals among mice that received DST at day 0 only ($19.8\% \pm 2.9$, $P = 0.21$) or DST at days -27 and 0 ($18.1\% \pm 2.1$, $P = 0.57$). These data once again suggest that the increases seen in animals pre-treated with 177/DST and, to a lesser extent with YTS177 only, were a specific effect of the pre-treatment rather than a result of cell activation.

3.2.7 *CTLA-4 expression by $CD4^+CD25^+$ cells*

Attention was next turned to CTLA-4, since $CD4^+CD25^+$ Treg have been shown to express this molecule in both mouse [216, 218, 288] and human [284-286, 301, 304,

306] studies. The importance of CTLA-4 expression for the functional activity of CD4⁺CD25⁺ Treg is, however, less clear since it appears to play a role in some studies [218, 222, 240, 288, 304] but not in others [241, 282, 283, 286, 293, 349].

In order to investigate the expression of CTLA-4 by different cell populations it was first necessary to determine whether its presence could be detected using flow cytometric analysis. It has previously been demonstrated that CTLA-4 is barely detectable on resting T cells, but is upregulated following cell activation [396, 397]. The surface expression of CTLA-4 by splenocytes that were either unmanipulated or stimulated *in vitro* was therefore compared (Figure 3.14), but it was not possible to demonstrate positive staining in either case.

Published data have shown CTLA-4 is stored primarily within intracellular vesicles and rapidly cycles between this intracellular compartment and the cell surface [398, 424, 425]. Thus cell surface expression of CTLA-4 is difficult to detect, and most studies instead examine intracellular expression [216, 218, 285, 286, 288, 301, 306]. Intracellular CTLA-4 staining was therefore performed; as shown in Figure 3.14, while *ex vivo* splenocytes were negative, stimulation of cells *in vitro* did lead to detectable expression.

Following the demonstration that the presence of intracellular CTLA-4 could be detected, CTLA-4 expression between *ex vivo* CD4⁺CD25⁺ and CD4⁺CD25⁻ cells from naïve mice was compared (Figure 3.15). While the CD25⁻ population was CTLA-4 negative, a significant proportion of CD25⁺ cells showed constitutive expression, a finding that is entirely consistent with the reports cited above. However, we wondered whether, like CD122, expression of CTLA-4 by CD4⁺CD25⁺ cells may increase following 177/DST pre-treatment and DST re-challenge. As shown in Figure 3.16 (n = 4 in each group), 45.4% ± 2.3 of CD4⁺CD25⁺ cells from animals

pre-treated in this way expressed CTLA-4, compared to $35.5\% \pm 0.63$ of those from naïve controls ($P = 0.0002$). A similar increase, to $42.3\% \pm 3.6$, was seen in mice pre-treated with YTS177 alone followed by day 0 DST ($P = 0.007$ compared to naïve animals, $P = 0.28$ compared to group receiving 177/DST followed by day 0 DST). However, as with CD122, no increase was seen in mice that received either day 0 DST only ($32.3\% \pm 3.5$, $P = 0.12$) or DST on days -27 and 0 ($36.2\% \pm 1.0$, $P = 0.24$), suggesting that once again the increases seen in the other groups was not simply a result of cell activation by alloantigen.

3.2.8 Attempted enumeration of alloantigen-specific $CD4^+CD25^+$ cells: capture assays

One of the limitations in studying changes in the size and phenotype of the whole $CD4^+CD25^+$ population following 177/DST pre-treatment is that any effect on the donor-specific subpopulation may be difficult to discern. For example, the relatively modest overall increase in the proportion of $CD4^+$ cells expressing CD25 following 177/DST pre-treatment (with or without additional DST at day 0) may be misleading since, if this consisted mainly of donor-specific cells, this could represent a major expansion in the donor-specific subpopulation. A method that would allow the identification and enumeration of donor-specific cells would thus be extremely valuable. In recent years there has been much progress made in the study of peptide-specific T cells using synthetic peptide-MHC tetramers that bind to the T cell receptors, thus allowing their detection by flow cytometry [426]. However, although MHC class II tetramers with the ability to bind to $CD4^+$ cells are now being developed [427], to date this approach has mainly been limited to the study of $CD8^+$ cells [428]. In addition, use of tetramer technology requires knowledge of the peptide(s)

recognised by the T cell population(s) under investigation, information that is not yet available for the transplantation model described in this chapter. Even if this information were available, identification of the entire range of donor-specific T cells would be likely to require too many different tetramers to make this approach feasible. An alternative method for the enumeration of antigen-specific T cells was therefore sought.

Davis and colleagues have previously developed a technique to examine interactions between T cells and APC [429]. In this system, incubation of CD4⁺ T cell clones together with APC pulsed with the appropriate peptide leads to the formation of “immunological synapses” between the two populations. This prompted us to ask whether the strength of the interactions between TCR of alloreactive T cells and peptide-MHC complexes on donor APC subsequently strengthened by adhesion molecule and coreceptor ligation could be sufficient to provide a basis for the enumeration of donor-specific T cells operating through the direct pathway of allorecognition. We speculated that if T cells of interest were added to immobilised donor APC, donor-reactive T cells may form conjugates with the immobilised APC, whereas other T cells would not stick and could be washed away. Captured T cells could then theoretically be enumerated using a colourimetric assay (Figure 3.17).

As shown in Figure 3.18, B10 splenocytes could be successfully coated with biotin (Figure 3.18b) and then captured on plates covered with streptavidin, with captured cells detected using a colourimetric assay (Figure 3.18c). However, attempts to capture and detect B10-reactive T cells were less successful. CBA spleen cells were coated with rat anti-mouse CD45 antibody and incubated in wells containing immobilised B10 cells, and the plates were then washed to remove any unbound cells. In order to detect captured CBA cells, HRP-conjugated mouse anti-rat IgG was added,

the plates washed, and the colourimetric developed as before. However, no colour change was detected. We wondered whether CBA splenocytes contained B10-specific cells at too low a precursor frequency to be detected in this way, and splenocytes from DES mice, a strain containing a high proportion of T cells expressing a transgenic T cell receptor specific for K^b [351] were therefore used instead; however, once again it was not possible to detect any captured T cells.

We next postulated that coating the CBA or DES cells with anti-CD45 antibody prior to incubating with the target B10 cells may cause steric hindrance and prevent cell capture. CBA or DES cells were therefore placed into wells without prior antibody coating, and then the anti-CD45 antibody added after the initial incubation. However, once again no bound cells were detected. Given the failure to detect bound T cells it seemed probable that, even if cells were being captured via TCR interactions with peptide-MHC complexes, the strength of these interactions would not be able to withstand the washing steps necessary for the subsequent detection process. It was therefore felt that further pursuit of this approach would be unlikely to yield a useful assay.

3.2.9 Attempted enumeration of alloantigen-specific CD4⁺CD25⁺ cells: conjugate assays

An alternative potential approach for the detection of antigen-specific T cells was provided by a method described by Schaefer and colleagues [366]. They labelled a T cell line with CFSE, a green fluorescent dye that binds to cytoplasmic proteins, and APC with PKH26, a red fluorescent dye that binds to cell membranes. Incubation of T cells with APC pulsed with appropriate peptides led to the formation of T cell-APC

“conjugates”, which could be detected as “double positive” (red and green) events when analysed by flow cytometry.

In an attempt to harness this approach, CBA splenocytes were labelled with PKH26, incubated for 30 minutes at 37°C with B10 or BALB splenocytes that had been labelled with CFSE, and flow cytometric analysis was then performed (Figure 3.19). A small but definite double positive population (0.5% for B10 assay, 0.9% for BALB assay) was present in both cases. Encouraged by this finding we next attempted to detect cell conjugate formation in a situation where the frequency of alloreactive T cells should be much higher. Labelled DES cells were incubated with splenocytes from B10 mice, for which the vast majority of DES cells possess specific T cell receptors, or from BALB mice, with which the DES T cells should make no specific interactions (negative control). We also wondered whether the detection of cell conjugates could be improved by fixing them with formalin prior to flow cytometric analysis, and hence increase the probability of their remaining intact during the acquisition process. Finally the incubation time for conjugate formation was varied to see whether this would improve assay sensitivity. The results of these experiments are summarised in table 3.1. Although these experiments were not replicated, it is apparent that the frequency of cell conjugates was similar in most cases. There was only one situation where there was an apparent increase in conjugate formation between DES and B10 cells compared to BALB cells. However, the magnitude of this difference (5.3% versus 2.0%) was small, and suggests that in the non-transgenic setting (e.g. CBA versus B10) that this type of assay is unlikely to provide adequate sensitivity. This is supported by the experience Schaefer and colleagues that robust detection of cell conjugates only occurs when using T cell lines and APC pulsed with

the appropriate peptide (B. Schaefer, personal communication). This approach was therefore not pursued any further.

T cells	APC	Formalin fixation	Incubation time	Conjugates
CBA	B10	No	30 minutes	0.5%
CBA	BALB	No	30 minutes	0.9%
DES	B10	No	30 minutes	0.9%
DES	BALB	No	30 minutes	1.2%
DES	B10	Yes	30 minutes	5.3%
DES	BALB	Yes	30 minutes	2.0%
DES	B10	Yes	2 hours	2.8%
DES	BALB	Yes	2 hours	2.3%
DES	B10	Yes	Overnight	2.1%
DES	BALB	Yes	Overnight	2.0%

Table 3.1. Results of cell conjugate assays

3.3 Discussion

The potential of regulatory cells could theretically be harnessed in clinical transplantation in two main ways. Firstly, if some way can be found to detect and quantify donor-reactive Treg in transplant recipients it may be possible to identify individuals whose immunosuppressive therapy may safely be reduced without a significant risk of precipitating graft rejection, thus minimising the unwanted effects of this immunosuppression both in terms of its non-specificity and the individual side effects of the drugs involved. Secondly, if a protocol can be developed that allows the

generation of donor-reactive Treg, this may enable the avoidance of non-specific immunosuppressive therapy in an even greater number of patients. However, these approaches require that Treg populations can be identified reliably and, if possible that their antigen specificity can be determined. As discussed earlier, CD25 does not possess sufficient specificity (nor possibly even sensitivity) to act as a perfect marker to identify Treg. Thus a major focus of research has been to try to identify other markers.

In Figure 3.1 it was shown that $CD4^+CD25^+$ cells lie almost entirely within the $CD45RB^{low}$ population, a finding consistent with the observations of others [209, 210, 216, 282, 369]. This has prompted speculation that regulatory activity of $CD4^+CD45RB^{low}$ cells may be confined to the $CD25^+$ subset. However, attempts to investigate this question have yielded conflicting results. Powrie's group have found that, unlike $CD4^+CD25^+$ cells, $CD4^+CD25^-CD45RB^{low}$ cells are unable to prevent disease in a colitis model [218, 227], suggesting that expression of CD25 serves to enrich further for cells with regulatory activity than expression of low levels of CD45RB. Conversely, in studies by Kullberg *et al.* [318] and Oida *et al.* [279], $CD4^+CD25^-CD45RB^{low}$ cells were able to prevent colitis. Interestingly, Annacker and colleagues found a difference in the behaviour of $CD4^+CD25^+$ and $CD4^+CD25^-CD45RB^{low}$ cells in a model of colitis mediated by $CD4^+CD45RB^{high}$ cells [223]. Although both populations were able to suppress the development of disease, only the $CD25^+$ cells inhibited expansion of the $CD4^+CD45RB^{high}$ population. The regulatory capacity of $CD4^+CD25^-CD45RB^{low}$ cells will be investigated further in Chapter 4.

The analyses shown in Figure 3.2 compared the expression of various cell surface markers between $CD4^+CD25^+$ and $CD4^+CD25^-$ cells, starting with CD44,

CD62L, and CD69, molecules whose level of expression is known to change following cell activation. Although CD44 is expressed at higher levels by the CD4⁺CD25⁺ population (Figure 3.2a), there is no difference in expression of CD62L (Figure 3.2b) or CD69 (Figure 3.2c). As discussed in Section 3.1.1, reports in the literature examining the expression of these markers by CD4⁺CD25⁺ Treg have yielded varying results. Most of these reports have found that, consistent with the observation in Figure 3.2a, most CD4⁺CD25⁺ Treg express CD44 [209, 293, 370, 389]. However, examination of the expression patterns of CD62L and CD69 in these reports has not yielded consistent results. Importantly, the finding that purifying CD4⁺CD25⁺ cells on the basis of their levels of expression of CD62L does not affect their ability to regulate *in vitro* [217, 224] but that only the CD62L^{high} fraction prevents autoimmune disease *in vivo* [224] suggests that although expression of such molecules may not affect the ability of cells to regulate *per se*, it may play a vital role in permitting their functional activity *in vivo*, for example by allowing homing to their site of action. This illustrates one of the difficulties in extrapolating results from *in vitro* studies to predict behaviour in more complex *in vivo* systems.

The finding shown in Figure 3.2d that a higher proportion of CD4⁺CD25⁺ than CD4⁺CD25⁻ cells express CD122 represents the first description of this in mice; however, it supports previous observations in human studies that CD122 is expressed by CD4⁺CD25⁺ Treg [283, 285, 286, 301, 306, 307]. Expression of CD122 by Treg may have various theoretical functional implications. Firstly, expression of both CD25 and CD122 (together with CD132, Figure 3.2e) may enable Treg to bind IL-2 with high affinity, thus sequestering this important growth factor from the population being regulated. Secondly, since CD122 also forms part of the receptor for IL-15, a cytokine that confers resistance to activation-induced cell death and prolongs cell survival *in*

vivo [430-435], CD122 expression by Treg may promote their survival. However, the finding that expression of the α chain of the IL-15 receptor is no different on CD4⁺CD25⁺ compared to CD4⁺CD25⁻ cells (Figure 3.2f) suggests that this may not be relevant. Indeed, the published studies examining the role of IL-15 on cell survival *in vivo* have predominantly demonstrated an effect on CD8⁺ cells [433-440]. This is supported by the observation in Figure 3.4 that cells expressing IL-15R α lie predominantly within the CD4⁻ rather than the CD4⁺ population.

The significance of TGF- β bound to the surface of CD4⁺CD25⁺ Treg remains controversial, with some studies suggesting that this plays an important role in their function [279, 304, 311], whereas others do not [314, 335]. The finding in Figure 3.2g that TGF- β could not be detected on the surface of CD4⁺CD25⁺ cells is consistent with the latter position.

In Figures 3.2h and 3.3 it was demonstrated that CD4⁺CD25⁺ cells are predominantly GITR⁺. This supports published findings that CD4⁺CD25⁺ Treg express GITR [308, 312-314]. However, as discussed in Section 3.1.1.5, GITR may not play a functional role in Treg activity but rather may actually provide a mechanism to inhibit their regulatory action under circumstances where a robust immune response is required.

Attention was next turned away from naturally-occurring CD4⁺CD25⁺ cells towards antigen-induced Treg, and more specifically to investigate whether such cells may play a role in the induction phase of tolerance in a transplantation model. In Figures 3.5 and 3.6 it was shown that CBA-Rag mice contain no T cells and are unable to reject skin allografts, but that rejection can be restored by the administration of naïve CD4⁺CD45RB^{high} cells. This provides a model to study the regulatory ability of putative Treg populations. In Figure 3.9 it was clearly shown that in mice

pre-treated with 177/DST, CD4⁺CD25⁺ Treg are present at the time point when a cardiac allograft would be performed in the primary heart model. Further work in our laboratory has demonstrated that equivalent numbers of CD4⁺CD25⁺ cells taken from naïve CBA mice or from CBA mice pre-treated with DST alone on day -27 or with YTS177 alone on days -28 and -27 are not able to regulate in this way [441]. Taken together these data clearly demonstrate that 177/DST pre-treatment leads to the generation of CD4⁺CD25⁺ Treg at a sufficient precursor frequency to overcome the rejection of skin allografts in this model, showing that Treg are present during the induction phase of tolerance. The fact that equal numbers of CD4⁺CD25⁻ cells from pre-treated mice are not able to regulate skin allograft rejection shows that the Treg lie mainly within the CD25⁺ population. However, it does not exclude the possibility that cells capable of preventing graft rejection are present within the CD25⁻ fraction, albeit at a lower precursor frequency. In a study by Graca *et al.* examining the regulatory properties of cells taken from mice tolerant to skin allografts it was shown that both CD4⁺CD25⁺ and CD4⁺CD25⁻ populations could regulate anti-donor responses, but that the number of CD25⁻ cells required to achieve this was tenfold higher than the number of CD25⁺ cells [241]. However, in that study the purity of the sorted CD25⁻ population was >98%, whereas that of the CD25⁺ population was >90%; this suggests that the regulatory potency of the CD25⁺ cells may have been underestimated in comparison to the CD25⁻ cells.

The finding that CD4⁺CD25⁺ Treg can be generated by 177/DST pre-treatment provides a powerful model to study antigen-induced Treg and will be used extensively in this thesis. In the context of this chapter it provides a system to investigate whether antigen-induced Treg differ phenotypically from naturally-occurring cells.

Given that 177/DST pre-treatment leads to the development of a $CD4^+CD25^+$ Treg population, a logical question to ask was whether this is accompanied by a detectable increase in the size of the $CD4^+CD25^+$ population. However, comparison of absolute cell numbers was hampered by the finding that administration of YTS177 led to partial depletion of the $CD4^+$ population (Figure 3.11a), and thus also the $CD4^+CD25^+$ population (Figure 3.11b). The relative balance of $CD25^+$ and $CD25^-$ cells within the residual $CD4^+$ population was therefore examined instead. In the animals pre-treated with 177/DST there was an increase in the proportion of $CD4^+$ cells expressing CD25 (Figure 3.11c). It could be argued that this was simply a result of cell activation (and hence upregulation of CD25) by the DST component of pre-treatment rather than a relative expansion of the Treg population. However, the fact that a similar increase was not seen in animals pre-treated with DST alone makes this possibility unlikely. The increase seen in animals pre-treated with YTS177 alone may at first seem to contradict the observation that $CD4^+CD25^+$ cells from animals pre-treated with YTS177 alone are unable to regulate the rejection of skin allografts [441]. However, if Treg generation is a result of antigen encounter by T cells under conditions of CD4 blockade, encounter of cognate antigen (such as environmental pathogens) by any T cell, and not just those specific for B10 alloantigen, during the presence of YTS177 would lead to the production of Treg. Thus the DST component of pre-treatment would lead to alloantigen specificity within the Treg population without being an essential requirement for the generation of Treg *per se*.

In the mice pre-treated with 177/DST and then given an antigen re-challenge in the form of a second DST at day 0, the increase in the proportion of $CD4^+$ cells expressing CD25 was further augmented (Figure 3.12). This is consistent with the hypothesis that pre-treatment leads to priming of the Treg but that full development

only occurs when antigen is re-encountered in a secondary response. Once again, this increase is unlikely to be due simply to cell activation in view of the fact that no increase was seen in the animals exposed to DST only using the same protocol (either at day 0 alone or days –27 and 0).

The observation that pre-treatment with 177/DST followed by a further DST challenge led to an increase in the proportion of CD4⁺CD25⁺ cells expressing CD122 (Figure 3.13) provides further circumstantial evidence to support the hypothesis earlier in this chapter that CD122 expression may play a role in the functional activity of CD4⁺CD25⁺ Treg. This issue will be addressed further in Chapters 4 and 6.

In Figure 3.15 it was demonstrated that a significant proportion of CD4⁺CD25⁺ cells express CTLA-4 constitutively; this is consistent with previous reports from both mouse [216, 218, 237, 288, 308] and human [284-287, 301, 302, 304, 306, 307, 310] studies. As with CD122, the proportion of CD4⁺CD25⁺ cells expressing CTLA-4 was higher in mice pre-treated with 177/DST followed by DST re-challenge (Figure 3.16), suggesting that CTLA-4 expression may play a role in the suppressive function of these cells. In support of this hypothesis, various groups have shown that CTLA-4 blockade abrogates regulation by CD4⁺CD25⁺ cells [218, 240, 288, 304]; indeed using the adoptive transfer system described in this chapter we have demonstrated that regulation of skin allograft rejection by CD4⁺CD25⁺ cells from mice pre-treated with 177/DST is abrogated by administration of anti-CTLA-4 antibody to the adoptive transfer recipients [441]. Although one consequence of CTLA-4 engagement is to inhibit cell activation and proliferation [399-404], a further effect is to stimulate the secretion of TGF- β [405, 406] which, as discussed above, may play a role in mediating the regulatory action of these cells.

In the final part of this chapter attempts were made to enumerate alloantigen-specific cells, since this would provide an extremely powerful tool for use in both experimental and clinical settings. The methods explored here (Figures 3.17, 3.18, and 3.19 and Table 3.1) were based on the premise that the interaction between MHC-peptide complexes on allogeneic APC and the T cell receptors on donor-specific T cells, once stabilised by adhesion molecule and coreceptor interactions) would be of sufficient affinity to allow the detection of cell conjugates. However, under the conditions tried this appeared not to be the case. Other approaches that may possibly allow the enumeration of antigen-specific cells exist. Radford and colleagues have developed a flow-based system that allows the study of binding of neutrophils to activated platelets [442]. It may be possible to adapt this approach to examine the binding of T cells to APC, although once again this would require an interaction of sufficient affinity. An alternative strategy may be to examine the production of cytokines by T cell populations following antigen stimulation. Data from our laboratory have shown that $CD4^+CD25^+$ cells from mice pre-treated with 177/DST produce IFN- γ when re-challenged with donor alloantigen (B.S. Sawitzki and C.I. Kingsley, manuscript submitted). Various methods have been described that allow measurement of cytokine production by T cells, including intracellular cytokine staining [443], capture of secreted cytokine on the cell surface followed by flow cytometric analysis [444], or the use of a solid phase enzyme-linked immunosorbent (ELISPOT) assay [445]. A further method that may allow the enumeration of antigen-specific T cells is the use of limiting dilution analysis [443, 446, 447].

In summary, the data presented in this chapter demonstrate that $CD4^+CD25^+$ capable of regulating the rejection (mediated by $CD4^+CD45RB^{high}$ cells) of skin allografts can be generated by pre-treatment of mice with donor-specific blood

transfusion under the cover of anti-CD4 antibody. Phenotypic analysis of these CD4⁺CD25⁺ cells shows that the pre-treatment leads to an increase in the proportion expressing CD122 and CTLA-4, suggesting that these molecules may play a role in the function of the Treg; this issue will be examined further in later chapters.

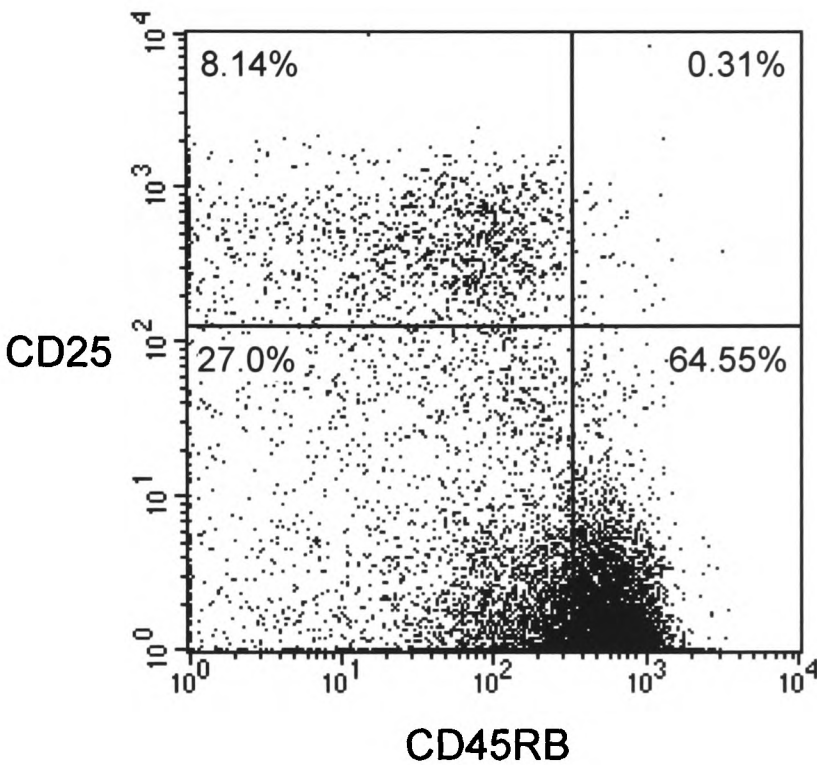


Figure 3.1. Relationship between CD4⁺CD25⁺ and CD4⁺CD45RB^{low} populations
Splenocytes from naïve CBA mice were stained with anti-CD4-PerCP, anti-CD45RB-PE, and biotinylated anti-CD25 followed by SA-APC, and were then analysed by flow cytometry. Plot shows expression of CD25 and CD45RB gated on CD4⁺ population. Percentages represent proportion of cells within each quadrant. Data are representative of four independent experiments.

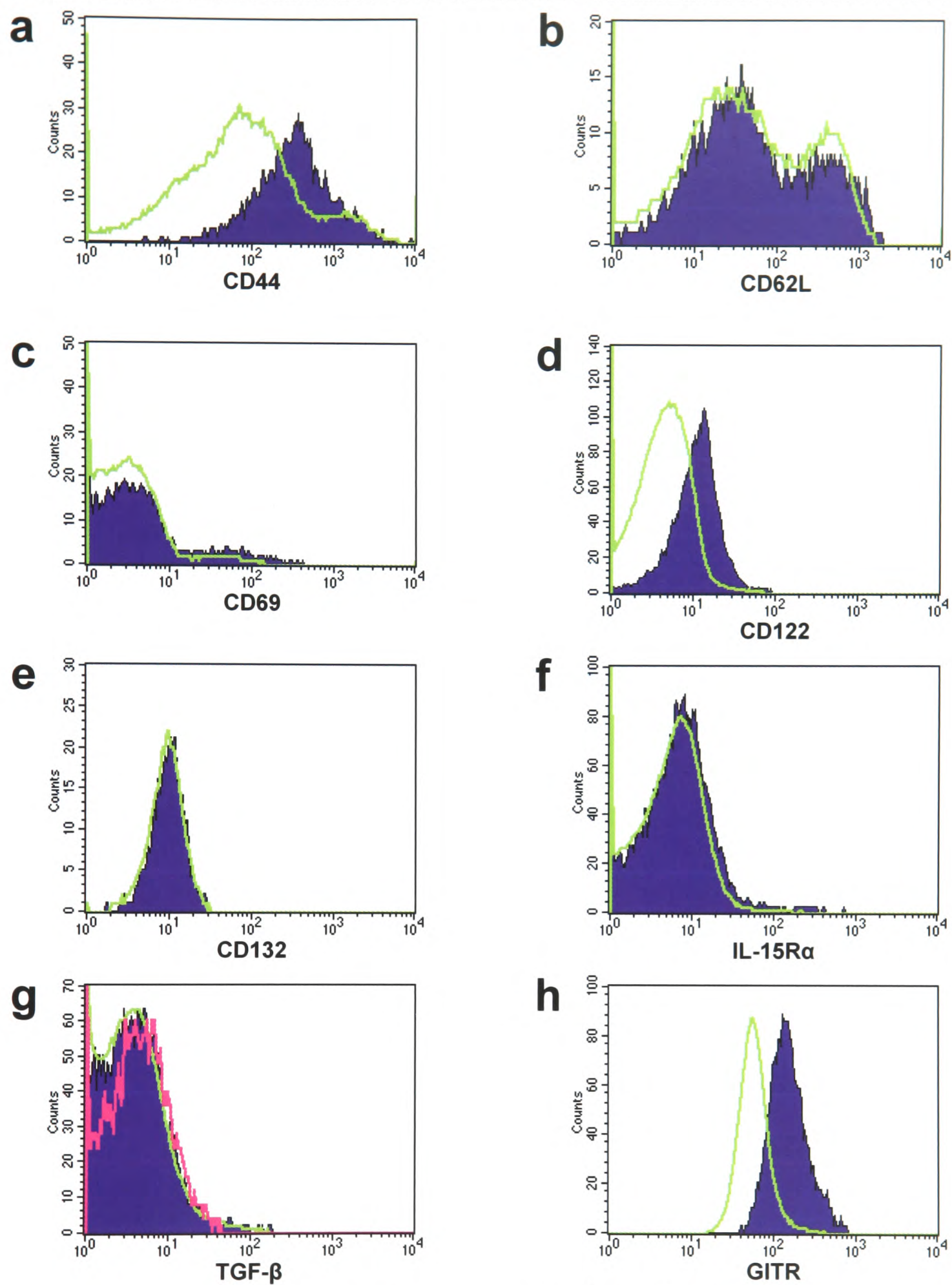


Figure 3.2. Phenotype of CD4⁺CD25⁺ cells

Spleens were harvested from naïve CBA mice and single cell suspensions stained with the appropriate reagents for flow cytometric analysis. Histogram plots show expression of the indicated cell surface marker by CD4⁺CD25⁺ (solid plots) and CD4⁺CD25⁻ (green outline plots) populations. Plots (a) to (e) show staining with anti-CD4-PerCP and biotinylated anti-CD25 together with PE-conjugated to anti-CD44, anti-CD62L, anti-CD69, anti-CD122, or anti-CD132 respectively, followed by SA-APC. Plots (f) to (h) show staining with anti-CD4-PerCP and anti-CD25-PE together with (f) biotinylated IL-15-Fc fusion protein followed by SA-APC, (g) biotinylated anti-TGF-β₁ followed by SA-APC, (h) anti-GITR followed by FITC-conjugated anti-rat IgG antibody. For TGF-β histogram red outline plot shows staining of CD4⁺CD25⁺ population with biotinylated control chicken IgY antibody followed by SA-APC. Data are representative of three independent experiments.

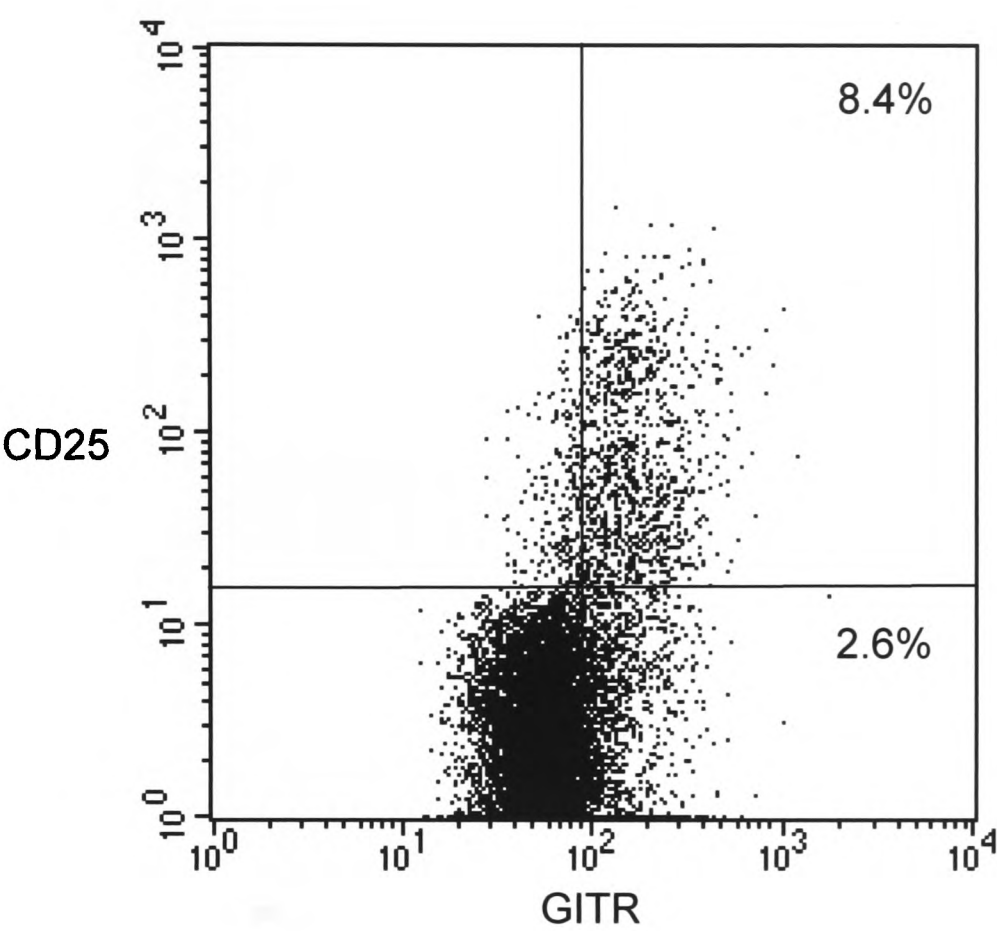


Figure 3.3. Expression of CD25 and GITR by CD4⁺ cells
Splenocytes from naïve CBA mice were stained with anti-CD4-PerCP, anti-CD25-PE, and anti-GITR followed by FITC-conjugated anti-rat IgG antibody. Flow cytometric analysis was then performed. Plot shows expression of CD25 and GITR gated on CD4⁺ population and is representative of three independent experiments.

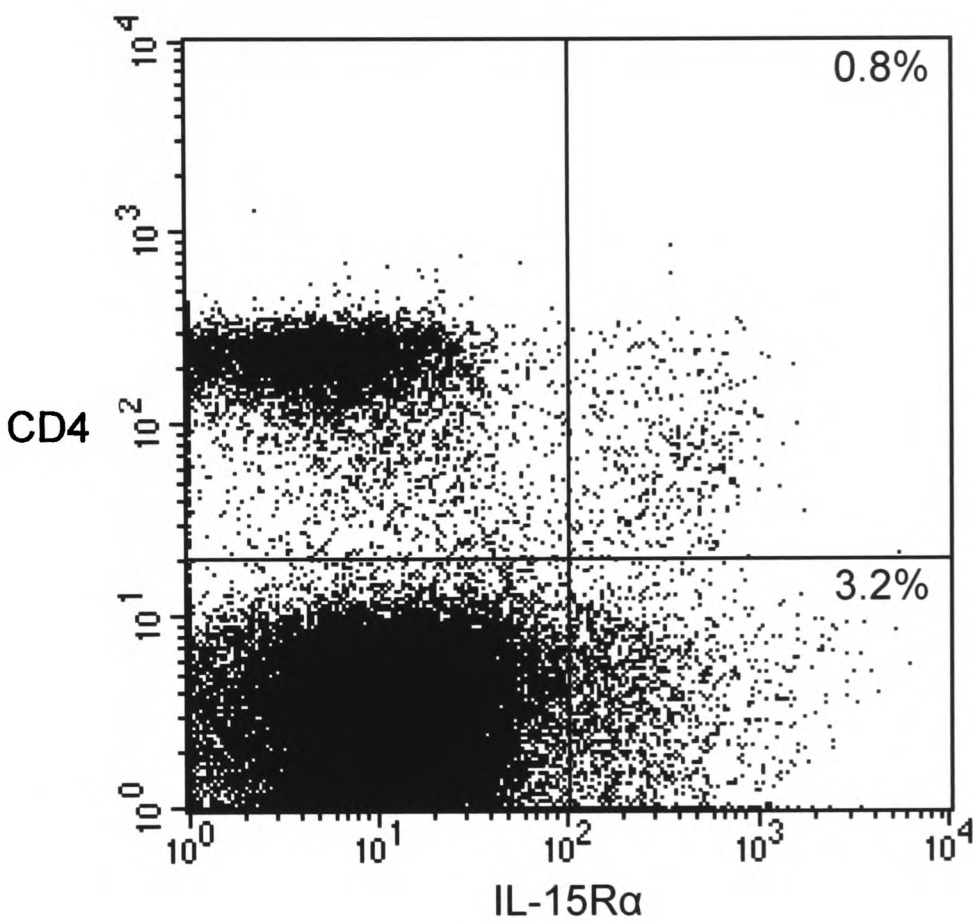


Figure 3.4. Expression of IL-15Rα by CD4⁺ and CD4⁻ cells

Splenocytes from naïve CBA mice were stained with anti-CD4-PerCP and biotinylated IL-15-Fc fusion protein followed by SA-APC. Flow cytometric analysis was then performed. Plot shows expression of CD4 versus IL-15Rα and is representative of three independent experiments.

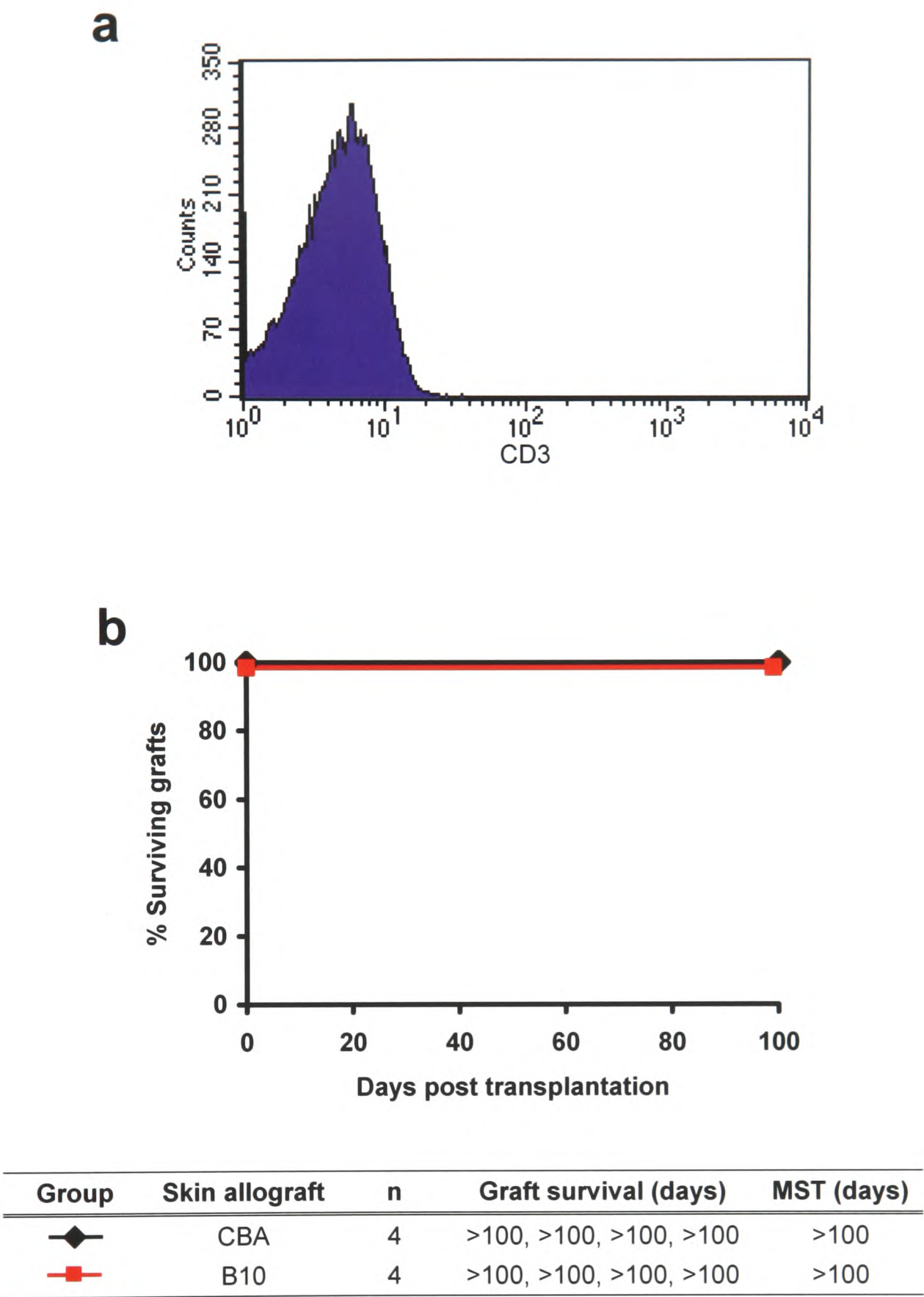
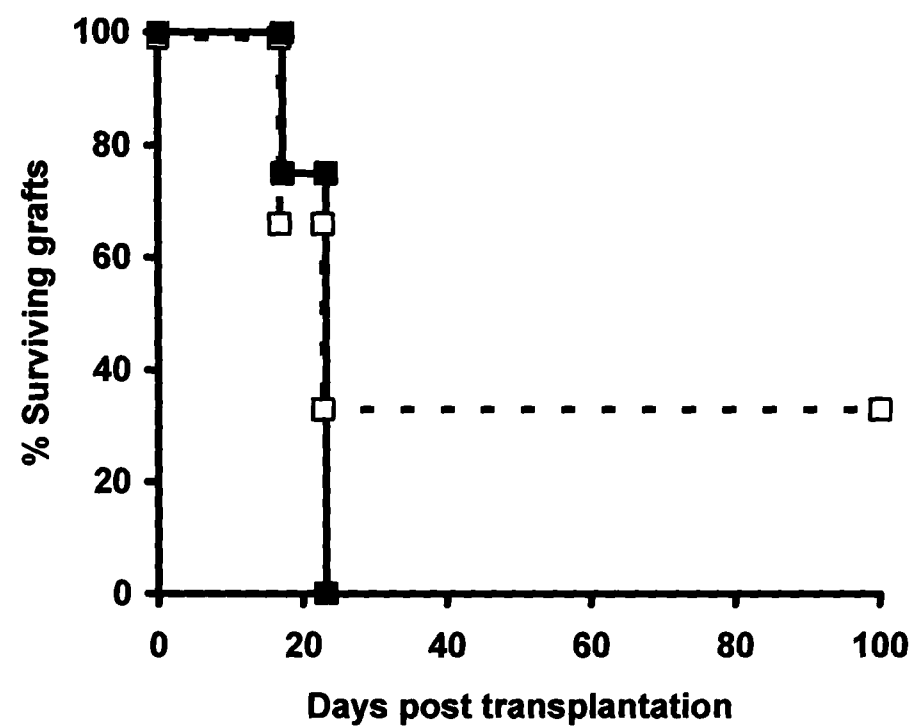


Figure 3.5. CBA-Rag mice contain no T cells and are unable to reject skin allografts

(a) Splenocytes from naïve CBA-Rag mice were stained with anti-CD3-FITC prior to flow cytometric analysis. Histogram shows CD3 expression. (b) Naïve CBA-Rag mice received a syngeneic CBA or allogeneic B10 allograft.



Group	Number of CD4 ⁺ CD45RB ^{high} cells transferred	n	Graft survival (days)	MST (days)
-□-	5×10 ⁴	3	17, 23, >100	23
■	10 ⁵	4	17, 23, 23, 23	23

Figure 3.6. Adoptive transfer of CD4⁺CD45RB^{high} cells reconstitutes the ability of CBA-Rag mice to reject B10 skin allografts
CBA-Rag mice received the indicated number of CD4⁺CD45RB^{high} cells from naive CBA mice intravenously. The following day B10 skin allografts were performed.

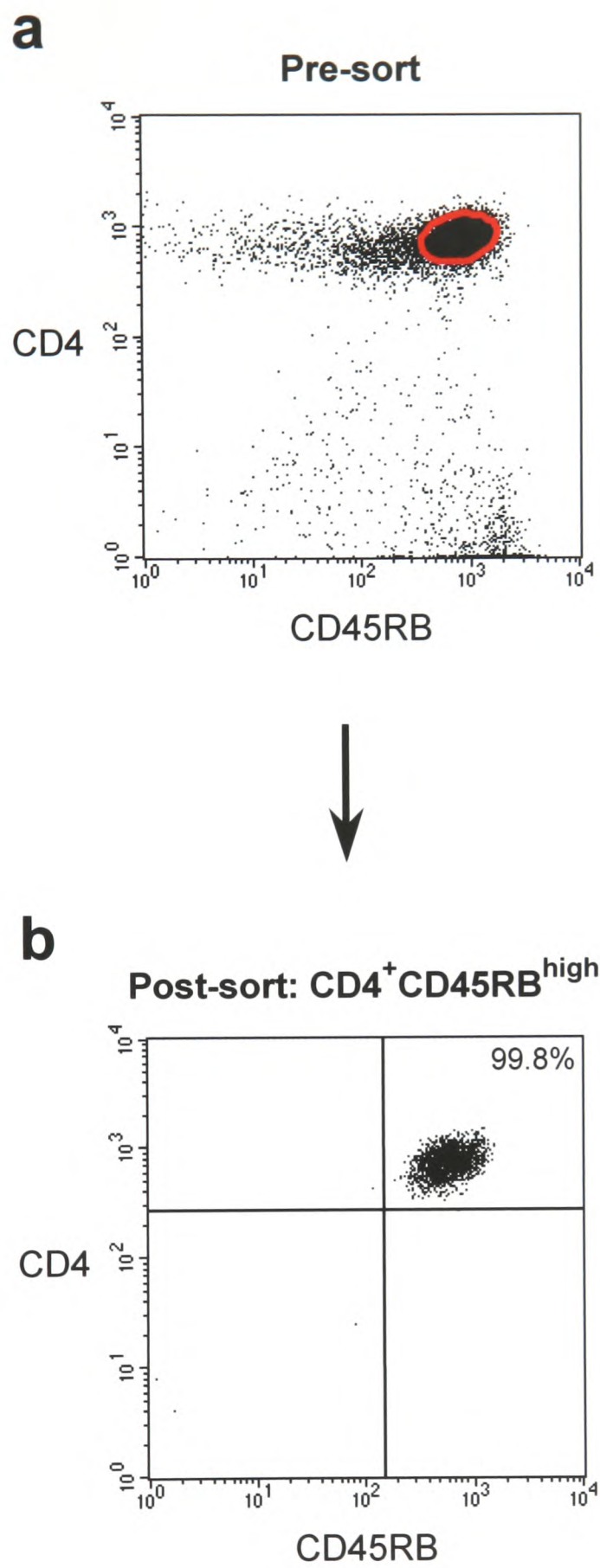


Figure 3.7. Purification of CD4⁺CD45RB^{high} population
(a) Cells were isolated from the spleens and lymph nodes of naïve CBA mice and enriched for CD4⁺ cells by negative selection using magnetic beads and stained with anti-CD4-CyC and anti-CD45RB-PE prior to purification of the CD4⁺CD45RB^{high} population by flow cytometry (gate used for sorting shown in red). (b) Re-analysis of purified CD4⁺CD45RB^{high} population. Percentage indicates sample purity. Plots are representative of the CD4⁺CD45RB^{high} cell purifications throughout this thesis.

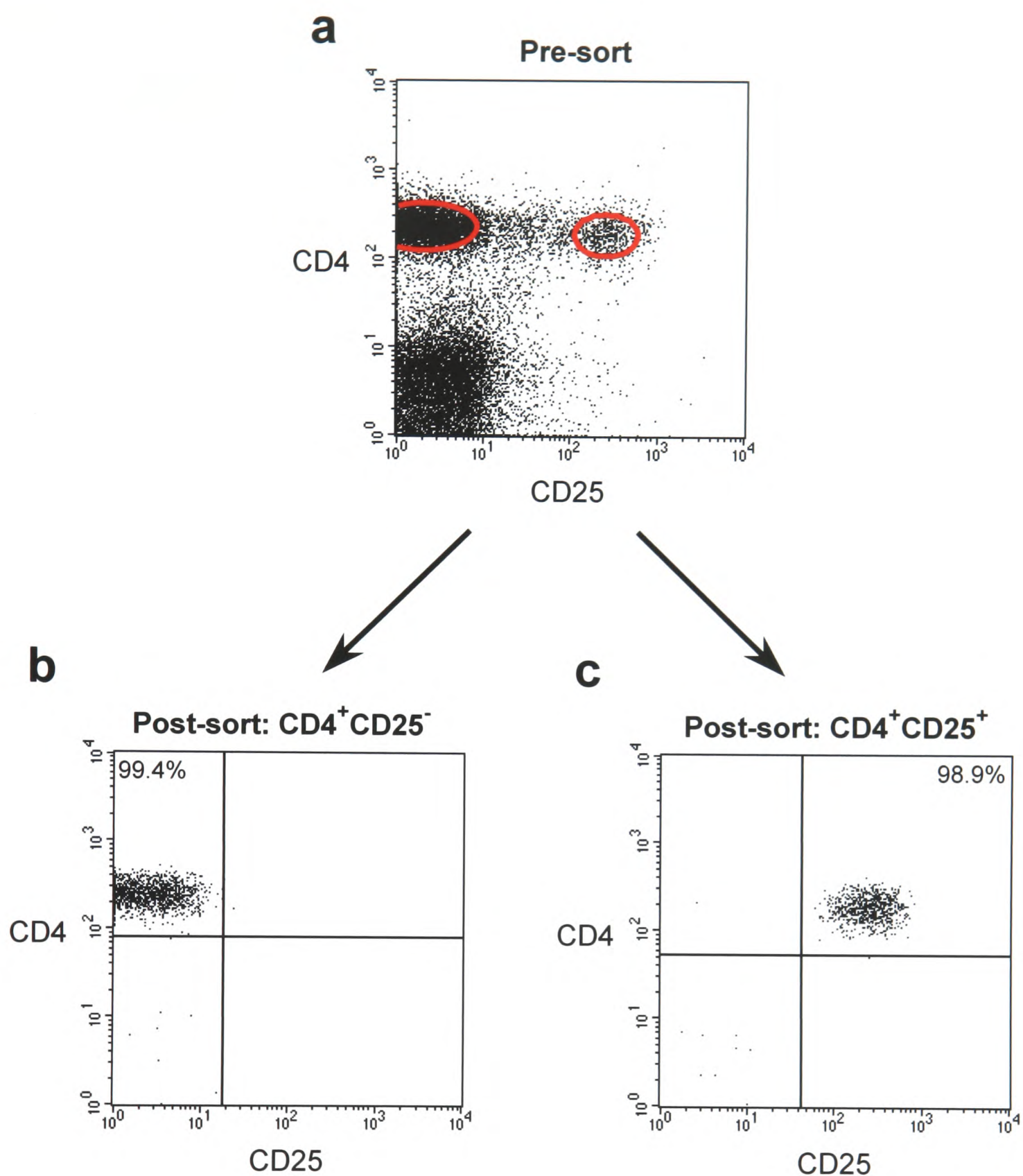


Figure 3.8. Purification of CD4⁺CD25⁺ and CD4⁺CD25⁻ populations
(a) Splenocytes from CBA mice pre-treated according to the 177/DST protocol underwent magnetic bead enrichment for CD4⁺ cells and then staining with anti-CD4-CyC and biotinylated anti-CD25 followed by SA-PE prior to purification by flow cytometry of CD4⁺CD25⁺ and CD4⁺CD25⁻ populations. Gates used for sorting are shown in red. (b) and (c) Re-analysis of purified CD4⁺CD25⁻ and CD4⁺CD25⁺ populations respectively. Percentages indicate sample purity. Plots are representative of the CD4⁺CD25⁻ and CD4⁺CD25⁺ purifications throughout this thesis.

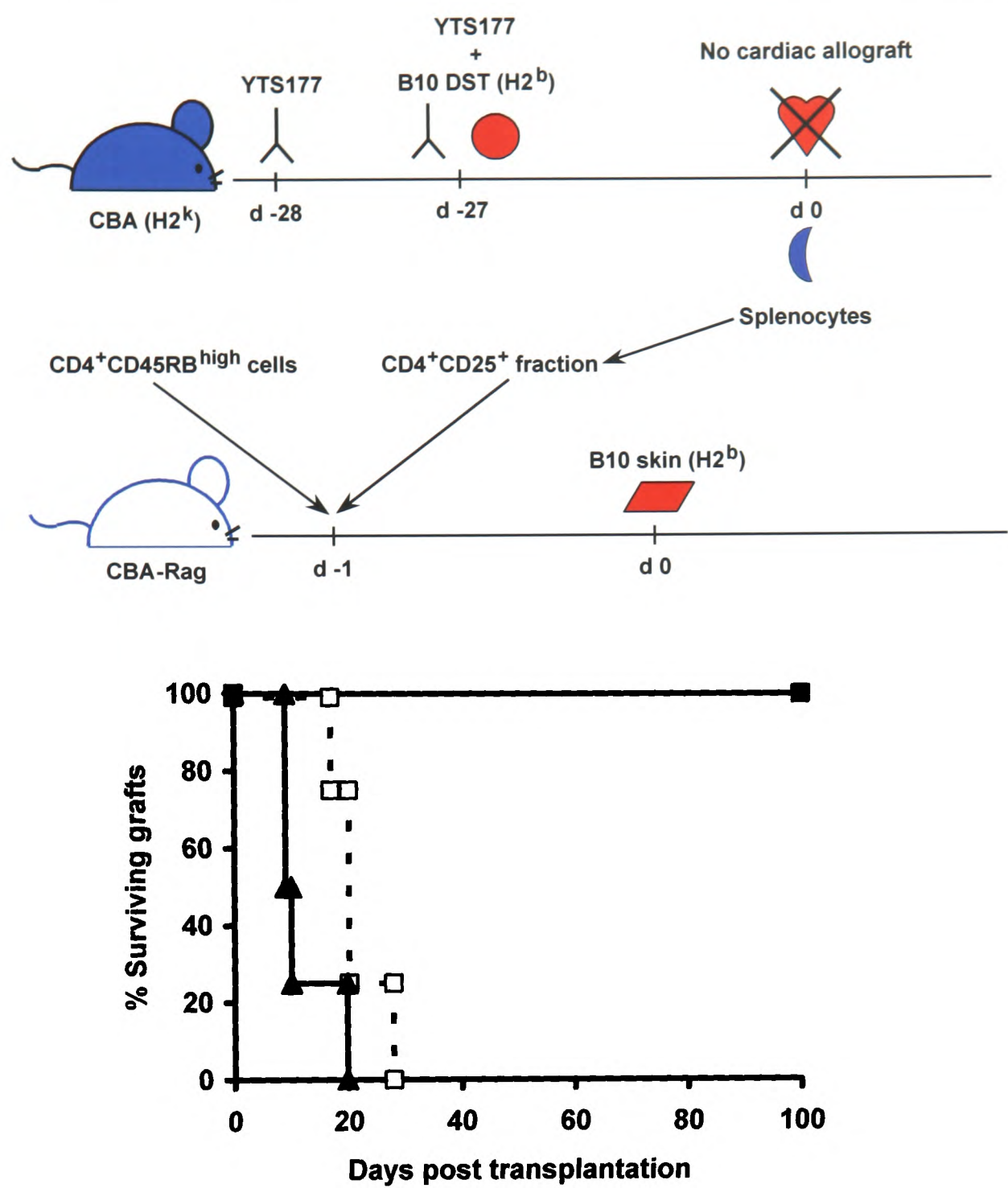


Figure 3.9. CD4⁺CD25⁺ cells from mice pre-treated with 177/DST regulate rejection of skin allografts

CBA-Rag mice were reconstituted with 10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice together with either 2×10⁵ CD4⁺CD25⁺ or CD4⁺CD25⁻ cells taken from CBA mice pre-treated with YTS177 (days -28 and -27) and B10 DST (day -27). The following day they received a B10 skin graft.

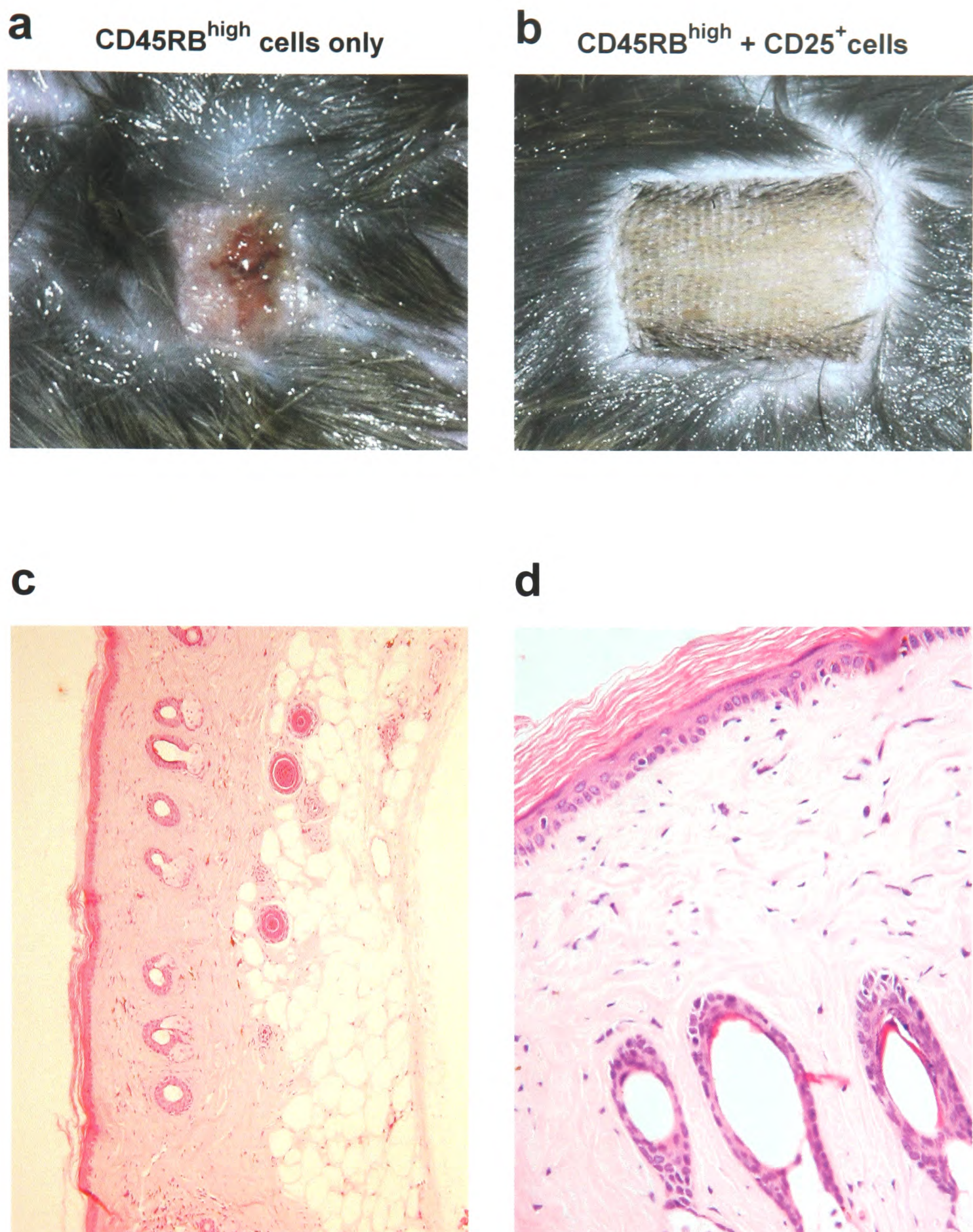


Figure 3.10. Macroscopic and histological appearance of skin allografts

(a) Rejected B10 skin allograft 14 days after transplantation onto CBA-Rag mouse reconstituted with 10^5 $CD4^+CD45RB^{high}$ cells from naïve CBA mice. (b) Accepted B10 skin allograft 14 days after transplantation onto CBA-Rag mouse reconstituted with 10^5 $CD4^+CD45RB^{high}$ cells from naïve CBA mice together with 2×10^5 $CD4^+CD25^+$ cells from CBA mice pre-treated with 177/DST. (c) Histological appearance of skin allograft in (b) (H&E, low power). (d) Histological appearance of skin allograft in (b) (H&E, high power).

