The Regulation of Peripheral T Cell Responses in TCR Transgenic Mice

Matthew E. Wikstrom

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Department of Experimental Medicine
The University of Sydney

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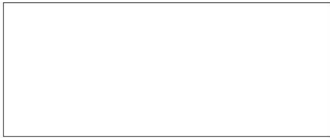
Preface

The work described in this thesis was designed to investigate the mechanisms of regulation of peripheral T cell responses in TCR transgenic mice. Unless otherwise stated, all experiments were carried out by the author at the Centenary Institute of Cancer Medicine and Cell Biology between January 1992 and October 1995. The work is entirely original and has not been presented previously for the purpose of obtaining any other degree.



Matthew Wikstrom

B.App.Sc. (Hons).

Abstract

Transgenic mice expressing a T cell receptor specific for cytochrome C in association with I-E^k were employed to study the mechanisms regulating the decision between tolerance and immunity. Tolerance and immunity to the same peptide antigen were induced by using different routes of administration. Thus tolerance was induced by the intravenous route and immunity by the subcutaneous route. The results presented in this thesis provide a detailed map of the cellular events that occur during these two distinct responses.

Intravenous immunisation induced activation of $CD4^+$ cells expressing the transgenic TCR ($CD4^+Tg\alpha^+$ cells), resulting in CD69 expression within two hours, priming of T cell proliferation and Th1 cytokine production by 24 hours. Clonal expansion peaked 3-4 days after immunisation. The number of $CD4^+Tg\alpha^+$ cells decreased rapidly after day four, such that only 50% of initial cell numbers remained 7-10 days after immunisation. Intravenous peptide also upregulated CD44 expression, increasing the proportion and number of $CD4^+Tg\alpha^+CD44^{hi}$ cells at the peak of the response, but having no net effect at the resolution of the response. When labelled $CD4^+Tg\alpha^+$ cells were adoptively transferred to syngeneic non-transgenic hosts, deletional tolerance to intravenous peptide was still seen, demonstrating that it was not an artefact of the high precursor frequency in TCR transgenic mice. Antigen re-challenge experiments demonstrated that $CD4^+Tg\alpha^+$ cells remaining at the resolution of the primary response to intravenous peptide could not respond as vigorously as naive cells either *in vitro* and *in vivo*. Interestingly, intravenous administration of intact cytochrome C also stimulated T cell activation, but failed to induce peripheral deletion, and resulted instead in a minor increase in the final proportion of $CD4^+Tg\alpha^+CD44^{hi}$ cells.

Subcutaneous immunisation with peptide emulsified in CFA induced T cell activation with a similar sequence of events to those observed during the intravenous response, namely CD69 expression, cytokine production, CD44 upregulation and clonal

expansion. However, significant differences were seen: first, the T cell response was localised to the draining lymph nodes; second, the spectrum of cytokines produced by $CD4^+Tg\alpha^+$ cells from the draining lymph nodes was influenced by the dose of peptide administered; third, the response exhibited slower kinetics than the intravenous response; and fourth, $CD4^+Tg\alpha^+$ cells retained their functional activity at the conclusion of the response and were more sensitive to low doses of antigen both *in vitro* and *in vivo*. This correlated with an elevation in the number of small $CD4^+Tg\alpha^+CD44^{hi}$ cells six weeks after immunisation, although there was no net increase in the number of antigen-specific T cells. Subcutaneous administration of peptide without adjuvant also increased the number of small $CD4^+Tg\alpha^+CD44^{hi}$ cells, though not to the extent seen with adjuvant. Thus subcutaneous immunisation of TCR transgenic mice appeared to generate a state of memory mediated by a change in phenotype in the absence of a change in the precursor frequency.

Two adoptive transfer models were used to test the ability of particular APC populations to present intravenous antigen, in an attempt to understand why peptide but not intact protein induced deletion. In the first system, enriched $I\text{-}E^+$ APCs were transferred into -D x 36-2 double transgenic mice, which possess an endogenous population of $CD4^+Tg\alpha^+$ cells but do not express I-E in the periphery. Intravenous immunisation with peptide induced only low levels of CD69 expression on $CD4^+Tg\alpha^+$ cells in recipients of either unfractionated $I\text{-}E^+$ spleen cells or enriched $I\text{-}E^+$ B cells. No increase in CD69 expression was seen in recipients of cultured $I\text{-}E^+$ splenic dendritic cells. Thus it appeared that repopulation by transferred APC was not efficient enough to stimulate effective T cell responses. In the second system, $CD4^+Tg\alpha^+$ cells and enriched $I\text{-}E^+$ APC populations were transferred into $I\text{-}E^+$ or $I\text{-}E^-$ homozygous *scid/scid* or Rag-1-deficient recipients. Intravenous immunisation induced high level CD69 expression and clonal expansion of $CD4^+Tg\alpha^+$ cells *in vivo*. However, cell numbers then returned to baseline, so that no net deletion was seen. Thus immunodeficient recipients appear to be defective in induction of tolerance via

peripheral deletion. Enriched B cells induced low levels of T cell activation, but were not as effective as unfractionated APCs.

The interaction between responses to intravenous and subcutaneous peptide was investigated by administering intravenous peptide at various times after subcutaneous immunisation. Simultaneous administration of intravenous peptide reduced the number of CD4+Tgα+ cells in the draining lymph nodes of mice six weeks after immunisation with subcutaneous peptide/CFA, although the reduction was not as great as that seen in unimmunised controls. When intravenous peptide was delayed four days, the number of $CD4^{+}Tg\alpha^{+}$ cells and the size of the $CD4^{+}Tg\alpha^{+}CD44^{hi}$ population in the draining lymph nodes six weeks later was unaffected, although $CD4^{+}Tg\alpha^{+}CD44^{lo}$ cells in other lymphoid organs were still susceptible to deletion. Administration of intravenous peptide six weeks after subcutaneous peptide/CFA reduced the total number of $CD4^{+}Tg\alpha^{+}$ cells, without changing the number of $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells either within the draining lymph nodes or systemically. Taken together, these results suggest that $CD4^{+}Tg\alpha^{+}$ cells acquire resistance to deletion by intravenous peptide within four days of subcutaneous immunisation, and that $CD4^{+}Tg\alpha^{+}CD44^{hi}$ generated by subcutaneous immunisation are resistant to deletion even at late time points.

Abbreviations

1°	Primary immunisation
2°	Secondary immunisation
APC	Antigen-presenting cell
BrdU	5-Bromo-2'-deoxy-uridine
$CD4^+Tg\alpha^+$	$CD4^+$ cells expressing both chains of the transgenic TCR
$CD4^+Tg\alpha^-$	$CD4^+$ cells expressing the β chain but not the α chain of the transgenic TCR
CDR	Complementarity determining region
CFA	Complete Freund's adjuvant
CFSE	5-carboxyfluorescein diacetate-succinimidyl ester
CLIP	Class II-associated invariant chain peptide
daPCC	Des-ala pigeon cytochrome C
DC	Dendritic cell
DTH	Delayed-type hypersensitivity
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte and macrophage colony stimulating factor
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL-2	Interleukin 2
IL-3	Interleukin 3
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-13	Interleukin 13

LC	Langerhans cell
LFA	Lymphocyte function-related antigen
MCC	Moth cytochrome C
MHC	Major histocompatibility complex
PCC	Pigeon cytochrome C
PE	Phycoerythrin
TCM	T cell medium (RPMI 1640 containing 20mM HEPES, 10mM sodium bicarbonate, 50mg/L penicillin, 100mg/L streptomycin, 10% FCS, 2mM glutamine and 50μM 2-mercaptoethanol)
TCR	T cell receptor
Tg	Transgenic
TGF	Transforming growth factor
Th	T helper
Th1	T helper subset 1
Th2	T helper subset 2
Tm	T memory
TNF	Tumour necrosis factor

Section 1. Introduction.

1.1. An overview of peripheral immunity.

Foreign organisms and the substances they produce pose a serious threat to the survival of the host, so it is important that an efficient system exists to identify and eliminate them. The mammalian immune system is characterised by its ability to identify foreign organisms and their components and to effect their removal and destruction without compromising the integrity of the host.

The most important aspect of this process is the specificity of the immune response. In the case of cell-mediated immunity, the specificity is dictated by T lymphocytes. T lymphocytes bear an antigen-sensitive receptor, the T cell receptor (TCR), which must be triggered in order to initiate an immune response. The TCR is usually triggered by a complex of a major histocompatibility complex (MHC) molecule in association with a peptide derived by processing foreign antigen. Endogenous antigen, derived for example from viruses, is usually processed for presentation in association with class I MHC expressed by most cell types. Exogenous antigens, such as bacteria and their products, are usually processed for presentation in association with class II MHC, which is expressed by a more restricted subset of cells.

T cells are activated when their receptor binds the peptide/MHC complex, and it is this step that regulates the specificity of T cell activation. The affinity of the interaction is such that an antigen-specific TCR must bind to both peptide and MHC residues in order to achieve sufficient strength of binding. Furthermore, the class of MHC molecule influences which subset of T cells recognises the peptide. $CD4^+$ cells usually recognise peptides presented in association with class II MHC, and $CD8^+$ cells usually recognise peptides in association with class I MHC. Ligation of peptide/MHC complexes stimulates antigen-specific T cells to produce cytokines and enter the cell cycle.

CD4+ T helper cells direct the development of the immune response both through the expression of surface molecules and via cytokine secretion. CD4+ T cells collaborate with antigen-presenting cells (APC) and B cells via surface molecules that costimulate activation, in addition to secreting the cytokines that influence cell growth and differentiation. Cytokines serve as intercellular messengers and are able to differentially stimulate T cell expansion, recruit B cells for antibody production, and promote inflammation. Three subsets of CD4+ T helper cells have been characterised on the basis of the different combinations of cytokines they secrete, and differential development of T helper subsets during an immune response influences the balance between inflammation and antibody mediated-responses. The nature of T cell collaboration, the effects of cytokines on the immune response and the role of T cell subsets will be discussed in Section 1.4.2.

Exposure of the immune system to antigen(s) can lead to two diametrically opposed long-term and antigen-specific consequences- memory and tolerance. Immunological memory is characterised by the ability of the immune system to generate a more effective response after a second encounter with the antigen. In direct contrast, the tolerant state is characterised by a less effective immune response after a second encounter with antigen.

Peripheral immune responses are influenced by a number of antigen-related variables including the route of administration, the form of the antigen, and the dose administered. By altering these variables, the same antigen can elicit either cell-mediated or antibody-mediated responses (termed immune deviation) (Parish and Liew, 1972) or alternatively can induce tolerance (Mitchison, 1965). The course of an immune response appears to be determined soon after antigen is encountered, and is directly dependent on the differential expression of antigen-specific T cell functions. The cells and processes that regulate the activation of T cells are described in more detail below.

1.2. Antigen processing pathways.

Protein antigens are usually presented to T cells as a peptide fragment associated with an MHC molecule. The peptides bound by MHC molecules are generated by two independent pathways: one pathway processes exogenous antigen for preferential association with class II MHC; the other pathway processes endogenous antigen for preferential association with class I MHC (Braciale et al., 1987). Class I and II MHC possess peptide-binding grooves in which peptides are bound in extended conformation (Bjorkman et al., 1987; Fremont et al., 1992; Brown et al., 1993). The peptide-binding groove of class I MHC molecule has closed ends (Bjorkman et al., 1987; Fremont et al., 1992), restricting the length of the bound peptide to nine or ten residues (Jardetzky et al., 1991; Matsumura et al., 1992). Class II MHC molecules can bind longer peptides with greater variation in length (13-25 residues) (Rudensky et al., 1991; Chicz et al., 1992) since they possess an open-ended binding-groove (Brown et al., 1993).

Endogenous antigens are processed into peptides by an ATP-dependent proteolytic system located in the cytosol. The proteolytic system is thought to consist of a large complex of proteinases used for both general protein breakdown and generation of peptides for class I-restricted presentation (Goldberg and Rock, 1992). It is believed that the peptides generated by the proteolytic complex are delivered to newly synthesised class I MHC molecules in the endoplasmic reticulum by peptide transporters located in the membrane of the endoplasmic reticulum (reviewed by Parham, 1992). Peptide-binding stabilises the class I molecule (reviewed by Elliott, 1991) and leads to rapid transport of the complex to the cell surface (Neefjes and Ploegh, 1988).

Exogenous antigens are processed after they have been endocytosed by the antigen-presenting cell. Pinocytosis, the endocytosis of small particles in the fluid phase, typically proteins and other macromolecules, is carried out by most cell types. Phagocytosis describes the internalisation of macromolecules and large particles,

including entire cells, and is a specialised function of a few cell types, such as macrophages. Both processes can deliver antigen to the class II MHC pathway.

The endocytic pathway delivers exogenous antigen to lysosomes via a sequence of increasingly acidic and degradative endosomes (Brodsky and Guagliardi, 1991). A battery of proteinases in the endosomes and lysosomes cleaves the native antigen into peptides. Class II molecules are assembled in the endoplasmic reticulum and delivered to a compartment that contains both antigen and proteinases (Guagliardi et al., 1990) and resembles lysosomes (Peters et al., 1991). The transport of class II molecules from the endoplasmic reticulum is directed by the invariant chain (Lotteau et al., 1990), which also blocks peptide-binding (Teyton et al., 1990). Invariant chain is cleaved in the late endosomes/lysosomes leaving the class II-associated invariant chain peptide (CLIP) bound in the groove of class II molecules. CLIP facilitates the formation of MHC/peptide complexes by an unknown mechanism (Reviewed by Brodsky and Guagliardi, 1991; Ceman and Sant, 1995). Class II molecules can be delivered to the cell surface in the absence of invariant chain, but they have an altered conformation, suggesting that invariant chain is also a chaperone (Anderson and Miller, 1992).

The association of endogenous antigen with class I MHC and exogenous antigen with class II MHC is not absolute. Endogenous antigens can be presented in association with class II MHC (Nuchtern et al., 1990; Weiss and Bogen, 1991) and exogenous antigen has been shown to be processed by a subset of splenic macrophages for class I-restricted presentation (Rock et al., 1992; Rock et al., 1993). The significance of this redundancy is not known, but it may reflect the ability of the immune system to find the most effective molecule for the presentation of peptide. In addition, it is important that viruses should not be able to evade the immune system simply by failing to infect the APCs that prime naive T cells. Thus viral antigens derived from outside the presenting cell also need to be effectively endocytosed and presented in association with both class I and II MHC.

1.3. T cell activation.

1.3.1. T cell requirements for activation.

The TCR is a heterodimer of disulfide-linked α and β (or γ and δ) chains that are associated with the CD3 complex. Each chain of the TCR has a constant and a variable domain; the variable domain contains at least three loops, the complementarity determining regions (CDRs), which are thought to make direct contact with the peptide/MHC complex (Jorgensen et al., 1992). However, the arrangement of the TCR-peptide-MHC interaction has yet to be determined in detail. Upon engagement of the TCR, signalling within the T cell is rapidly initiated, driving it to proliferate and produce cytokines. The affinity of the TCR-peptide-MHC complex is very low with a K_D of ~5 x 10^{-5} M (Jorgensen et al., 1992). Together with the mutual repulsion of cell membranes, this suggests that any interaction of the TCR with peptide/MHC will be short-lived in the absence of accessory molecules that increase the avidity of binding (Makgoba et al., 1989). It has been estimated using hybridomas that only ~200 specific peptide/MHC complexes are required on the surface of the presenting cell to activate a T cell (Demotz et al., 1990; Harding and Unanue, 1990) although data from antigen-specific TCR transgenic T cells suggests an even lower figure (Fazekas de St. Groth, unpublished observations).

Adhesion between T cells and APCs is probably initiated by transient TCR ligation, triggering an adhesion amplification mechanism with the capacity to stabilise the T cell/APC interaction. LFA-1 is expressed on resting T cells in a low avidity state capable of binding to ICAM-1 on APC, but unable to stabilise cell adhesion. If the TCR is ligated at any time, an intracellular signal converts LFA-1 to a high avidity state, amplifying and stabilising cell adhesion (Dustin and Springer, 1989). In the absence of TCR ligation, the APC and T cell dissociate. High avidity LFA-1/ICAM-1 interaction allows stabilisation of the TCR/APC interaction so that "zippering-up" of other receptor-ligand pairs can occur at the membrane interface (Kupfer and Singer, 1989).

Once the T cell and APC are "zipped-up", T cell activation is initiated by full-scale, long-term TCR ligation. CD2 is thought to contribute to T cell adhesion by interacting with CD58 (LFA-3) on APCs (Makgoba et al., 1989) and synergises with TCR signals to enhance T cell stimulation (Altman et al., 1990).

CD4 and CD8 are accessory molecules that coaggregate with the TCR upon ligation of peptide/MHC. CD4 binds a conserved region of class II MHC (Cammarota et al., 1992) and CD8 an analogous region of class I MHC (Salter et al., 1990; Konig et al., 1992), accounting for the preferential recognition of class II-associated peptides by $CD4^+$ T cells, and class I-associated peptides by $CD8^+$ T cells. CD4 is also postulated to provide a signalling function to supplement TCR signals (Julius et al., 1993). TCR ligation stimulates CD8-mediated adhesion, indicating that CD8 has an early role in stabilising TCR-binding (O'Rourke and Mescher, 1992).

TCR ligation initiates a cascade of events, referred to as signal transduction, that culminates in IL-2 production and subsequent proliferation. The earliest events of signal transduction occur at the cell membrane where the TCR is ligated. Signalling molecules (ZAP-70, lck, fyn) are recruited from the cytoplasm and congregate at the membrane after recognition of activation sequences contained in the cytoplasmic tails of the TCR ζ chain and the CD3 chains. Each molecule is activated as it is recruited, leading to a cascade of recruitment and activation involving protein kinase C, calcineurin and ras. Thus a signal is passed from the cell membrane to the nucleus (reviewed by Crabtree and Clipstone, 1994; Weiss and Littman, 1994).

A second group of molecules have been suggested to provide costimulatory signals to the T cell upon ligation. Costimulatory signals are postulated to act independently of TCR-mediated signals although the effect of TCR-mediated and costimulatory signals may be cooperative. Examples of costimulatory molecules include IL-1 (Weaver and Unanue, 1990), B7-1(CD80) and B7-2(CD86) (CD28 ligands) (Linsley et al., 1991; Bluestone 1995), heat-stable antigen (Liu et al., 1992), and probably others that have

yet to be characterised (Weaver et al., 1988). A role for costimulators in T cell activation has been deduced from experiments in which a decreased level of activation is seen when the costimulators are either absent, destroyed by fixation, or blocked by soluble ligands (Jenkins and Schwartz, 1987; Weaver et al., 1988; Linsley et al., 1992; Liu et al., 1992). Induction of a hyporesponsive state has also been attributed to activation in the absence of costimulation (Lamb and Feldmann, 1984; Jenkins and Schwartz, 1987). These results led Jenkins and Schwartz (1987) to propose a two-signal model for T cell activation (which was similar to the earlier model of Lafferty and Cunningham (1975), itself a modification of Bretscher and Cohn's two signal model of B cell activation (Bretscher and Cohn, 1970)). In the Jenkins and Schwartz model, optimal T cell activation requires both TCR ligation (signal 1) and costimulatory signals (signal 2) and T cells are rendered tolerant if they are stimulated by signal 1 in the absence of signal 2. This model has focused attention on the events occurring at the cell membrane during T cell activation, and biochemical evidence of costimulatory pathways has been found (for example, see Rudd et al., 1994). However the role of costimulation in preventing tolerance has been called into question by *in vivo* studies that have demonstrated induction of both tolerance and immunity in the absence of costimulatory interactions (reviewed by Bluestone, 1995). Induction of hyporesponsiveness *in vitro* appears to be limited to a subset of T cell clones, and has not been demonstrated for naive T cells stimulated in the absence of costimulation. Further work is required to establish whether costimulatory molecules provide a unique signal that cannot be generated by TCR ligation, or whether costimulation simply augments the response to TCR signalling.

1.3.2. Antigen-presenting cells.

Antigen-presenting cells (APC) are the first point of contact between antigen and T cells. APCs process antigen into peptides and present them in association with MHC molecules, as described in Section 1.2 above. Most cell types appear to process and present endogenous antigens for presentation in association with class I MHC. The

ability to process and present exogenous antigen in association with class II MHC is a function of specialised APCs. The features of three specialised APCs, macrophages, dendritic cells (DC) and B cells, will be discussed below.

Macrophages are responsible for clearing insoluble material from the periphery and are essential for the initiation of immune responses against particulate antigens in the spleen (Claassen et al., 1986; Delemarre et al., 1990). Macrophages do not constitutively express class II MHC, so their role in initiating immune responses against particulates may be limited to capturing and processing them for presentation by other cell types (van Rooijen, 1992). However macrophages do appear to be required as APC during some infections, particularly those caused by intracellular pathogens such as *Mycobacteria* and *Listeria*. Infectious agents elicit IFN-γ, probably from natural killer cells (Scharton and Scott, 1993), leading to upregulation of class II MHC expression and microbicidal activity of macrophages *in vivo* (Dalton et al., 1993; Huang et al., 1993). Macrophages exposed to *Listeria monocytogenes* amplify the development of IFN-γ-producing T cells *in vitro* via the production of IL-12 (Hseih et al., 1993). In contrast, some populations of macrophages inhibit T cell responses. The T-dependent response to intratracheal antigen is enhanced by depletion of alveolar macrophages prior to antigen administration (Thepen et al., 1989). Macrophages directly inhibit antigen-presentation by lung dendritic cells *in vitro* via the production of nitric oxide (Holt et al., 1993).

Dendritic cells (DC) are distributed throughout lymphoid and non-lymphoid tissues, and are referred to by different names, depending on their location: Langerhans cells (LC) reside in the skin, veiled cells/dendritic leukocytes in blood and lymph, and dendritic cells, or interdigitating dendritic cells, in the spleen, lymph nodes, heart, lungs and gut (Steinman, 1991). The phagocyte responsible for capturing particulates in the skin is thought to be the LC. Phagocytosis appears to be a function of immature LC, and is lost upon maturation *in vitro* (Reis e Sousa et al., 1993). Even at the immature stage, LC have less phagocytic capacity than macrophages (Reis e Sousa et

al., 1993). The loss of phagocytosis by LC coincides with an increase in ability to present antigen via class II MHC, and migration to regional lymph nodes (Pure et al., 1990; Streilein et al. 1990; Dai et al., 1993). Dendritic cell precursors in bone marrow and blood behave in a similar manner to LC. Their limited phagocytic activity is lost on maturation as presenting ability is enhanced (Inaba et al., 1993). The loss of phagocytic activity with maturation of LC and dendritic cells in the lymph nodes may serve to prolong presentation of exogenous antigens captured at distal sites such as the skin.

Mature DC constitutively express high levels of class I and class II MHC, and a range of accessory molecules including LFA-1, LFA-3 (CD58) and ICAM-1 (CD11a) (Steinman, 1991; Austyn, 1992), all of which are necessary for, or enhance, T cell activation. DCs also constitutively express B7-2 (CD86) and low levels of B7-1(CD80) that have been identified as important costimulatory molecules for T cell activation (reviewed by Bluestone, 1995). Purified DC are potent APCs for naive T cells *in vitro* (Steinman et al., 1983; Steinman, 1991) and *in vivo* (Britz et al., 1982; Boog et al., 1985; Sornasse et al., 1992). In addition, antigen is found on DC soon after immunisation in a form capable of stimulating antigen-specific T cell lines or antigen-primed T cells *in vitro* (Bujdoso et al., 1989; Crowley et al., 1990; McKeever, 1992; Liu and MacPherson, 1993; Moll et al., 1993). Thus DC are well equipped to initiate a primary response against antigen.

B cells have been postulated to function as antigen-presenting cells because they can efficiently trap specific antigen via uptake by surface immunoglobulin. Lanzavecchia (1985) demonstrated that antigen-specific B cells require antigen at 10,000-fold lower concentrations than non-specific B cells in order to stimulate T cell clones. However, this advantage is available to only a tiny fraction of B cells for any given antigen. Thus B cell presentation of antigen may serve only to direct cognate T cell help to antigen-specific B cells. Alternatively, since the frequency of antigen-specific B cells and the level of antigen-specific antibody is increased by challenge with antigen, antibody-assisted antigen capture may increase the efficiency of antigen capture during the

second encounter with antigen, and possibly play a role in increasing the kinetics of the recall response.

B cells constitutively express class I and class II MHC, ICAM-1, and LFA-3 (Clark and Lane, 1991). Resting B cells also express low levels of B7-1(CD80) but not B7-2(CD86) (Reviewed by Bluestone, 1995). B7-1 and B7-2 are upregulated by surface immunoglobulin engagement, with expression of B7-2 being more rapid than B7-1 (Freeman et al., 1993; Nabavi et al., 1992; Lenschow et al., 1993), improving the ability of B cells to present antigen to T cells *in vitro* (Ranheim and Kipps, 1993; Ho et al., 1994). Resting B cells can stimulate T cell lines and freshly-isolated antigen-primed T cells *in vitro* (Ashwell et al., 1984). However, in comparison with DC, resting B cells require 100-fold more non-cognate antigen to stimulate naive $CD4^+$ T cells *in vitro*, whilst B cells activated by mitogenic stimulation require only ten-fold more antigen than DC (Croft et al., 1992). The ability of B cells to present antigen and initiate an immune response *in vivo* has been tested in several systems, and when care is taken to limit antigen presentation to B cells, they appear to induce tolerance rather than immunity (Lassila, et al., 1988; Eynon and Parker, 1992; Fuchs and Matzinger, 1992; Ronchese and Hausmann, 1993). In contrast, there is evidence that B cells contribute to localised immune responses in the lymph nodes (Ron and Sprent, 1987; Janeway et al., 1987; Constant et al., 1995; Liu et al., 1995). The detailed experiments of Kosco et al. (1988) have shown that lymph node B cells process and present antigen after it is carried into the lymph node by follicular dendritic cells, but these observations were limited to antigen complexed with antibody (ie. during a secondary immune response). Taken together, these studies support a role for B cells as APCs, if only to amplify the response induced by other APCs (see below).

1.4. T cell responses in immunity.

T cell activation leads to cytokine production and clonal expansion, which in turn drives the immune response as T cells communicate with APCs, B cells, and other accessory cell types. The cytokines secreted by activated T cells influence T cell proliferation and differentiation (IL-2 and IL-4), B cell growth and differentiation (IL-4 and IL-5), and APC activation and function (TNF-α and IFN-γ). In addition, T cells collaborate with B cells and APC through surface molecules which affect their growth, differentiation and/or function. The function of specific surface molecules and the role of cytokines in the immune response will be discussed below in Sections 1.4.1 and 1.4.2, respectively.

The immune response expands and accelerates with the activation and proliferation of antigen-specific T cells. The majority of activated T cells are destined to die, and as the antigen is cleared from the host, the immune response diminishes. However, a small population of antigen-specific T cells, known as memory T cells, persists. Memory T cells are more sensitive to antigen and mediate a faster and larger secondary response. The characteristics of memory T cells will be discussed in detail in section 1.4.4.

1.4.1. Collaboration between T cells, B cells and APCs.

T cells communicate directly with B cells and APC through the ligation of surface molecules. Ligation generates accessory or costimulatory signals in both the T cell and the apposing B cell or APC, allowing the two cells to collaborate in their activation, growth and differentiation. Two systems of collaboration have been characterised in great detail: the interaction of CD28/CTLA-4 with the B7 family of molecules and the interaction of CD40 with its ligand CD40-L.

The family of B7 molecules (B7-1/CD80 and B7-2/CD86) has been mentioned previously (see Section 1.3.1) as costimulatory molecules for T cell activation. The B7 molecules interact with two receptors on T cells: CD28 and CTLA-4. The function of

CD28 has been characterised in detail and is essential for T cell activation and proliferation except when the TCR/peptide/MHC interaction is of very high avidity. CD28 delivers or supplements signals that stimulate production of IL-2 and other cytokines (reviewed by Bluestone, 1995). Thus, T cell activation is usually dependent on B7 expression by APCs. B7-1 is absent or expressed at low levels on DC, macrophages and B cells, and is upregulated following activation by cytokines or receptor engagement (eg. class II or CD40). B7-2, in contrast, is constitutively expressed on DC and macrophages, and is rapidly upregulated on B cells. In contrast to CD28, CTLA-4 appears to inhibit T cell activation and has an important role in downregulation of responses (Tivol et al., 1995; Waterhouse et al., 1995).

CD40 is expressed on all mature B cells and most APC types including DC. CD40-L is expressed by activated, but not resting T cells, chiefly $CD4^+$ T cells. In addition, some activated $CD8^+$ T cells, mast cells and basophils express CD40-L. CD40 ligation costimulates B cell activation, proliferation, and cytokine production, and synergises with IL-4 and IL-13 to sustain B cell growth *in vitro*. CD40 ligation also induces immunoglobulin production and isotype switching in the presence of IL-4, IL-5, and IL-10. There is also some evidence that ligation of CD40 induces cytokine production on APCs, for example the production of IL-12 by DCs (reviewed by Banchereau et al., 1994).

A model to explain T cell collaboration with B cells and APCs on the basis of the B7/CD28/CTLA-4 and CD40/CD40-L systems has been proposed by Hodgkin and Kehry (1993). The expression of B7 on APC, including B cells, after antigen capture, processing and presentation costimulates efficient T cell activation. The expression of CD40-L on activated T cells in turn provides costimulation for B cell and APC activation and function. As a result, there is further T cell activation and expansion, escalating the T cell response, which in turn perpetuates the immune response.

1.4.2. T cell cytokines and subsets.

T helper (Th) cells can secrete a vast array of cytokines including IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, GM-CSF, TNF-α, TNF-β and TGF-β. Three T cell subsets have been defined in the mouse on the basis of their cytokine profiles: Th0 cells secrete IL-2, IL-3, IL-4, IFN-γ and TNF-α (Firestein et al., 1989); Th1 cells secrete IL-2, IL-3, GM-CSF, TNF-α and IFN-γ, but no IL-4 or IL-10; Th2 cells secrete IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, GM-CSF, TNF-α, little or no IL-2, and no IFN-γ (Mosmann et al., 1986; Cherwinski et al., 1987; Fiorentino et al., 1989). However, these subsets were defined *in vitro* using a selection of long-term T cell clones and do not represent the only possible cytokine profiles for T helper cells. *In vivo* studies have shown that the frequency of T cells producing Th1 (eg. IFN-γ) or Th2 (eg. IL-4) cytokines correlates exactly with that predicted if each cytokine were regulated independently within each cell (Kelso et al., 1995), ruling out the possibility of coordinated production of a panel of cytokines by T cell subsets. These results have led Kelso et al. (1995) to conclude that T helper cytokine production is a stochastic process and that the classification of T cells into three subsets is an oversimplification. Nonetheless, a population of T cells can be biased towards a Th1 or Th2 profile. In the same way, human Th clones defy simple classification into distinct subsets, but display Th1- or Th2-like cytokine profiles (Del Prete et al., 1993).

Cytokines themselves appear to direct the development of differentiated Th subsets from a common precursor. Th2 development *in vitro* is solely dependent on IL-4 (Le Gros et al., 1990; Swain et al., 1990; Seder et al., 1992), which dominates the effect of any other cytokine (Hseih et al., 1992; Seder et al., 1992; Hseih et al., 1993) and specifically inhibits Th1 development. Th1 development appears to proceed as a default pathway in the absence of IL-4 (Le Gros et al., 1990), and is amplified by IL-12 (Hseih et al., 1993; Seder et al., 1993). The development of Th subsets and the effects of the cytokines they secrete have been studied extensively in mice infected with

Leishmania major. Mouse strains that are resistant (healer phenotype) to *Leishmania major* produce large amounts of IFN-γ and little IL-4. In contrast, susceptible mouse strains (non-healer phenotype) produce IL-4, antibody and little IFN-γ (Heinzel et al., 1989). IL-4 and IFN-γ appear to act early in the development of responses to *Leishmania major*: non-healer mice are protected by administration of anti-IL-4 prior to infection, whilst healer mice succumb to infection if they are given anti-IFN-γ prior to infection (Sadick et al., 1990; Chatelain et al., 1992). The protective activity of IFN-γ appears to be limited to its role as an effector molecule, rather than as an inducer of Th1 development, since early administration of IFN-γ does not cure non-healer mice on its own (Sadick et al., 1990). IL-12 has been shown to direct Th1 development via an IFN-γ-independent pathway *in vitro* (Seder et al., 1993), and, in contrast to IFN-γ, IL-12 administration protects non-healer mice, enhancing IFN-γ production and inhibiting IL-4 production when administered during the first week of infection (Heinzel et al., 1993; Sypek et al., 1993). Another study using anti-IL-4 treatment prior to immunisation has extended the early role of IL-4 in the development of Th2 responses to include soluble antigens (Gross et al., 1993). These results obtained *in vivo* confirm the critical roles of IL-4 and IL-12 in Th development.

Long-term Th1 and Th2 clones appear to have different activation requirements, including a distinct preference for particular costimulatory molecules and possibly distinct APC subsets. Th2 clones depend on the expression of IL-1 by the antigen-presenting cell, whereas Th1 clones respond well to paraformaldehyde-fixed macrophages if they have been induced to express class II MHC by IFN-γ. Th1 clones also respond well to fixed B cells treated with IFN-γ and anti-immunoglobulin prior to fixation (Weaver et al., 1988). Bone marrow macrophages derived in culture with GM-CSF initiate protective responses against *L. major* in non-healer mice, whereas macrophages stimulated by M-CSF do not influence the course of infection (Doherty and Coffman, 1993). The different APC requirements of Th clones may reflect differential expression of B7 molecules. B7-1 and B7-2 equivalently costimulate IL-2

and IFN-γ production *in vitro*. On the other hand, B7-2 preferentially costimulates IL-4 production (Freeman et al., 1995). When blocking antibodies against B7-1 and B7-2 were administered *in vivo* during induction of experimental autoimmune encephalitis (EAE), anti-B7-1 inhibited the onset of disease, while anti-B7-2 exacerbated disease (Kuchroo et al., 1995). In this situation, it is hypothesised that anti-B7-1 limits Th1 development and inhibits inflammation, whereas anti-B7-2 blocks Th2 development, allowing Th1 cells to induce disease.

As well as influencing Th differentiation, the cytokines secreted by Th cells directly influence the response of immune effector cells. For example, IFN-γ is an essential effector molecule for resistance to infection. Macrophages from IFN-γ- or IFN-γ-receptor-deficient mice demonstrate diminished microbicidal activity *in vitro* and the mice themselves succumb to infection (Dalton et al., 1993; Huang et al., 1993), indicating that production of significant amounts of IFN-γ *in vivo* is necessary to activate macrophages for resistance to infectious organisms. Susceptibility to infections requiring a strong cell-mediated response, particularly those caused by intracellular bacteria and parasites, is associated with predominant IL-4 production and little or no IFN-γ production *in vivo* (Sadick et al., 1990; Yamamura et al., 1991; Al-Ramadi et al., 1992; Bretscher et al., 1992; Chatelain et al., 1992). Furthermore, IL-4 can directly inhibit microbicidal mechanisms (Al-Ramadi et al., 1992).

Immune deviation is a term used to describe the predominance of either antibody production or DTH-type responses following immunisation. Immune deviation may be a function of the dose of antigen used during immunisation: in the studies of Parish and Liu (1972), a high antigen dose elicited a large antibody response and poor DTH, whereas a low antigen dose led to DTH responses and poor antibody production. Diminishing the avidity of B cell recognition of flagellin or sheep red blood cells by acetoacetylation of the antigen also reduced antibody production and enhanced DTH (Parish, 1971; Parish, 1972; Parish, 1973). Reducing the dose of *Leishmania major* can elicit protective DTH responses in non-healer mice that would otherwise make large

amounts of antibody but poor DTH responses (Bretscher et al., 1992). Hosken et al. (1995) have clearly shown the influence of antigen dose on IL-4 and IFN-γ production *in vitro*. Using a naive monoclonal T cell population derived from TCR transgenic mice, they demonstrated that the ratio of IL-4 to IFN-γ secreted upon restimulation with antigen was dependent on the dose of antigen in the primary culture. Secondary IL-4 production increased as a function of the primary dose of antigen, coincident with a decrease in IFN-γ production, suggesting that differentiation to an IL-4 producing phenotype is regulated by antigen-dependent IL-4 production in the primary cultures (ie. IL-4 positive feedback) as first suggested by Seder et al. (1992). However, they also observed IL-4 production at very low doses of antigen, indicating a second mechanism for the regulation of IL-4 production, and hence, Th subset development. This activation mechanism may be dependent on a very low avidity TCR stimulus (Sloan-Lancaster et al., 1993).

1.4.3. T cell death.

The majority of T cells activated by antigen *in vivo* are destined to die. This process was first described in detail by Sprent and Miller two decades ago (Sprent, 1976; Sprent and Miller, 1976a; Sprent and Miller, 1976b). In their experiments, T cells were activated *in vivo* by transfer into allogeneic hosts. Activated T cells were recovered from the thoracic duct lymph 4 days after transfer and labelled with iododeoxyuridine, thymidine, or chromium before return to syngeneic hosts. The labelled cells homed to the lymph nodes and spleen, and the majority disappeared over a two-week period, either dying *in situ* to be engulfed by macrophages or excreted into the lumen of the gut (Sprent, 1976; Sprent and Miller, 1976a). A small population of transferred cells persisted and retained sensitivity to alloantigen, suggesting that they were memory T cells (Sprent and Miller, 1976b).

T cell death has become an area of intense interest in recent years and an understanding of the mechanism(s) responsible for the death of activated T cells is starting to emerge

(reviewed by Cohen et al., 1992). Many terms have been used to refer to T cell death, including programmed cell death, apoptosis, and activation-induced cell death. Cohen et al. (1992) define programmed cell death as death that is a physiological feature of the life cycle of the cell, and is not related to the death of the organism as a whole. Apoptosis was first used to describe the morphology of a cell undergoing programmed cell death to distinguish it from the morphology of necrotic cells undergoing accidental death. Apoptotic cells are characterised firstly by cell shrinkage, then membrane blebbing, and finally, collapse of the nucleus. In addition, apoptotic cells are phagocytosed before they lyse. Thus apoptotic T cells are engulfed by macrophages (Cohen et al., 1992). Activation-induced cell death is another term used to describe the death of T cells following activation via the TCR (reviewed by Lenardo et al., 1995).

The pathways of T cell apoptosis have yet to be established clearly *in vivo*, but experiments *in vitro* have shown that the cell surface molecule Fas (also known as APO-1 and CD95) is required for T cell death (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995). Singer and Abbas (1994) used the mutant *lpr* mouse strain, in which Fas expression is disrupted, to show that Fas was necessary for T cell death in response to intravenous peptide immunisation. However, recent experiments in a similar system have found that T cell death is delayed but not prevented by the *lpr* mutation (B. Fazekas de St. Groth, personal communication), suggesting that more than one molecule is involved in the induction of peripheral T cell death *in vivo*.

1.4.4. T cell memory.

Encounter with antigen can lead to the generation of immunological memory, a state in which the immune system exhibits increased sensitivity to antigen after the resolution of the primary immune response. The memory (or recall) response is elicited by a second challenge with antigen and in comparison to the primary response, is of greater magnitude and occurs more quickly. The phenomenon of immunological memory is systemic and long-lived, and is thought to be mediated by a population of memory cells

that are generated at some point during the first encounter with the antigen (reviewed by Vitetta et al., 1991).

Memory T cells (Tm) have been identified by phenotypic changes which appear to have resulted from T cell activation. The most common markers used are CD44 and CD45. CD44 (Pgp-1) is upregulated upon T cell activation and remains upregulated ($CD44^{hi}$) on Tm (Budd et al., 1987a). CD45 has several isoforms of different molecular weight due to differential splicing of extracellular domains. Activated T cells and Tm express low molecular weight isoforms (Lee et al., 1990). Thus both activated T cells and Tm express a $CD44^{hi}$ $CD45R^{lo}$ phenotype whereas naive T cells are $CD44^{lo}$ $CD45R^{hi}$. Tm differ from activated T cells in their size, since Tm are small cells, resting in interphase, whereas activated T cells are traversing the cell cycle and are therefore large in size. Both Tm and activated cells also show increased expression of adhesion molecules such as CD2, LFA-1 and ICAM-1 (Vitetta et al., 1991; Mackay 1991).

Whilst phenotypic markers serve as a convenient method to identify Tm, they must be used with caution for two reasons. Firstly, the memory phenotype described above ($CD44^{hi}$ $CD45R^{lo}$) is also shared with activated T cells, and thus, any analysis of Tm *in vivo* is prone to interference by ongoing T cell responses. Secondly, the expression of memory markers is not entirely stable. Transfer studies in rats have demonstrated that $CD45R^{lo}$ T cells can spontaneously revert to a $CD45R^{hi}$ phenotype, and vice versa, in the absence of exogenous antigenic stimulation (Bell and Sparshott, 1990; Sparshott and Bell, 1994). Murine T cells expressing a $CD44^{hi}$ phenotype are also thought to revert to a $CD44^{lo}$ phenotype, although at a far lower frequency than is seen for CD45 isoforms (Tough and Sprent, 1994). Thus CD44 remains the most reliable marker of Tm in mice.