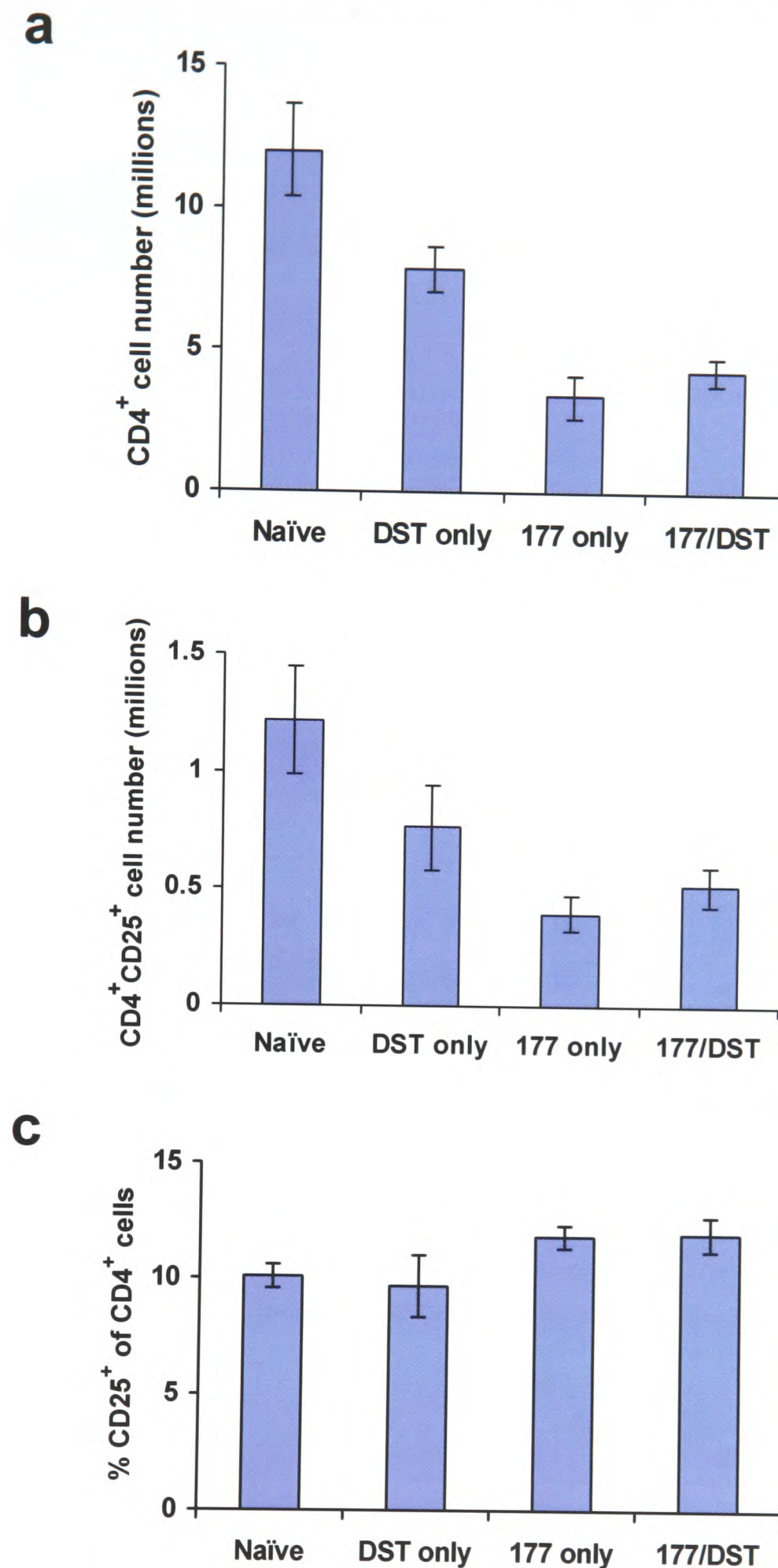


Figure 3.11. CD25 expression following 177/DST pre-treatment

Spleens from naïve CBA mice or mice pre-treated with the indicated combinations of YTS177 (days -28 and -27) and B10 DST (day -27) were stained with anti-CD4-PerCP and biotinylated anti-CD25 followed by SA-APC prior to flow cytometric analysis. (a) Absolute number of CD4⁺ cells recovered from spleen. (b) Absolute number of CD4⁺CD25⁺ cells. (c) Percentage of CD4⁺ cells expressing CD25. Results are shown as mean $\pm$ standard deviation. $n = 3$ in each group. Data are representative of three independent experiments.

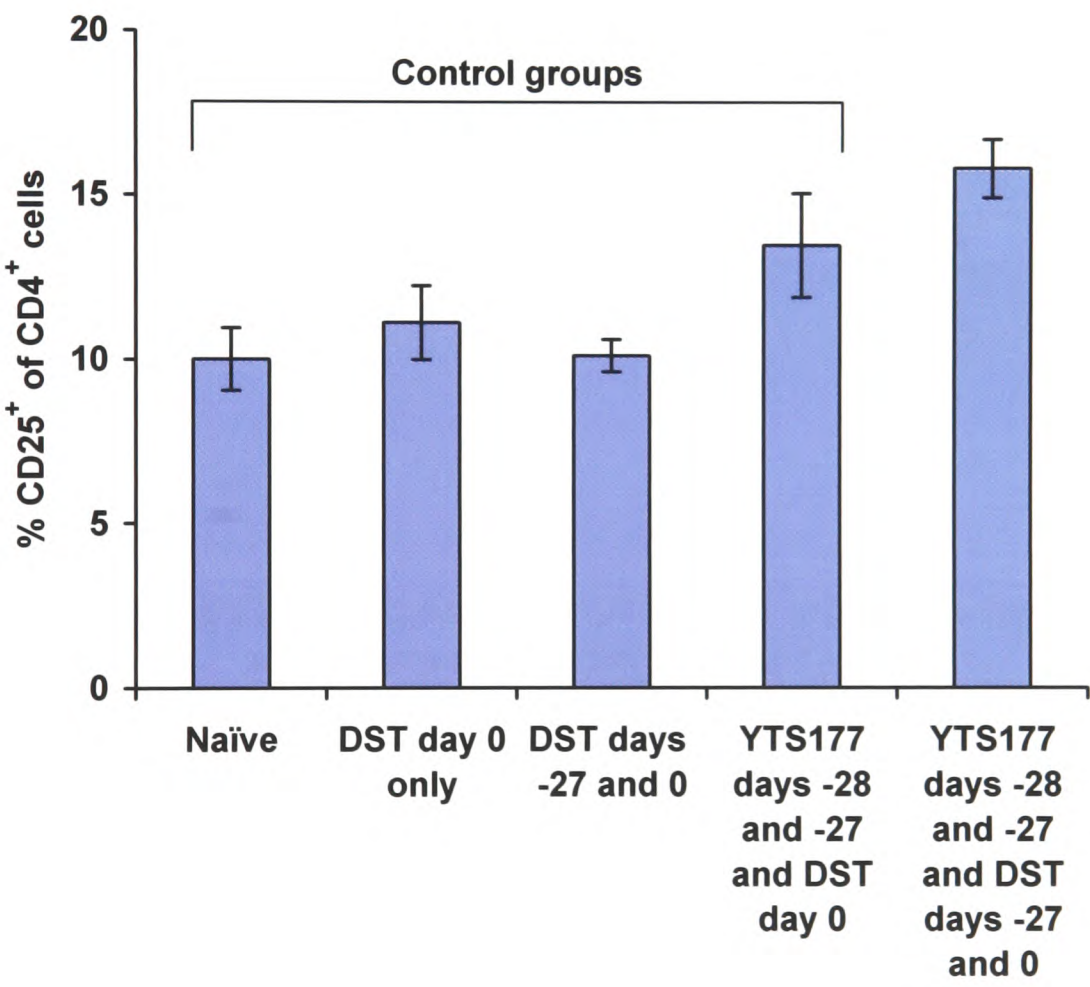
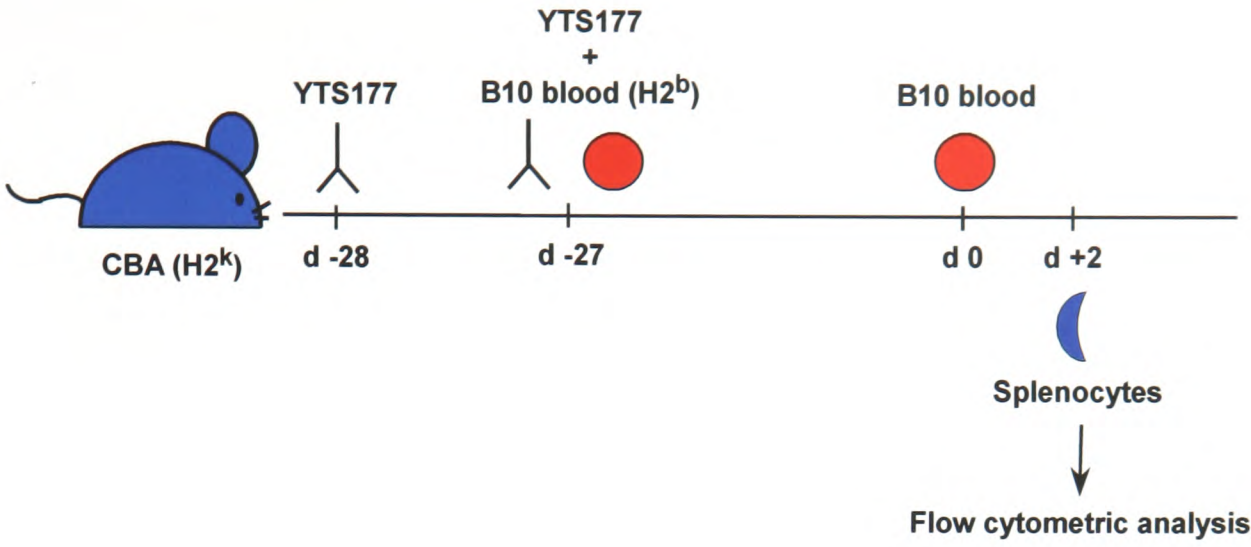


Figure 3.12. CD25 expression by CD4⁺ cells following 177/DST pre-treatment and DST re-challenge
CBA mice were pre-treated with the indicated combinations of YTS177 and B10 DST and at day +2 the spleens were harvested and stained with anti-CD4-PerCP and biotinylated anti-CD25 followed by SA-APC prior to flow cytometric analysis. The proportion of CD4⁺ cells expressing CD25 was measured and the results shown as mean ± standard deviation. n = 4 in each group. Data are representative of three independent experiments.

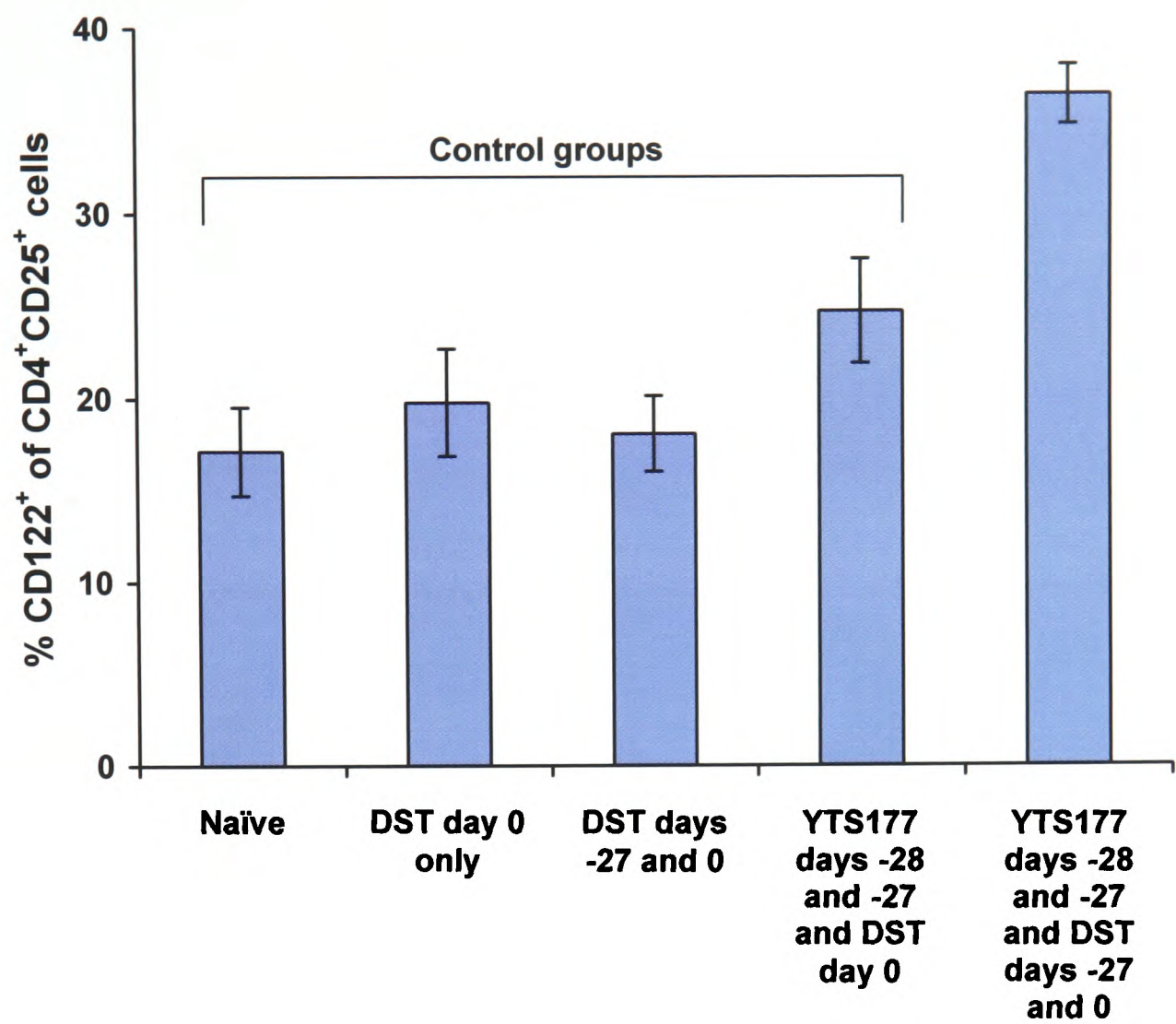


Figure 3.13. CD122 expression by CD4⁺CD25⁺ cells following 177/DST pre-treatment and DST re-challenge
CBA mice were pre-treated with the indicated combinations of YTS177 and B10 DST and at day +2 the spleens were harvested and stained with anti-CD4-PerCP, anti-CD122-FITC, and biotinylated anti-CD25 followed by SA-APC prior to flow cytometric analysis. The proportion of CD4⁺CD25⁺ cells expressing CD122 was measured and the results shown as mean ± standard deviation. n = 4 in each group. Data are representative of three independent experiments.

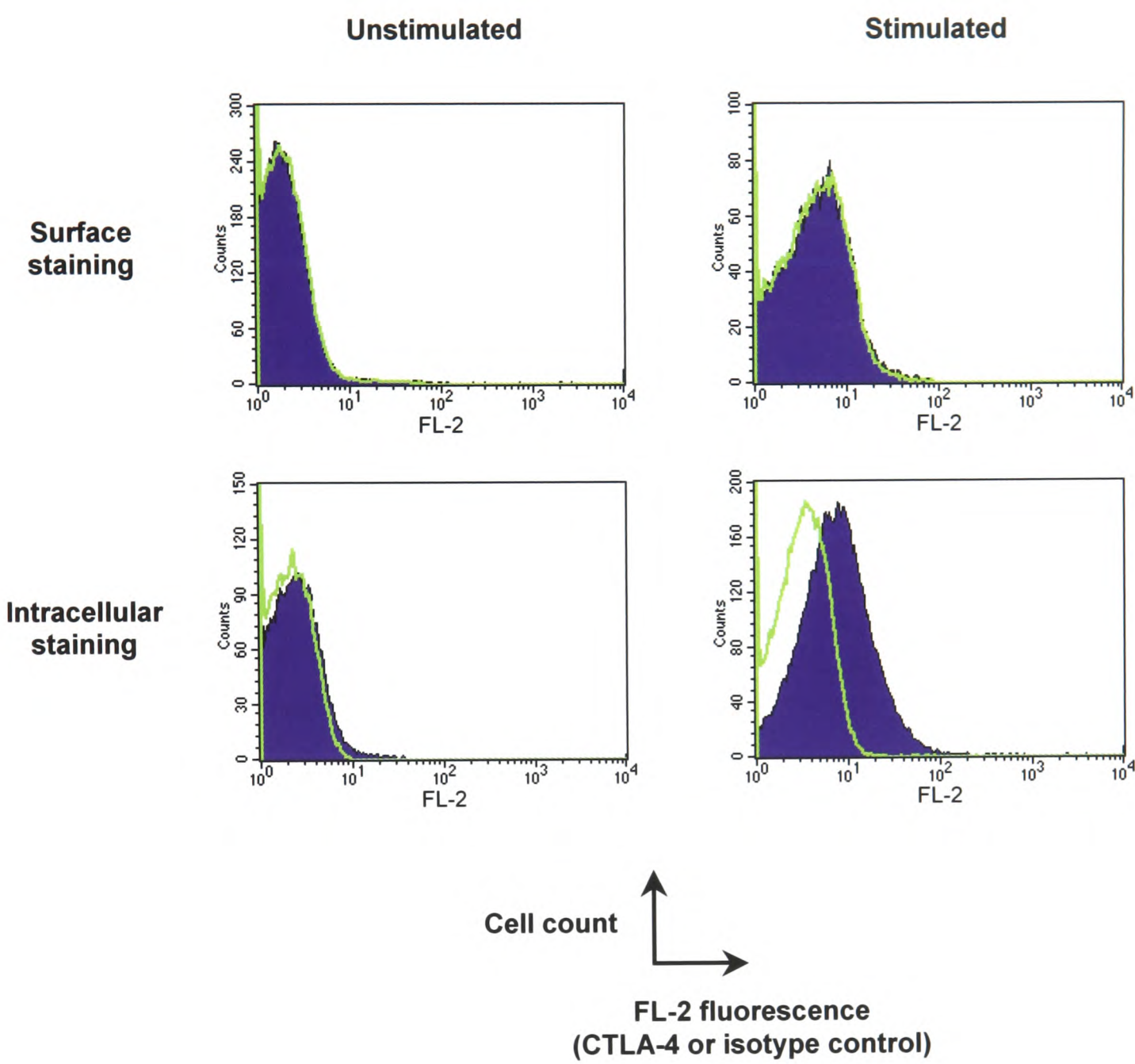


Figure 3.14. Staining of unstimulated and stimulated splenocytes for surface and intracellular CTLA-4 expression

CBA splenocytes were isolated and either analysed immediately or following stimulation *in vitro* with PMA and ionomycin. Surface or intracellular CTLA-4 expression was then measured by flow cytometric analysis after staining with anti-CTLA-4-PE (solid plots) or Armenian hamster IgG group 1, κ isotype control antibody (green outline plots). Data are representative of three independent experiments.

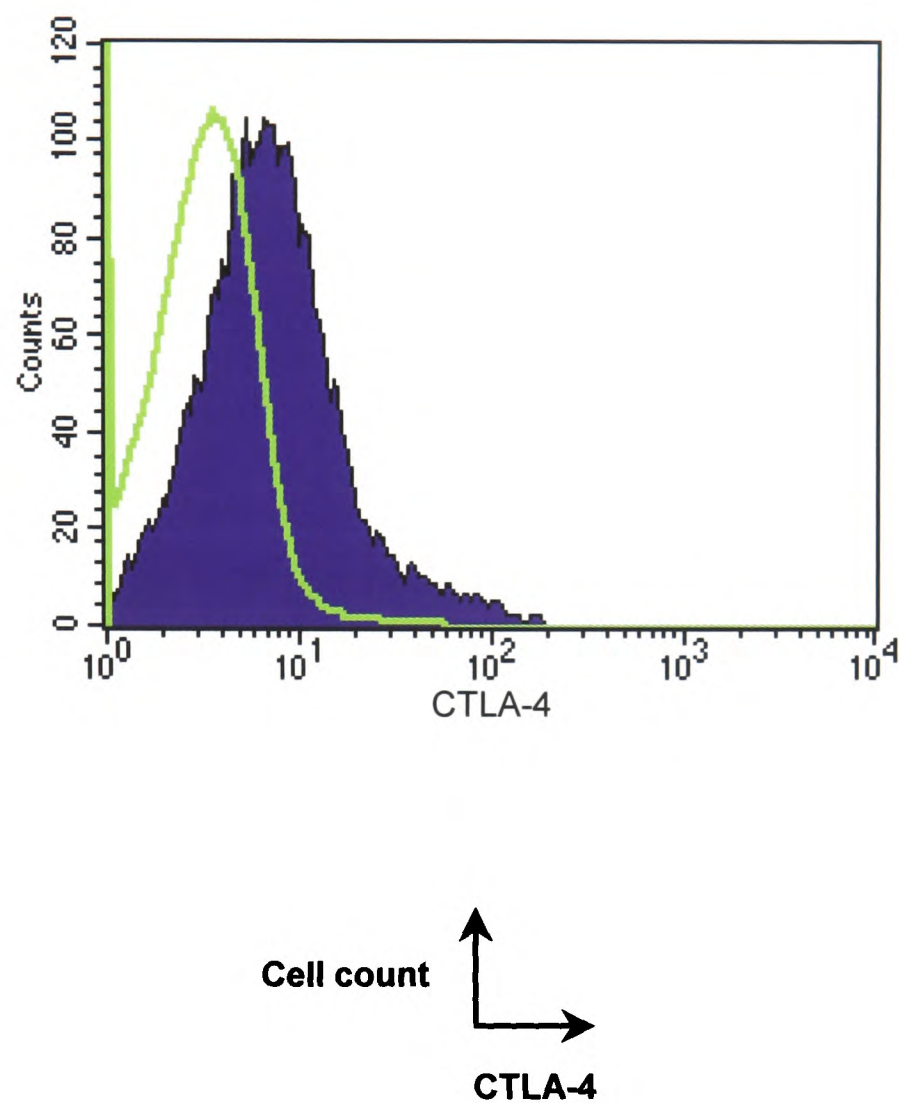


Figure 3.15. $CD4^+CD25^+$ cells show constitutive expression of intracellular CTLA-4

CBA splenocytes were stained for surface expression of CD4 and CD25 using anti-CD4-PerCP and biotinylated anti-CD25 followed by SA-APC. They were then permeabilised and stained with anti-CTLA-4-PE. Flow cytometric analysis was then performed. Solid plot shows expression of intracellular CTLA-4 by $CD4^+CD25^+$ cells, and outline plot expression by $CD4^+CD25^-$ cells. Data are representative of three independent experiments.

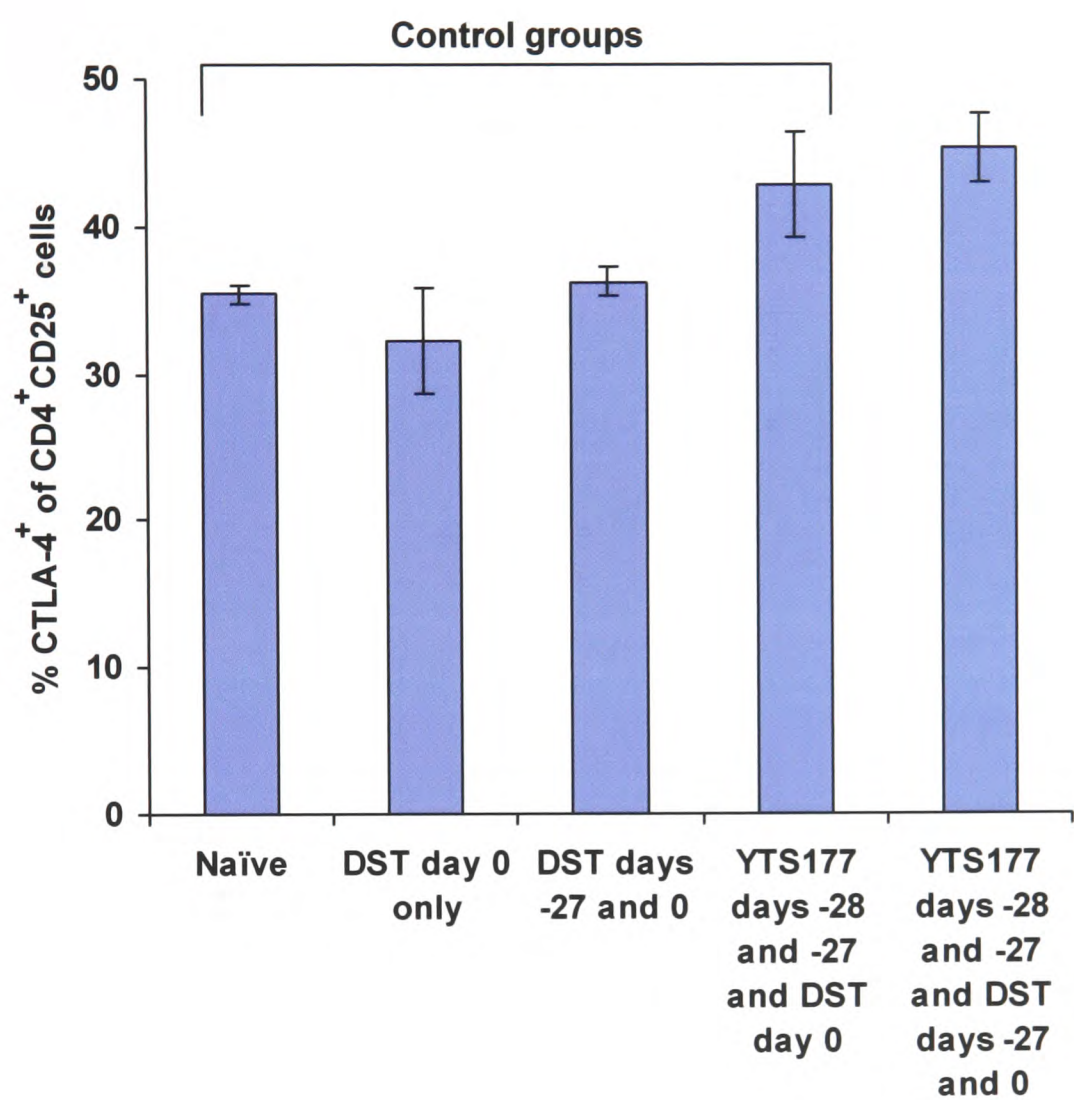


Figure 3.16. Intracellular CTLA-4 expression by CD4⁺CD25⁺ cells following 177/DST pre-treatment and DST re-challenge
CBA mice were pre-treated with the indicated combinations of YTS177 and B10 DST and at day +2 the spleens were harvested and stained with anti-CD4-PerCP and biotinylated anti-CD25 followed by SA-APC. The cells were then permeabilised and intracellular staining with anti-CTLA-4-PE performed prior to flow cytometric analysis. The proportion of CD4⁺CD25⁺ cells expressing CTLA-4 was measured and the results shown as mean ± standard deviation. n = 4 in each group. Data are representative of three independent experiments.

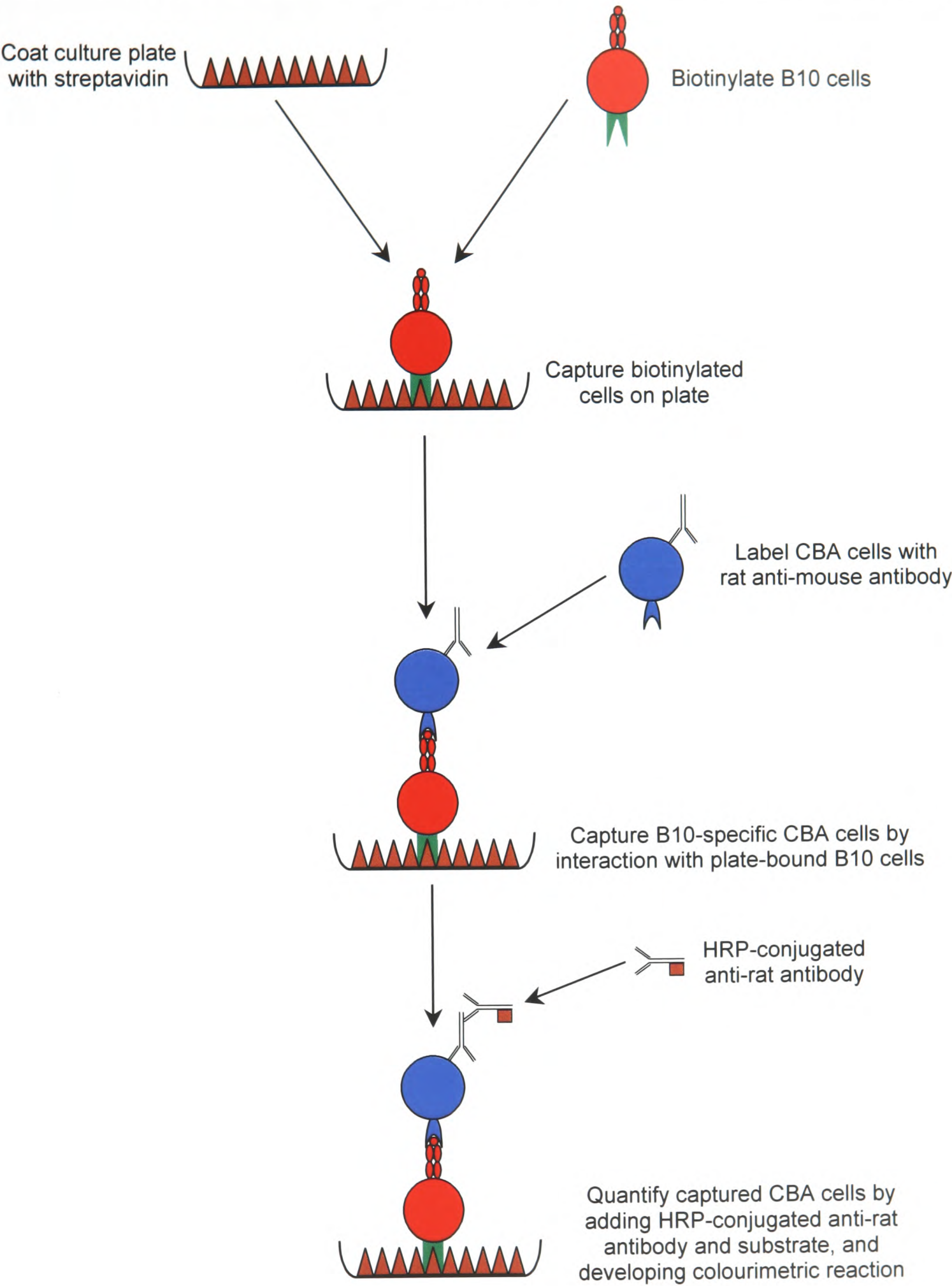


Figure 3.17. Proposed protocol for quantification of B10-specific CBA cells
B10 cells are biotinylated and then captured onto streptavidin-coated 96 well plates. CBA cells are coated with rat anti-mouse antibody and then added to the wells. The plates are then incubated to allow binding of B10-specific CBA cells to the captured B10 cells via TCR-MHC / peptide interactions. The plates are then washed to remove unbound CBA cells and the number of bound CBA cells quantified by the addition of HRP-conjugated anti-rat antibody followed by development of a colourimetric reaction by the addition of the substrate ABTS.

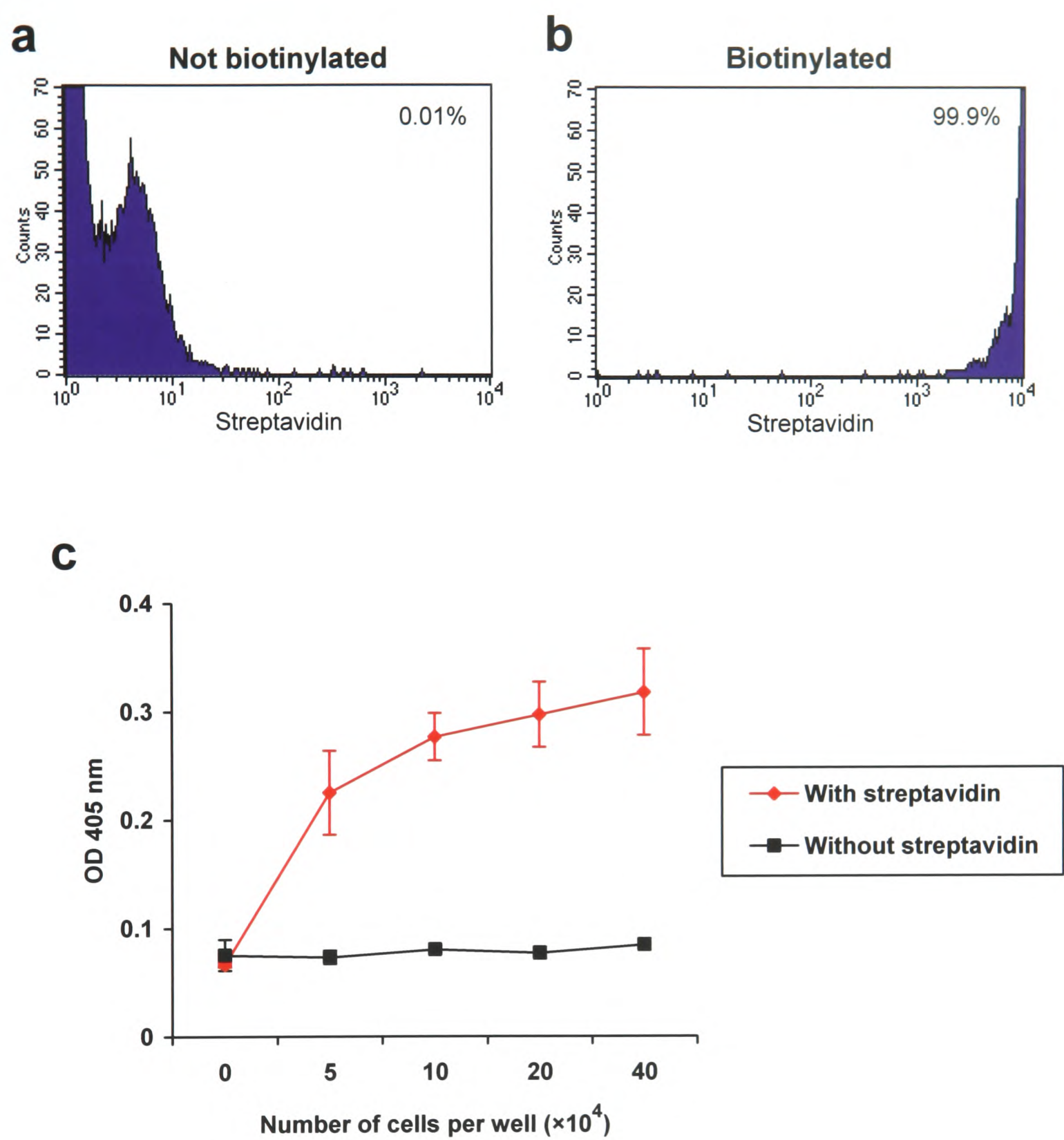


Figure 3.18. Capture and detection of biotinylated B10 splenocytes
(a) Unmodified or (b) biotinylated B10 splenocytes were incubated with SA-APC and analysed by flow cytometry. Percentages represents proportion of cells positive for SA-APC. (c) Biotinylated B10 splenocytes were added at the numbers shown to uncoated or streptavidin-coated 96 well plates. The plates were then incubated with anti-mouse CD45 followed by HRP-conjugated mouse anti-rat Ig and then ABTS substrate and the optical density at 405 nm was measured. Results are presented as the mean of triplicate wells $\pm$ standard deviation and are representative of two independent experiments.

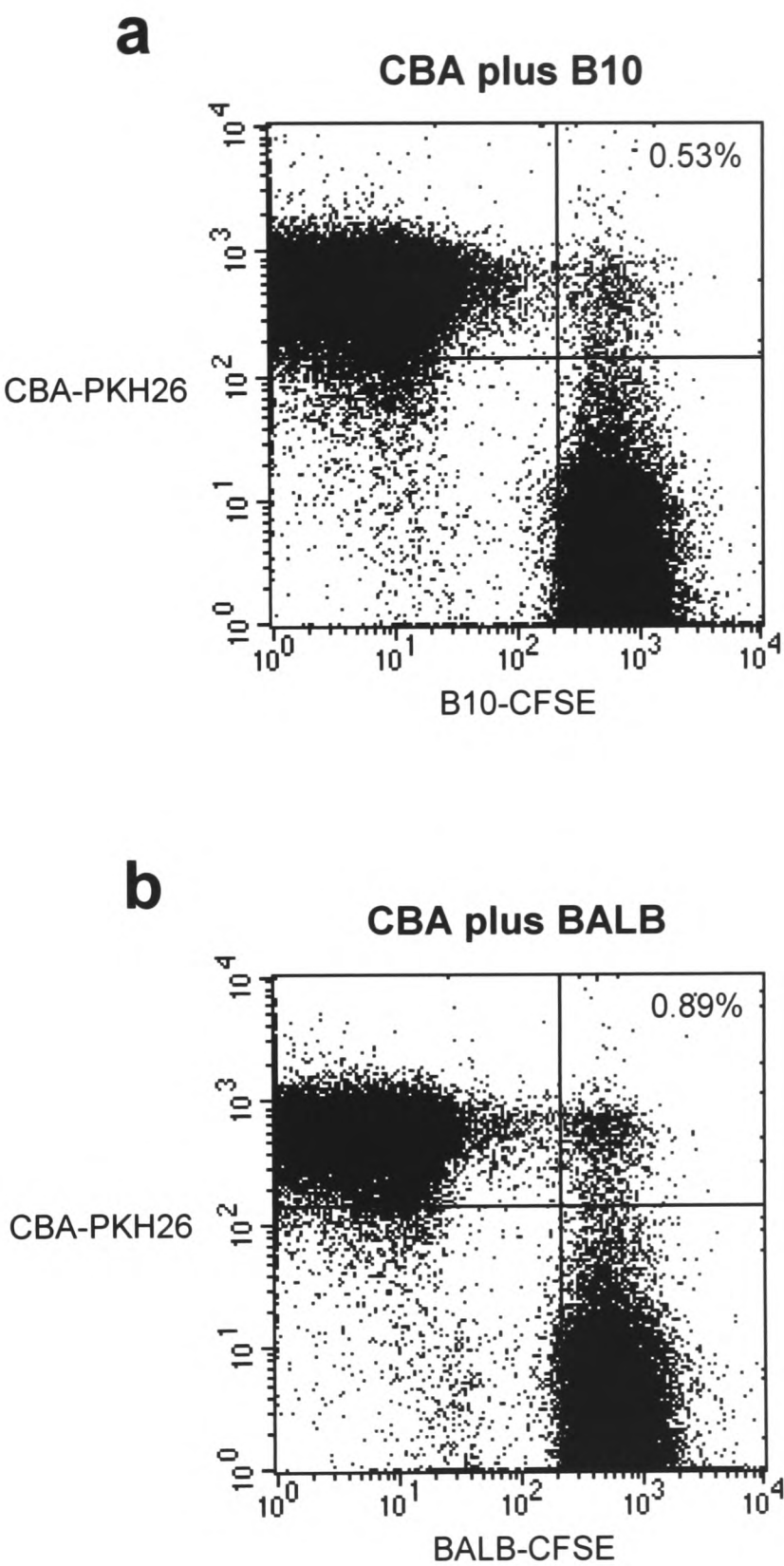


Figure 3.19. Analysis of cell conjugate formation

CBA splenocytes were stained with PKH26 and B10 and BALB splenocytes with CFSE (0.1 μ M). Stained CBA cells were then mixed with stained B10 (a) or BALB (b) cells and incubated for 20 minutes at 37°C prior to flow cytometric analysis. Percentages indicate proportion of double positive events. Data are representative of two independent experiments.

CHAPTER 4

MODELLING OF REGULATION *IN VITRO*

4.1 Introduction

In Chapter 3 it was demonstrated that pre-treatment of mice with DST under the cover of YTS177 leads to the generation of CD4⁺CD25⁺ Treg with the capacity to regulate allogeneic responses, as shown by their ability to prevent the rejection of skin allografts mediated by CD4⁺CD45RB^{high} cells *in vivo* in an adoptive transfer system. This adoptive transfer system provides a robust model to test the ability of putative Treg to control alloresponses *in vivo* and will be used again in later chapters. However, this approach has the major disadvantage that regulation is demonstrated by the long term survival of skin allografts, which takes several months to achieve in any single experiment. A system that could provide a quicker readout would therefore have a major advantage; this is particularly true if assays of Treg activity are to be used in clinical practice, since a system that takes several months to provide a result would be of little practical use.

4.1.1 Regulatory properties of CD4⁺CD25⁺ cells in vitro

Published studies examining the properties of CD4⁺CD25⁺ cells from mice [276, 277, 282] and humans [283-287] have shown that these cells do not proliferate when stimulated via their T cell receptors *in vitro*, but that this non-proliferative state may be overcome by the addition of exogenous IL-2. However, it is also well documented that CD4⁺CD25⁺ cells are able to regulate the proliferation of CD4⁺CD25⁻ responder cell populations *in vitro* in mouse [217, 224, 276, 277, 282, 288, 289, 293] and human [283-287, 301, 302, 304, 305] models. Given that CD4⁺CD25⁺ cells do not normally proliferate, regulation of CD4⁺CD25⁻ responder cell proliferation in these studies was assessed by measuring total ³H-thymidine incorporation in culture wells containing responder cells with or without putative CD4⁺CD25⁺ Treg. Although the human

studies have shown that naturally-occurring $CD4^+CD25^+$ cells can regulate the proliferation of $CD4^+CD25^-$ cells in response to stimulation with polyclonal agents [283, 284, 286, 287, 301, 302, 305] or with defined antigens (including alloantigen) [284-286, 304], studies examining the regulatory properties *in vitro* of naturally-occurring mouse $CD4^+CD25^+$ cells have predominantly employed systems using polyclonal stimulation [217, 224, 276, 282, 288]. The mouse models demonstrating regulation of responses driven by defined antigen have used $CD4^+CD25^+$ cell lines or TCR transgenic cells rather than bulk populations from normal mice [217, 289]. There is thus, to date, a dearth of data regarding the ability of *ex vivo* mouse $CD4^+CD25^+$ cells to regulate responses of responder cell populations to defined antigens *in vivo*.

4.1.2 Aims of this chapter

The overall aim of this chapter was to develop an *in vitro* assay system that would model the regulation by $CD4^+CD25^+$ cells of $CD4^+CD45RB^{high}$ -mediated skin allograft rejection seen in the *in vivo* adoptive transfer system, but that would also provide a basis for an assay system that may be useful in clinical practice. Given that, as described above, $CD4^+CD25^+$ cells have been demonstrated to have the capacity to regulate the proliferative response of responder cell populations *in vitro*, a logical starting point seemed to be to investigate whether a simple proliferation assay of this sort would meet this objective. However, in order to reflect the adoptive transfer system accurately, this proliferation assay would need to differ from published studies in that it would need to demonstrate regulation by *ex vivo* mouse $CD4^+CD25^+$ T cells (rather than cell lines) of a $CD4^+CD45RB^{high}$ (rather than $CD4^+CD25^-$) responder cell population in response to alloantigen-driven (rather than polyclonal) stimulation.

4.2 Results

4.2.1 Kinetics of proliferation of $CD4^+CD45RB^{high}$ cells in response to anti-CD3 stimulation *in vitro*

Since the goal of this chapter was to develop an *in vitro* system to model the regulation by $CD4^+CD25^+$ cells of $CD4^+CD45RB^{high}$ cell activity *in vivo*, we first wished to optimise the conditions for proliferation *in vitro* of the responder $CD4^+CD45RB^{high}$ population. The majority of studies investigating the regulatory properties of mouse $CD4^+CD25^+$ cells *in vitro* have used systems based on responses to polyclonal stimulation such as that provided by anti-CD3 antibody [217, 224, 277, 282, 288, 376]. As a first step to developing a model of regulation *in vitro* we therefore wished to set up a similar system investigating regulation of responses driven by anti-CD3 antibody. $CD4^+CD45RB^{high}$ cells were thus stimulated *in vitro* with soluble anti-CD3 antibody in the presence of irradiated syngeneic spleen cells, and the effects of varying the number of responder cells per well, concentration of anti-CD3 antibody, and time in culture were examined (Figure 4.1). Optimal proliferation was achieved by culturing 2×10^5 responder cells per well in the presence of anti-CD3 antibody for 4 days; the three concentrations of anti-CD3 antibody used yielded similar proliferative responses. Subsequent experiments for assays assessing proliferation of anti-CD3-stimulated cultures by measurement of 3H -thymidine incorporation were therefore performed using these parameters and anti-CD3 antibody at a concentration of 2 $\mu g/ml$.

4.2.2 The non-proliferative state of CD4⁺CD25⁺ cells in vitro is reversed by exogenous IL-2

We next wished to examine whether, in common with the studies cited above, *ex vivo* CD4⁺CD25⁺ cells from normal mice in our laboratory would exhibit a lack of proliferation when stimulated *in vitro* and, if so, whether this could be overcome by the addition of exogenous IL-2. As shown in Figure 4.2, in contrast to CD4⁺CD45RB^{high} and CD4⁺CD25⁻ cells, the CD4⁺CD25⁺ population did not proliferate in response to stimulation with anti-CD3 antibody. However, while the addition of exogenous IL-2 had no effect on proliferation of CD4⁺CD45RB^{high} or CD4⁺CD25⁻ cells, it restored proliferation of the CD4⁺CD25⁺ population to the same level as that displayed by the reciprocal CD4⁺CD25⁻ population.

4.2.3 CD4⁺CD25⁺ cells display regulatory capacity in vitro

Having established that CD4⁺CD45RB^{high} and CD4⁺CD25⁻ cells proliferate *in vitro* in response to anti-CD3 stimulation but that CD4⁺CD25⁺ cells do not, we next wished to determine whether CD4⁺CD25⁺ cells could regulate the proliferation of responder cell populations. As described above, published studies have shown that CD4⁺CD25⁺ cells can suppress responses of CD4⁺CD25⁻ cells *in vitro*; CD4⁺CD25⁻ cells were thus initially used as the responder population to determine whether these findings could be replicated. Figure 4.3 demonstrates that addition of CD4⁺CD25⁺ cells did indeed regulate the anti-CD3-driven proliferation of CD4⁺CD25⁻ responder cells in a dose-dependent manner. Suppression was evident even when using a regulator : responder ratio of 0.125:1, and reached a maximum level of 75% suppression at a ratio of 1:1.

In the *in vivo* skin allograft model described in Chapter 3, the target population for regulation consists of $CD4^+CD45RB^{high}$ rather than $CD4^+CD25^-$ cells. This thus raised the question of whether $CD4^+CD25^+$ cells possess the capacity to regulate the activity of the former as well as the latter population *in vitro*. Figure 4.4 demonstrates that $CD4^+CD25^+$ cells were also able to regulate the proliferation of $CD4^+CD45RB^{high}$ responder cells. As in the situation where $CD4^+CD25^-$ cells were used as responders, the use of a 1:1 regulator : responder ratio led to a 75% suppression of proliferation. Given that the aim of this chapter was to develop an *in vitro* assay to model regulation seen in the *in vivo* skin allograft system and the fact that we have previously demonstrated that, in this *in vivo* system, $CD4^+CD25^+$ cells from mice pre-treated with 177/DST are able to regulate the rejection of skin allografts whereas equal numbers of $CD4^+CD25^+$ cells from naïve animals are not [441], we wondered whether $CD4^+CD25^+$ cells from these two sources would display differences in their potential to regulate anti-CD3-driven responses *in vitro*. However, as shown in Figures 4.5 and 4.6, regulation by cells from 177/DST pre-treated animals followed the same dose-response curve as that displayed by cells from naïve animals, once again achieving maximal regulation of 75% at a regulator : responder ratio of 1:1.

4.2.4 $CD4^+CD25^+CD45RB^{low}$ cells appear not to display regulatory capacity in vitro

Although the anti-CD3-stimulated system does not provide an assay capable of demonstrating the effect of 177/DST pre-treatment on regulatory cell function (since regulation by cells from naïve animals is equivalent to that seen by cells from animals pre-treated with 177/DST), it does allow specific aspects of regulatory cell activity *in vitro* to be examined. As discussed in Chapter 3, there has been controversy over the

question of whether $CD4^+CD25^-CD45RB^{low}$ cells possess regulatory capacity [218, 223, 227, 279, 318]. In order to determine whether this population is able to regulate *in vitro*, $CD4^+CD45RB^{high}$ and / or $CD4^+CD25^-CD45RB^{low}$ cells were stimulated with anti-CD3 antibody (Figure 4.7). $CD4^+CD25^-CD45RB^{low}$ cells proliferated to a similar extent to $CD4^+CD45RB^{high}$ cells, and mixing these two populations did not appear to reveal any suppression. Although consistent with the hypothesis that $CD4^+CD25^-CD45RB^{low}$ cells do not possess regulatory potential, these data do not exclude the possibility that, in the co-cultures, suppression of the $CD4^+CD45RB^{high}$ population was occurring and that the observed proliferation originated solely from the $CD4^+CD25^-CD45RB^{low}$ cells. The issue of whether regulation attributed to $CD4^+CD45RB^{low}$ cells in fact lies within the $CD25^+$ subpopulation will be addressed later in this chapter.

4.2.5 The regulatory capacity of $CD4^+CD25^+$ cells in vitro is contained within the $CD25^{high}$ fraction

While analysing the expression of CD25 we observed that $CD4^+$ cells do not simply segregate into $CD25^-$ and $CD25^+$ fractions, but that there is also a population expressing intermediate levels of CD25 (Figure 4.8). This is in accordance with previous observations both in the mouse [217] and in humans [283]. In both these studies regulatory capacity was displayed by $CD25^{high}$ but not $CD25^{intermediate}$ cells. We therefore sought to determine whether differential regulatory capacity of these two populations could be demonstrated *in vitro*. Since $CD4^+CD25^{intermediate}$ cells form a relatively small population, it was not possible to purify these cells in sufficient numbers to perform a suppression assay. An alternative approach was therefore employed. We reasoned that if whole $CD4^+$ cells from normal mice contain

subpopulations of regulatory cells, then removal of these Treg prior to polyclonal stimulation should lead to augmented proliferation of the remaining cells. Whole CD4⁺ cells or CD4⁺ cells depleted of the CD25^{intermediate} and / or CD25^{high} fractions were therefore purified and then stimulated *in vitro* with anti-CD3 antibody (Figure 4.9). Depletion of CD25^{high} cells led to an increase in proliferation of the remaining population ($P = 0.001$), whereas removal of the CD25^{intermediate} fraction resulted in a diminution ($P = 0.008$). These data are consistent with the suggestion by Kuniyasu *et al.* that Treg lie within the CD25^{high} population, whereas the CD25^{intermediate} fraction consists of activated cells [217].

4.2.6 CD4⁺CD25⁺ cells purified on the basis of lack of CD122 expression display regulatory capacity in vitro

It has been demonstrated that human CD4⁺CD25⁺ Treg express CD122, the β chain of the IL-2 receptor [283, 285, 286, 301, 306, 307]; however, it has not yet been established whether this is the case in the mouse. The observations in Chapter 3 that a significant proportion of CD4⁺CD25⁺ cells from naïve mice express CD122 constitutively (Figure 3.2) and that this proportion is higher in mice pre-treated with 177/DST followed by DST re-challenge (Figure 3.13) prompted us to ask whether the regulatory properties of CD4⁺CD25⁺ cells show any segregation to the CD122⁺ or CD122⁻ fractions. The ability of these subpopulations to regulate the proliferation of CD4⁺CD45RB^{high} cells in response to anti-CD3 stimulation was therefore tested (Figure 4.10). Neither CD4⁺CD25⁺CD122⁺ nor CD4⁺CD25⁺CD122⁻ cells proliferated in response to anti-CD3 stimulation, and while CD4⁺CD25⁺CD122⁻ cells suppressed the proliferation of CD4⁺CD45RB^{high} responders the CD4⁺CD25⁺CD122⁺ fraction did not. However, there are a number of factors that may obscure any regulatory capacity

of the CD122⁺ fraction in this setting. Firstly, the antibody used for CD122 sorting (TM-β1) blocks the binding of IL-2 to its receptor [448], and may thus potentially interfere with the regulatory function of CD122⁺ cells. The second potentially confounding factor is that, as demonstrated in Figure 4.11, the CD122⁺ cells lie predominantly within the CD25^{intermediate} population, and thus when cells are sorted the CD4⁺CD25⁺CD122⁺ fraction contains a much higher proportion (40%) of CD25^{intermediate} cells (which, as shown in Figure 4.9, are unable to regulate) than is present within the CD122⁻ fraction (20%). Thus, although these data clearly demonstrate that CD122⁻ cells possess regulatory properties *in vitro*, no conclusions may be drawn regarding the CD122⁺ fraction.

4.2.7 CTLA-4 blockade partially abrogates regulation by CD4⁺CD25⁺ cells in vitro

As discussed in Chapter 3, there have been conflicting reports of the effect of CTLA-4 blockade on the function of CD4⁺CD25⁺ Treg [218, 222, 240, 241, 282, 283, 286, 288, 293, 304, 309, 349]. In models examining the function of mouse CD4⁺CD25⁺ Treg *in vitro*, neither Thornton and Shevach [282] nor Chai *et al.* [293] observed any abrogation of regulation in cultures to which anti-CTLA-4 antibody was added. In contrast, Takahashi and colleagues observed that addition of anti-CTLA-4 antibody in a similar situation led to a 50% abrogation of regulation [288]. A potential explanation for the disparity between these different studies is that the latter two used a much higher dose of intact anti-CTLA-4 antibody (100 µg/ml) than the former two (10-30 µg/ml). We therefore wished to ascertain whether the addition of anti-CTLA-4 antibody at this high dose would have any effect on *in vitro* regulation in our hands. As shown in Figure 4.12, neither anti-CTLA-4 antibody nor hamster Ig control

antibody had any effect on the proliferation of $CD4^+CD45RB^{high}$ or $CD4^+CD25^+$ cells alone. As demonstrated previously, suppression was observed when these two populations were mixed. However, the addition of anti-CTLA-4 antibody led to restoration of proliferation to 50% of the level observed for $CD4^+CD45RB^{high}$ cells in the absence of the $CD4^+CD25^+$ population (precisely the same level observed by Takahashi *et al.*), whereas normal regulation occurred when hamster Ig control antibody was added. This suggests that regulation is, at least in part, CTLA-4-dependent.

4.2.8 Addition of $CD4^+CD25^+$ cells to allogeneic MLR leads to augmented total cell proliferation

The data presented so far in this chapter have examined the regulatory properties of $CD4^+CD25^+$ cells *in vitro* in responses to polyclonal stimulation by anti-CD3 antibody. The subsequent studies in this chapter will focus more closely on attempts to develop an *in vitro* assay that would model the regulation *in vivo* observed in the skin allograft system described in Chapter 3, and, specifically, to ask whether $CD4^+CD25^+$ cells can regulate the proliferation of $CD4^+CD45RB^{high}$ cells in response to allogeneic stimulation.