Tm have yet to be characterised in detail, and this reflects the difficulty in reliably identifying and isolating antigen-specific Tm. Tm are commonly suggested to have a lower threshold of activation than naive T cells. This difference may be the result of

increased expression of adhesion molecules, resulting in increased avidity for antigen-presenting cells (Vitteta et al., 1991; Swain and Bradley, 1992; Croft et al., 1994). CD44^{hi} cells also produce more IFN-γ than CD44^{lo} or naive cells when stimulated with antigen *in vitro* (Budd et al., 1987b; Swain et al. 1991), although the amount of IFN-γ is small and somewhat variable compared to that produced by activated effector T cells. More recently, Tm have been characterised in TCR transgenic mice, since the transgenic TCR can be selected so that the exposure to antigen is controlled. Such studies are in their infancy, but early experiments revealed that when Tm and naive T cells were both stimulated *in vitro* under optimal conditions using potent APCs such as DC, they appeared to be functionally equivalent (Croft et al., 1994). However, Tm were found to be less dependent on costimulation, and thus were able respond better to antigen presented by weak APC or at low concentrations (Croft et al., 1994).

The systemic distribution of memory may be accounted for by altered migration of Tm. Unlike naive T cells, Tm are not confined to blood, lymph and peripheral lymphoid tissue, but recirculate through peripheral non-lymphoid tissues in anticipation of antigen (Mackay et al., 1990). Expression of the lymph node homing molecule L-selectin (also referred to as Mel-14 and LECAM-1) is required for T cells to enter lymph nodes via high endothelial venules (Gallatin et al., 1983). Mel-14 is not expressed by a substantial fraction of activated and memory T cells in the mouse, and has therefore been exploited as a memory marker (Bradley et al., 1992). However, experiments in aged mice have demonstrated that both Mel-14^{-} and Mel-14^{+} cells can display memory characteristics (Dobber et al., 1994). The preferential migration of Tm into non-lymphoid tissues (Mackay et al., 1990) can be attributed to the selective expression of homing molecules, some of which have been characterised on human Tm (Picker et al., 1991; Picker et al., 1994).

The longevity of T cell memory may be due to long-lived Tm, or alternatively, continuing division within the memory population. Experiments in mice, sheep and humans have demonstrated that T cells expressing activation/memory markers have a

rapid turnover (Mackay et al., 1990; Michie et al., 1992; Tough and Sprent, 1994), although no distinction was made between activated T cells and Tm in these experiments. Tm have been found to disappear from the host in the absence of antigen in some experimental systems (Gray and Matzinger, 1991; Oehen et al., 1993) leading to the suggestion that Tm renew themselves by periodic stimulation from long-term antigen depots (Gray and Matzinger, 1991). However, results from other experimental systems have shown that T cell memory can be maintained in the absence of antigen (Mullbacher, 1994; Bruno et al., 1995) and a significant proportion of T cells expressing memory markers remain in interphase for several weeks (Tough and Sprent, 1994).

In summary, all of the available evidence suggests that Tm are small resting T cells that express an activated phenotype, have a low threshold of activation and can migrate into non-lymphoid tissues. Tm respond to antigen by producing increased levels of cytokines under suboptimal conditions, particularly those cytokines characteristic of the Th1 and Th2 subsets. Tm are members of a long-lived population, but it is not clear whether each cell remains in interphase for long periods or divides occasionally.

1.5. T cell tolerance.

The immune system is regulated in such a way that most foreign agents are differentiated from the components of the host, that is, there is effective discrimination between self and non-self. As described in Section 1.3.1 above, protein antigen is recognised by T cells in the form of processed peptide in association with MHC. The majority of peptide eluted from surface MHC is derived from self-proteins (Rudensky et al., 1991; Chicz et al., 1992), although the proportion of self-proteins presented by APCs has yet to be quantitated *in vivo*. Burnet (1959) was the first to propose that tolerance was induced through the elimination of self-reactive lymphocytes during development. The first clear demonstration of deletion was the work of Kappler et al. (1987), who employed monoclonal antibodies and superantigens to show that immature

T cells were eliminated from the thymus after interaction with antigen. Thymic contact with antigen can also result in tolerance via the induction of anergy or nonresponsiveness (Ramsdell et al., 1992). However, some self-antigens may be poorly represented in the thymus, due to low levels of expression at that site, to delayed expression (eg. upon sexual maturation), or to sequestration in the periphery. Under these circumstances, self-reactive T cells may be exported to the periphery before they encounter the corresponding antigen, necessitating extrathymic mechanisms of tolerance to regulate self-reactivity.

Tolerance in mature T cell populations, like immunity to exogenous antigen, is influenced by the dose, form and route of antigen administration (reviewed by Weigle, 1973). Mitchison (1965) induced tolerance by high or cumulative doses of antigen, while Dresser (1962) used intravenous injection of soluble or deaggregated antigens. The tolerogenic effect of antigens could be overcome by subcutaneous administration with adjuvant containing bacterial components (Nauciel et al., 1974; Bullock et al., 1975). Parish converted immunogenic antigens into tolerogens by chemical treatment (Parish 1971; Parish 1972). It is difficult to account for the way in which the route of administration, and the dose and form of the antigen affect T cell responses, given that antigen is presented in the form of peptide in association with MHC, regardless of its form or route of administration. However, APCs differ in their ability to present antigen delivered in different forms. Their ability to activate T cells differentially may play a crucial role in regulating T cell responses (discussed in Section 1.3.2 above) and may be responsible both for self-non-self discrimination and the regulation of peripheral tolerance.

The mechanisms of tolerance induction in the periphery have been difficult to study at the cellular level due to the small number of T cells specific for any given antigen. In the last ten years, two experimental models in which there is a high frequency of antigen-specific T cells have become available. Superantigens are recognised by a large proportion of the natural T cell population, since they bind to most T cell receptors

incorporating a particular germline-encoded Vβ. Alternatively, a high frequency of antigen-specific T cells can generated by expressing a transgenic TCR of known specificity. In both models, antigen-specific T cells can be readily identified and quantitated, allowing their fate to be determined after they encounter peripheral antigen.

Administration of superantigens has been shown to induce tolerance by deletion and/or anergy of reactive T cells in the periphery (Rammensee et al., 1989; Kawabe and Ochi, 1990; Rellahan et al., 1990; Webb et al., 1990; Kawabe and Ochi, 1991; Ramsdell et al., 1992). T cell deletion was an early consequence of superantigen administration, and was preceded by vigorous expansion of reactive T cells (Webb et al., 1990; Kawabe and Ochi, 1991; MacDonald et al., 1991). The mechanism of peripheral T cell deletion has been attributed to apoptosis of activated T cells (Kawabe and Ochi, 1991; MacDonald et al., 1991; Miethke et al., 1994). Superantigen administration could also induce anergy in reactive T cells. Anergic T cells proliferated poorly when stimulated with superantigen *in vitro*, produced little IL-2 and responded poorly to anti-TCR ligation even in the presence of added IL-2 (Rammensee et al., 1989; Kawabe and Ochi, 1990; Rellahan et al., 1990). Anergy has been shown to be dependent on antigen persistence since anergic T cells recover within 20 days of transfer to untreated recipients (Ramsdell and Fowlkes, 1992). The relevance of these observations has been challenged since superantigens are not conventional exogenous antigens, do not require processing for presentation, and are not presented as a peptide in association with MHC (Hewitt et al., 1992; Jorgensen et al., 1992; Jardetzky et al., 1994). Importantly, superantigens caused obligatory tolerance even when administered using protocols that induce immunity to conventional antigens (Rellahan et al., 1990).

T cell tolerance to tissue-specific antigens has been investigated using transgenic mice expressing a foreign antigen (eg. alloantigen) under the control of a tissue-specific promoter. In each instance, functional tolerance was indicated by the lack of an immune response directed to the particular tissue. There was little evidence that tolerance was due to deletion of antigen-specific T cells, since T cells from tolerant mice could

respond to the antigen *in vitro*, although the response was significantly reduced in some cases (Lo et al., 1989a; Lo et al., 1992). This notion was confirmed by experiments performed in double-transgenic mice expressing foreign antigen and a transgenic TCR that recognised the antigen. In this model, the fate of the antigen-specific T cells could be accurately measured since the population of antigen-specific T cells was easily detected. There was no evidence of T cell deletion, and instead, downregulation of the transgenic TCR was seen (Schonrich et al., 1991; Schonrich et al., 1992). Reduced responsiveness of self-reactive T cells appeared to be dependent on the continued presence of antigen (Morahan et al., 1989) and there is evidence that the level of antigen expressed in the periphery influenced the extent of hyporesponsiveness, such that high levels of antigen expression induce greater hyporesponsiveness than low levels of antigen (Ferber et al., 1994). TCR downregulation probably represents a minor mechanism of peripheral tolerance, since there are very few T cells with low TCR levels in normal mice.

Peripheral tolerance to conventional antigens has been studied using TCR-transgenic mice. Class-I restricted antigens were the first to be analysed in detail, and in each case, antigen-reactive T cells were rapidly deleted in the periphery after encountering their antigen (Carlow et al., 1992; Zhang et al., 1992; Kyburz et al., 1993a; Moskophidis et al., 1993a), lending weight to the notion that the majority of T cells activated in the periphery are destined to die. This phenomenon was studied in detail using TCR transgenic mice recognising an epitope of lymphocytic choriomeningitis virus (LCMV) (Kyburz et al., 1993a; Moskophidis et al., 1993a). T cell deletion was induced by large doses of live virus or repeated administration of peptide, and was preceded by marked expansion of antigen-specific $CD8^+$ cells, in a manner resembling the induction of tolerance by superantigens (Kyburz et al., 1993a; Moskophidis et al., 1993a). The mechanism of T cell death appeared to be apoptosis of activated cells (Kyburz et al., 1993a). When transgenic mice were infected with a low dose of virus, a strong immune response was elicited, and no deletion was observed (Moskophidis et al., 1993a).

When LCMV-derived peptide was administered to normal mice, the dose and route of administration affected the development of protective immunity against the virus (Aichele et al., 1995). In these experiments, high doses or repeated administration of peptide induced tolerance, whilst lower doses or subcutaneous administration were protective (Aichele et al., 1995). However, interpretation of the results in the LCMV peptide model is confounded by the observation that LCMV-specific $CD8^+$ cells kill each other in the presence of free peptide *in vitro* and *in vivo* (Kyburz et al., 1993b).

More recently, two studies have used transgenic mice expressing a TCR capable of recognising class II-restricted soluble antigens. These studies employed an adoptive transfer strategy in which transgenic TCR T cells were transferred to non-transgenic syngeneic hosts and then challenged with antigen. This strategy was used since it was considered that the recipients would resemble normal hosts more closely than transgenic mice, in which up to 100% of $CD4^+$ cells express the transgenic TCR. Intravenous administration of antigen, in the form of intact protein or peptide, resulted in deletion of the majority of T cells expressing the transgenic TCR (Critchfield et al., 1994; Kearney et al., 1994). T cell deletion was preceded by expansion in the periphery as had previously been observed for superantigens and class I-restricted antigens in TCR transgenic mice. Other experiments using class II-restricted TCR transgenic mice suggested that T cell deletion in response to intravenous peptide was mediated by Fas ligation (Singer and Abbas, 1994), identifying apoptosis as the most likely cause of T cell deletion. Kearney et al. (1994) went on to demonstrate that T cell tolerance was not an inevitable consequence of T cell activation in their model, since subcutaneous administration of peptide in adjuvant generated T cells that were more sensitive to antigen than naive cells (Kearney et al., 1994).

While there appears to be more than one active mechanism to maintain peripheral T cell tolerance, organ-specific autoimmunity can be induced in normal animals after immunisation with self-antigen in adjuvant (eg. experimental autoimmune encephalomyelitis), suggesting that self-reactive T cells may simply ignore tissue-

specific antigens (reviewed by Fazekas de St. Groth, 1995). Organ-specific antigens may be accessible to self-reactive T cells, but if cells in the organ are poorly equipped to present antigen, they will fail to stimulate peripheral T cells. Most tissues do not express class II MHC or costimulatory molecules and are unable to produce cytokines that attract and/or perpetuate T cell activation. For those organs in which professional APCs are present, induction of peripheral tolerance will operate to limit T cell activation and autoimmunity.

The distinction between tolerance and immunity to antigen appears to lie in the functional status of the cells that remain after resolution of the primary response. In tolerance, if any antigen-specific T cells persist, they are functionally hyporesponsive or anergic, whilst those that remain after activation exhibit memory capability. This paradigm was first suggested by the early experiments of Sprent and Miller (see Section 1.4.3 above). However, the mechanisms that determine how T cells choose between death, anergy, and memory have yet to be elucidated.

Section 2. Studying Peripheral T Cell Responses in TCR Transgenic Mice.

In the past, study of peripheral T cell regulation *in vivo* was limited to gross observations of tolerance and immunity at the level of the whole animal, since the low frequency of antigen-specific T cells in normal animals made them difficult to detect and characterise during the inductive phase of the response. Indeed, the primary T cell response to most antigens in normal animals could not be directly measured, although its consequences could be defined in terms of the secondary response of the animal. A measurable secondary response was characteristic of immunity, whilst the absence of a measurable response indicated tolerance. Furthermore, the characteristics of the primary T cell response could be inferred only from the kinetics and characteristics of the secondary response. Thus, it was important to find a way to measure primary T cell responses *in vivo*.

A detailed study of primary T cell responses *in vivo* was first made possible by the use of superantigens that stimulated a large proportion of T cells in normal animals, as described above (see Section 1.5 above). The experiments of Webb et al. (1990) elegantly characterised the primary response of T cells to a tolerogenic antigen, introducing the concept that peripheral deletion and tolerance were obligatory consequences of a vigorous T cell response, which up until then, was believed to be an exclusive characteristic of immunity. However, superantigens induce tolerance, regardless of the protocol used for immunisation, in contrast to conventional antigens, for which tolerance or immunity can be elicited by using different immunisation protocols. Superantigens also differ from conventional antigens in that they are presented to T cells only in an unprocessed form (Hewitt et al., 1992), which binds to MHC molecules outside the peptide-binding groove (Jardetzky et al., 1994). Thus, it was important to repeat these experiments in a model that employed a conventional antigen.

For most conventional antigens, the identification of antigen-specific T cells *in vivo* is made difficult by their low frequency and the variety of TCR α and β chains expressed by an antigen-specific population. For some antigens, such as pigeon cytochrome C (PCC), responder T cells in certain mouse strains express only a limited subset of TCRs (Hedrick et al., 1988), but junctional diversity of the TCR chains means that not all T cells identified by monoclonal antibodies to V_α and V_β determinants encoded in the germline will have a high affinity for the antigen. Thus, antigen-specific T cells cannot be accurately quantitated either in naive mice or at the resolution of the response when T cells are not sufficiently activated to be easily detected by functional tests.

A high frequency of antigen-specific T cells with a monoclonal TCR can be generated by expressing a transgenic TCR of known specificity, and T cells expressing the transgenic TCR can be easily identified and accurately quantitated using monoclonal antibodies. TCR transgenic mice have been used to characterise the primary T cell response to class I- and class II-restricted antigens *in vivo* (Carlow et al., 1992; Zhang et al., 1992; Kyburz et al., 1993a; Moskophidis et al., 1993a; Critchfield et al., 1994) and they are a valuable source of monoclonal T cells for *in vitro* studies (Seder et al., 1992; Hsieh et al., 1992; Croft et al., 1994; Hosken et al., 1995).

The abnormally high frequency of responder cells in TCR transgenic mice has led to the suggestion that, like models employing superantigens, they are regulated differently from normal mice. For example, deletion caused by superantigens could represent a homeostatic mechanism that limits what would otherwise be an overwhelming response (Webb et al., 1990). Kearney et al. (1994) reported that intact TCR transgenic mice respond abnormally to antigen *in vivo*, concluding that this resulted from the high frequency of responder T cells. They therefore transferred T cells expressing a transgenic TCR into syngeneic hosts to lower the frequency of responder T cells. However, the results and conclusions of Kearney et al. can be criticised on two key points: firstly, their assessment of intact TCR transgenic mice was flawed because they compared them directly with non-transgenic mice without giving any consideration to

the obvious effects of responder cell frequency. Naive TCR transgenic mice respond vigorously to antigen *in vitro* due to the high frequency of responder cells, and therefore background responses are much higher than non-transgenic mice. As a consequence, cell numbers have a greater impact on results and thus need to be titrated and normalised to draw out functional differences. Secondly, the low frequency of TCR transgenic T cells in non-transgenic recipients compromises the ability to accurately quantitate responder T cells, particularly when they are not pre-labelled to distinguish them from host cells. The role of responder frequency in peripheral T cell responses needs to be rigorously tested before intact TCR transgenic mice are ruled out as valid experimental models.

The experiments described in this thesis were performed using transgenic mice expressing an $\alpha\beta$ TCR that recognises residues 87-103 of moth cytochrome C (MCC) in association with I-E^k (Fazekas de St. Groth et al., 1992; Seder et al., 1992). One line of these mice, termed -D, expresses the β chain of the transgenic TCR ($Tg\beta^+$) on over 90% of peripheral $CD4^+$ T cells (Figure 2.1). In -D mice homozygous for H-2^k, approximately 80% of $CD4^+Tg\beta^+$ cells also express the α chain of the transgenic TCR ($Tg\alpha^+$) at weaning (3-4 weeks of age). The percentage of $CD4^+Tg\alpha^+Tg\beta^+$ cells declines after weaning, the rate of decrease being faster in male than female mice. The reason for this decline is unknown, but it necessitates the inclusion of an age- and sex-matched control group in every experiment. $Tg\alpha$ is not expressed on $CD4^+$ cells in the absence of $Tg\beta$, and thus, $CD4^+Tg\alpha^+Tg\beta^+$ cells can be accurately identified by $Tg\alpha$ alone (Figure 2.1). The remainder of $CD4^+Tg\beta^+$ cells express only endogenous TCR α chains ($Tg\alpha^-$). Thus, $CD4^+Tg\alpha^+Tg\beta^+$, but not $CD4^+Tg\alpha^-Tg\beta^+$, T cells recognise residues 87-103 of MCC in association with I-E^k. For simplicity, $CD4^+Tg\alpha^+Tg\beta^+$ cells and $CD4^+Tg\alpha^-Tg\beta^+$ cells will be referred to as $CD4^+Tg\alpha^+$ and $CD4^+Tg\alpha^-$, respectively.

The -D TCR transgenic mice offer several advantages over other lines TCR transgenic mice. The transgenic TCR has been characterised in detail and its interaction with

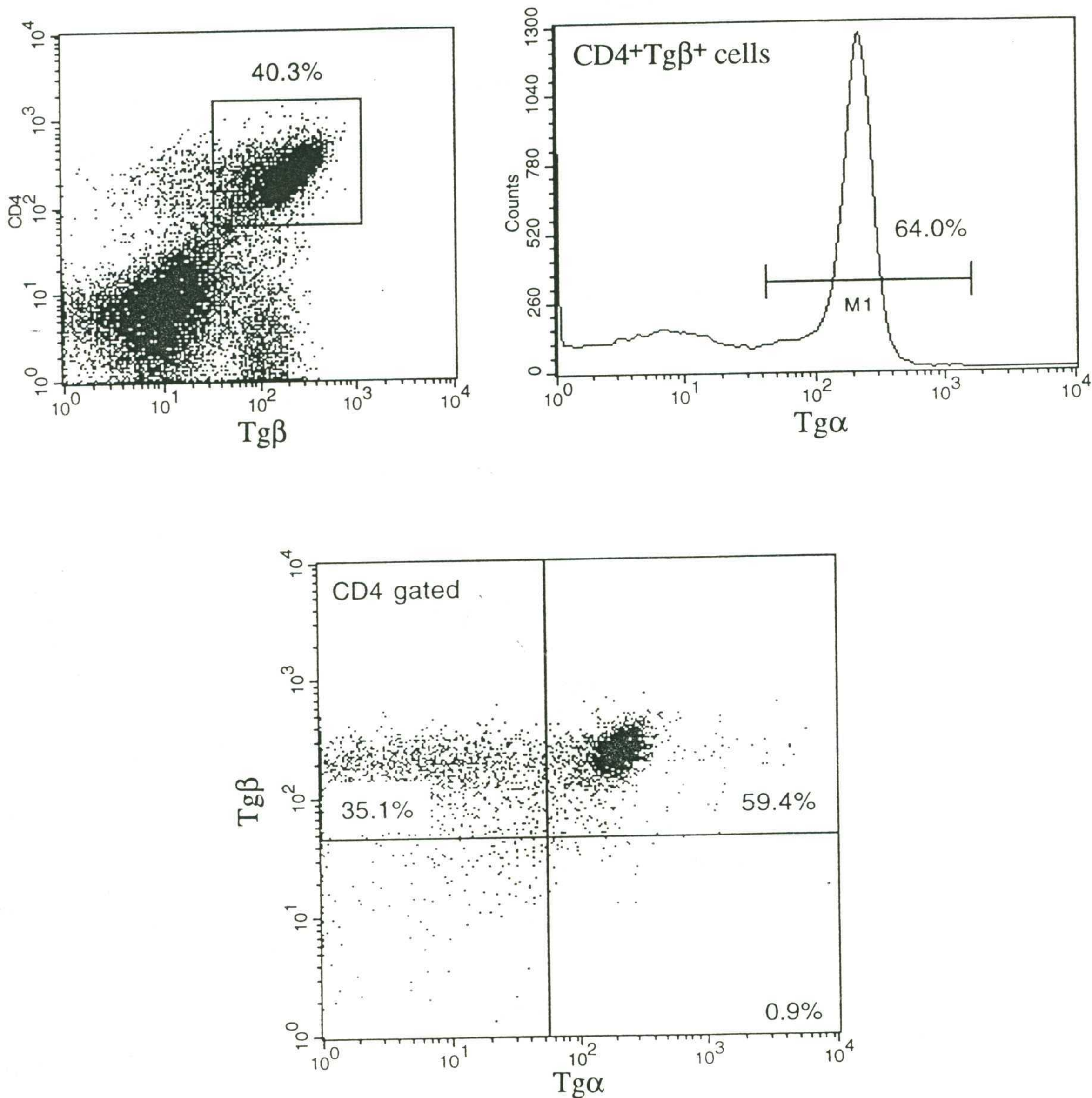


Figure 2.1. Expression of the transgenic TCR in the periphery of -D mice. Lymph node cells were removed from an untreated adult -D TCR transgenic mouse to determine surface expression of the transgenic TCR chains by $CD4^+$ cells using immunostaining and flow cytometry (see Section 3.5). The upper left panel shows the expression of CD4 and the β chain of the transgenic TCR (Tgβ). Over 30% of total lymph node cells express CD4 and Tgβ, and over 90% of $CD4^+$ cells express Tgβ. The upper right panel shows that over 60% of $CD4^+Tg\beta^+$ cells coexpress the α chain of the transgenic TCR (Tgβ), giving rise to two populations of $CD4^+Tg\beta^+$ cells, those that are $Tg\alpha^+$, and the remainder that are $Tg\alpha^-$. The lower panel indicates that the vast majority of $CD4^+Tg\alpha^+Tg\beta^+$ cells can be identified on the basis of CD4 and Tgα expression alone.

peptide and MHC is well understood (Hedrick et al., 1988; Jorgensen et al., 1992). It is one of the few transgenic models that employs a foreign class II-restricted antigen. The antigen is well-defined and is available as a peptide or intact protein. In addition, lower affinity variants have been extensively characterised (Reay et al., 1992). Both chains of the transgenic TCR can be identified with specific monoclonal antibodies and the high frequency of $CD4^+Tg\alpha^+$ cells allows their number to be accurately measured. The presence of a significant and quantifiable population of $CD4^+Tg\alpha^-$ cells provides a valuable internal control for specificity and is unique within the class II-restricted transgenic models currently in use. Previous experiments using the -I line created with the same constructs as -D have shown that both Th1 and Th2 cells can be derived from $CD4^+Tg\alpha^+$ cells *in vitro* (Seder et al., 1992), proving that there is no intrinsic bias in the cytokine response to peptide. A range of other transgenic mice are available for use in conjunction with the -D line, including Rag-1-deficient mice which have been used to breed mice with a monoclonal T cell population, and a wide range of I-E transgenics to study antigen presentation. Thus, -D TCR transgenic mice offer a wide scope to dissect key aspects of peripheral T cell responses *in vivo.*

Peripheral T cell responses were studied in -D TCR transgenic mice in the following way: firstly, the route of antigen administration and the form of the antigen administered were exploited to induce tolerance or immunity so that the two processes could be characterised and compared. The experimental protocol was based on well-established data from conventional animal models in which intravenous immunisation induces tolerance and subcutaneous immunisation generates immunity and memory. Secondly, the T cell response was characterised in detail to map out the events that occurred during the induction of tolerance or immunity. In addition, the capacity of different APC populations to stimulate T cell activation *in vivo*, was studied using an adoptive transfer strategy in which TCR transgenic T cells were immunised *in vivo* in the presence of purified APC populations.

The following parameters were measured in each experiment:

i. The number of $CD4^{+}Tg\alpha^{+}$ cells.

ii. Expression of activation and memory markers.

iii. Proliferation and cytokine production following re-stimulation with peptide *in vitro*.

Thirdly, all functional measurements were normalised on the basis of cell number and expressed per cell (see Section 3) so that meaningful comparisons could be made between cell populations containing different percentages of responder cells. Finally, the responses of peripheral T cells were measured in a closed system, particularly when assessing the long-term effects of antigen. Preliminary experiments performed by Dr. Barbara Fazekas de St. Groth showed that peripheral T cell depletion was quickly reversed in euthymic animals (Figure 2.2), suggesting that thymic emigrants replenished the peripheral pool. Therefore all mice were thymectomised so that the effects of immunisation could be assessed without interference from subsequent thymic emigrants.

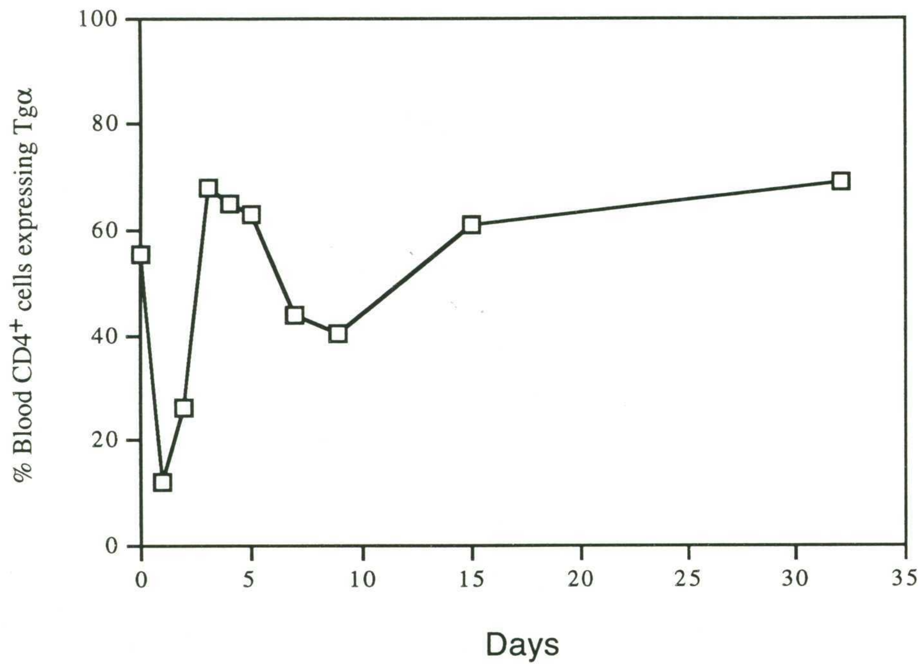


Figure 2.2. Reversion of peripheral T cell deletion in euthymic TCR transgenic mice. -D TCR transgenic mice were immunised intravenously with 1μg peptide, and the percentage of blood $CD4^+$ cells expressing the transgenic TCR was determined by immunostaining and flow cytometry (see Section 3.5). Reversion of T cell deletion is seen by an increase in the percentage of $CD4^+Tg\alpha^+$ cells after day 9.

Section 3. Materials and Methods.

3.1. Mice.

TCR transgenic mice were created using rearranged $V_{\alpha}11.1$ and $V_{\beta}3$ chain genes isolated from the 5C.C7 T cell clone (Fink et al., 1986). The transgenes were cointegrated and expressed under the control of the endogenous 3' β chain enhancer (Fazekas de St. Groth et al., 1992; Seder et al., 1992). The specificity and structure of the 5C.C7 TCR has been described in detail elsewhere (Matis et al., 1983; Fink et al., 1986; Hedrick et al., 1988). 5C.C7 proliferates in response to the C-terminal region (minimal determinant residues 94-103) of moth cytochrome C (MCC) in association with I-E^k. 5C.C7 also recognises residues 81-103 of MCC in association with I-$E\alpha^k\beta^b$ or I-$E\alpha^d\beta^b$ and residues 81-104 of pigeon cytochrome C (PCC) in association with I-E^k. In addition, 5C.C7 is alloreactive to I-A^s and to superantigens such as Staphylococcal enterotoxin A and mls-2^a3^a which bind to $V_{\beta}3$.

The -D line used in all experiments was established by microinjection of fertile C57BL/6J eggs and maintained by backcrossing to B10.BR mice obtained from the Animal Resources Centre (Perth, Australia). All experimental mice were derived from between the seventh and fourteenth backcross and were homozygous for H-2^k and heterozygous for integration of the transgene.

Transgenic mice were thymectomised as required at 4-6 weeks of age by aspiration under anaesthesia induced by intraperitoneal injection of 8mg/kg ketamine (Ketapex, Apex Laboratories, Australia) and 1.6mg/kg xylazine (Rompun, Bayer, Australia) in PBS. Thymectomised mice were splenectomised as required at least two weeks after thymectomy. Mice were anaesthetised as for thymectomy, then the spleen was removed after tying off both sets of vessels supplying the spleen. All mice undergoing operative procedures were administered 0.5mg/kg intraperitoneal Temgesic post-operatively.

Two transgenic I-$E\alpha^d$ lines (kindly supplied by Dr. David Lo) on an H-2^b background were used in adoptive transfer experiments. 107-1 transgenic mice express I-$E\alpha^d$ in a wild-type manner and 36-2 transgenic mice exclusively express of I-$E\alpha^d$ on cells in the

thymus (Widera et al., 1987; Burkly et al., 1990). Transgenic TCR mice (-D) were backcrossed with C57BL/10 mice to yield transgenic mice homozygous for H-2^b, and then crossed with 36-2 mice to generate double transgenic mice. -D x 36-2 double transgenic mice showed positive selection of the transgenic TCR, although selection was not as efficient as in crosses with 107-1 transgenic mice (unpublished observations).

Immunodeficient mice were used as recipients in adoptive transfer experiments. *Scid/scid* homozygotes on an H-2^b background were derived from C.B-17 *scid/scid* founders. Dr. C. Sidman at Jackson Laboratories (USA) kindly provided C.B-17 *scid/scid* mice backcrossed with C57BL/6J mice for six generations, which were then crossed with B10.BR mice a further three generations to yield [H-2^b x H-2^k]F1 (which will be referred to as H-2^{bk}) *scid/scid* homozygotes. Rag-1-deficient mice were created as detailed by Spanopoulou et al. (1994) and kindly supplied by Dr. L.M. Corcoran (Walter and Eliza Hall Institute, Melbourne, Australia) as heterozygotes at the 6th backcross with C57BL/6. They were then crossed with B10.BR mice to yield [H-2^b x H-2^k]F1 (H-2^{bk}) mice or H-2^k Rag-1-deficient homozygotes.

All mice were maintained at the Centenary Institute of Cancer Medicine and Cell Biology animal house facilities following A.C.E.C. guidelines and approved protocols.

3.2. Immunisation protocols.

Transgenic mice were immunised with a synthetic peptide comprising residues 87-103 of MCC (see Figure 3.1 for sequence) biotinylated at the N-terminus (prepared by Dr. P. Peake (Centenary Institute) or by the Queensland Institute of Medical Research, Australia). Des-ala-pigeon cytochrome C (daPCC) was engineered by Dr. M. Cook (Centenary Institute) by mutagenising recombinant PCC to remove alanine 103 from the C-terminus, effectively replacing the C-terminal epitope of PCC with that of MCC, as shown in Figure 3.1. Schwartz (1985) has previously shown that daPCC generates an identical response to MCC. daPCC was expressed with a six-histidine tag in *E. coli* to facilitate affinity purification on a nickel-agarose column. Affinity-purified daPCC

Position	81																							104
Pigeon cytochrome C 81-104	Ile	Phe	Ala	Gly	Ile	Lys	Lys	Lys	Ala	Glu	Arg	Ala	Asp	Leu	Ile	Ala	Tyr	Leu	Lys	Gln	Ala	Thr	Ala	Lys
Moth cytochrome C 81-103	Val	Phe	Ala	Gly	Leu	Lys	Lys	Ala	Asn	Glu	Arg	Ala	Asp	Leu	Ile	Ala	Tyr	Leu	Lys	Gln	Ala	Thr	Lys	
Des-ala-pigeon cytochrome C 81-103	Ile	Phe	Ala	Gly	Ile	Lys	Lys	Lys	Ala	Glu	Arg	Ala	Asp	Leu	Ile	Ala	Tyr	Leu	Lys	Gln	Ala	Thr	Lys	

Figure 3.1. C-terminal sequences of moth-, pigeon- and Des-ala-pigeon cytochrome C.

was further purified on a Sepharose G-200 (Pharmacia, Australia) column to separate proteolytic fragments, then passed over a de-toxi column (Pierce, USA) to remove residual LPS.

For intravenous injection, peptide was dissolved in 200µl PBS, while for subcutaneous immunisation, peptide in PBS was emulsified 1:1 in Complete Freund's Adjuvant (CFA) and a total of 200µl was injected into both hind footpads and base of tail.

3.3. Re-stimulation of transgenic T cells *in vitro*.

Mice were sacrificed and spleen and pooled lymph nodes (popliteal, inguinal, para-aortic, brachial, axillary, cervical) were collected. Draining lymph nodes (popliteal, inguinal, para-aortic) were collected separately if the mouse had been immunised subcutaneously. The organs were passed through a sieve, washed twice with TCM (RPMI 1640 containing 20mM HEPES, 10mM sodium bicarbonate, 50mg/L penicillin, 100mg/L streptomycin, 10% foetal calf serum, 2mM glutamine and 50µM 2-mercaptoethanol) and cells counted in a haemocytometer. For *in vitro* restimulation, cells were resuspended in TCM containing 50µg/ml gentamicin and 100U/ml nystatin and plated out in a two-fold dilution series, in quadruplicate wells, starting at 1×10^5 cells per well in flat-bottom 96-well microtitre plates (Falcon or Nunc). 1×10^5 irradiated (1500R) syngeneic (B10.BR) spleen cells were added, with or without 1µM biotinylated MCC peptide (87-103), to a total of 200µl per well. Peptide dose responses of $1\text{-}2 \times 10^5$ cells/well were measured in triplicate using a 10-fold dilution series of MCC peptide (87-103), starting at 1µM. Cells were routinely incubated at 37°C for 72 hours, then 100µl of culture supernatant was collected from each well for lymphokine assay. Plates were pulsed with 0.5µCi of ^{3}H-TdR/well and harvested six hours later for β-scintillation counting.

Proliferation was routinely normalised for the proportion of $CD4^+Tg\alpha^+$ cells in culture. Normalisation was necessary for accurate comparison of proliferation exhibited by individual mice in different experimental groups, since the proportion of $CD4^+Tg\alpha^+$ cells varied between mice and proliferation was not a linear function of cell number.

The number of CD4$^+$Tgα^+ cells/well was calculated by determining the proportion of cultured cells which expressed CD4 and both chains of the transgenic TCR by immunostaining and flow cytometry (see Section 3.5). Proliferation was calculated per 10^4 or 10^3 CD4$^+$Tgα^+ cells by generating a log-log plot of cpm versus number of CD4$^+$Tgα^+ cells/well (see Figure 3.2). The plot is described by the equation $y=bx^a$, where y is proliferation, x is cell number, b is the intercept, and a is the slope of the line. Plotted points deviate from a straight line at low cell numbers as they approach background counts and at high cell numbers when culture conditions are exhausted. However, the linear part of the curve can be used to interpolate counts incorporated for a specific cell number.

3.4. Cytokine assays.

IL-2 was measured using the IL-2-dependent cell lines, HT-2 (Watson, 1979) or CTLL (Gillis et al., 1978) in the presence of anti-IL-4 mAb (11B11; Ohara and Paul, 1985) to block any IL-4-induced proliferation. 4-5x10^3 HT-2 or CTLL cells were added in 50µl of TCM to two-fold dilutions of culture supernatant in 50µl in flat-bottom 96-well microtitre plates (Falcon or Nunc). Cells were cultured for 24 hours, then pulsed and harvested as for T cell cultures. IL-2 was quantitated by comparison with recombinant human IL-2 (Cetus Corp., USA). 1U/ml was defined as the concentration of IL-2 that stimulated 50% of maximal ^{3}H-TdR incorporation. HT-2 cells were maintained in TCM supplemented with 50-200U/ml recombinant IL-2 (Cetus Corp., USA). CTLL cells were maintained in HEPES-free TCM containing 50-200U/ml IL-2.

IL-3 was measured by bioassay using the IL-3-dependent cell line R6X (Schrader et al., 1983). 4-5x10^3 R6X cells were added in 50µl of TCM to two-fold dilutions of culture supernatant in 50µl in flat-bottom 96-well microtitre plates (Falcon or Nunc). Cells were cultured for 48 hours, then pulsed and harvested as described above. IL-3 was quantitated by using a standard source of IL-3 (conditioned medium from the WEHI-3 cell line (Warner et al., 1969); 1U/ml was defined as the concentration of IL-3

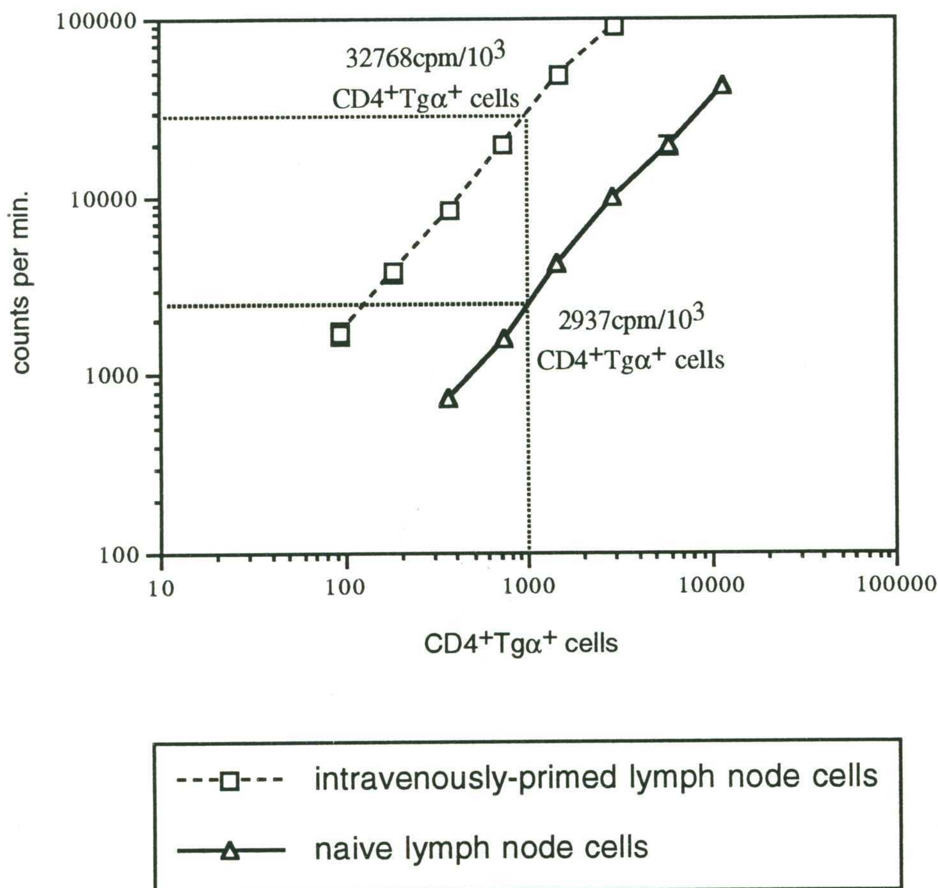


Figure 3.2. Quantitation of T cell proliferation *in vitro* using a log-log plot. The proliferative response of lymph node cells from a naive -D TCR transgenic mouse is plotted together with that of lymph node cells harvested 1 day after intravenous immunisation with 15µg of peptide 87-103. Cells were cultured with 1µM peptide and 10^5 irradiated syngeneic B10.BR spleen cells/well for 72 hours, then pulsed with 3H-TdR for six hours, as described in Section 3.3. The counts incorporated were log transformed and plotted against the log of potential responder cells/well to linearise the curve. The number of cells/well was adjusted for each individual mouse according to the proportion of $CD4^+Tg\alpha^+$ cells present in each cell suspension, as determined by immunostaining and flow cytometry (Section 3.5). Each point represents the average of four replicates with error bars indicating standard deviation. Proliferation for each sample was expressed as counts incorporated per 10^3 $CD4^+Tg\alpha^+$ cells. In this case, the interpolated values were 2937cpm and 32768cpm for the naive control and the immunised mouse, respectively.

that stimulated 50% of maximal ^{3}H-TdR incorporation. R6X was maintained in TCM supplemented with 30% WEHI-3 conditioned medium as a source of IL-3.

IL-4 was measured using the IL-4 dependent cell line, CT.4S (Hu-Li et al., 1989). Serial dilutions of culture supernatant were assayed with and without anti-IL-4 mAb (11B11, Ohara and Paul, 1985), to confirm that stimulation was due to IL-4 alone. 4-5x10^3 CT.4S cells were added in 50μl of TCM to two-fold dilutions of culture supernatant in 50μl in flat-bottom 96-well microtitre plates (Falcon or Nunc). Cells were cultured for 48 hours, then pulsed overnight and harvested as described above. IL-4 was quantitated by using a standard source of IL-4 (supernatant from Con A-stimulated D10.G4.1 cells (Kaye et al., 1983); 1U/ml was defined as the concentration of IL-4 that stimulated 50% of maximal ^{3}H-TdR incorporation. CT.4S was maintained in TCM supplemented with D10.G4.1 supernatant containing 20U/ml IL-4.

IFN-γ was measured using the IFN-γ-sensitive cell line WEHI-279 (Reynolds et al., 1987). Serial dilutions of culture supernatant were assayed with and without anti-IFN-γ (XMG1.2, Cherwinski et al., 1987; or R4-6A2, Spitalny and Havell, 1984) to ensure that inhibition was due to IFN-γ alone. 1x10^4 WEHI-279 cells were added in 50μl of TCM to two-fold dilutions of culture supernatant in 50μl in flat-bottom 96-well microtitre plates (Falcon or Nunc). Cells were cultured for 48 hours, then pulsed and harvested as described above. IFN-γ was quantitated with reference to a defined quantity of recombinant murine IFN-γ (kindly supplied by J. Ruby, John Curtin School of Medical Research, Canberra, Australia). WEHI-279 was maintained in TCM.

In contrast to proliferation, cytokine production was a linear function of responder cell number. Thus all estimates of cytokine titres were expressed per 10^4 $CD4^+Tg\alpha^+$ cells by multiplying the amount of cytokine produced by the total number $CD4^+Tg\alpha^+$ cells present, then dividing by 10^4.

3.5. Immunostaining and flow cytometry.

Single cell suspensions were prepared for immunostaining by gently teasing lymphoid organs through a sieve as described above, and washing with 0.45µ-filtered PBS containing 5% foetal calf serum and 5mM sodium azide (FACS wash). 200µl aliquots of cells at ~10^7 cells/ml were then pelleted in 96-well round-bottom microtitre plates for immunostaining. The transgenic TCR was detected using biotinylated RR8.1 (rat anti-$V_{\alpha}11$) (Jameson et al., 1991) and unconjugated KJ25-606.7 (hamster anti-$V_{\beta}3$) (Pullen et al., 1988), followed by streptavidin-conjugated Quantum Red (Sigma) and FITC-conjugated goat anti-hamster immunoglobulin (Caltag). CD4 was detected using PE-conjugated YTS 191.1 (Caltag). CD44 was detected with FITC-conjugated IM7 (Budd et al., 1987a) (Pharmingen) and CD69 was detected using FITC-conjugated H1.2F3 (Yokoyama et al., 1988) (Pharmingen). B220 was detected using PE-conjugated RA3-6B2 (Caltag). I-E was detected using biotin-conjugated 14.4.4S (Ozato et al., 1980), followed by streptavidin-conjugated Quantum Red (Sigma) or FITC (Molecular Probes). All antibodies were diluted in FACS wash and used at optimal concentrations. Samples were hard-gated for lymphocytes on the basis of forward- and side-scatter profiles, and collected using a Becton Dickinson FACScan. 50,000 events were routinely collected and analysed with Lysys II software.

Alternatively, four-colour flow cytometry was used so that dead cells could be excluded from analysis. The same staining protocols were used as described above, except that streptavidin-conjugated APC (Molecular Probes) was substituted for streptavidin-conjugated Quantum Red. Dead cells were detected by incubating for one minute in 1µg/ml propidium iodide in FACS wash at the completion of immunostaining. Samples were collected using a Becton Dickinson FACStar plus and gated to exclude dead cells detected in channel three. 50,000 events were routinely collected and analysed with Lysys II software.

3.6. Adoptive transfer of transgenic T cells to syngeneic non-transgenic hosts.

Adoptive transfer of transgenic T cells to non-transgenic hosts was performed using donor cells labelled with a fluorescein dye. Spleen and lymph nodes were harvested from TCR transgenic mice and a single cell suspension was prepared as described above. The cells were washed once with TCM and once with RPMI (without added serum). $5x10^7$ cells/ml were labelled with 5μM 5-carboxyfluorescein diacetate-succinimidyl ester (CFSE, Molecular Probes) (Lyons and Parish, 1994) in warm serum-free RPMI for ten minutes at 37°C, inverting every 3-4 minutes. Several volumes of cold TCM were added to stop labelling and the cells were then washed twice with TCM, before resuspending in PBS for transfer into unirradiated syngeneic B10.BR recipients. Each recipient received $50x10^6$ labelled cells via the tail vein. Transferred cells were detected in the recipient animals using a Becton Dickinson FACStar plus after excluding dead cells by propidium iodide staining. Transgenic T cells within the $CFSE^+$ fraction were identified using biotinylated RR8.1 followed by streptavidin-conjugated allophycocyanin in conjunction with PE-conjugated rat anti-mouse CD4.

3.7. 5-Bromo-2'-deoxy-uridine labelling studies.

1mg/ml of 5-Bromo-2'-deoxy-uridine (BrdU, Sigma) was administered to mice in their drinking water for three days. Six weeks later, spleen and lymph nodes were harvested and prepared for immunostaining and flow cytometry. BrdU incorporation was detected using the method of Tough and Sprent (1994). Briefly, cells were stained with biotinylated RR8.1, PE conjugated rat anti-mouse CD4, and streptavidin-conjugated Quantum Red prior to overnight fixation and permeabilisation in 1% paraformaldehyde, 0.01% Tween 20 in PBS. Cells were then treated with 50 Kunitz units DNase I (Sigma) in 0.15M NaCl, 4.2mM $MgCl_2$, pH5, for ten minutes at 37°C. BrdU was detected using FITC conjugated rat anti-BrdU (Becton Dickinson). Cells were collected and analysed as described above.

3.8. Purification of B cells.

B cells were purified from spleen cell suspensions after complement-mediated lysis of T cells using a combination of monoclonal rat anti-mouse CD4 (RL172.4; Ceredig et al., 1985), CD8 (3.155; Sarmiento et al., 1980) and Thy-1.2 (HO-13-4; Marshak-Rothstein et al., 1979). Cells were incubated at a concentration of $5x10^7$ cells/ml in the mAb cocktail for 30 minutes on ice, then guinea pig or rabbit complement was added and the cells were incubated for 15 minutes at 37°C. Cells were washed and layered onto a discontinuous gradient of 50%, 60%, 65%, 70% and 80% Percoll in PBS (Pharmacia). Purified B cells were harvested from the 65/70% interface, washed, and resuspended in PBS for adoptive transfer.

The purity of the B cell population was assessed by immunostaining and flow cytometry. An aliquot of purified cells was stained for B220 and I-E, and dead cells were excluded with propidium iodide. All preparations were at least 80% pure B cells, as determined by coexpression of B220 and I-E, with less than 20% contamination by T cells and $I-E^-$ cells. Contamination by other $I-E^+$ cells was less than 5%.

3.9. Derivation of dendritic cells *in vitro*.

Purified murine DC were derived from B10.BR spleen cell suspensions after culturing in 0.5ng/ml GM-CSF (Serotec) for nine days as described by Lu et al. (1995). Briefly, a spleen cell suspension was prepared and diluted to $2x10^6$ cells/ml in TCM supplemented with 0.5ng/ml GM-CSF. Cells were cultured in 1ml volumes in 24-well culture plates (Falcon) at 37°C, and non-adherent cells were removed by gentle swirling and aspiration every three days. On day nine floating clumps of cells containing DC were harvested. The purity of the harvested cells was assessed by flow cytometry after staining for B220 and I-E. All viable cells were large, $B220^-$ and $I-E^+$. The GM-CSF-derived population was ten-fold more potent on a per cell basis than irradiated spleen cells in stimulating peptide-dependent proliferation of transgenic T cells from -D x 36.2 double transgenic mice.

3.10 Antigen-presentation of intravenous peptide *in vivo*.

The ability of different antigen-presenting cells to present intravenous peptide was investigated *in vivo* using an adoptive transfer system in congenic mice. Antigen presenting cells were purified from 107-1 spleen and lymph nodes and adoptively transferred to -D x 36-2 double transgenic mice. Mice were immunised intravenously one or more days after adoptive transfer.

Alternatively, unfractionated spleen and lymph node cells were harvested from [H-2^b x H-2^k]F1 -D transgenic mice and adoptively transferred into homozygous *scid/scid* or Rag-1-deficient mice. In some experiments, spleen and lymph node cells from -D transgenic mice were incubated in plastic dishes for two hours at 37°C to deplete adherent cells, ensuring that the only I-E^+ APCs were B cells. Contamination by other APC populations was assessed by staining with B220 and I-E. In each case, >98% of all I-E^+ cells were small $B220^+$ cells. In other experiments, transferred cells were labelled with CFSE (as described above) to assess cell division *in vivo*. All recipients were immunised intravenously or intraperitoneally one or more days after adoptive transfer.

Section 4. Characterisation of the Effects of Intravenous Administration of Peptide to TCR Transgenic Mice.

4.1. Antigen-specific T cells are deleted from the periphery after intravenous immunisation of TCR transgenic mice.

Adult thymectomised TCR transgenic mice were immunised intravenously with PBS or 15μg of MCC peptide and the number of $CD4^{+}Tg\alpha^{+}$ and $CD4^{+}Tg\alpha^{-}$ cells in the spleen and lymph nodes was determined. Intravenous peptide induced a rapid response in the $CD4^{+}Tg\alpha^{+}$ compartment (Figure 4.1). The number of $CD4^{+}Tg\alpha^{+}$ cells in the spleen and lymph nodes was reduced by approximately 50% within 18 hours of immunisation. This effect was a manifestation of a specific response of $CD4^{+}Tg\alpha^{+}$ cells to peptide, since there was no significant change in the number of $CD4^{+}Tg\alpha^{-}$ cells, nor in the number of $CD4^{+}Tg\alpha^{+}$ cells in mice that received intravenous PBS. There was minor (two-fold) TCR downregulation at this timepoint (data not shown), consistent with T cell activation. The loss of $Tg\alpha^{+}$ cells corresponded with an equal reduction in the number of $CD4^{+}$, $CD3^{+}$ and Thy-1^{+} cells (data not shown), ruling out the possibility that TCR downregulation accounted for the change in T cell numbers. Curiously, the number of $B220^{+}$ cells increased by up to three-fold in the lymph nodes and 20% in the spleen one day after intravenous administration of peptide (data not shown).

On day three after immunisation, the number of $CD4^{+}Tg\alpha^{+}$ cells had doubled with respect to the PBS control group in both the spleen and lymph nodes. Thereafter the number declined to 50% of control by day seven. There were no corresponding changes in the number of $CD4^{+}Tg\alpha^{-}$ cells, indicating that deletion of $CD4^{+}Tg\alpha^{+}$ cells was antigen-specific. There was a slow decline in T cell numbers in both groups of mice over the course of the experiment, reflecting the loss of peripheral T cells in adult thymectomised mice, but the loss of $CD4^{+}Tg\alpha^{+}$ cells was consistently accelerated in the peptide group, suggesting that deletion continued after day seven. T cell deletion

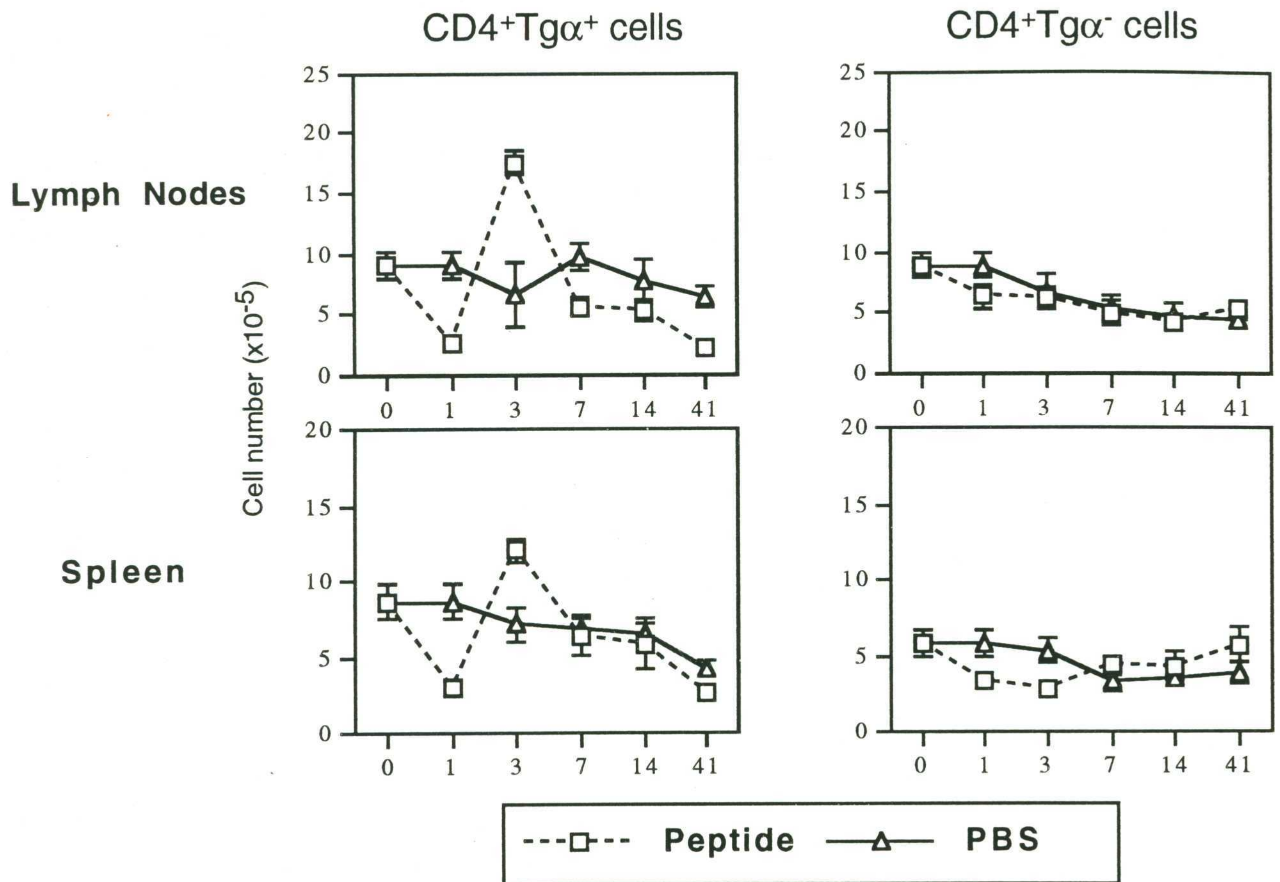


Figure 4.1. The number of CD4$^+$Tgα^+ cells in the periphery of -D TCR transgenic mice after intravenous immunisation. Adult thymectomised -D TCR transgenic mice were immunised intravenously with PBS or 15μg of peptide, as described in Section 3.2. The number of CD4$^+$Tgα^+ (left panels) and CD4$^+$Tgα^- cells (right panels) in the lymph nodes and spleen of PBS- and peptide-treated mice was calculated from the percentages determined by flow cytometry (Section 3.5). All points represent the average of at least 3-4 mice with error bars indicating SEM.

was an exclusive property of $CD4^{+}Tg\alpha^{+}$ cells in mice immunised with peptide, as shown in Figure 4.2, in which pooled results from four separate experiments are plotted. The number of $CD4^{+}Tg\alpha^{+}$ and $CD4^{+}Tg\alpha^{-}$ cells in the spleen and lymph nodes six weeks after immunisation were normalised with respect to the PBS control for each experiment, so that results from several experiments could be compared. There was a similar distribution of values about the mean for each T cell subset in each experimental group.

4.2. Antigen-specific T cells are activated in response to peptide administered intravenously to TCR transgenic mice.

Spleen and lymph node cells were restimulated with peptide 87-103 *in vitro* at various times following intravenous immunisation. Results of proliferation and cytokine assays were normalised as described in Section 3.3 and Figure 3.2, to take into account the variation in the proportion of antigen-specific $CD4^{+}Tg\alpha^{+}$ cells in each animal, allowing a more meaningful comparison to be made between individual mice.

Spleen and lymph node cells re-stimulated with 1μM peptide 87-103 one day after intravenous immunisation exhibited markedly enhanced proliferation with respect to the PBS control, and produced large amounts of IL-2, IL-3, and IFN-γ per cell (Figure 4.3). IL-4 production was never detected (data not shown). By day three after intravenous immunisation, the response of spleen and lymph node cells to peptide challenge *in vitro* had returned to baseline. Similarly, cells taken at several later timepoints were not significantly different from the control group in their ability to proliferate and secrete cytokines *in vitro*. When the kinetics of cytokine production were compared with the kinetics of $CD4^{+}Tg\alpha^{+}$ cell expansion, it was clear that priming of T cell function preceded cell expansion *in vivo* by 1-2 days (Figure 4.4).

The expression of two activation markers, CD69 (a very early activation marker) and CD44 (Pgp-1, an intermediate activation/memory marker), was characterised following intravenous immunisation. CD69 expression was rapidly stimulated by intravenous

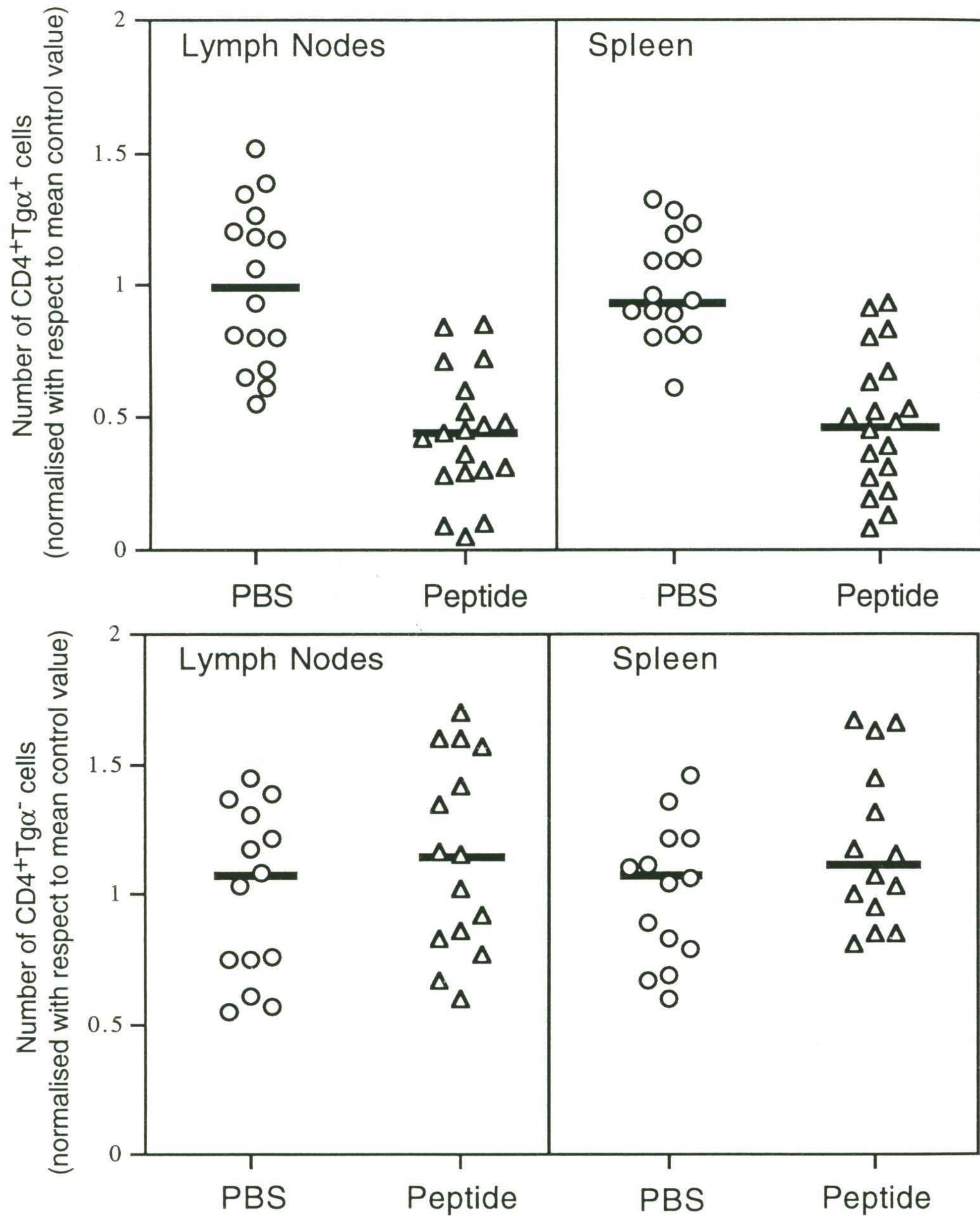


Figure 4.2. Peripheral deletion after intravenous immunisation of -D TCR transgenic mice. The number of $CD4^{+}Tg\alpha^{+}$ (upper panel) and $CD4^{+}Tg\alpha^{-}$ (lower panel) cells remaining in the lymph nodes and spleen six weeks after intravenous immunisation of thymectomised -D TCR transgenic mice is shown as pooled data from four experiments. Data is normalised within each experiment with respect to the mean number of cells present in the PBS control to remove variation in cell numbers due to lack of age- and sex-matching between the individual experiments. The mean value is represented by the bold bar.

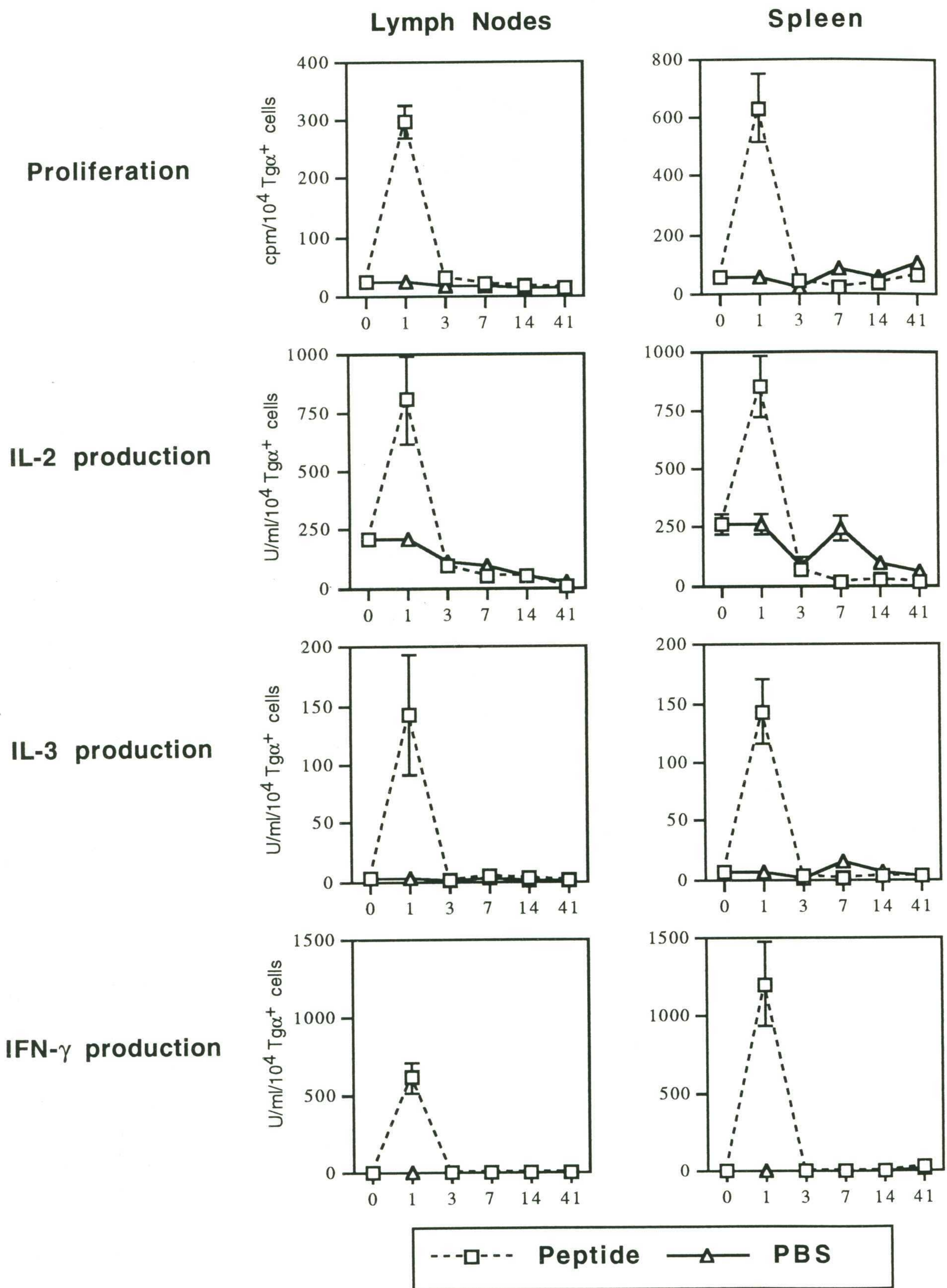


Figure 4.3. Activation of CD4$^+$Tgα^+ cells following intravenous immunisation. Adult thymectomised -D TCR transgenic mice were immunised intravenously with PBS or 15µg of peptide, as described in Section 3.2. Lymph nodes and spleen cells were harvested from PBS- and peptide-treated mice at each timepoint shown and restimulated with peptide *in vitro*, as described in Section 3.3. Proliferation was measured as described in Section 3.3, and the amount of IL-2, IL-3 and IFN-γ produced during culture was measured as described in Section 3.4. No IL-4 was detected in the cultures. Each point represents the average of 3-4 mice with error bars indicating SEM.

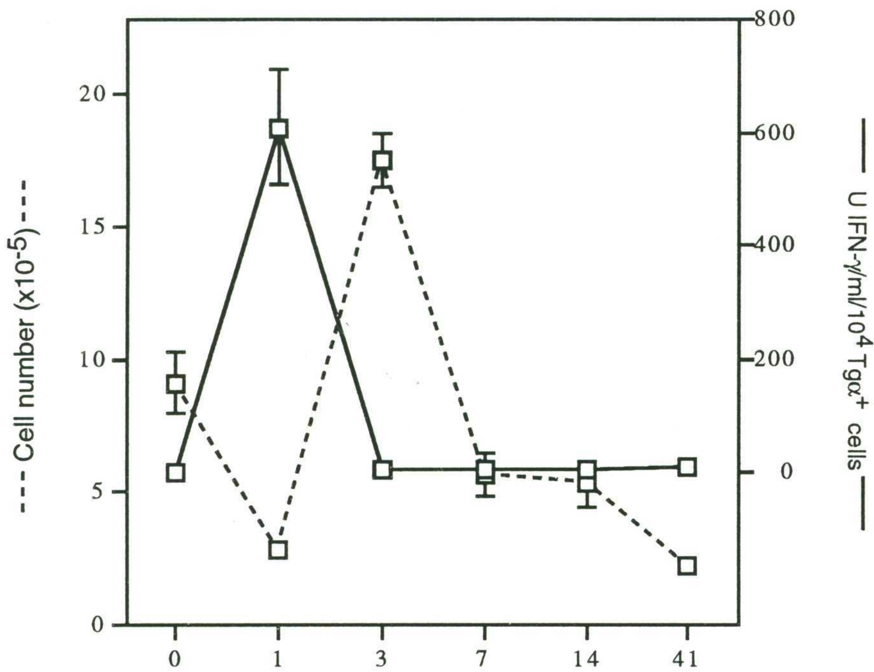


Figure 4.4. Relationship between T cell responses *in vitro* and *in vivo*. Lymph node cells from adult thymectomised -D TCR transgenic mice immunised intravenously with peptide exhibited an increase in IFN-γ production (bold line) upon restimulation *in vitro* which preceded the increase in the number of $CD4^{+}Tg\alpha^{+}$ cells (broken line) *in vivo*. A similar relationship was seen between $CD4^{+}Tg\alpha^{+}$ cell number and proliferation, IL-2 and IL-3 (data not shown). Each point represents the average of 3-4 mice with error bars indicating SEM.

immunisation of transgenic mice, with up to 80% of splenic $CD4^{+}Tg\alpha^{+}$ cells expressing CD69 two hours after intravenous immunisation (Figure 4.5A). The level of splenic CD69 expression on day one had declined, coincident with the loss of $CD4^{+}Tg\alpha^{+}$ cells from the periphery (Figure 4.1), suggesting that most of the $CD4^{+}Tg\alpha^{+}$ cells lost from the periphery during this period had already been activated by peptide. By day three only a small percentage of the expanded $CD4^{+}Tg\alpha^{+}$ population expressed CD69, although the total number of $CD4^{+}Tg\alpha^{+}CD69^{+}$ cells was at its peak. The percentage of $CD4^{+}Tg\alpha^{+}$ cells expressing CD69 was generally higher in the spleen than the lymph nodes, which may reflect either a difference in the amount of antigen reaching $CD4^{+}Tg\alpha^{+}$ cells in each organ, or a delay in antigen presentation in the lymph nodes.

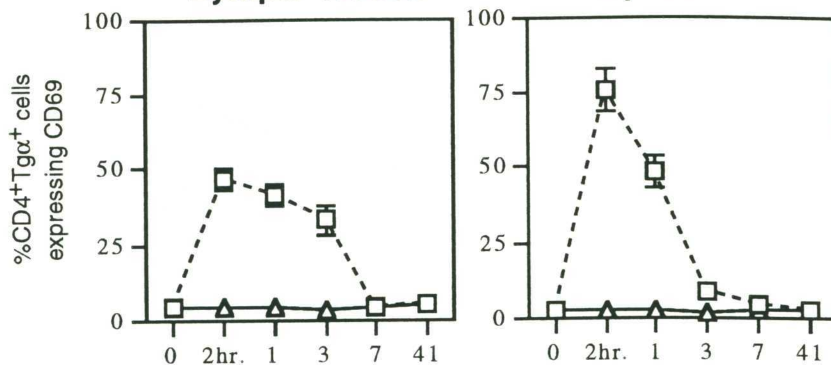
The percentage of $CD4^{+}Tg\alpha^{+}$ cells expressing high levels of CD44 also increased in response to intravenous immunisation (Figure 4.5B). A small percentage (around 10%) of $CD4^{+}Tg\alpha^{+}$ cells in both groups were $CD44^{hi}$ prior to immunisation, probably due to prior responses to environmental antigens. This degree of priming appeared to be dependent on co-expression of a second endogenous TCR α chain (see below), since TCR transgenic mice on a homozygous Rag-1 knockout background consistently expressed CD44 at high levels on fewer than 1% of $CD4^{+}Tg\alpha^{+}$ cells, whereas in naive Rag-1 sufficient transgenic mice, between 5 and 10% of $CD4^{+}Tg\alpha^{+}$ cells were $CD44^{hi}$ (B. Fazekas de St. Groth, personal communication). In addition, the percentage of $CD4^{+}Tg\alpha^{-}$ cells that were $CD44^{hi}$ at ages greater than eight weeks was always greater than 20% (data not shown). An early increase in the percentage of $CD44^{hi}$ cells was seen one day after immunisation, but there was no change in the total number of $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells (Figure 4.5D). A corresponding decrease in the number of $CD4^{+}Tg\alpha^{+}CD44^{lo}$ cells (Figure 4.5E) on day one indicated that the majority of $CD4^{+}Tg\alpha^{+}$ cells that disappeared from the periphery during the first 24 hours of the response (see Figure 4.1) were derived from the $CD44^{lo}$ population

Figure 4.5. Expression of activation markers after intravenous immunisation. Adult thymectomised -D TCR transgenic mice were immunised intravenously with PBS or 15μg of peptide, as described in Section 3.2. Lymph nodes and spleens were harvested from PBS- and peptide-treated mice at each timepoint shown and the expression of CD69 and CD44 determined by immunostaining and flow cytometry, as described in Section 3.3. **A.** CD69 expression shown as a percentage of total $CD4^+Tg\alpha^+$ cells. **B.** $CD44^{hi}$ expression shown as a percentage of total $CD4^+Tg\alpha^+$ cells. **C.** Mean level of CD44 expression by $CD4^+Tg\alpha^+$ cells, shown as the mean channel number normalised with respect to the mean control value to remove variation in the brightness of staining between different days. **D.** Total number of $CD4^+Tg\alpha^+CD44^{hi}$ cells. **E.** Total number of $CD4^+Tg\alpha^+CD44^{lo}$ cells.

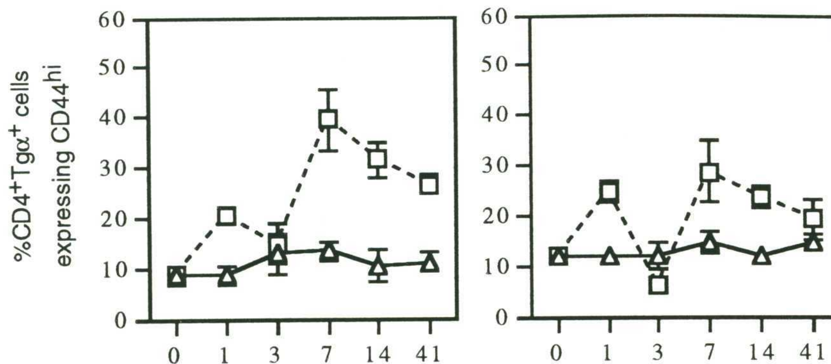
Each point represents the average of 3-4 mice with error bars indicating SEM.

Lymph Nodes　Spleen

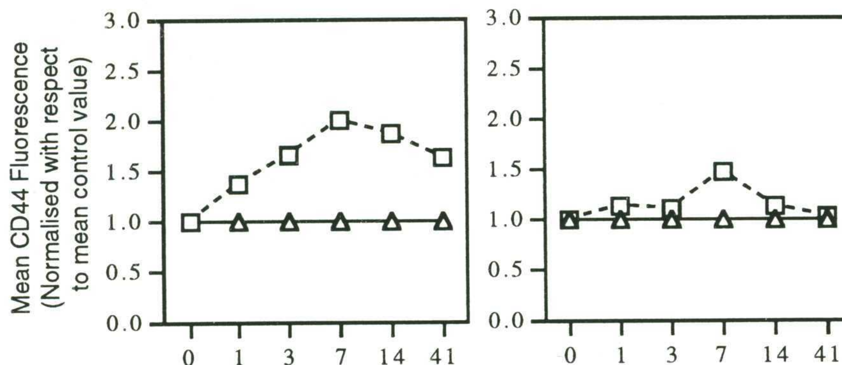
A. CD69 Expression



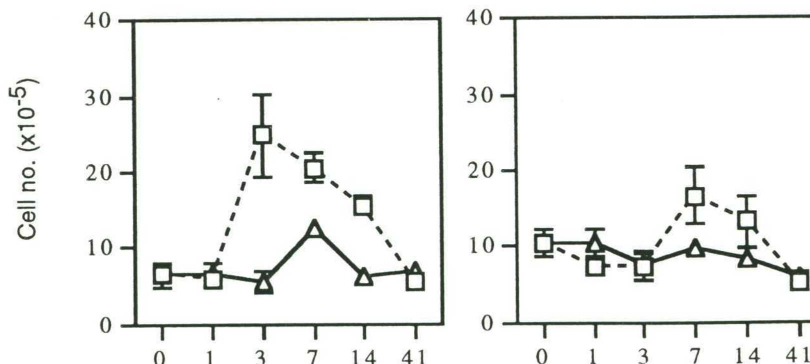
B. $CD44^{hi}$ Expression



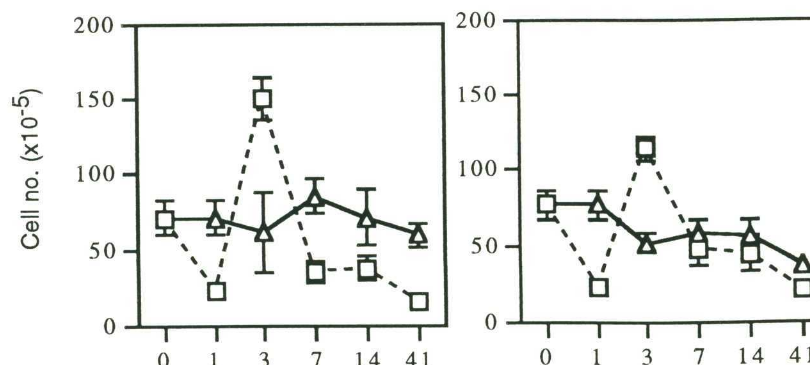
C. Mean Level of CD44 Expression



D. No. of $CD4^+Tg\alpha^+$ $CD44^{hi}$ cells



E. No. of $CD4^+Tg\alpha^+$ $CD44^{lo}$ cells



Peptide　PBS

The number of $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells was elevated in the lymph nodes but not the spleen three days after immunisation (Figure 4.5D). Curiously, at this time there appeared to be a decline in the percentage of $CD44^{hi}$ cells within the $CD4^{+}Tg\alpha^{+}$ population in the lymph nodes and spleen (Figure 4.5B). By day seven however, the percentage of $CD44^{hi}$ cells had increased again in the spleen and lymph nodes, and the number of $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells was also elevated. A comparison of CD44 profiles for the two experimental groups (Figure 4.6) revealed that the decline in the percentage of $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells on day three was an artefact of gating during FACS analysis. On day one, both experimental groups exhibited similar CD44 profiles, in which the majority of cells were $CD44^{lo}$, with a small percentage of $CD44^{hi}$ cells. On day three there was an increase in CD44 expression on the majority of $CD4^{+}Tg\alpha^{+}$ cells from the peptide group, resulting in a large population of $CD44^{med}$ cells. Most cells within the $CD44^{med}$ population were contained within the $CD44^{lo}$ gate during analysis, and thus the percentage of $CD44^{hi}$ cells was actually smaller on day three than day one. A substantial increase in the proportion of $CD44^{hi}$ cells was seen by day seven, suggesting that the level of CD44 expression by $CD4^{+}Tg\alpha^{+}$ cells continued to increase after day three. To confirm this hypothesis, the mean channel number for CD44 fluorescence was determined for $CD4^{+}Tg\alpha^{+}$ cells in the spleen and lymph nodes of mice immunised with peptide and normalised with respect to the mean value in control mice so that channel numbers from different days could be compared (Figure 4.5C). There was a gradual increase in the mean expression level between days one and seven, confirming that CD44 was slowly upregulated on $CD4^{+}Tg\alpha^{+}$ cells after intravenous peptide administration.