We first examined the effect of $CD4^+CD25^+$ cells from naïve animals (Figure 4.13). As with stimulation with anti-CD3 antibody, $CD4^+CD45RB^{high}$ and $CD4^+CD25^+$ cells proliferated in response to allogeneic stimulation whereas $CD4^+CD25^+$ cells did not. However, surprisingly, addition of $CD4^+CD25^+$ cells to $CD4^+CD45RB^{high}$ cultures not only failed to reveal any suppression, but conversely lead to a 3-fold augmentation in total proliferation. In order to determine whether this was a unique feature of $CD4^+CD25^+$ cells from naïve animals, the experiment was repeated using

CD4⁺CD25⁺ cells from mice pre-treated with 177/B10 DST. Cultures were stimulated with splenocytes from B10 (donor strain) and BALB (third party) mice. In addition, (CBA×B6)F₁ stimulators were also used since, in previous study from our laboratory, it was shown that *in vitro* regulation of allostimulated responses by Treg from mice with long term surviving cardiac allografts was only observed when cultures were stimulated with (recipient × donor)F₁ cells (providing antigen presentation via both the direct and indirect pathways) and not when donor stimulators alone were used (presentation via direct pathway only) [236]. However, as shown in Figure 4.14, once again addition of CD4⁺CD25⁺ cells failed to reveal any regulation and instead led to augmentation of the proliferative response to all three stimulator cell types.

The inability to demonstrate a difference between the *in vitro* regulatory capacity of CD4⁺CD25⁺ cells from naïve mice (Figure 4.4) and mice pre-treated with 177/DST (Figure 4.5), combined with the findings presented in Chapter 3 that the phenotypic changes seen in CD4⁺CD25⁺ cells were accentuated following alloantigen re-exposure, led us to hypothesise that regulation of alloantigen-driven responses *in vitro* would only be revealed following a similar antigen re-exposure. The regulatory ability of cells from mice pre-treated with 177/B10 DST followed by DST re-challenge was therefore tested (Figure 4.15). However, once again an augmentation of proliferation was seen when CD4⁺CD25⁺ cells were added to CD4⁺CD45RB^{high} cells.

The fact that additional cell proliferation was observed when CD4⁺CD25⁺ populations from different sources were added to cultures containing CD4⁺CD45RB^{high} cells and allogeneic stimulators was disappointing. However, we speculated that at least part of this augmentation may be due to proliferation of the CD4⁺CD25⁺ cells themselves, perhaps driven by IL-2 produced by the

CD4⁺CD45RB^{high} responders. In order to address this question the fluorescent label CFSE was used [449, 450]. This binds to cytoplasmic proteins and is detectable by flow cytometry. In addition it has the useful property that when labelled cells undergo division, the CFSE is divided equally between the two daughter cells which therefore exhibit half the fluorescence intensity of the parent cell, thereby allowing analysis of cell division (Figure 4.16). CD4⁺CD25⁺ cells were thus labelled with CFSE, stimulated *in vitro* with allogeneic cells in the presence or absence of CD4⁺CD45RB^{high} cells, and their division history then analysed. As shown in Figure 4.17, CD4⁺CD25⁺ cells did not divide when cultured with allogeneic stimulators alone (Figure 4.17a), but did divide when CD4⁺CD45RB^{high} cells were added (Figure 4.17b), confirming our original speculation.

Given the observations described above (Section 4.2.2) by ourselves and others [276, 277, 282-287], one obvious possible reason that CD4⁺CD25⁺ cells only proliferate when in the presence of stimulators and CD4⁺CD45RB^{high} cells is that the CD4⁺CD45RB^{high} population produces IL-2 in sufficient quantity to overcome the non-proliferative state of the CD4⁺CD25⁺ cells. Attempts were made to investigate this by measuring the concentration of IL-2 in supernatants from culture wells containing CD4⁺CD45RB^{high} cells plus allogeneic stimulators with or without CD4⁺CD25⁺ cells by means of ELISA. However, the optical density readings obtained were below the detection limit of the assay. There are several potential reasons for this. Firstly, it is possible that the assay has insufficient sensitivity to detect IL-2 at the levels present in these cultures. Secondly, it is possible that the majority of IL-2 produced in these cultures had already been consumed by cells and so was not measurable in the supernatant. Finally, some soluble factor other than IL-2 may have been responsible for causing the proliferation of the CD4⁺CD25⁺ cells.

An alternative approach to investigate this phenomenon was therefore employed (Figure 4.18). $CD4^+CD45RB^{high}$ and / or $CD4^+CD25^+$ cells were cultured in the presence of allogeneic stimulators and then, 4 days later, the supernatants from these cultures were harvested and transferred to secondary cultures containing fresh $CD4^+CD25^+$ cells alone plus allogeneic stimulators; proliferation of the secondary cultures was then measured. Supernatants from primary cultures containing $CD4^+CD25^+$ and stimulator cells alone were unable to overcome the non-proliferative state of the $CD4^+CD25^+$ cells in the secondary cultures. However, transfer of supernatants from wells containing $CD4^+CD45RB^{high}$ and stimulator cells did reverse this non-proliferative state. A lesser effect was observed with supernatants from cultures containing $CD4^+CD25^+$ cells in addition to $CD4^+CD45RB^{high}$ and stimulator populations. These data support the hypothesis that a soluble factor is produced in co-cultures of $CD4^+CD45RB^{high}$ and stimulator cells that is able to drive the proliferation of $CD4^+CD25^+$ cells. The lower potency of supernatants from cultures containing $CD4^+CD45RB^{high}$, $CD4^+CD25^+$, and stimulator cells could be explained by the likelihood that much of the soluble factor(s) had already been consumed during the proliferation of the $CD4^+CD25^+$ cells in the primary culture. An additional possible explanation is that the presence of the $CD4^+CD25^+$ cells in the primary culture led to inhibition of growth factor production by the $CD4^+CD45RB^{high}$ cells.

Although these data do not identify the factor responsible for driving $CD4^+CD25^+$ cell proliferation, IL-2 remains a likely candidate. In these cultures, the IL-2 is likely to have originated from the $CD4^+CD45RB^{high}$ cells, though it is also possible that some production may have occurred from T cells within the irradiated allogeneic spleen cell population used as stimulators. However, this latter explanation seems unlikely. Firstly, previous work has shown that the dose of irradiation used

(3600 rad) causes a substantial, albeit not complete, reduction in the ability of leukocytes to secrete IL-2 [451]. Secondly, theoretically, $CD4^+CD25^+$ and $CD4^+CD45RB^{high}$ cells should be as capable of stimulating IL-2 production by allogeneic T cells within the stimulator population, but in these experiments the presence of the latter population was required for $CD4^+CD25^+$ cell proliferation. We wished to assess the first point, the effect of irradiation on IL-2 secretion. As already discussed, attempts to detect IL-2 by ELISA in allogeneic MLR were unsuccessful. However, anecdotal observations in our laboratory have shown that IL-2 can be detected by ELISA when cells from DES mice (which possess a transgenic TCR specific for K^b) are stimulated *in vitro*. Unmodified or irradiated DES cells were therefore stimulated with anti-CD3 and anti-CD28 antibodies and then IL-2 production was measured. As shown in Figure 4.19, non-irradiated cells produce large quantities of IL-2, but this was prevented by cell irradiation. This result would support the view that any IL-2 produced in co-cultures of $CD4^+CD45RB^{high}$, $CD4^+CD25^+$, and allogeneic stimulator cells originated from the $CD4^+CD45RB^{high}$ T cells rather than the stimulator population.

4.2.9 Irradiated $CD4^+CD25^+$ cells do not display regulatory capacity in vitro

Given the finding that $CD4^+CD25^+$ cells proliferate in the presence of $CD4^+CD45RB^{high}$ and allogeneic stimulator cells, it is possible that regulation of $CD4^+CD45RB^{high}$ proliferation is occurring in these co-culture experiments but that it is masked as 3H -thymidine-based assays are unable to distinguish between proliferation of individual cell populations. This problem could potentially be overcome if irradiation of the $CD4^+CD25^+$ population prevented their proliferation but still allowed them to perform their regulatory function. $CD4^+CD45RB^{high}$ cells were

therefore stimulated with B10 splenocytes in the presence of irradiated $CD4^+$, $CD4^+CD25^-$, or $CD4^+CD25^+$ cells from mice pre-treated with 177/B10 DST (Figure 4.20a). However, although no augmentation of $CD4^+CD45RB^{high}$ proliferation was seen when the various irradiated cell fractions from the pre-treated animals were added, there was also no evidence of suppression. In order to test the effect of irradiation in a situation in which it has been demonstrated in this chapter that regulation can be detected (Section 4.2.3), the effect of irradiation on the ability of $CD4^+CD25^+$ cell to regulate the proliferation of $CD4^+CD45RB^{high}$ cells in response to stimulation with anti-CD3 antibody was examined (Figure 4.20b). However, irradiation of the $CD4^+CD25^+$ population completely prevented their ability to regulate the proliferation of the $CD4^+CD45RB^{high}$ responder population. These data support the view that regulation is not a passive process, but an active one requiring, for example, the synthesis of cytokines or other proteins. This would be consistent with previous observations that the suppressive activity of lymphocyte populations can be prevented by their pre-treatment with inhibitors of DNA synthesis such as mitomycin [452], or of protein synthesis such as cycloheximide [303, 453].

4.2.10 Depletion of the $CD4^+CD25^+$ population does not allow assessment of regulation in allogeneic MLR

In Section 4.2.5 it was demonstrated that depletion of the $CD25^+$ fraction leads to enhanced proliferation of $CD4^+$ cells in response to stimulation with anti-CD3 antibody. Attempts were therefore made to use this depletion approach as an alternative method for detecting regulation of allogeneic responses. Whole or $CD25^+$ -depleted $CD4^+$ splenocytes from mice pre-treated with 177/B10 DST were stimulated with B10 (donor-specific) or BALB (third party) stimulators. We predicted

that if donor-specific regulation were occurring, depletion of the CD25⁺ fraction should lead to an enhanced proliferative response to B10 but not BALB stimulators. However, as shown in Figure 4.21, this did not occur. Once again we speculated that, when the whole CD4⁺ population was stimulated, proliferation of CD4⁺CD25⁺ cells occurred in response to the production of growth factors by other CD4⁺ subsets, thus concealing any regulatory effect.

4.2.11 Kinetics of proliferation of CFSE-labelled CD4⁺CD45RB^{high} cells in vitro

In view of the data presented so far, it was felt that attempts to detect regulation by the use of ³H-thymidine incorporation assays was unlikely to be fruitful since this method is unable to distinguish between the proliferation of responder and regulatory populations. Attention was therefore instead turned to the use of a CFSE-based assay since this would allow the selective analysis of responder cell proliferation. In order to use this approach it was first necessary to determine the optimal time point for the analysis of proliferation of CFSE-labelled responder cells. Labelled CD4⁺CD45RB^{high} cells were therefore stimulated with anti-CD3 antibody or irradiated splenocytes from a range of allogeneic strains, and then cell proliferation was measured at different time points. As shown in Figure 4.22, a significant response to allogeneic stimulation was not detectable until day 7, whereas it occurred much earlier in the case of anti-CD3 stimulation. Based on these findings, analysis in subsequent experiments was performed at day 7 for allogeneic cultures and day 4 for cultures stimulated with anti-CD3 antibody.

4.2.12 CD4⁺CD25⁺ cells regulate the proliferation of CFSE-labelled CD4⁺CD45RB^{high} cells in vitro in response to anti-CD3 stimulation

Before attempting to demonstrate regulation of allogeneic responses using a CFSE-based method we wished to validate this approach in an anti-CD3-driven system, where it has already been shown in this chapter that regulation can be detected by other means (Figures 4.4, 4.5). CFSE-labelled CD4⁺CD45RB^{high} cells were therefore stimulated with anti-CD3 antibody in the presence or absence of CD4⁺CD25⁺ or CD4⁺CD25⁻ cells. Figure 4.23 demonstrates that the addition of CD4⁺CD25⁻ cells did not inhibit CD4⁺CD45RB^{high} proliferation whereas, in contrast, the presence of CD4⁺CD25⁺ cells led to striking suppression.

This demonstration that regulation can be detected using this CFSE-based method provided an opportunity to validate some of the results obtained earlier in this chapter. First the finding that the regulatory capacity of CD4⁺CD25⁺ cells lies predominantly within the CD25^{high} rather than the CD25^{intermediate} fraction was examined further. Figure 4.24 demonstrates that the addition of CD25^{high} cells suppressed the proliferation of labelled CD4⁺CD45RB^{high} responder cells, whereas CD25^{intermediate} cells did not; this is entirely consistent with the data presented in Section 4.2.5.

The next issue to be re-examined was the finding in Section 4.2.7 that CTLA-4 blockade inhibits regulation *in vitro*. Figure 4.25 shows that, while the addition of anti-CTLA-4 antibody had no effect on the proliferation of CD4⁺CD45RB^{high} cells, it partially reversed regulation mediated by CD4⁺CD25⁺ cells, supporting the data described earlier.

4.2.13 $CD4^+CD25^-CD45RB^{low}$ cells do not display regulatory capacity in vitro

In Section 4.2.4 it was demonstrated that the addition of $CD4^+CD25^-CD45RB^{low}$ cells to $CD4^+CD45RB^{high}$ cells in the presence of anti-CD3 stimulation does not suppress total cell proliferation as assessed by incorporation of 3H -thymidine. However, as discussed, it is possible that suppression of the response of the $CD4^+CD45RB^{high}$ population was occurring but was masked by proliferation of the $CD4^+CD25^-CD45RB^{low}$ cells. In order to address this possibility CFSE-labelled $CD4^+CD45RB^{high}$ cells were therefore stimulated with anti-CD3 antibody in the presence of varying numbers of unlabelled $CD4^+CD25^-CD45RB^{low}$ cells (Figure 4.26). However, no regulation was observed, confirming that $CD4^+CD25^-CD45RB^{low}$ cells do not show suppressive properties *in vitro* in our hands.

4.2.14 $CD4^+CD25^+$ cells regulate the proliferation of CFSE-labelled $CD4^+CD45RB^{high}$ cells in vitro in response to allogeneic stimulation

The validation of the use of CFSE-based proliferation assays in the setting of polyclonal stimulation with anti-CD3 antibody, permitted the use of this approach to examine regulation of allogeneic responses *in vitro*. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were harvested from mice pre-treated with 177/B10 DST and added at varying ratios to CFSE-labelled $CD4^+CD45RB^{high}$ responder cells. The cultures were then stimulated with B10, (CBA×B6) F_1 , or BALB splenocytes and proliferation of the responder population analysed. In order to take account of the difference in proliferation occurring in response to stimulators from the different strains (Figure 4.22), values were normalised so that proliferation of $CD4^+CD45RB^{high}$ cells alone was designated as 100%. Figure 4.27 shows that addition of $CD4^+CD25^+$ cells led to suppression of responses to all three stimulator types. However, while the

dose-response curve of suppression achieved in response to stimulation with donor (B10) and (recipient $\times$ donor) F_1 cells was similar, regulation of the response to third party (BALB) stimulation appeared to be less potent.

Previous studies examining the properties of $CD4^+CD25^+$ Treg *in vitro* have demonstrated that activation of these cells allows them to regulate in an antigen non-specific manner [276, 289, 291, 293]. The demonstration in Chapter 3 that the administration of a re-challenge DST at day 0 following 177/DST pre-treatment leads to changes in the size and phenotype of the $CD4^+CD25^+$ population raised the possibility that this may be associated with cell activation that may allow antigen non-specific regulation. Mice were therefore pre-treated with 177/B10 DST on days –28 and –27 followed by a further B10 DST at day 0, and $CD4^+CD25^+$ cells were isolated on day 2 and their regulatory capacity tested *in vitro* (Figure 4.28). There now no longer appeared to be a difference between regulation of third party (BALB) and donor-specific (B10 and F_1) responses.

Although these CFSE-based assessments of regulation of allogeneic responses appear promising, they must, however, be interpreted with caution. As shown in Figure 4.29, there was a wide variation both within individual assays and between separate experiments. This probably occurred for two main reasons. Firstly, the proportion of cells dividing in response to allogeneic stimulation was low (Figure 4.22), and thus the ability to detect changes in the presence of putative Treg populations had limited sensitivity. Secondly, since the yield of $CD4^+CD25^+$ cells obtained during each experiment was limited, there were insufficient cells to set up more than one well for each data point, and thus it was not possible to assess the degree of variation. Taken together, these experiments suggest that, although this

approach appears promising, in its current form this assay is unlikely to provide data that are sufficiently robust to allow reliable conclusions to be drawn.

4.3 Discussion

The results presented in the first part of this chapter demonstrate that *ex vivo* mouse CD4⁺CD25⁺ cells isolated in our laboratory possess similar functional properties to those previously described in both mouse and human models [217, 224, 276, 277, 282-289, 293, 301, 302, 304, 305]. Thus, in Figure 4.2 it was shown that CD4⁺CD25⁺ cells do not proliferate in response to polyclonal stimulation, but that this anergic state can be reversed by the addition of exogenous IL-2, and in Figure 4.3 that the addition of CD4⁺CD25⁺ cells reduced the proliferation of CD4⁺CD25⁻ cells in response to stimulation with anti-CD3 antibody. The published studies have gone on to demonstrate that, in such *in vitro* assays, the addition of exogenous IL-2 restores proliferation to the levels seen in wells containing stimulated CD4⁺CD25⁻ cells in the absence of the CD4⁺CD25⁺ regulatory population; the various authors concluded that exogenous IL-2 abrogated regulation. However, Shevach and colleagues have presented anecdotal data demonstrating that, although addition of IL-2 to co-cultures of CD4⁺CD25⁺ regulatory and CD4⁺CD25⁻ responder cells restores proliferation of the responder population to levels seen in the absence of Treg, production of IL-2 by the responder cells themselves (as assessed by IL-2 mRNA production) remains suppressed. These results thus suggest that the addition of exogenous IL-2 does not strictly abrogate regulation, and demonstrate that interpretation of simple proliferation assays can be difficult, an issue that is clearly relevant to later results in this chapter.

Notwithstanding this reservation about the use of proliferation assays to assess regulation, the next aim of this chapter was to examine whether CD4⁺CD25⁺ cells can regulate the proliferation of polyclonally-stimulated CD4⁺CD25⁻ responder populations *in vitro*; it was demonstrated in Figure 4.3 that this is indeed the case. Furthermore, it was also shown that CD4⁺CD25⁺ cells can regulate the proliferation (again polyclonally-stimulated using anti-CD3 antibody) of CD4⁺CD45RB^{high} cells, the effector population in the adoptive transfer skin allograft system, with regulation *in vitro* being demonstrated in both a ³H-thymidine incorporation assay (Figure 4.4) and a CFSE-based proliferation assay (Figure 4.23). However, this approach did not demonstrate any difference in the regulatory potency of CD4⁺CD25⁺ cells from naïve mice (Figure 4.4) compared to mice pre-treated with the 177/DST protocol (Figure 4.5). This finding is not entirely surprising. In these proliferation assays, a fixed number of CD4⁺CD25⁺ cells was used. Since polyclonal stimulation with anti-CD3 antibody would lead to activation of all CD4⁺CD25⁺ cells (and not just an antigen-specific subpopulation), the same number of activated Treg should be present whether using cells from naïve or pre-treated animals. The fact that no difference in regulation was observed between these two settings would support the hypothesis that 177/DST pre-treatment leads to the relative expansion of an antigen-specific subpopulation of CD4⁺CD25⁺ Treg rather than an increase in the regulatory potency of all CD4⁺CD25⁺ cells. Thus the anti-CD3 system in this form does not satisfy the requirement of providing a surrogate assay for the *in vivo* adoptive transfer system.

Although the anti-CD3 assay does not achieve the overall objective of this chapter, it does, however, provide a means to examine other properties of regulation *in vitro*. As discussed in Chapter 3, CD4⁺CD25⁺ cells lie within the CD45RB^{low} fraction, whereas CD25⁺ cells constitute a minority of the CD4⁺CD45RB^{low} population. This

has prompted speculation that regulatory activity of $CD4^+CD45RB^{low}$ cells may be confined to the $CD25^+$ subset. However, attempts to investigate this question have yielded conflicting results. Read *et al.* found that, unlike $CD4^+CD25^+$ cells, $CD4^+CD25^-CD45RB^{low}$ cells were unable to prevent disease in a colitis model [218], whereas conversely, in studies by Kullberg *et al.* [318] and Oida *et al.* [279], $CD4^+CD25^-CD45RB^{low}$ cells were able to prevent colitis. Interestingly, Annacker and colleagues have found a difference in the behaviour of $CD4^+CD25^+$ and $CD4^+CD25^-CD45RB^{low}$ cells in a model of colitis mediated by $CD4^+CD45RB^{high}$ cells [223]. Although both populations were able to suppress the development of disease, only the $CD25^+$ cells inhibited expansion of the $CD4^+CD45RB^{high}$ population. Taken together these results suggest that $CD4^+CD25^+$ and $CD4^+CD25^-CD45RB^{low}$ cells represent populations with functionally distinct properties, although some of these properties may overlap. Figures 4.7 and 4.26 provide data supporting this view, since they demonstrate that $CD4^+CD25^-CD45RB^{low}$ cells do not display regulatory capacity *in vitro* using the anti-CD3 system.

The next question addressed was whether there is a difference in regulatory capacity between $CD4^+$ cells that express intermediate or high levels of CD25. Figures 4.9 and 4.24 suggest that this is indeed the case and that Treg lie predominantly in the latter rather than the former population. This supports the findings of two previous studies. In the first [217], Kuniyasu and colleagues found that $CD4^+CD25^{intermediate}$ cells from normal mice did not display regulatory capacity *in vitro* and that *in vivo* they caused rather than suppressed autoimmune disease. In addition they demonstrated that polyclonal stimulation of $CD4^+CD25^+$ cells led to sustained expression of high levels of CD25, whereas similar stimulation of $CD4^+CD25^-$ cells led to only transient expression of intermediate levels of CD25. The

authors suggested that $CD4^+CD25^{high}$ cells represent true Treg, whereas $CD4^+CD25^{intermediate}$ cells consist of activated “effector” cells. In a parallel human study [283], Baecher-Allan *et al.* also showed that although $CD4^+CD25^+$ cells displayed regulatory capacity *in vitro*, $CD4^+CD25^{intermediate}$ cells did not, supporting this hypothesis.

In Chapter 3 it was shown that 177/DST pre-treatment leads to an increase in the proportion of $CD4^+CD25^+$ cells expressing CD122, the β chain of the IL-2 receptor. Similarly, published studies have demonstrated that $CD4^+CD25^+$ cells from humans express CD122 constitutively [285, 286, 301]. These findings raise the question of whether CD122 expression may help to identify Treg, and, moreover, whether this molecule may play a mechanistic role in regulation. For example, it could be hypothesised that if Treg express both the α and β chains of the IL-2 receptor, they may preferentially bind IL-2 and thus sequester it from the “effector” population. Surprisingly however, Figure 4.10 shows that $CD4^+CD25^+CD122^-$ cells clearly possess regulatory capacity *in vitro*. As discussed in Section 4.2.6, potential confounding factors (the presence of cell surface antibody that blocks IL-2 binding, and an increased proportion of $CD25^{intermediate}$ cells) mean that no conclusions can be drawn from this experiment about the regulatory properties of the $CD4^+CD25^+CD122^+$ population. If both $CD122^+$ and $CD122^-$ subfractions of the $CD4^+CD25^+$ population do indeed possess regulatory capacity, this would suggest that CD122 expression is not directly involved in the regulatory function of these cells *per se*. However, this would not necessarily mean that CD122 expression has no relevance. CD122 not only forms part of the IL-2 receptor, but is also the β chain of the IL-15 receptor. These two cytokines have contrasting effects on T cells: whilst IL-2 makes cells more susceptible to activation-induced cell death, IL-15 confers

CD8⁺ cells with resistance [430-433, 435, 436, 454-458]. Although a similar effect of IL-15 on promoting the survival of CD4⁺ cells has not yet been demonstrated, it is possible that the expression of CD122 by CD4⁺CD25⁺ Treg (and the increase in expression following 177/DST pre-treatment) may provide them with a survival advantage *in vivo*, increasing their regulatory efficacy. Further circumstantial evidence supporting a role for CD122 expression in the role of Treg is provided by the observation that CD122-deficient mice develop autoimmune disease [326, 459-461]. The role of CD122 expression by CD4⁺CD25⁺ Treg on their function *in vivo* will be addressed further in Chapter 6.

It is now well established both in mouse [216, 218, 288] and human [284-287, 301, 302, 304, 306, 307, 310] studies that CD4⁺CD25⁺ Treg express CTLA-4 constitutively. We have previously shown that, in the adoptive transfer skin allograft system, regulation of CD4⁺CD45RB^{high}-mediated skin allograft rejection by CD4⁺CD25⁺ cells from animals pre-treated with 177/DST is abrogated by CTLA-4 blockade [441]. Other groups have also demonstrated that regulation by CD4⁺CD25⁺ Treg *in vivo* is CTLA-4-dependent [218, 222, 240], although not all investigators have found this to be the case [241]. Similarly, studies of regulation by CD4⁺CD25⁺ cells *in vitro* have also yielded conflicting results, with some studies suggesting that this process is CTLA-4-dependent [240, 288, 304, 309] while others have not [282, 283, 286, 293, 349]. As discussed earlier, this may at least in part be due to differences in the dose of anti-CTLA-4 antibody used in these various studies. Another related factor to consider is whether complete CTLA-4 blockade was achieved in all these models. Takahashi *et al.* found that F_{ab} fragments of anti-CTLA-4 antibody were more effective at abrogating regulation than whole antibody [288]. Furthermore, in their study in which they could not demonstrate any effect on regulation when using whole

antibody, Jonuleit and colleagues showed that F_{ab} fragments of anti-CTLA-4 antibody did lead to partial abrogation of regulation *in vitro* [284]. These results are explicable by the fact that although the antibodies used may block the binding site of CTLA-4, multivalent antibodies still have the capacity to cross-link and thus exert an agonistic effect, whereas F_{ab} fragments are not able to do so and are therefore truly antagonistic. Thus the published literature taken as a whole would suggest that CTLA-4 does play an important role in regulation both *in vitro* and *in vivo*, a view supported by the data presented in this chapter.

Having used the anti-CD3 system to examine the regulatory properties of $CD4^+CD25^+$ cells *in vitro*, the focus was then moved to the main aim of this chapter, namely attempts to develop a system to model the regulation of allogeneic responses *in vitro*. The finding that the addition of $CD4^+CD25^+$ cells (whether from naïve or pre-treated animals) to wells containing $CD4^+CD45RB^{high}$ and allogeneic stimulator cells led to an increase in total proliferation (Figures 4.13, 4.14, 4.15) was a surprise. However, the use of CFSE labelling demonstrated that this increase was due to proliferation of the $CD4^+CD25^+$ cells themselves (Figure 4.17). It was further demonstrated in Figures 4.18 and 4.19 that this proliferation of the $CD4^+CD25^+$ cells was driven by one or more soluble factors produced by the $CD4^+CD45RB^{high}$ cells; although IL-2 seems to be the most obvious candidate for the factor in question, others such as IL-15 may also be involved.

Although the finding that $CD4^+CD25^+$ cells proliferate in the presence of $CD4^+CD45RB^{high}$ and allogeneic stimulator cells was frustrating, it may provide a basis for the enumeration of antigen-specific T cells to add to those methods proposed in Chapter 3. It has previously been demonstrated that if $CD4^+$ cell populations are labelled with CFSE and then stimulated *in vitro*, analysis of the CFSE division

profiles allows estimation of the precursor frequency of cells that have divided [38]. It would be possible to use this method to compare the precursor frequency of $CD4^+CD25^+$ cells from naïve mice or from mice pre-treated with 177/DST in response to stimulation with donor-specific or third party alloantigen; this may allow determination of whether 177/DST pre-treatment leads to an increase in the precursor frequency of donor-specific $CD4^+CD25^+$ cells.

The final part of this chapter demonstrated that, in regulation assays using allogeneic stimulation, the confounding effect of proliferation of the $CD4^+CD25^+$ population can be removed by analysing the proliferation of the $CD4^+CD45RB^{high}$ responder population alone by means of CFSE labelling. Using this approach it was shown that $CD4^+CD25^+$ cells from animals pre-treated with 177/DST were indeed able to regulate the proliferation (to allogeneic stimulation) of $CD4^+CD45RB^{high}$ cells and, although regulation of third party responses was also manifest, this was less potent than regulation of donor-specific responses (Figure 4.27). In these assays, “donor-specific” stimulation was achieved by using not only donor strain splenocytes, but also with (recipient $\times$ donor) F_1 cells. This is because previous work from our laboratory has demonstrated that Treg isolated from mice with long term surviving cardiac allografts (i.e. the maintenance phase of tolerance) were able to regulate the proliferation *in vitro* of responder $CD4^+CD45RB^{high}$ cells in response to stimulation with (recipient $\times$ donor) F_1 cells (providing stimulation via both the direct and indirect pathways), but not when using donor strain cells only (stimulation via the direct pathway), suggesting that indirect stimulation was necessary in order to achieve regulation in this setting. [236]. In contrast, Figure 4.27 showed that, when using $CD4^+CD25^+$ cells from mice pre-treated with 177/DST, regulation was equally potent whether the stimulation was provided by cells from

donor or (recipient $\times$ donor) F_1 mice. However, this apparent dichotomy can be explained by the hypothesis that the maintenance of $CD4^+CD25^+$ Treg populations *in vivo* requires the continued presence of cognate antigen [462]. In mice with long term surviving grafts, it is likely that the majority of donor passenger APC are no longer present, and that presentation of donor alloantigen occurs mainly through the indirect pathway, leading to the preferential survival of Treg recognising donor alloantigen via this pathway. In contrast, in mice pre-treated with 177/DST 28 days previously donor APC are still present, allowing alloantigen presentation via the direct pathway.

Although the CFSE-based approach appears to provide a promising surrogate *in vitro* assay, a major problem is the variability of the assay (Figure 4.29). One potential way of reducing this would be to perform replicate wells. However, this would require greater numbers of $CD4^+CD25^+$ cells. Although for animal experiments this could theoretically be achieved by pre-treating more animals, the vastly increased time required for the flow cytometric purification of the cells would be likely to render them non-viable. Furthermore, it would not be possible to obtain such high numbers of cells from patients, thus making this approach impractical for clinical use, a longer term aim for the development of *in vitro* regulation assays.

In summary, the overall aim of this chapter was to develop a surrogate *in vitro* regulation assay that would model the regulation of skin allograft rejection seen *in vivo* by $CD4^+CD25^+$ cells from mice pre-treated with 177/DST. Although the use of a CFSE-based approach may potentially provide such an assay, in its present form it is unlikely to be of practical use. However, the surprising finding that $CD4^+CD25^+$ cells proliferate when mixed with $CD4^+CD45RB^{high}$ and allogeneic stimulator cells may provide a means to enumerate the precursor frequency of Treg. Although this would not provide a direct functional assay, it would at least provide a means to quantify

donor-specific Treg, a tool that, as discussed in Chapter 3, would be extremely valuable.

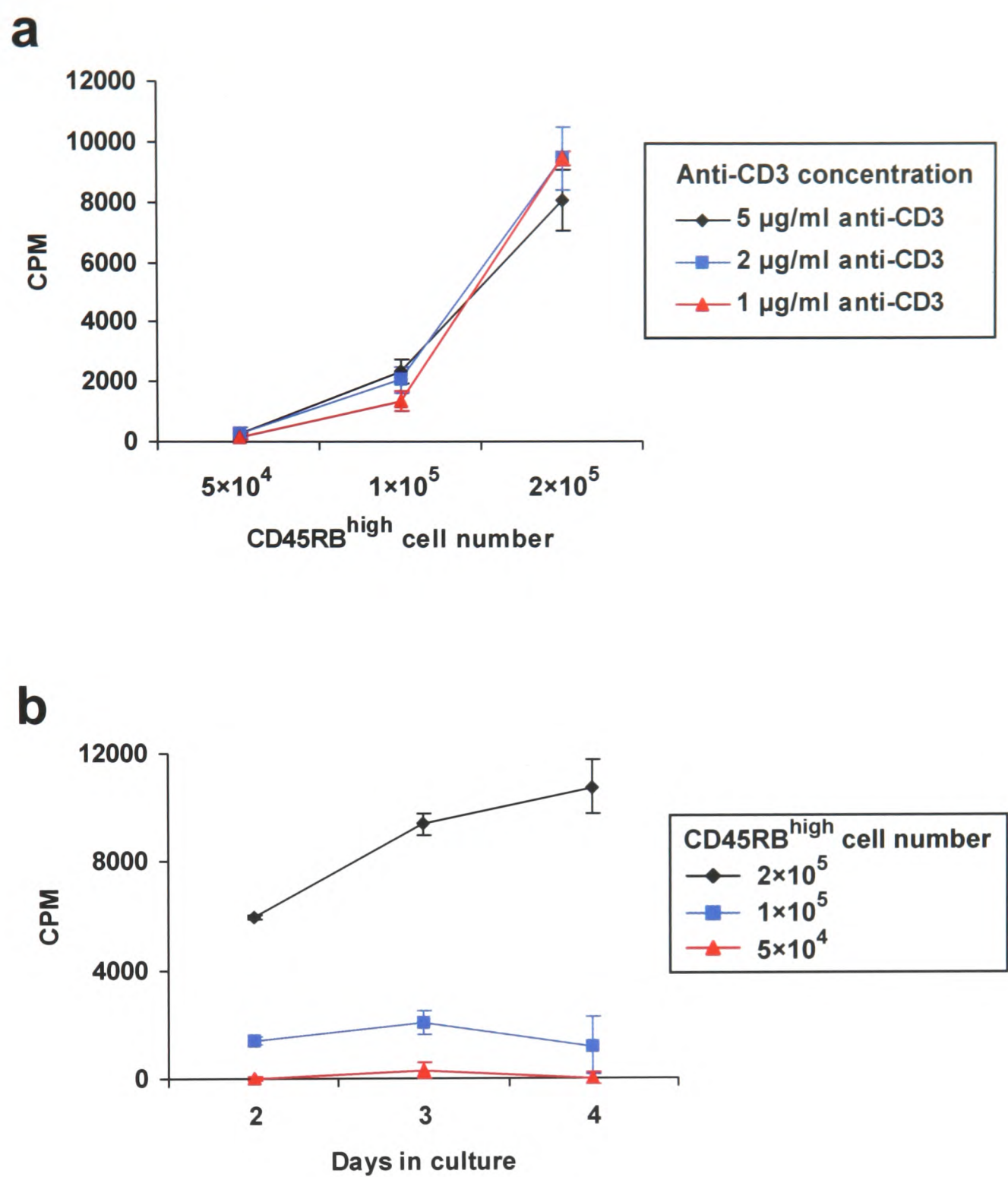


Figure 4.1. Kinetics and titration of proliferative response of CD4⁺CD45RB^{high} cells in response to anti-CD3 stimulation

(a) CD4⁺CD45RB^{high} cells from naïve CBA mice were cultured for 4 days at the numbers shown in the presence of 5 × 10⁵ irradiated CBA spleen cells and anti-CD3 antibody at the concentrations indicated. (b) CD4⁺CD45RB^{high} cells were cultured in the presence of 5 × 10⁵ irradiated CBA spleen cells and anti-CD3 antibody at a concentration of 2 µg/ml and then harvested at the time point indicated. In both cases ³H-thymidine was added to wells for the final 18 hours of culture and then ³H incorporation measured. Data are expressed as mean cpm ± standard deviation of triplicate cultures and are representative of two independent experiments.

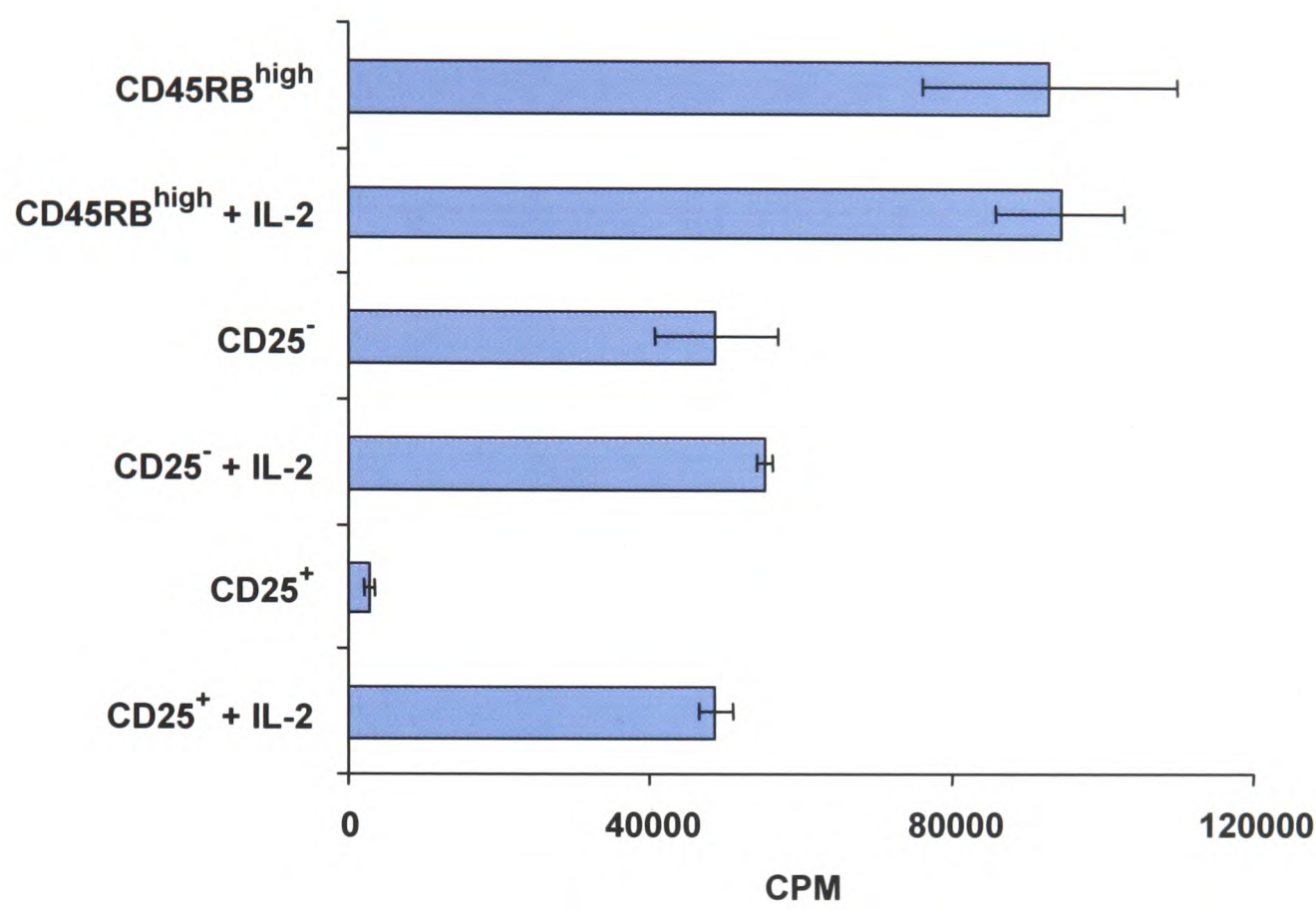


Figure 4.2. Exogenous IL-2 overcomes the non-proliferative state of CD4⁺CD25⁺ cells *in vitro*

2×10^5 CD4⁺CD45RB^{high}, CD4⁺CD25⁻, or CD4⁺CD25⁺ cells from naïve CBA mice were cultured for 4 days with anti-CD3 antibody (2 µg/ml) and 5×10^5 irradiated CBA spleen cells in the presence or absence of exogenous IL-2 (200 units/ml). ³H-thymidine was added to wells for the final 18 hours of culture and then ³H incorporation measured. Data are expressed as mean cpm ± standard deviation of triplicate cultures and are representative of two independent experiments.

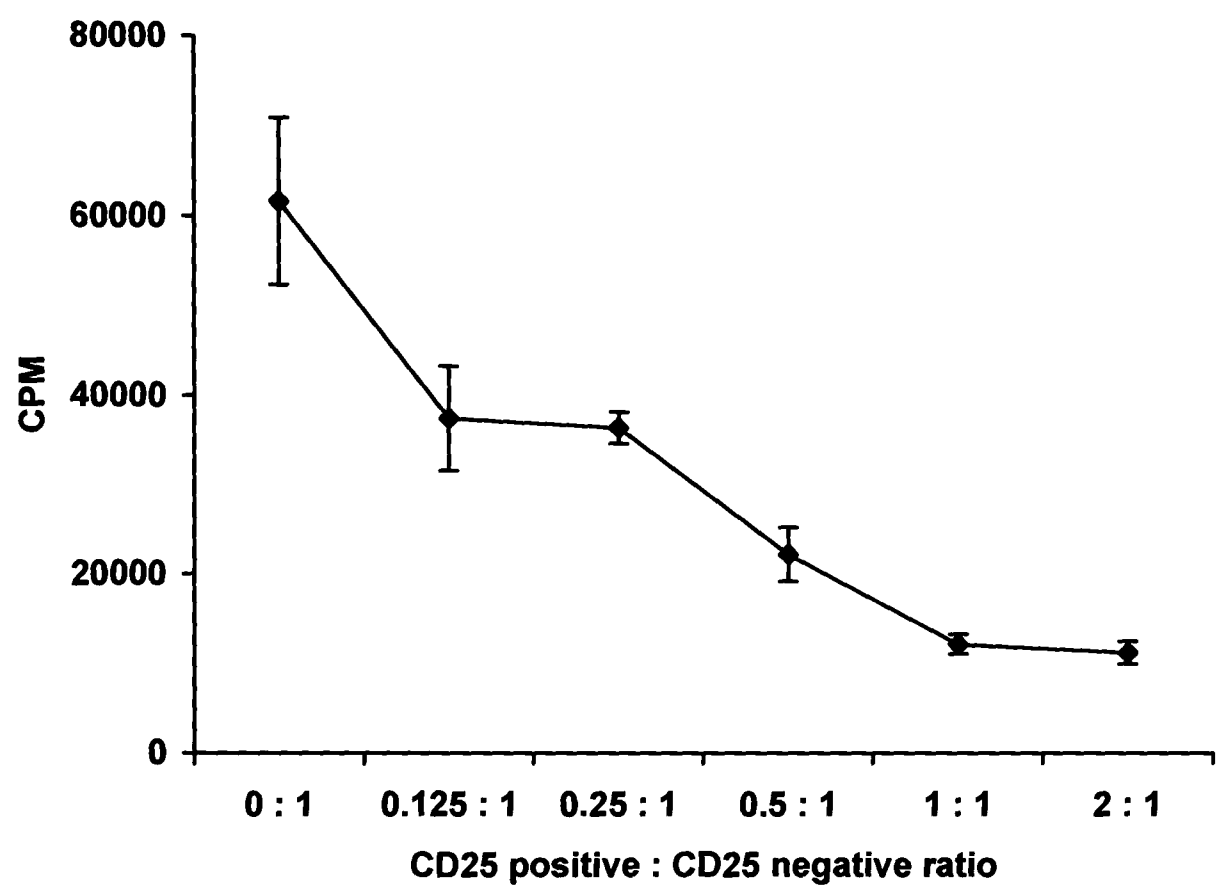


Figure 4.3. $CD4^+CD25^+$ cells can regulate the proliferation of $CD4^+CD25^-$ cells *in vitro*
 $CD4^+CD25^-$ (2×10^5 per well) and / or $CD4^+CD25^+$ (at ratio shown) cells from naïve CBA mice were cultured for 4 days with anti-CD3 antibody ($2 \mu\text{g/ml}$) and irradiated CBA spleen cells (5×10^5 per well). ^3H -thymidine was added to wells for the final 18 hours of culture and then ^3H incorporation measured. Data are expressed as mean $\text{cpm} \pm$ standard deviation of triplicate cultures and are representative of two independent experiments.

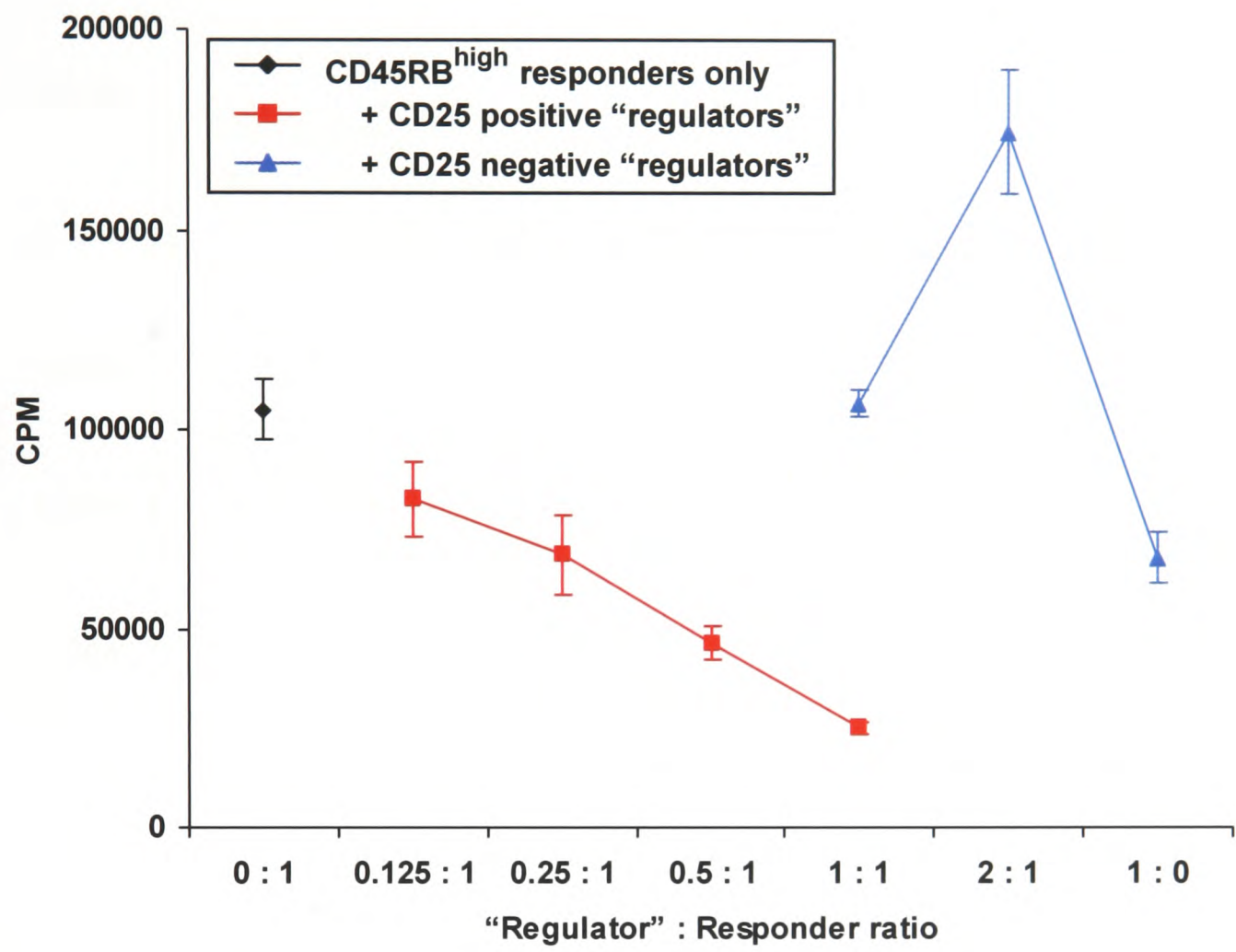


Figure 4.4. CD4⁺CD25⁺ cells from naïve mice can regulate the proliferation of CD4⁺CD45RB^{high} cells *in vitro*
2×10⁵ CD4⁺CD45RB^{high} responder cells and / or putative regulatory (CD4⁺CD25 positive or negative) cells (at ratios shown) from naïve CBA mice were cultured for 4 days with anti-CD3 antibody (2 µg/ml) and 5×10⁵ irradiated CBA spleen cells. ³H-thymidine was added to wells for the final 18 hours of culture and then ³H incorporation measured. Data are expressed as mean cpm ± standard deviation of triplicate cultures and are representative of two independent experiments.

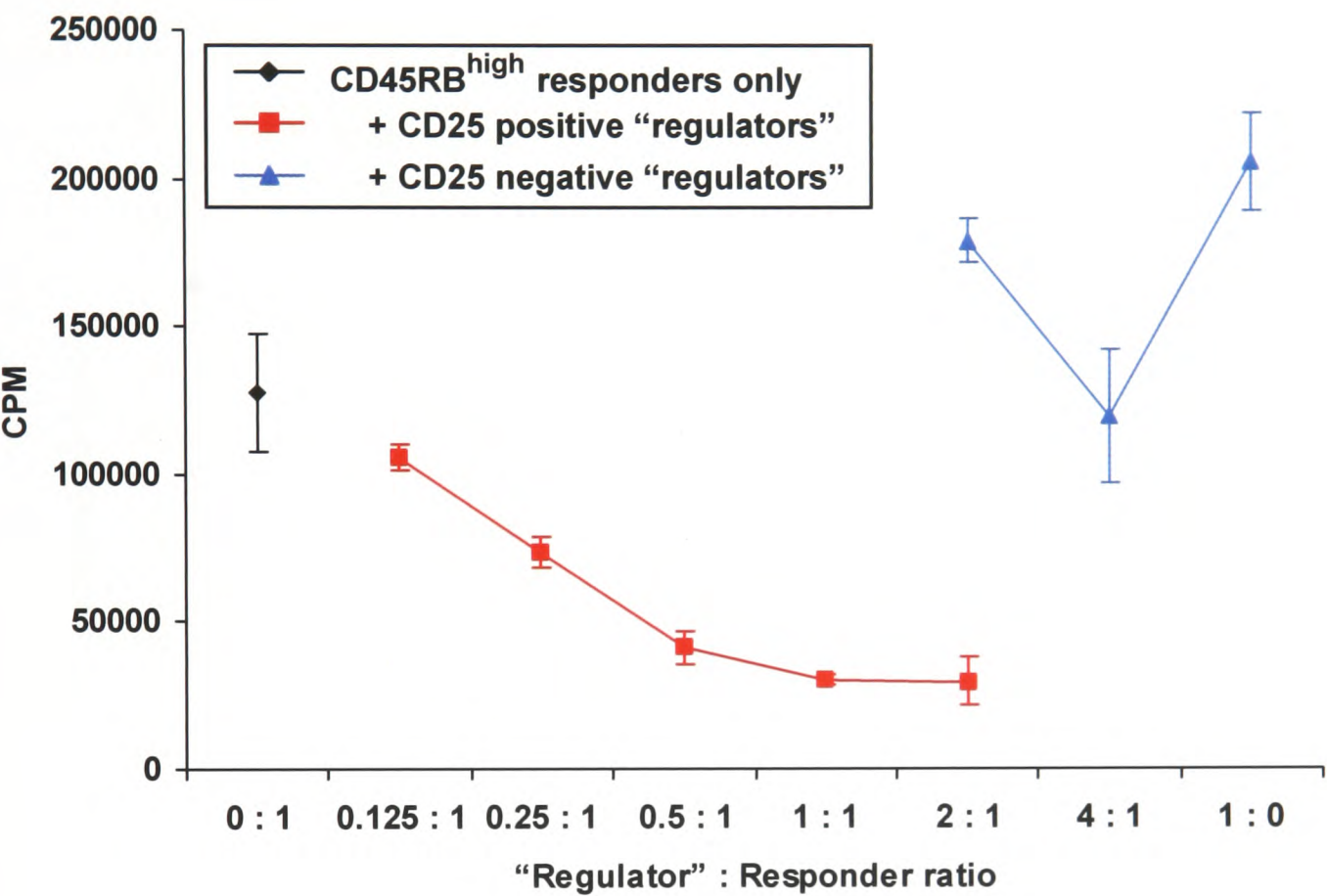


Figure 4.5. CD4⁺CD25⁺ cells from 177/DST pre-treated mice can regulate the proliferation of CD4⁺CD45RB^{high} cells *in vitro*
CD4⁺CD45RB^{high} responder cells (2×10⁵ per well) from naïve CBA mice and / or putative regulatory (CD4⁺CD25 positive or negative) cells (at ratios shown) from CBA mice pre-treated with 177/DST were cultured for 4 days with anti-CD3 antibody (2 µg/ml) and irradiated CBA spleen cells (5×10⁵ per well). ³H-thymidine was added to wells for the final 18 hours of culture and then ³H incorporation measured. Data are expressed as mean cpm ± standard deviation of triplicate cultures and are representative of two independent experiments.

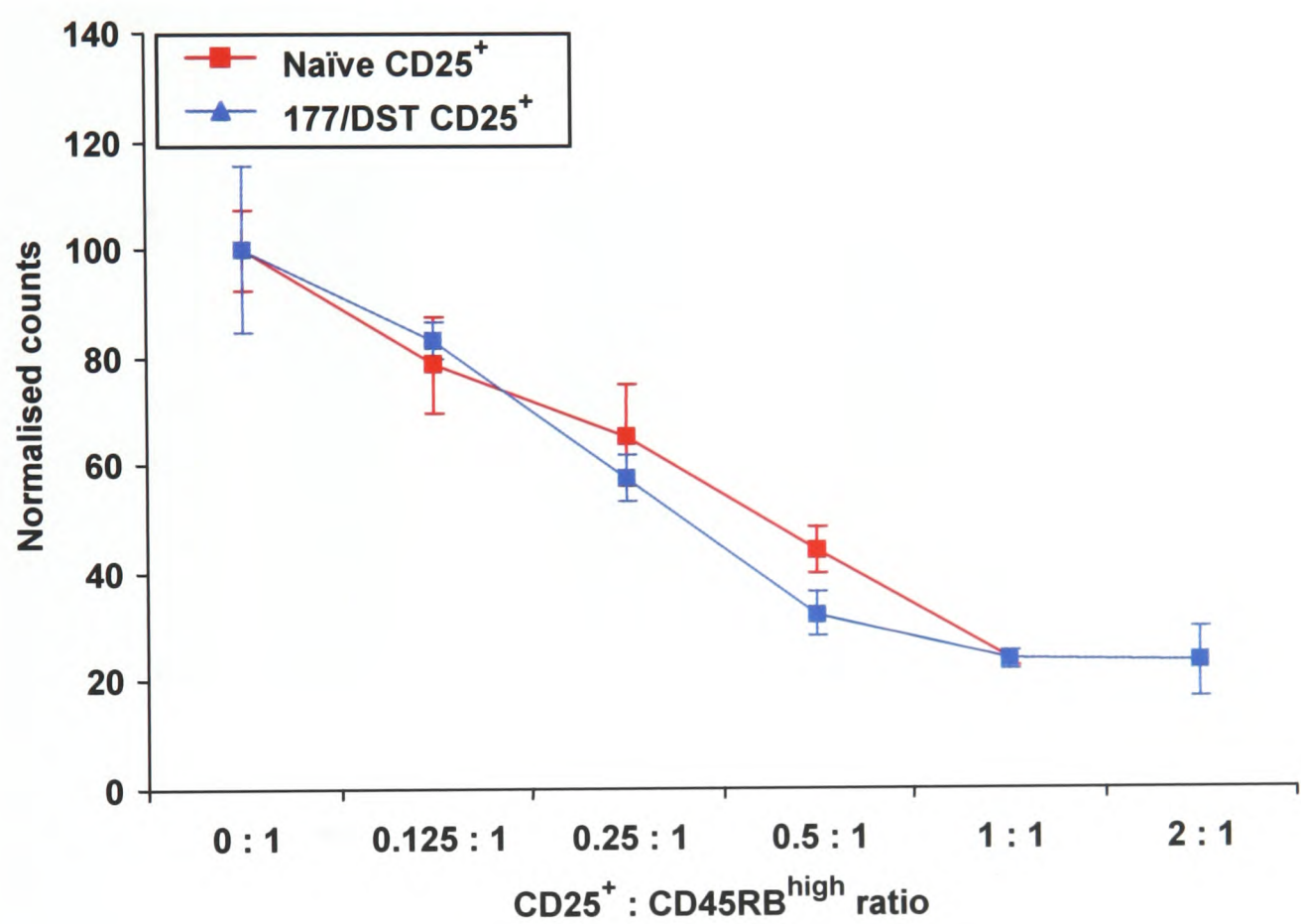


Figure 4.6. *In vitro* suppressive activity of CD4⁺CD25⁺ cells from naïve and 177/DST pre-treated mice is similar
To allow comparison of regulatory effect of CD4⁺CD25⁺ cells from naïve and 177/DST pre-treated animals, data from figures 4.4 and 4.5 were normalised so that proliferation of CD4⁺CD45RB^{high} cells alone was designated as 100.