The number of $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells peaked on day three in the lymph nodes and day seven in the spleen (Figure 4.5D), then slowly declined, reaching baseline levels several weeks after immunisation. There was no increase in the number of $CD4^{+}Tg\alpha^{+}CD44^{lo}$ cells after day seven (Figure 4.5E), demonstrating that the decline

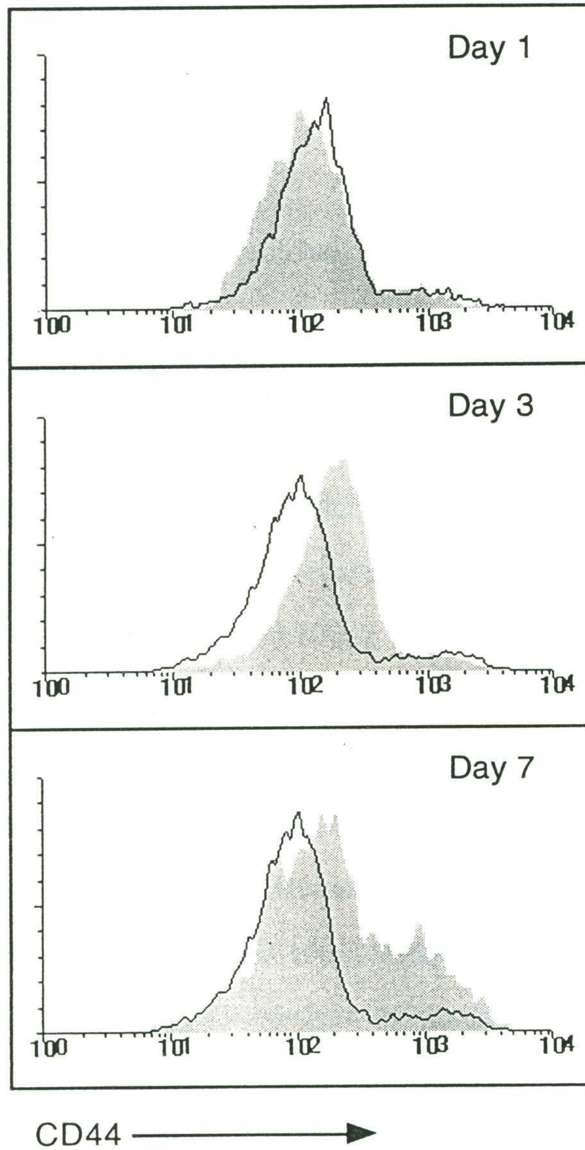


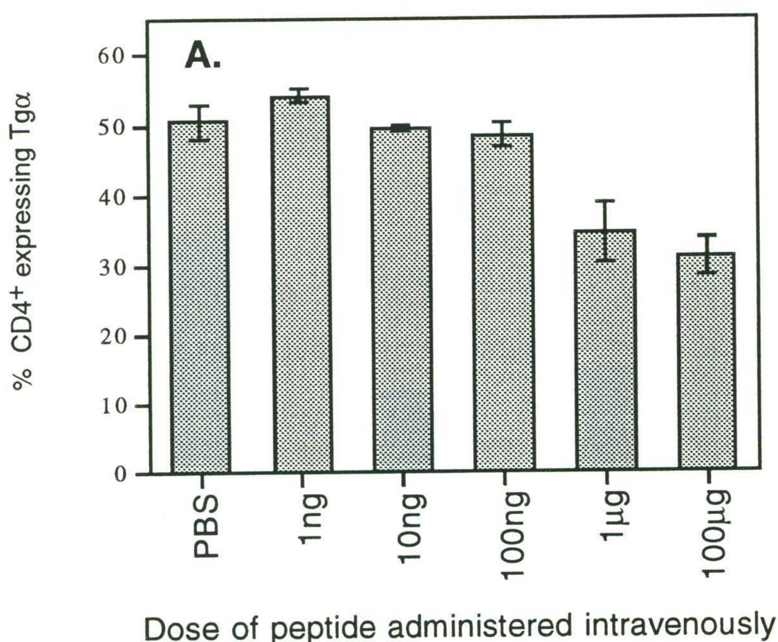
Figure 4.6. Upregulation of CD44 expression on CD4$^+$Tgα^+ cells following intravenous immunisation. CD44 was slowly upregulated on CD4$^+$Tgα^+ cells following intravenous immunisation, giving rise to a transient population of CD44^{med} cells on day three. Representative CD44 profiles are shown for CD4$^+$Tgα^+ cells from the spleens of PBS controls (outline) and peptide-treated mice (shaded) one, three and seven days after immunisation.

in the number of $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells could not be accounted for by reversion to low CD44 expression.

4.3. Peripheral deletion requires a threshold dose of peptide and correlates with T cell activation.

The correlation between T cell activation and peripheral deletion was tested by administering increasing doses of peptide to TCR transgenic mice. Adult thymectomised -D TCR transgenic mice were immunised intravenously with doses of peptide ranging from 1ng to 100μg per mouse, and the degree of deletion was determined six weeks after administration (Figure 4.7). Doses of 1-100ng failed to reduce the percentage of $CD4^{+}$ cells expressing the transgenic TCR (Figure 4.7A). However, mice that received 1 or 100μg of peptide showed a reduction in the proportion of $CD4^{+}Tg\alpha^{+}$ cells. There was a correlation between activation and deletion since intravenous doses of peptide above 500ng stimulated expression of CD69 on $CD4^{+}Tg\alpha^{+}$ cells one day after immunisation, as shown in Figure 4.7B. Furthermore, there was no difference in either the amount of CD69 expression or the level of deletion resulting from injection of 1μg and 10μg of peptide (data not shown), suggesting a causal relationship between T cell activation and peripheral deletion.

When peptide was delivered intraperitoneally, $CD4^{+}Tg\alpha^{+}$ T cell activation was again seen on day one, followed by deletion detectable on day seven (Figure 4.8). Intraperitoneal administration was slightly less efficient than intravenous administration as a route of peptide delivery, since the threshold dose for substantial $CD4^{+}Tg\alpha^{+}$ T cell activation was 1μg, rather than 500ng (compare Figure 4.8B with Figure 4.7B). These results demonstrate that intraperitoneal administration of peptide induced peripheral deletion in an analogous manner to intravenous peptide.



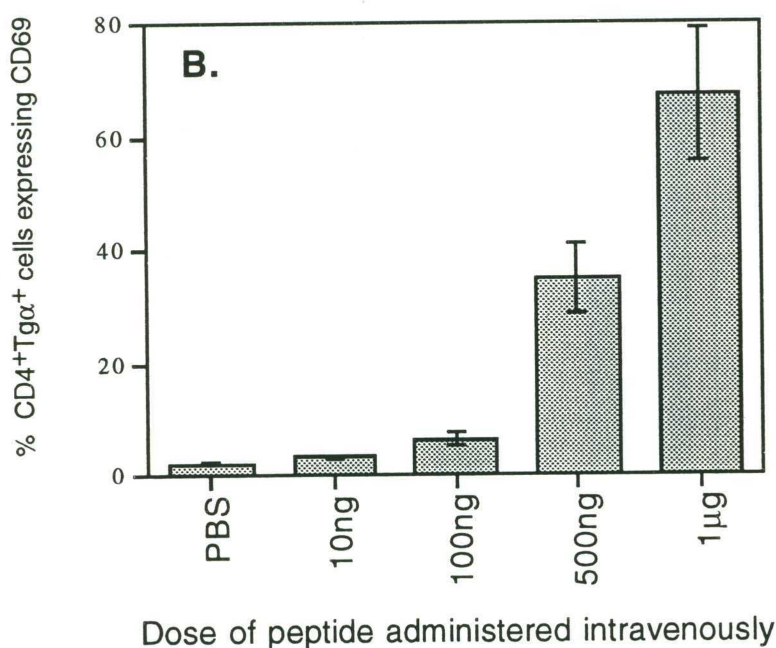


Figure 4.7. Correlation between peripheral deletion and T cell activation *in vivo*. Adult thymectomised -D TCR transgenic mice were immunised intravenously with PBS or a dose of peptide ranging from 1ng to 1µg. **A.** Six weeks after immunisation, the proportion of blood $CD4^+$ cells expressing the transgenic TCR (Tgα) was determined by flow cytometry as described in Section 3.5. Each point represents the average of four mice with error bars indicating SEM. **B.** In a second experiment, CD69 expression by spleen and lymph node cells (not shown) was measured one day after immunisation and is shown as a percentage of total $CD4^+Tg\alpha^+$ cells in the organ. Each point represents the average of two mice with error bars indicating the range of values.

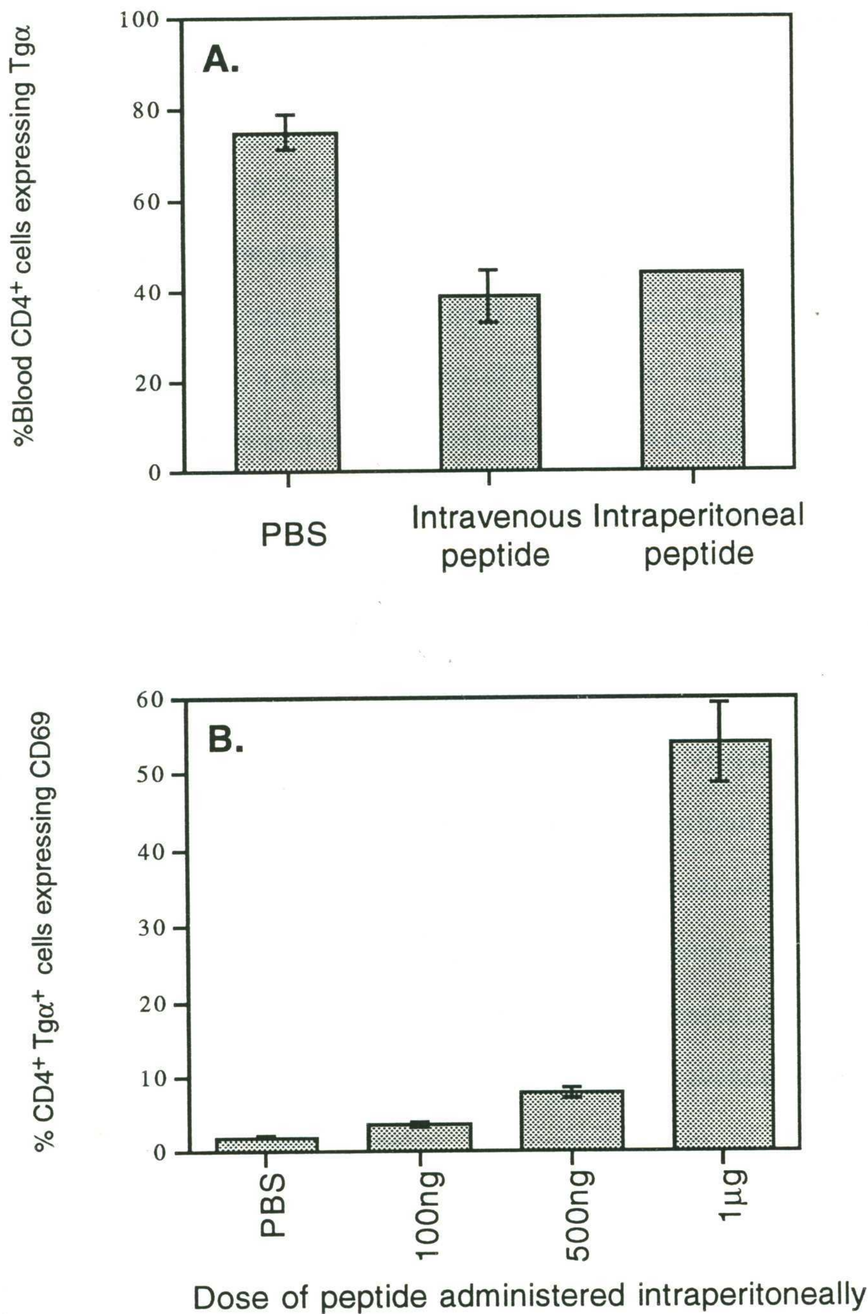


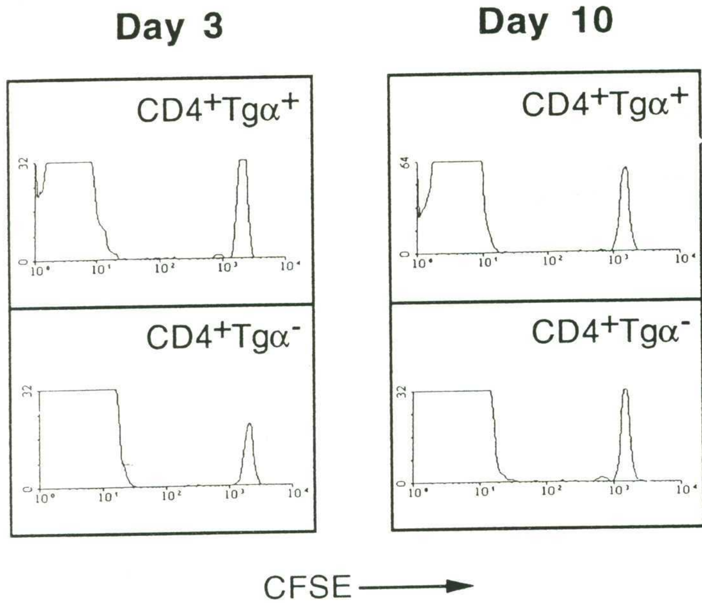
Figure 4.8. Comparison of intraperitoneal immunisation with intravenous immunisation. Adult thymectomised -D TCR transgenic mice were immunised with PBS or 1μg peptide intravenously or intraperitoneally. **A.** The proportion of blood $CD4^+$ cells expressing the transgenic TCR (Tgα) was determined by flow cytometry seven days after immunisation, as described in Section 3.5. Each point represents the average of 1-4 mice with error bars indicating SEM. **B.** In a second experiment, CD69 expression was measured on spleen cells one day after intraperitonal administration of PBS or a dose of peptide ranging from 100ng to 1μg. CD69 expression is shown as a percentage of total $CD4^+Tg\alpha^+$ cells. Each point represents the average of two mice with error bars indicating the range of values.

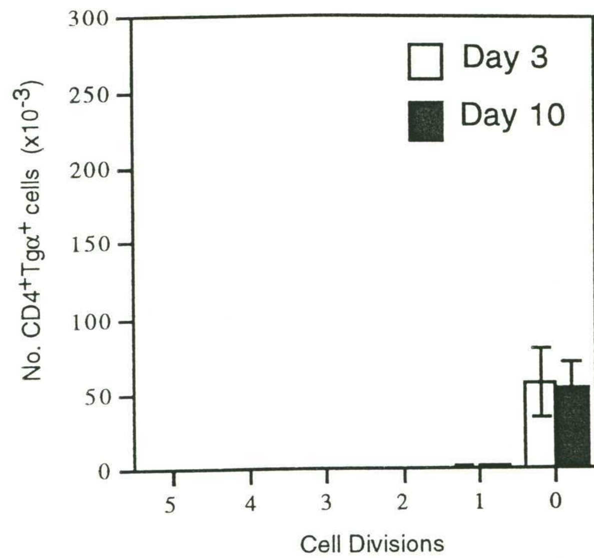
4.4. Peripheral deletion is not an artefact of the high frequency of antigen-specific T cells in the periphery.

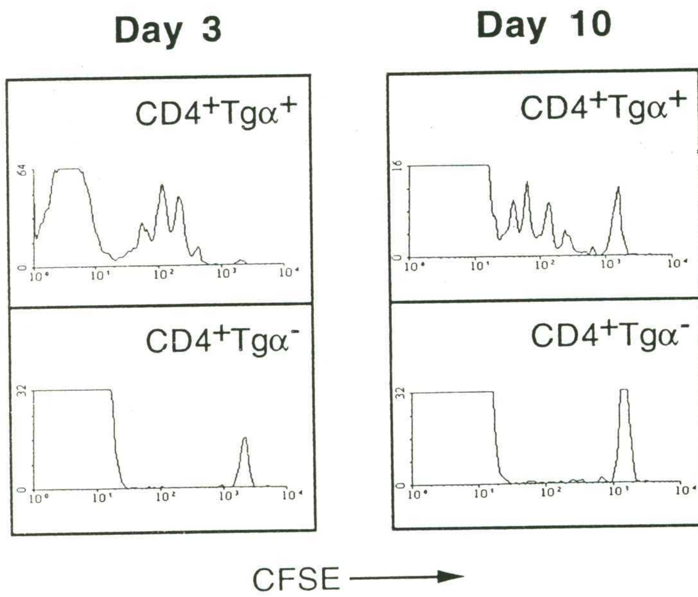
As mentioned in Section 2, transgenic TCR models are frequently criticised as unphysiological due to the abnormally high frequency of antigen-specific T cells. For this reason, it was important to determine if intravenous peptide induced peripheral deletion when there was a low frequency of $CD4^{+}Tg\alpha^{+}$ cells in the periphery. The frequency of $Tg\alpha^{+}$ cells was reduced from 80% to approximately 2% of $CD4^{+}$ cells by adoptive transfer of -D TCR transgenic cells to syngeneic non-transgenic (B10.BR) recipients. Transferred cells were identified in recipients by first labelling them with a fluorescein conjugate. 5-carboxyfluorescein diacetate-succinimidyl ester (CFSE) was chosen for two reasons: firstly, it irreversibly labels cells by covalently binding cellular components, and secondly, it allows cell division to be visualised, since labelling intensity is highly uniform, so that daughter cells which exhibit half the fluorescence intensity of the parent cell can be clearly distinguished (Lyons and Parish, 1994). Each recipient received $50x10^{6}$ pooled unfractionated -D transgenic spleen and lymph node cells. Recipients were bled seven days later to confirm transfer of labelled cells and to check the uniformity of labelling, and then immunised intravenously with PBS or 1µg of peptide on day ten after transfer. The consequences of immunisation were determined three and ten days later.

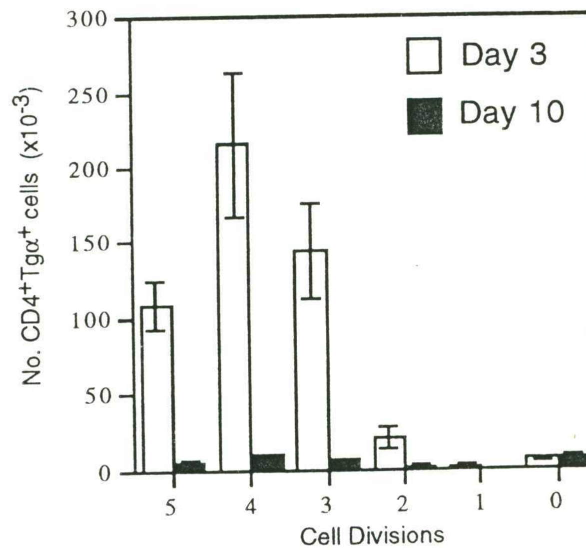
$CD4^{+}Tg\alpha^{+}$ and $CD4^{+}Tg\alpha^{-}$ cells from PBS-treated recipients displayed a single peak of high intensity fluorescence on days three and ten, demonstrating that neither subset had undergone cell division (Figure 4.9A, left panels). In contrast, 95% of $CD4^{+}Tg\alpha^{+}$ cells had divided in recipients of intravenous peptide. Divided cells appeared as several peaks of lower fluorescence intensity, whilst $CD4^{+}Tg\alpha^{-}$ cells remained undivided (Figure 4.9B, left panels). Up to six distinct peaks of fluorescence were detected above the background, corresponding to the original population plus five cell divisions. The number of $CD4^{+}Tg\alpha^{+}$ cells at each cell division (0-5) on days three and ten was

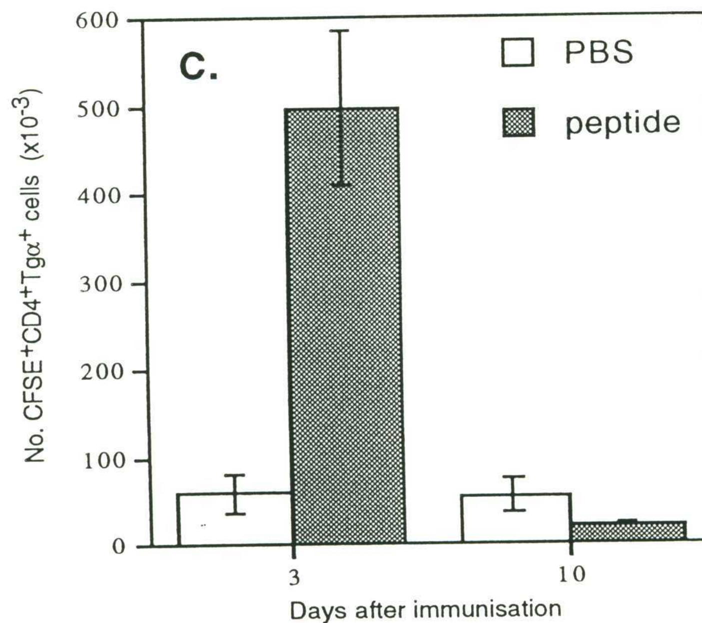
Figure 4.9. Peripheral deletion of CD4$^+$Tgα^+ cells in adoptive recipients of transgenic T cells. Spleen and lymph nodes cells were harvested from adult -D TCR transgenic mice, pooled and labelled with 5μM 5-carboxyfluorescein diacetate-succinimidyl ester, as described in Section 3.6. 50x10^6 CFSE-labelled cells were adoptively transferred to syngeneic nonotransgenic B10.BR hosts. Ten days later, recipients were immunised intravenously with PBS or 1μg peptide. Cellular division of CFSE-labelled cells was determined by flow cytometry three and ten days after immunisation, as described in Section 3.6. Representative plots of CFSE-labelled CD4$^+$Tgα^+ and CD4$^+$Tgα^- cells are shown in the left panels for **A.** PBS control and **B.** peptide-treated mice. The number of CD4$^+$Tgα^+ cells at each cell division was calculated and plotted in the right panels. Panel **C** shows the total number of CFSE-labelled CD4$^+$Tgα^+ cells on days three and ten for each group. Each point represents the average of four mice with error bars indicating SEM.


Day 3
Day 10
CD4+Tgα+
CD4+Tgα-
CFSE


A. Intravenous PBS
Day 3
Day 10
No. CD4+Tgα+ cells (x10-3)
Cell Divisions


Day 3
Day 10
CD4+Tgα+
CD4+Tgα-
CFSE


B. Intravenous peptide
Day 3
Day 10
No. CD4+Tgα+ cells (x10-3)
Cell Divisions


C.
PBS
peptide
No. CFSE+CD4+Tgα+ cells (x10-3)
Days after immunisation

calculated for each group (Figures 4.9A and 4.9B, right panels). There was no significant change in the number of undivided $CD4^+Tg\alpha^+$ cells in the PBS control between days three and ten. Similarly, there was no change in the number of undivided $CD4^+Tg\alpha^+$ cells in the peptide group, suggesting that intravenous peptide did not persist in the periphery to continually stimulate $CD4^+Tg\alpha^+$ cells. In contrast, 95% of divided $CD4^+Tg\alpha^+$ cells had disappeared by day ten. Despite the substantial loss of divided $CD4^+Tg\alpha^+$ cells, they still comprised the majority (~80%) of all $CD4^+Tg\alpha^+$ cells remaining in the peptide group. In addition, there was no change in the proportion of cells at each cell division (the median of the cell division distribution being the fourth division on both days), indicating random loss of divided cells irrespective of the number of divisions they had undergone.

The total number of labelled $CD4^+Tg\alpha^+$ cells was calculated for each group at each timepoint (Figure 4.9C). There was a ten-fold increase in the number of $CD4^+Tg\alpha^+$ cells three days after intravenous peptide, followed by 50% net deletion in the lymph nodes and 75% net deletion in the spleen by day ten. These kinetics are very similar to those observed in intact TCR transgenic mice (Figure 4.1). However, the degree of proliferation was more marked as was the extent of deletion. Thus, peripheral deletion is not dependent on the high frequency of antigen-specific T cells in TCR transgenic mice, but appears to be an obligatory consequence of intravenous administration of peptide.

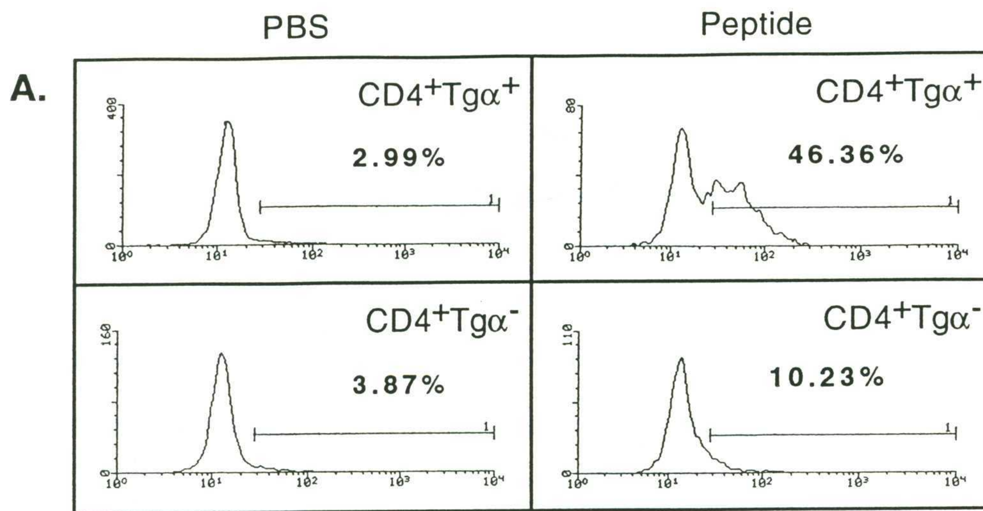
4.5. Not all antigen-specific T cells that are activated by intravenous peptide are deleted.

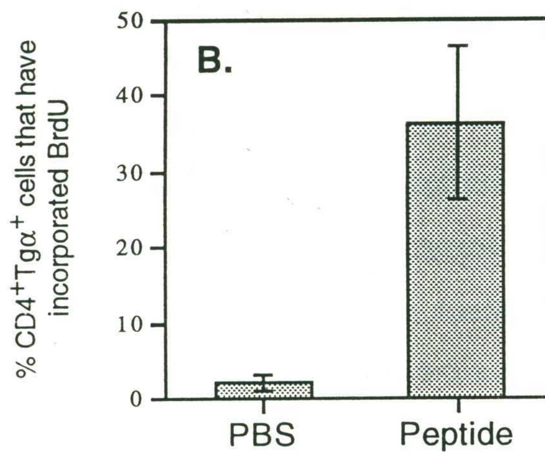
The CFSE studies in adoptively transferred mice demonstrated that a large proportion of $CD4^+Tg\alpha^+$ cells remaining ten days after intravenous immunisation had divided in response to antigen. In order to examine the relationship between cell division and deletion in intact transgenic mice, a different labelling strategy was employed. Bromodeoxyuridine (BrdU) is incorporated into the DNA of cells traversing the cell cycle, and

can be detected within the nucleus using a monoclonal antibody. Thus, cells that have entered into S-phase of mitosis can be identified by flow cytometry.

Adult thymectomised -D transgenic mice were administered PBS or 1μg peptide intravenously. At the same time, both groups were started on a three day course of BrdU, administered in the drinking water. Six weeks later, BrdU incorporation was measured. Figure 4.10 shows that only $CD4^{+}Tg\alpha^{+}$ cells from mice immunised with intravenous peptide incorporated a significant amount of BrdU. Approximately 40% of $CD4^{+}Tg\alpha^{+}$ cells had incorporated BrdU, compared to less than 5% in mice that received PBS. Surprisingly, $CD4^{+}Tg\alpha^{-}$ cells from peptide-treated mice had also incorporated low levels of BrdU (10% compared to <5% in the PBS control). Tighter gating of $Tg\alpha^{-}$ cells did not alter the BrdU profile, suggesting that the increase in incorporation was not an artefact. The total number of $CD4^{+}Tg\alpha^{-}$ cells did not increase detectably following intravenous immunisation (see Figure 4.1), but the low level of BrdU incorporation seen here may indicate a minor bystander response (see Discussion below).

The data from this experiment confirm previous results using CFSE (Section 4.4 above), namely that a proportion of $CD4^{+}Tg\alpha^{+}$ cells activated by intravenous peptide are not deleted from the periphery. The proportion of divided cells detected within the $CD4^{+}Tg\alpha^{+}$ population by BrdU incorporation was 40-50% in intact transgenic mice, whereas around 80% of adoptively transferred cells had divided in the CFSE study. The reason for this difference lies in the timepoints examined in each experiment: the CFSE study concluded ten days after immunisation, whereas BrdU incorporation was measured six weeks after immunisation. Previous results (see Figure 4.1) have suggested that T cell deletion continues after day seven, albeit at a slower rate, accounting for the lower proportion of divided cells in the BrdU study.





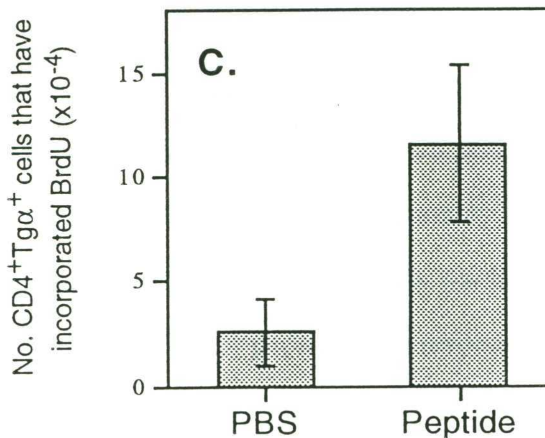


Figure 4.10. Incorporation of BrdU by CD4$^+$Tgα^+ cells following intravenous immunisation. Adult thymectomised -D TCR transgenic mice were intravenously immunised with PBS or 1μg peptide, and administered 1mg/ml BrdU in their drinking water for three days. Spleen and lymph node cells (not shown) were harvested six weeks later and BrdU incorporation was measured by immunostaining and flow cytometry, as described in Section 3.7. **A.** Representative plots of BrdU incorporation by CD4$^+$Tgα^+ and CD4$^+$Tgα^- cells from PBS- and peptide-treated mice. **B.** BrdU incorporation by each group is shown as the percentage of CD4$^+$Tgα^+ cells that were BrdU$^+$, as gated in panel A. **C.** The total number of CD4$^+$Tgα^+BrdU$^+$ cells in each group. Each point represents the average of two mice with error bars indicating the range of values.

4.6. Intravenously immunised transgenic mice are hyporesponsive to secondary challenge with peptide.

The functional characteristics of CD4$^+$Tgα^+ cells remaining after intravenous immunisation were tested by measuring the response of peptide- and PBS-treated mice to a secondary challenge with peptide *in vitro* and *in vivo*. The secondary response to peptide was measured *in vitro* in the following way: lymph node cells were harvested from PBS- and peptide-treated mice six weeks after immunisation and cultured with peptide doses ranging from 1pM to 1µM to measure proliferation. Lymph node cells from PBS-treated mice exhibited dose-dependent proliferation at peptide concentrations greater than 100pM (Figure 4.11A). Cells from peptide-treated mice appeared to be less responsive to peptide *in vitro* since there was a seven-fold reduction in proliferation measured at 1µM and a two-fold reduction at 10nM. However, the frequency of CD4$^+$Tgα^+ cells in peptide-treated mice was 3.5-4.2% of total lymph node cells, compared to 7.1-7.7% for the PBS group. Previous proliferation experiments showed no significant decrease in proliferation per precursor cell at 1µM peptide (Figure 4.3).

The secondary response of immunised mice was characterised *in vivo* using the parameters defined for the primary response. Two groups of adult thymectomised -D transgenic TCR mice were immunised intravenously with PBS or peptide (denoted 1°PBS and 1°peptide, respectively), and then six weeks later the groups were split in two and rechallenged with PBS or peptide. The response was measured at one, three, and 42 days in order to assess T cell activation, expansion and deletion respectively.

Figure 4.11B shows that the response of the 1°peptide group to intravenous peptide rechallenge was reduced in comparison to the 1°PBS group. CD69 expression by CD4$^+$Tgα^+ cells was elevated one day after 2° peptide immunisation, but the percentage of CD4$^+$Tgα^+ cells expressing CD69 was lower for the group that had previously received peptide (Figure 4.11B, upper panel). A comparison of staining profiles for each group showed that there was no difference in the level of CD69 expressed per cell

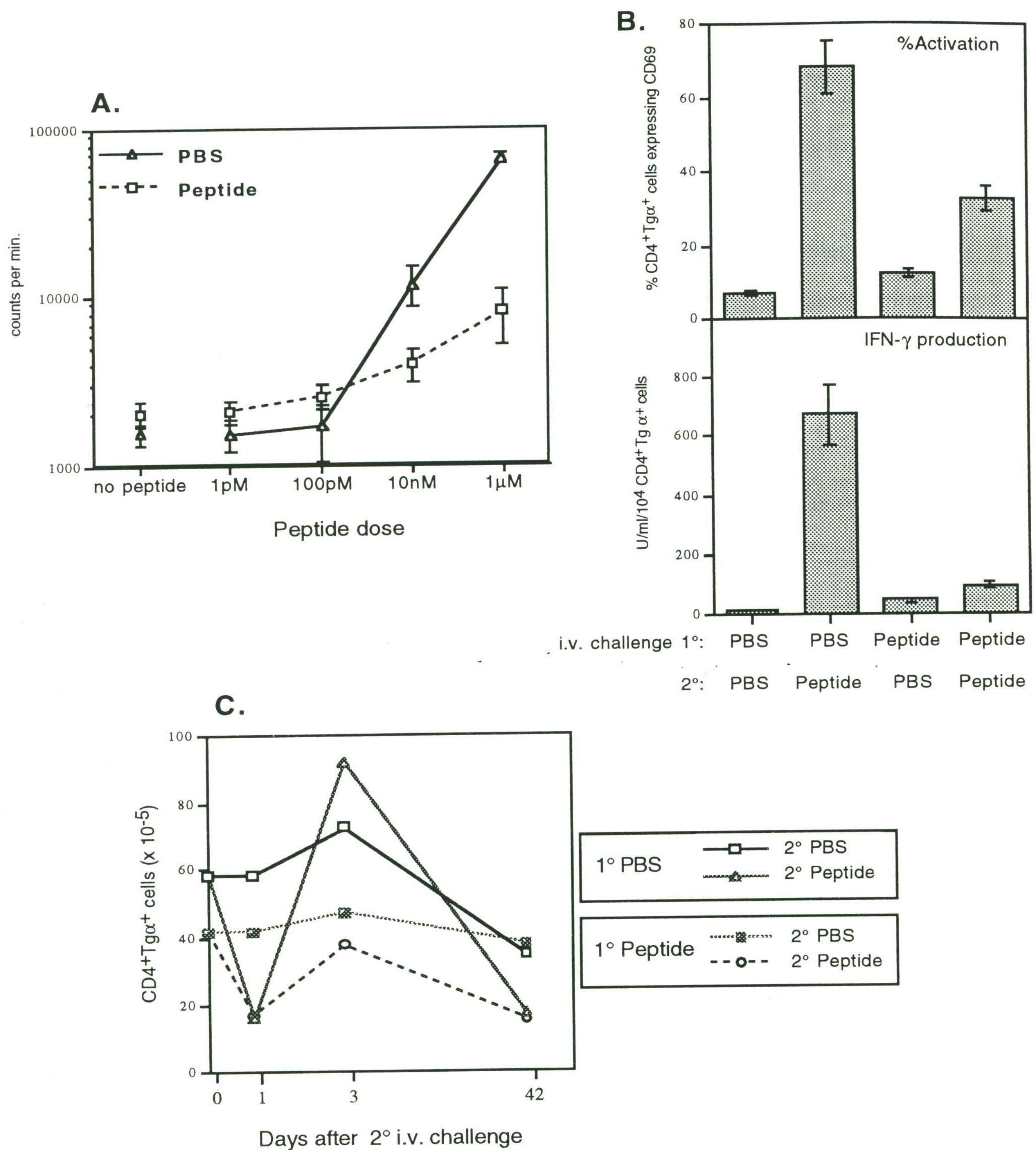


Figure 4.11. Intravenous re-challenge of intravenously immunised TCR transgenic mice *in vitro* and *in vivo*. Adult thymectomised -D TCR transgenic mice were immunised intravenously with PBS (1°PBS) or 1μg peptide (1°Peptide). **A.** Lymph node cells were harvested six weeks after immunisation and restimulated with 1pM-1μM peptide *in vitro*. Proliferation was measured and expressed as counts/min./culture. Each point represents the average of three mice with error bars indicating SEM.

B and **C.** Mice were immunised as described for **A**. Six weeks later, each group was divided into two for secondary intravenous immunisation, with one half receiving PBS (2°PBS) and the other 1μg peptide (2°Peptide). Spleen and lymph nodes were harvested one, three and 42 days later. **B.** On day one, CD69 was measured on $CD4^{+}Tg\alpha^{+}$ cells in the lymph nodes (top panel) and IFN-γ production was measured after restimulation with peptide *in vitro* (bottom panel). CD69 expression is shown as a percentage of total $CD4^{+}Tg\alpha^{+}$ cells. Each point represents the average of two mice with error bars indicating SEM. **C.** The number of $CD4^{+}Tg\alpha^{+}$ cells in the lymph nodes is shown for each timepoint. Each point represents the mean of two mice, with less than 10% variation between mice in each group.

(not shown). When lymph node or spleen cells (not shown) were restimulated with peptide *in vitro*, 2° peptide immunisation elicited a large amount of IFN-γ from the 1°PBS but not the 1°peptide group (Figure 4.11B, lower panel). Similar results were obtained when proliferation, IL-2 and IL-3 production were measured, though the reduction was not as marked as that seen for IFN-γ production (data not shown). Both groups showed a drop in the number of $CD4^{+}Tg\alpha^{+}$ cells one day after 2° peptide, followed on day three by expansion of $CD4^{+}Tg\alpha^{+}$ cells. In the 1°PBS group, four-fold expansion was seen, in comparison with only two-fold expansion in the 1°peptide group (Figure 4.11C). However, by day 42, cell numbers in both 2° peptide groups had returned to the low level seen on day one. The mice receiving 2° PBS had relatively stable cell numbers, although there was a drop in cell number in the mice which received only PBS, consistent with the gradual loss of $CD4^{+}Tg\alpha^{+}$ cells over time in the absence of immunisation (see Figure 4.1). The reduced T cell response observed in the 1°peptide group suggests that $CD4^{+}Tg\alpha^{+}$ cells that are not deleted by intravenous peptide are functionally hyporesponsive although whether this is due to selection of cells with low receptor levels or induction of a new functional phenotype is not clear. However, those cells which do respond to peptide rechallenge are still deleted as a consequence.

4.7. Further evidence for T cell hyporesponsiveness *in vivo*.

The results obtained so far demonstrated the profound effect of intravenous peptide on TCR transgenic mice - a single dose induced deletion of half of the antigen-specific T cell compartment, and the remainder appeared to be functionally hyporesponsive. It was therefore of interest to determine the effects of multiple doses of peptide. The response of adult thymectomised -D transgenic mice was monitored by weekly bleeding prior to injection with either PBS or 1μg peptide. Peptide was administered intraperitoneally since the mice were tail-bled each week. The proportion of blood $CD4^{+}$ cells expressing transgenic TCR was determined seven days after each dose of peptide, as shown in Figure 4.12A. The first dose of peptide reduced the proportion of

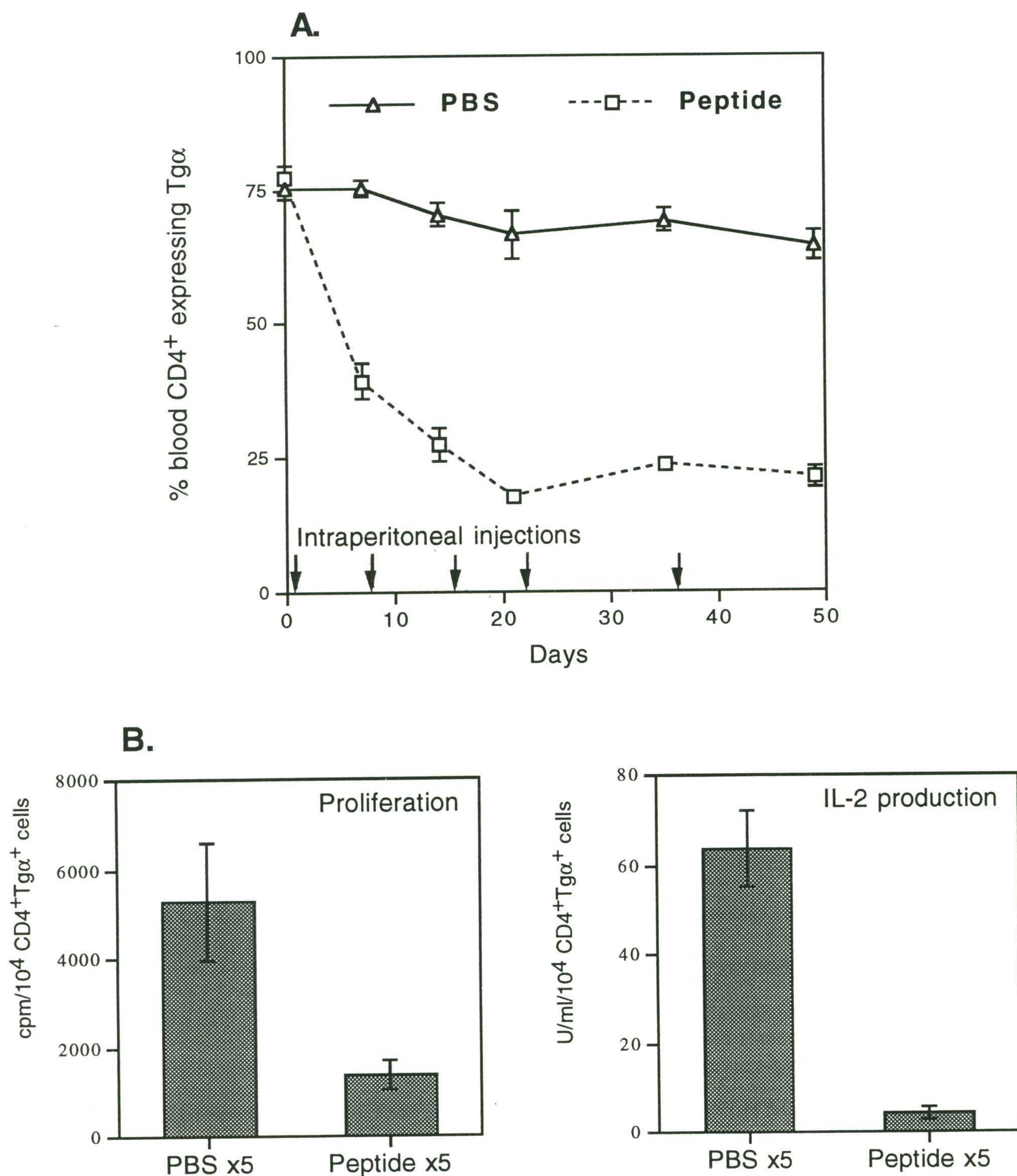


Figure 4.12. The effect of repeated intraperitoneal peptide immunisation. Adult thymectomised -D TCR transgenic mice were immunised intraperitoneally with PBS or 1μg peptide at weekly intervals. **A.** The percentage of blood $CD4^+$ cells expressing the transgenic TCR (Tgα) was determined by bleeding the mice prior to each peptide injection. **B.** Spleen cells were harvested from each group (PBS x5 and Peptide x5) on day 50 and restimulated with peptide *in vitro*. Proliferation is shown in the left panel and IL-2 production in the right panel. Each point represents the average of 4-5 mice with error bars indicating SEM.