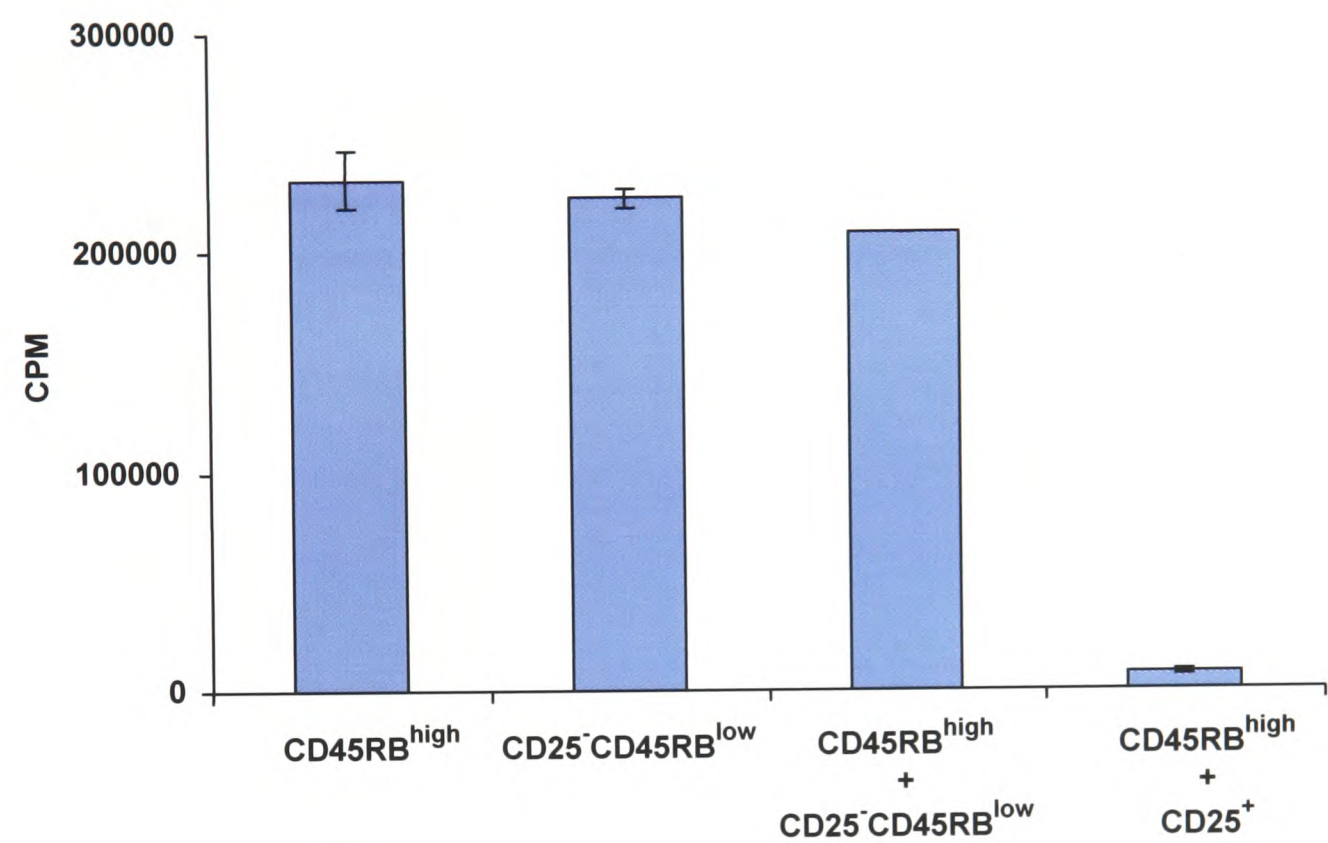


Figure 4.7. $CD4^+CD25^-CD45RB^{low}$ cells appear not to possess regulatory capacity *in vitro*

2×10^5 $CD4^+CD45RB^{high}$, $CD4^+CD25^-CD45RB^{low}$, and $CD4^+CD25^+$ cells from naïve CBA mice were cultured for 4 days in the combinations shown in the presence of anti-CD3 antibody (2 μ g/ml) and 5×10^5 irradiated CBA spleen cells. 3H -thymidine was added to wells for the final 18 hours of culture and then 3H incorporation measured. Data are expressed as mean cpm $\pm$ standard deviation of triplicate cultures and are representative of two independent experiments.

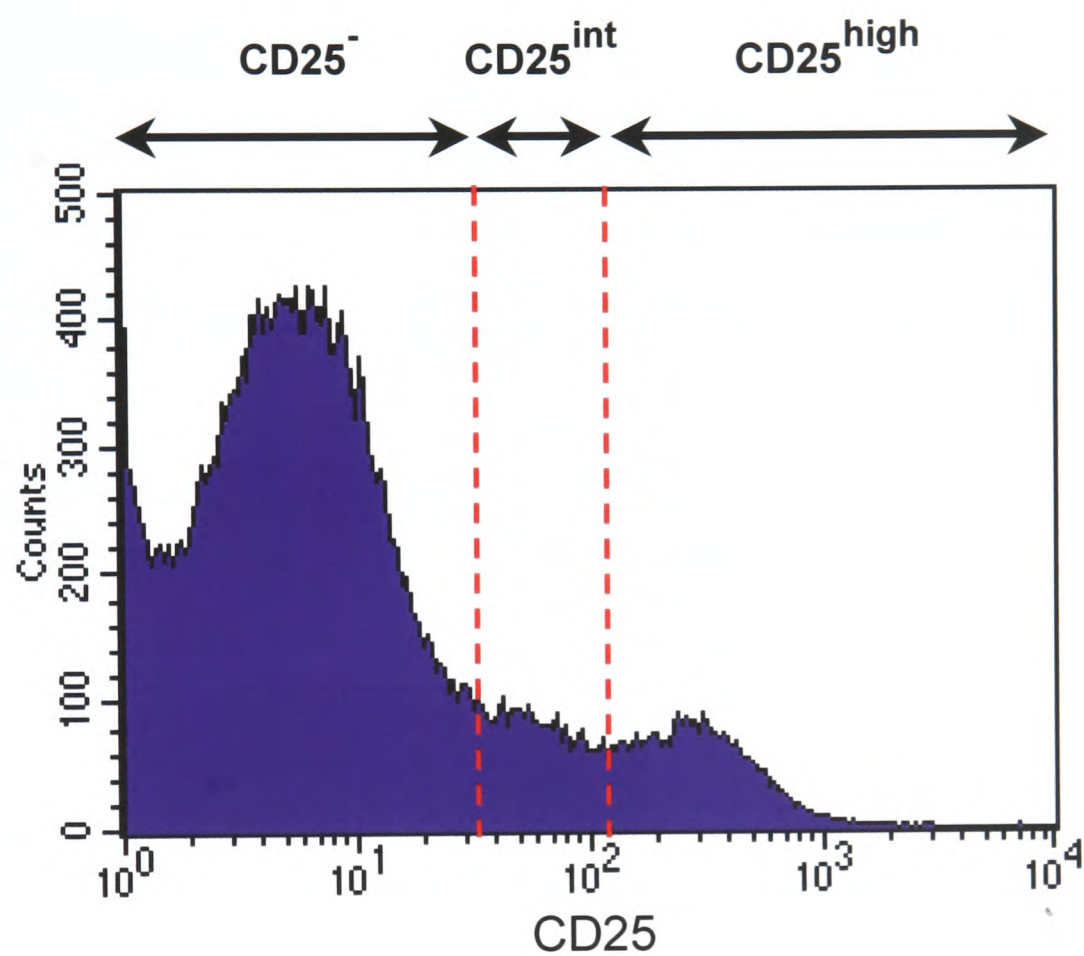


Figure 4.8. CD4⁺ cells show three levels of expression of CD25
Spleen cells from naïve CBA mice were stained with anti-CD4-PerCP and biotinylated anti-CD25 followed by SA-APC prior to flow cytometric analysis. Plot shows CD25 expression by CD4⁺ population. CD25⁻, CD25^{intermediate} (CD25^{int}), and CD25^{high} populations are indicated. Data are representative of four independent experiments.

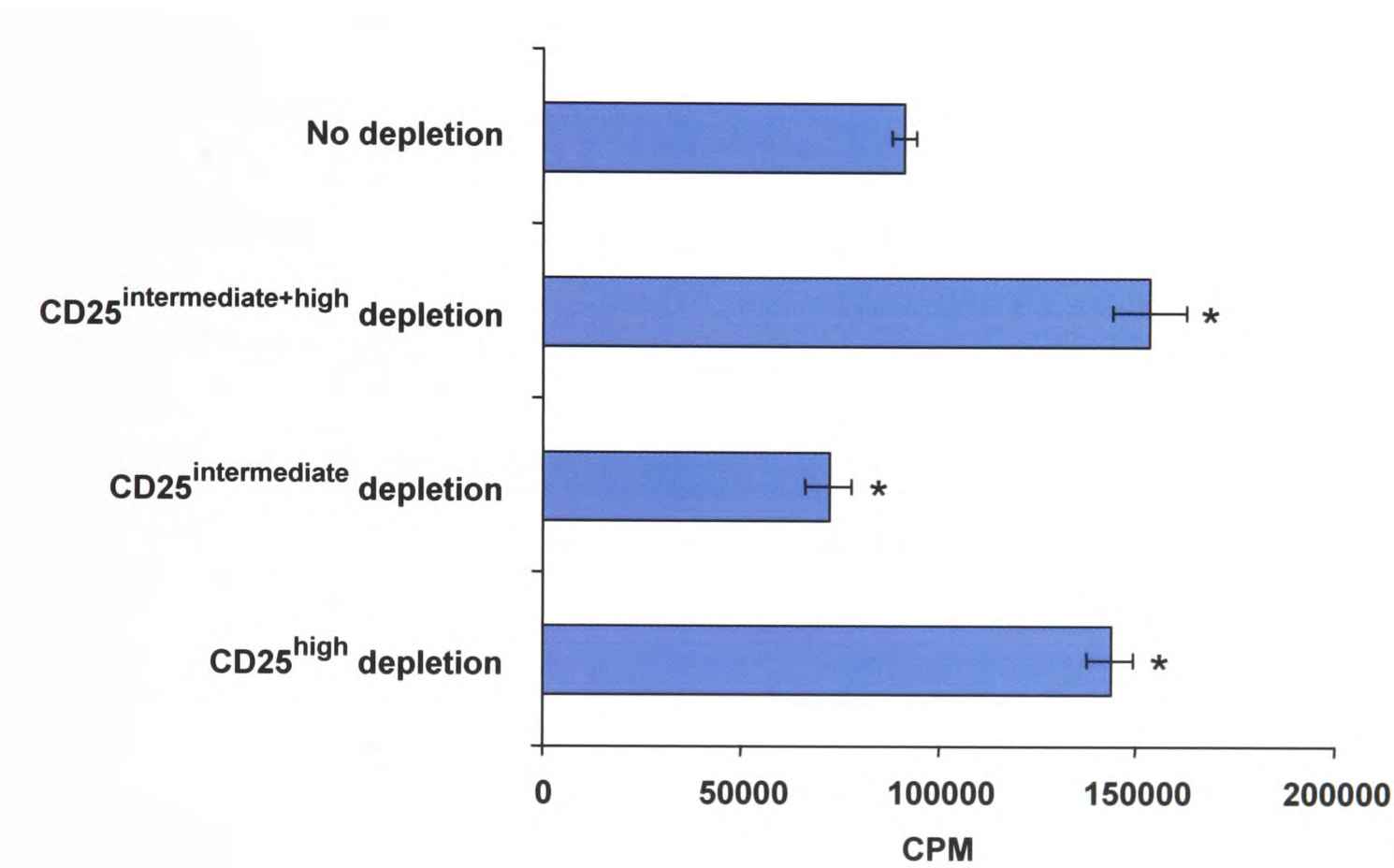


Figure 4.9. Depletion of CD25^{high}, but not CD25^{intermediate}, population enhances proliferation of CD4⁺ cells in response to anti-CD3 stimulation
2×10⁵ whole CD4⁺ cells, or CD4⁺ cells depleted of the indicated subpopulations, from naïve CBA mice were cultured for 4 days in the presence of anti-CD3 antibody (2 µg/ml) and irradiated CBA spleen cells (5×10⁵ cells per well). ³H-thymidine was added to wells for the final 18 hours of culture and then ³H incorporation measured. Data are expressed as mean cpm ± standard deviation of triplicate cultures and are representative of two independent experiments.
* : *P* < 0.01 compared to no depletion group.

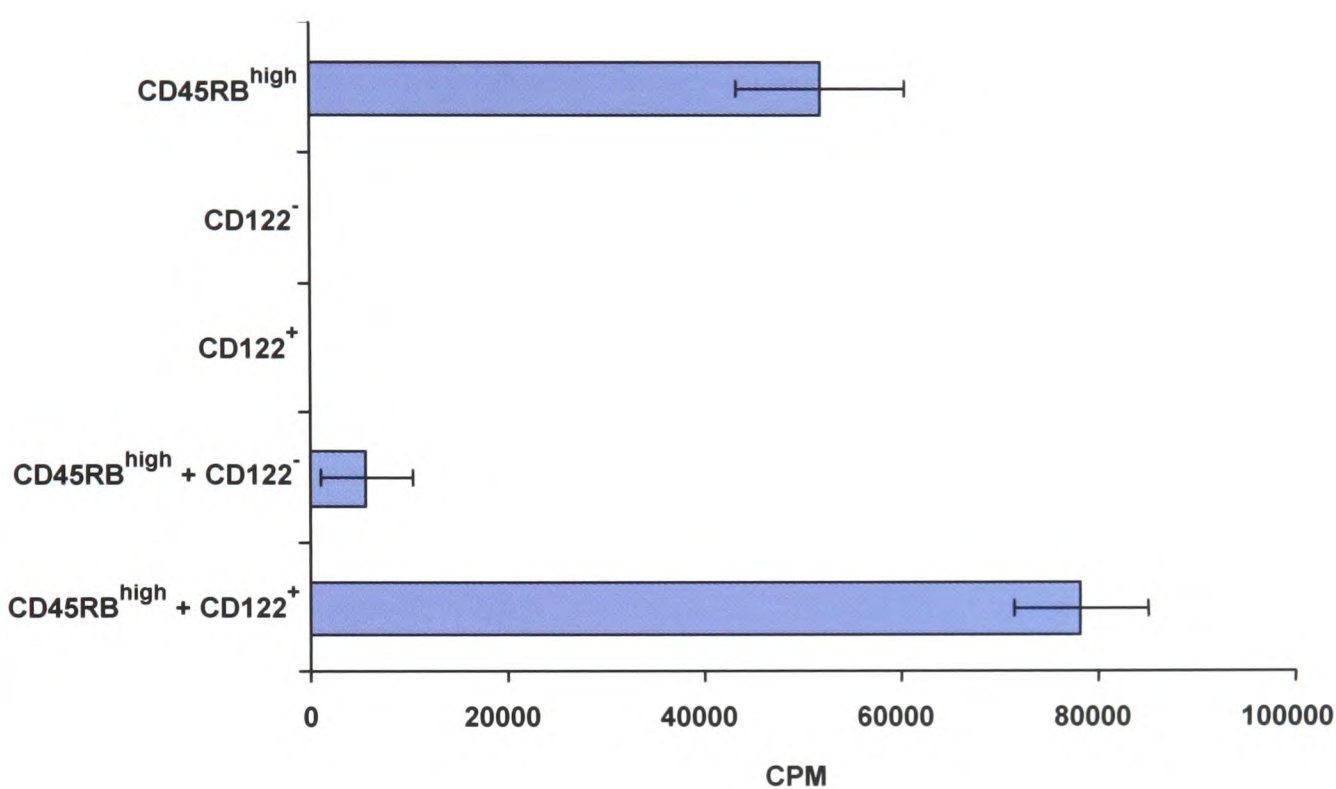


Figure 4.10. CD4⁺CD25⁺CD122⁻ cells possess regulatory properties *in vitro*
The combinations indicated of CD4⁺CD45RB^{high}, CD4⁺CD25⁺CD122⁺, and CD4⁺CD25⁺CD122⁻ cells (2×10^5 of each cell fraction per well) from naïve CBA mice were cultured for 4 days with anti-CD3 antibody (2 μ g/ml) and 5×10^5 irradiated CBA spleen cells. ³H-thymidine was added to wells for the final 18 hours of culture and then ³H incorporation measured. Data are expressed as mean cpm $\pm$ standard deviation of triplicate cultures and are representative of two independent experiments.

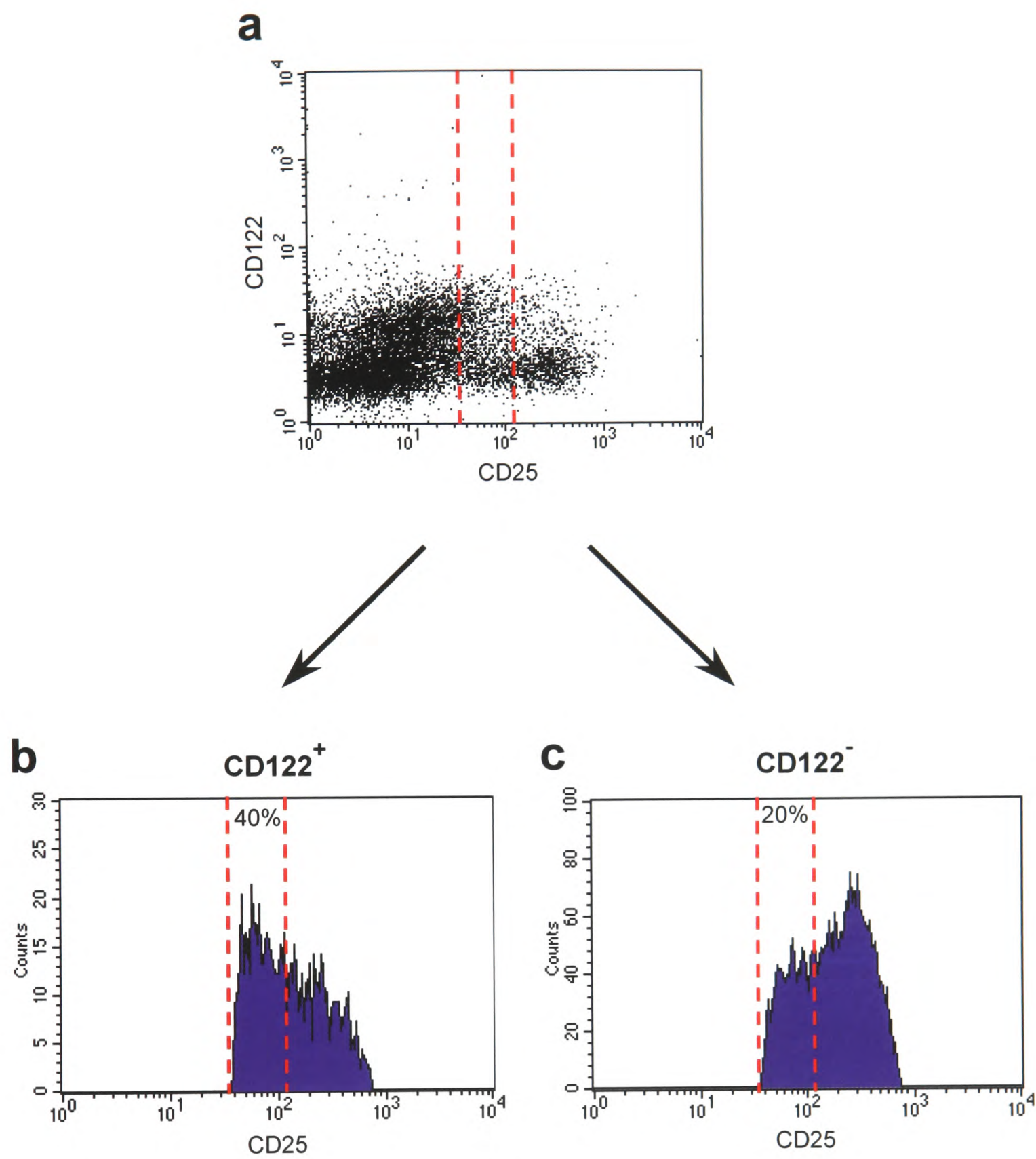


Figure 4.11. $CD4^+CD25^+CD122^+$ population contains a higher proportion of $CD25^{intermediate}$ cells than $CD4^+CD25^+CD122^-$ population

Spleens from naïve CBA mice were stained with anti-CD4-PerCP, and-CD122-PE, and biotinylated anti-CD25 followed by SA-APC prior to flow cytometric analysis. (a) Expression of CD25 and CD122 by $CD4^+$ population. (b) Expression of CD25 by $CD4^+CD25^+CD122^+$ population. (c) Expression of CD25 by $CD4^+CD25^+CD122^-$ population. Dotted lines represent boundaries between $CD25^-$, $CD25^{intermediate}$, and $CD25^{high}$ populations. Percentages indicate proportion of $CD25^{intermediate}$ cells. Data are representative of two independent experiments.

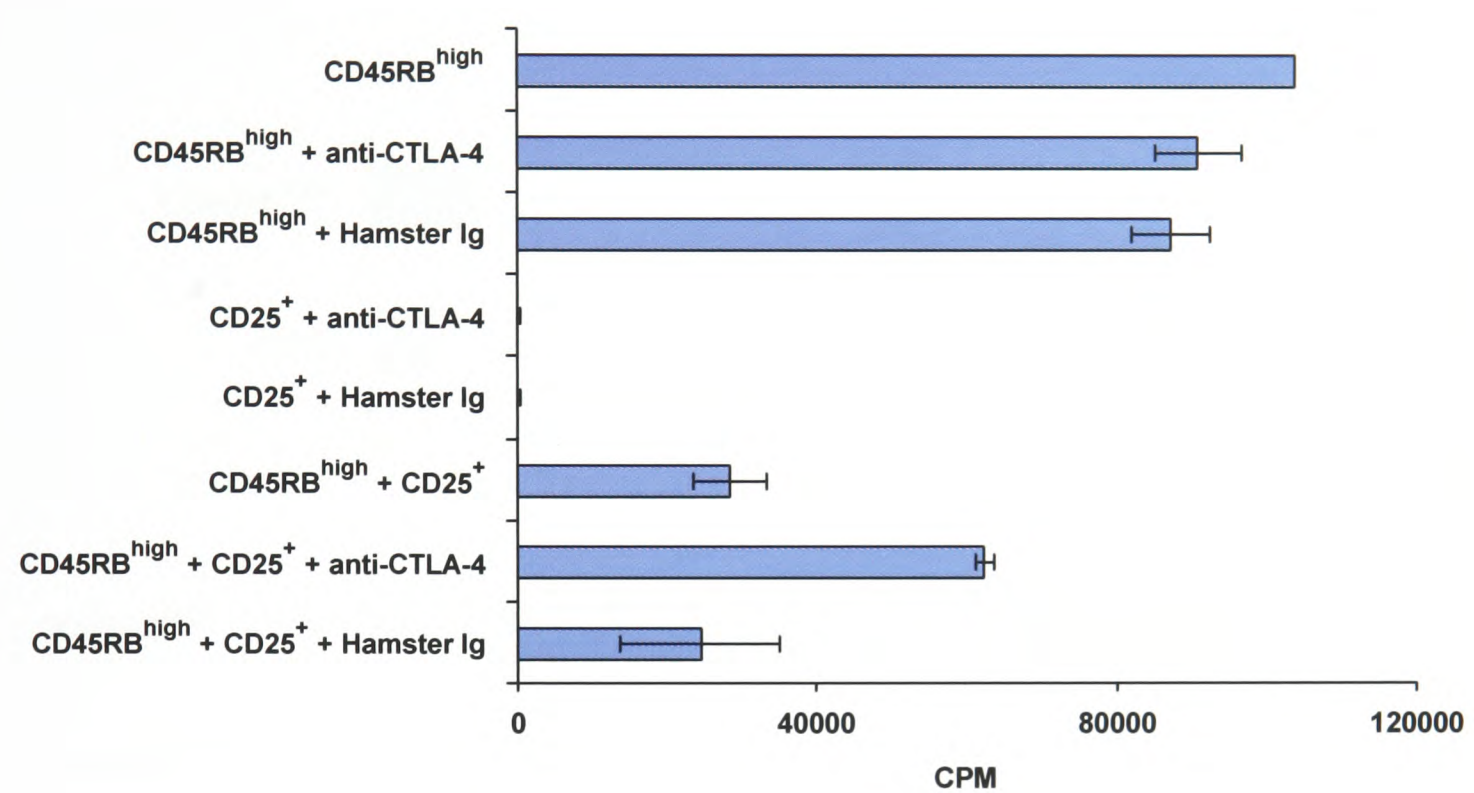


Figure 4.12. CTLA-4 blockade partially abrogates regulation *in vitro* by CD4⁺CD25⁺ cells

2×10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice were stimulated with anti-CD3 antibody (2 µg/ml) and 5×10⁵ irradiated CBA spleen cells with or without 2×10⁵ CD4⁺CD25⁺ cells from naïve CBA mice and / or 100 µg/ml of the anti-CTLA-4 antibody 4F10 or hamster IgG control. ³H-thymidine was added to wells for the final 18 hours of culture and then ³H incorporation measured on day 4. Data are expressed as mean cpm ± standard deviation of triplicate cultures and are representative of two independent experiments.

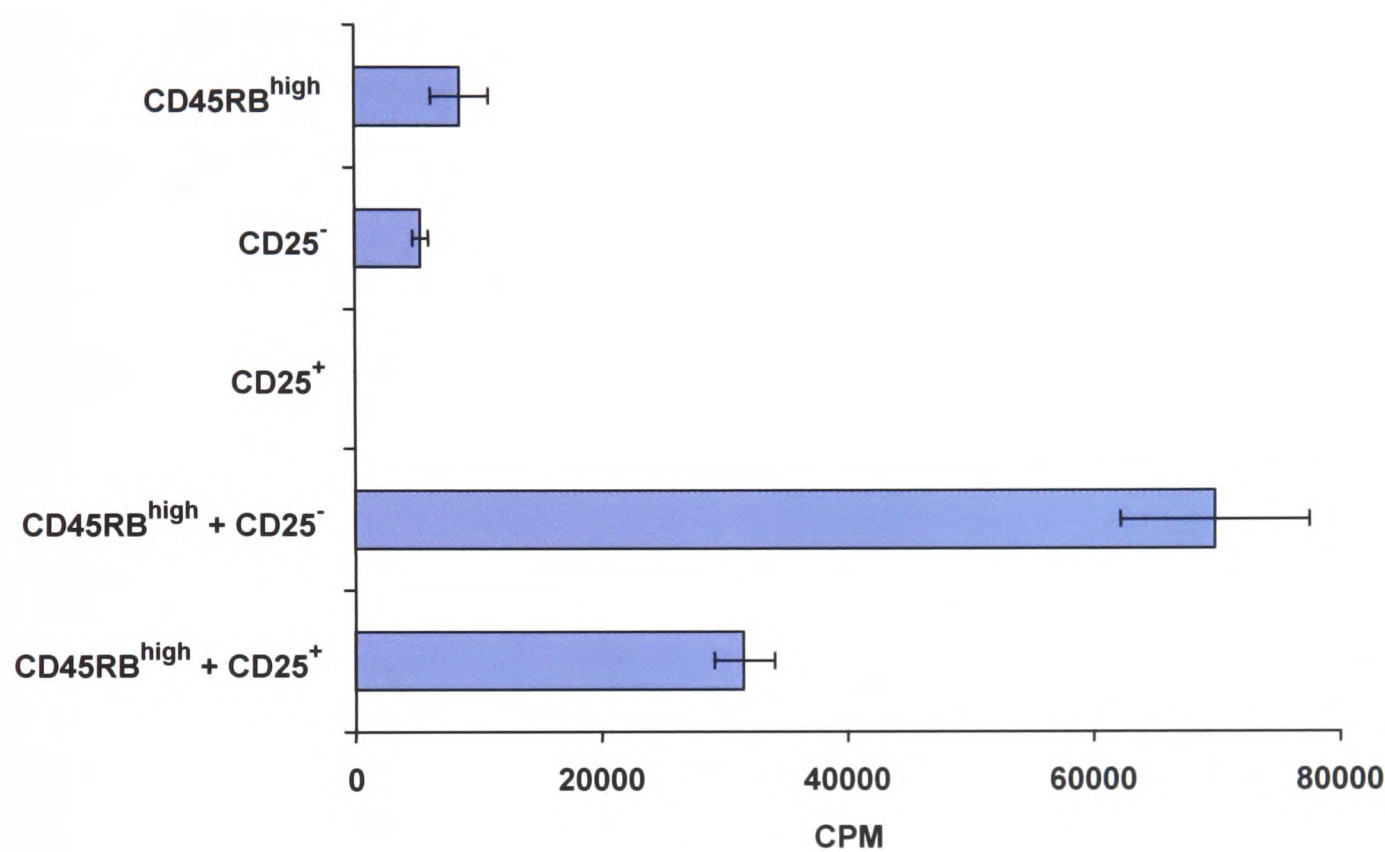


Figure 4.13. Effect of the addition of naïve CD4⁺CD25⁺ cells to *in vitro* MLR
2×10⁵ CD4⁺CD45RB^{high}, CD4⁺CD25⁻, and CD4⁺CD25⁺ cells from naïve CBA mice were cultured for 5 days in the combinations indicated together with 5×10⁵ irradiated B10 spleen cells. ³H-thymidine was added to wells for the final 18 hours of culture and then ³H incorporation measured. Data are expressed as mean cpm ± standard deviation of triplicate cultures and are representative of three independent experiments.

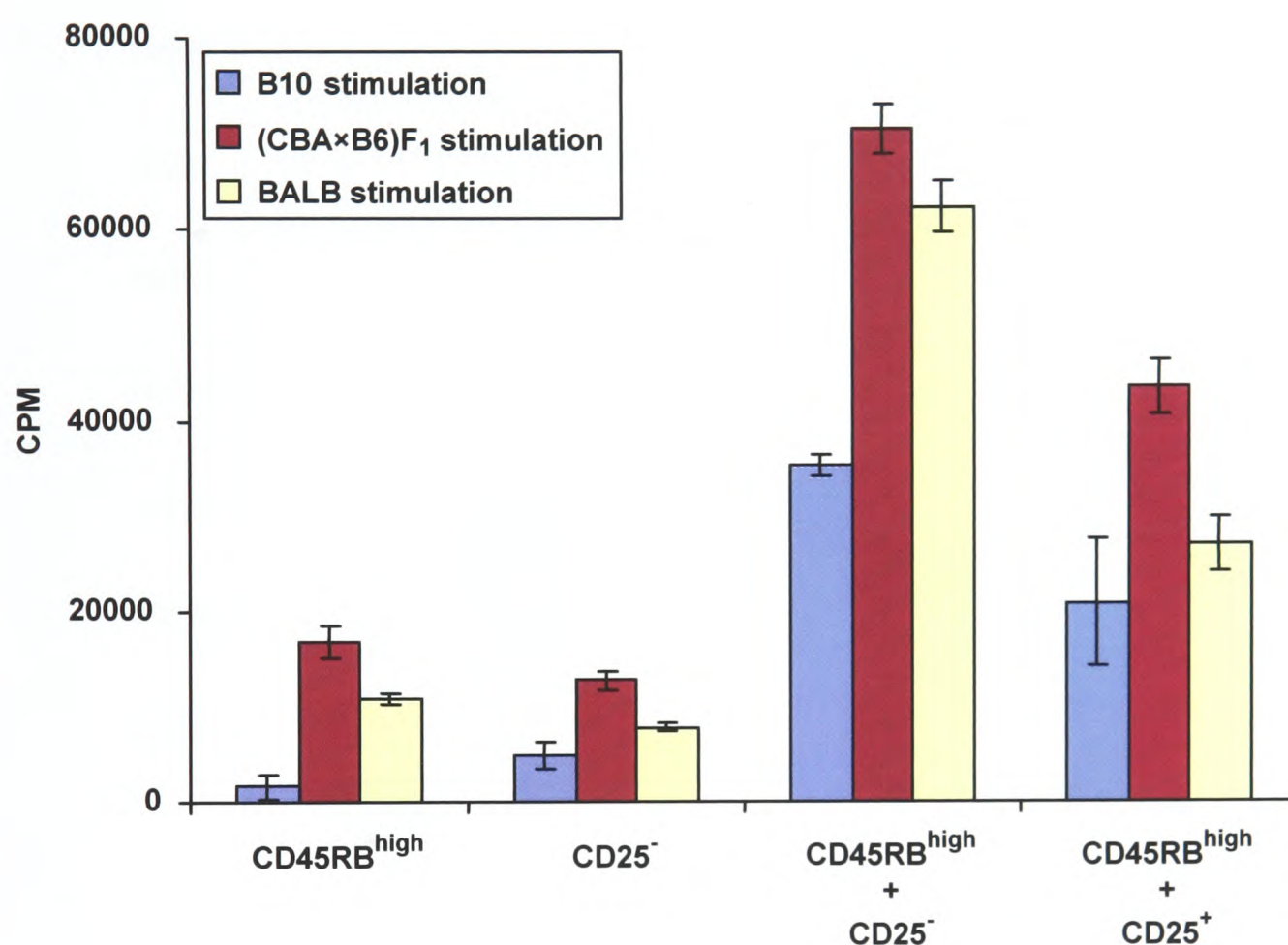


Figure 4.14. Effect of the addition of CD4⁺CD25⁺ cells from 177/DST mice to *in vitro* MLR

2×10^5 CD4⁺CD45RB^{high} cells from naïve CBA mice and / or 2×10^5 CD4⁺CD25⁻ or CD4⁺CD25⁺ cells from CBA mice pre-treated 28 days previously with 177/B10 DST were cultured for 5 days in the combinations indicated together with 5×10^5 irradiated allogeneic (B10, (CBA x B6)F₁, or BALB) spleen cells. ³H-thymidine was added to wells for the final 18 hours of culture and then ³H incorporation measured. Data are expressed as mean cpm $\pm$ standard deviation of triplicate cultures and are representative of two independent experiments.

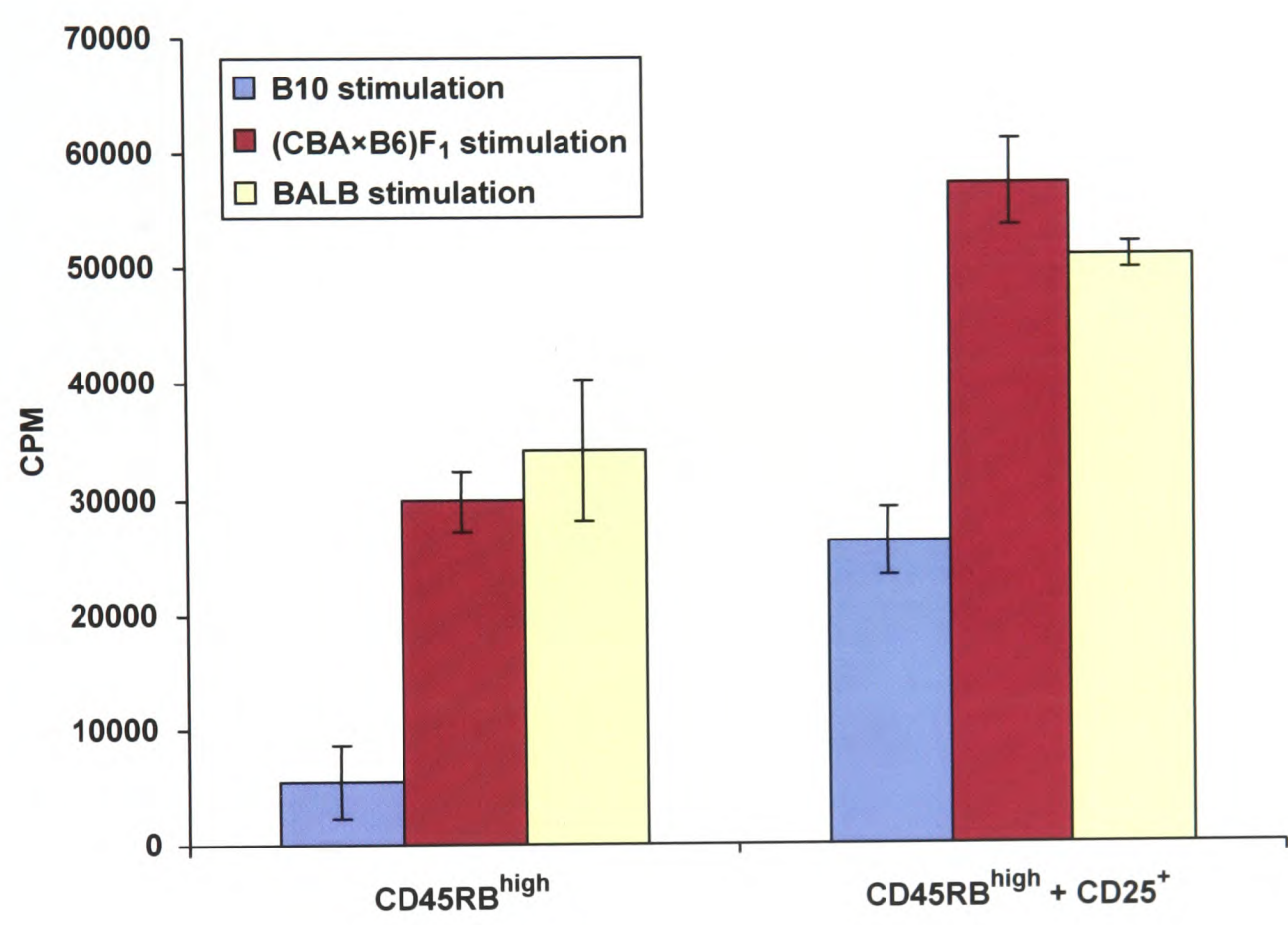


Figure 4.15. Effect of the addition to *in vitro* MLR of CD4⁺CD25⁺ cells from mice pre-treated with 177/B10 DST plus DST re-challenge
2×10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice were cultured for 5 days with 5×10⁵ irradiated allogeneic (B10, (CBA×B6)F₁, or BALB) spleen cells in the presence or absence of 2×10⁵ CD4⁺CD25⁺ cells harvested at day +2 from CBA mice pre-treated on days -28 and -27 with 177/B10 DST and given a further B10 DST on day 0. ³H-thymidine was added to wells for the final 18 hours of culture and then ³H incorporation measured. Data are expressed as mean cpm ± standard deviation of triplicate cultures and are representative of two independent experiments.

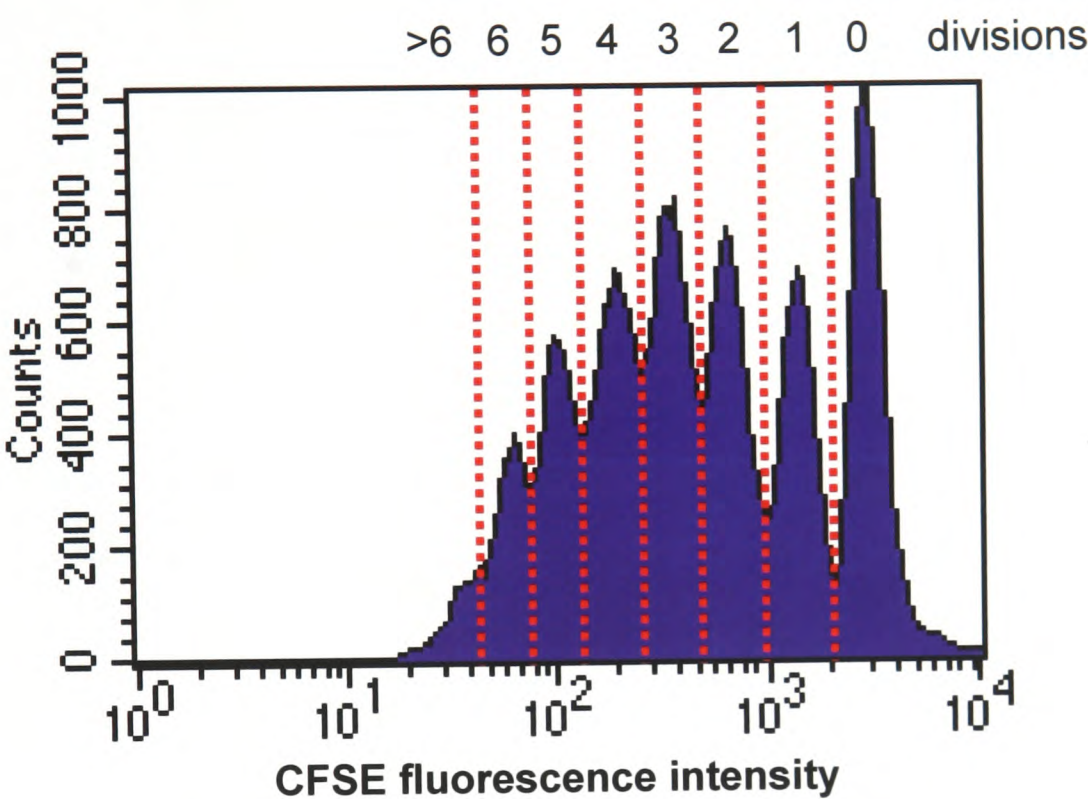


Figure 4.16. Assessment of cell division by analysis of CFSE fluorescence intensity
CBA CD4⁺CD45RB^{high} cells were stimulated *in vitro* with anti-CD3 antibody in the presence of irradiated syngeneic spleen cells. 4 days later cells were harvested and CFSE fluorescence intensity measured by flow cytometric analysis. Right hand peak contains cells that have not divided, while each peak to the left represents one round of cell divisions. Data are representative of three independent experiments.

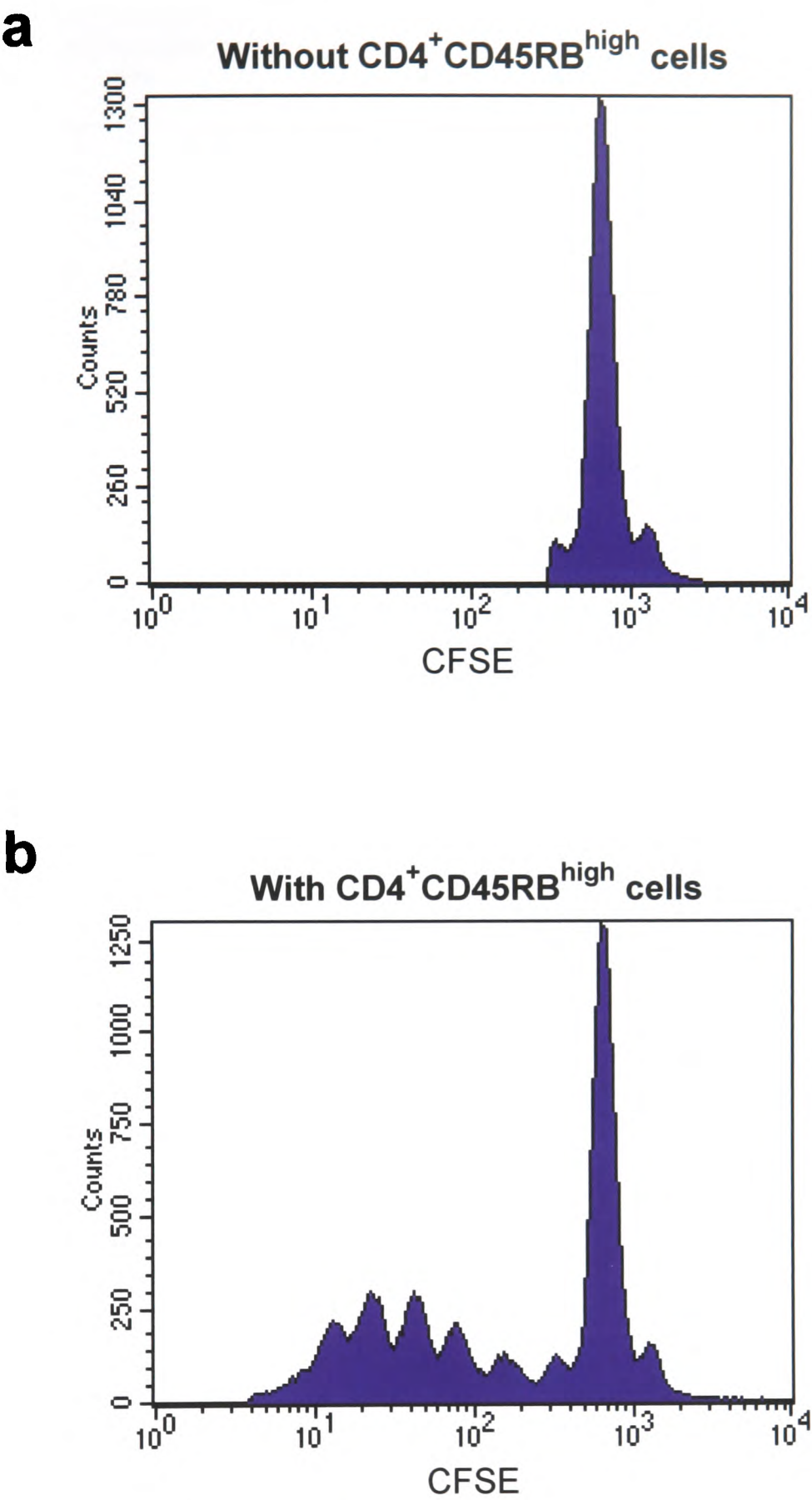


Figure 4.17. CD4⁺CD25⁺ cells proliferate in response to allogeneic stimulation when cultured in the presence of CD4⁺CD45RB^{high} cells
2×10⁵ CFSE-labelled CD4⁺CD25⁺ cells from naïve CBA mice were stimulated with 5×10⁵ irradiated B10 splenocytes in the absence (a) or presence (b) of 2×10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice. Cultures were harvested on day 4 and flow cytometric analysis was performed. Data are representative of two independent experiments.

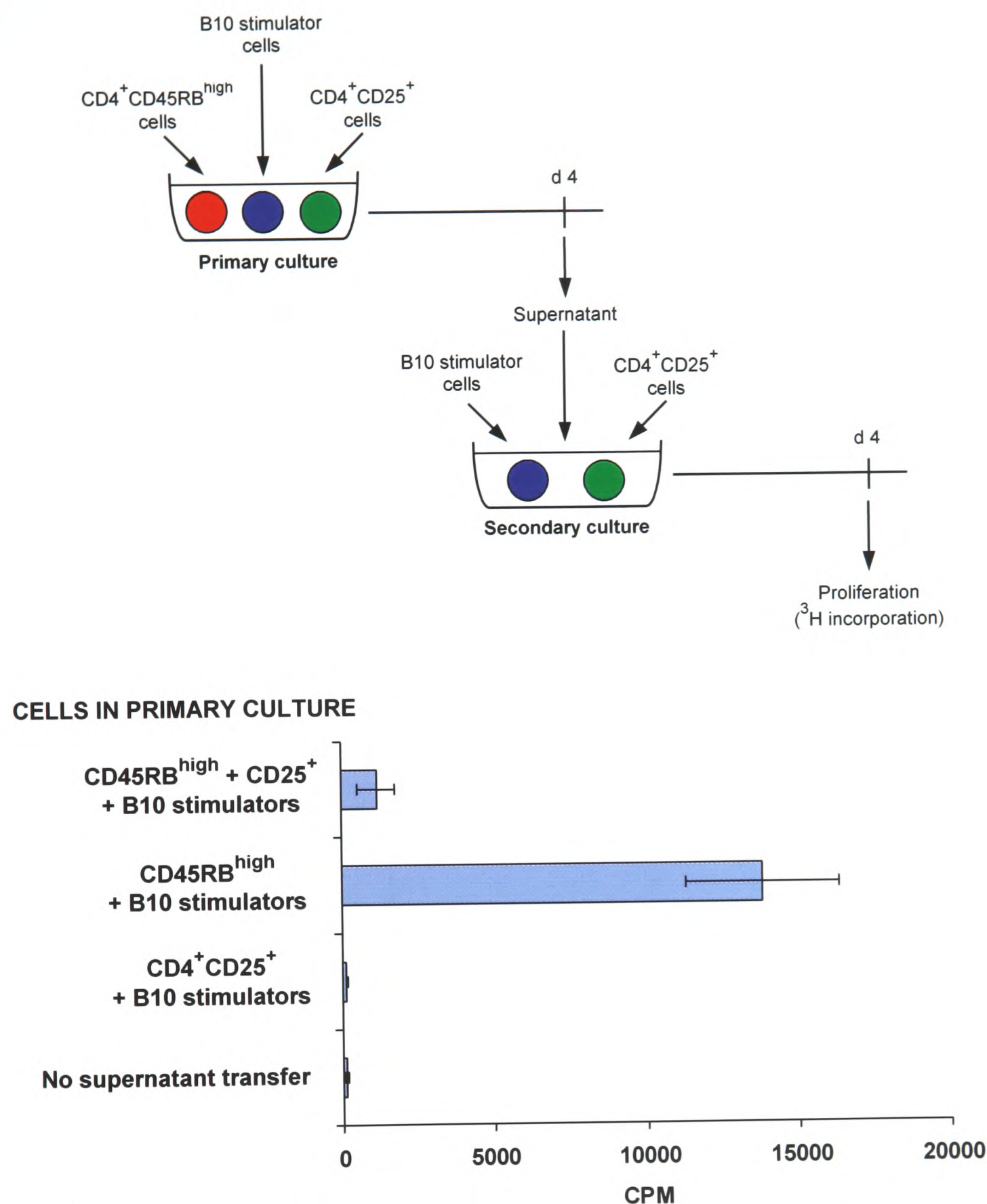
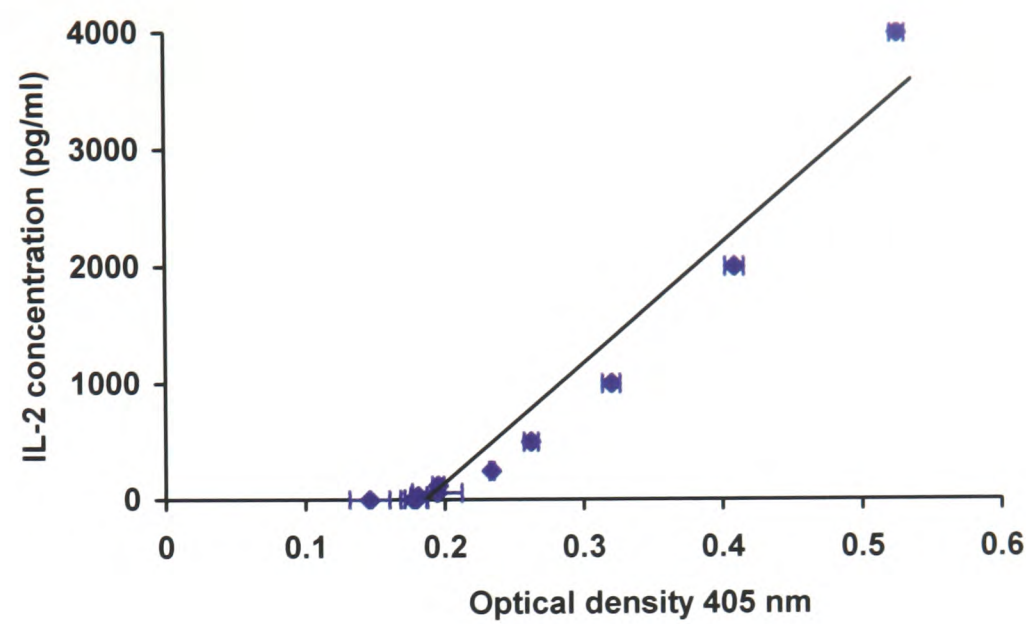


Figure 4.18. Co-culture of CD4⁺CD45RB^{high} cells with CD4⁺CD25⁺ cells and allogeneic splenocytes generates soluble factors that are able to overcome the non-proliferative state of CD4⁺CD25⁺ cells *in vitro*

Primary cultures (total volume 200 μ l per well) were set up containing 2×10^5 CD4⁺CD45RB^{high} and / or CD4⁺CD25⁺ cells from naïve CBA mice and stimulated with 5×10^5 irradiated B10 spleen cells. On day 4, 100 μ l of supernatant were transferred from these wells into secondary cultures (also with a total volume of 200 μ l per well) containing 2×10^5 fresh CD4⁺CD25⁺ cells and 5×10^5 irradiated B10 spleen cells. ³H-thymidine was added to the secondary cultures for the final 18 hours of culture and then ³H incorporation measured on day 4. Data are expressed as mean cpm $\pm$ standard deviation of triplicate cultures and are representative of two independent experiments.

a



b

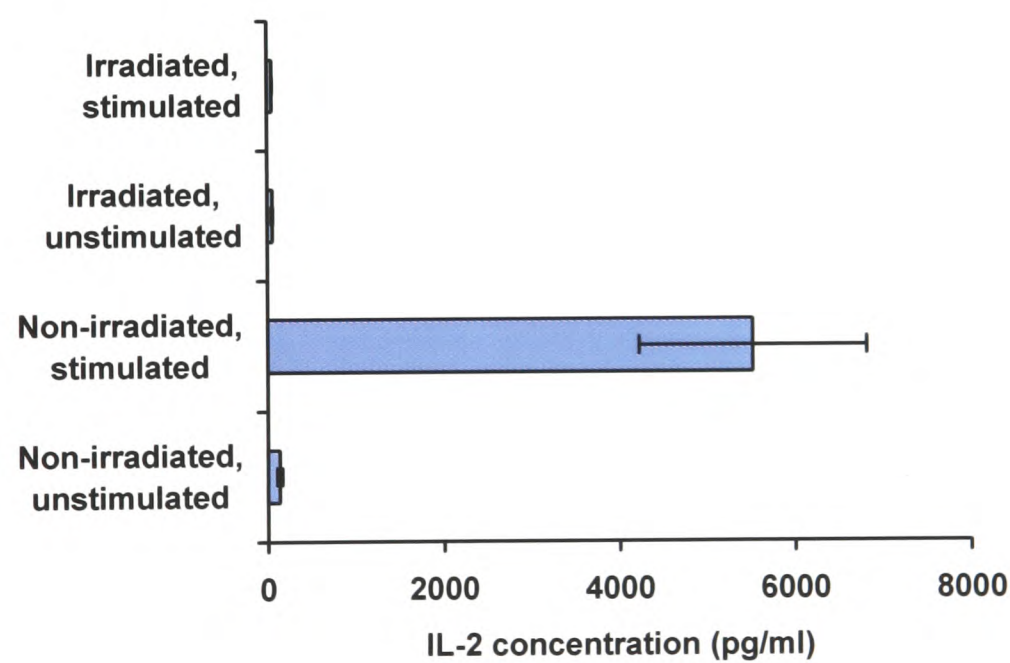


Figure 4.19. Irradiation prevents production of IL-2 by transgenic spleen cells 2×10^5 spleen cells from DES mice were cultured. In some cases the cells underwent 3600 rad irradiation prior to culture. Where indicated, cultures were stimulated by the addition of anti-CD3 and anti-CD28 antibodies (each at a final concentration of $2 \mu\text{g/ml}$). On day 3 culture supernatants were harvested and IL-2 concentration measured by ELISA. (a) IL-2 standard curve. (b) IL-2 concentration in culture supernatants. Results are presented as mean $\pm$ standard deviation of triplicate cultures and are representative of two independent experiments.

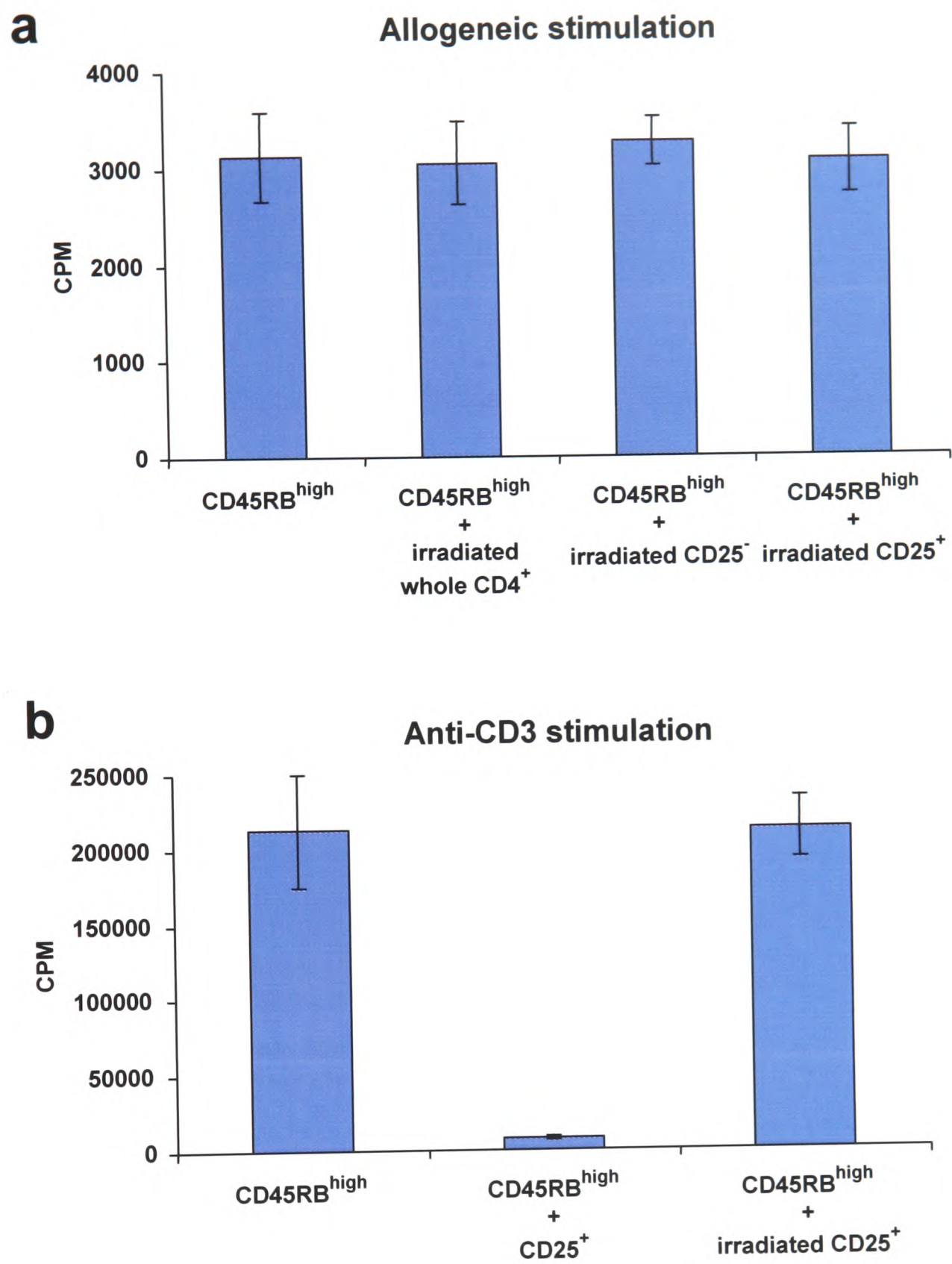


Figure 4.20. Irradiated CD4⁺CD25⁺ cells are unable to regulate *in vitro*
(a) 2×10^5 CD4⁺CD45RB^{high} cells from naïve CBA mice were cultured for 5 days with 5×10^5 irradiated B10 spleen cells together with 2×10^5 irradiated (3600 rad) whole CD4⁺, CD4⁺CD25⁻, or CD4⁺CD25⁺ cells from CBA mice pre-treated with 177/DST 28 days previously. (b) 2×10^5 CD4⁺CD45RB^{high} cells from naïve CBA mice were cultured for 4 days with anti-CD3 antibody (2 μ g/ml) and 5×10^5 irradiated CBA spleen cells together with 2×10^5 non-irradiated or irradiated (3600 rad) CD4⁺CD25⁺ cells from naïve CBA mice. In both cases ³H-thymidine was added to wells for the final 18 hours of culture and then ³H incorporation measured. Data are expressed as mean cpm $\pm$ standard deviation of triplicate cultures and are representative of two independent experiments.

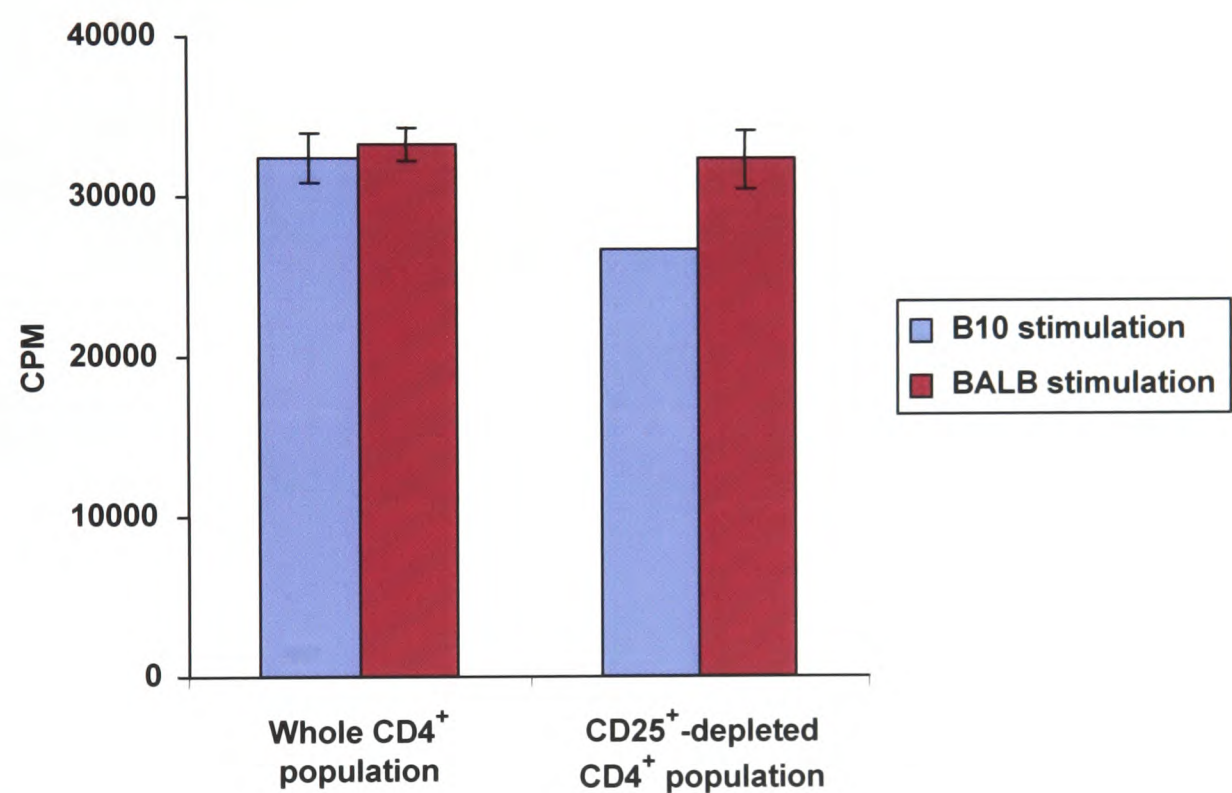


Figure 4.21. Depletion of the CD4⁺CD25⁺ population does not lead to augmented proliferation in response to allogeneic stimulation of CD4⁺ cells from 177/DST pre-treated mice

2×10^5 CD4⁺ or CD4⁺CD25⁻ cells from CBA mice pre-treated with 177/B10 DST were cultured for 5 days in the presence of 5×10^5 irradiated B10 or BALB spleen cells. ³H-thymidine was added to wells for the final 18 hours of culture and then ³H incorporation measured. Data are expressed as mean cpm $\pm$ standard deviation of triplicate cultures and are representative of two independent experiments.

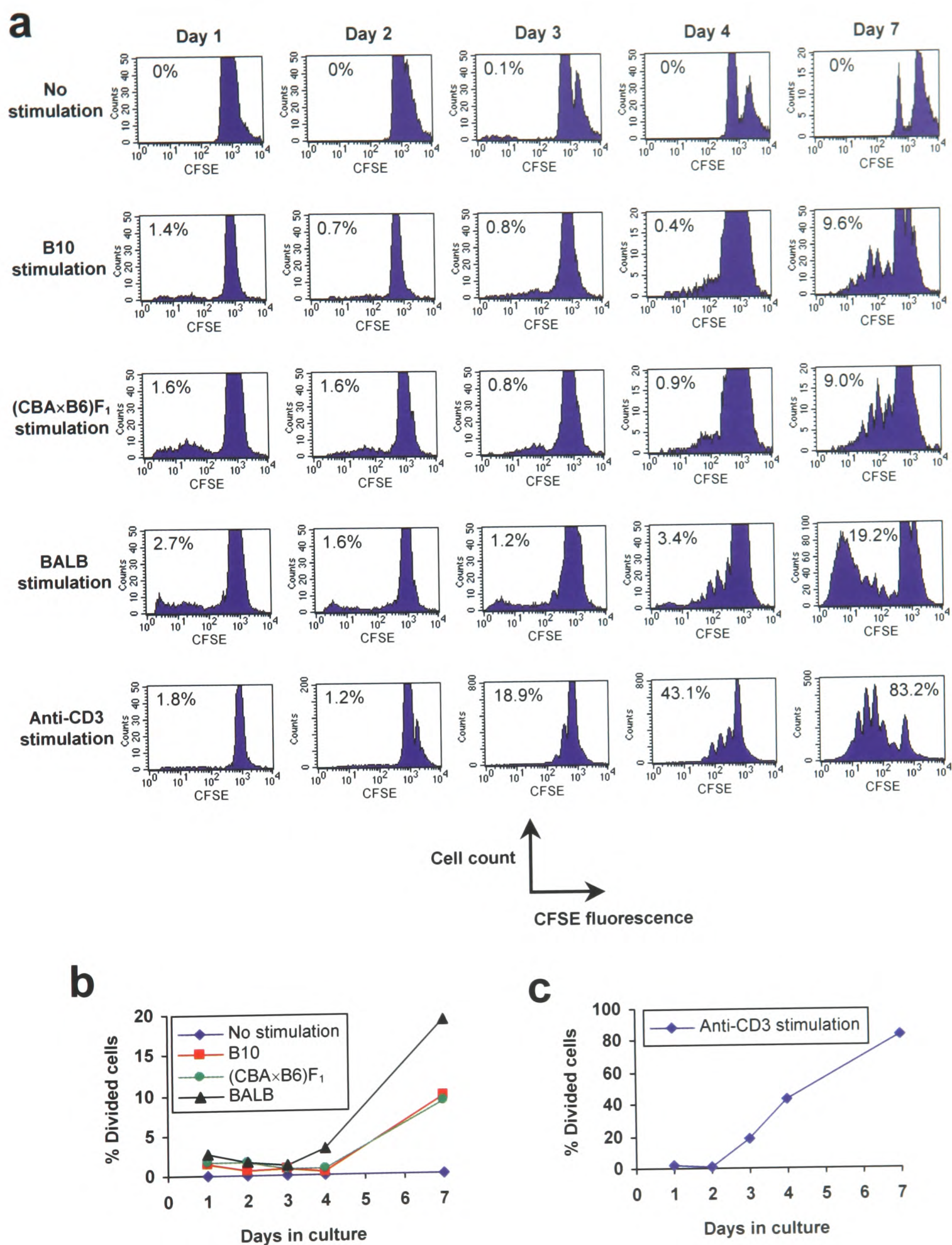


Figure 4.22. Time course of proliferation of CFSE-labelled $CD4^+CD45RB^{high}$ cells in response to allogeneic or anti-CD3 stimulation
 2×10^5 CFSE-labelled $CD4^+CD45RB^{high}$ responder cells from naïve CBA mice were stimulated with 5×10^5 irradiated allogeneic (B10, (CBA $\times$ B6) F_1 , or BALB) spleen cells or with anti-CD3 antibody (2 μ g/ml) in the presence of 5×10^5 irradiated CBA spleen cells. Cells were harvested at time points shown for flow cytometric analysis. (a) Histogram plots showing CFSE fluorescence profile of labelled responder cells. Percentages represent proportion of cells that have divided at least once. (b) and (c) Time course of proliferation of labelled responder cells in response to allogeneic (b) and anti-CD4 (c) stimulation. Data are representative of two independent experiments.

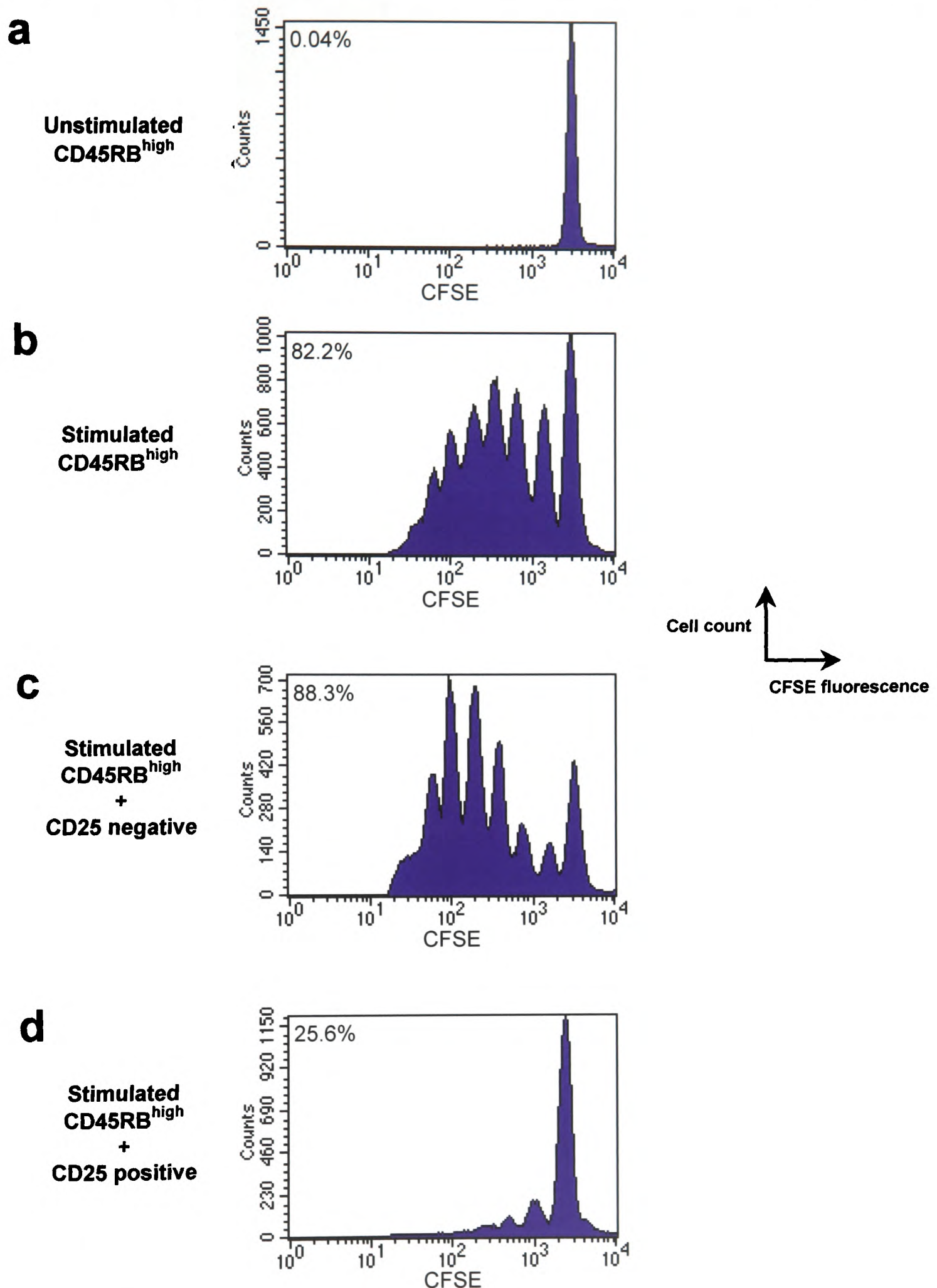


Figure 4.23. $CD4^+CD25^+$ cells can regulate the proliferation of CFSE-labelled $CD4^+CD45RB^{high}$ cells *in vitro*

CFSE-labelled $CD4^+CD45RB^{high}$ responder cells (2×10^5 cells per well) from naïve CBA mice were cultured for 4 days (a) alone; (b) stimulated with anti-CD3 antibody ($2 \mu\text{g/ml}$) and irradiated CBA spleen cells (5×10^5 cells per well); (c) with stimulation plus $CD4^+CD25^-$ cells from naïve CBA mice (2×10^5 cells per well); (d) with stimulation plus $CD4^+CD25^-$ cells from naïve CBA mice (2×10^5 cells per well). At the end of the culture period cells underwent flow cytometric analysis and results are presented as CFSE fluorescence profile of responder cells. Data are representative of three independent experiments.

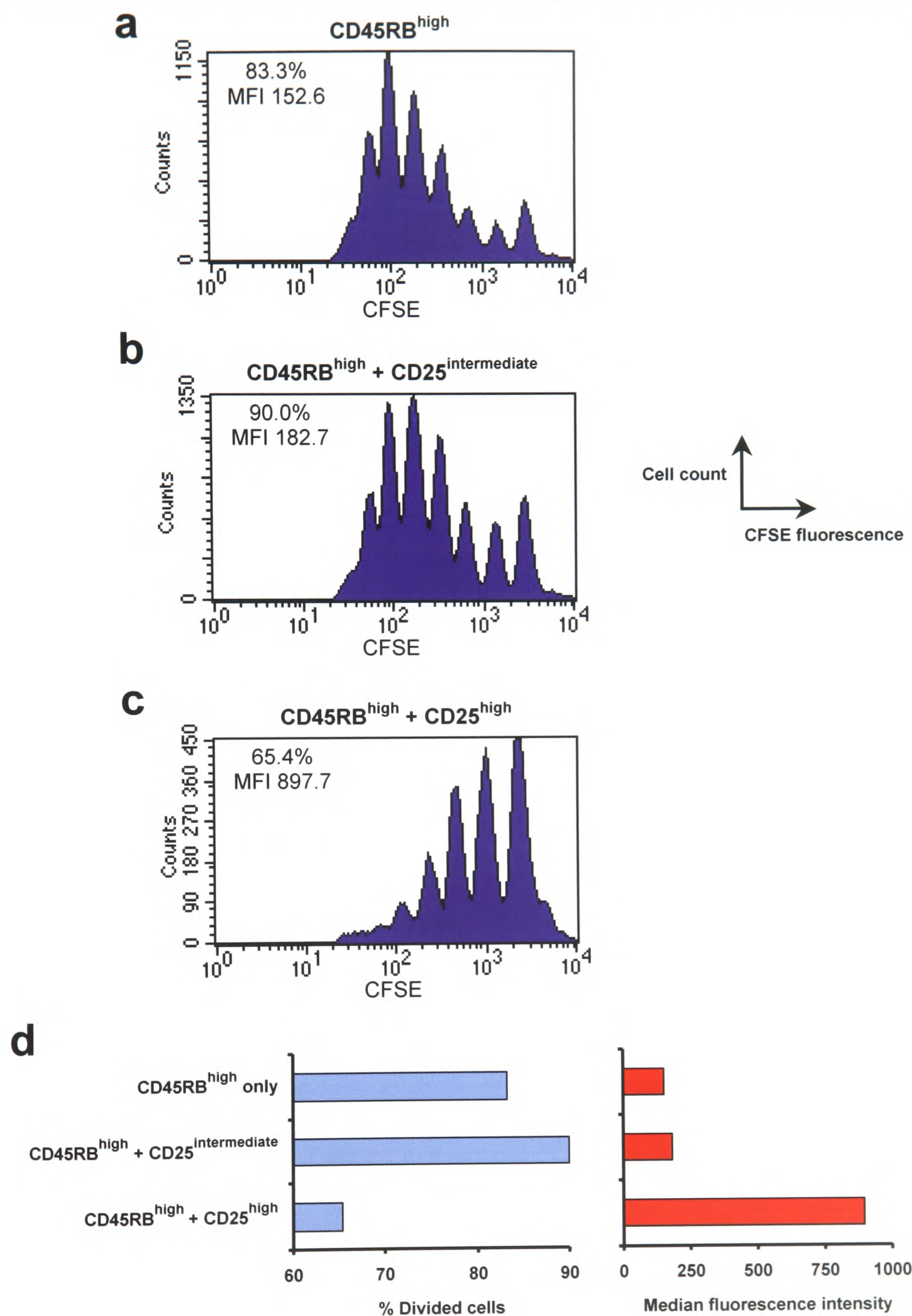


Figure 4.24. Regulatory effect of CD4⁺CD25^{intermediate} and CD4⁺CD25^{high} cells
(a) to (c) 2×10⁵ CFSE-labelled CD4⁺CD45RB^{high} cells were stimulated with anti-CD3 antibody (2 µg/ml) in the presence of irradiated CBA spleen cells (5×10⁵ per well) with or without 2×10⁵ CD4⁺CD25^{intermediate} or CD4⁺CD25^{high} cells from naïve CBA donors. Wells were harvested on day 4 for flow cytometric analysis. Percentages represent proportion of cells that have divided at least once, and MFI median CFSE fluorescence intensity of whole cell populations. (d) Percentage of cells that have divided (left) and median CFSE fluorescence intensity (right) of cells in plots (a) to (c). Data are representative of two independent experiments.

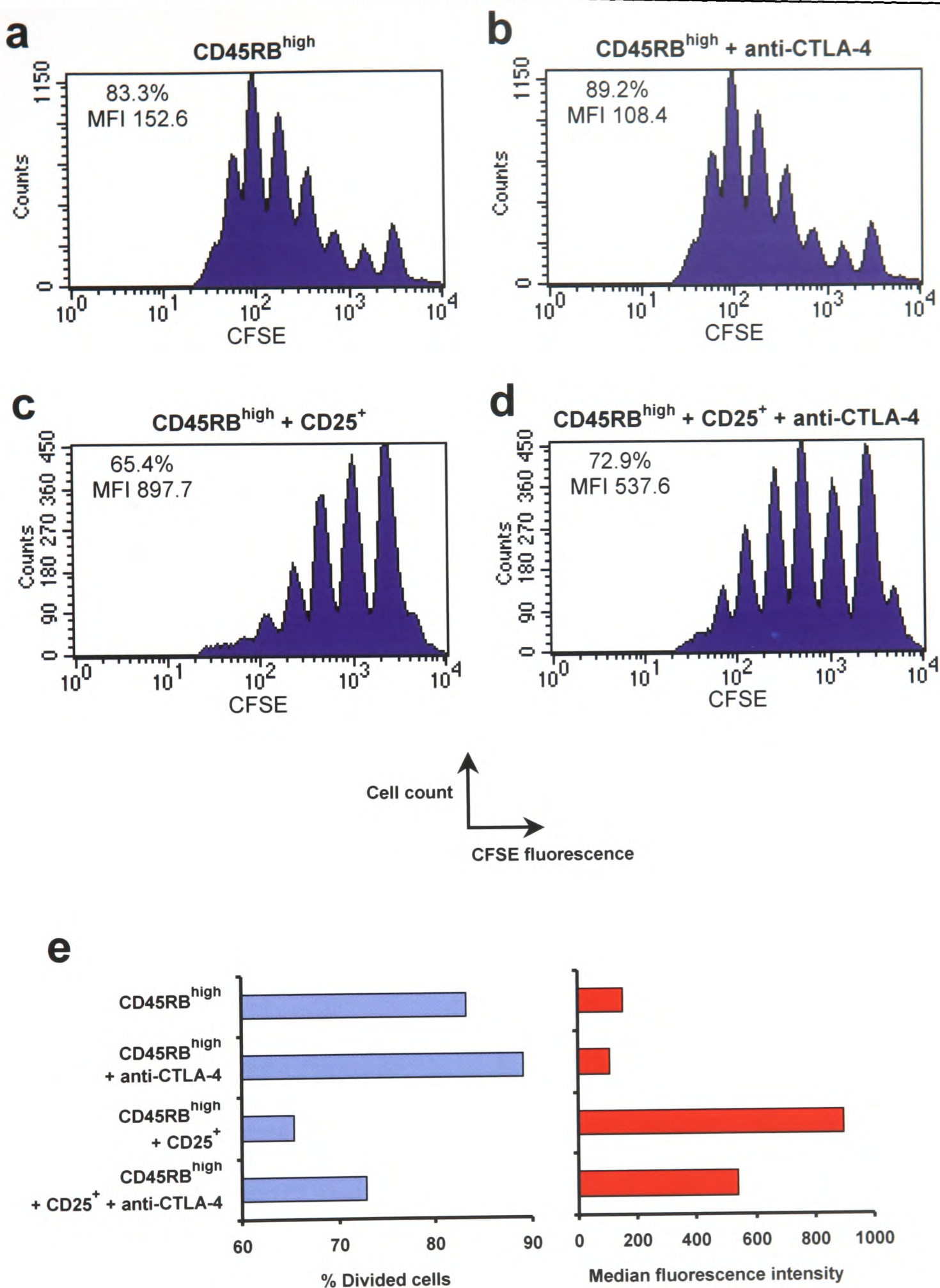


Figure 4.25. CTLA-4 blockade partially abrogates regulation *in vitro* by $CD4^+CD25^+$ cells

(a) to (d) 2×10^5 CFSE-labelled $CD4^+CD45RB^{high}$ responder cells were stimulated with anti-CD3 antibody (2 μ g/ml) in the presence of irradiated CBA spleen cells (5×10^5 per well) together with the combinations indicated of the anti-CTLA-4 antibody 4F10 (100 μ g/ml) and / or 2×10^5 $CD4^+CD25^+$ cells from naïve CBA donors. Wells were harvested on day 4 for flow cytometric analysis. (e) Percentage of responder cells that have divided (left) and median CFSE fluorescence intensity (right) of responder cells in plots (a) to (d). Data are representative of two independent experiments.

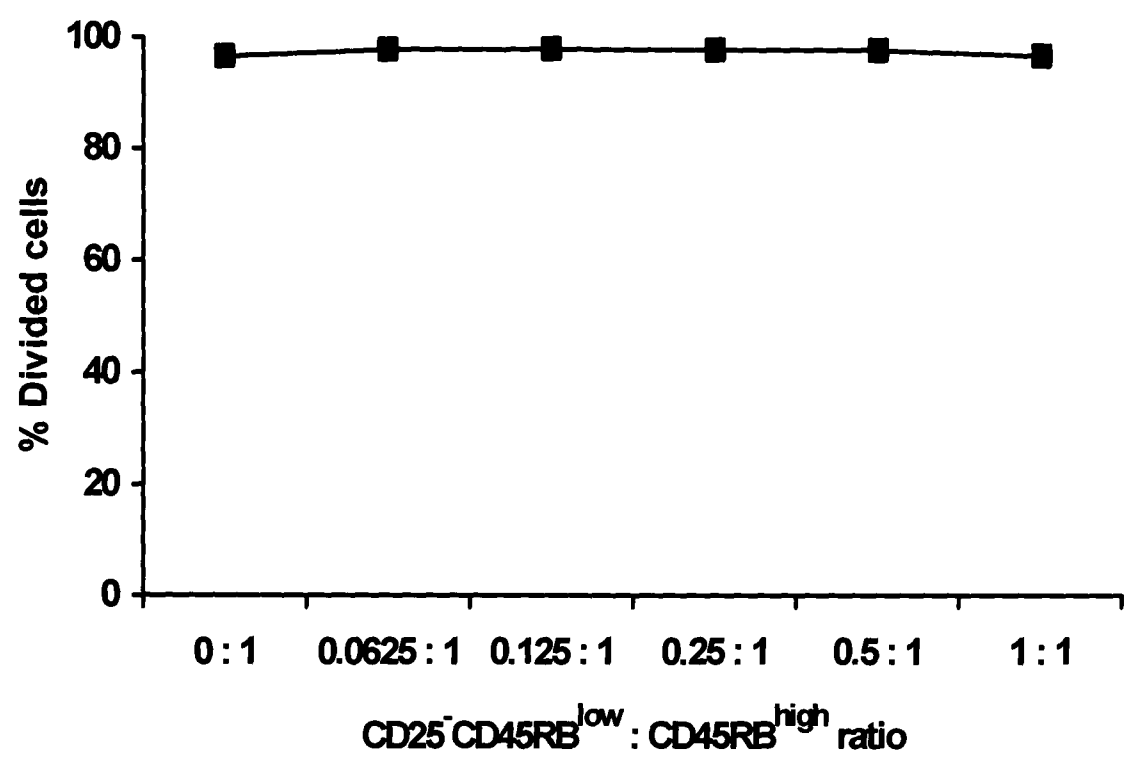


Figure 4.26. CD4⁺CD25⁻CD45RB^{low} cells do not possess regulatory capacity *in vitro*
2×10⁵ CFSE-labelled CD4⁺CD45RB^{high} cells from naïve CBA mice were cultured in the presence of anti-CD3 antibody (2 µg/ml) and 5×10⁵ irradiated CBA spleen cells together with CD4⁺CD25⁻CD45RB^{low} cells from naïve CBA mice at the ratios indicated. On day 4 cultures underwent flow cytometric analysis. Graph shows the percentage of labelled cells that have undergone at least one round of division. Data are representative of two independent experiments.

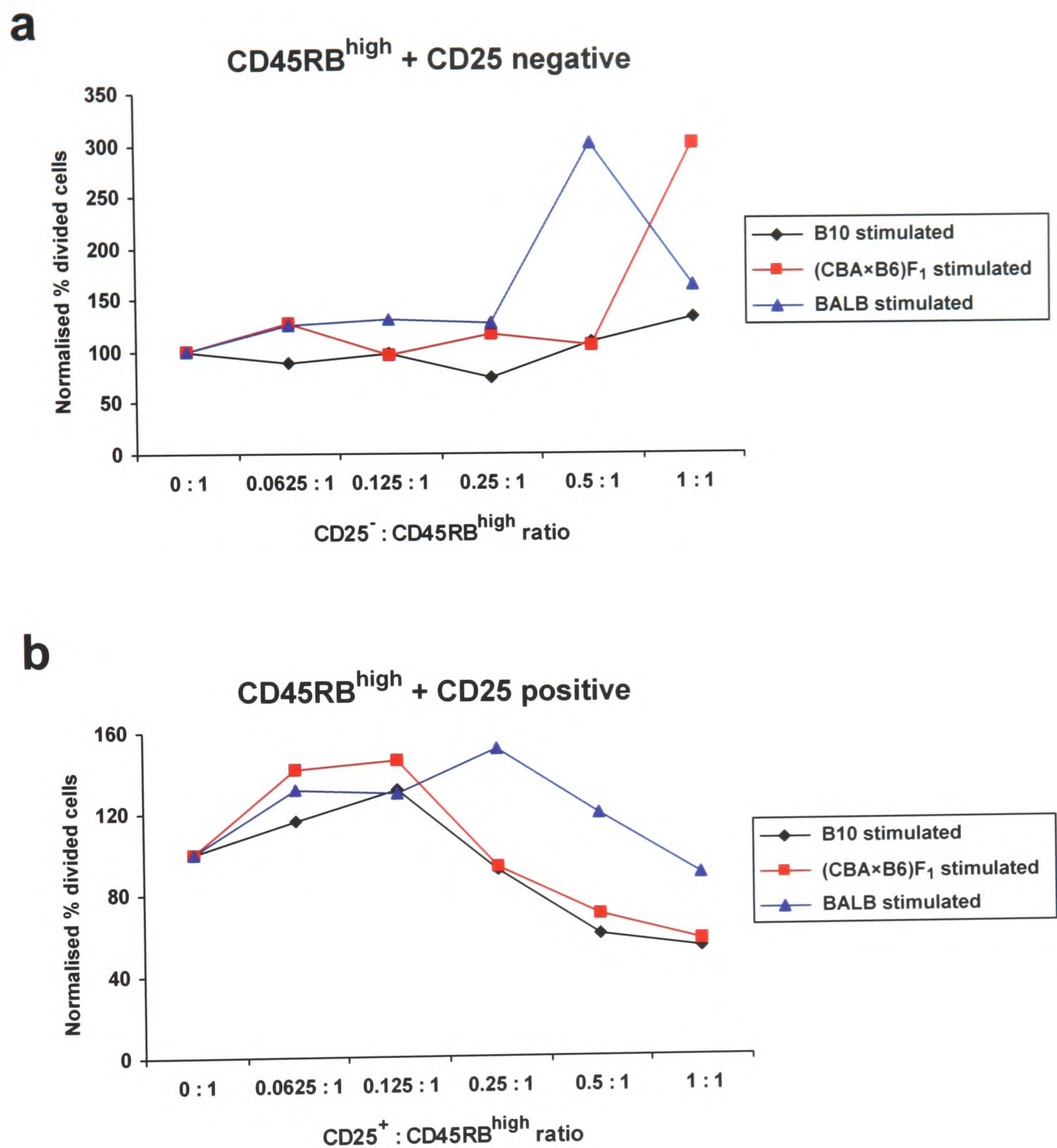


Figure 4.27. CD4⁺CD25⁺ cells from 177/DST pre-treated mice regulate proliferation of CD4⁺CD45RB^{high} cells in response to allogeneic stimulation *in vitro*

2×10⁵ CFSE-labelled CD4⁺CD45RB^{high} responder cells from naïve CBA mice were cultured in the presence of the indicated number of CD4⁺CD25⁻ (a) or CD4⁺CD25⁺ (b) cells from CBA mice pre-treated with 177/B10 DST. Cultures were stimulated with 5×10⁵ irradiated B10, (CBA×B6)F₁, or BALB spleen cells. Cells were harvested on day 7 for flow cytometric analysis. Graphs show normalised percentage of responder cells that have undergone cell division, 100% being responder cell division in the absence of CD4⁺CD25⁻ or CD4⁺CD25⁺ cells.

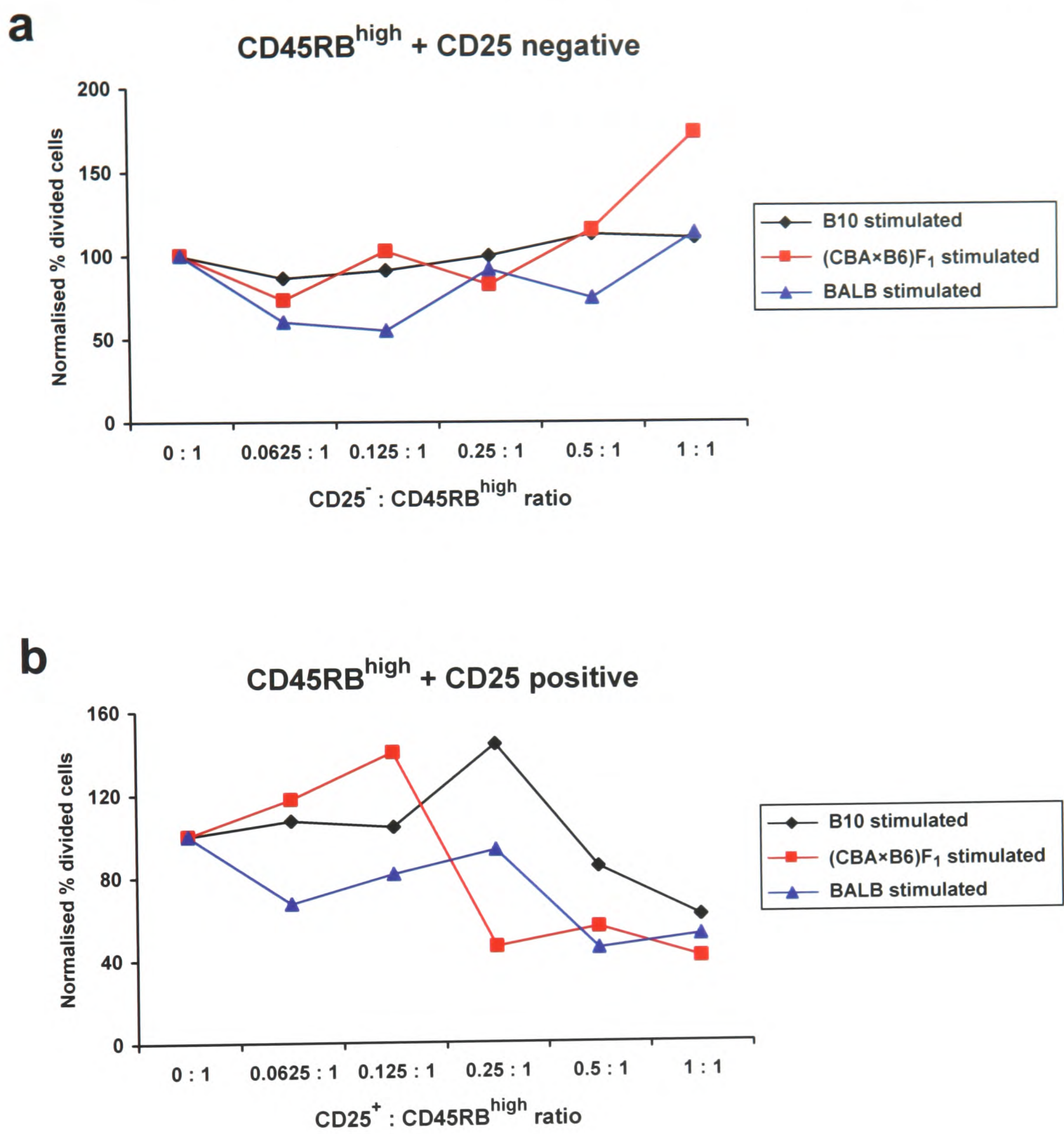


Figure 4.28. DST re-challenge overcomes antigen specificity of regulation *in vitro* by CD4⁺CD25⁺ cells from 177/DST pre-treated mice

2×10⁵ CFSE-labelled CD4⁺CD45RB^{high} responder cells from naïve CBA mice were cultured in the presence of the indicated number of CD4⁺CD25⁻ (a) or CD4⁺CD25⁺ (b) cells isolated on day +2 from CBA mice pre-treated on days -28 and -27 with 177/B10 DST followed by a second B10 DST on day 0. Cultures were stimulated with 5×10⁵ irradiated B10, (CBA×B6)F₁, or BALB spleen cells. Cells were harvested on day 7 for flow cytometric analysis. Graphs show normalised percentage of responder cells that have undergone cell division, 100% being responder cell division in the absence of CD4⁺CD25⁻ or CD4⁺CD25⁺ cells.

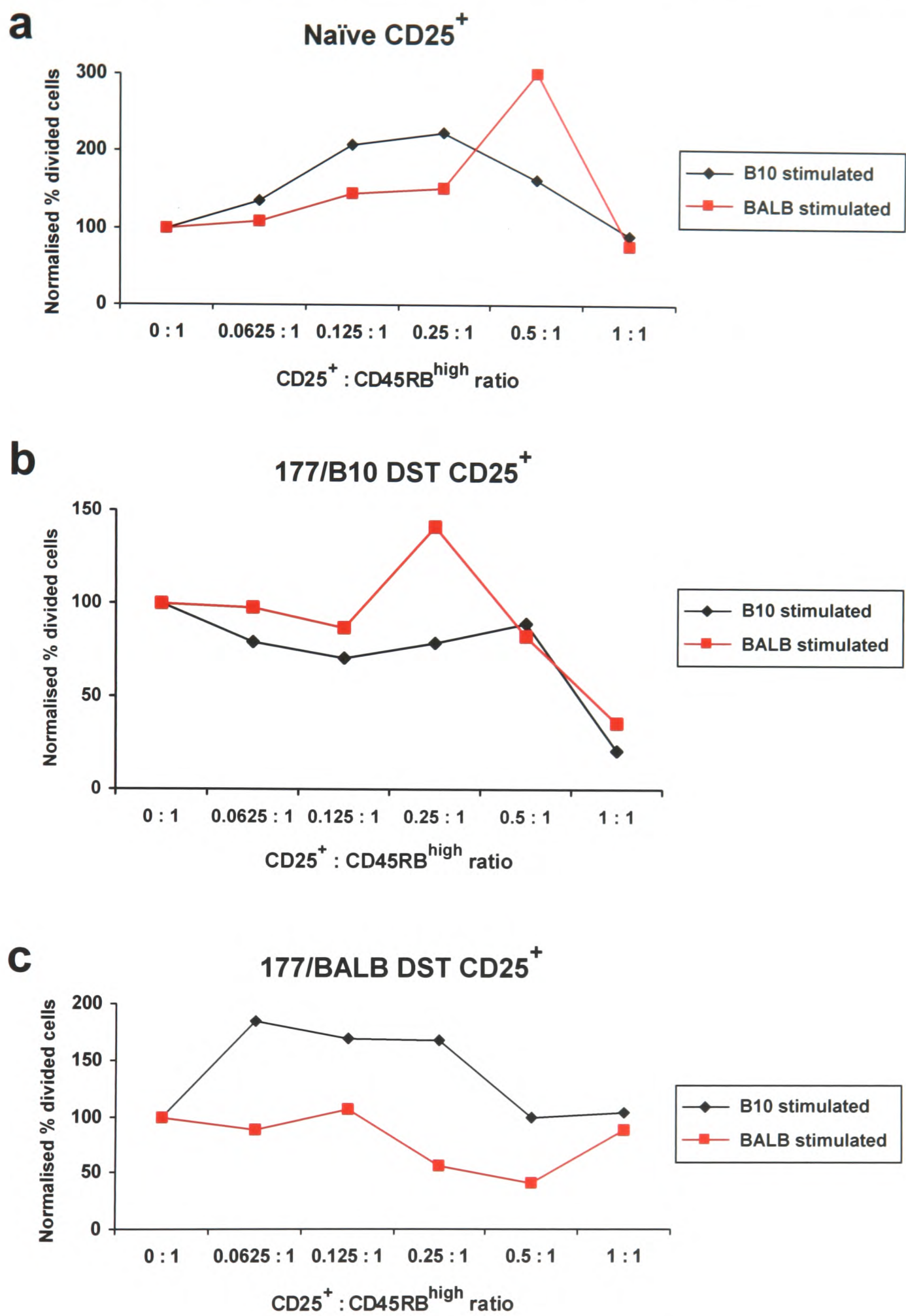


Figure 4.29. Effect of CD4⁺CD25⁺ cells from naïve and 177/BALB DST pre-treated mice on proliferation of CD4⁺CD45RB^{high} cells in response to allogeneic stimulation *in vitro*

2×10⁵ CFSE-labelled CD4⁺CD45RB^{high} responder cells from naïve CBA mice were cultured in the presence of the indicated number of CD4⁺CD25⁺ cells from (a) naïve CBA mice, (b) CBA mice pre-treated 28 days previously with 177/B10 DST, or (c) CBA mice pre-treated 28 days previously with 177/BALB DST. Cultures were stimulated with 5×10⁵ irradiated B10 or BALB spleen cells. Cells were harvested on day 7 for flow cytometric analysis. Graphs show normalised percentage of responder cells that have undergone cell division, 100% being responder cell division in the absence of CD4⁺CD25⁺ cells.

CHAPTER 5

PATHWAYS OF GENERATION OF

REGULATORY CELLS *IN VIVO*

5.1 Introduction

In Chapter 4 the *in vitro* properties of alloantigen-induced $CD4^+CD25^+$ Treg generated by the 177/DST pre-treatment protocol were examined. The remainder of this thesis will aim to study the characteristics of these cells in an *in vivo* setting. Chapter 6 will address some of the mechanistic aspects of Treg function, with particular emphasis on their activation and antigen specificity requirements. However, this chapter will examine an earlier aspect of Treg biology, namely the mechanisms by which these cells may be generated following 177/DST pre-treatment.

5.1.1 Intrathymic development of T cells

In order to gain some insight into the pathways by which 177/DST pre-treatment may generate $CD4^+CD25^+$ Treg, a brief consideration of the pathway via which normal $\alpha\beta$ T cells develop, reviewed in [171, 463-466], is required. $CD4^-CD8^-$ (“double negative”) $\alpha\beta$ TCR⁺ precursors leave the bone marrow and enter the thymus. Within the thymus these cells upregulate both CD4 and CD8 to become “double positive” cells; during this process they also begin to express the $\alpha\beta$ TCR. By default these double positive cells undergo programmed death unless rescued by an appropriate signal provided by contact between the TCR and self peptide-MHC complexes presented by thymic cortical epithelial cells. If the affinity of this interaction is sufficiently high death is prevented, the process of positive selection [163, 164]. Cells that survive the positive selection process then downregulate expression of CD4 or CD8 (depending on whether the positive selection signal was provided predominantly by MHC class I or class II respectively) to become “single positive” $CD8^+$ or $CD4^+$ cells [163, 467-470].

Following the process of positive selection, single positive cells then undergo a negative selection process. Once again, this is dependent upon contact between the TCR and self peptide-MHC complexes. However, in contrast to positive selection, cells that undergo high affinity interactions are deleted during the process of negative selection [165-169], a process that is IL-2-dependent [471] and may be glucocorticoid-mediated [472]. Thus, cells that survive both positive and negative selection and are subsequently exported to the periphery possess TCR that have experienced interactions of intermediate affinity with peptide-MHC complexes within the thymus (Figure 1.5a,b).

The processes of positive and negative selection play an important role in ensuring that mature T cells exported from the thymus respond appropriately when presented with antigen in the periphery. Positive selection ensures that encounter of antigen presented in the context of self MHC can provide a signal sufficiently strong to lead to T cell activation. In contrast, negative selection prevents the export of T cells that may be activated by self peptide antigens and thus potentially mediate autoimmunity; negative selection therefore forms the basis for central tolerance. Recently it has become apparent that central tolerance may be responsible for controlling responses to a wider range of self antigens than previously thought, since it has been shown that self antigens that had thought to be expressed only in the periphery can be expressed within the thymus under the control of the AIRE (autoimmune regulator) gene [473, 474]. Nevertheless, it is recognised that intrathymic negative selection is not the only way in which self tolerance is achieved, and other mechanisms also exist including peripheral deletion of autoreactive T cells [172-174], ignorance of antigens to which the immune system is not normally exposed [193-196], anergy [190-192], and development of Treg.

5.1.2 *Intrathymic development of naturally-occurring CD4⁺CD25⁺ Treg*

Published studies have demonstrated that neonatal thymectomy can result in the development of autoimmune disease [475] that may be prevented by the adoptive transfer of thymocytes [476, 477]. More recent observations that CD4⁺CD25⁺ cells isolated from the thymus possess regulatory properties in both mouse [277] and human [304] studies have led to speculation that naturally-occurring CD4⁺CD25⁺ Treg with the capacity to control responses against self may be generated within the thymus. In order to address this question, Jordan and colleagues used a transgenic system in which mice with TCR specific for haemagglutinin were crossed with animals expressing haemagglutinin [321]. They found that haemagglutinin-specific CD4⁺CD25⁺ Treg developed within the thymus from cells bearing TCR with high affinity for haemagglutinin, but that cells with low affinity TCR did not develop along this pathway. Other groups have similarly shown that intrathymic expression of appropriate antigen in transgenic mice can lead to the development of CD4⁺CD25⁺ Treg within the thymus [317, 323, 327]. It has been demonstrated that the intrathymic positive selection of CD4⁺CD25⁺ Treg is mediated by MHC class II positive thymic cortical epithelium [322]. The finding that this intrathymic selection requires high affinity interactions between TCR and peptide-MHC complexes (Figure 1.5c) is also supported by the findings of others [323-325]; indeed, it has been demonstrated that this affinity may, under other circumstances, be sufficient to lead to the deletion by negative selection of autoreactive T cells [323, 478]. Further evidence to support the hypothesis that, during intrathymic development, interaction between TCR and peptide-MHC complexes with sufficient affinity to lead to T cell activation may result in Treg generation is provided by the observations of Stephens *et al.*, who

demonstrated that lowering the activation threshold of T cells within the thymus biases towards the development of CD4⁺CD25⁺ cells [328].

IL-2 appears to play an important role in the intrathymic generation of CD4⁺CD25⁺ Treg. Papiernik and colleagues have demonstrated that IL-2 knockout mice lack CD4⁺CD25⁺ cells both within the thymus and the periphery [320]. Similarly, Malek *et al.* have shown that mice deficient in CD122 lack CD4⁺CD25⁺ cells and develop autoimmune disease, but that transgenic expression of CD122 within the thymus restores the presence of the CD4⁺CD25⁺ population and prevents disease development [326]. A further factor that may contribute to the intrathymic selection of CD4⁺CD25⁺ cells is expression of GITR (discussed in Chapters 1 and 3). Since GITR positive cells are less susceptible to apoptosis, it has been postulated that resistance to intrathymic deletion is conferred by expression of this molecule [312]. This may provide a mechanism by which the fate of immature T cells that possess receptors with high affinity for self peptide-MHC complexes is determined, namely either deletion through negative selection or development into CD4⁺CD25⁺ Treg.

5.1.3 Extrathymic development of CD4⁺CD25⁺ Treg

Although the data described above demonstrate that CD4⁺CD25⁺ Treg can develop within the thymus, they do not address the issue of whether such Treg are also generated in the periphery. Several studies have shown that administration of antigen by the oral route can lead to the development of CD4⁺CD25⁺ Treg [309, 330-332]. Similarly, Taams *et al.* found that normal humans contain CD4⁺CD25⁺ Treg specific for dietary antigens [479]. They postulated that, since these dietary antigens are unlikely to enter the thymus, it is probable that CD4⁺CD25⁺ Treg can develop in the

periphery. However these data provide circumstantial rather than direct evidence for extrathymic generation.

One approach to addressing this issue is to determine whether mature peripheral $CD4^+CD25^-$ cells can be converted to $CD4^+CD25^+$ Treg. Although work both in our laboratory (V. Oliveira and B. Sawitzki, unpublished observations) and by others [334] has shown that this is possible *in vitro*, there is to date no direct evidence to demonstrate that this can occur *in vivo*.

5.1.3 Aims of this chapter

The 177/DST model examined in this thesis, in which pre-treatment with allogeneic blood transfusion under the cover of anti-CD4 antibody leads to the generation of $CD4^+CD25^+$ Treg, provides a robust system to study the mechanisms by which peripheral alloantigen administration generates these Treg. We speculate that there are three theoretical mechanisms by which this may occur [480] (Figure 5.1). Firstly, alloantigen may migrate from the periphery to the thymus where it then mediates the positive selection of $CD4^+CD25^+$ Treg. Secondly, alloantigen may act in the periphery to convert “normal” $CD4^+$ T cells, i.e. cells that did not leave the thymus already committed as $CD4^+CD25^+$ Treg (predominantly $CD25^-$), to a $CD4^+CD25^+$ Treg phenotype. Finally, alloantigen may act in the periphery to expand a population of naturally-occurring $CD4^+CD25^+$ Treg generated within the thymus that cross-react with alloantigen. The aim of this chapter was to examine which of these theoretical pathways may be operating *in vivo* in the 177/DST system.

5.2 Results

5.2.1 *CD4⁺CD25⁺ Treg can be generated in a thymus-independent process*

The first question considered here is whether generation of CD4⁺CD25⁺ Treg by the 177/DST pre-treatment protocol might be dependent upon migration of donor antigen into the thymus and subsequent positive selection of donor-reactive CD25⁺CD4⁺ Treg. CBA mice were therefore thymectomised prior to receiving 177/DST pre-treatment, and the ability of CD4⁺CD25⁺ cells from these animals to regulate the rejection of skin allografts mediated by naive CD4⁺CD45RB^{high} cells was then tested *in vivo* (Figure 5.2). While mice reconstituted with CD4⁺CD45RB^{high} cells alone acutely rejected their B10 skin allografts (MST = 16 days, n = 7), co-transfer of CD4⁺CD25⁺ cells from athymic donors led to long term graft survival in the majority of cases (MST > 100 days, n = 6; *P* = 0.008), clearly demonstrating that the generation of CD4⁺CD25⁺ Treg by the administration of 177/DST is not thymus-dependent.

5.2.2 *The monoclonal antibody PC61 depletes CD25⁺ cells*

Having established that generation of CD4⁺CD25⁺ Treg can occur in the periphery in a thymus-independent process, the next question to consider was whether this process is reliant upon the expansion of pre-existing CD4⁺CD25⁺ cells that cross-react with alloantigen. One way to address this issue would be to deplete CD25⁺ cells from mice prior to pre-treating them with 177/DST. It has previously been demonstrated that administration of the anti-CD25 mAb PC61 to normal mice leads to the loss of detectable CD25⁺ cells from the periphery [294]. However, although this may be due to the deletion of CD25⁺ cells, it is also possible that PC61 instead leads to the modulation of CD25 from the cell surface, which would give a similar result on flow cytometric analysis. In order to distinguish between these two possibilities PC61 was

administered to mice and seven days later splenocytes were stained for CD25 expression both on the surface and intracellularly using the anti-CD25 antibody 7D4, which recognises a distinct epitope from PC61 [481]. Figure 5.3 depicts results obtained from 1 of 2 mice in each group from 1 of 2 independent experiments. Among splenocytes isolated from normal CBA mice, a mean of $2.38\% \pm 0.07$ of cells expressed CD25 both on the surface and intracellularly, whereas $3.20\% \pm 0.17$ showed intracellular expression only (Figure 5.3a). If PC61 treatment led to modulation of CD25 from the cell surface, it would be predicted that PC61-treated animals would show a loss of the population expressing surface CD25, but that the proportion of cells showing only intracellular expression would increase by a corresponding percentage. Conversely, if PC61 depleted CD25⁺ cells, then treated animals would again show a loss of the population expressing surface CD25 but without any corresponding increase in the number of cells showing intracellular expression. As shown in Figure 5.3b, mice that received PC61 did indeed show a loss of cells expressing surface CD25 ($0.35\% \pm 0.07$, $P < 0.05$ compared to naïve group); however, the proportion of cells expressing intracellular CD25 ($3.66\% \pm 0.09$, $P = 0.14$) was indistinguishable from naïve animals, demonstrating that PC61 does indeed deplete CD25⁺ cells.

5.2.3 *CD4⁺CD25⁺ Treg can be generated in the absence of CD25⁺ precursors*

The fact that PC61 depletes CD25⁺ cells allowed investigation of the possibility that the generation of alloantigen-induced Treg depends on the peripheral expansion of cross-reactive naturally-occurring CD4⁺CD25⁺ Treg generated in thymus. If this were the case then depletion of these thymic emigrants prior to administration of 177/DST should render this protocol ineffective. CBA mice were therefore thymectomised in

order to prevent export of new $CD4^+CD25^+$ thymic emigrants, given PC61 to deplete $CD25^+$ cells, and then rested for three weeks to allow antibody clearance [294] before receiving pre-treatment with 177/DST. As shown in Figure 5.4, $CD4^+CD25^+$ cells taken from these cell donors following 177/DST pre-treatment maintained their ability to regulate B10 skin allograft rejection mediated by naïve $CD4^+CD45RB^{high}$ cells in CBA-Rag recipients (MST > 100 days, $n = 5$; $P = 0.013$ compared to mice reconstituted with $CD4^+CD45RB^{high}$ cells alone), clearly showing that alloantigen-induced $CD4^+CD25^+$ Treg can be generated in the absence of pre-existing $CD25^+$ T cells.

5.2.4 Treg can be generated from $CD4^+CD25^-$ precursors

As an alternative approach to investigating whether alloantigen-induced Treg can be generated in the absence of $CD25^+$ precursors, CBA-Rag mice were reconstituted with only $CD4^+CD25^-$ cells and then given 177/DST pre-treatment. Then, in order to test for the presence of Treg with the capacity to suppress graft rejection mediated by effector cells, 28 days after the 177/DST pre-treatment these animals received a dose of naïve $CD4^+CD45RB^{high}$ cells followed by a B10 skin allograft the next day (Figure 5.5). Control mice reconstituted with $CD4^+CD25^-$ and $CD4^+CD45RB^{high}$ cells but not given the 177/DST protocol all rejected their B10 skin grafts acutely (MST = 9 days, $n = 5$), whereas animals in the group that did receive 177/DST all accepted their grafts long term (MST > 100 days, $n = 4$; $P = 0.007$). This graft acceptance cannot have been due to effects such as deletion of B10-reactive cells in the 177/DST group, because $CD4^+CD45RB^{high}$ cells from naïve mice were given the day prior to skin transplantation to provide an abundant source of effector cells. The fact that regulation

was seen in this situation clearly confirms that Treg can be generated from CD4⁺CD25⁻ precursors.

5.3 Discussion

The thymus is well known to play an important role in achieving self-tolerance, and several studies have shown that administration of exogenous antigen directly into the thymus can lead to the development of tolerance [482, 483]. More specifically, in the transplantation setting intrathymic alloantigen administration has been shown to prolong the survival of rat liver [484], cardiac [485-490], pancreatic islet [491], small bowel [492], and kidney [493-495], and mouse cardiac [496-499] and skin [327, 500] allografts. Even in models where allograft acceptance is achieved by means other than direct intrathymic antigen administration, the thymus may play an important role in this process. This is exemplified by the miniature swine model described by Sachs and colleagues, in which thymus-dependent mechanisms facilitate the development of tolerance to cardiac [501] and renal [501-503] allografts. It has been shown that donor alloantigen can be detected in the thymuses of rodents that have received cardiac [504, 505] and liver [506] allografts. However, it is possible that this represents an epiphenomenon rather than implying that antigen migration to the thymus plays a role in prolonging graft survival. Indeed, in the liver study [506], Kobayashi *et al.* found that although donor alloantigen could be detected within the thymus, this process was not necessary for the development of graft tolerance. Similarly, in one of the cardiac allograft models, the authors demonstrated that intrathymic mechanisms alone were not sufficient for the development of tolerance [505].

The observation that encounter of antigen by immature T cells during thymic development can result in negative selection [165] led to the hypothesis that tolerance induced by intrathymic antigen administration is mediated by the clonal deletion of developing T cells. However, although deletion of donor-reactive T cells has been demonstrated following intrathymic alloantigen administration [484, 496-498, 507], it has also been established that other, non-deletional mechanisms contributing to tolerance also occur. Previous work in our laboratory has shown that acceptance of mouse cardiac allografts can be induced by intrathymic alloantigen administration in the absence of intrathymic deletion of donor-reactive cells [497] and has suggested that mechanisms such as the generation of regulatory cells may play a role [499]. This is supported by the observation of Trani and colleagues that intrathymic administration of donor peptides can lead to the development of CD4⁺CD25⁺ cells capable of preventing the rejection of skin grafts mismatched for minor antigens [327].

The finding that CD4⁺CD25⁺ cells from athymic mice pre-treated with 177/DST can regulate skin allograft rejection (Figure 5.2) demonstrates that generation of the Treg population in this model is not thymus-dependent and can take place in the periphery, but this result does not exclude the possibility that intrathymic Treg generation can occur in parallel. However, this seems unlikely since previous work in our laboratory using PCR analysis has been unable to detect donor alloantigen within the thymuses of mice pre-treated with the 177/DST protocol [508]. This may seem to be at odds with the data cited above in which donor alloantigen could be detected in the thymuses of rodents with long term surviving allografts [504-506]. However, in those situations the grafts provided a continuous supply of alloantigen (possibly including cells within the graft with the capacity to migrate to the thymus),

whereas this is not the case in the 177/DST protocol in which donor alloantigen is administered as a single intravenous bolus; this is likely to be of insufficient quantity and / or longevity to migrate to the thymus and mediate positive selection of Treg. Nevertheless, one potential approach to determine whether intrathymic selection does occur in the 177/DST protocol would be to isolate thymic $CD4^+CD8^-CD25^+$ ($CD4$ single positive) cells, the population that would migrate to the periphery as $CD4^+CD25^+$ thymic emigrants, from naïve and 177/DST pre-treated mice and compare their ability to regulate skin allograft rejection upon adoptive transfer. If the latter were able to regulate but the former were not, this would support the hypothesis that intrathymic selection does occur. However, attempts to purify such populations yielded cells in insufficient quantity to allow adoptive transfer.