$CD4^+Tg\alpha^+$ cells by half, but subsequent doses were progressively less effective, so that the fourth and fifth doses caused no further deletion of the remaining $CD4^+Tg\alpha^+$ cells. When spleen cells were harvested two weeks after the fifth dose of peptide and restimulated with peptide *in vitro*, proliferation and IL-2 production were substantially reduced when compared to the PBS control on a per cell basis (Figure 4.12B).

Several hypotheses could account for a residual "anergic" population after administration of multiple peptide doses. The remaining population may contain a substantial number of $CD4^+$ cells expressing $V_\alpha 11$ derived solely from endogenous rearrangement, and thus incapable of recognising MCC peptide, although in non-transgenic B10.BR mice such cells comprise less than 0.5% of $CD4^+$ cells (data not shown). Alternatively, the remaining cells may have been selected for low T cell receptor expression. $CD4^+Tg\alpha^+$ cells from mice that had received four doses of peptide at weekly intervals exhibited 2-3-fold downregulation of both CD4 and the α and β chains of the TCR, in comparison with the PBS-treated control (Figure 4.13A). Although spontaneously low levels of receptor expression could have been responsible for a primary failure to react to intravenous peptide, this degree of downregulation is also seen on activated and memory T helper cells (Hayakawa and Hardy, 1991; see Section 5) and could therefore have been induced by exposure to intravenous antigen. When the expression of CD44 was measured, 10% of the residual $CD4^+Tg\alpha^+$ cells were $CD44^{hi}$, compared to 2% in the PBS control, but there was no significant increase in the absolute number of $CD4^+Tg\alpha^+CD44^{hi}$ cells (Figure 4.13C), suggesting that these $CD44^{hi}$ T cells were present prior to peptide administration and had proven resistant to deletion. This notion is supported by the very low level of $Tg\alpha$ expression on $CD44^{hi}$ cells from PBS- and peptide-treated mice (Figure 4.13B), suggesting co-expression of endogenous α chains in all the $CD44^{hi}$ cells that fall within the $Tg\alpha^+$ gate. Co-expression of endogenous α chains would bestow alternative specificities on these cells, and thus their memory phenotype was most likely due to previous encounter with environmental antigens.

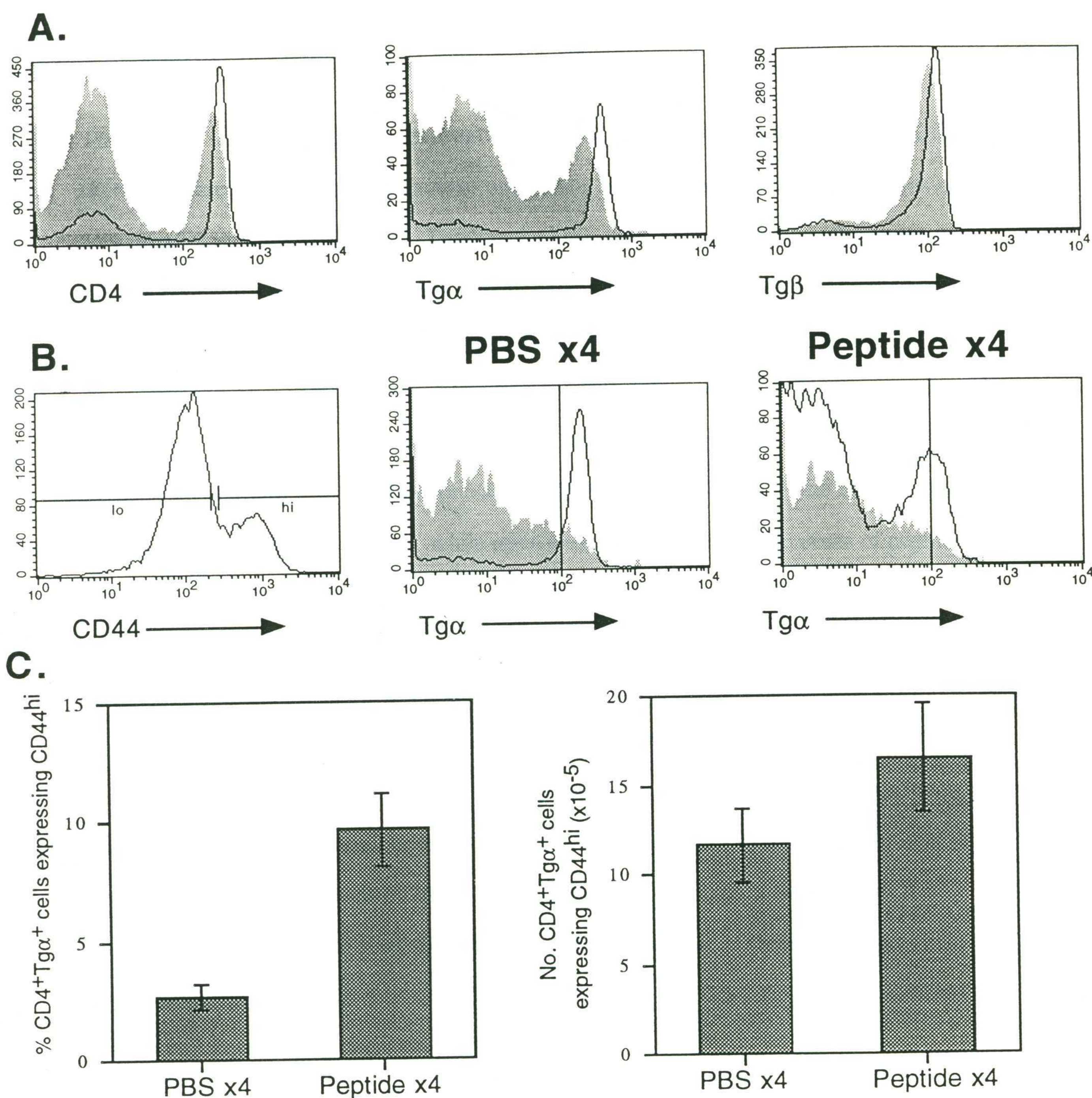


Figure 4.13. Phenotype of deletion-resistant CD4$^+$Tgα$^+$ cells. Lymph node cells were harvested from adult thymectomised -D TCR transgenic mice two weeks after the last of four intraperitoneal injections of 1μg peptide at weekly intervals. **A.** Representative plots (shaded) of the level of CD4 expression by Tgα$^+$ cells (left panel) and the level of expression of the Tgα and Tgβ chains on CD4$^+$ cells (middle and right panels, respectively). Expression of CD4, Tgα and Tgβ on cells from the PBS control are shown in outline. **B.** The level of Tgα expression was determined on CD4$^+$CD44^{hi} and CD4$^+$CD44^{lo} cells gated as shown in the left panel. Representative plots of Tgα expression on CD4$^+$CD44^{hi} cells (shaded) and CD4$^+$CD44^{lo} cells (outline) are shown for mice immunised with PBS (middle panel) and peptide (right panel). **C.** The expression of CD44^{hi} is shown for PBS- (PBS x4) and peptide-treated (Peptide x4) mice as a percentage of CD4$^+$Tgα$^+$ cells (left panel). In addition, the absolute number of CD4$^+$Tgα$^+$CD44^{hi} cells is shown in the right panel. Each point represents the average of 3-4 mice with error bars indicating SEM.

To test the capacity of the residual cells to respond to peptide *in vivo*, adult thymectomised mice were given zero, one, three, or five intraperitoneal injections of 1μg peptide at weekly intervals. Two weeks after the final injection, each group was immunised with 1μg of peptide intravenously. Spleen and lymph node cells were harvested one day later and CD69 expression, proliferation and IFN-γ production measured. Figure 4.14A (left panel) shows that intravenous peptide induced expression of CD69 by $CD4^{+}Tg\alpha^{+}$ cells in all groups of mice. There was little difference in the percentage of $CD4^{+}Tg\alpha^{+}$ cells expressing CD69 in the PBS control and in mice that had received one dose of peptide, while mice treated with three or five doses of peptide showed a reduction. Clearly however, mice that had received three or five doses of peptide still retained a substantial number of T cells capable of recognising peptide *in vivo*. Despite the evidence T cell activation in each group, mice that had received three or five weekly injections of peptide failed to exhibit enhanced proliferation and IFN-γ production *in vitro* (Figure 4.14A, right panel) when compared to naive controls. Mice that had received only one previous peptide dose responded to a second dose in a similar manner to the unimmunised control group apart from a decrease in IFN-γ production. In this experiment the decrease was not as great as that seen in previous experiments (see Figure 4.11), indicating that a single dose of peptide induces hyporesponsiveness with variable efficiency.

Subcutaneous immunisation of mice that had received multiple doses of intraperitoneal peptide once again stimulated a proportion of $CD4^{+}Tg\alpha^{+}$ cells in the draining lymph nodes to express CD69, but failed to prime significantly increased IFN-γ production (Figure 4.14B). Thus, at least a proportion of deletion-resistant $CD4^{+}Tg\alpha^{+}$ cells were capable of recognising antigen *in vitro* and *in vivo*, but were profoundly hyporesponsive at the functional level. This may explain why they could not be deleted from the periphery by further doses of peptide.

Figure 4.14. Intravenous and subcutaneous immunisation of mice after multiple injections of peptide. Adult thymectomised -D TCR transgenic mice were given 1-5 intraperitoneal injections of PBS or 1μg peptide (Peptide x1, x3, x4, or x5) at weekly intervals or left untreated. Two weeks after the final dose, each group was challenged with peptide intravenously or subcutaneously. **A.** Spleen cells were harvested one day after intravenous immunisation with 1μg peptide and compared to spleen cells from naive mice. CD69 expression was measured and expressed as a percentage of total $CD4^{+}Tg\alpha^{+}$ cells (left panel). In addition, proliferation and IFN-γ production were measured after restimulation with peptide *in vitro* (right panels). **B.** Draining lymph node cells were harvested four days after subcutaneous immunisation with PBS or 1μg peptide emulsified in CFA. CD69 expression was measured and expressed as a percentage of total $CD4^{+}Tg\alpha^{+}$ cells (left panel). In addition, IFN-γ production was measured after restimulation with peptide *in vitro* (right panel).

Each point represents the average of 3-4 mice with error bars indicating SEM.

A. 1 day after intravenous peptide challenge

% Activation

%CD4+Tgα+ expressing CD69

0
20
40
60
80

Unchallenged
PBS
Peptide x1
Peptide x3
Peptide x5

Proliferation

cpm/10⁴ CD4+Tgα+ cells

0
50000
100000
150000

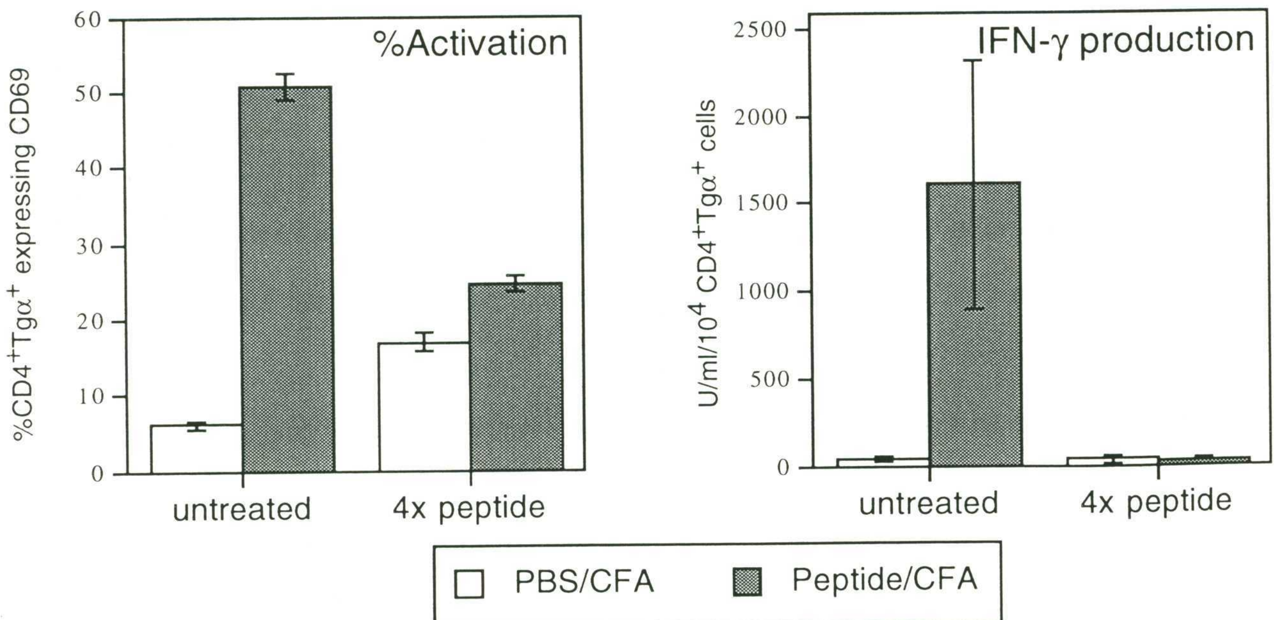
IFN-γ production

U/ml/10⁴ CD4+Tgα+ cells

0
400
800
1200

Unchallenged
PBS
Peptide x1
Peptide x3
Peptide x5

B. 4 days after subcutaneous challenge



4.8. Intravenous administration of intact antigen fails to induce peripheral deletion.

In order to further explore the impact of the intravenous route of antigen administration on the T cell response, TCR transgenic mice were immunised intravenously with intact cytochrome C, which requires processing to liberate the T cell epitope corresponding to the synthetic peptide used in the experiments described above. Since purified MCC was not available, a recombinant antigen containing an epitope of identical affinity to MCC 87-103 was prepared. Using the available construct for PCC, in which the minimal T cell epitope (94-104) differs from MCC by insertion of a single alanine residue at position 103 (see Figure 3.1), a synthetic des-ala-pigeon cytochrome C (daPCC) was engineered in *E. coli* by removing alanine 103 (engineered and kindly provided by Dr. M. Cook).

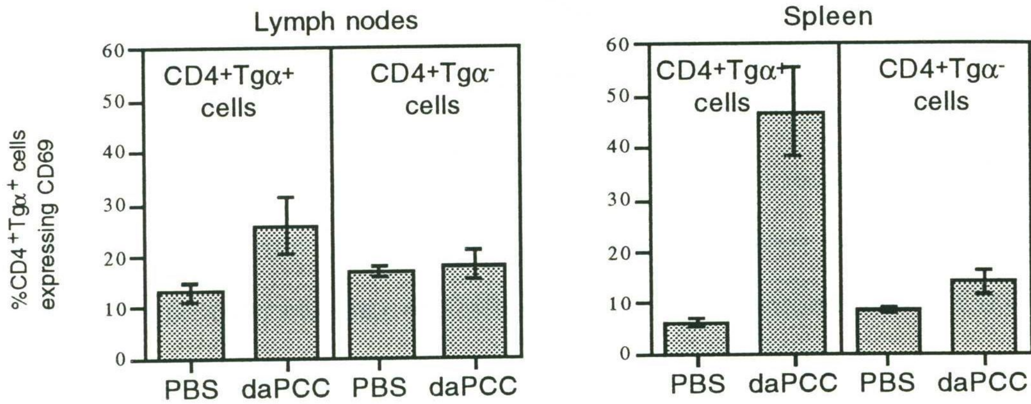
Adult thymectomised -D TCR transgenic mice were administered PBS or 10μg daPCC intravenously. A dose of 10μg was chosen because the molecular weight of daPCC is approximately ten-times greater than the synthetic peptide. The synthetic peptide was routinely administered in 1μg doses to induce peripheral deletion (see Section 4.1) and thus, a 10μg dose of daPCC would contain an equivalent dose of T cell epitope for presentation to T cells.

T cell activation was assessed in the spleen and lymph nodes by measuring the expression of CD69 by $CD4^{+}Tg\alpha^{+}$ and $CD4^{+}Tg\alpha^{-}$ cells one day after immunisation. Figure 4.15A shows that there was a marked increase in CD69 expression by $CD4^{+}Tg\alpha^{+}$ cells in the spleens of mice that received daPCC, in comparison with the PBS control, and a smaller increase in the lymph nodes. No significant change in the expression of CD69 on $CD4^{+}Tg\alpha^{-}$ cells was seen, demonstrating that T cell activation was antigen-specific. When the T cell response to rechallenge *in vitro* was measured one day after immunisation, spleen cells from daPCC-treated mice exhibited increased IFN-γ production when compared to the PBS control (Figure 4.15B), although the

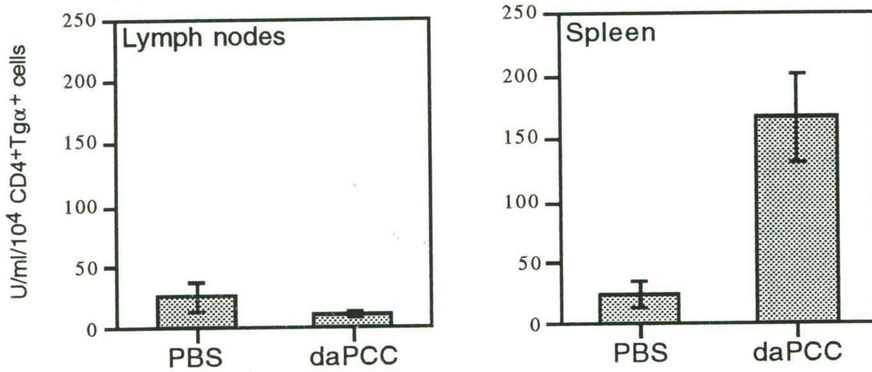
Figure 4.15. Peripheral T cell activation but not deletion after intravenous administration of des-ala pigeon cytochrome C. Adult thymectomised -D TCR transgenic mice were immunised intravenously with PBS or 10μg des-ala-pigeon cytochrome C (daPCC), as described in Section 3.2. **A.** CD69 was measured on spleen and lymph node cells one day after immunisation and expressed as a percentage of $CD4^+Tg\alpha^+$ and $CD4^+Tg\alpha^-$ cells. **B.** Spleen and lymph node cells were harvested one day after immunisation and restimulated with peptide *in vitro* to measure IFN-γ production. **C.** The total number of $CD4^+Tg\alpha^+$ cells in the lymph nodes and spleen was determined 11 days after immunisation. **D.** CD44 expression was measured 25 days after immunisation and expressed as a percentage of $CD4^+Tg\alpha^+$ cells.

Each point represents the average of 2-3 mice with error bars indicating SEM.

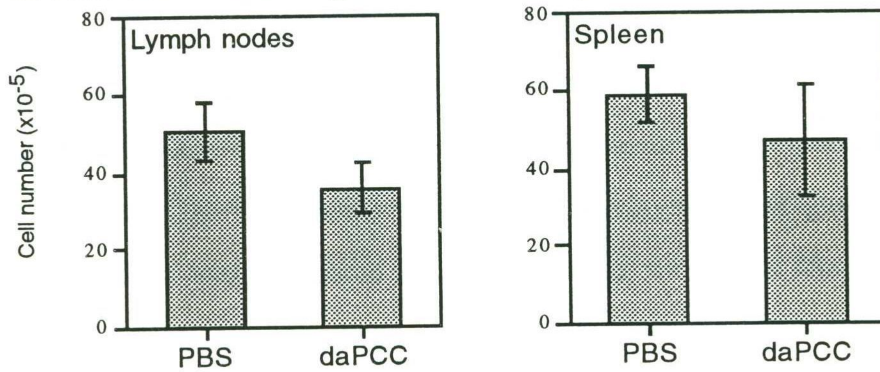
A. CD69 expression 1 day after immunisation



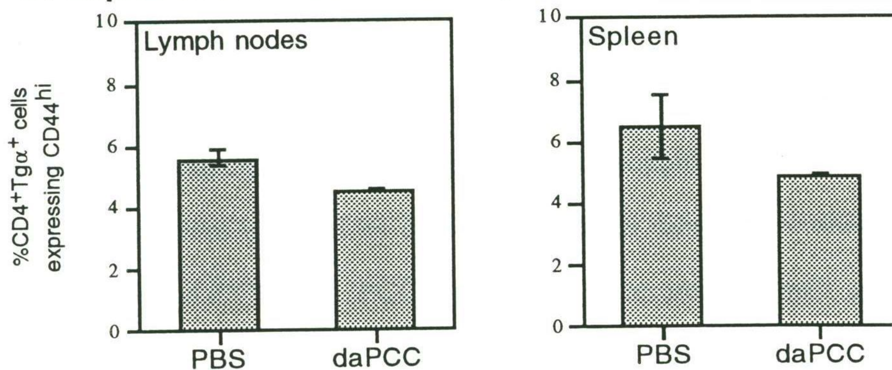
B. IFN-γ production 1 day after immunisation



C. Number of CD4+Tgα+ cells 11 days after immunisation



D. Expression of CD44hi 25 days after immunisation



amount of IFN-γ production per cell was lower than that seen after intravenous peptide. Moreover, lymph node cells from daPCC-treated mice failed to produce more IFN-γ than the PBS control (Figure 4.15B), and the proliferative responses of spleen and lymph node cells from immunised and control groups were equivalent (data not shown). Despite clear evidence of T cell activation on day one, deletion of $CD4^{+}Tg\alpha^{+}$ cells 11 (Figure 4.15C) or 25 days (data not shown) after immunisation did not reach statistically significant levels. Intravenous administration of a commercial preparation of PCC (which has a lower affinity T cell epitope than MCC) also failed to induce peripheral deletion (Figure 4.16A), confirming the results obtained with daPCC. Thus, intravenous administration of intact antigen to TCR transgenic mice did not induce peripheral deletion, despite evidence that it stimulated T cell activation *in vivo*.

Generation of T cells with a memory phenotype in response to intravenous administration of intact antigen was variable. There was no difference in the percentage of $CD4^{+}Tg\alpha^{+}$ cells expressing high levels of CD44 in the PBS and daPCC groups 25 days after immunisation (Figure 4.15D) but a small increase was apparent six weeks after intravenous PCC (Figure 4.16B). Based on the previous observation that peptide deletion-resistant $CD44^{hi}$ cells express very low levels of Tgα (Figure 4.13B), the expression of Tgα was compared for $CD44^{hi}$ cells six weeks after intravenous administration of PBS, peptide, PCC and daPCC. Figure 4.17 shows there was little difference in the Tgα profiles for $CD44^{hi}$ cells from PBS- and peptide-treated mice (consistent with the data shown in Figure 4.13B), but a clear increase in the proportion of $Tg\alpha^{hi}$ cells in daPCC- and PCC-treated mice. These profiles resemble those for subcutaneous immunisation (see Figure 5.8B) and suggests that the high levels of CD44 expression were induced by cytochrome rather than environmental antigens. Thus intravenous daPCC and PCC may have generated a small population of cytochrome-specific memory T cells. Taken together, these results demonstrate that tolerance is not an obligatory consequence of T cell activation following intravenous

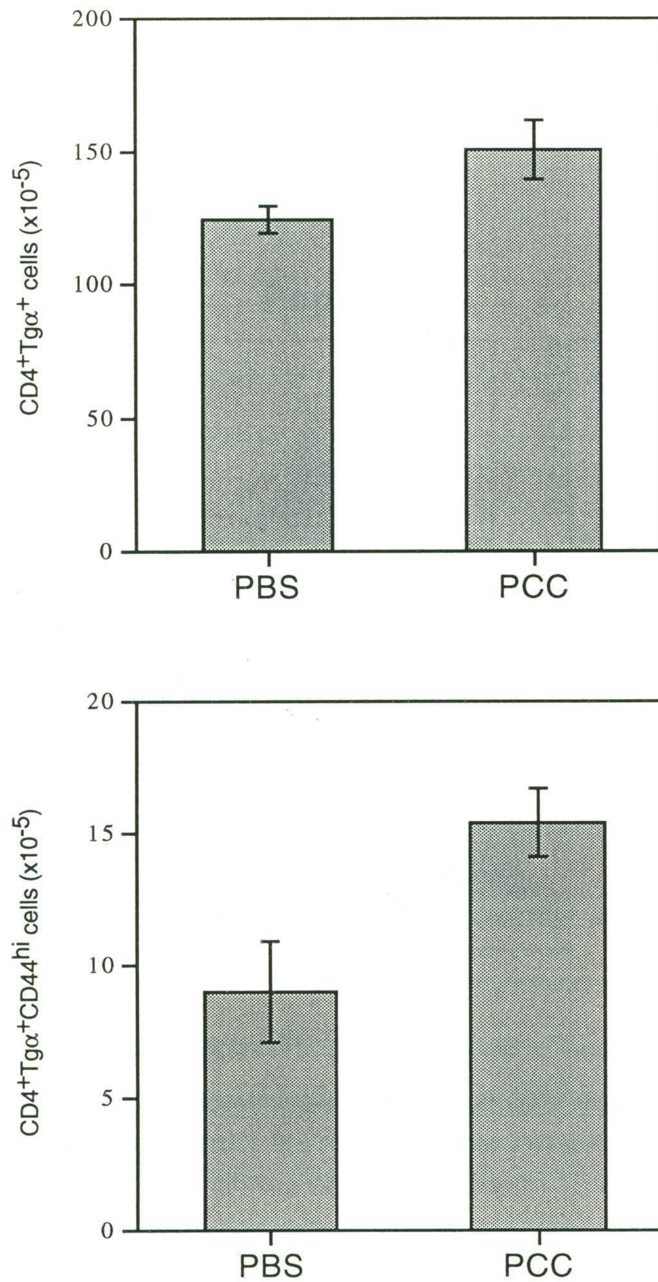


Figure 4.16. Lack of deletion after intravenous administration of pigeon cytochrome C. Adult thymectomised -D TCR transgenic mice were immunised intravenously with PBS or 10μg pigeon cytochrome C (PCC), as described in Section 3.2. 50 days later, the number of $CD4^+Tg\alpha^+$ (upper panel) and $CD4^+Tg\alpha^+CD44^{hi}$ cells (lower panel) in the spleens of PBS- and PCC-treated mice was calculated from the percentages determined by flow cytometry. Each point represents the average of three mice with error bars indicating SEM.

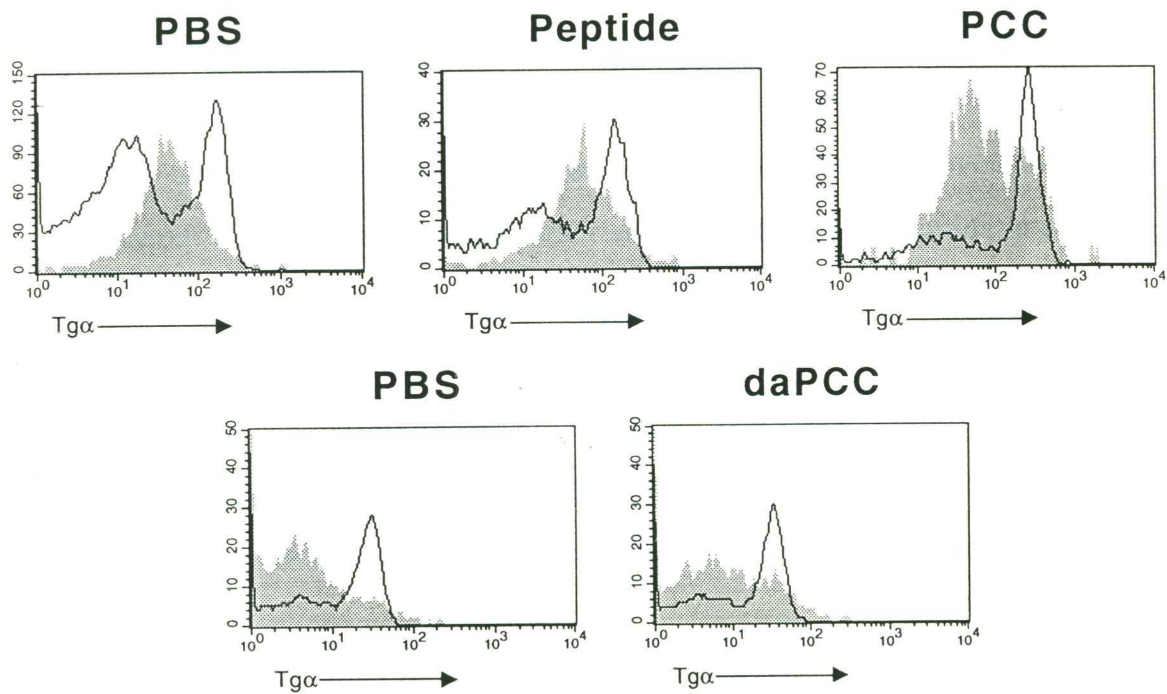


Figure 4.17. Expression of Tgα on CD44^{hi} cells from mice immunised intravenously with PBS, peptide, PCC or daPCC. Adult thymectomised -D TCR transgenic mice were immunised intravenously with PBS, 1μg peptide or 10μg PCC (upper panels). In a second experiment adult thymectomised -D TCR transgenic mice were immunised intravenously with PBS or 10μg daPCC (lower panels). Lymph node cells were harvested six weeks after immunisation in both experiments and the level of Tgα expression was determined on CD4$^+$CD44^{hi} (shaded plots) and CD4$^+$CD44^{lo} cells (outlined plots). Each plot is representative of 2-4 mice in each experimental group. The level of fluorescence in the Tgα$^-$CD44^{hi} population was higher than expected in the experiment shown in the upper panels because of incorrect compensation in that particular experiment.

immunisation and emphasise that the form of the antigen can have a profound effect on the T cell response.

4.9. Investigation of presentation of intravenously-administered peptide.

The results presented in this chapter demonstrated that the form of antigen (peptide versus intact antigen) administered to transgenic mice affected the final outcome of the peripheral T cell response. Since intact antigen differs from peptide in requiring processing by APC for presentation to T cells (see section 1.2), it appeared that the APCs which induced peripheral tolerance to intravenous antigen were incapable of presenting foreign antigen requiring processing. This could result from inability to either internalise or process antigen. In addition, the APC which did process and present intact exogenous antigen appeared to be unable to induce tolerance.

Two adoptive transfer protocols were designed to assess the ability of different APC populations to induce peripheral tolerance after administration of intravenous peptide. In order to distinguish the functional capacity of various APCs to present peptide administered intravenously, expression of I-E, required for MCC peptide presentation, was restricted to donor APCs. In the first system, -D x 36-2 double transgenic mice were used as recipients of purified APCs expressing I-E^{db}, which can serve as the restriction element for MCC peptide recognised by -D transgenic TCR T cells. -D x 36-2 double transgenic mice express I-E^{db} exclusively in the thymus and are thus tolerant of I-E^{db} but have no endogenous I-E^{db+} peripheral APC (Widera et al., 1987; Burkly et al., 1990). The congenic 107-1 transgenic line served as the APC donor expressing transgenic I-E^{db} with a wildtype distribution (Widera et al., 1987). In the second system, immunodeficient homozygous *scid/scid* or Rag-1-deficient mice were used as recipients of transgenic T cells and purified APC populations. Recipients were immunised intravenously with peptide after adoptive transfer and the response measured as for intact TCR transgenic mice.

4.9.1. Adoptive transfer of APC into TCR transgenic mice.

The first strategy used to study the antigen-presenting ability of different APCs *in vivo* was to transfer I-E^{db+} APC into -D x 36-2 TCR transgenic mice. The absence of I-E^{db} in the periphery of unmanipulated recipients was confirmed by failure to activate CD4$^+$Tgα^+ cells by intravenous administration of peptide (Figure 4.18).

Adult -D x 36-2 double transgenic mice received 80x10^6 unfractionated 107-1 spleen cells or 30x10^6 purified 107-1 splenic B cells in adoptive transfer. Figure 4.19A shows that adoptive transfer of each APC population was inefficient, since less than 6% of spleen and lymph node cells in hosts of I-E$^+$ cells expressed I-E, of which 3-4% could be attributed to background staining (see also Figure 4.20A). The number of I-E$^+$ cells recovered in each recipient thus represented only 10-15% of the number of I-E$^+$ cells transferred. Each group was administered PBS or 1μg peptide intravenously one day after adoptive transfer of APCs. Spleen and lymph nodes were harvested one day after immunisation and T cell activation was assessed by measuring the expression of CD69 by CD4$^+$Tgα^+ cells. Despite the low proportion of I-E$^+$ cells, intravenous peptide stimulated CD69 expression on a significant number of CD4$^+$Tgα^+ cells (Figure 4.19B), although the percentage of CD69$^+$ cells was significantly lower than previously seen in intact transgenic mice (Figure 4.5), especially in the lymph nodes. Spleen cells did not exhibit increased proliferation when restimulated with peptide *in vitro* one day after immunisation, and there was no decline in the number of CD4$^+$Tgα^+ cells in the spleen or lymph nodes seven days after immunisation (data not shown). These results clearly demonstrate that both unfractionated spleen cells and purified B cells were capable of presenting intravenous peptide to CD4$^+$Tgα^+ cells *in vivo*, although they did not appear to be capable of stimulating detectable cell division or deletion in this experimental model.

The capacity of different APC to activate CD4$^+$Tgα^+ cells *in vivo* was explored further by comparing enriched populations of dendritic cells (DC) or B cells. The DC

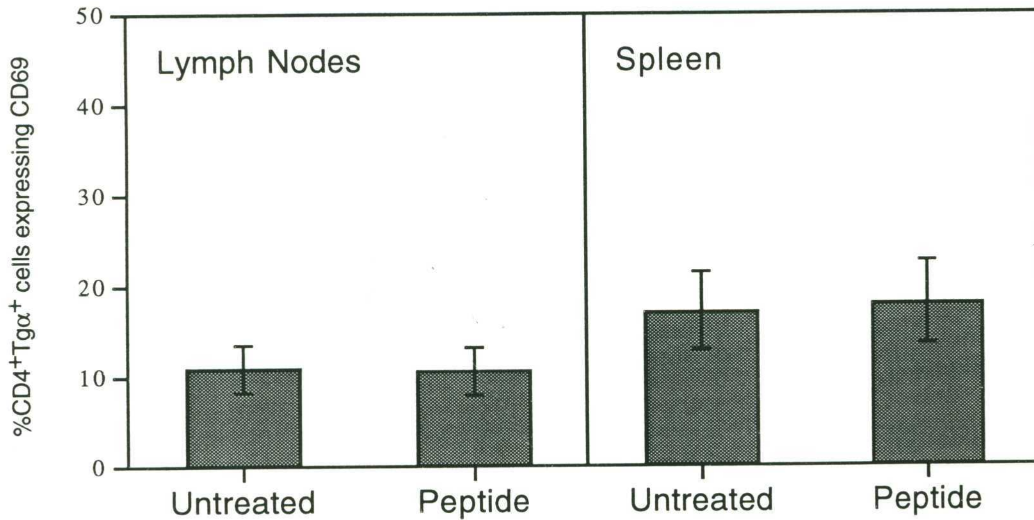


Figure 4.18. Intravenous immunisation of -D x 36-2 double transgenic mice. Adult -D x 36-2 double transgenic mice (see Section 3.1) were immunised intravenously with 20μg peptide. 20 hours later, spleen and lymph node cells were harvested, along with those from untreated controls, and CD69 was measured by flow cytometry. CD69 expression is shown as a percentage of total $CD4^{+}Tg\alpha^{+}$ cells. Each point represents the average of two mice with error bars indicating the range of values.

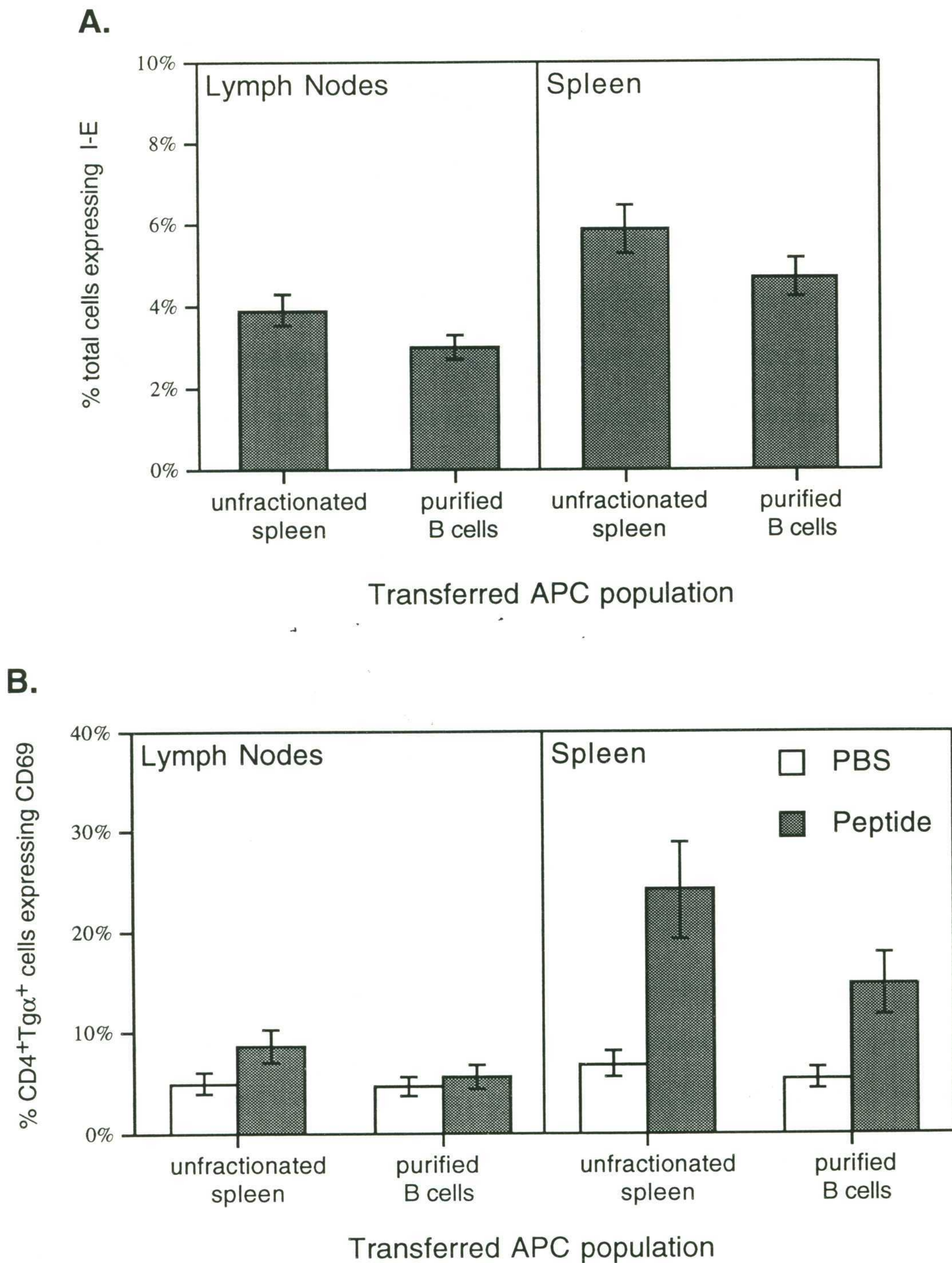


Figure 4.19. Intravenous immunisation of -D x 36-2 double transgenic mice after adoptive transfer of APC. Adult -D x 36-2 double transgenic mice were administered 80x106 unfractionated spleen cells or $30x10^6$ purified B cells (purified as described in Section 3.8) from 107-1 donors in adoptive transfer. **A.** The percentage of cells expressing I-E in the lymph nodes and spleen was determined three days after transfer by immunostaining and flow cytometry. **B.** Recipients were immunised intravenously with PBS or 1μg peptide one day after adoptive transfer. CD69 was measured on spleen and lymph node cells one day after immunisation and expressed as a percentage of total $CD4^+Tg\alpha^+$ cells.