Since it is well established that a large proportion of T cells cross-react with alloantigen [32, 39, 509, 510], we hypothesised that naturally-occurring $CD4^+CD25^+$ Treg generated within the thymus may also show such cross-reactivity, and that one mechanism by which the 177/DST protocol may generate Treg may be to expand populations of autospecific $CD4^+CD25^+$ thymic emigrants that are also reactive to donor alloantigen. The finding that PC61 depletes $CD25^+$ cells rather than modulating expression of CD25 from the cell surface (Figure 5.3) allowed the demonstration that $CD4^+CD25^+$ precursors are not necessary for the generation of $CD4^+CD25^+$ Treg following 177/DST pre-treatment (Figure 5.4). This is supported by the finding that 177/DST can induce regulation in CBA-Rag mice containing only $CD25^-CD4^+$ cells (Figure 5.5), although in this latter case we have not demonstrated that the Treg generated are $CD25^+$. Although it has been suggested that generation of $CD4^+CD25^+$ Treg from $CD4^+CD25^-$ precursors is likely to take place *in vivo* [479] and has been demonstrated to be possible *in vitro* [334], to our knowledge these data provide the

first direct demonstration that this can occur *in vivo*. However, as with the experiments examining the role of the thymus in the generation of alloantigen-induced Treg, these data do not exclude the possibility that expansion of naturally-occurring CD4⁺CD25⁺ Treg that cross-react with donor alloantigen also takes place. Again, a potential way of addressing this question (if cells could be purified in sufficient quantity) would be to repeat the experiments in which CBA-Rag mice were reconstituted with cells from naïve CBA donors and then given 177/DST, but using CD4⁺CD8⁻CD25⁺ (CD4 single positive) thymocytes instead of peripheral CD4⁺CD25⁻ cells. If Treg capable of preventing skin allograft rejection could not be generated from these thymocytes, it would suggest that expansion of cross-reactive cells is unlikely to make a significant contribution to the development of Treg in the 177/DST system.

In summary, previous work has demonstrated the importance of intrathymic positive selection in the development of naturally-occurring CD4⁺CD25⁺ Treg specific for self peptide [277, 304, 317, 320-328, 478]. However, the mechanisms by which Treg can be generated against non-self antigens have been less thoroughly investigated. In the 177/DST model, peripheral administration of alloantigen leads to the development of CD4⁺CD25⁺ Treg which, as will be demonstrated in Chapter 6, are alloantigen-specific. This model therefore provides a robust system to study the generation of CD4⁺CD25⁺ Treg following the peripheral administration of defined antigen. The results presented in this chapter clearly demonstrate that such Treg can be generated by the conversion of peripheral CD4⁺CD25⁻ T cells to a CD4⁺CD25⁺ Treg phenotype. Given that previous work in our laboratory has been unable to demonstrate donor alloantigen within the thymus following 177/DST pre-treatment [508], it seems unlikely that intrathymic positive selection makes a significant

contribution to the generation of Treg in this model, although further investigation would be required to confirm this. Similarly, on the basis of the results presented here the possibility that 177/DST pre-treatment leads to the expansion of naturally-occurring $CD4^+CD25^+$ Treg that cross-react with alloantigen cannot be excluded. Nevertheless, the observation that peripheral $CD4^+CD25^-$ T cells can be converted to a $CD4^+CD25^+$ Treg phenotype may have important implications for the design of protocols for potential clinical use since it clearly demonstrates that *in vivo* generation of Treg by peripheral antigen administration is possible in adults with limited thymic function. This may have relevance not only to the field of transplantation, but also to other areas where induction of tolerance to defined antigens would be useful, such as therapy of autoimmune disease.

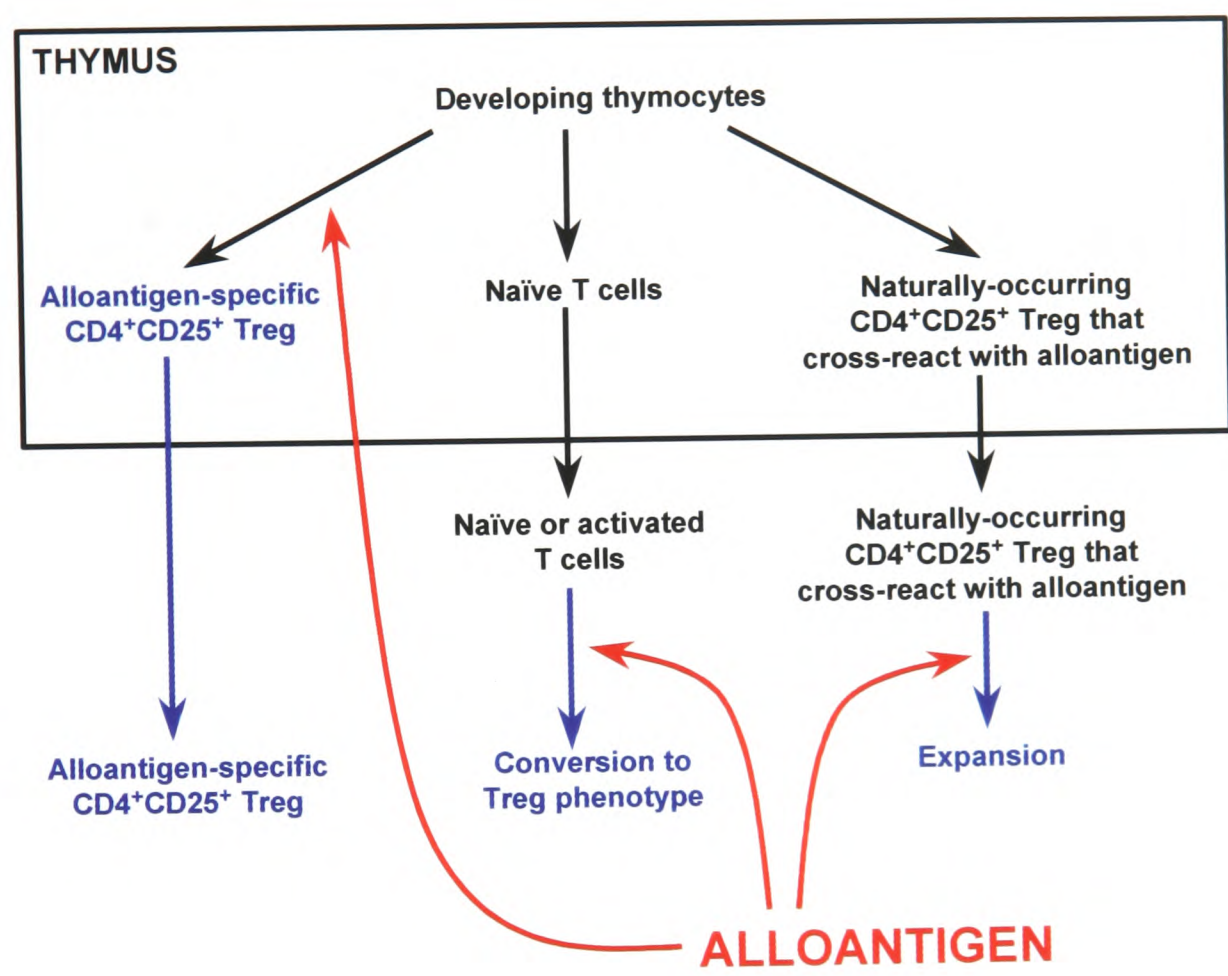
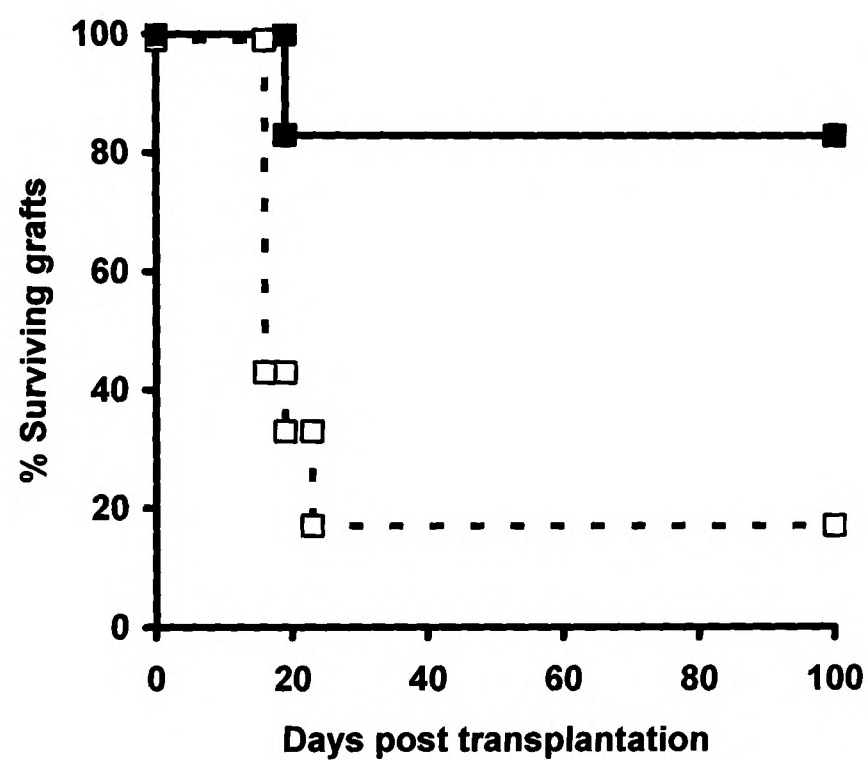
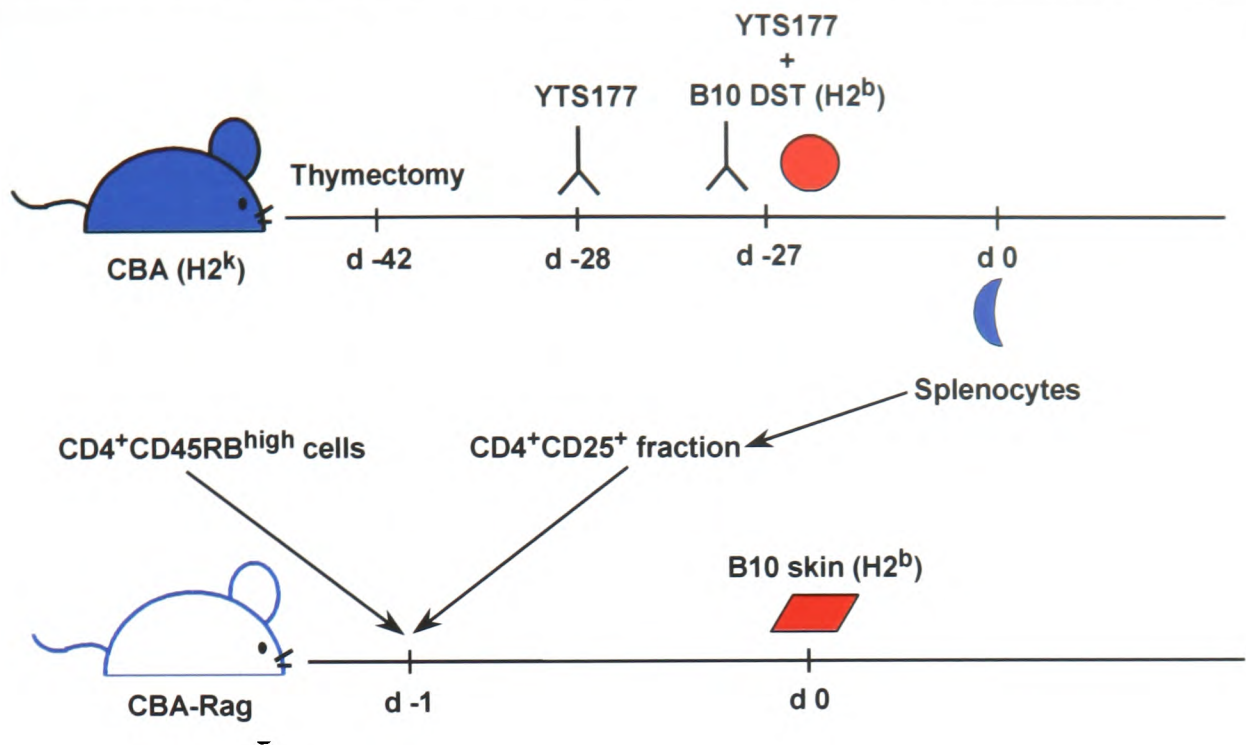


Figure 5.1. Theoretical routes of generation of alloantigen-specific CD4⁺CD25⁺ Treg by peripheral administration of alloantigen.

Peripherally-administered alloantigen may theoretically induce Treg development by three pathways: (1) Alloantigen may enter the thymus and mediate the positive selection of Treg. (2) Alloantigen may convert peripheral T cells to a Treg phenotype. (3) Alloantigen may expand populations of naturally-occurring autoreactive Treg that cross-react with alloantigen.



Group	Cells transferred	n	Graft survival (days)	MST (days)
-□-	CD45RB ^{high} only	7	16, 16, 16, 16, 19, 23, >100	16
-■-	CD45RB ^{high} + CD25 ⁺	6	19, >100, >100, >100, >100, >100	>100

P = 0.008

Figure 5.2. Generation of CD4⁺CD25⁺ regulatory cells by 177/DST pre-treatment is not thymus-dependent

CBA-Rag mice were reconstituted with 10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice together with 2×10⁵ CD4⁺CD25⁺ cells from CBA mice that had undergone thymectomy 14 days prior to pre-treatment with 177/DST. The following day they received a B10 skin graft.

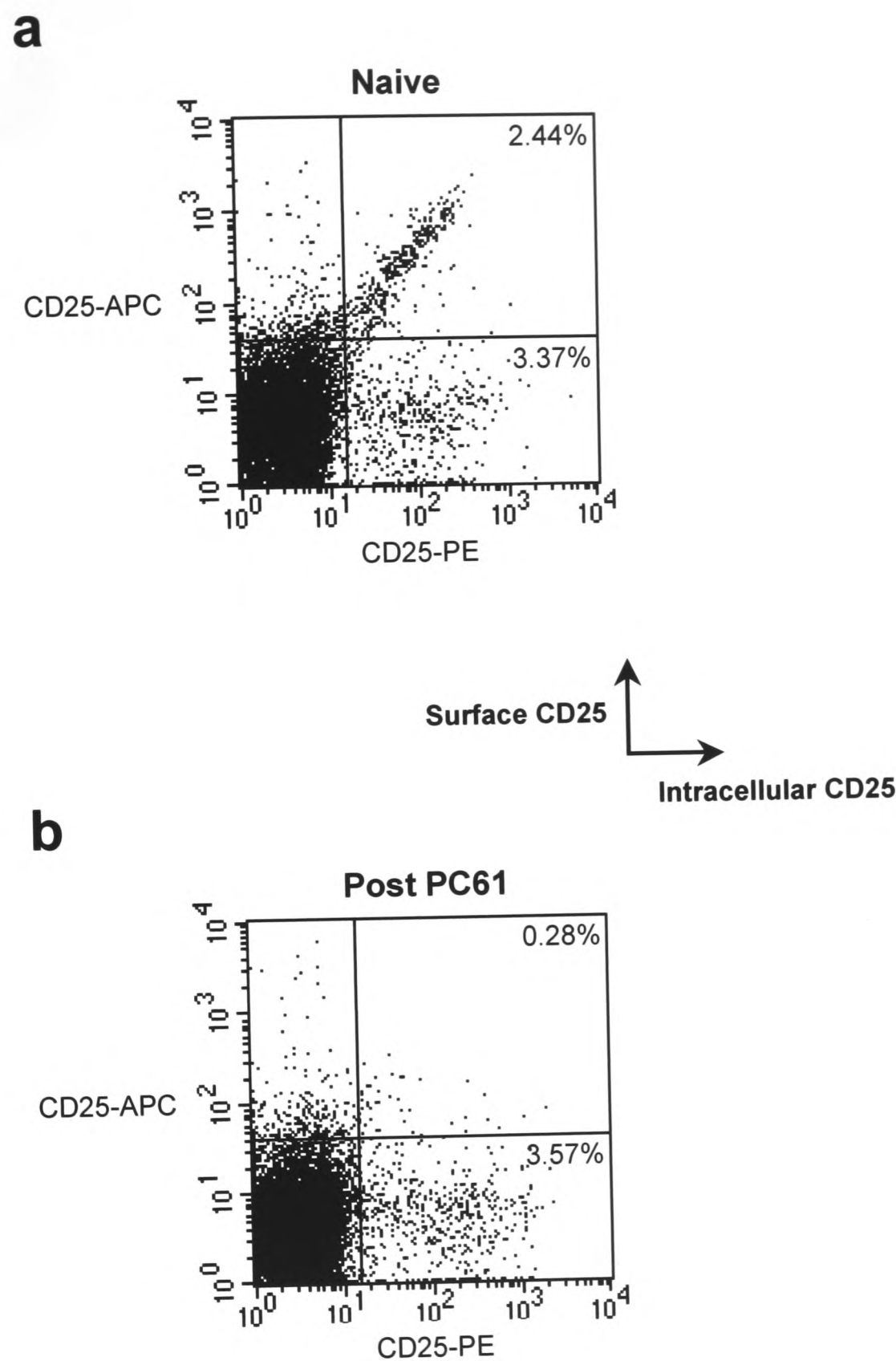
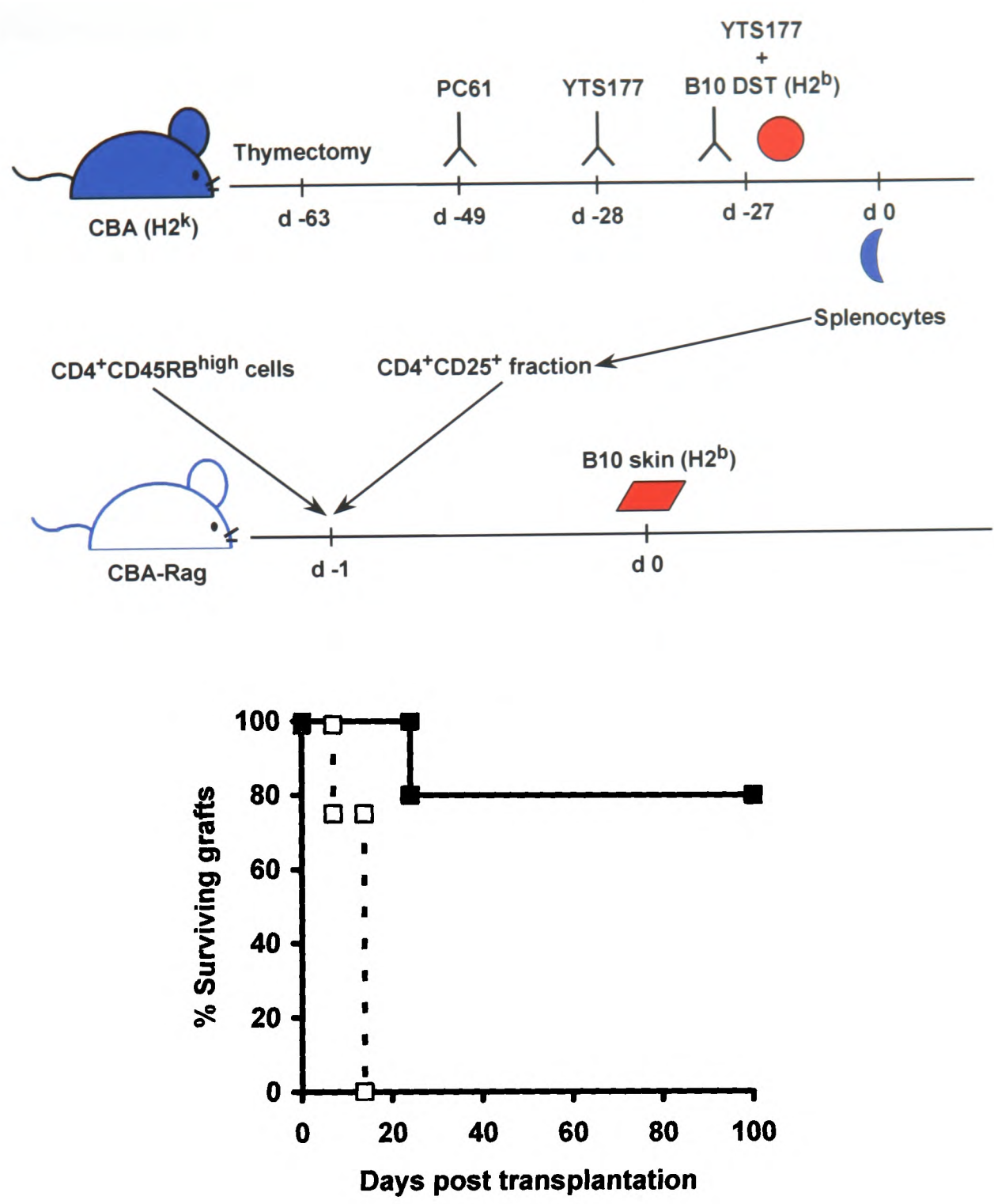


Figure 5.3. The mAb PC61 depletes CD25⁺ cells

Splenocytes from (a) naïve CBA mice or (b) CBA mice 7 days after treatment with 1 mg PC61 intravenously were stained with biotinylated anti-CD25 followed by SA-APC. They were then permeabilised and stained for intracellular CD25 expression using anti-CD25-PE prior to flow cytometric analysis. Percentages indicate proportion of cells in each quadrant. Data are representative of two independent experiments (n = 2 in each group).



Group	Cells transferred	n	Graft survival (days)	MST (days)
-□-	CD45RB ^{high} only	4	7, 14, 14, 14	14
■	CD45RB ^{high} + CD25 ⁺	5	24, >100, >100, >100, >100	>100

P = 0.013

Figure 5.4. Generation of CD4⁺CD25⁺ regulatory cells by 177/DST pre-treatment does not require CD4⁺CD25⁺ precursors

CBA-Rag mice were reconstituted with 10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice together with 2×10⁵ CD4⁺CD25⁺ cells isolated on day 0 from CBA mice that had undergone thymectomy on day -63, depletion of CD25⁺ cells by administration of the anti-CD25 antibody PC61 at a dose of 1 mg intravenously on day -49, and pre-treatment with 177/DST on days -28 and -27. The day after reconstitution they received a B10 skin graft.

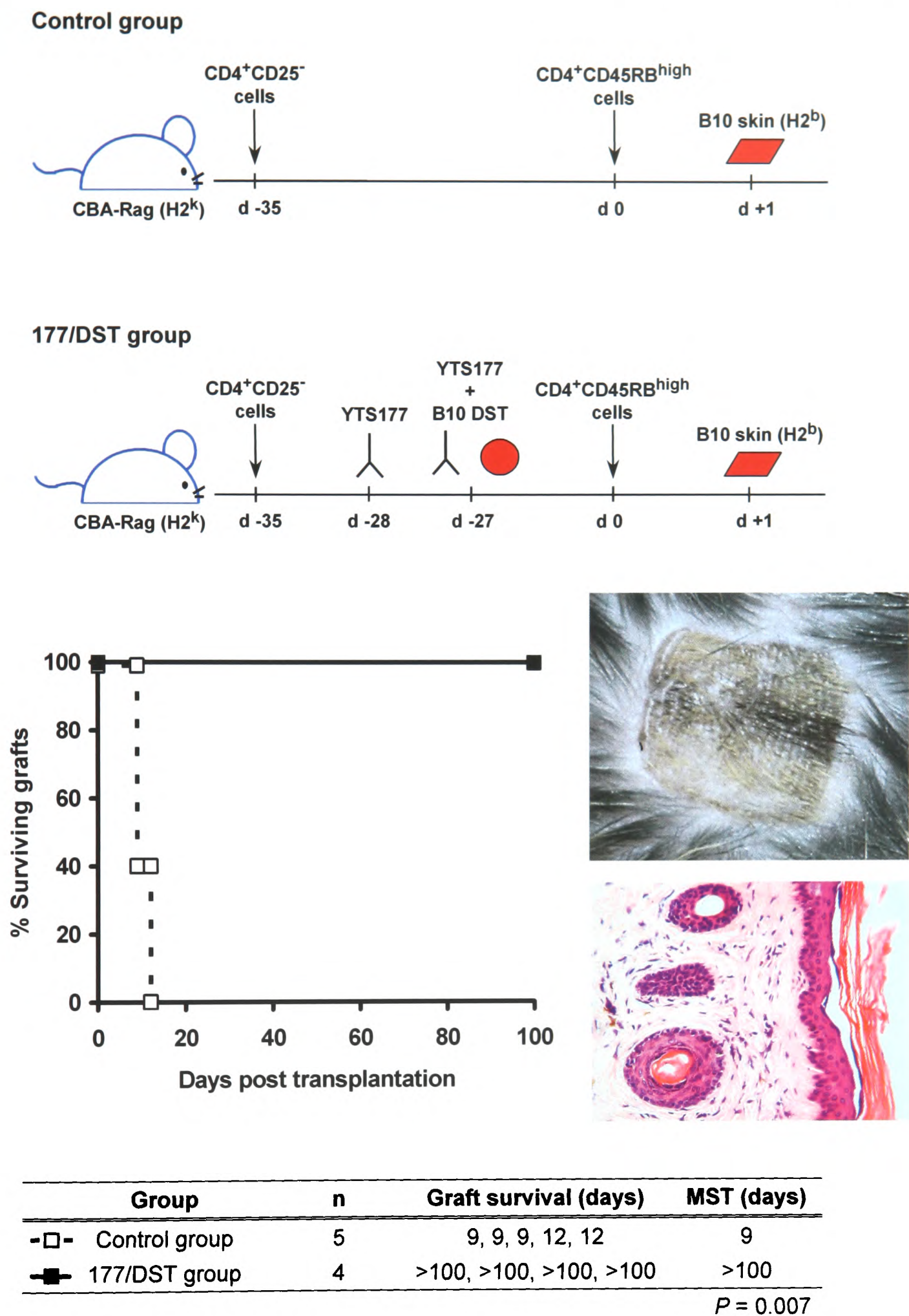


Figure 5.5. Regulatory cells can be generated from CD4⁺CD25⁻ precursors Control CBA-Rag mice were reconstituted with 10⁶ CD4⁺CD25⁻ cells from naïve CBA mice on day -35 followed by 10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice on day 0, and on day +1 received a B10 skin graft. Mice in the 177/DST experimental group received, in addition, 177/DST pre-treatment (YTS177 200 μg on days -28 and -27 and B10 blood 250 μg on day -27). Photographs show macroscopic and microscopic (H&E) appearances of a representative skin graft 100 days after transplantation onto a mouse from the 177/DST experimental group.

CHAPTER 6

MECHANISTIC ASPECTS OF REGULATORY

CELL FUNCTION *IN VIVO*

6.1 Introduction

Having examined the phenotypic properties of the $CD4^+CD25^+$ population containing cells capable of regulation generated by the 177/DST pre-treatment protocol in Chapter 3, their *in vitro* properties in Chapter 4, and the mechanisms by which pre-treatment generates these cells in Chapter 5, the aim of this final results chapter was to study the *in vivo* regulation phase itself. In particular three main areas were investigated: whether $CD4^+CD25^+$ T cells need to express CD122 in order to exert their regulatory effect, the antigen specificity and activation requirements of regulation mediated by $CD4^+CD25^+$ cells, and the fate of $CD4^+CD45RB^{high}$ and $CD4^+CD25^+$ populations *in vivo* after adoptive transfer.

6.1.1 CD122 expression by $CD4^+CD25^+$ Treg

As discussed in previous chapters, various groups have attempted to determine whether $CD4^+CD25^+$ Treg express CD122, the β chain of the receptor for IL-2 (and IL-15). It has been shown that a significant proportion of human $CD4^+CD25^+$ cells do indeed express CD122 [283, 285, 286, 301, 306, 307] but, to date, no published data have established whether the same is true in mice. Furthermore, the human studies did not examine the functional importance of CD122 expression or ask whether CD122 expression is actually required for $CD4^+CD25^+$ cells to exert their regulatory effect. Thus the significance of CD122 expression for Treg activity is at present unclear.

In Chapter 3 it was shown that a significant proportion of *ex vivo* mouse $CD4^+CD25^+$ cells express CD122 constitutively (Figure 3.2d) and that this proportion is increased in mice pre-treated with 177/DST and then re-challenged with alloantigen in the form of a further DST (Figure 3.13); this would support the hypothesis that mouse $CD4^+CD25^+$ Treg express CD122. However, in Chapter 4 it was demonstrated

that $CD4^+CD25^+CD122^-$ cells can regulate proliferative responses *in vitro* (Figure 4.9). In that experiment the $CD4^+CD25^+CD122^+$ population did not appear to regulate; however, as discussed in Section 4.2.6, there are various confounding factors that make this latter result difficult to interpret, including the fact that the anti-CD122 antibody (TM- β 1) on the surface of the $CD122^+$ population may block binding of IL-2 to its receptor and so inhibit the regulatory function of these cells. Nevertheless, it was suggested in Section 4.3 that although CD122 expression may not be required for $CD4^+CD25^+$ cells to exert their regulatory function *in vitro*, it may still play a role *in vivo*, for example by promoting cell survival through IL-15 binding. Thus one aim of this chapter was to test the regulatory ability of $CD4^+CD25^+CD122^+$ and $CD4^+CD25^+CD122^-$ cells *in vivo* by using the 177/DST pre-treatment and adoptive transfer system.

6.1.2 Antigen specificity of regulation by $CD4^+CD25^+$ cells

As discussed in Chapter 4, it has been shown by several groups that, although $CD4^+CD25^+$ Treg require activation via their T cell receptors in order to regulate *in vitro*, once activated they are able to regulate responses in an antigen non-specific manner, the process of bystander regulation [276, 289, 291, 293]. The study by Thornton and Shevach [289] examined the ability of $CD4^+CD25^+$ cells specific for pigeon cytochrome C (PCC) peptide presented in the context of E^k to regulate the proliferation of $CD4^+CD25^-$ cells specific for a different cognate ligand, haemagglutinin (HA) peptide presented by E^d . When these two populations were mixed and stimulated with $H2^d$ APC pulsed with HA peptide, proliferation of the $CD4^+CD25^-$ responder population was not inhibited. However, when $H2^k$ APC pulsed with PCC peptide were also added, suppression of the HA-specific E^d -restricted

CD4⁺CD25⁻ responders did occur, demonstrating that CD4⁺CD25⁺ regulatory cells can regulate responses against third party antigens provided that they are activated by their cognate peptide-MHC ligand. The experiments described by Takahashi *et al.* [276] and Chai *et al.* [293] yielded similar results. Although these three studies clearly show that bystander regulation can occur *in vitro*, this phenomenon has not yet been directly demonstrated *in vivo*. However, indirect evidence to support the hypothesis that bystander regulation may occur *in vivo* is provided by an early mouse study by Sakaguchi and colleagues [209] in which it was shown that depletion of CD4⁺CD25⁺ cells led to enhanced immune responses to non-self antigens. It was felt that the 177/DST pre-treatment and adoptive transfer skin allograft system may provide a model to test whether bystander regulation can occur *in vivo*. Furthermore, if there were evidence of bystander regulation in this model, it might be possible to exploit this phenomenon in a protocol which may have relevance to clinical transplantation.

6.1.3 Tracking adoptively transferred cells in vivo

The final part of this chapter describes attempts to develop a system to track the fate of effector and regulatory cell populations *in vivo* following adoptive transfer into immunodeficient CBA-Rag recipients. Such a system would be valuable for two main reasons. Firstly it may allow investigation of the anatomical location where regulation takes place in the 177/DST pre-treatment and adoptive transfer skin allograft system, and secondly it may provide mechanistic insights into the regulation occurring in this model.

In the late 1960s Barker and Billingham demonstrated that an intact afferent lymphatic system was required for mice to reject skin allografts [197]. Similarly, more recently Lakkis *et al.* showed that mice lacking secondary lymphoid organs were

unable to reject cardiac allografts [198]. These data suggest that in allograft recipients activation of the recipient immune system by alloantigen (the first stage in initiating graft rejection) occurs within secondary lymphoid organs rather than within the graft itself. It could therefore be postulated that Treg that prevent graft rejection may also exert their effects within secondary lymphoid organs. However, Graca and colleagues have recently demonstrated that in mice tolerant to skin allografts regulatory cells can be found within the grafts themselves [511]. Whether this is a general phenomenon common to all tolerated grafts is at present unclear. The ability to track cells *in vivo* in the 177/DST pre-treatment and adoptive transfer system may allow this issue to be examined in more detail.

In recent years it has become apparent that normal T cell populations adoptively transferred into lymphopenic hosts undergo expansion to fill “space” in the recipients, a process known as homeostatic proliferation [512-516]. Studies examining the fate of regulatory cell populations following adoptive transfer into lymphopenic hosts have shown that although these cells do proliferate [227, 517] they do so to a lower degree than effector populations [207, 219]. It has also been shown that the presence of Treg can inhibit the expansion of effector populations in this setting [207, 219, 223]. This has led to the suggestion that the suppressive effect of Treg populations observed in *in vivo* models using lymphopenic hosts is a result of non-antigen-specific inhibition of effector cell expansion rather than specific regulation of particular immune responses [518, 519]. Once again, the ability to track effector and regulatory populations *in vivo* would allow this question to be addressed in the 177/DST pre-treatment and adoptive transfer skin allograft system.

6.1.4 Aims of this chapter

In summary the overall aims of this chapter were as follows: firstly to investigate whether $CD4^+CD25^+$ cells with regulatory capacity are confined to the $CD122^+$ or $CD122^-$ subsets; secondly to examine antigen specificity of regulation of skin allograft rejection by cells from mice pre-treated with YTS177 and allogeneic blood; and thirdly to attempt to devise a system to track $CD4^+CD45RB^{high}$ effector and $CD4^+CD25^+$ Treg populations *in vivo* following their adoptive transfer into immunodeficient CBA-Rag recipients.

6.2 Results

6.2.1 Separating $CD4^+CD25^+$ cells on the basis of $CD122$ expression does not distinguish Treg populations

In order to address the issue of whether expression of $CD122$ distinguishes subsets within the $CD4^+CD25^+$ population that display regulatory properties *in vivo*, splenocytes were isolated from mice pre-treated with 177/DST, $CD4^+CD25^+CD122^+$ and $CD4^+CD25^+CD122^-$ populations were purified, and the ability of these two cell fractions to regulate the rejection of skin allografts *in vivo* was then tested (Figure 6.1). In these experiments, since $CD4^+CD25^+CD122^+$ cells form a small population, putative Treg were adoptively transferred at a lower cell number (10^5 per animal) than the dose of 2×10^5 used elsewhere in this thesis. However, as shown in Figure 6.1, both the $CD122^+$ and $CD122^-$ populations were able to regulate in this setting (MST > 100 days for both groups; $n = 4$, $P = 0.02$, and $n = 6$, $P = 0.008$ respectively compared to mice receiving naïve $CD4^+CD45RB^{high}$ cells only), thus demonstrating that

CD4⁺CD25⁺ Treg do not lie exclusively within either the CD122⁺ or CD122⁻ subfractions.

6.2.2 Regulation by CD4⁺CD25⁺ cells in vivo is donor-specific

Although data obtained from the 177/DST pre-treatment and adoptive transfer system demonstrate the ability of CD4⁺CD25⁺ cells to regulate the rejection of skin allografts (Figure 3.9, [441]), it is possible that this effect may be an idiosyncrasy confined to the B10 to CBA strain combination. In order to test this, a second donor / recipient combination was used. Recipients were once again CBA mice, but as donors BALB (H2^d) mice were used; these are fully MHC-mismatched with respect to both the CBA recipients and to the B10 donors used previously in this thesis. CBA mice were pre-treated with YTS177 and BALB blood, and then CD4⁺CD25⁺ cells were isolated and their ability to regulate the rejection of donor-specific BALB skin allografts mediated by naïve CD4⁺CD45RB^{high} cells was tested (Figure 6.2). As in the B10 to CBA combination, CBA-Rag mice reconstituted with naïve CD4⁺CD45RB^{high} cells alone all rejected their BALB skin grafts acutely (MST = 12 days, n = 4), whereas this was prevented by co-transfer of CD4⁺CD25⁺ cells from animals pre-treated with 177/(BALB) DST (MST > 100 days, n = 4; *P* = 0.008).

Having demonstrated the efficacy of the 177/DST pre-treatment protocol in a second strain combination, it was possible to ask whether regulation is donor-specific by testing whether Treg generated by pre-treatment with blood from one donor strain are able to regulate the rejection of skin allografts from a third party strain. CBA mice were therefore pre-treated with YTS177 and BALB blood, and the ability of CD4⁺CD25⁺ cells from these mice to regulate the rejection of B10 skin grafts was tested. However, as shown in Figure 6.3, all mice that received both CD4⁺CD45RB^{high}

cells from naïve animals and CD4⁺CD25⁺ cells from mice pre-treated with YTS177 and BALB blood rejected their third party B10 skin grafts acutely (MST = 25 days, n = 4; *P* = 0.75 compared to group receiving CD4⁺CD45RB^{high} cells alone), showing that regulation mediated by CD4⁺CD25⁺ cells in this setting is donor-specific.

6.2.3 CD4⁺CD25⁺ cells generated by pre-treatment with a single donor alloantigen can regulate the rejection of fully allogeneic grafts

It has previously been demonstrated that linked unresponsiveness / suppression facilitates the development of tolerance in both the induction and maintenance phases of the response *in vivo* [154, 244-247, 422, 423, 520, 521]. The induction of specific unresponsiveness to fully allogeneic grafts by pre-treatment with donor alloantigen therefore does not require pre-treatment with the full complement of graft alloantigens, and exposure to a limited subset is sufficient [154, 246, 247, 422, 423]. We wondered whether regulatory cells may play a role in this phenomenon, and therefore asked whether Treg generated by pre-treatment with a subset of graft antigens are able to regulate the rejection of fully allogeneic grafts. CBA mice were pre-treated with blood from CBK donors (H2^k but also expressing the single MHC class I alloantigen K^b as a transgene) under the cover of YTS177, and CD4⁺CD25⁺ cells were isolated for adoptive transfer. As shown in Figure 6.4, these cells were able to regulate the rejection of fully allogeneic B10 skin grafts expressing not only the K^b antigen used for pre-treatment but also all the other major and minor histocompatibility antigens of the H2^b haplotype (MST > 100 days, n = 5; *P* = 0.008 compared to group receiving naïve CD4⁺CD45RB^{high} cells only). These results clearly demonstrate that pre-treatment with a single donor MHC alloantigen can generate CD25⁺CD4⁺ cells capable of regulating the rejection of a fully allogeneic graft.

The fact that $CD4^+CD25^+$ cells generated by pre-treatment with K^b antigen were able to regulate the rejection of fully allogeneic $H2^b$ skin grafts provided an opportunity to investigate the role of $CD4^+CD25^+$ Treg in achieving tolerance to self MHC. It is likely that the tolerance to K^b of normal CBK mice (which naturally express this antigen) is predominantly due to the deletion of T cells reactive to K^b presented either directly or indirectly during thymic ontogeny. However, it could be postulated that, in addition, these mice may contain $CD4^+CD25^+$ Treg with receptors specific for K^b peptide (presented in the context of MHC class II), positively selected within the thymus (as discussed in Chapter 5). In order to test this, the ability of $CD4^+CD25^+$ cells from naïve CBK mice to regulate the $CD4^+CD45RB^{high}$ -mediated rejection of B10 skin grafts was examined (Figure 6.5). However, all the CBA-Rag mice reconstituted with both naïve CBA $CD4^+CD45RB^{high}$ and naïve CBK $CD4^+CD25^+$ cells rejected their B10 skin allografts acutely (MST = 17 days, $n = 5$; $P = 0.58$ compared to mice reconstituted with naïve CBA $CD4^+CD45RB^{high}$ cells alone), demonstrating that naïve CBK mice do not contain K^b peptide-reactive $CD4^+CD25^+$ Treg at sufficient precursor frequency to be detected in this assay, and supporting the view that clonal deletion rather than Treg generation is the predominant mechanism of achieving tolerance to self MHC.

6.2.4 Donor specificity exhibited by $CD4^+CD25^+$ Treg in vivo can be overcome by re-activation prior to adoptive transfer

The ability of $CD4^+CD25^+$ cells from mice pre-treated with YTS177 and CBK blood to regulate the rejection of B10 skin grafts (Figure 6.4) could be an *in vivo* manifestation of the phenomenon of bystander regulation that has previously been described *in vitro* [276, 289, 293]. It seemed likely that Treg generated by

administration of cells expressing K^b would be re-activated at the time of transplantation by K^b expressed by the graft and thereby acquire the ability to inhibit effector cell responses against the other alloantigens expressed by the B10 skin graft through bystander regulation *in vivo*. The next logical question to ask, therefore, was whether re-activation of Treg generated against non-graft antigens would allow them to regulate allograft rejection. The observations that $CD4^+CD25^+$ cells generated by pre-treatment with YTS177 and BALB blood were able to regulate the rejection of donor-specific BALB (Figure 6.2) but not third party B10 (Figure 6.3) skin allografts provided an ideal opportunity to test this.

Data from our laboratory have demonstrated that $CD4^+CD25^+$ cells generated by pre-treatment with 177/DST produce an early, transient IFN- γ response when they re-encounter donor alloantigen. This response is at its peak one day after antigen re-challenge, and appears to play an important role in regulation (B.S. Sawitzki and C.I. Kingsley, manuscript submitted). CBA mice were therefore pre-treated with BALB blood under the cover of YTS177 and then, one day before isolation of $CD4^+CD25^+$ cells, received a second dose of BALB blood with the objective of re-activating any BALB-specific $CD4^+CD25^+$ regulatory cells generated by the pre-treatment protocol. The ability of $CD4^+CD25^+$ cells from these mice to regulate the rejection of skin grafts from B10 mice, completely mismatched to both the CBA recipients and the BALB blood donors, was then tested. Figure 6.6 shows that, in distinct contrast to the situation where $CD4^+CD25^+$ cells from mice pre-treated with YTS177 and a *single* dose of BALB blood were unable to regulate the rejection of B10 skin grafts (Figure 6.3), administration of a second BALB blood transfusion the day before $CD4^+CD25^+$ cell isolation and adoptive transfer overcame the donor-specificity of regulation and allowed the long term survival of B10 skin grafts

in all cases (MST > 100 days, $n = 4$; $P = 0.008$ compared to group receiving naïve $CD4^+CD45RB^{high}$ cells alone). These data appear to support the hypothesis that bystander regulation by activated Treg can operate *in vivo*.

6.2.5 $CD4^+CD25^+$ cells generated against a non-graft protein antigen and then re-activated can regulate skin allograft rejection

The demonstration that re-activation of Treg prior to adoptive transfer prevented the rejection of third party grafts could have important implications for the design of tolerance induction strategies in man, but concerns over the potential for transmission of blood-borne pathogens may limit the utility of blood or other allogeneic cells as a source of tolerising antigen. Generating Treg specific for a non-cellular protein antigen could offer a potential alternative strategy if such cells could regulate responses to alloantigens *in vivo* once re-activated. It has previously been demonstrated that tolerance to soluble antigens such as albumin [522, 523] or immunoglobulin [355, 365, 524, 525] can be induced when administered to mice under the cover of anti-CD4 antibody. More specifically, Waldmann and colleagues have shown that administration of human gamma globulin (HGG) to mice under the cover of YTS177, the same antibody used in the model described in this thesis, induces tolerance to HGG as judged by the lack of anti-HGG antibody production in response to subsequent challenge with HGG [355, 365]. Therefore, HGG was chosen as a candidate for the tolerising antigen in these experiments.

An important initial experiment was to determine firstly whether using the same protocol as Qin *et al.* [355] we could see attenuated antibody responses against HGG, and then secondly whether mice given HGG under the cover of YTS177 according to the time schedule of the 177/DST protocol were also tolerant to HGG as

judged by their antibody response. Various combinations of YTS177 and HGG were therefore administered to mice and then tolerance to HGG was assessed by the measurement of serum anti-HGG antibody titres by ELISA (Figure 6.7, $n = 2$ in each group). Positive control mice that received a priming protocol (where HGG was given *without* the cover of YTS177) produced high levels of anti-HGG antibody whereas mice given HGG under the cover of YTS177 gave low antibody titres identical to those from unprimed naïve mice. Importantly, mice pre-treated according to the standard pre-treatment and re-challenge schedule (antigen under the cover of YTS177 followed by a second dose of antigen the day prior to analysis) produced the same background levels of anti-HGG antibody.

Following confirmation that tolerance to HGG can be induced in this way, the next objective was to ask whether $CD4^+CD25^+$ cells generated by pre-treatment with HGG under the cover of YTS177 and then re-activated with a further dose of HGG could regulate the rejection of B10 skin allografts. Figure 6.8 demonstrates that animals reconstituted with naïve $CD4^+CD45RB^{high}$ effector cells alone all acutely rejected their grafts (MST 13 days, $n = 4$) but, in contrast, all mice that received co-transfer of $CD4^+CD25^+$ cells from animals pre-treated with YTS177 and HGG and then given a second dose of HGG the day prior to cell isolation (with the aim of re-activating the regulatory population) accepted their B10 skin allografts long term (MST > 100 days, $n = 5$; $P = 0.003$). All five grafts in this group had a macroscopic and histological appearance which was essentially identical to that seen with the basic 177/DST protocol (Figures 3.10 and 6.2), indicating that at equivalent cell doses $CD4^+CD25^+$ cells generated against nominal non-graft antigen and then re-activated regulate as effectively as their donor-specific counterparts. As important controls, the regulatory capacities of $CD4^+CD25^+$ cells from animals pre-treated with YTS177 and

HGG but without the second (re-activating) dose of HGG, and from animals pre-treated with HGG in the absence of YTS177 followed by a second dose of HGG prior to cell isolation were also tested. In both these situations all B10 skin allografts were rejected acutely (MST = 21 days and 20 days respectively, $n = 5$ for both groups $n = 5$). These data demonstrate that the regulation mediated by the cells from animals pre-treated with 177/HGG plus HGG re-challenge was a specific phenomenon and not an artefact related to the adoptive transfer of activated cells (group pre-treated with two doses of HGG) or of Treg that had not been re-activated prior to adoptive transfer (group pre-treated with YTS177 and a single dose of HGG).

6.2.6 Non-specific regulation by activated $CD4^+CD25^+$ cells is IL-10-dependent

It has previously been demonstrated that regulation by $CD4^+CD45RB^{low}$ cells of colitis mediated by $CD4^+CD45RB^{high}$ cells is IL-10-dependent [214]. Similarly, we have shown that the ability of $CD4^+CD25^+$ cells generated by the 177/DST protocol to regulate skin allograft rejection is abrogated by IL-10 blockade [441]. These data demonstrate that naturally-occurring $CD4^+CD25^+$ Treg and those generated following 177/DST pre-treatment share a common IL-10-dependent mechanism. The 177/HGG protocol provides a model with which to ask whether *bystander* regulation by $CD4^+CD25^+$ cells is similarly IL-10-dependent. In order to test this, $CD4^+CD25^+$ cells from CBA mice pre-treated with 177/HGG plus HGG re-challenge were adoptively transferred into CBA-Rag recipients together with naïve $CD4^+CD45RB^{high}$ cells, and a B10 skin allograft was performed the next day as usual. However, in addition, in this experiment the adoptive transfer recipients also received therapy with either an antibody directed against the IL-10 receptor or an isotype control antibody (Figure 6.9). As expected, mice treated with the isotype control antibody all accepted their

skin allografts long term (MST > 100 days, n = 4). However, regulation was completely abrogated in the group treated with anti-IL-10R antibody in which all the grafts were rejected acutely (MST = 10 days, n = 4; $P = 0.008$ compared to isotype control group, $P = 0.81$ compared to mice reconstituted with naïve $CD4^+CD45RB^{high}$ cells alone), showing that bystander regulation by $CD4^+CD25^+$ cells is indeed IL-10-dependent.

6.2.7 Attempted cell tracking in vivo

Attention was finally turned to a separate aspect of Treg function, namely in which anatomical location they exert their regulatory effect *in vivo*, and whether they have an effect on the expansion and / or homing pattern of the naïve $CD4^+CD45RB^{high}$ population. In order to address these questions, attempts were made to develop a system that would allow tracking of the fate of $CD4^+CD45RB^{high}$ and $CD4^+CD25^+$ cells *in vivo* when reconstituted into CBA-Rag recipients. As a prelude to this, to confirm that adoptively transferred cells can be detected, an “*in vivo* MLR” was performed, a technique in which the division of CFSE-labelled $CD4^+$ cells can be followed upon reconstitution into immunodeficient allogeneic recipients [526]. B10 splenocytes were labelled with CFSE and adoptively transferred into CBA-Rag recipients at a dose of 50×10^6 cells per mouse. The spleens were harvested from these recipients at various time points, and the CFSE division profile of splenocytes was analysed by flow cytometry. As shown in Figure 6.10, cells that had undergone different numbers of cell divisions could clearly be detected. The expression of markers whose levels are known to change upon cell activation (discussed in Chapter 3) was then analysed. Figure 6.11 demonstrates that, as expected, expression

of CD25, CD44, CD69, and CD122 progressively increased as cells underwent more rounds of division, whereas levels of CD45RB and CD62L fell.

Although these results show that the proliferation of adoptively transferred allogeneic CD4⁺ cells could be followed *in vivo*, the 50×10^6 dose of cells used in these experiments was clearly much high than the numbers of CD4⁺CD45RB^{high} (10^5) and CD4⁺CD25⁺ (2×10^5) cells used in the standard adoptive transfer system in this thesis. An attempt was therefore made to track lower numbers of cells in the *in vivo* MLR setting. However, as shown in Figure 6.12, when doses of between 5×10^4 and 5×10^6 CD4⁺ B10 cells were adoptively transferred into CBA-Rag recipients, very few cells could be detected four days later. Despite this lack of success, we wondered whether CFSE-labelled CD4⁺CD45RB^{high} cells may be easier to track *in vivo* than whole CD4⁺ cells. Naïve CBA CD4⁺CD45RB^{high} cells were therefore labelled with CFSE and adoptively transferred into CBA-Rag recipients at a dose of either 10^5 or 10^6 per animal, and a B10 skin allograft was then performed the following day. The spleens, skin grafts, liver, and lungs were then harvested from these mice at various time points between 1 and 14 days after skin transplantation. However, CFSE positive cells could not be reliably detected at any time point either by flow cytometry or by fluorescence microscopy.

Not deterred by this negative result, we hypothesised that the failure to detect CFSE positive cells may be a result of the loss of CFSE fluorescence intensity during cell division: at early time points CFSE positive cells may have been present but at a frequency below the detection limit of the assay, whereas at the later time points examined there may have been cells that originated from the original adoptively transferred population, but these cells may have divided to the point where they expressed insufficient CFSE to be detected. We reasoned that, since CBA-Rag

recipients contain no T cells of their own (Figure 3.5) CD4 could be used as a marker instead, as any CD4⁺ cells would have originated from the CD4⁺CD45RB^{high} population used for reconstitution. Naive CBA CD4⁺CD45RB^{high} cells were therefore purified and transferred at a dose of 10⁵ or 10⁶ cells into CBA-Rag recipients, and a B10 skin allograft was performed the following day. Ten days after skin grafting the spleens of these mice were harvested and CD4⁺ cells quantified by flow cytometry. As shown in Figure 6.13, even at the lower dose of 10⁵ cells (the number used in the normal adoptive transfer model), CD4⁺ cells were easily detectable. However, as would be expected, some of these cells had become activated and thus up-regulated expression of CD25 (Figure 6.13c). Thus this approach cannot be used as it stands to distinguish between cells arising from CD4⁺CD45RB^{high} or CD4⁺CD25⁺ precursors when both populations are transferred together. It was therefore not possible to proceed further with this line of investigation. However, one approach currently being pursued is the use of CD4⁺CD45RB^{high} cells from CBA mice transgenic for human CD52 [527]. The use of such a marker would allow CD4⁺CD25⁺ and CD4⁺CD45RB^{high} cells to be tracked separately and thus enable further study of the interactions between these populations *in vivo*.

6.3 Discussion

One of the problems encountered in transplantation research is the phenomenon of so-called “split tolerance”, in which *in vitro* and *in vivo* assays assessing the presence of tolerance yield opposing results [187, 418, 528-534]. This may not be too surprising given the fact that even the most sophisticated *in vitro* assay systems cannot hope to replicate the complex *in vivo* environment. One example of split tolerance in

this thesis is the difference demonstrated in the regulatory potential of $CD4^+CD25^+CD122^+$ cells *in vitro* and *in vivo*. In the *in vitro* assay shown in Figure 4.9, $CD4^+CD25^+CD122^+$ cells did not regulate, whereas they did do so in the *in vivo* assay in Figure 6.1. It was proposed in Chapter 4 that any suppressive capacity of these cells *in vitro* may have been obscured by two confounding factors: firstly the presence of anti-CD122 antibody (used for cell purification) on their surface, and secondly the relatively high proportion of $CD25^{intermediate}$ cells in the $CD122^+$ population. The fact that $CD122^+$ cells could regulate *in vivo* suggests that their lack of effect *in vitro* was not due to the presence of $CD25^{intermediate}$ cells, and that the disparity was more likely to be related to the presence of cell surface antibody. The anti-CD122 antibody (TM- β 1) used for cell sorting prevents IL-2 from binding to its receptor [448], whereas the anti-CD25 antibody (7D4) does not [114]. Immediately after purification, the ability of the $CD4^+CD25^+CD122^+$ population to bind IL-2 would therefore probably be impaired. It has been shown that during *in vitro* stimulation, signs of T cell activation (such as formation of the immunological synapse) appear within minutes [535, 536]. It is thus likely that *in vitro* successful suppression requires Treg to exert their function early, at a time when in this context cell surface antibody is still likely to be present on the $CD4^+CD25^+CD122^+$ population and IL-2 binding therefore remains impaired. In contrast, in the *in vivo* assay, alloantigen encounter (in the form of the skin graft) does not occur until one day after cell purification and adoptive transfer. Although the half life of the cell surface anti-CD122 antibody is unknown, it is possible that by the time of skin transplantation its inhibitory effect has declined to the extent that it no longer has a significant effect on IL-2 binding and thus regulation. If the presence of cell surface antibody is indeed

responsible for the lack of regulation observed *in vitro*, this implies that IL-2 binding by Treg plays an important mechanistic role in their suppressive function.

In Figure 4.9 it was shown that $CD4^+CD25^+CD122^-$ cells could regulate *in vitro*. It was postulated that although CD122 expression may not be directly required for regulation, the presence of CD122 on Treg may promote their survival *in vivo* (and thus enhance their regulatory potential) by making them more responsive to IL-15. However, the fact that the $CD122^-$ population could regulate *in vivo* (Figure 6.1) suggests that, in this particular model, Treg are not reliant upon such a survival signal provided by IL-15. Although the ability of $CD122^-$ cells to regulate both *in vitro* and *in vivo* would be consistent with the view that CD122 expression is not required for Treg to exert their suppressive function, this would appear to be at odds with the suggestion in the previous paragraph that the presence of anti-CD122 antibody (that blocks IL-2 binding) abrogates regulation. However, there are two potential explanations for this apparent disparity. Firstly, it is possible that when the $CD122^-$ population is activated *in vitro* or *in vivo* CD122 is upregulated, so that the cells do express CD122 at the time they exert their regulatory effect. Secondly it may be that *signalling* via the IL-2 receptor on Treg is not required for regulation, but rather that *binding* of IL-2 to Treg and consequent sequestration from the effector population is important. CD25 mediates high affinity IL-2 binding, whereas CD122 is required for receptor signalling [274]. Cells that express CD25 in the absence of CD122 would therefore still be able to sequester IL-2 (but not signal), whereas cells that express both CD25 and CD122 but that also have blocking anti-CD122 antibody bound to their surface would show impaired sequestration activity.

The focus of this chapter next moved on to examine the antigen specificity of regulation in the adoptive transfer system. In Figure 6.2 it was shown that the

177/DST pre-treatment protocol was able to generate $CD4^+CD25^+$ cells capable of regulating skin allograft rejection in a second strain combination, namely BALB to CBA. The data in Figure 6.3 demonstrate that regulation in the unmodified 177/DST protocol is donor-specific, since $CD4^+CD25^+$ cells from mice pre-treated with YTS177 and BALB blood were not able to regulate the rejection of third party B10 skin grafts. However, although pre-treatment with donor alloantigen was required for the generation of Treg, administration of the single MHC class I donor alloantigen K^b was sufficient to generate cells capable of regulating the rejection of fully allogeneic grafts (Figure 6.4), showing that exposure to the full repertoire of donor alloantigens is not necessary in this system. The fact that $CD4^+CD25^+$ cells from naïve CBK mice (naturally expressing and therefore tolerant to K^b) were unable to regulate in the same way (Figure 6.5) implies that these mice do not contain naturally-occurring $CD4^+CD25^+$ Treg reactive against K^b peptide (presented in the context of MHC class II) at a significant precursor frequency. It thus seems likely that $CD4^+CD25^+$ Treg do not contribute significantly to the maintenance of tolerance to self MHC, and that deletion of T cells possessing receptors with high affinity for self MHC is the predominant mechanism. More importantly in the context of this thesis, this observation demonstrates that antigen presence *per se* is not sufficient for the generation of Treg, and provides further evidence that the full pre-treatment protocol is required for Treg generation in this model.