Each point represents the average of two mice, with error bars indicating the range of values.

population was derived *in vitro* from unfractionated 107-1 spleen cells cultured with GM-CSF, as described in the Section 3.9. The resulting population expressed high levels of I-E, no B220 or CD3, and was 10-fold more potent on a per cell basis than irradiated spleen in stimulating proliferation of $CD4^{+}Tg\alpha^{+}$ cells *in vitro* (data not shown). An enriched population of B cells from 107-1 transgenic mice was prepared by adherence-depletion of spleen cells, as described in the Section 3.10, and contained ~30% $CD3^{+}$ cells and less than 0.1% $B220^{-}$ $I\text{-}E^{+}$ cells (data not shown). Each -D x 36-2 host received $2x10^{6}$ dendritic cells or $90x10^{6}$ enriched B cells. Three days later, both groups of recipients, plus a third group of unmanipulated -D x 36-2 mice, were immunised intravenously with 1μg peptide. CD69 expression by $CD4^{+}Tg\alpha^{+}$ cells was measured one day later, and peripheral deletion was assessed on day eight. Figure 4.20A shows that there was again a low frequency of $I\text{-}E^{+}$ cells in the spleens of APC recipients. Transferred B cells were easily detected, whilst transferred DC could not be detected by this technique. Figure 4.20B shows that both groups of APC recipients showed an increase in CD69 expression after immunisation with peptide, but the percentage of positive cells was low in B cell recipients and even lower in DC recipients. Thus there was evidence of T cell responsiveness to intravenous peptide presented by both DC and B cells. However, there was no evidence of a decrease in the number of $CD4^{+}Tg\alpha^{+}$ cells on day one (data not shown) or of peripheral deletion on day eight, as shown in Figure 4.20C. Repeat experiments yielded similar results and it was concluded that the low frequency of transferred APC limited the potential of this system, since it was not possible to distinguish whether deletion was undetectable because too few T cells were activated, or because the transferred APC were incapable of inducing deletion.

4.9.2. Adoptive transfer of TCR transgenic T cells and APC populations into immunodeficient recipients.

A second adoptive transfer system was developed to study the presenting ability of different APC *in vivo* with the emphasis on efficient transfer of APCs. Homozygous

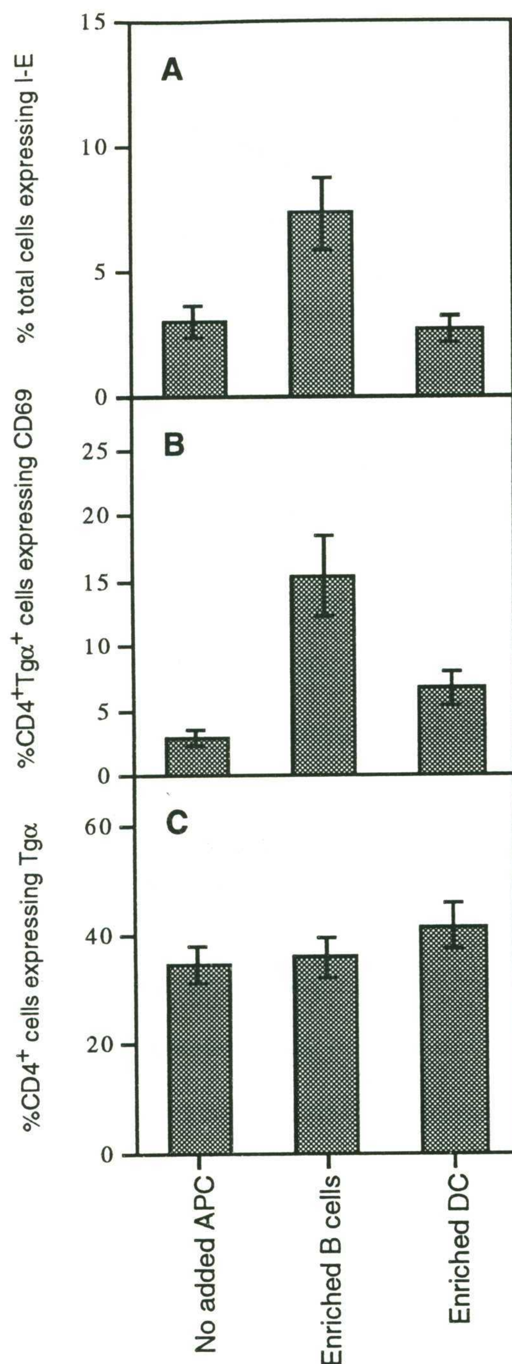


Figure 4.20. Intravenous immunisation of -D x 36-2 double transgenic mice after adoptive transfer of cultured dendritic cells or B cells. Adult -D x 36-2 double transgenic mice were administered 90×10^6 enriched B cells (prepared as described in Section 3.10) or 2×10^6 enriched dendritic cells (DC, derived *in vitro* as described in Section 3.9) from 107-1 donors in adoptive transfer. A third group of -D x 36-2 double transgenic mice served as unmanipulated controls. **A.** The percentage of cells expressing I-E in the spleen and lymph nodes (not shown) was determined four days after transfer. **B.** Recipients were immunised intravenously with PBS or 1µg peptide one day after adoptive transfer. CD69 was measured on spleen cells one day after immunisation and expressed as a percentage of total $CD4^+Tg\alpha^+$ cells. **C.** The percentage of $CD4^+$ cells expressing the transgenic TCR (Tgα) in the spleen was determined eight days after immunisation.

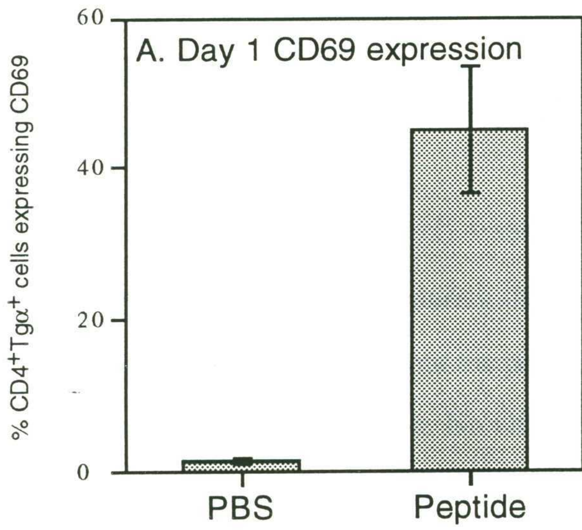
Each point represents the average of two mice, with error bars indicating the range of values.

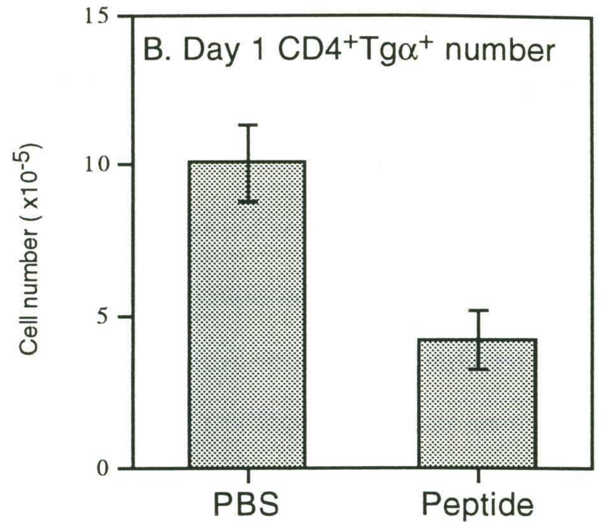
scid/scid mice lack a significant population of peripheral T and B cells, and thus they can be populated with a high frequency of transferred cells despite a low efficiency of transfer. In addition, they cannot mount a host-versus-graft response against cells expressing a foreign MHC molecule. *Scid/scid* mice with a H-2^b haplotype were used as recipients to avoid antigen presentation by endogenous cells. To avoid a graft-versus-host response, I-E^+ donor cells were from H-2^{bk} mice tolerant of H-2^b.

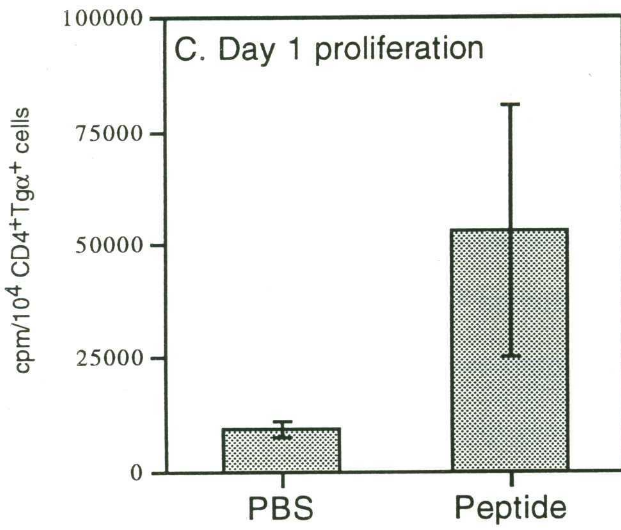
50×10^6 unfractionated pooled spleen and lymph node cells from TCR transgenic donors were adoptively transferred into *scid/scid* recipients, providing both T cells expressing the transgenic TCR and APC expressing I-E. Recipients were immunised intraperitoneally with PBS or 1μg peptide three days after transfer, and the response was characterised as shown in Figure 4.21. Intravenous peptide clearly activated $CD4^+Tg\alpha^+$ cells, as shown by a significant increase in the percentage of $CD4^+Tg\alpha^+$ cells expressing CD69 in spleen (Figure 4.21A) and lymph nodes (not shown). Activation was associated with a decrease in the number of $CD4^+Tg\alpha^+$ cells on day one (Figure 4.21B) and the proliferative response to rechallenge *in vitro* was significantly increased (Figure 4.21C), hallmarks of the response to intravenous peptide in intact transgenic mice (see Figures 4.1, 4.3 and 4.5 for comparison). The number of $CD4^+Tg\alpha^+$ cells increased in the spleen (Figure 4.21D) and lymph nodes (not shown) three days after immunisation, as seen previously in intact transgenic mice (see Figure 4.1). The number of $CD4^+Tg\alpha^+$ cells returned to baseline by day seven, but no peripheral deletion seen then, or at later timepoints, in five separate experiments (Figure 4.21E and data not shown). These results conflicted with those in intact transgenic mice and previous adoptive transfer experiments in B10.BR recipients (Figure 4.9) in which significant deletion was manifested by days 7-10. An obvious difference between the B10.BR and *scid/scid* systems was the presence of endogenous I-E^+ APC. For this reason, the experiment was repeated using H-2^{bk} *scid/scid* recipients, thus providing endogenous non-B cell APC expressing I-E. Once again, there was no evidence of deletion at eight (Figure 4.21F) or 21 days (data not shown) after intravenous

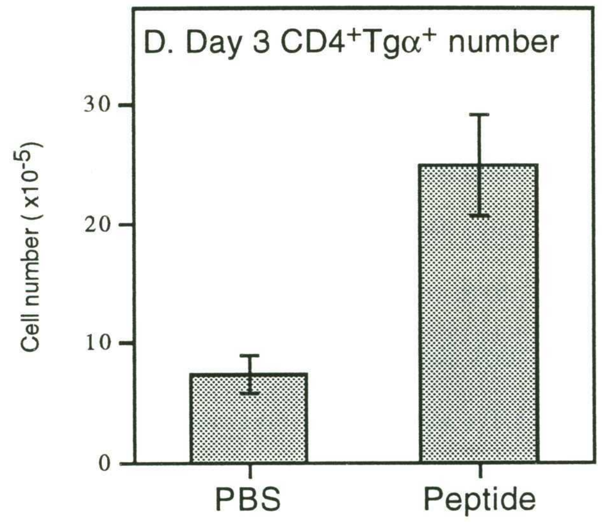
Figure 4.21. Intravenous immunisation of *scid/scid* recipients after adoptive transfer. Homozygous *scid/scid* (H-2^b) mice received 50x10^6 unfractionated pooled spleen and lymph node cells from -D TCR transgenic H-2^{bk} donors in adoptive transfer (as described in Section 3.10). Recipients were immunised intraperitoneally with PBS or 1µg peptide three days after adoptive transfer. **A.** CD69 was measured on spleen cells one day after immunisation and expressed as a percentage of $CD4^+Tg\alpha^+$ cells. **B.** The number of $CD4^+Tg\alpha^+$ cells present in the spleens of recipients was determined one day after immunisation. **C.** Spleen cells were restimulated with peptide *in vitro* one day after immunisation and proliferation was measured. **D.** The number of $CD4^+Tg\alpha^+$ cells in the spleens of recipients was determined three days after immunisation. **E.** The percentage of blood $CD4^+$ cells expressing the transgenic TCR (Tgα) was determined seven days after immunisation. **F.** Homozygous *scid/scid* H-2^{bk} mice received 50-100x10^6 unfractionated spleen and lymph node cells from -D TCR transgenic H-2^{bk} donors in adoptive transfer, as above, and were immunised intraperitoneally with PBS or 1µg peptide three days later. The percentage of blood $CD4^+$ cells expressing the transgenic TCR (Tgα) was determined eight days after immunisation.

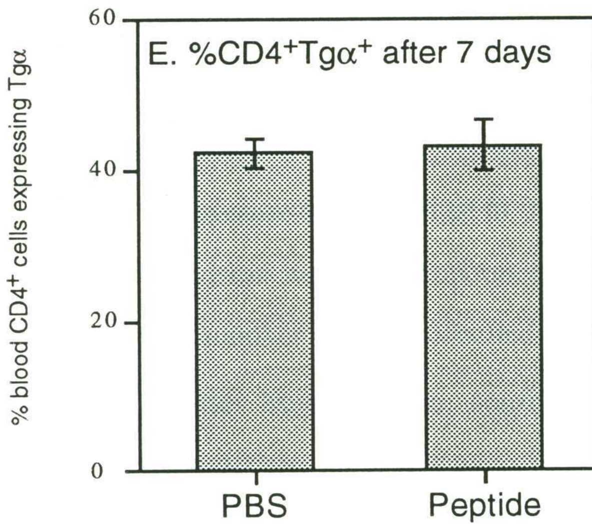
Each point represents the average of 3-4 mice with error bars indicating SEM.

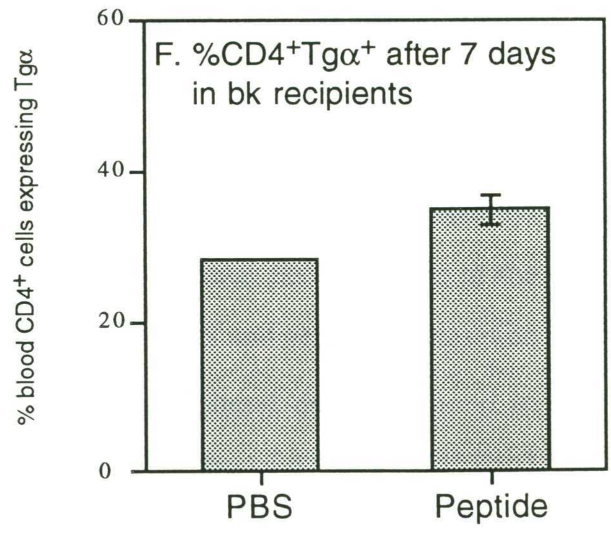

A. Day 1 CD69 expression
% CD4+Tgα+ cells expressing CD69
60
40
20
0
PBS
Peptide


B. Day 1 CD4+Tgα+ number
Cell number (x10-5)
15
10
5
0
PBS
Peptide


C. Day 1 proliferation
cpm/10^4 CD4+Tgα+ cells
100000
75000
50000
25000
0
PBS
Peptide


D. Day 3 CD4+Tgα+ number
Cell number (x10-5)
30
20
10
0
PBS
Peptide


E. %CD4+Tgα+ after 7 days
% blood CD4+ cells expressing Tgα
60
40
20
0
PBS
Peptide


F. %CD4+Tgα+ after 7 days
in bk recipients
% blood CD4+ cells expressing Tgα
60
40
20
0
PBS
Peptide

immunisation, indicating that *scid/scid* mice appear to possess a defect in induction of peripheral deletion.

The response of adoptively-transferred $CD4^+Tg\alpha^+$ cells in *scid/scid* recipients was further tested by administering three doses of 1μg peptide intraperitoneally at weekly intervals in order to rule out the possibility that anergy rather than deletion was induced by peptide administration. No change in the percentage of $CD4^+Tg\alpha^+$ cells was seen seven days after the final dose of peptide (Figure 4.22A). Furthermore, there was no evidence of hyporesponsiveness, since the PBS and peptide groups exhibited equivalent T cell activation and proliferation one day after intravenous peptide rechallenge (Figure 4.22B).

To confirm these observations in a system in which T cell division could be measured, $20x10^6$ CFSE-labelled unfractionated spleen cells from $H\text{-}2^k$ -D TCR transgenic mice were transferred into $H\text{-}2^k$ homozygous Rag-1-deficient recipients (Spanopoulou et al., 1994). The recipients were immunised intravenously with PBS or 1μg peptide four days after cell transfer. Spleen and lymph nodes were harvested nine days after immunisation, and the distribution of CFSE-labelled $CD4^+Tg\alpha^+$ cells was determined by flow cytometry. Figure 4.23A shows that there was significant division of $CD4^+Tg\alpha^+$ cells in $H\text{-}2^k$ recipients immunised with peptide, but not PBS. Limited division of $CD4^+Tg\alpha^-$ cells in both PBS and peptide groups proved to be a feature of adoptive transfer into T cell-deficient recipients in repeat experiments in *scid/scid* mice (data not shown), in contrast to immunosufficient B10.BR hosts in which background cell division was less pronounced (see Figure 4.9). There was no difference in the overall number of CFSE-labelled $CD4^+Tg\alpha^+$ cells in the PBS and peptide groups nine days after immunisation, confirming the defect in peripheral deletion found in immunodeficient *scid/scid* mice. However, a substantial number of divided $CD4^+Tg\alpha^+$ cells had disappeared between days three and seven, since the total number of CFSE-labelled $CD4^+Tg\alpha^+$ cells in peptide-treated recipients had not increased.

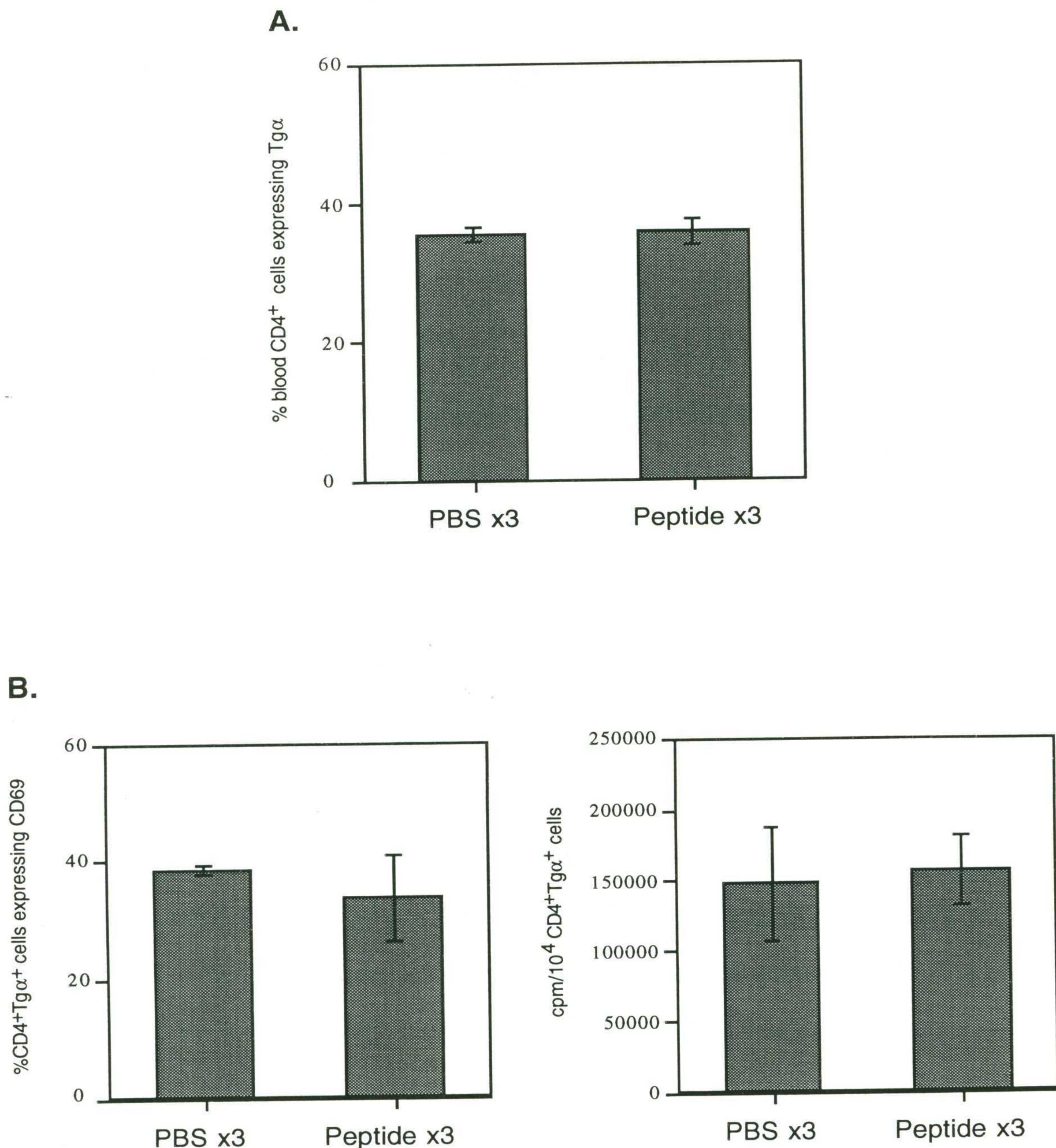
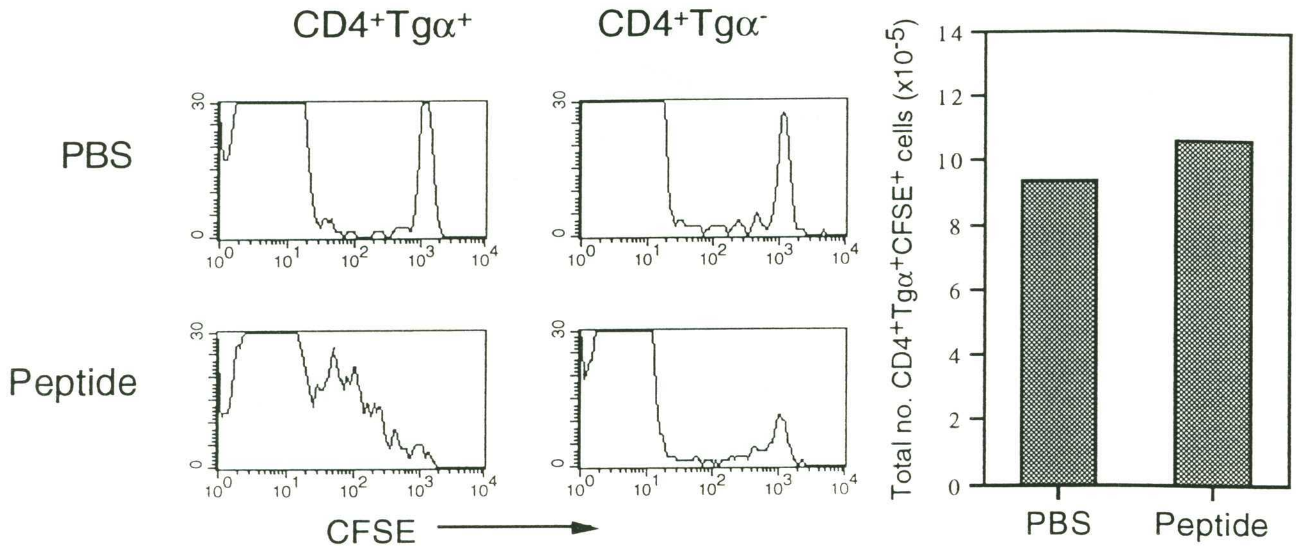


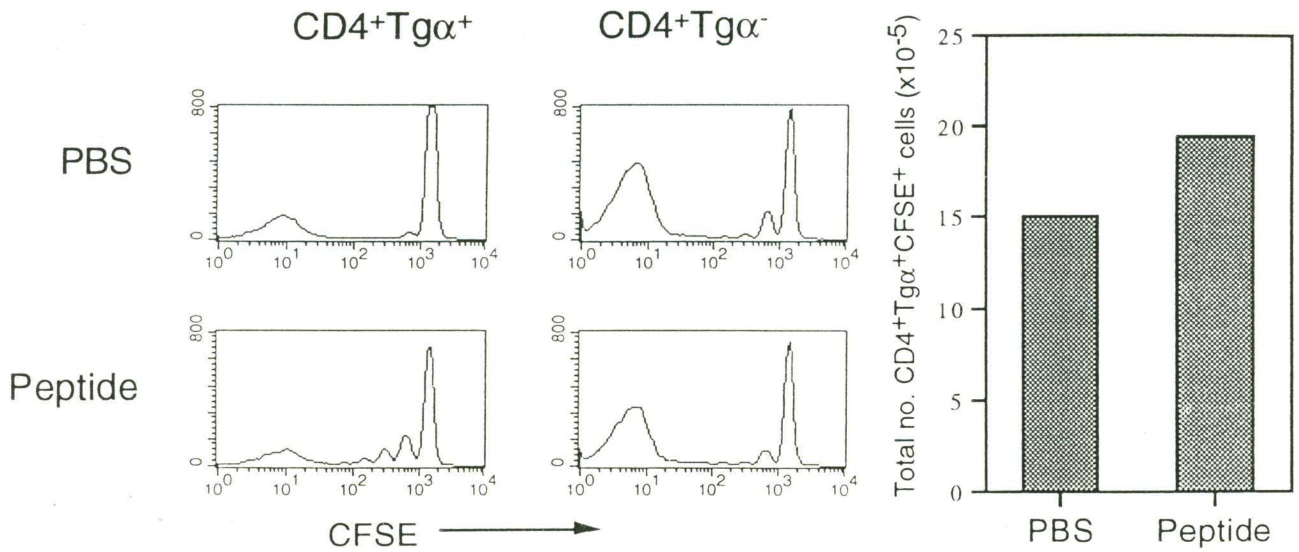
Figure 4.22. Repeated intraperitoneal immunisation of *scid/scid* recipients after adoptive transfer. Homozygous *scid/scid* (H-2^b) mice received 50-100x10^6 unfractionated pooled spleen and lymph node cells from -D TCR transgenic [H-2^b x H-2^k]F1 donors in adoptive transfer. Three days later, recipients were immunised intraperitoneally with PBS or 1μg peptide, followed by two doses at weekly intervals. **A.** The percentage of blood $CD4^+$ cells expressing the transgenic TCR (Tgα) was determined seven days after the third immunisation. **B.** Both groups of recipients were challenged with 1μg peptide intravenously seven days after the third immunisation. One day later, spleen cells were harvested and CD69 was measured and expressed as a percentage of total $CD4^+Tg\alpha^+$ cells (left panel). In addition, the cells were restimulated with peptide *in vitro* to measure proliferation (right panel).

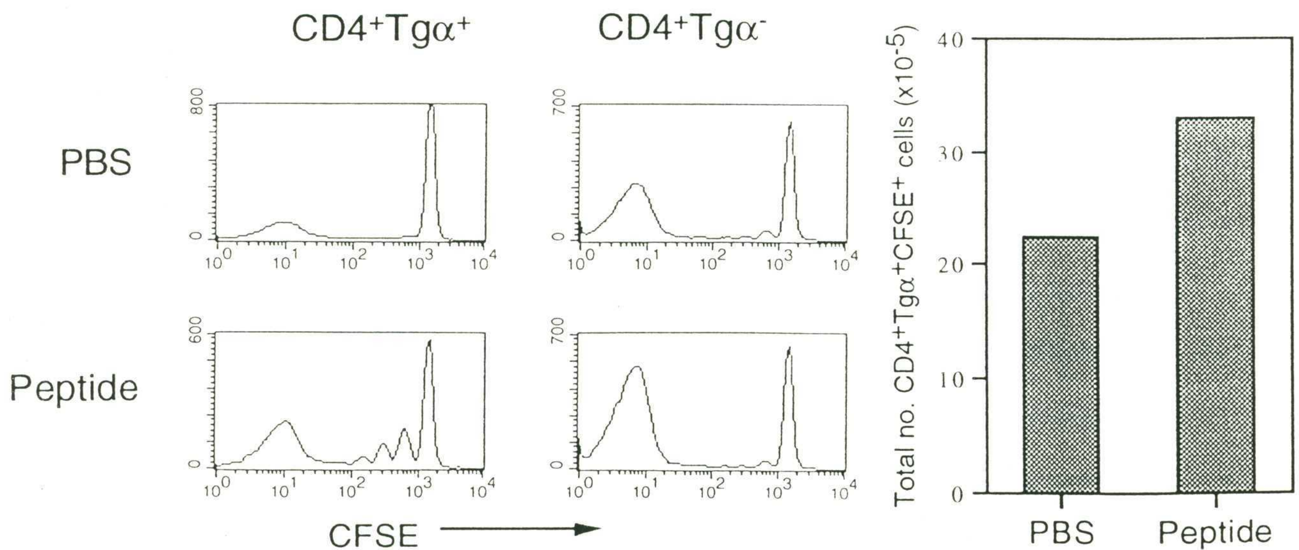
Each point represents the average of three mice with error bars indicating SEM.

Figure 4.23. Intravenous immunisation of homozygous Rag-1-deficient mice after adoptive transfer. Homozygous Rag-1-deficient mice (see Section 3.1 for details) received 20x10^6 CFSE-labelled (see Section 3.6) pooled spleen and lymph node cells from -D TCR transgenic mice in adoptive transfer in the following combinations: **A.** H-2^k recipients received unfractionated spleen and lymph node cells from H-2^k donors. **B.** H-2^{bk} recipients received adherent-depleted spleen and lymph node cells from H-2^{bk} donors. **C.** H-2^b recipients received adherent-depleted spleen and lymph node cells from H-2^{bk} donors.

Recipients were immunised intravenously with PBS or 1μg peptide four days after adoptive transfer. The division of CFSE-labelled $CD4^+Tg\alpha^+$ and $CD4^+Tg\alpha^-$ cells was determined in the lymph nodes ten days after intravenous immunisation with PBS or 1μg peptide by flow cytometry (as described in Section 3.6). Representative plots are shown on the left for each group of recipients. The total number of CFSE-labelled $CD4^+Tg\alpha^+$ cells in the spleen and lymph nodes was calculated for each group and is shown on the right. Each point represents the average of two mice, with less than 20% variation between individual mice.


A. H-2k recipients
CD4+Tgα+
CD4+Tgα-
PBS
Peptide
CFSE
Total no. CD4+Tgα+CFSE+ cells (x10-5)
PBS
Peptide


B. H-2bk recipients
CD4+Tgα+
CD4+Tgα-
PBS
Peptide
CFSE
Total no. CD4+Tgα+CFSE+ cells (x10-5)
PBS
Peptide


C. H-2b recipients
CD4+Tgα+
CD4+Tgα-
PBS
Peptide
CFSE
Total no. CD4+Tgα+CFSE+ cells (x10-5)
PBS
Peptide

Adoptive transfer of CFSE-labelled TCR transgenic cells was also performed in H-2^{bk} and H-2^{b} homozygous Rag-1-deficient recipients (Figure 4.23B and 4.23C, respectively). In this case, the hosts received adherent-depleted pooled spleen and lymph node cells from H-2^{bk} -D TCR transgenic mice, and thus the only I-E^{+} cells transferred into the hosts were B cells. There was limited division of $CD4^{+}Tg\alpha^{+}$ cells in both H-2^{bk} and H-2^{b} recipients, confirming earlier results (Figure 4.19) that B cells can present peptide to activate T cells *in vivo*. However, the level of cell division was not as great as that seen in H-2^{k} recipients (Figure 4.23A). This was not surprising in the case of H-2^{b} recipients, since the only I-E^{+} cells were the donor B cells. However, H-2^{bk} recipients possess an endogenous population of I-E^{+} cells, and thus a greater level of cell division was expected, approximating that seen in H-2^{k} recipients. The reasons for the low level of cell division observed in H-2^{bk} recipients will be considered in the discussion below.

4.10. Discussion

4.10.1. Intravenous immunisation of TCR transgenic mice.

Intravenous administration of antigen was first used by Dresser in 1962 to induce tolerance in adult animals. By the end of the decade, immunologists had defined the properties which conferred antigenicity and the routes of administration necessary to induce tolerance (reviewed by Dresser and Mitchison, 1968; Weigle, 1973). The intravenous route of immunisation has generally proven to be the most effective route for the induction of tolerance in adult animals, but antigen must be prepared in a form that is "non-immunogenic" or "weakly immunogenic" (Dresser and Mitchison, 1968). For example, aggregated human or bovine gamma-globulins induce immunity when administered intravenously, whereas deaggregated preparations are tolerogenic (Dresser, 1962). The use of adjuvants in the antigen preparation also enhances the immunogenicity of an antigen. Mitchison (1965) demonstrated the importance of antigen dose in the induction of tolerance, inducing tolerance with high doses of non-immunogenic antigens and low doses of weakly immunogenic antigens.

Despite the early characterisation of tolerogenicity of antigens, very little progress was made in understanding the cellular events that occurred during the induction of tolerance. Immunologists were forced to wait two decades before the technology was available to visualise tolerance induction at the cellular level *in vivo*. Tolerance induction was originally defined in functional terms as "paralysis", since the normal responsiveness of the host to antigen in adjuvant was reduced or abolished by the tolerogenic protocol. Thus, the misconception arose that immunity was produced by an active response of the immune system and tolerance was the product of a non-response. It was therefore something of a surprise when Webb et al. (1990) showed that peripheral tolerance to superantigen was the consequence of a brief but vigorous T cell response. However, instead of viewing such a response as the initial phase of classical immunological tolerance, Webb et al. suggested that deletion eventuated only because

the precursor frequency of superantigen-reactive cells was unusually high. The results presented in this chapter confirm the observations of Webb and others, extending current understanding of the cellular events that occur during the induction of peripheral T cell tolerance while suggesting an alternative explanation to that previously hypothesised in the literature.

Intravenous immunisation of TCR transgenic mice with peptide induced a reproducible sequence of antigen-specific changes in the total number of $CD4^{+}Tg\alpha^{+}$ T cells. There was an early disappearance of 50% of $CD4^{+}Tg\alpha^{+}$ cells from the spleen and lymph nodes seen most clearly 24 hours after immunisation (Figure 4.1). This was not an artefact due to downregulation of surface receptors such as CD4 and TCR used to identify these cells. Tracking $CD4^{+}Tg\alpha^{+}$ cells with CFSE suggested that at least some of these cells were sequestered rather than deleted since almost 100% of total CFSE could be detected at three days by calculating the amount within the divided and undivided populations. $CD4^{+}Tg\alpha^{+}$ cells expanded two-fold over baseline by day three, and then rapidly disappeared from the periphery, resulting in a 50% reduction below baseline 7-10 days after immunisation (Figure 4.1). The kinetics of the intravenous response observed here closely resembled those seen in normal mice following superantigen administration (Webb et al., 1990; Kawabe and Ochi, 1991; MacDonald et al., 1991; Miethke et al., 1994) with an early phase of cell loss on day one, a phase of expansion peaking on day three, and a late phase of irreversible deletion with a net loss of T cells and/or the onset of T cell anergy after day seven. Kyburz et al. (1993a) characterised the same response in TCR transgenic mice after immunisation with a class I-restricted peptide, demonstrating for the first time that such a response was not peculiar to superantigens.

Experiments with superantigens and TCR transgenic mice have been criticised for the unphysiologically high frequency of antigen-specific T cells. Kearney et al. (1994) addressed these concerns by using class II-restricted TCR transgenic cells in adoptive transfer, such that the frequency of antigen-specific T cells in the periphery of the

recipients was reduced by 20-50-fold. Intravenous immunisation of recipients with peptide induced a phase of expansion that peaked on day three, and was followed by deletion. The remaining cells were 'hyporesponsive' to restimulation at day 17. The authors concluded that this indicated anergy. However their data is entirely consistent with ours, which clearly shows that proliferation per cell (Figure 4.3) is unaffected by prior intravenous peptide. Had Kearney et al. taken into account the exponential nature of proliferative responses, rather than assuming them to be linear, their conclusions would most likely have agreed with ours.

Intravenous immunisation of syngeneic non-transgenic recipients of -D TCR transgenic cells induced a response with kinetics identical to those seen in intact TCR transgenic mice (compare Figures 4.1 and 4.9 and see later). The only difference observed was a greater degree of expansion and subsequent deletion in mice with fewer precursors. Further studies using this model have shown that the number of cells which divide in transgenic versus non-transgenic hosts of transgenic T cells is 2-3-fold lower, and the average number of divisions per precursor is one rather than four, accounting for the difference in peak cell number. Since only divided cells are sensitive to deletion, less deletion is seen in transgenic versus non-transgenic recipients (A.L. Smith and B. Fazekas de St. Groth, personal communication). Thus although there are quantitative differences related to precursor frequency, induction of deletion rather than memory is independent of precursor frequency, suggesting that peripheral deletion is a physiological mechanism for regulating peripheral T cell responses. Moreover, the extent of deletion is not a direct function of the degree of proliferation, although only those cells that have proliferated are destined to die.

Intravenous immunisation rapidly induced a series of systemic antigen-specific T cell activation events. The majority of $CD4^{+}Tg\alpha^{+}$ cells in the spleen and lymph nodes upregulated surface expression of CD69 within two hours of immunisation (Figure 4.5). Subsequently, priming of cytokine production could be detected one day after immunisation. Cells restimulated with peptide *in vitro* produced large amounts of IL-3

and IFN-γ and proliferated significantly more than naive controls, although IL-4 was never detected in these experiments, in contrast to subcutaneous immunisation (see next Section 5, Figure 5.3). By day three, the peak of $CD4^+Tg\alpha^+$ T cell expansion *in vivo*, responses to *in vitro* restimulation had returned to baseline, indicating that T cell activation was short-lived. This data is consistent with the very early appearance of T-cell-derived cytokines in the spleens of mice immunised with superantigens (Bette et al., 1993).

The early loss of $CD4^+Tg\alpha^+$ cells on day one was puzzling. The decline in $CD69^+$ $CD4^+Tg\alpha^+$ cells at 24 hours may indicate that at least some activated cells were involved, since cell numbers had not declined at two hours when >50% of cells were $CD69^+$. On the other hand, CD69 expression is reversible in the face of continued stimulation and some cells may already have downregulated CD69 by 24 hours. Miethke et al. (1994) have previously characterised the early loss of T cells following subcutaneous immunisation of normal mice with the superantigen staphylococcal enterotoxin B (SEB). They observed a decline in the percentage of SEB-reactive $V\beta8^+$ T cells within 20 hours of immunisation and found that it was preceded by downregulation of TCR expression, in contrast to -D TCR transgenic mice in which no significant TCR downregulation was seen. They went on to show that TCR downregulation was reversible *in vitro* and that a proportion of $V\beta8^-$ cells underwent apoptosis *in vivo*, whilst the remainder presumably re-expressed their TCR (Miethke et al., 1994). Further work is required to examine the phenotype of $CD4^+Tg\alpha^+$ cells and their fate during the first 24 hours of intravenous immunisation, but the observations of Miethke et al. offer an intriguing insight into the cellular events that may be occurring during the early phase of deletion.

CD44 expression was slowly upregulated on $CD4^+Tg\alpha^+$ cells after intravenous immunisation. By day three the majority of responding cells expressed medium levels of CD44 and a substantial increase in the proportion of $CD44^{hi}$ cells was achieved only by day seven (Figure 4.6). There was an early increase in the percentage but not the

total number of $CD44^{hi}$ cells on day one, probably due to the disappearance of $CD44^{lo}$ $CD4^+Tg\alpha^+$ cells. The total number of $CD4^+Tg\alpha^+CD44^{hi}$ cells peaked between days 3-7, and then slowly declined to reach baseline by day 41 (Figure 4.5). Comparison of the number of $CD4^+Tg\alpha^+CD44^{hi}$ and $CD4^+Tg\alpha^+CD44^{lo}$ cells over the course of the experiment showed that the number of $CD44^{lo}$ cells did not increase as $CD44^{hi}$ cells were disappearing, suggesting that $CD44^{hi}$ cells did not revert to $CD44^{lo}$ status at a high rate (Figure 4.5D-E). However, the number of $CD4^+Tg\alpha^+CD44^{lo}$ cells could not be calculated accurately early in the response because of the difficulties in gating as the level of CD44 increased (Figure 4.6). Therefore a low level of CD44 reversion cannot be ruled out, as has been suggested previously by Tough and Sprent (1994). More recent studies have indicated that all cells upregulate CD44 before division, although the level per cell continues to increase during the first four rounds of cell division (A.L. Smith and B. Fazekas de St. Groth, personal communication). Moreover, the number of $CD4^+Tg\alpha^+CD44^{lo}$ undivided cells remained steady between days 7-14, all the cell loss occurring within the $CD44^{hi}$ compartment, as indicated in Figure 4.5D-E. It is not clear from these experiments whether $CD4^+Tg\alpha^+CD44^{hi}$ cells are physically deleted or sequestered in non-lymphoid organs, although clearly they do not impart a state of memory to the animal (see below). The possibility exists that all $CD4^+Tg\alpha^+CD44^{hi}$ cells generated during the intravenous response were deleted by six weeks, leaving the original population that had not participated in the peptide response because their level of $Tg\alpha$ expression was insufficient. Comparison of $Tg\alpha$ expression on $CD44^{hi}$ cells before immunisation and six weeks later showed that there was no clearly defined $Tg\alpha^+$ population at either time (Figure 4.17), in contrast to the population induced by subcutaneous immunisation (see next section). Further experiments are required using cell sorting to determine the responsiveness of $CD4^+Tg\alpha^+CD44^{hi}$ cells *in vitro* and *in vivo* six weeks after intravenous immunisation.

There was a strong correlation between induction of T cell activation and subsequent deletion. When a range of peptide doses was administered to -D TCR transgenic mice,

T cell deletion occurred only at doses that induced T cell activation (Figure 4.7). CFSE studies of cell division *in vivo* reinforced the correlation between T cell activation and deletion, since cells that had undergone division were the only ones to be deleted (Figure 4.9). Moreover, the cell division number profile was similar on days three and ten, suggesting that any number of cell divisions was sufficient to target a T cell for deletion. More recent experiments (A.L. Smith and B. Fazekas de St. Groth, personal communication) using a Ly5 adoptive transfer model have shown that cell division stops on day three and no cells divide more than six times in response to intravenous antigen.

The results from the CFSE studies in adoptively transferred mice indicated that not all divided $CD4^{+}Tg\alpha^{+}$ cells were deleted from the periphery. Although 95% of the divided $CD4^{+}Tg\alpha^{+}$ cells present on day three had disappeared by day ten, divided cells still represented 80% of the remaining $CD4^{+}Tg\alpha^{+}$ cells (Figure 4.9). At later time points (A.L. Smith and B. Fazekas de St. Groth, personal communication) up to 40% of remaining $CD4^{+}Tg\alpha^{+}$ cells had undergone division. BrdU labelling studies in intact -D TCR transgenic mice also showed that 40% of $CD4^{+}Tg\alpha^{+}$ cells remaining six weeks after intravenous immunisation had incorporated BrdU (Figure 4.10), although these results are not directly comparable, since the CFSE studies were performed in adoptive transfer, in which a higher percentage of antigen-specific precursors undergo division (see above). Regardless, they indicate that a significant proportion of $CD4^{+}Tg\alpha^{+}$ cells remaining in the periphery after day ten had undergone cell division during the intravenous response. Furthermore, this proportion appeared to decrease with time after immunisation, consistent with the continual loss of $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells after day seven (Figure 4.5D). When taken together with the constant number of $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells at the start and finish of the response, and the very low $Tg\alpha$ expression in this population, a paradox emerges. As mentioned earlier, CFSE experiments performed by A.L. Smith and B. Fazekas de St. Groth (personal communication) showed that all cells upregulate CD44 before division and 40% of the

$CD4^+Tg\alpha^+$ population remaining at late timepoints after intravenous immunisation had divided. Moreover, the BrdU experiment showed 40% of $CD4^+Tg\alpha^+$ cells had incorporated BrdU (Figure 4.10), presumably as a consequence of cell division. Thus both the CFSE and BrdU data indicate that the divided cells are contained within the $CD44^{lo}$ fraction. One possibility is that BrdU may be incorporated by cells that prepare for cell division but do not divide. This question could be answered by double labelling of cells with BrdU and CFSE. Alternatively, 5-colour flow cytometry with simultaneous detection of CFSE, CD4, Tgα, Ly5.1 and CD44 will allow this issue to be resolved in the adoptive transfer model.

The use of CFSE promises to be a powerful tool in visualising cellular responses *in vivo* . Future experiments in the adoptive transfer model with earlier timepoints and the inclusion of non-lymphoid tissues in the analyses would allow a detailed examination of the fate of cells that disappear from the periphery (both early and late phases) to determine if they die by apoptosis or migrate elsewhere. The early work of Sprent and Miller (Sprent, 1976; Sprent and Miller, 1976a; Sprent and Miller, 1976b) demonstrated that activated T cells migrated to the gut and entered the lumen to be excreted. Cell sorting would allow the fate of specific subpopulations, such as cells at each division, to be assessed *in vitro* and *in vivo*.

Re-challenge of intravenously immunised -D TCR transgenic mice allowed the function of the $CD4^+Tg\alpha^+$ cells that had been activated but not deleted by the first dose of peptide to be assessed. Spleen and lymph node cells harvested six weeks after intravenous immunisation and restimulated with peptide *in vitro* made equivalent responses to naive cells when measured on a per cell basis. At the population level the deficit in $CD4^+Tg\alpha^+$ cells reduced the responsiveness in comparison to naive mice (Figure 4.11A). A second intravenous dose of peptide, administered six weeks after the first, induced some CD69 expression on $CD4^+Tg\alpha^+$ cells *in vivo*, but both *in vitro* IFN-γ production and *in vivo* clonal expansion were reduced with respect to naive mice (Figure 4.11B-C) in this experiment. These results suggested that $CD4^+Tg\alpha^+$ cells that

had been activated by intravenous peptide, but not deleted, were hyporesponsive to restimulation although the effect was somewhat variable after a single dose of peptide (compare Figures 4.11 and 4.14). Whether hyporesponsiveness was due to selection for low avidity, or induction of a distinct functional phenotype, remains unclear. However, more than half of the $CD4^+Tg\alpha^+$ cells remaining in intravenously immunised -D TCR transgenic mice could be defined as naive, since the BrdU study discussed above showed that they had never incorporated BrdU. Therefore it was important to administer multiple doses of peptide to activate all $CD4^+Tg\alpha^+$ cells at least once.

Weekly administration of peptide to -D TCR transgenic mice failed to delete all $CD4^+Tg\alpha^+$ cells from the periphery (Figure 4.12). Each dose was progressively less efficient at inducing deletion, and the residual population of $CD4^+Tg\alpha^+$ cells were refractory to peptide administration. These cells could be defined as anergic since they responded to peptide *in vitro* by proliferating weakly and producing only small amounts of IL-2 (Figure 4.12). Anergic T cells have previously been characterised in other systems *in vitro* as having a defect in IL-2 production (Lamb and Feldmann, 1984; Jenkins and Schwartz, 1987) such that TCR signals fail to induce IL-2 transcription via AP-1. In contrast, anergic T cells produced *in vivo* by superantigen administration were refractory to stimulation even in the presence of IL-2 (Rammensee et al., 1989; Kawabe and Ochi, 1990; Rellahan et al., 1990). In our model, deletion-resistant $CD4^+Tg\alpha^+$ cells could be induced to express CD69 by intravenous or subcutaneous peptide administration, but proliferation and cytokine production were not augmented over unchallenged controls (Figure 4.14). Deletion-resistant $CD4^+Tg\alpha^+$ cells exhibited 2-3-fold lower CD4 and TCR levels than naive cells, but only 10% were $CD44^{hi}$ (compared to 3% in the PBS control) and there was no change in the total number of $CD4^+Tg\alpha^+CD44^{hi}$ cells (Figure 4.13). A much higher proportion of $CD44^{hi}$ cells would be expected if all the residual $CD4^+Tg\alpha^+$ cells had been activated by peptide at least once, unless all activated cells reverted to $CD44^{lo}$ status. Thus the possibility

remains that deletion-resistant $CD4^{+}Tg\alpha^{+}$ cells were poorly activated, or not all, by multiple doses of peptide. Deletion-resistant $CD44^{hi}$ cells were also characterised by low levels of Tgα expression (Figure 4.13B), suggesting that they co-expressed endogenous TCR α chains and acquired their memory phenotype from previous encounter with environmental antigens. It is currently unclear whether this subset of cells responds to peptide *in vivo*. Regardless, cells with low TCR levels would manifest an "anergic" phenotype at the level of IL-2 production and proliferation *in vitro*. The observation that $CD44^{hi}$ cells from intravenously immunised mice express only low level of Tgα is significant since a large proportion of $CD44^{hi}$ cells with demonstrated memory function from subcutaneously immunised express high levels of Tgα (see Section 5). Further experiments with cell sorting are required to determine the responsiveness of deletion-resistant $CD44^{hi}$ and $CD44^{lo}$ cells *in vitro* and *in vivo*.

There was no evidence that deletion-resistant T cells persisting after multiple doses of peptide were undergoing a slow death since their numbers remained stable for weeks. In contrast, anergic B cells generated by low avidity interactions with antigen, are destined to die in a few days in the absence of T cell help (Fulcher et al., 1996). High avidity interaction with antigen causes B cells to be deleted from the periphery within 24 hours (Hartley et al., 1993). It would be of interest to determine the lifespan of deletion-resistant T cells in comparison with naive T cells and cells undergoing active peripheral deletion. Yet another possibility is that this deletion-resistant phenotype results specifically from intraperitoneal immunisation. The experiments with multiple doses of peptide employed the intraperitoneal route for convenience. While a intraperitoneal dose of peptide appeared to induce peripheral deletion in the same manner as the intravenous route (Figure 4.8), the effects of intraperitoneal peptide have yet to be characterised in the same detail as intravenous peptide. It is possible that some intraperitoneal peptide is delivered to T cells in a manner which mimics mucosal tolerisation, since intranasal and intraperitoneal peptide are both known to induce bystander suppression of Th1 responses (D. Wraith, personal communication).

The results presented in this chapter demonstrate that intravenous and intraperitoneal peptide induced peripheral tolerance in -D TCR transgenic mice through two mechanisms: deletion and anergy. The relationship between T cell anergy and deletion has yet to be elucidated, but antigen dose, TCR avidity and/or APCs are likely to play a role in directing the fate of peripheral T cells in tolerance. T cell deletion and anergy have also been observed during negative selection in the thymus, and studies with superantigens and transgenic TCR models have suggested that the fate of immature T cells is determined by the amount of antigen present, such that low doses induce anergy and high doses induce deletion (Ramsdell and Fowlkes, 1990; Fazekas de St. Groth et al., 1992). Thus, TCR avidity and T cell costimulation by APCs could play a role in determining the balance of anergy versus deletion. Further work is required to understand the molecular mechanisms that regulate the induction of anergy and deletion in this model.

Intravenous administration of intact cytochrome C failed to induce peripheral deletion in -D TCR transgenic mice. Nonetheless, there was evidence of significant T cell activation in the spleen one day after immunisation, as indicated by elevation of CD69 expression by $CD4^{+}Tg\alpha^{+}$ cells *in vivo* and priming of IFN-γ production *in vitro* (Figure 4.15A-B) Thus, cytochrome C was efficiently processed for presentation. However, the long-term consequences of T cell activation by intravenous cytochrome C were unclear, since the number of $CD4^{+}Tg\alpha^{+}$ cells and the proportion expressing high levels of CD44 were unchanged or slightly increased by the procedure (Figures 4.15 and 4.16). However, the increase in the proportion of $Tg\alpha^{hi}$ cells within the $CD44^{hi}$ population (Figure 4.17) suggested some cytochrome-specific memory T cells were generated, as was observed following subcutaneous immunisation (Section 5). Further experiments are required to explore this possibility, along with dose response studies to determine the efficiency of T cell activation by intact cytochrome C. Despite these limitations, the results suggest that intravenous administration of intact antigen does not

induce peripheral tolerance via deletion, and therefore, that antigen processing and APC function are important determinants of the decision between tolerance and immunity.

4.10.2. Assessment of APC that present intravenous peptide.

It has long been known that purified B cells are able to activate T cells *in vitro* (Ashwell et al., 1984), but in the absence of surface immunoglobulin of appropriate specificity, they require at least ten-fold more antigen per cell than dendritic cells to achieve the same level of T cell activation (Croft et al., 1992). When B cells utilise surface immunoglobulin to concentrate antigen, a 10,000-fold reduction in the dose required to stimulate T proliferation is seen (Lanzavecchia, 1985; Ho et al., 1994), giving B cells great potential as APC *in vivo*. However, several *in vivo* experiments have indicated that all B cells induce tolerance to antigen when they are the sole APC (Lassila et al., 1988; Fuchs and Matzinger, 1992; Ronchese and Hausmann, 1993). There is some controversy about whether antigen-activated B cells are tolerogenic; some evidence suggests that they induce immunity instead (Eynon and Parker, 1992; Finkelman et al., 1992). In contrast, dendritic cells are well-recognised as potent inducers of immunity *in vivo* (Britz et al., 1982; Boog et al., 1985; Sornasse et al., 1992) and this may be related to the constitutive expression of costimulatory molecules such as the B7 family members (reviewed by Bluestone, 1995). However activated B cells also express high levels of costimulatory molecules.

Two adoptive transfer models were developed to test the APC function of different cell types *in vivo*. The advantage of -D x 36-2 double transgenic mice was that they possess a large endogenous population of $CD4^{+}Tg\alpha^{+}$ cells in the periphery in the absence of I-E^{+} APC. Intravenous immunisation of -D x 36-2 double transgenic mice confirmed that endogenous APC could not activate $CD4^{+}Tg\alpha^{+}$ cells (Figure 4.18). Adoptive transfer of unfractionated I-E^{+} spleen cells and subsequent intravenous immunisation showed that exogenous APC, whether they were unfractionated spleen cells or purified B cells, were able to capture and present peptide to stimulate CD69 expression on $CD4^{+}Tg\alpha^{+}$

cells *in vivo* (Figure 4.19). However, the proportion of activated CD4$^+$Tgα^+ cells was always lower than that observed in intact -D TCR transgenic mice (Figure 4.5A) and was often localised to the spleen, regardless of the APC population or the protocol used (Figure 4.20). Preferential APC homing to the spleen may have accounted for localisation of T cell activation. The extent of T cell activation may have been limited by the number of I-E$^+$ cells successfully transferred to recipients, since only a small percentage of I-E$^+$ cells was detected in APC recipients. Alternatively, the adoptively transferred APC population may have contained only poor stimulators of naive T cells An additional problem with this experimental model was the extent of positive selection of the transgenic TCR. The 36-2 I-E transgene induces only low levels of positive selection of the -D transgenic TCR, due to poor expression on thymic epithelium, and the majority of selected cells have low levels of Tgα, expressed together with the endogenous receptor responsible for their positive selection. The average avidity of I-E/cytochrome peptide/transgenic TCR interactions in these mice is therefore low. These results suggest that the best way to study the function of APCs *in vivo* is to employ a system in which APCs are in excess and the T cells are purified from mice that express high levels of I-E in the thymus, such as -D x 107-1 double transgenic mice.

The second adoptive transfer system involved transfer of both CD4$^+$Tgα^+ cells and I-E$^+$ APCs into immunodeficient mice and promised to be a useful system to study the roles of different APCs *in vivo*. Intravenous immunisation of *scid/scid* recipients after adoptive transfer of T cells and APCs from TCR transgenic mice induced a response that was comparable in intensity with that in intact -D transgenic TCR mice. A large increase in CD69 expression, T cell priming to produce cytokines *in vitro* and a drop in the number of CD4$^+$Tgα^+ cells were detected in both lymph nodes and spleen on day one, followed by a four-fold expansion of CD4$^+$Tgα^+ cells by day three (Figure 4.21A-D). Subsequently, the number of CD4$^+$Tgα^+ cells returned to baseline. However, no net deletion was seen (Figure 4.21E-F). Repeated peptide administration

induced neither deletion nor hyporesponsiveness of $CD4^{+}Tg\alpha^{+}$ cells (Figure 4.22). Thus, peripheral tolerance could not be induced in *scid/scid* mice, and experiments in homozygous Rag-1-deficient recipients suggested that it was a characteristic of all immunodeficient hosts (Figure 4.23). Subsequent experiments by A.L. Smith and B. Fazekas de St. Groth (personal communication) in intact -D TCR transgenic mice on a homozygous Rag-1-deficient background have confirmed that deletion of $CD4^{+}Tg\alpha^{+}$ cells is not seen seven days after intravenous administration of peptide. Williams et al. (1994) have previously reported that SEB administration failed to induce peripheral deletion or anergy in T cell-reconstituted nude mice, and thus a defect in peripheral tolerance may be a common feature of T cell-deficient hosts. This may be due either to overriding control of cell number independent of specific antigen activation, or absence of a tolerogenic (T-dependent) APC population in T cell-deficient hosts.

The CFSE studies in homozygous Rag-1-deficient mice were performed in hosts with different MHC haplotypes in an additional attempt to measure the presenting capacity of different APC populations. Adherent-depleted -D cells were transferred into $H\text{-}2^{bk}$ or $H\text{-}2^{b}$ recipients to assess the presenting capacity of B cells. In $H\text{-}2^{b}$ recipients, donor B cells were able to stimulate $CD4^{+}Tg\alpha^{+}$ cells in the absence of endogenous $I\text{-}E^{+}$ APC (Figure 4.23C). However, the level of proliferation was only a fraction of that seen when unfractionated -D cells were transferred into $H\text{-}2^{k}$ Rag-1-deficient recipients (Figure 4.23A). The majority of $CD4^{+}Tg\alpha^{+}$ cells divided 3-5 times in $H\text{-}2^{k}$ Rag-1-deficient mice, and the division profile was identical to that seen in previous experiments in B10.BR mice (compare Figure 4.23A with Figure 4.9B). Surprisingly, there was no difference between the level of division seen in $H\text{-}2^{bk}$ versus $H\text{-}2^{b}$ Rag-1-deficient mice, suggesting that endogenous $I\text{-}E^{+}$ APC in $H\text{-}2^{bk}$ hosts did not supplement peptide presentation to T cells. It is possible that the level of proliferation seen in $H\text{-}2^{bk}$ and $H\text{-}2^{b}$ Rag-1-deficient mice was limited by the level of I-E expressed on donor and/or recipient APC, since both groups of recipients received cells from MHC heterozygous rather the homozygous donors used in the transfer into $H\text{-}2^{k}$ Rag-

1-deficient hosts. Alternatively, T cell proliferation in H-2^b and H-2^{bk} hosts may have been induced only by donor APC, implying that Rag-1-deficient mice were deficient in dendritic cells or other adherent APC. Further experiments are required to examine these possibilities.

While the usefulness of the adoptive transfer experiments in *scid/scid* or homozygous Rag-1-deficient mice were limited by defective peripheral tolerance induction, they still provide a valuable system to study T cell activation *in vivo*. Ronchese et al. (1994) previously employed *scid/scid* recipients of adoptively transferred purified T cells to show that T cells can be activated to produce IFN-γ and IL-4 after subcutaneous immunisation in the absence of B cells. This experimental protocol may elucidate mechanisms by which APCs modify T cell activation and its consequences.

Section 5. Characterisation of the Effects of Subcutaneous Administration of Peptide to TCR Transgenic Mice.

5.1. Antigen-specific T cells are not deleted from the periphery following subcutaneous immunisation.

The results presented in the previous section demonstrated that peripheral deletion was induced by intravenous immunisation of TCR transgenic mice with peptide. The kinetics of activation and deletion closely resembled those reported for superantigens (Webb et al., 1990; MacDonald et al., 1991; Miethke et al., 1994), and therefore it was important to determine whether peptide was also an obligatory tolerogen. Subcutaneous administration of antigen emulsified in adjuvant is a potent immunisation protocol for conventional antigens in normal mice, but induces tolerance to superantigen (Rellahan et al., 1990). Therefore, the tolerogenicity of peptide was tested by subcutaneous administration with adjuvant.

Thymectomised TCR transgenic mice were immunised subcutaneously with peptide 87-103 emulsified in complete Freund's adjuvant (CFA), while control thymectomised transgenic mice were immunised with PBS in CFA. As shown in Figure 5.1, subcutaneous immunisation of transgenic mice led to a ten-fold increase in the number of $CD4^+Tg\alpha^+$ cells in the draining lymph nodes seven days after immunisation. There was also a small increase in the number of $CD4^+Tg\alpha^-$ cells, not seen in the PBS/CFA control. No corresponding increases in cell number were seen in the lymph nodes distal to the site of immunisation (distant lymph nodes) or the spleen. The number of $CD4^+Tg\alpha^+$ cells in the draining lymph nodes slowly declined after day seven, reaching the control level by day 59. The baseline number of both $CD4^+Tg\alpha^+$ and $CD4^+Tg\alpha^-$ cells in all lymphoid organs in both test and control groups also declined, reflecting the effect of adult thymectomy. It is important to emphasise that there was no evidence of specific deletion of $CD4^+Tg\alpha^+$ cells following subcutaneous immunisation in at least four separate experiments, as summarised in Figure 5.2 in which cell numbers were

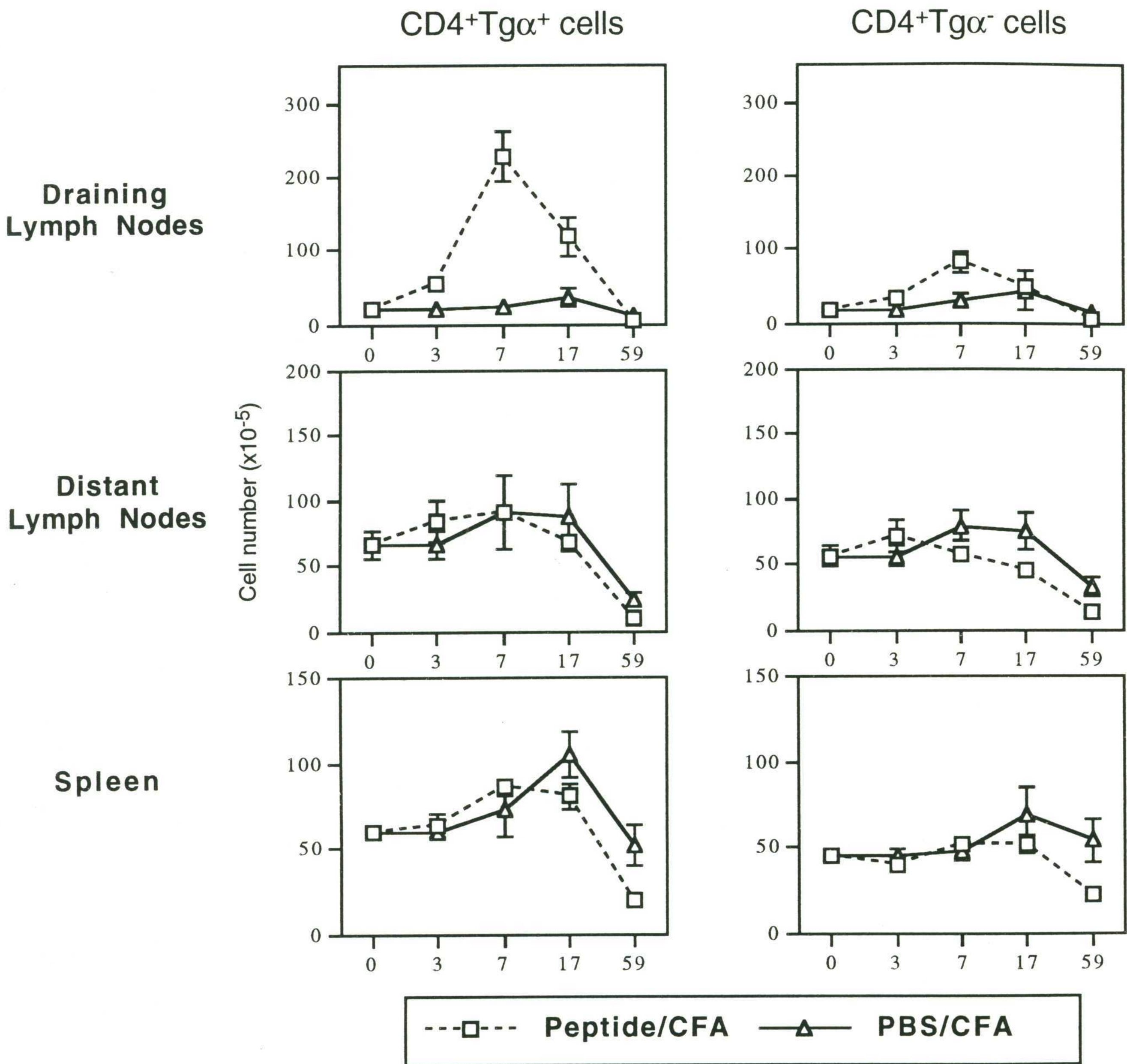


Figure 5.1. The number of CD4$^+$Tgα^+ cells in the periphery of -D TCR transgenic mice after subcutaneous immunisation. Adult thymectomised -D TCR transgenic mice were immunised subcutaneously with PBS or 15μg of peptide emulsified in CFA, as described in Section 3.2. The number of CD4$^+$Tgα^+ (left panels) and CD4$^+$Tgα^- cells (right panels) in the draining lymph nodes, distant lymph nodes and spleens of PBS- and peptide-treated mice was calculated from the percentages determined by flow cytometry. All points represent the average of 3-4 mice with error bars indicating SEM.

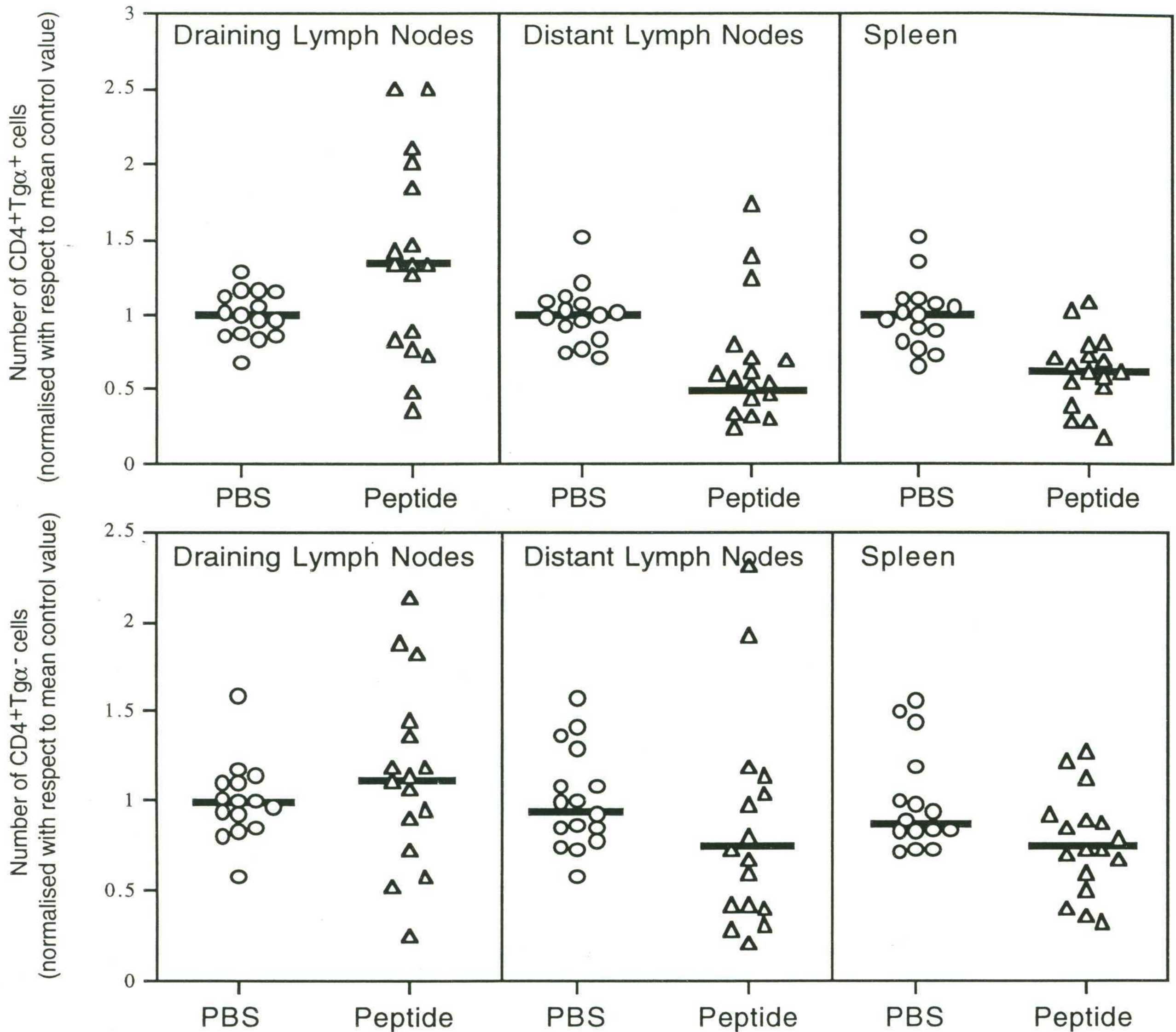


Figure 5.2. The number of CD4$^+$Tgα^+ cells in the periphery of -D TCR transgenic mice after subcutaneous immunisation. The number of CD4$^+$Tgα^+ (upper panel) and CD4$^+$Tgα^- (lower panel) cells in the lymph nodes and spleen six weeks after subcutaneous immunisation of thymectomised -D TCR transgenic mice is shown as pooled data from four experiments. Data is normalised within each experiment with respect to the mean number of cells present in the PBS control to remove variation in cell numbers due to lack of age- and sex-matching between the individual experiments. The mean value is represented by the bold bar.

normalised with respect to the PBS control for each experiment. The number of CD4$^+$Tgα^+ cells in the draining lymph nodes of the peptide-treated mice were generally increased by peptide immunisation, whereas the numbers in the distant lymph nodes and spleen were significantly decreased. These changes were mirrored by similar changes of smaller magnitude in the number of CD4$^+$Tgα^- cells, in contrast to intravenous immunisation (see Figure 4.2) in which CD4$^+$Tgα^- cell number was unchanged by peptide. The non-specific effects of subcutaneous peptide on T cell number may be related to a bystander effect of CD4$^+$Tgα^+ cell help on mycobacteria-responsive CD4$^+$Tgα^- cells. The numbers of CD4$^+$Tgα^+ and CD4$^+$Tgα^- cells in the draining lymph nodes of the PBS control group were tightly grouped about the mean, whereas the range of values for the peptide group was far wider, indicating that the response of each animal to subcutaneous immunisation with peptide was somewhat variable.

5.2. Antigen-specific T cells are activated in the periphery by subcutaneous immunisation of TCR transgenic mice.

The effect of subcutaneous peptide immunisation of TCR transgenic mice on T cell responsiveness to *in vitro* rechallenge was tested in the experiment shown in Figure 5.3. No consistent increase in proliferation per CD4$^+$Tgα^+ cell for the draining lymph nodes, distant lymph nodes and spleen was seen. However, draining lymph node cells produced large amounts of IFN-γ and IL-3 per cell and detectable amounts of IL-4 when restimulated *in vitro* three days after subcutaneous immunisation. There was no evidence of elevated cytokine production prior to day three (data not shown). At later timepoints, IFN-γ production by draining lymph node cells was only slightly increased over baseline levels and IL-4 production could not be detected. The amount of IFN-γ, but not IL-4, produced by distant lymph node and spleen cells was increased seven and 17 days after immunisation, though these increases were small in comparison to those observed in the draining lymph nodes. IL-4 was detected in cultures of spleen cells from both PBS- and peptide-treated mice, and may have been a non-specific effect of

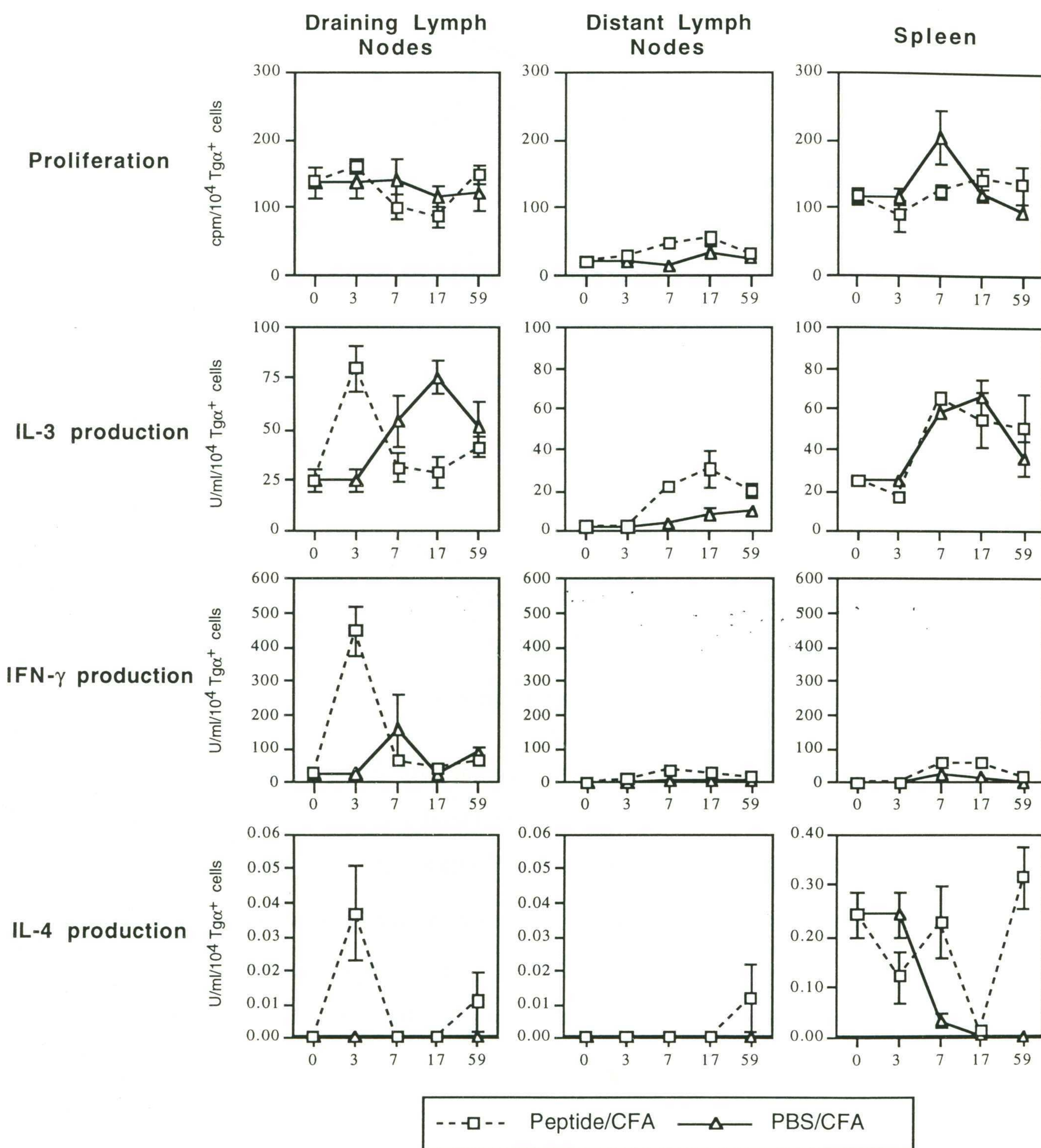


Figure 5.3. Activation of CD4$^+$Tgα^+ cells following subcutaneous immunisation. Adult thymectomised -D TCR transgenic mice were immunised subcutaneously with PBS or 15μg of peptide emulsified in CFA, as described in Section 3.2. Draining lymph nodes, distant lymph nodes and spleen cells were harvested from PBS- and peptide-treated mice at each timepoint shown and restimulated with peptide *in vitro*, as described in Section 3.3. Proliferation was measured as described in Section 3.3, and the amount of IL-3, IFN-γ and IL-4 produced during culture was measured as described in Section 3.4. Each point represents the average of 3-4 mice with error bars indicating SEM.

the adjuvant or may reflect production by NK1.1^{+} T cells. Although detectable amounts of IL-4 were produced by both draining and distant lymph node cells in two out of three peptide-treated mice 59 days after immunisation in this experiment, no IL-4 was seen at late time points when the experiment was repeated (data not shown). When the kinetics of cytokine production were compared with those of CD4^{+}Tgα^{+} cell expansion, it was clear that priming of T cell function preceded cell expansion in the draining lymph nodes by 2-3 days (Figure 5.4).

Up to 25% of CD4^{+}Tgα^{+} cells in the draining lymph nodes expressed CD69 one day after subcutaneous immunisation (Figure 5.5A). The percentage of CD69^{+} CD4^{+}Tgα^{+} cells continued to increase until day four, and then slowly declined, but was still elevated above the PBS/CFA control six weeks after immunisation. In the distant lymph nodes and spleen, there was a small increase in the percentage of CD69^{+} CD4^{+}Tgα^{+} cells on day one which was maintained for the duration of the experiment.

Subcutaneous immunisation also induced upregulation of CD44 expression by CD4^{+}Tgα^{+} cells in the draining lymph nodes, distant lymph nodes and spleen. The percentage and number of CD44^{hi} CD4^{+}Tgα^{+} cells rapidly increased in the draining lymph nodes after day one, reached a peak of 30-40 times background at day eight, then slowly declined. The percentage was still elevated about three-fold above the control on day 43 (Figure 5.5B) and represented a real increase in the number of CD44^{hi} cells in the draining lymph nodes (Figure 5.5C). Pooled data from four experiments showed that the percentage (and number) of CD44^{hi} CD4^{+}Tgα^{+} cells was consistently elevated in the draining lymph nodes six or more weeks after immunisation (Figure 5.6). There were smaller elevations in the percentage and number of CD44^{hi} CD4^{+}Tgα^{+} cells in the distant lymph nodes and spleen, following the same kinetics as the draining lymph nodes. Changes in the number of CD4^{+}Tgα^{+}CD44^{lo} cells mirrored changes in the total number of CD4^{+}Tgα^{+} cells (Figure 5.5D) as a result of the gating problems clearly demonstrated in Figure 5.7. However the failure of CD44lo cells to

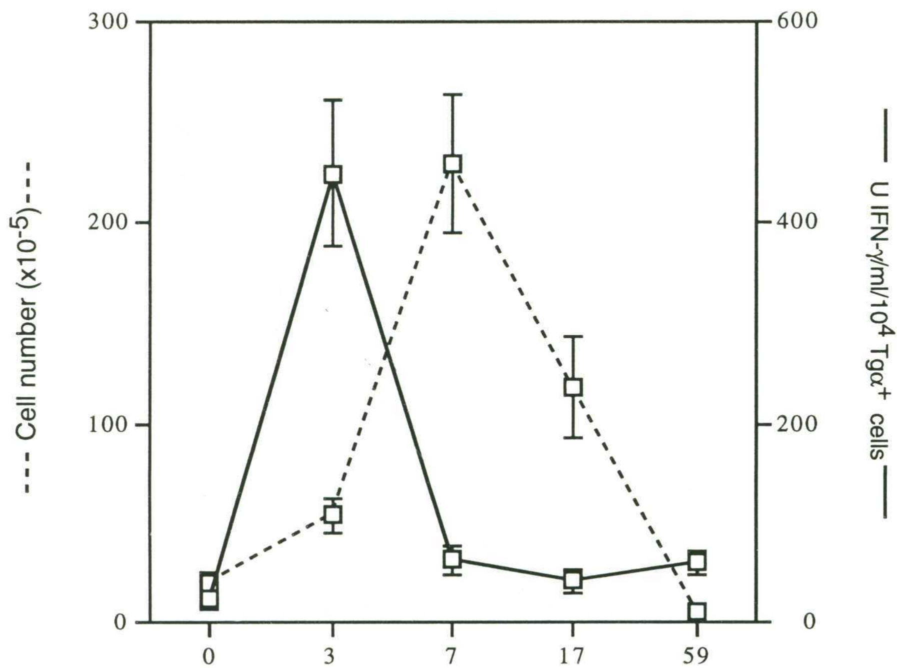


Figure 5.4. Relationship between T cell responses *in vitro* and *in vivo*. Draining lymph node cells from adult thymectomised -D TCR transgenic mice immunised subcutaneously with peptide exhibit an increase in IFN-γ production (bold line) upon restimulation *in vitro* which precedes the increase in the number of $CD4^{+}Tg\alpha^{+}$ cells (broken line) *in vivo*. A similar relationship was seen for IL-3 and IL-4 (data not shown). Each point represents the average of 3-4 mice with error bars indicating SEM.