The fact that $CD4^+CD25^+$ cells generated by pre-treatment with a single donor alloantigen were able to regulate the rejection of fully MHC-mismatched skin allografts (Figure 6.4) could be explained on the basis of bystander regulation occurring *in vivo*. Stronger evidence for bystander regulation *in vivo* is provided by the data shown in Figure 6.6, in which $CD4^+CD25^+$ cells from mice pre-treated with

177/BALB blood that were then re-activated prior to purification were able to prevent the rejection of third party B10 skin allografts, overcoming the antigen specificity of regulation demonstrated in Figure 6.3. It could be argued that the regulation seen in this experiment was due to cross-reactivity between BALB and B10, and that the re-challenge with the second dose of BALB blood served to potentiate the effect of cross-reactive Treg sufficiently to allow regulation of rejection of the B10 grafts. However, the fact that $CD4^+CD25^+$ cells generated by pre-treatment with HGG (an antigen completely unrelated to the subsequent graft) and then re-activated could also prevent skin allograft rejection (Figure 6.8) makes cross-reactivity an unlikely explanation, and would support the view that bystander regulation was instead responsible. In order to ensure that this property is not unique to HGG, experiments are in progress in the laboratory to determine whether pre-treatment with YTS177 and other non-graft antigens (such as ovalbumin) followed by antigen re-challenge similarly generates $CD4^+CD25^+$ cells capable of regulating the rejection of skin allografts.

In the adoptive transfer model both the effector and regulatory populations are $CD4^+$. Since $CD4^+$ cells are highly unlikely to recognise MHC class I antigens via the direct pathway of alloantigen presentation, the observation that $CD4^+CD25^+$ cells from mice pre-treated with a single MHC class I alloantigen are able to regulate the rejection of skin allografts (Figure 6.4) clearly demonstrates that antigen presentation via the indirect pathway is sufficient for the generation and activation of $CD4^+CD25^+$ Treg. Further evidence for this is provided by the finding that re-activated $CD4^+CD25^+$ cells from mice pre-treated with the exogenous non-cellular protein antigen HGG (processed and then presented in the context of self MHC class II) can also regulate skin allograft rejection (Figure 6.8). The potential importance of the

indirect pathway of alloantigen presentation for the activity of Treg has previously been indicated by a study in which it was shown that regulation *in vitro* by $CD4^+CD45RB^{low}$ cells from mice with long term surviving cardiac allografts was only observed in the presence of indirect stimulation [236]. In addition, if the generation of Treg requires indirect antigen presentation, this may partially explain the observation that tolerance to semiallogeneic grafts is easier to achieve than to those from fully mismatched donors [500, 537, 538], since in the former situation alloantigen presentation by donor cells can occur via the indirect as well as the direct pathway, theoretically providing a stimulus facilitating Treg generation.

In Figures 3.9 and 6.2 it was shown that $CD4^+CD25^+$ cells from mice pre-treated with 177/DST were able to regulate the rejection of skin allografts, in Figure 6.6 that $CD4^+CD25^+$ cells from mice pre-treated with YTS177 and allogeneic blood and then given a second dose of allogeneic blood of the same strain prevented the rejection of third party skin grafts, and in Figure 6.8 that $CD4^+CD25^+$ cells from mice pre-treated with 177/HGG followed by HGG re-challenge also regulated skin allograft rejection. However, it is possible that the regulation occurring in the first scenario, using pre-treatment with donor alloantigen, is more potent than that in the latter two situations. One way to test this would be to titrate down the number of $CD4^+CD25^+$ cells adoptively transferred to see whether the dose-response curve differs in the three situations. However, practical considerations, in particular limited yields of $CD4^+CD25^+$ cells, meant that this was not possible within the scope of this thesis.

We have previously shown that, in common with naturally-occurring $CD4^+CD25^+$ cells, regulation by $CD4^+CD25^+$ cells from mice pre-treated with 177/DST is abrogated by IL-10 blockade [441]. Further evidence supporting a critical

mechanistic role for IL-10 in regulation by $CD4^+CD25^+$ cells is provided by the data depicted in Figure 6.9 demonstrating that bystander regulation by activated $CD4^+CD25^+$ cells is also IL-10-dependent.

The final part of this chapter set out to track the fate of cells adoptively transferred into lymphopenic recipients. Figures 6.10 to 6.12 demonstrated that although CFSE-labelled cells could be tracked *in vivo*, this was only possible at cell doses that were much higher than those used in the standard adoptive transfer assay. However, Figure 6.13 showed that cells adoptively transferred at these lower standard doses could be followed if detected by CD4 staining rather than by CFSE expression. There are two factors that may stimulate the proliferation of $CD4^+CD45RB^{high}$ cells adoptively transferred into CBA-Rag recipients that subsequently receive skin allografts. The first is homeostatic proliferation due to the lymphopenic environment, and the second is the alloantigen stimulus provided by the graft. By adoptively transferring $CD4^+CD45RB^{high}$ cells into CBA-Rag mice with or without skin allografts the following day, the relative contributions of these two stimuli could be assessed. As discussed earlier, although $CD4^+CD25^+$ cells do not proliferate *in vitro* under normal circumstances (Chapter 4), it has been shown that they can do so *in vivo* when adoptively transferred into lymphopenic hosts [207, 219, 227, 517]. However, these *in vivo* studies examined the proliferation of naturally-occurring $CD4^+CD25^+$ cells in autoimmune disease models. Tracking the proliferation *in vivo* of $CD4^+CD25^+$ cells adoptively transferred alone (without effector cells) into CBA-Rag recipients with and without skin grafts would quantify the expansion of this population and, once again, determine how much of this expansion was due to the lymphopenic environment rather than alloantigen stimulation. Furthermore, comparison of the properties of $CD4^+CD25^+$ cells from naïve and 177/DST pre-treated

animals would establish whether the pre-treatment leads to enhanced proliferation *in vitro*.

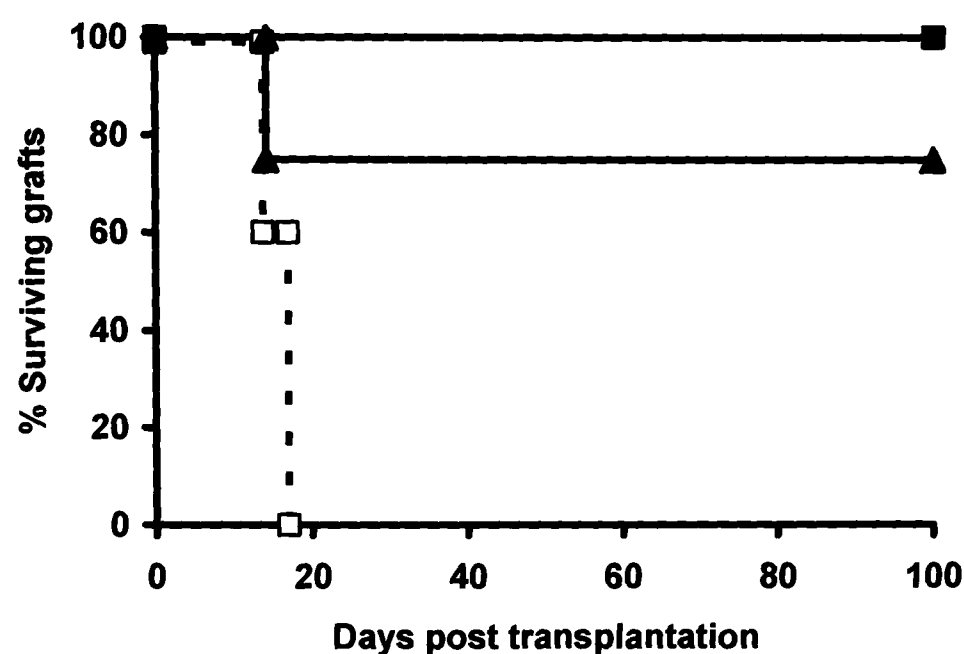
It has previously been shown that one potential effect of Treg can be to suppress the expansion of effector cell populations [207, 219, 223]. However, in a transplantation model Lin *et al.* have shown that CD4⁺CD25⁺ cells can inhibit the effector function of CD8⁺ cells without suppressing their proliferation [539]. It would therefore be valuable to know whether CD4⁺CD25⁺ cells in the 177/DST model have any effect on the expansion of the CD4⁺CD45RB^{high} population. This would require the ability to track these two populations *in vivo* when adoptively transferred together. However, the data depicted in Figure 6.13 show that following adoptive transfer a significant proportion of the CD4⁺CD45RB^{high} population (which, as shown in Figure 3.1, are CD25⁻ at the time of adoptive transfer) become CD25⁺. Thus, as mentioned in Section 6.2.7, the ability to track effector and regulatory populations separately would require the ability to distinguish between different cell populations from mice of the same strain, for example by using a transgenic marker such as human CD52. This approach would determine not only whether CD4⁺CD25⁺ cells do indeed affect the expansion of the CD4⁺CD45RB^{high} population, but also the relative size of the effect on homeostatic and alloantigen-driven proliferation.

As discussed earlier, it has been postulated that the regulation seen in models using lymphopenic hosts is not the result of true suppression, but rather due to non-specific inhibition of effector cell proliferation [518, 519]. If this were the case then CD4⁺CD25⁺ cells from any source, which should be able to inhibit CD4⁺CD45RB^{high} cell expansion, should therefore also be able to regulate skin allograft rejection. However, regulation is not seen when using CD4⁺CD25⁺ cells from naïve mice or from mice pre-treated with YTS177 or DST alone [441], from mice

pre-treated with YTS177 and third party blood (Figure 6.3), or from mice pre-treated with 177/HGG without HGG re-challenge or with HGG alone followed by HGG re-challenge (Figure 6.8). The tracking experiments described above would address this issue. Further evidence that the regulation by cells from mice pre-treated with 177/DST is not simply due to a non-specific effect on effector cell proliferation in lymphopenic hosts is provided by the observation that adoptive transfer of CD4⁺ cells from mice pre-treated with 177/DST into normal naïve CBA recipients is able to suppress the rejection of donor type cardiac allografts (A.R. Bushell, unpublished observations). Taken together these data would suggest that cells from mice pre-treated with antigen under the cover of anti-CD4 therapy do indeed exert a genuine regulatory effect *in vivo*.

In summary, the data presented in this chapter demonstrate that both the CD122⁺ and CD122⁻ subsets of the CD4⁺CD25⁺ population are able to regulate *in vivo*, and provide a basis for assays to track effector and regulatory populations *in vivo*. More importantly they provide insights into the functional activity of Treg *in vivo*, in particular showing that bystander regulation, a phenomenon previously mainly demonstrated *in vitro* can also occur *in vivo*. A hypothetical model to explain the antigen specificity data presented in this chapter is presented in Figure 6.14. If rejection of fully allogeneic grafts is to be prevented by the generation of regulatory cell populations, one way to do this is to generate Treg specific for the whole range of graft alloantigens. Alternatively, however, if Treg specific for a single graft antigen are generated, these will be activated by the graft and may then overcome rejection responses directed against the other graft alloantigens through the process of bystander regulation. Furthermore, bystander regulation may allow regulatory cells generated against non-graft antigens to prevent rejection provided that the Treg are

activated in some way. The potential implications of these observations will be discussed further in Chapter 7.



Group	CD25 ⁺ population transferred	n	Graft survival (days)	MST (days)
□	No CD25 ⁺ cells	5	14, 14, 17, 17, 17	17
■	CD25 ⁺ CD122 ⁻ cells	6	>100, >100, >100, >100, >100, >100	>100
▲	CD25 ⁺ CD122 ⁺ cells	4	14, >100, >100, >100	>100

P = 0.008 (□ vs ■), 0.02 (□ vs ▲), 0.22 (■ vs ▲)

Figure 6.1. Both CD122⁺ and CD122⁻ fractions of CD4⁺CD25⁺ cells from mice pre-treated with 177/DST regulate rejection of skin allografts

Splenocytes from CBA mice pre-treated 28 days previously with 177/DST were stained with anti-CD4-CyC, anti-CD122-FITC, and biotinylated anti-CD25 followed by SA-PE. CD4⁺CD25⁺CD122⁺ or CD4⁺CD25⁺CD122⁻ cell fractions were then purified by flow cytometry. CBA-Rag mice were reconstituted with 10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice together with either 10⁵ CD4⁺CD25⁺CD122⁺ or CD4⁺CD25⁺CD122⁻ cells. The following day they received a B10 skin graft.

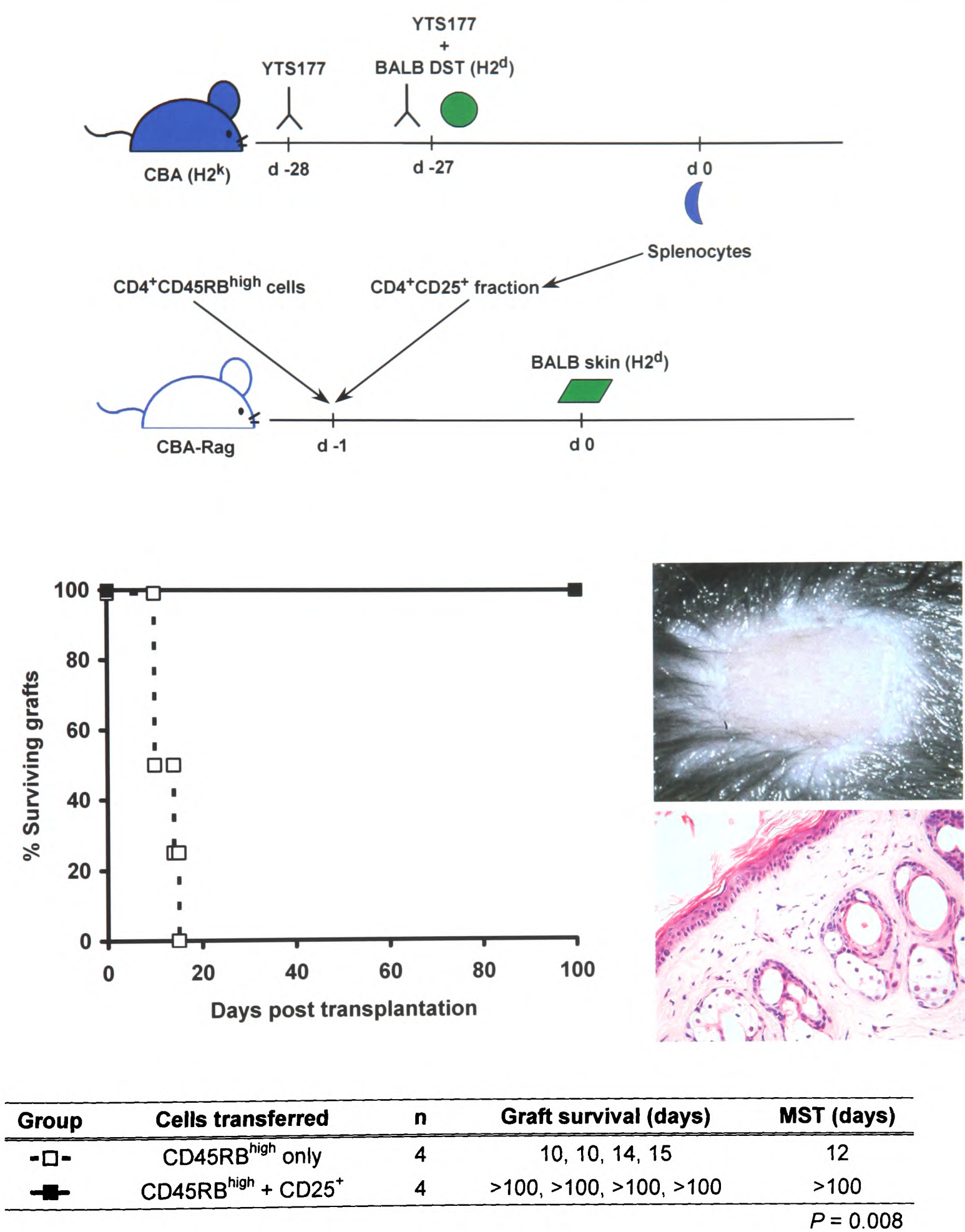


Figure 6.2. CD4⁺CD25⁺ cells from mice pre-treated with 177/BALB DST regulate rejection of BALB skin allografts

CBA-Rag mice were reconstituted with 10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice together with 2×10⁵ CD4⁺CD25⁺ cells taken from CBA mice pre-treated with YTS177 (days -28 and -27) and BALB DST (day -27). The following day they received a BALB skin graft. Photographs show macroscopic and microscopic (H&E) appearances of a representative skin graft 100 days after transplantation onto a mouse reconstituted with both CD4⁺CD45RB^{high} and CD4⁺CD25⁺ cells.

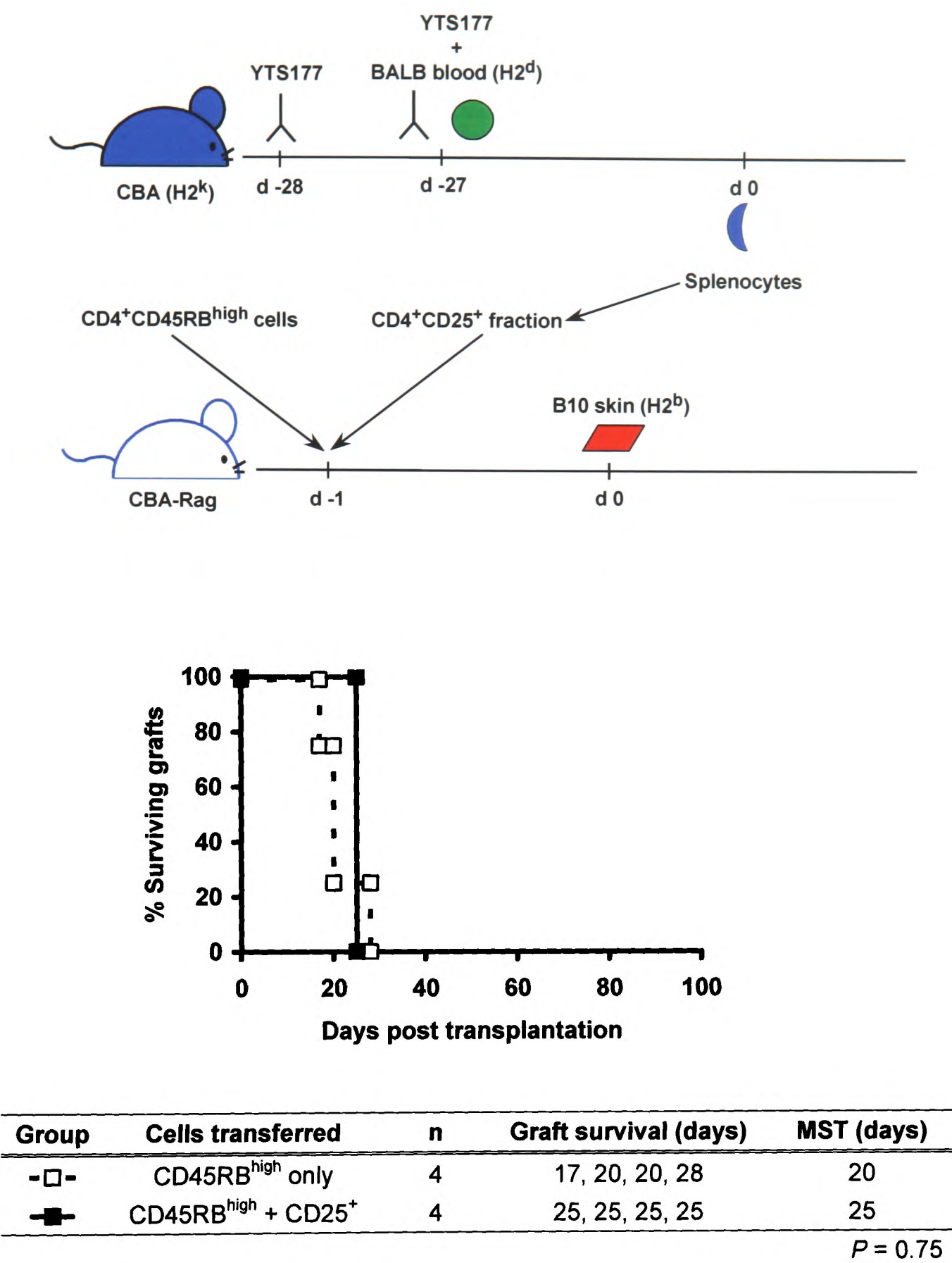


Figure 6.3. CD4⁺CD25⁺ cells from mice pre-treated with 177/BALB blood do not regulate rejection of B10 skin allografts

CBA-Rag mice were reconstituted with 10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice together with 2×10⁵ CD4⁺CD25⁺ cells taken from CBA mice pre-treated with YTS177 (days -28 and -27) and BALB blood (day -27). The following day they received a B10 skin graft.

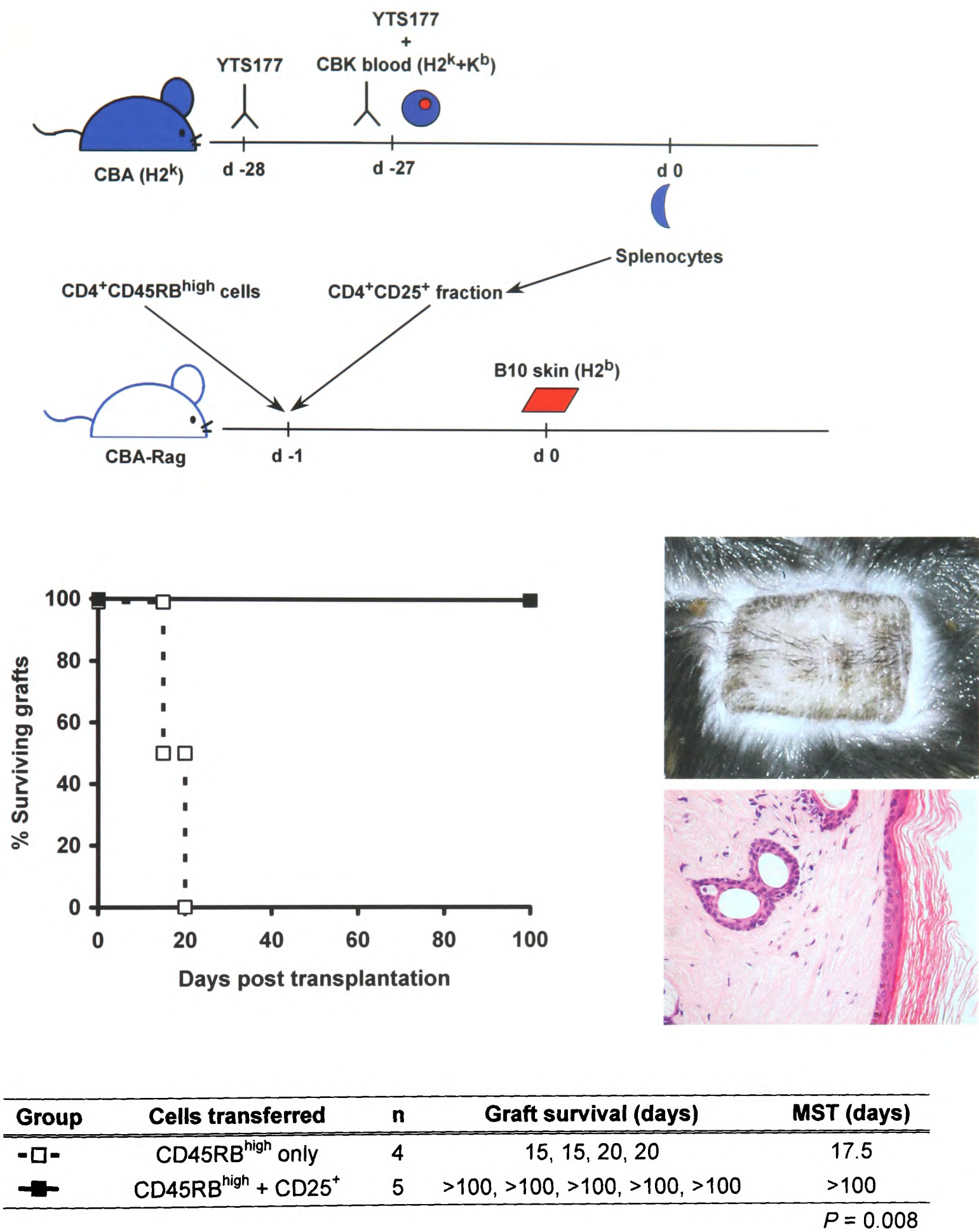
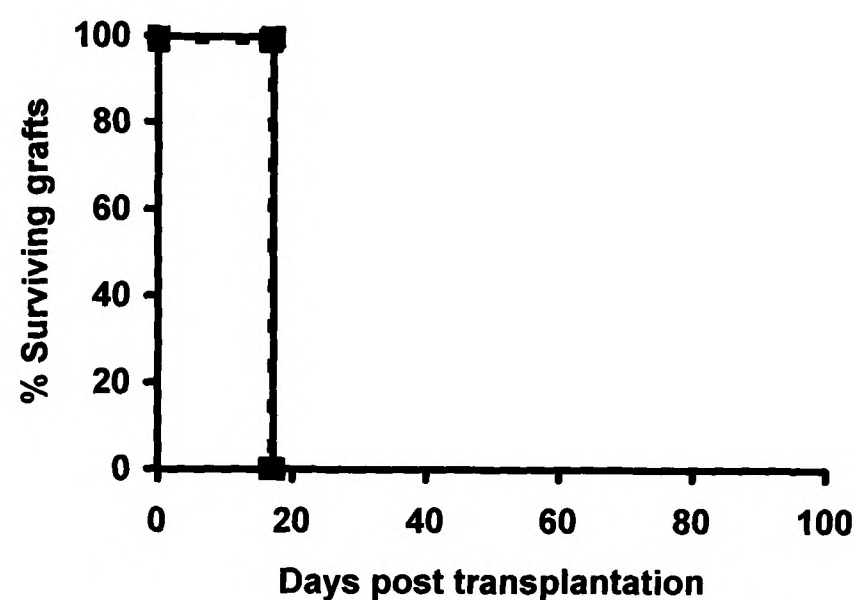
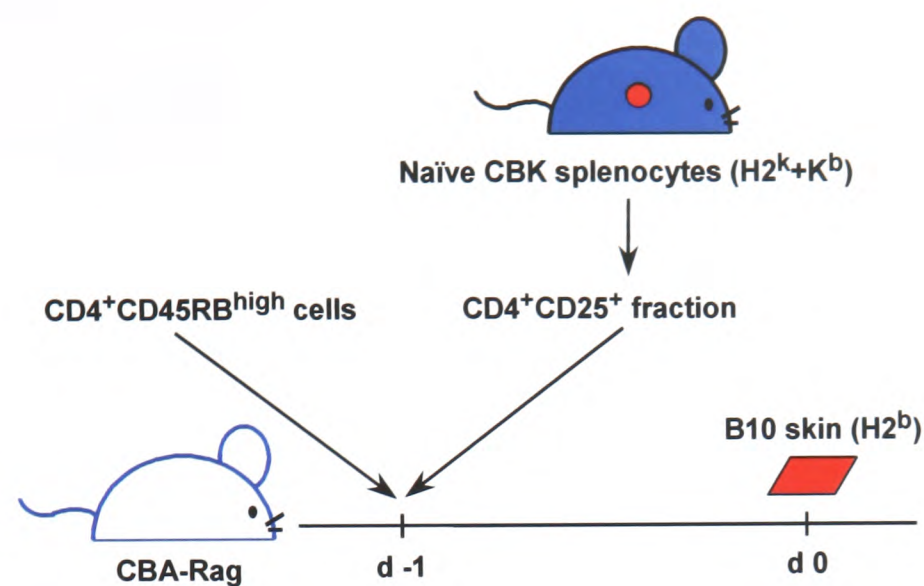


Figure 6.4. CD4⁺CD25⁺ cells from mice pre-treated with YTS177 and a single donor MHC class I alloantigen regulate rejection of fully allogeneic skin grafts CBA-Rag mice were reconstituted with 10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice with or without 2×10⁵ CD4⁺CD25⁺ cells taken from CBA mice pre-treated with YTS177 (days -28 and -27) and CBK blood (day -27). The following day they received a B10 skin graft. Photographs show macroscopic and microscopic (H&E) appearances of a representative skin graft 100 days after transplantation onto a mouse reconstituted with both CD4⁺CD45RB^{high} and CD4⁺CD25⁺ cells.



Group	Cells transferred	n	Graft survival (days)	MST (days)
-□-	CD45RB ^{high} only	4	17, 17, 17, 17	17
■	CD45RB ^{high} + CD25 ⁺	5	17, 17, 17, 17, 17	17

P = 0.58

Figure 6.5. CD4⁺CD25⁺ cells from naïve CBK mice do not regulate the rejection of B10 skin allografts

CBA-Rag mice were reconstituted with 10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice together with 2×10⁵ CD4⁺CD25⁺ cells from naïve CBK mice. The following day they received a B10 skin graft.

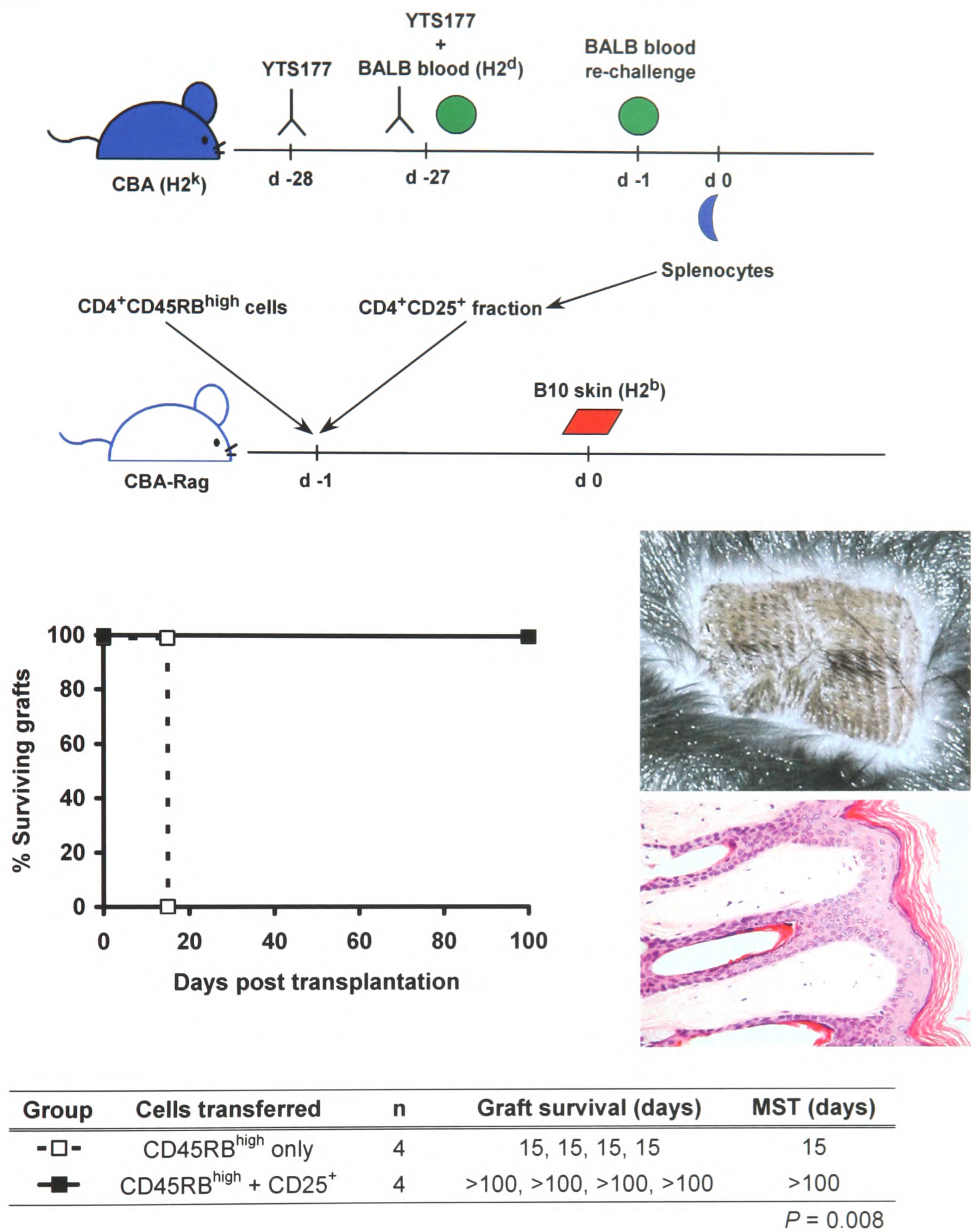


Figure 6.6. Re-activated CD4⁺CD25⁺ cells from mice pre-treated with 177/BALB blood regulate the rejection of B10 skin allografts

CBA-Rag mice were reconstituted with 10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice together with 2×10⁵ CD4⁺CD25⁺ cells taken from CBA mice pre-treated with YTS177 (days -28 and -27) and BALB blood (day -27) followed by BALB blood re-challenge (day -1). The following day they received a B10 skin graft. Photographs show macroscopic and microscopic (H&E) appearances of a representative skin graft 100 days after transplantation onto a mouse reconstituted with both CD4⁺CD45RB^{high} and CD4⁺CD25⁺ cells.

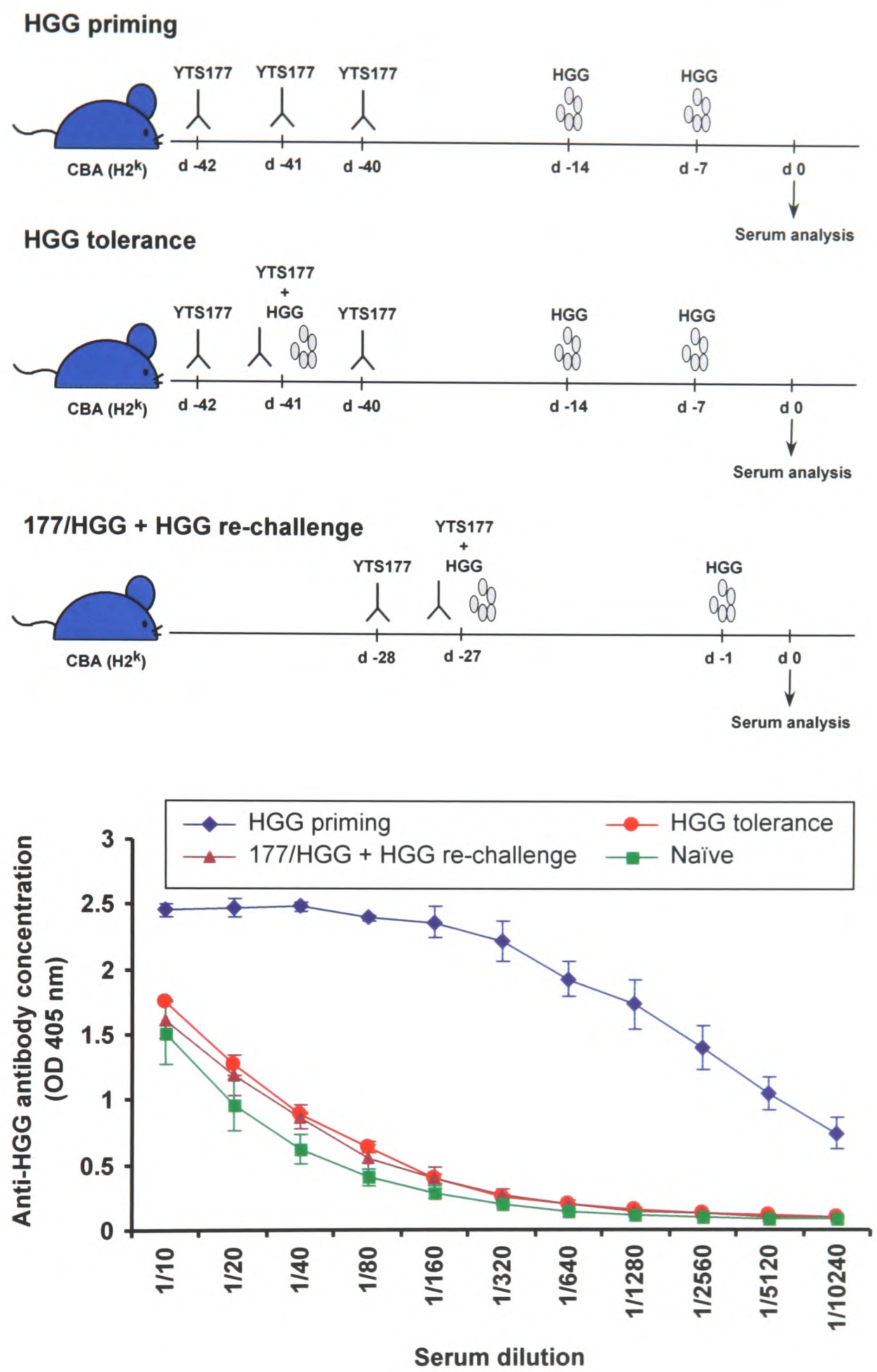


Figure 6.7. Tolerance to HGG can be induced by intravenous administration under the cover of YTS177

CBA mice were pre-treated with YTS177 (200 µg per dose) and HGG (500 µg per dose) at the time points shown. On day 0 serum was taken from the pre-treated mice or from naïve controls and anti-HGG antibodies measured by ELISA. Results are presented as mean OD ± standard deviation of triplicate wells. n = 2 in each group.

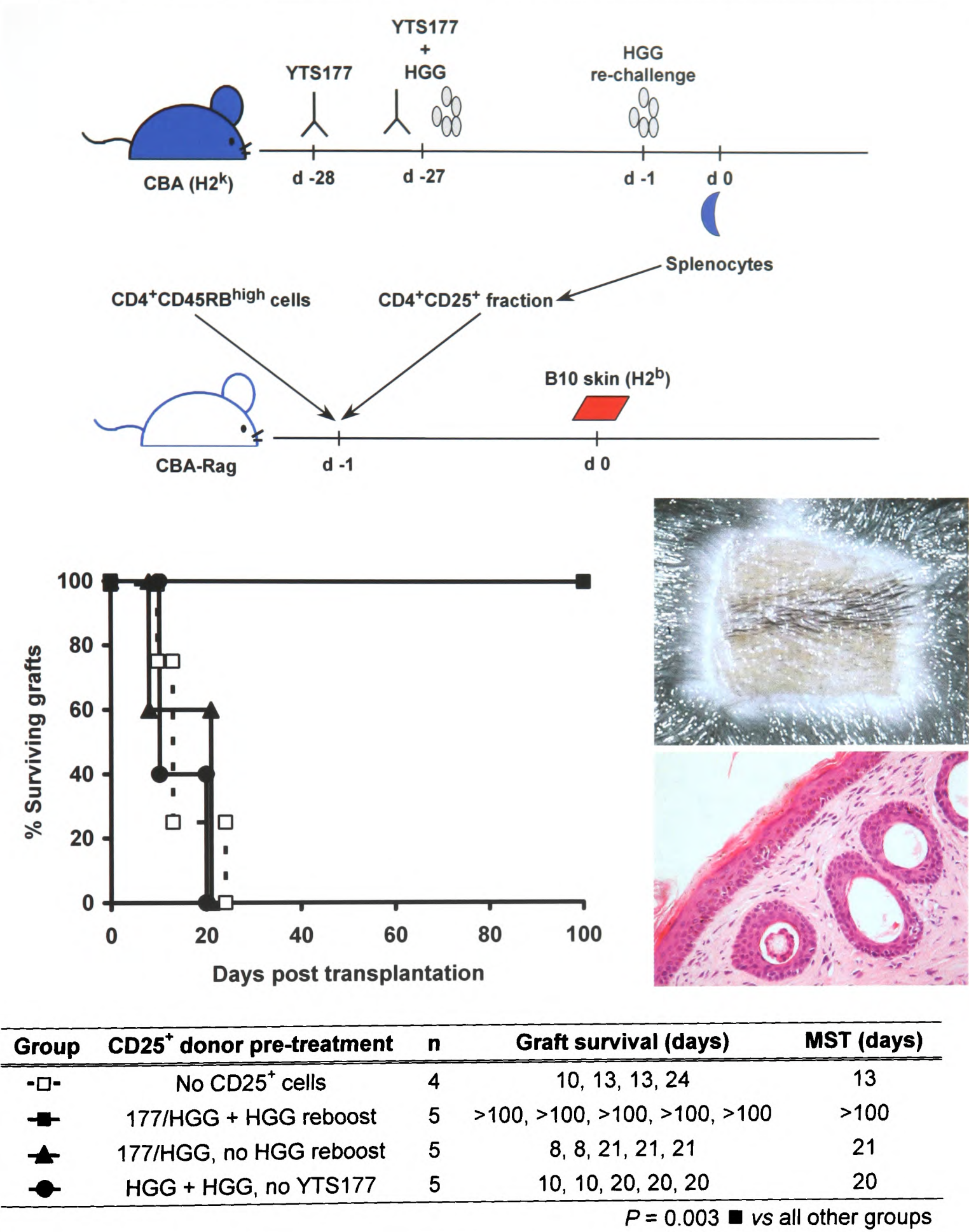


Figure 6.8. Re-activated CD4⁺CD25⁺ cells from mice pre-treated with 177/HGG regulate rejection of B10 skin allografts

CBA-Rag mice were reconstituted with 10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice together with 2×10⁵ CD4⁺CD25⁺ cells taken from CBA mice pre-treated with YTS177 (days -28 and -27), HGG (500 µg day -27), and HGG reboost (day -1) in the combinations indicated. The following day they received a B10 skin graft. Photographs show macroscopic and microscopic (H&E) appearances of a representative skin graft 100 days after transplantation onto a mouse reconstituted with both CD4⁺CD45RB^{high} and CD4⁺CD25⁺ cells from 177/HGG + HGG reboost group.

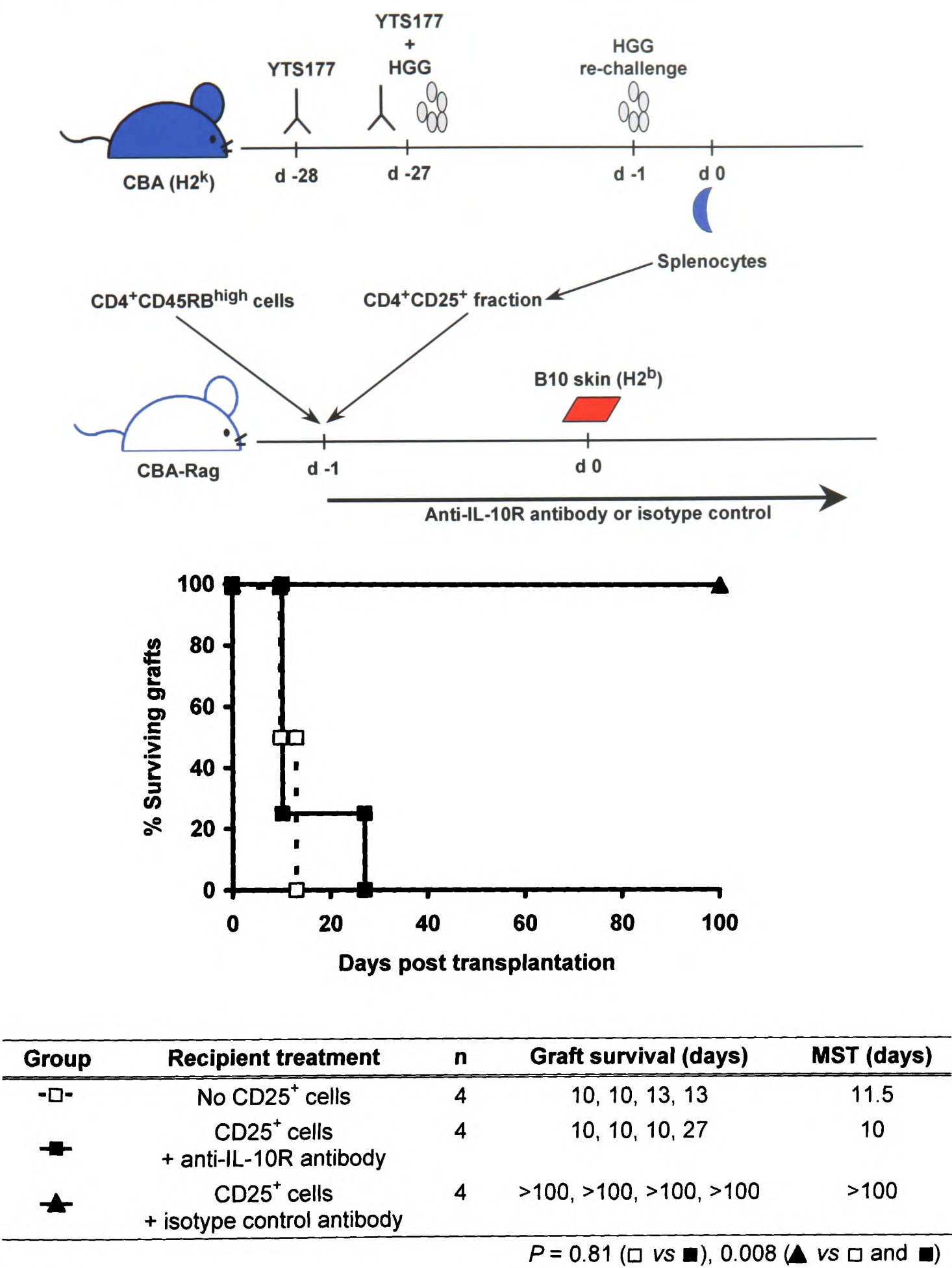


Figure 6.9. IL-10 blockade prevents bystander regulation by CD4⁺CD25⁺ cells *in vivo*

CBA-Rag mice were reconstituted with 10⁵ CD4⁺CD45RB^{high} cells from naïve CBA mice with or without 2×10⁵ CD4⁺CD25⁺ cells from CBA mice pre-treated with YTS177 (days –28 and –27) and HGG (500 µg day –27) followed by a further dose of HGG on day –1. The day after adoptive transfer B10 skin allografts were performed. Mice reconstituted with both CD4⁺CD45RB^{high} and CD4⁺CD25⁺ cells also received antibody therapy with the anti-IL-10R antibody 1B1.2 or isotype control antibody GL113, each administered at a dose of 1 mg via the intraperitoneal route at the time of cell adoptive transfer and then 0.5 mg weekly thereafter for 6 weeks or until graft rejection occurred.

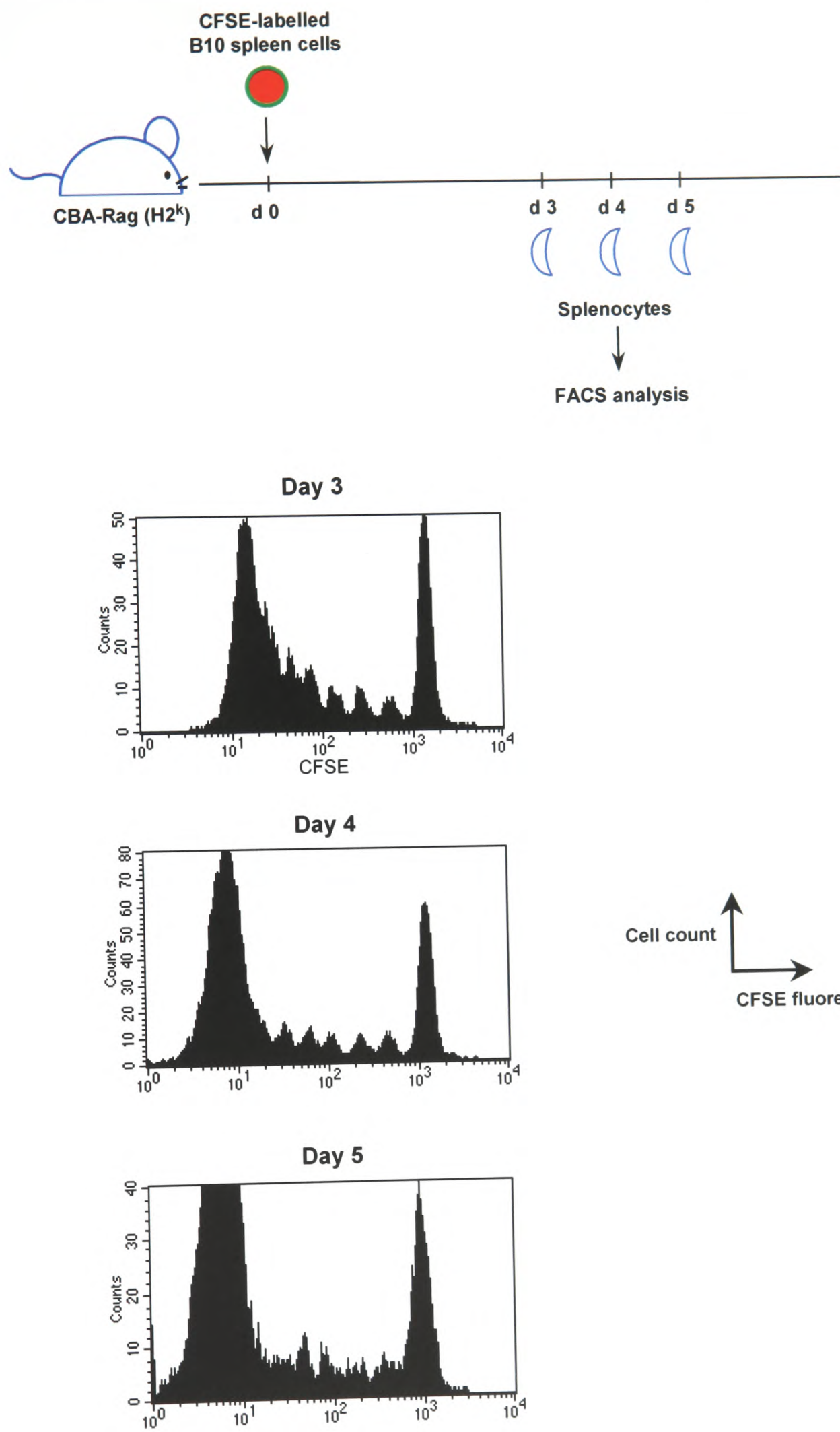


Figure 6.10. Division of allogeneic cells *in vivo*

CBA-Rag mice received 50×10^6 CFSE-labelled B10 spleenocytes intravenously on day 0. On day 3, 4, or 5 the spleens were harvested and the cells stained with anti-CD4-PerCP prior to flow cytometric analysis. Histogram plots show CFSE expression by CD4⁺ cells. Data are representative of two independent experiments.

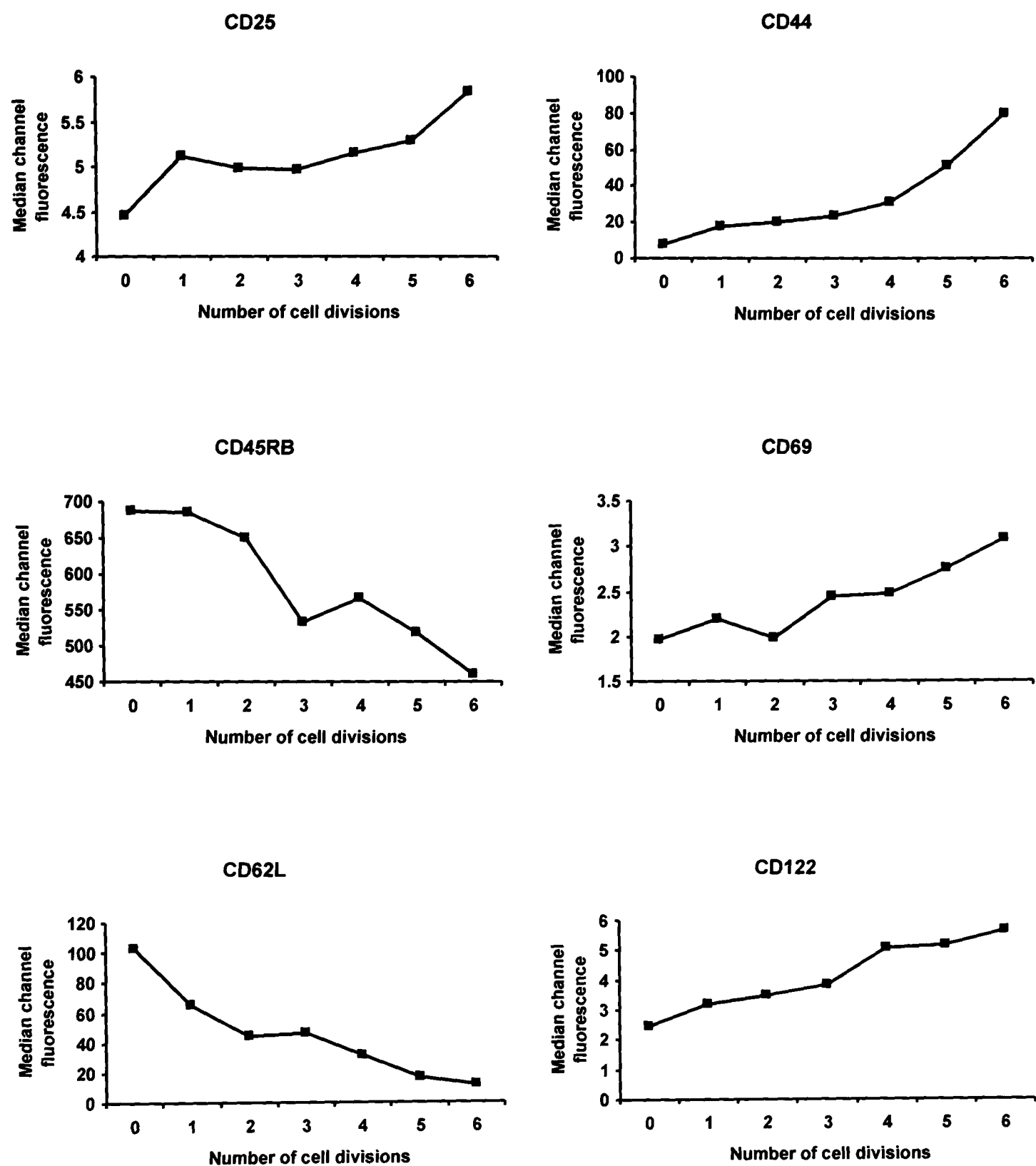


Figure 6.11. Expression of cell surface markers following cell division *in vivo* CBA-Rag mice received 50×10^6 CFSE-labelled B10 spleen cells intravenously. 4 days later splenocytes were isolated and stained with anti-CD4-PerCP together with anti-CD25-PE, anti-CD44-PE, anti-CD45RB-PE, anti-CD62L-PE, anti-CD69-PE, or anti-CD122-PE as appropriate. Flow cytometric analysis was then performed, and the number of cell divisions determined by analysis of CFSE expression by CD4⁺ cells. For each CFSE division peak median channel fluorescence for the indicated markers is shown. Data are representative of two independent experiments.

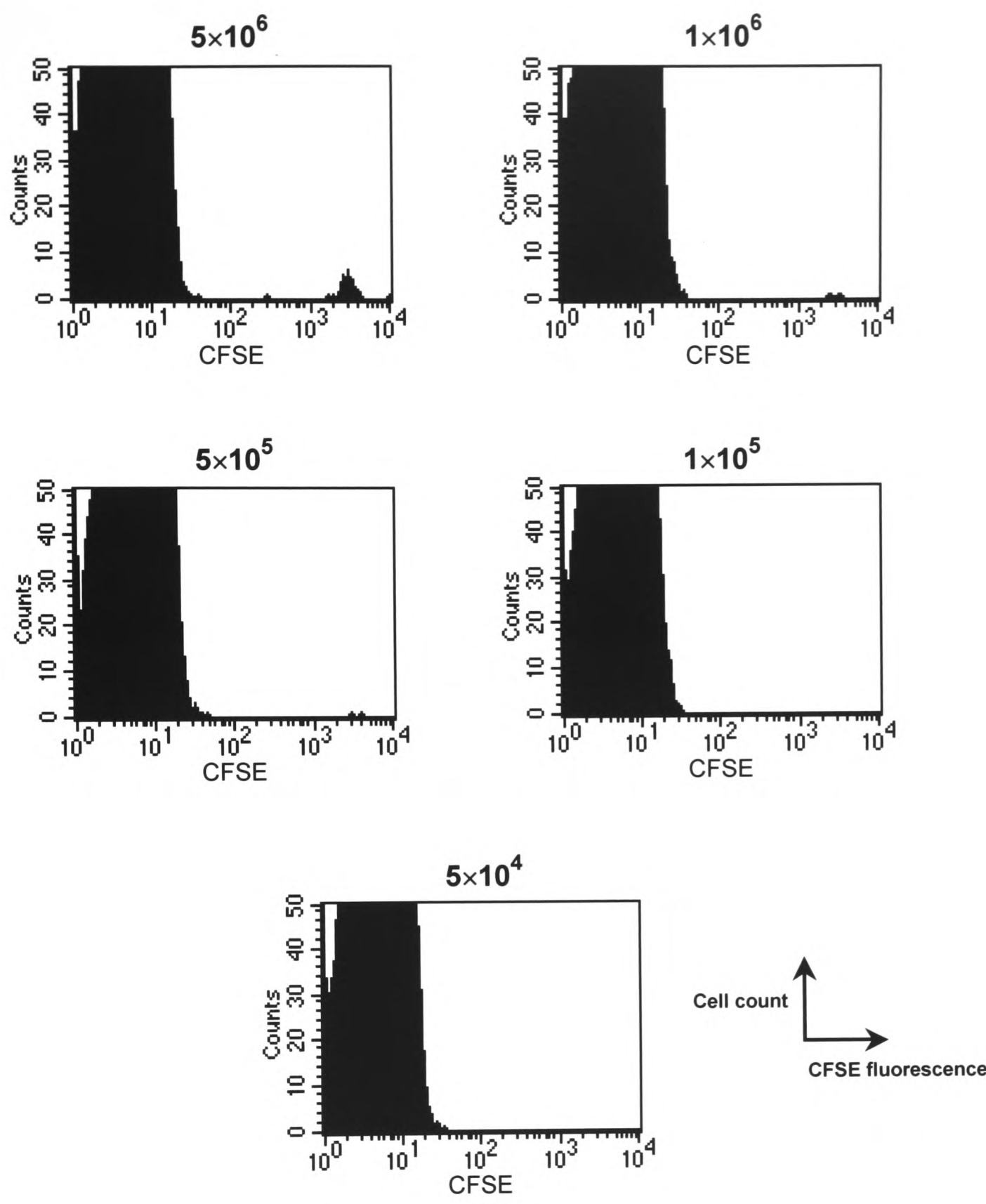


Figure 6.12. Detection of allogeneic CD4⁺ cells following adoptive transfer *in vivo* CBA-Rag mice received the indicated number of CFSE-labelled CD4⁺ B10 splenocytes intravenously. 4 days later the spleens were harvested and stained with anti-CD4-PerCP prior to flow cytometric analysis. Histograms show CFSE expression profile of CD4⁺ population. Data are representative of two independent experiments.

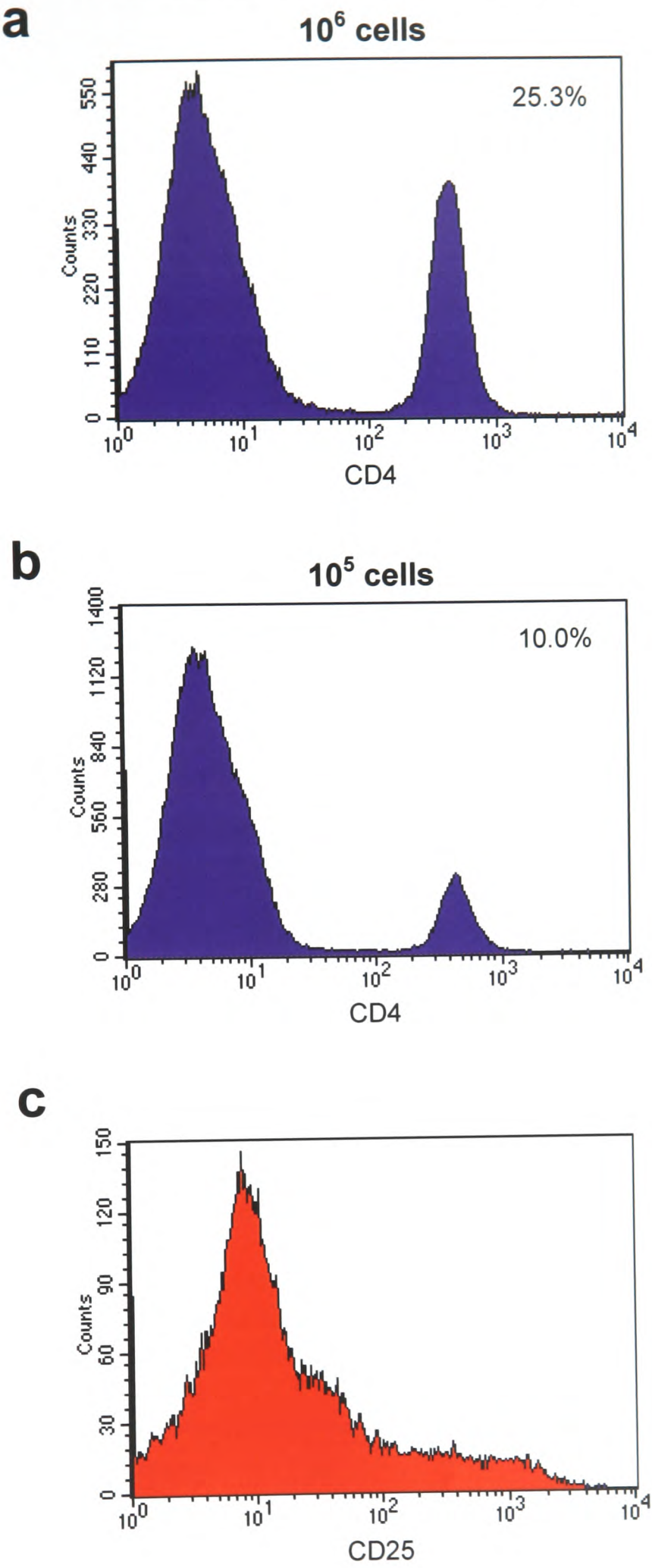


Figure 6.13. $CD4^+CD45RB^{high}$ cells can be detected following adoptive transfer into CBA-Rag recipients

CBA-Rag mice were reconstituted with (a) 10^6 or (b) 10^5 $CD4^+CD45RB^{high}$ cells from naïve CBA donors and the following day received a B10 skin allograft. 10 days after grafting splenocytes were isolated and stained with anti-CD4-FITC and biotinylated anti-CD25 followed by SA-APC prior to flow cytometric analysis. (a) and (b) show CD4 expression; percentages represent proportion of cells expressing CD4. (c) shows CD25 expression by $CD4^+$ cells from (a). Data are representative of two independent experiments.

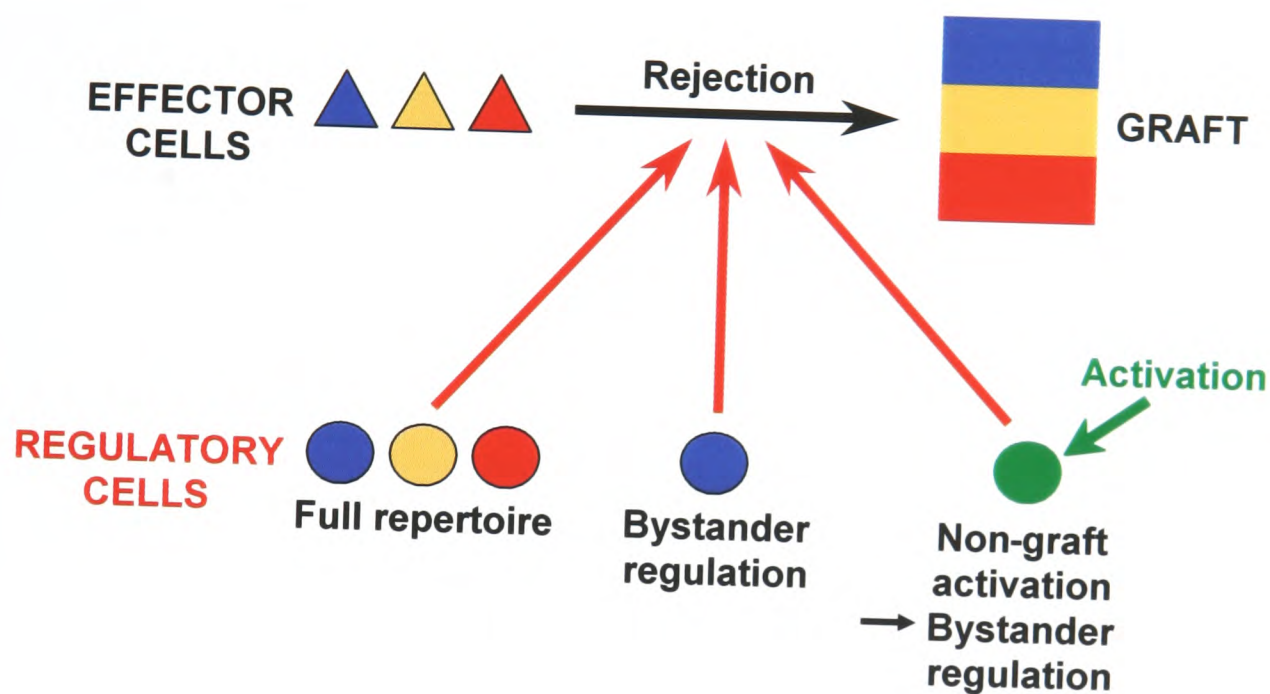


Figure 6.14. Proposed models for regulation of allograft rejection by Treg
Rejection may be overcome by the generation of Treg specific for all of the alloantigens expressed on a graft (full repertoire). Alternatively Treg may be generated against a single graft antigen; these Treg undergo activation by the graft and then suppress responses against other graft antigens (bystander regulation). Finally Treg generated against third party or even completely unrelated antigen can suppress graft rejection by bystander regulation provided that they are first activated before their functional activity is tested.

CHAPTER 7

DISCUSSION

7.1 Summary of results

This thesis has attempted to examine various aspects of the biology of CD4⁺CD25⁺ regulatory T cells in the context of a transplantation model. In this chapter the main findings will be summarised, following which the potential implications will be discussed.

7.1.1 Chapter 3

The main focus of Chapter 3 was to analyse the phenotypic characteristics of CD4⁺CD25⁺ cells capable of preventing the rejection of allogeneic grafts *in vivo*. Initially the phenotype of *ex vivo* CD4⁺CD25⁺ cells from normal unmanipulated mice (a cell population that has been shown by many groups to possess regulatory properties) was analysed. It was demonstrated that they have the phenotypic characteristics of activated cells and, more specifically, when compared to the reciprocal CD4⁺CD25⁻ population, a significant proportion shows constitutive expression of CTLA-4, CD122, and GITR. However, in contrast to some previous reports, no cell surface expression of TGF- β could be detected.

Next an *in vivo* model to examine the role of CD4⁺CD25⁺ T cells generated during the induction phase of transplantation tolerance was developed. It was shown that administration of donor-specific blood transfusion under the cover of anti-CD4 antibody (177/DST pre-treatment), a protocol that has previously been demonstrated to allow the long term survival of primary cardiac allografts, leads to the development of CD4⁺CD25⁺ cells that can regulate the rejection of donor strain skin allografts mediated by CD4⁺CD45RB^{high} cells in secondary recipients.

Having demonstrated that 177/DST pre-treatment does indeed lead to the generation of CD4⁺CD25⁺ cells capable of preventing graft rejection, the effect of this

pre-treatment on cell phenotype was examined. In mice that receive pre-treatment with 177/DST followed by a DST re-challenge, there is a modest increase in the proportion of CD4⁺ cells expressing CD25. Furthermore, within the CD4⁺CD25⁺ population, a greater proportion of cells expresses CTLA-4 and CD122, raising the possibility that these two molecules may act as phenotypic markers for Treg. Attempts to develop an assay to enumerate antigen-specific T cells, to allow more detailed examination of the effect of pre-treatment, were however unsuccessful.

7.1.2 Chapter 4

The overall aim of Chapter 4 was to develop an *in vitro* assay that would model the regulation of skin allograft rejection seen *in vivo*. As a prelude to this the *in vitro* characteristics of CD4⁺CD25⁺ cells were examined. It was shown that, as demonstrated in published studies, CD4⁺CD25⁺ cells do not proliferate when stimulated via their T cell receptors, but that this non-proliferative state is reversed by the addition of exogenous IL-2. It was next confirmed that *ex vivo* CD4⁺CD25⁺ cells can regulate the proliferation of responder CD4⁺ subpopulations in response to polyclonal stimulation, and this regulation was shown to be CTLA-4-dependent. Regulatory capacity is confined to cells expressing high (rather than intermediate) levels of CD25, and cells within the CD4⁺CD25⁻CD45RB^{low} population (about which published studies have yielded conflicting results) do not share this regulatory capacity. In view of the finding in Chapter 3 that a significant proportion of CD4⁺CD25⁺ cells from normal mice expresses CD122 and that this proportion rises following 177/DST pre-treatment, an attempt was made to determine whether regulatory cells within the CD4⁺CD25⁺ population are confined to the CD122⁺ subfraction; however, this was found not to be the case.

Having shown that $CD4^+CD25^+$ cells can regulate polyclonal responses *in vitro*, the aim was next to investigate whether similar regulation of responses to alloantigen stimulation could be demonstrated. Surprisingly, it became apparent that addition of $CD4^+CD25^+$ cells to cultures containing $CD4^+CD45RB^{high}$ responder cells and allogeneic stimulators leads to an increase in overall proliferation as measured by the incorporation of 3H -thymidine. Further investigation of this phenomenon revealed that this is due to proliferation of the $CD4^+CD25^+$ cells themselves, most likely driven by a soluble factor produced by the $CD4^+CD45RB^{high}$ population. An alternative method of measuring cell proliferation *in vitro* was therefore employed, using the fluorescent label CFSE. Using this approach it was shown that $CD4^+CD25^+$ cells can indeed regulate the proliferation of $CD4^+CD45RB^{high}$ cells in response to allogeneic stimulation. The data obtained suggested that, when using $CD4^+CD25^+$ cells from mice pre-treated with 177/DST, regulation of anti-donor responses may be more potent than of those against third party stimulation, but that this antigen specificity is abrogated by prior activation of the $CD4^+CD25^+$ population, an observation consistent with the phenomenon of bystander regulation described by others.

7.1.3 Chapter 5

Chapter 5 investigated the pathways by which 177/DST pre-treatment may generate $CD4^+CD25^+$ Treg *in vivo*. It has previously been established that naturally-occurring $CD4^+CD25^+$ Treg in mice develop predominantly through positive selection within the thymus. It was proposed that 177/DST pre-treatment may generate $CD4^+CD25^+$ Treg in three ways: via a similar intrathymic process, through the peripheral expansion of naturally-occurring $CD4^+CD25^+$ Treg that cross-react with donor alloantigen, or by conversion of peripheral $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells to a

CD4⁺CD25⁺ Treg phenotype. It was shown that the latter pathway, conversion of peripheral CD4⁺CD25⁻ T cells to a regulatory phenotype in a thymus-independent process, is sufficient for the generation of CD4⁺CD25⁺ Treg following administration of the 177/DST pre-treatment protocol.

7.1.4 Chapter 6

In Chapter 6, several aspects of regulation *in vivo* by CD4⁺CD25⁺ cells generated by antigen pre-treatment were examined. First, it was shown that Treg within the CD4⁺CD25⁺ population are not confined to the CD122⁺ or CD122⁻ subfractions, since both these populations are able to regulate the rejection of skin allografts.

Next the antigen specificity of regulation was investigated. It was found that the generation of CD4⁺CD25⁺ cells capable of regulating the rejection of skin allografts *in vivo* is not confined to a single strain combination, but that regulation in this setting is donor-specific. However, pre-treatment with a single donor alloantigen under the cover of anti-CD4 antibody is sufficient to generate cells capable of regulating the rejection of fully allogeneic grafts. Furthermore, CD4⁺CD25⁺ cells generated by pre-treatment with 177/DST can regulate the rejection of third party grafts if the cell donors are re-exposed to the original tolerising antigen, a step designed to re-activated putative Treg. These two latter observations would be consistent with the occurrence of bystander regulation *in vivo*. An even more compelling demonstration of bystander regulation was the finding that CD4⁺CD25⁺ cells that are generated by pre-treatment with the non-cellular protein antigen HGG and then re-activated are able to regulate the rejection of skin allografts, in a process that is IL-10-dependent.

In the final part of the chapter a system was described that may allow the tracking of effector and regulatory populations *in vivo*.

7.2 Discussion

The increased interest in the study of regulatory T cells in recent years has in part been stimulated by the recognition that a fuller understanding of their properties may enable the design of protocols allowing their clinical application in the therapy of autoimmune disease and in the prevention of allograft rejection.

As already discussed in this thesis, one major obstacle in the characterisation of Treg remains the lack of one or more phenotypic markers that identify regulatory populations with a high degree of sensitivity and specificity. Although CD25 is the most widely used marker at present, it is not specific to regulatory cells since it is also expressed on activated T cells, and its sensitivity is not absolute since some regulatory cells may lie within the CD25⁺ population. Other molecules, such as CTLA-4 and GITR, that have been shown to be expressed on Treg also lack specificity since they can be expressed on activated cells. The most promising marker identified to date is the transcription factor Foxp3, whose presence has been shown in the mouse to correlate well with regulatory activity [249, 315, 316]. Although a similar correlation has not yet been demonstrated in humans, the fact that Foxp3 deficiency in man leads to disease characterised by immune dysregulation [415-417] suggests that it may indeed also be a useful marker in man. The ability to identify regulatory T cells reliably by phenotypic analysis would be an extremely valuable tool since it may enable the success of tolerance induction regimens to be evaluated prior to transplantation, and following transplantation may allow the identification of patients in whom

conventional immunosuppressive therapy may safely be reduced. The utility of this approach would be even greater if the phenotypic identification of Treg could be combined with a method to assess their antigen specificity. As an alternative to the generation of regulatory cells *in vivo*, the ability to identify regulatory cells reliably could also allow the purification of cell populations *ex vivo* for therapeutic administration.

Although at present the precise identification of regulatory cells simply on the basis of phenotypic characteristics may not be an easily achievable goal, a more realistic option may be to use functional assays. As discussed in Chapter 4, the results of *in vitro* assays do not always correlate well with the behaviour of *in vivo* systems. However, it may be possible to design a functional *in vitro* assay that is sufficiently sophisticated to reflect the *in vivo* situation and that could therefore be used for the applications described in the previous paragraph.

The mechanisms by which naturally-occurring regulatory cells develop within the thymus have now been well studied. However, the demonstration that CD4⁺CD25⁺ Treg can also be generated in the periphery from CD4⁺CD25⁻ precursors has significant implications. Firstly, it shows that an intact thymus is not necessary for Treg generation, and thus clinical protocols need not exclude adults in whom thymic function is limited. Secondly, it suggests that the potential of regulatory cells may be harnessed to control immune responses to a wide range of antigens, and not to just those for which specific naturally-occurring Treg exist. Further investigation of the mechanisms by which CD4⁺CD25⁺ Treg are generated following administration of the 177/DST protocol *in vivo* remains an ongoing focus of research in our laboratory. It is well established that administration of antigen via the intravenous route may result in the development of tolerance rather than sensitisation, perhaps due to the lack of a

“danger” signal [540], and it is possible that the co-administration of anti-CD4 antibody facilitates this natural process. Indeed, we have shown that administration of a single DST at day -27 alone in the absence of antibody is ineffective at generating CD4⁺CD25⁺ cells capable of regulating skin allograft rejection [441], whereas multiple DSTs can do so [204]. It is possible that the anti-CD4 antibody facilitates persistence of donor alloantigen, since antigen persistence has been shown to have a profound effect on the induction of tolerance to primary cardiac allografts following anti-CD4/DST pre-treatment. Whilst a single DST under the cover of anti-CD4 antibody is sufficient to allow long term graft survival, the same dose of irradiated (non-viable) blood does not; however, tolerance induction can be restored if multiple doses of irradiated blood are given to ensure prolonged antigen persistence [157]. A further effect of the anti-CD4 antibody may be to attenuate signalling events in T cells, since it has been shown that antigen encounter by T cells in the presence of blockade with YTS177, the anti-CD4 antibody used in this thesis, results in an altered signalling pattern resembling that of anergic T cells [541]; this may facilitate the generation of Treg.

In Chapter 1 it was hypothesised that the beneficial effect on cadaveric renal allograft survival of pre-operative blood transfusions may, at least in part, be the result of the generation of regulatory cells. However, this raises the question of how transfusion of random blood can confer protection. One potential explanation may be provided by the data in Chapter 6 showing that generation of Treg against a single donor alloantigen can be sufficient to suppress the rejection of fully allogeneic grafts. It is thus possible that the beneficial effect of “random” transfusion is the result of fortuitous sharing of alloantigen(s) with the subsequent graft without the requirement for all graft alloantigens to be present in the transfusion. This would also explain the

observation that the beneficial effect of pre-operative transfusion increases with the number of transfusions given, since this would increase the probability of fortuitous antigen sharing. The high degree of cross-reactivity in the recognition of allogeneic MHC antigens may also be a factor, since giving more transfusions would increase the probability of providing epitopes that cross-react sufficiently with graft antigen. Indeed, recent work from our laboratory would support this view since it has been shown that pre-treatment with pooled blood mice of several allogeneic strains under the cover of anti-CD4 antibody can generate $CD4^+CD25^+$ cells capable of regulating the rejection of skin grafts from a distinct donor strain unrelated either to the recipient or to the blood donors [204].

Since $CD4^+$ cells predominantly recognise antigen presented in the context of MHC class II, the finding that $CD4^+CD25^+$ cells generated by pre-treatment with cells expressing the MHC class I molecule K^b as the only alloantigen or with the non-cellular protein antigen HGG are able to regulate the rejection of skin allografts demonstrates that Treg generation and activation via the indirect pathway of antigen recognition is sufficient for their function *in vivo* (Figure 7.1). These observations also have implications for the design of clinical protocols for the generation of Treg, discussed in more detail below.

As discussed in Chapter 1, Treg may exert their regulatory function in several ways: by direct interaction with APC mediated via T cell receptor binding to peptide-MHC complexes; through the secretion of soluble factors that act upon APC and target T cells; or possibly via a (currently hypothetical) direct but non-antigen-specific interaction with target T cells and / or APC. However, during the process of bystander regulation direct antigen-specific interaction with APC is not possible, and the HGG model thus demonstrates that regulation mediated

predominantly through the secretion of soluble factors is sufficient to prevent the rejection of skin allografts (Figure 7.2).

In addition to demonstrating the occurrence of bystander regulation *in vivo*, the HGG model also provides a system that allows different aspects of Treg generation and function to be studied separately. As already discussed, the pathway by which 177/DST pre-treatment generates $CD4^+CD25^+$ Treg is distinct from that by which naturally-occurring Treg develop within the thymus. In the majority of autoimmune disease models examining Treg function, naturally-occurring Treg are adoptively transferred together with effector cell populations, following which the Treg become activated and subsequently exert their regulatory function; however, these two processes cannot easily be studied separately (Figure 7.3a). In the 177/DST model, the generation of Treg can be studied in the cell donors, and the cell re-activation and regulation phases can be examined following skin transplantation in the recipients (Figure 7.3b). However, uniquely, in the HGG protocol re-exposure to HGG prior to cell adoptive transfer is required in order to achieve regulation; thus Treg generation (following pre-treatment), Treg re-activation (following HGG re-challenge), and regulation (following skin transplantation) can be studied separately (Figure 7.3c), providing an extremely valuable experimental system.

In the model shown in Figure 6.4 where the rejection of fully allogeneic grafts is prevented by $CD4^+CD25^+$ cells from mice pre-treated with cells expressing the single graft antigen K^b as the only alloantigen, the Treg can be activated *in situ* by graft antigen, and this activation can be maintained by continuous antigenic stimulation. However, the mechanisms involved in the HGG model are less straightforward. Firstly, the Treg in this system are not activated *in situ*, but instead are pre-activated prior to adoptive transfer and are then administered systemically,

following which they have to travel to the site at which regulation takes place. It is likely that these cells express surface molecules that facilitate their migration to sites of inflammation. One such molecule may be the integrin CD103 which, as has already been discussed in Chapter 3, is expressed by some Treg populations. Another example is the homing receptor CD62L (L-selectin): Szanya and colleagues have recently demonstrated that, in an autoimmune diabetes model, both the CD62L⁺ and CD62L⁻ subfractions of the CD4⁺CD25⁺ population show regulatory properties *in vitro*, whereas only the CD62L⁺ cells regulate the development of disease *in vivo* [224]. Similarly, CD4⁺CD25⁺ Treg have been shown to express chemokine receptors such as CCR4 [542], CCR5 [543], CCR7 [224], and CCR8 [542], which may affect their homing pattern. However, bystander suppression raises the concern that the presence of activated Treg may also lead to the unwanted suppression of protective immune responses, for example against infection. Furthermore, it is possible that even graft-specific Treg may exert some non-specific immunosuppressive effects in this way. In a study by Iwashiro *et al.*, it was shown that mice chronically infected with retrovirus developed an increase in the size of the CD4⁺CD25⁺ population and showed impaired ability to reject tumours [544]. Similarly, in a transplantation model Williams and colleagues found that induction of tolerance to allografts by the administration of costimulatory blockade led to an impaired immune response to viral infection [545]. However, perhaps more reassuringly, data from our laboratory suggest that Treg generated by the 177/DST protocol do not impair anti-viral responses (A.R. Bushell, E. Jones, A. Gallimore, manuscript in preparation). This is clearly an area that requires further investigation.

A further issue raised by the HGG model relates to the duration of Treg activation. When pre-activated CD4⁺CD25⁺ cells generated against HGG are

administered to mice, it is unlikely that, following adoptive transfer, they encounter cognate antigen that would allow their activation status to be maintained. In contrast, the $CD4^+CD45RB^{high}$ effector population should be capable of undergoing stimulation by graft antigen long after the activation status of the $CD4^+CD25^+$ Treg has waned. Although the length of time for which the $CD4^+CD25^+$ cells remain activated is at present unknown, preliminary data suggest that this is unlikely to be longer than a few days (B.S. Sawitzki and C.I. Kingsley, manuscript submitted). This raises the question of how these cells can enable long term graft survival rather than simply delay acute rejection. There are several potential explanations for this. The simplest is that there is sufficient cross-reactivity between HGG and mouse gamma globulin to maintain the activation status of the Treg; ongoing work in the laboratory using other model antigens will allow this possibility to be addressed. A second potential explanation is that the Treg mediate the deletion of effector cells; although this seems unlikely, it could be investigated by using the *in vivo* tracking system described in Chapter 6. Alternatively, Treg activity may lead to conversion of effector cells to a regulatory phenotype in a process akin to infectious tolerance; this could be examined by isolating $CD4^+CD25^+$ cells derived from the $CD4^+CD45RB^{high}$ population (purified on the basis of markers such as transgenic human CD52 as discussed in Chapter 6) from mice following skin transplantation and then testing their ability to regulate skin allograft rejection upon further adoptive transfer, although the cell yields obtainable may render this approach impractical. A fourth possibility is based on the fact that there are various mechanisms by which tolerance to allografts may develop naturally [546], some of which are summarised in Figure 7.4. Under normal circumstances, rejection occurs before these mechanisms become effective. However, pre-activated Treg may provide a window of protection sufficient for natural tolerance to develop.

This hypothesis would be consistent with the suggestion from clinical studies in Cambridge [547, 548] and Pittsburgh [549] that the use of certain peri-operative immunosuppressive protocols may reduce the requirement for maintenance therapy.

Although, as discussed in Chapter 1, pre-operative blood transfusion has been shown to have a protective effect on graft survival in clinical renal transplantation, this approach raises concern over the potential for transmission of infectious agents [550-557]. More specifically, in view of the perceived risk of transmission of prion-related spongiform encephalopathies [553, 554], blood used for transfusion in the United Kingdom is now depleted of leukocytes [553]. Although elective blood transfusion may carry some risk, it is probable that this is outweighed by the potential advantages obtained by reducing the need for long term non-specific immunosuppressive therapy in transplant patients. However, the demonstration that cells capable of regulating allograft rejection can be generated by administration of a non-cellular non-graft antigen (presented via the “indirect” pathway) may provide the basis for an even more attractive strategy. If this approach is to be useful it will be necessary to develop a protocol in which pre-treatment with such an antigen prevents the rejection of allografts in *primary* recipients rather than in an adoptive transfer system; this is currently under investigation in the laboratory. A further potential advantage of using a defined non-graft antigen for pre-treatment is that, provided that the antigen in question is not encountered by the recipient under normal circumstances, the timing of Treg re-activation can be carefully controlled.

If clinical protocols could be developed that lead to the generation of Treg capable of preventing allograft rejection, these could be applied in several ways. In live donor transplantation, where both the identity of the donor and the timing of the procedure are known in advance, it may be possible to generate Treg by pre-treatment

with donor alloantigen (Figure 7.5a). However, this is not possible in cadaveric transplantation, where an alternative strategy would therefore be necessary. Instead of pre-treating with donor alloantigen, Treg could be potentially generated by pre-treatment with a defined antigen and then re-activated immediately prior to transplantation by antigen re-challenge (Figure 7.5b). It may also be possible to extend this type of protocol for use outside the field of transplantation for the therapy of autoimmune disease.

At present there are two anti-CD25 antibodies, basiliximab and daclizumab, licensed for use in the United Kingdom for the prevention of acute allograft rejection. The rationale behind their use is that they target activated cells (which express CD25) and thus reduce the risk of rejection. This clearly raises the concern that they may also impair the development and / or function of regulatory cells within recipients. However, there are several considerations that make it difficult to predict whether this concern is justified. Firstly, these antibodies are used as an adjunct to conventional immunosuppressive therapy, and it is possible that this conventional therapy may impair the development of Treg [558] such that regulation does not normally play a significant role in promoting graft survival. Next, assuming that Treg do develop despite the use of conventional agents, these Treg may be less susceptible than the effector population to the effects of anti-CD25 antibody. There is experimental evidence that would support this scenario since, in a mouse cardiac allograft model, it has been shown that Treg are more resistant to depletion by anti-CD4 antibody than are effector T cells [156]. Finally, assuming that Treg are depleted by the anti-CD25 therapy, it is hypothetically possible that the CD25⁺ population that subsequently reconstitutes is skewed towards either a regulatory or effector phenotype. Thus on theoretical grounds alone it is not possible to predict the effect of anti-CD25 antibody

therapy on Treg populations. Clinical studies have failed to shed further light on this issue.

Table 7.1 summarises the results of the major clinical trials investigating the use of basiliximab and daclizumab in renal transplantation. All but one of these trials demonstrated a reduction in the rate of acute rejection episodes. However, analysis of longer term outcome yielded less clear results. The majority of the studies did not show a significant effect on graft function or on rates of graft loss or patient survival. Although this may have been due to lack of statistical power, a meta-analysis has also failed to show any long term benefit of anti-CD25 antibody therapy [559]. Interestingly, a study investigating the effect of another anti-CD25 antibody, BT563, on outcome following orthotopic liver transplantation suggested that the use of this agent actually led to an increase in the rate of chronic rejection, an effect that could be due to an adverse effect of treatment on regulatory cells. Thus overall, the effect of the clinical use of anti-CD25 antibody therapy on Treg activity remains unclear, and this

Reference	Antibody	Acute rejection	Graft function	Graft loss	Patient survival
[101]	Basiliximab	↓ 6 months	NS 1 year	NS 1 year	NS 1 year
[102]	Daclizumab	↓ 6 months	NS 6 months	NS 1 year	NS 1 year
[103]	Basiliximab	↓ 1 year	↑ 1 year	NS 1 year	NS 1 year
[104]	Daclizumab	↓ 6 months	↑ 6 months	NS 3 years	↑ 3 years
[107]	Basiliximab	↓ 6 months	NS 1 year	NS 1 year	NS 1 year
[111]	Daclizumab	↓ 6 months	NS 1 year	NS 1 year	NS 1 year
[112]	Basiliximab	NS 6 months	NS 4 weeks	NS 6 months	NS 1 year

Table 7.1. Clinical studies investigating the effect of the use of anti-CD25 antibodies compared to placebo in renal transplant recipients at time points indicated

NS = no significant difference

remains an area that merits further investigation if the true potential of Treg is to be harnessed. In particular, if $CD4^+CD25^+$ Treg are to be generated prior to transplantation, it will be important to know whether the peri-operative use of anti-CD25 antibodies negates any protective effect of these cells on graft survival.

7.3 Conclusions

As shown in Figure 7.6, the immune system has evolved to allow the elimination of pathogenic organisms and to play a role in tumour surveillance. However, if immune responses occur against self antigens this can result in the development of autoimmune disease, with potentially dire consequences. Similarly, if immune responses develop that are against appropriate targets but that are inappropriately severe in scale, tissue damage may occur, for example in some hypersensitivity reactions. Finally, the rejection of allografts also represents an unwanted immune response. It is clear that in mice naturally-occurring regulatory T cells play an important role in the prevention of autoimmunity. A fuller understanding of the properties of Treg may allow the design of clinical protocols to control deleterious responses (that cause autoimmune disease or allograft rejection) more effectively than current therapy allows, and potentially with fewer adverse side effects. The data presented in this thesis may assist in achieving this tantalising goal.

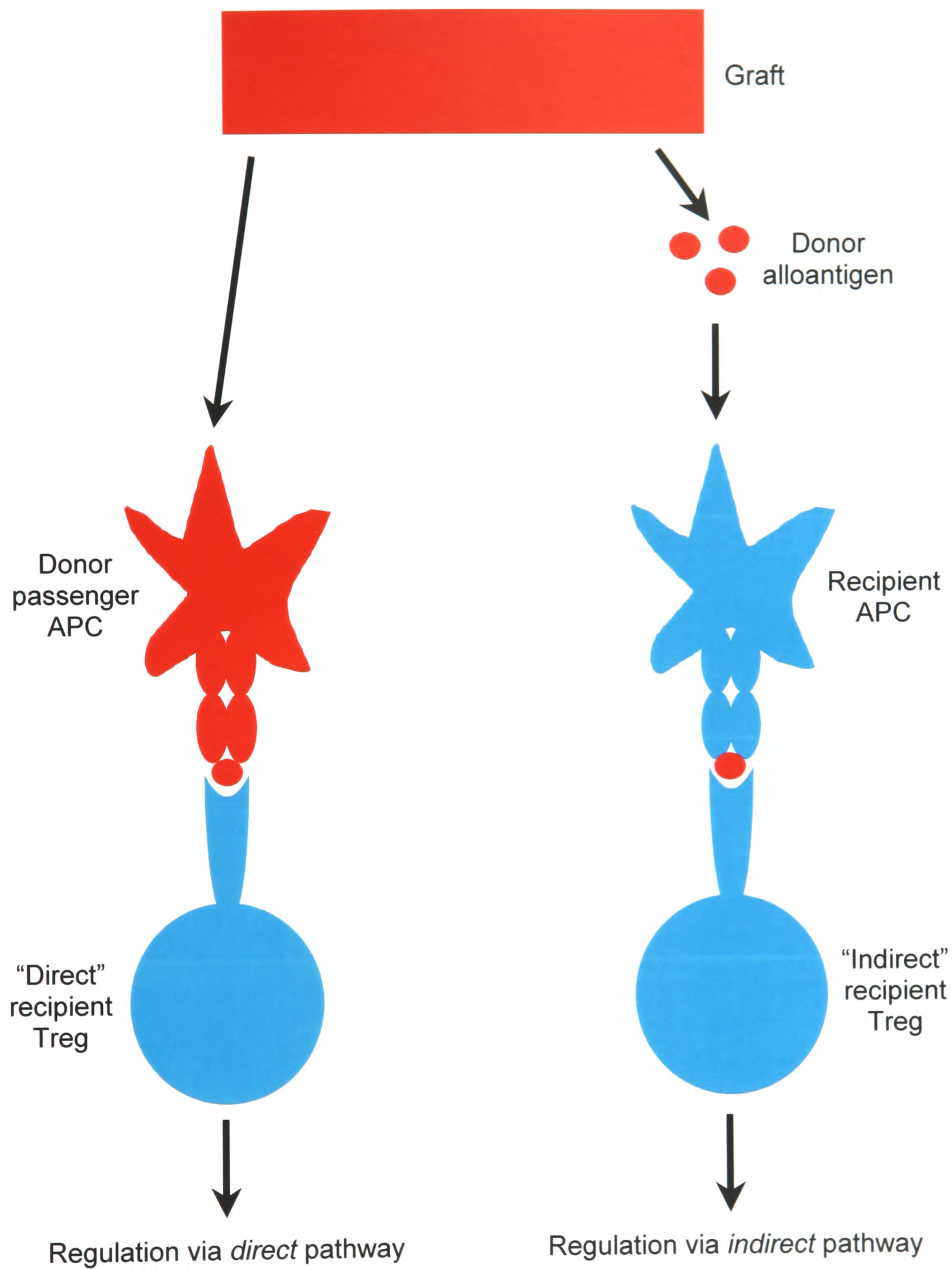


Figure 7.1. Activation of regulatory cells via the direct and indirect pathways
Recipient Treg capable of recognising alloantigen via the direct pathway may be activated by donor passenger APCs, whereas those that recognise alloantigen via the indirect pathway require activation by recipient APCs that present donor antigen presented in the context of self MHC.

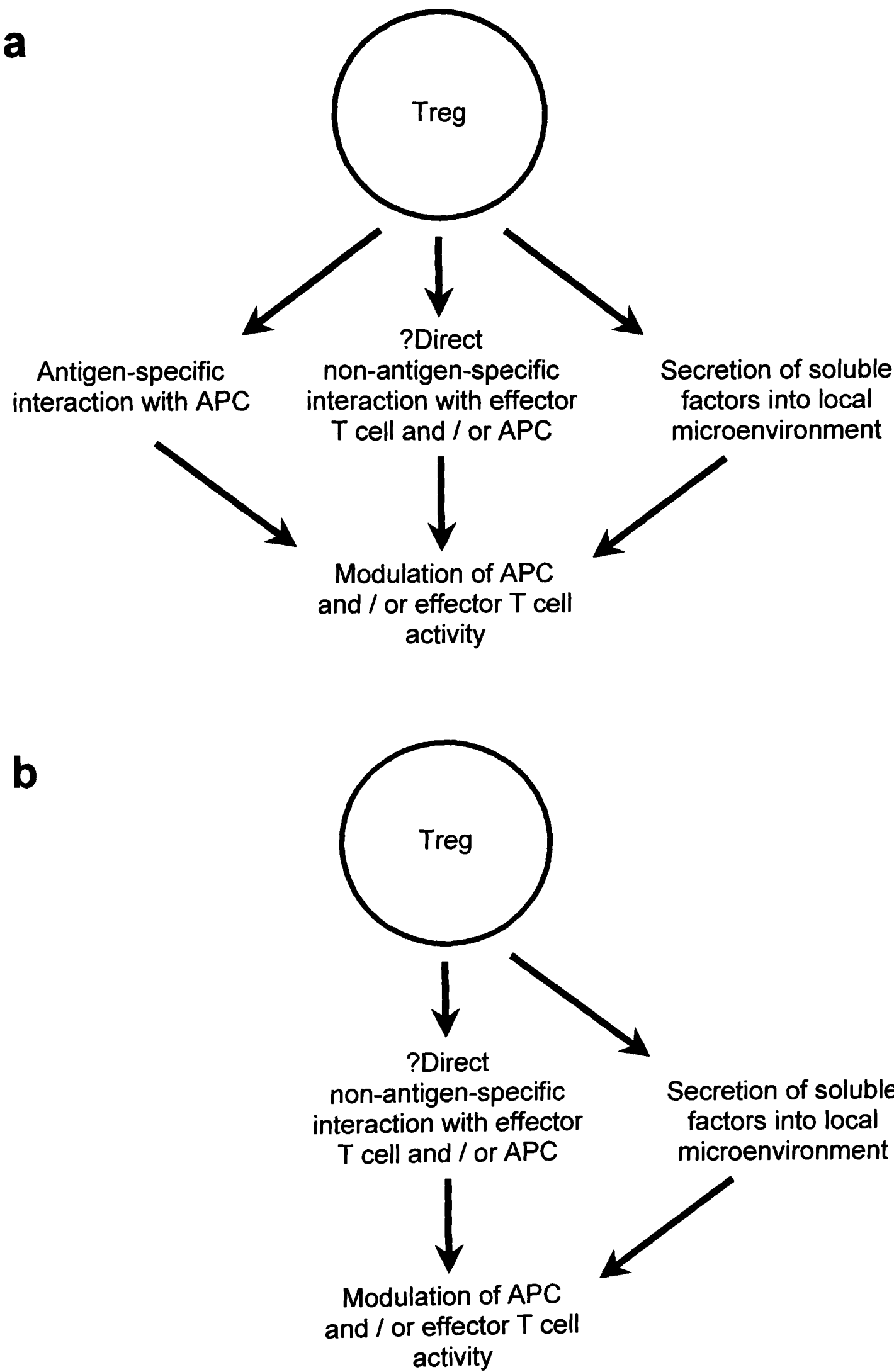


Figure 7.2. Mechanism of bystander suppression

(a) Under normal circumstances Treg may exert their regulatory effects either directly (via antigen-specific interaction with antigen presenting cells or possibly via direct contact with effector T cells or non-antigen-specific interaction with antigen presenting cells) or indirectly through the secretion of soluble factors. (b) Bystander regulation is likely to be mediated predominantly through the secretion into the local microenvironment of soluble factors which then modulate the activity of antigen presenting cells and / or effector T cells.

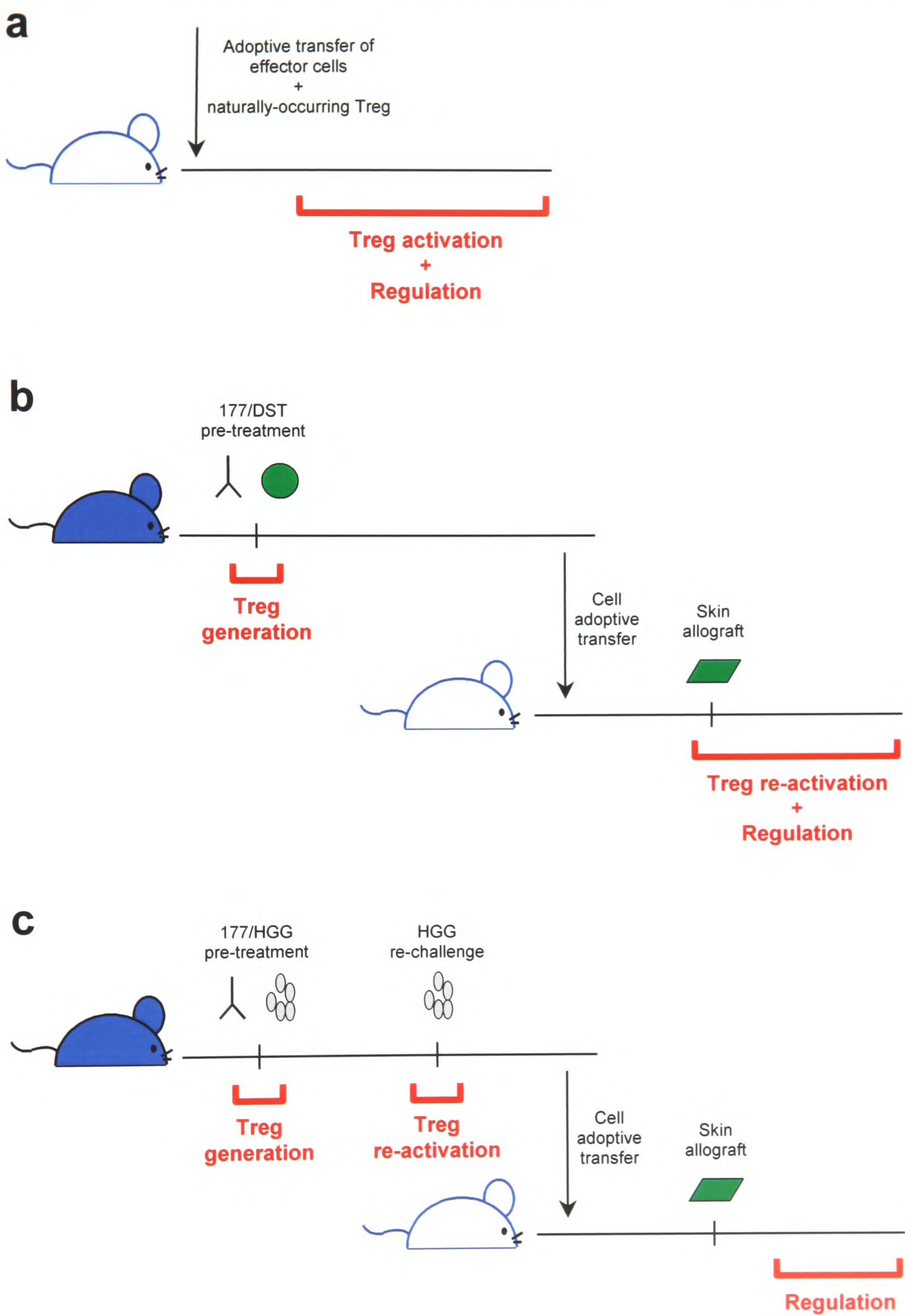


Figure 7.3. Investigation of the stages of regulatory cell generation and function
(a) Most autoimmune disease models employ naturally-occurring regulatory cells. Activation of Treg and regulation occur together following cell adoptive transfer.
(b) The 177/DST model allows generation of Treg (which occurs following 177/DST pre-treatment) to be studied separately from Treg re-activation and regulation (following skin grafting).
(c) The HGG model allows the separate study of Treg generation (following pre-treatment), Treg re-activation (following HGG re-challenge), and regulation (following skin transplantation).

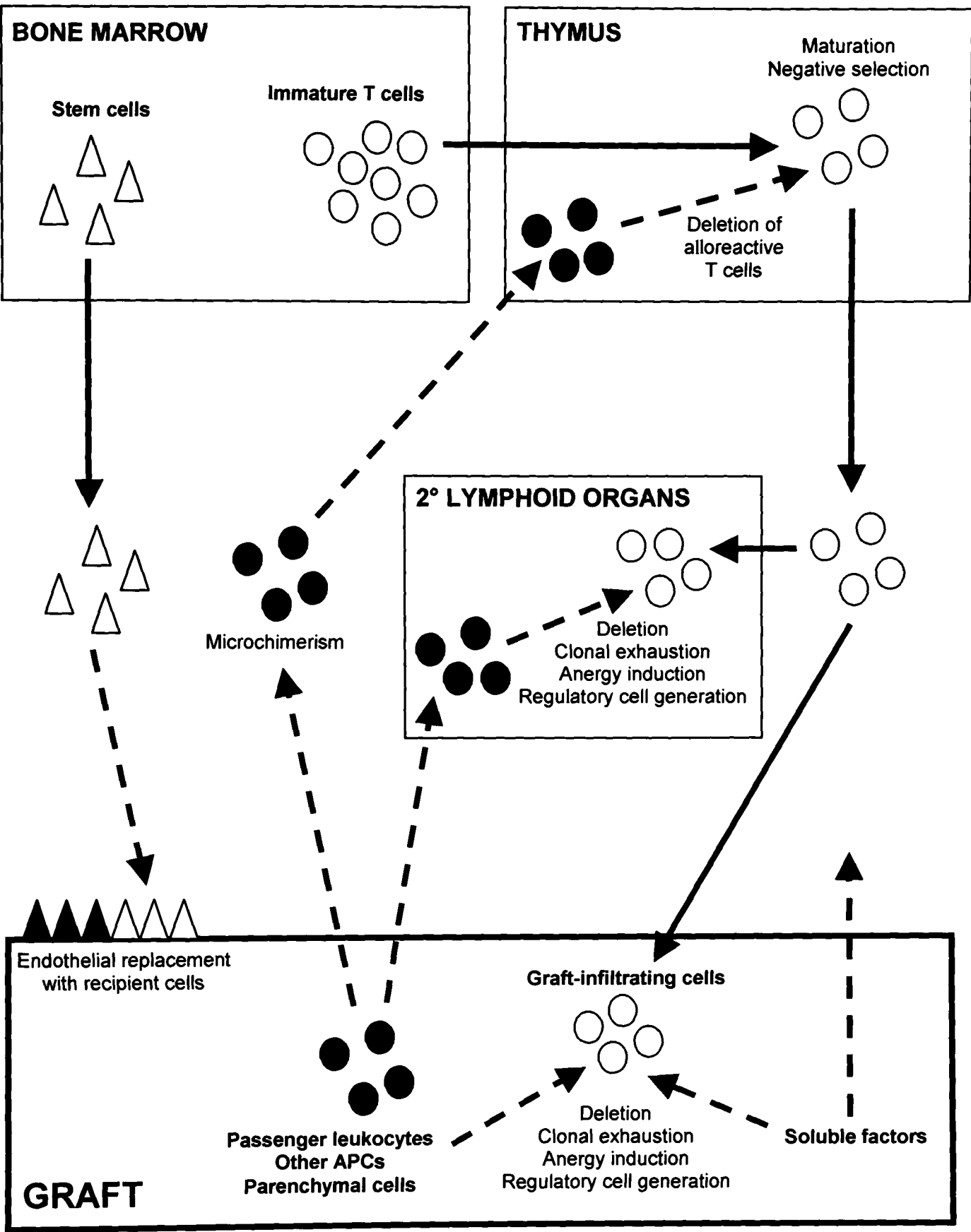


Figure 7.4. Potential mechanisms by which graft tolerance may develop
Following transplantation graft tolerance may develop through a number of mechanisms (solid symbols represent donor cells and open symbols recipient cells). The presence of regulatory cells at the time of transplantation may provide sufficient graft protection to allow the development of tolerance through such mechanisms.

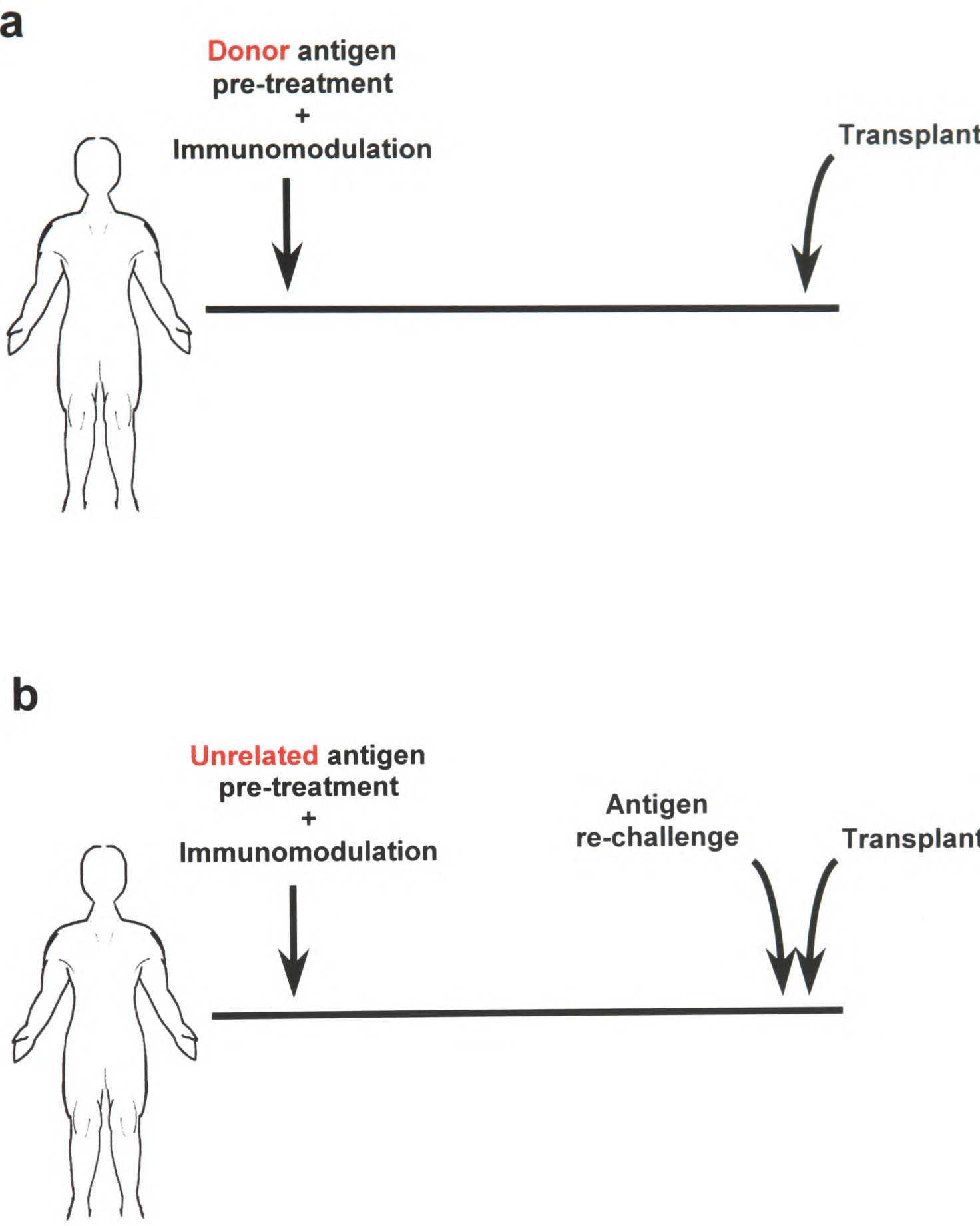


Figure 7.5. Potential generation of regulatory cells in clinical transplantation
(a) If the identity of the donor is known prior to transplantation (as in the case of live donors) it may be possible to generate regulatory cells by recipient pre-treatment with donor alloantigen. (b) If the identity of the donor is not known in advance it may be instead be possible to generate regulatory cells by pre-treatment with a defined non-graft antigen and then to re-activate these cells immediately prior to transplantation by giving an antigen re-challenge.

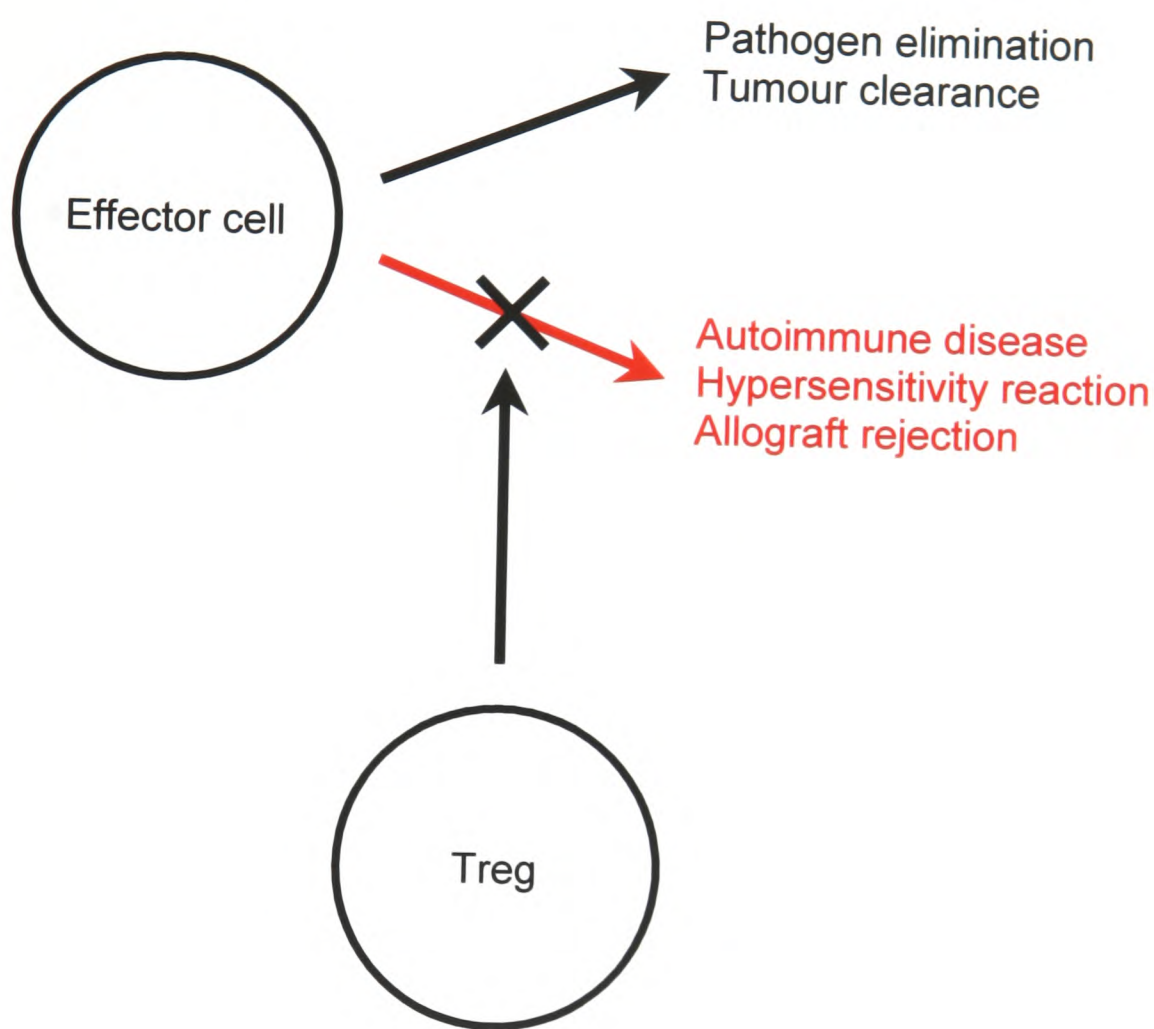


Figure 7.6. Control of immune responses by regulatory T cells

Protective immune responses can mediate the elimination of pathogenic organisms and of tumours. However, immune responses may also have deleterious consequences such as the development of autoimmune disease or hypersensitivity reactions, or the rejection of allografts. The use of regulatory T cell populations may allow control of these undesirable responses.

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