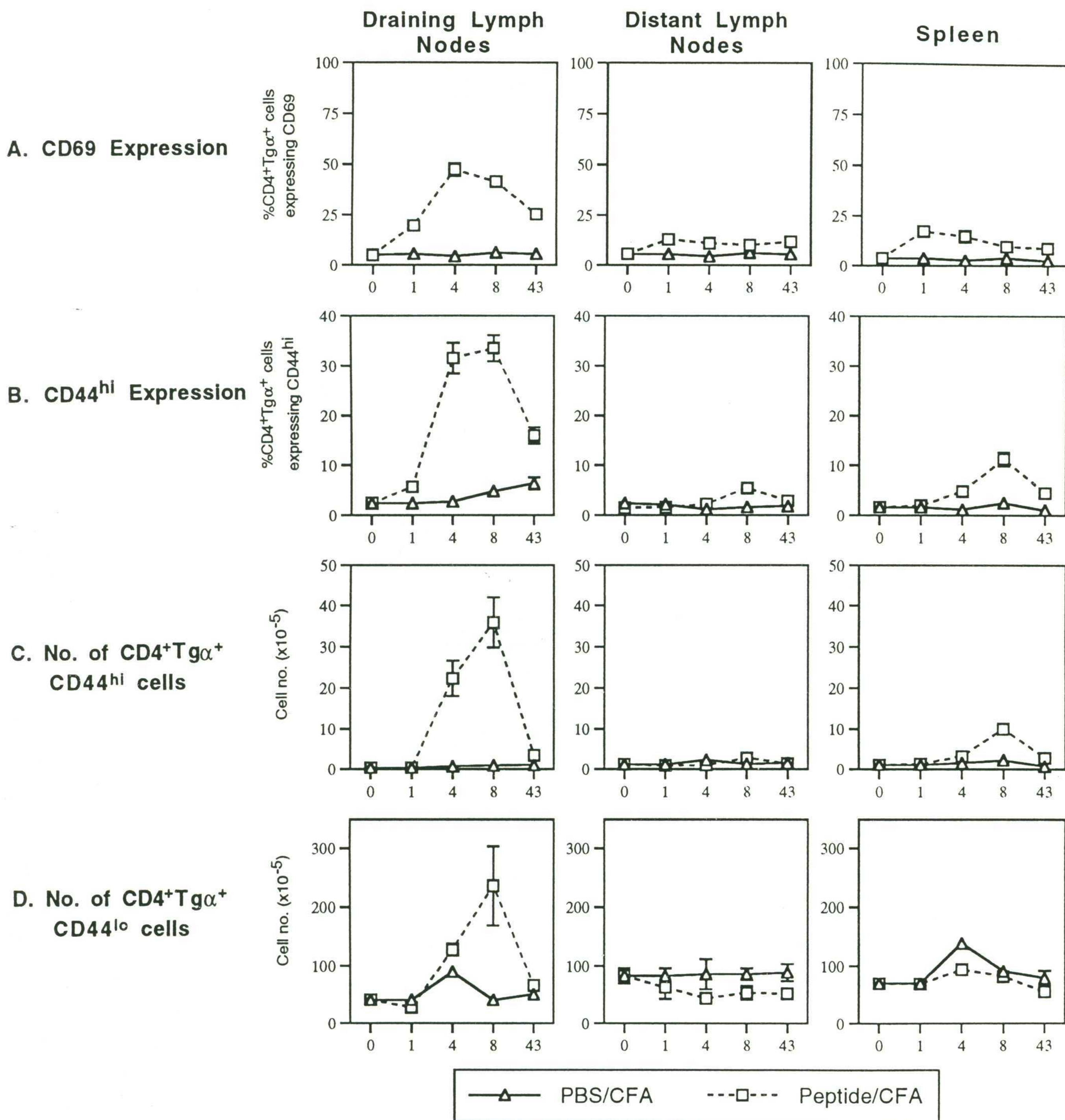


Figure 5.5. Expression of activation markers after subcutaneous immunisation. Adult thymectomised -D TCR transgenic mice were immunised subcutaneously with PBS or 1μg of peptide emulsified in CFA. Draining lymph nodes, distant lymph nodes and spleens were harvested from PBS- and peptide-treated mice at each timepoint shown and the expression of CD69 and CD44 was determined by immunostaining and flow cytometry. **A.** CD69 expression is shown as a percentage of total CD4+Tgα+ cells. **B.** $CD44^{hi}$ expression shown as a percentage of total CD4+Tgα+ cells. **C.** Total number of CD4+Tgα+$CD44^{hi}$ cells. **D.** Total number of CD4+Tgα+$CD44^{lo}$ cells.

Each point represents the average of 1-3 mice with error bars indicating SEM.

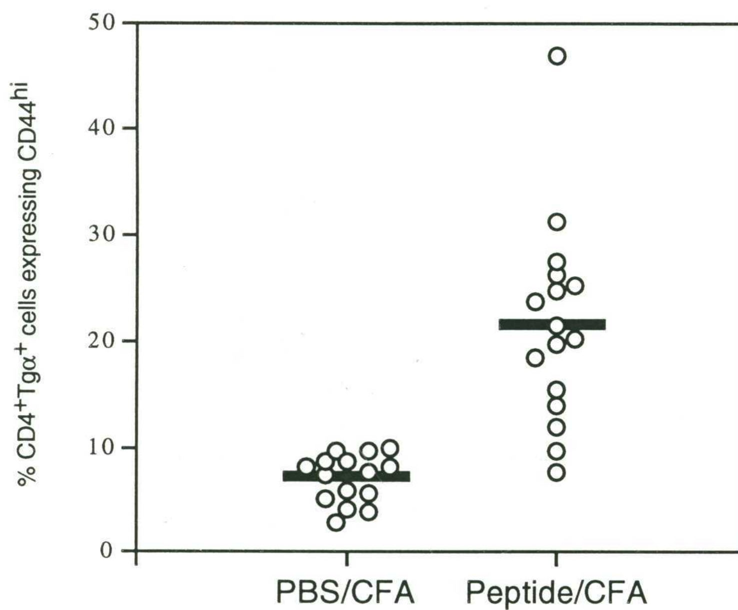


Figure 5.6. The percentage of $CD4^{+}Tg\alpha^{+}$ cells expressing a $CD44^{hi}$ phenotype is elevated six weeks after subcutaneous immunisation of -D TCR transgenic mice. Adult thymectomised -D TCR transgenic mice were subcutaneously immunised with PBS or 1.5-15μg peptide in CFA. Six weeks later, the percentage of $CD4^{+}Tg\alpha^{+}$ expressing a $CD44^{hi}$ phenotype in the draining lymph nodes was determined. Data was pooled from four separate experiments with the mean value represented by the bold bar.

increase at the expense of CD44hi at late stages of the response demonstrated that the decline in $CD44^{hi}$ cells was not due to reversion to a $CD44^{lo}$ phenotype.

The size of $CD44^{hi}$ cells distinguishes recently activated from memory T cells (see Section 1.4.4). Figure 5.7 shows the size of $CD4^{+}Tg\alpha^{+}CD44^{hi}$ and $CD4^{+}Tg\alpha^{+}CD44^{lo}$ cells in the draining lymph nodes during the course of the subcutaneous response. Both cell populations were a little larger than unactivated cells, consistent with the presence of a few activated $CD69^{+}$ cells which had not yet upregulated CD44. By day four, the size of $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells had increased substantially, whereas the remaining $CD4^{+}Tg\alpha^{+}CD44^{lo}$ cells had a resting size profile. After that time, the size of $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells declined such that the majority overlay the $CD4^{+}Tg\alpha^{+}CD44^{lo}$ cell profile by day 43. However, a proportion of large $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells remained, consistent with the minor elevation in the number of $CD69^{+}$ cells (Figure 5.5A). Similar size profiles were also seen for $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells in the distant lymph nodes and spleen on day 43 (data not shown). Thus, the population of $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells remaining six weeks after subcutaneous immunisation was made up of a minority of large cells and a majority of small resting memory cells.

5.3. Antigen-specific T cells primed by subcutaneous immunisation exhibit memory characteristics.

The net increase in the number of $CD4^{+}Tg\alpha^{+}$ cells with a $CD44^{hi}$ activated/memory phenotype following subcutaneous immunisation of transgenic mice was consistent with the generation of T cell memory. It has been demonstrated that memory T cells have a lower threshold for activation than naive T cells (Vitteta et al., 1991; Swain and Bradley, 1992; Croft et al., 1994), suggesting that memory T cells are more sensitive to small doses of antigen. To test whether the cells from subcutaneously primed animals exhibited enhanced responsiveness to antigen *in vitro*, draining lymph nodes were removed from adult thymectomised mice six weeks after subcutaneous immunisation

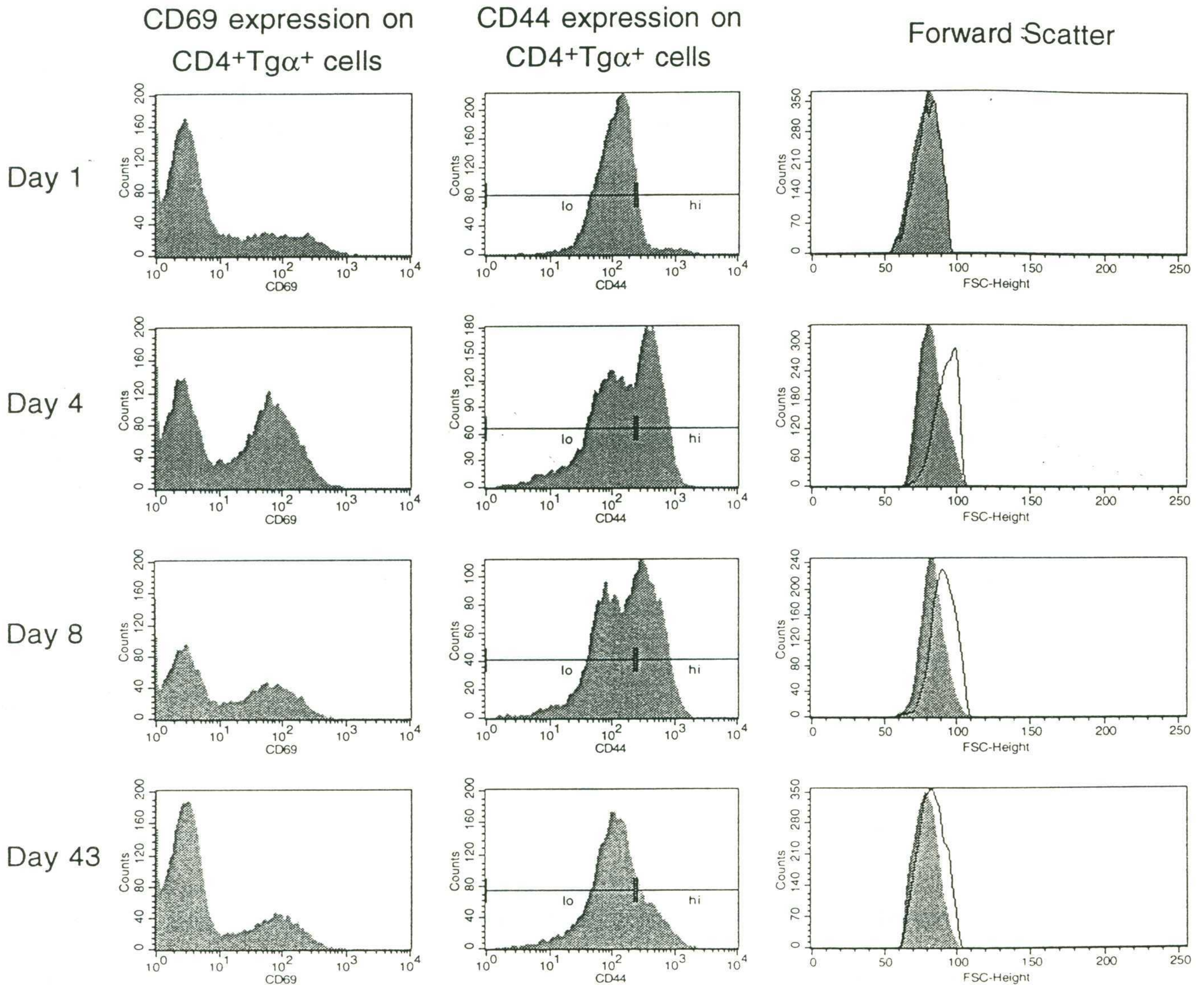


Figure 5.7. Size analysis of $CD4^+Tg\alpha^+CD44^{hi}$ and $CD4^+Tg\alpha^+CD44^{lo}$ cells in the draining lymph nodes following subcutaneous immunisation. Adult thymectomised -D TCR transgenic mice were immunised subcutaneously with 1μg peptide emulsified in CFA. Draining lymph nodes were harvested at each timepoint shown and the expression of CD44 and CD69 was determined by immunostaining and flow cytometry. Representative CD44 and CD69 profiles are shown for $CD4^+Tg\alpha^+$ in the centre and left panels, respectively. $CD44^{lo}$ and $CD44^{hi}$ cells were gated as shown for size analysis, and forward scatter profiles are shown in the right panels for $CD4^+Tg\alpha^+CD44^{lo}$ (shaded) and $CD4^+Tg\alpha^+CD44^{hi}$ cells (outline).

with PBS/CFA or peptide/CFA and proliferation was measured after re-stimulation with a range of peptide doses. The phenotype of the harvested cells was also determined.

Figure 5.8C shows that cells primed by subcutaneous immunisation exhibited a shift in the antigen dose-response curve. The frequency of $CD4^{+}Tg\alpha^{+}$ cells in the PBS-treated group ranged from 11.9-17.9% of total cells in culture, while in the peptide-treated group it ranged from 11.3-14.7% of total cells. Thus, the increased sensitivity of peptide-primed cells was a due to increased responsiveness per cell rather than an increased precursor frequency.

Figure 5.8A shows that $CD4^{+}Tg\alpha^{+}$ cells harvested six weeks after subcutaneous peptide immunisation expressed 2-fold lower levels of both CD4 and the α and β chains of the transgenic TCR when compared to cells from the PBS control, consistent with an activated or memory T helper cell phenotype (Hayakawa and Hardy, 1991). In addition, a significant $Tg\alpha^{+}$ peak was seen within the $CD44^{hi}$ population in both peptide- and PBS-treated mice (Figure 5.8B) in comparison to untreated mice (cf Figure 4.17). The size of the $CD44^{hi}Tg\alpha^{hi}$ population was clearly greater in the peptide/CFA group.

5.4. Subcutaneous administration of peptide without adjuvant increases the number of antigen-specific cells with a memory phenotype.

The results presented above demonstrated that subcutaneous administration of peptide in adjuvant, in contrast to intravenous administration, induces accumulation of hyper-responsive $CD44^{hi}$ antigen-specific cells. This phenomenon could result from the use of adjuvant, the subcutaneous route of administration or a combination of the two. In order to separate the effects of these factors, adult thymectomised -D TCR transgenic mice were immunised subcutaneously with 1μg peptide without adjuvant, and the numbers of $CD4^{+}Tg\alpha^{+}$ and $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells that were determined six weeks

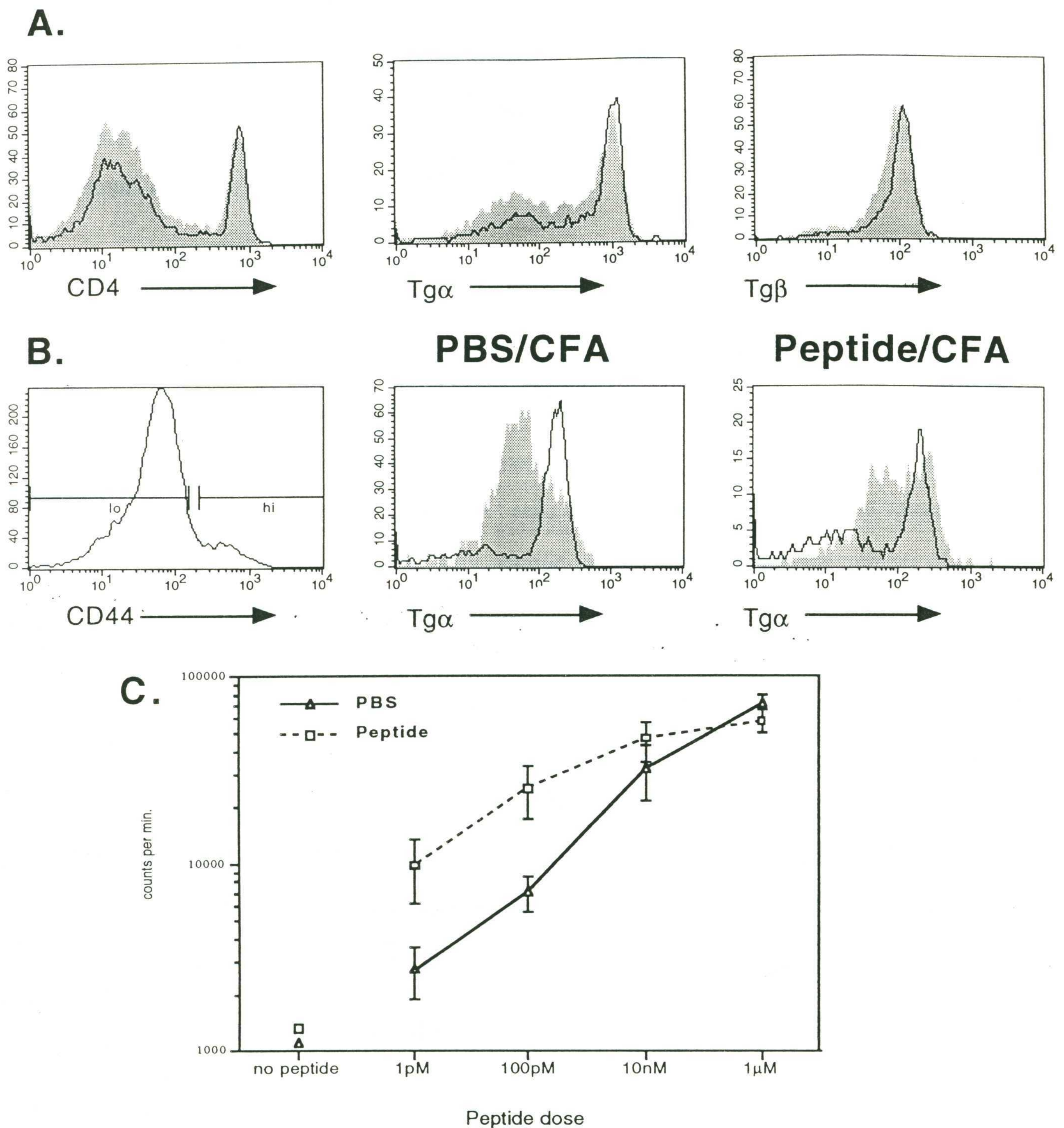


Figure 5.8. Phenotype and *in vitro* responsiveness of CD4$^+$Tgα$^+$ cells after subcutaneous immunisation. Adult thymectomised -D TCR transgenic mice were immunised subcutaneously with PBS or 15μg peptide emulsified in CFA and draining lymph node cells were harvested six weeks after immunisation **A.** The level of CD4 and TgTCR expression was determined by flow cytometry. Representative plots of CD4 expression on Tgα$^+$ cells (left panel) and the level of expression of Tgα and Tgβ on CD4$^+$ cells (middle and right panels, respectively) are shown as shaded plots for peptide-treated mice and in outline for the PBS control. **B.** The level of Tgα expression was determined on CD4$^+$CD44^{hi} and CD4$^+$CD44^{lo} cells. The gates used for CD44 expression are shown in the left panel. Representative plots of Tgα expression on CD4$^+$CD44^{hi} cells (shaded) and CD4$^+$CD44^{lo} cells (outline) are shown for mice immunised with PBS/CFA (middle panel) and peptide/CFA (right panel). The level of fluorescence in the Tgα$^-$CD44^{hi} population was higher than the Tgα$^-$CD44^{lo} population in this experiment because of incorrect compensation (see Figure 4.17 also) **C.** Harvested cells were restimulated with 1pM-1μM peptide *in vitro*. Proliferation was measured and expressed as counts/min./culture. Each point represents the average of three mice with error bars indicating SEM.

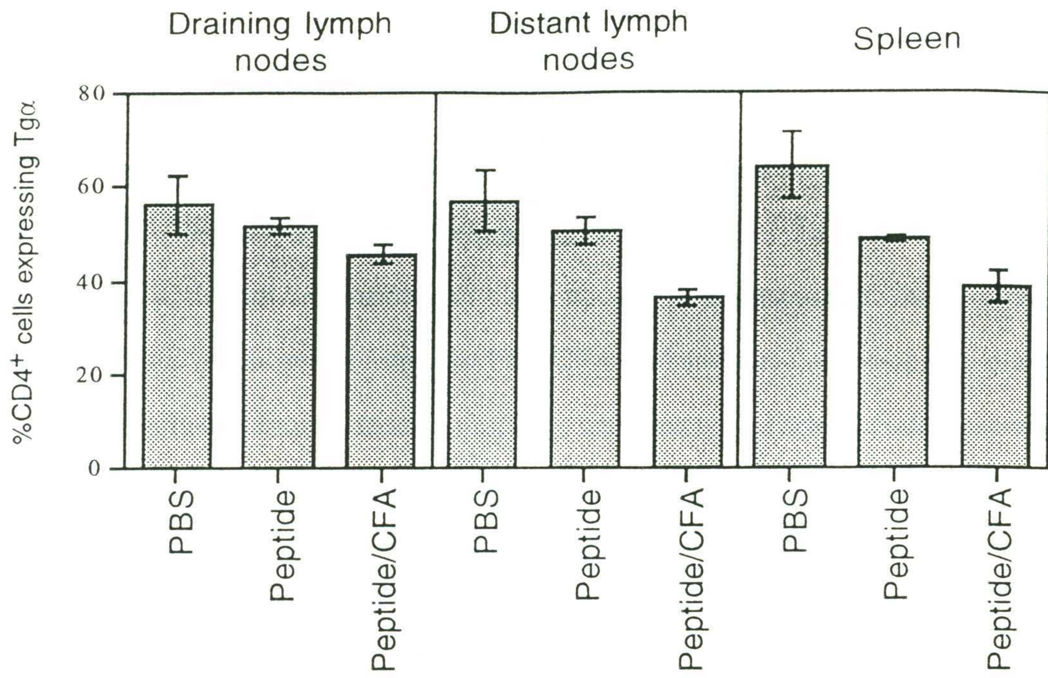
later. Control groups received either subcutaneous PBS or 1μg subcutaneous peptide emulsified in CFA.

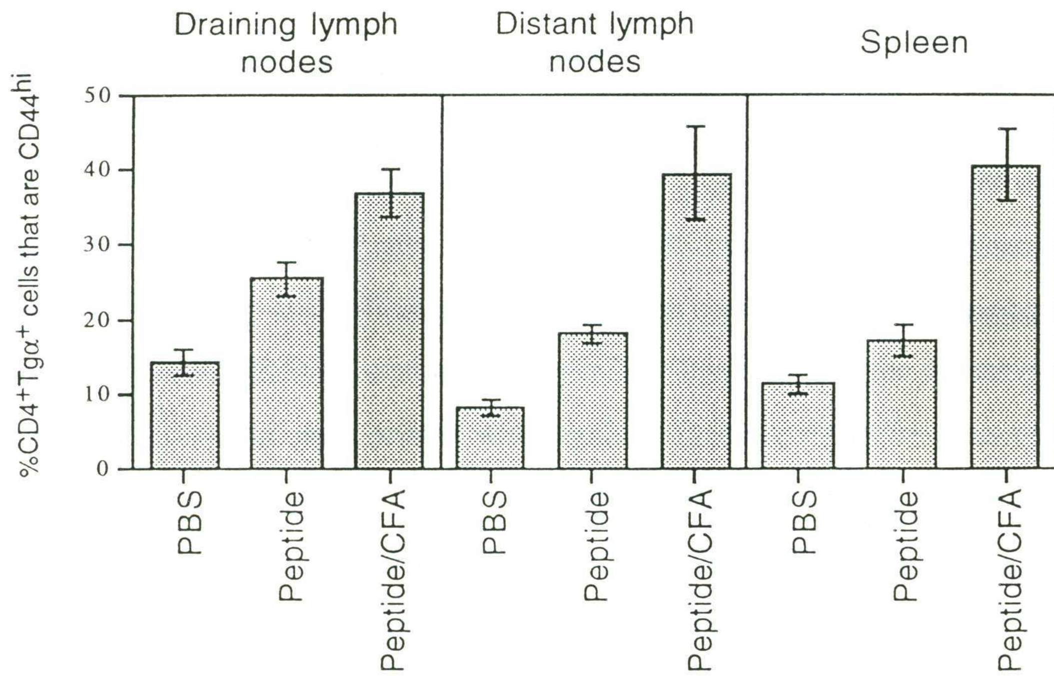
Figure 5.9A shows the percentage of $CD4^+$ cells expressing the transgenic TCR in the draining lymph nodes, distant lymph nodes, and spleen six weeks after subcutaneous immunisation. Administration of peptide without adjuvant did not induce significant peripheral deletion in lymph nodes compared with the PBS control although a 15% decrease was seen in the spleen. As shown previously (Figure 5.2), a significant decrease in the percentage of $CD4^+Tg\alpha^+$ cells in the distant lymph nodes and spleen was seen in the mice immunised with peptide in CFA. Once again, the number of $CD4^+Tg\alpha^-$ cells in this group was also decreased (data not shown). The percentage and number of $CD4^+Tg\alpha^+$ cells expressing high levels of CD44 was increased by peptide when compared to the PBS control, although the increase was not as great as that seen following immunisation with peptide in CFA (Figure 5.9B). The Tgα profile of $CD44^{hi}$ cells after subcutaneous soluble peptide was consistent with this finding, since a significant increase in $Tg\alpha^{hi}$ cells was seen within the $CD44^{hi}$ population (Figure 5.9C). Thus the route of administration of soluble peptide has a profound effect on the final outcome of the T cell response, independent of the presence of adjuvant.

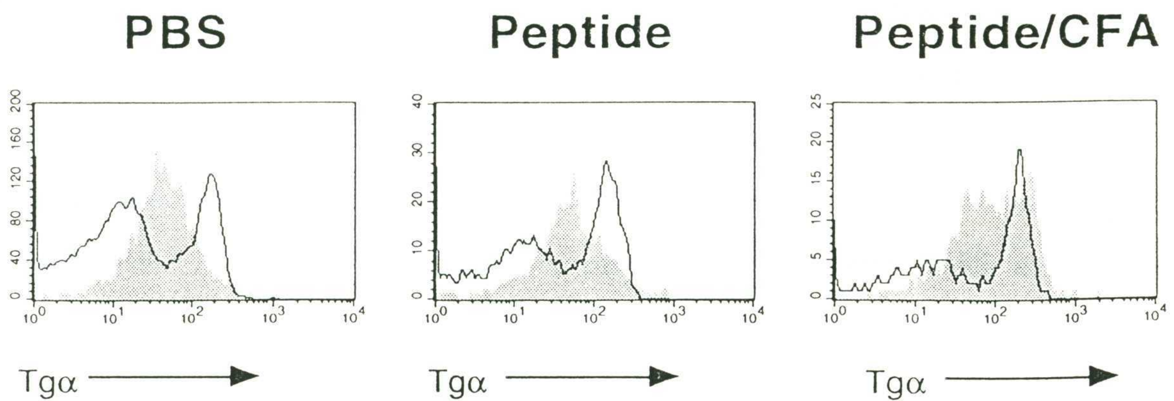
5.5. Characterisation of the secondary response of subcutaneously immunised transgenic mice.

The ability of subcutaneous peptide to increase the number of $CD44^{hi}$ antigen-specific cells, combined with evidence of increased sensitivity to antigen challenge in cell populations derived from subcutaneously immunised mice, suggested that subcutaneous immunisation induced a state of immunological memory in TCR transgenic mice. To test the *in vivo* response of subcutaneously immunised mice to a recall dose of antigen, a criss-cross experiment was performed in which adult thymectomised -D TCR transgenic mice were immunised subcutaneously with PBS or 10μg peptide 87-103 in CFA. Six weeks later, each group was divided into two for

Figure 5.9. Subcutaneous administration of peptide without adjuvant generates CD44^{hi} memory cells. Adult thymectomised -D TCR transgenic mice were immunised intravenously with PBS or subcutaneously with 1μg peptide, either alone or emulsified in CFA. **A.** Draining lymph nodes (left panel), distant lymph nodes (middle panel) and spleen (right panel) were harvested six weeks after immunisation and the percentage of CD4$^+$ cells expressing the transgenic TCR (Tgα) was determined. Each point represents the average of 3-4 mice with error bars indicating SEM. **B.** The expression of CD44 in the draining lymph nodes, distant lymph nodes and spleen is shown for each group of mice as the percentage of CD4$^+$Tgα$^+$ cells that were CD44^{hi}. Each point represents the average of 3-4 mice with error bars indicating SEM. **C.** The level of Tgα expression was determined on CD4$^+$CD44^{hi} and CD4$^+$CD44^{lo} cells. The gates used for CD44 expression are shown in Figure 5.8B. Representative plots of Tgα expression on CD4$^+$CD44^{hi} (shaded) and CD4$^+$CD44^{lo} cells (outline) are shown for mice immunised intravenously with PBS (left panel), or subcutaneously with peptide, either alone (middle panel) or emulsified in CFA (right panel). The level of fluorescence in the Tgα$^-$CD44^{hi} population was higher than the Tgα$^-$CD44^{lo} population because of incorrect compensation.


A.
Draining lymph nodes
Distant lymph nodes
Spleen
%CD4+ cells expressing Tgα
80
60
40
20
0
PBS
Peptide
Peptide/CFA
PBS
Peptide
Peptide/CFA
PBS
Peptide
Peptide/CFA


B.
Draining lymph nodes
Distant lymph nodes
Spleen
%CD4+Tgα+ cells that are CD44hi
50
40
30
20
10
0
PBS
Peptide
Peptide/CFA
PBS
Peptide
Peptide/CFA
PBS
Peptide
Peptide/CFA


C.
PBS
Peptide
Peptide/CFA
Tgα
Tgα
Tgα

rechallenge with PBS or 10μg peptide in CFA at the same site as the primary immunisation. In this way, the secondary peptide response of the primed group could be compared both with the response of naive mice to peptide and with the background responses of naive and primed mice to PBS/CFA rechallenge. Cells from the draining lymph nodes, distant lymph nodes and spleen were harvested one day after secondary immunisation and CD69 expression was measured. Further mice were sacrificed on day three for measurement of proliferation and IFN-γ production after restimulation with peptide *in vitro*.

Secondary challenge with peptide/CFA, but not PBS/CFA, stimulated expression of CD69 on 30% of $CD4^{+}Tg\alpha^{+}$ cells in the draining lymph nodes of mice, irrespective of whether they received PBS or peptide in the primary immunisation (Figure 5.10, upper panels). The small elevation in CD69 expression on $CD4^{+}Tg\alpha^{+}$ cells in the distant lymph nodes and spleens of mice which received PBS in the primary immunisation (Figure 5.10, middle left and lower left panels), was consistent with the response of naive mice (Figure 5.5). In contrast, 30% of $CD4^{+}Tg\alpha^{+}$ cells in the distant lymph nodes and spleens of mice primed with peptide expressed CD69 after secondary immunisation with peptide (Figure 5.10, middle and lower right panels). Thus, mice primed with peptide exhibited systemic T cell activation to localised antigen rechallenge, in contrast to mice which had never previously been immunised with peptide.

As expected from previous subcutaneous immunisation experiments (Section 5.3), draining lymph nodes from mice primed with peptide (1°peptide) or PBS (1°PBS) exhibited only small increases in their proliferative responses upon secondary peptide challenge at day three, when compared with the background response of the 1°peptide-2°PBS groups (Figure 5.11, upper left panel), but there were no significant increases in proliferation in the distant lymph nodes or spleen (Figure 5.11, upper middle and upper right panels, respectively). IFN-γ production was increased several-fold in the draining lymph nodes of the 1°PBS-2°peptide group when compared with the 1°PBS-2°PBS control (Figure 5.11, lower left panel), but not in the distant lymph nodes or spleen

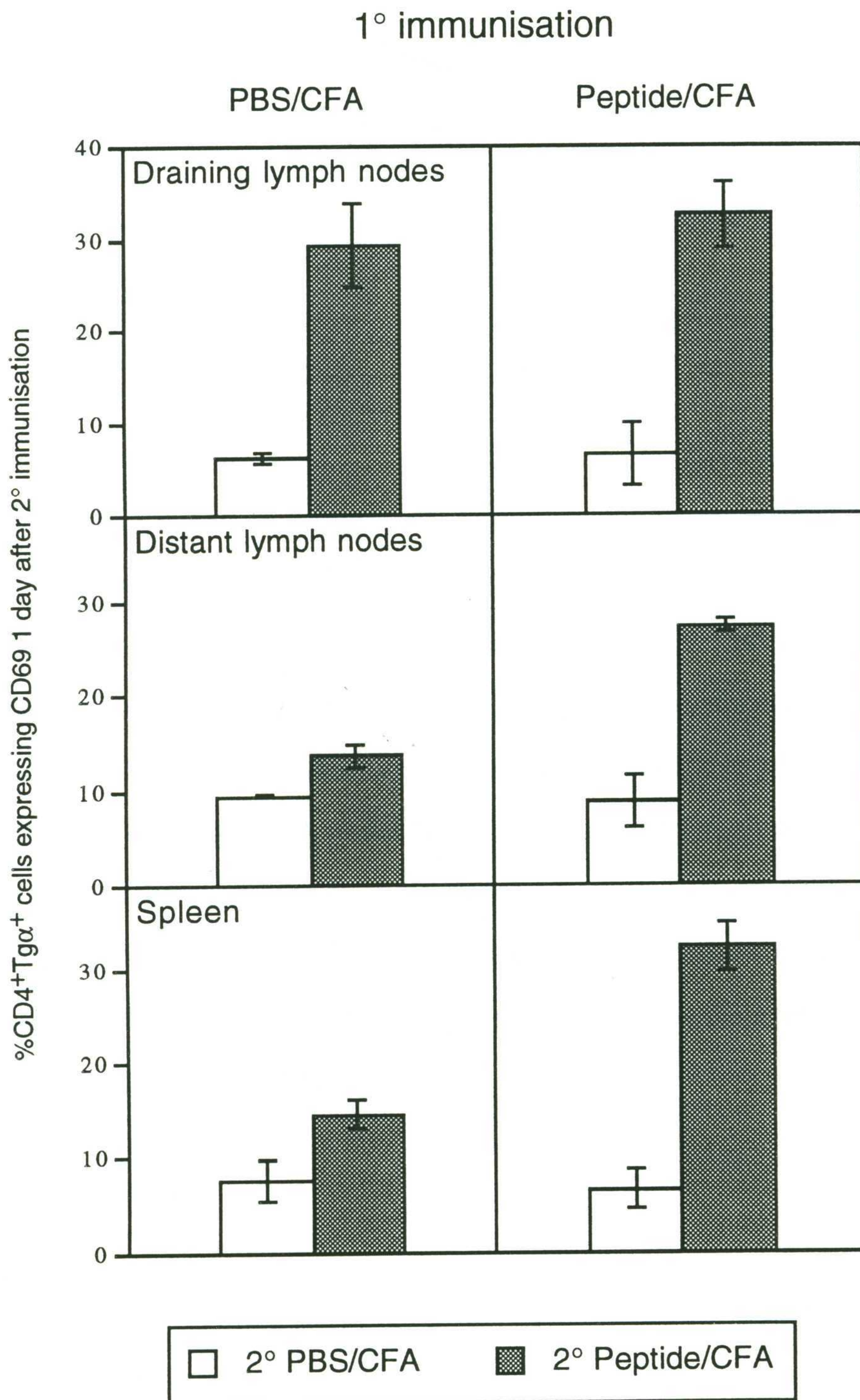
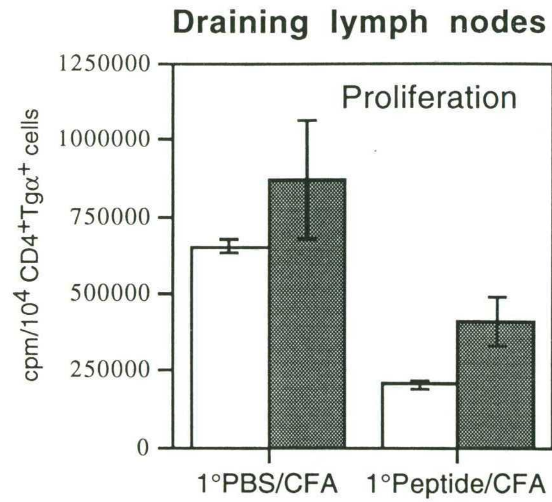
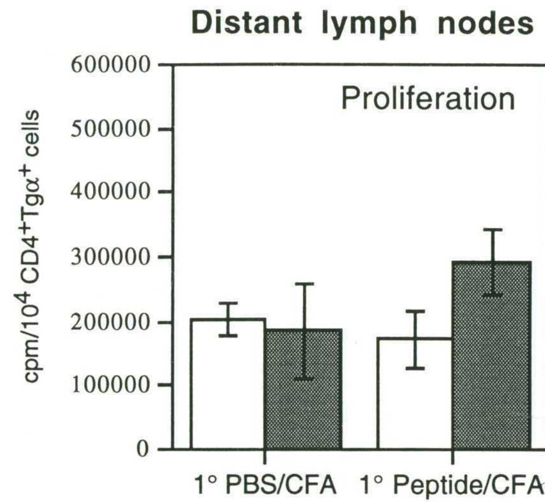
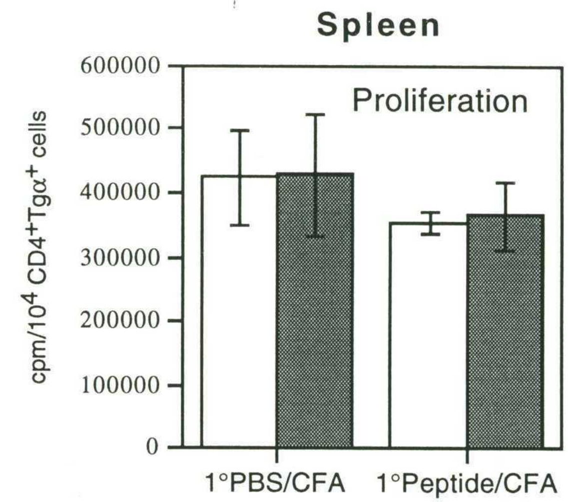


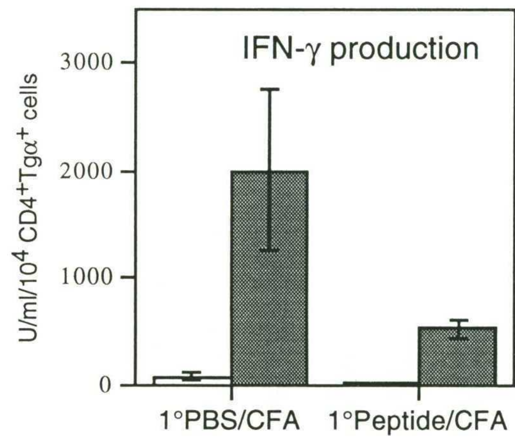
Figure 5.10. Subcutaneously immunised TCR transgenic mice exhibit systemic T cell activation after local re-challenge with peptide. Adult thymectomised -D TCR trangenic mice were immunised subcutaneously with PBS or 10μg peptide emulsified in CFA (1° immunisation). Six weeks later, each group was divided into two for subcuaneous re-challenge with PBS (2°PBS/CFA) or 10μg peptide emulsified in CFA (2°Peptide/CFA). CD69 expression was measured on $CD4^{+}Tg\alpha^{+}$ cells in the draining lymph nodes (upper panels), distant lymph nodes (middle panels), and spleen (lower panels) one day after re-challenge. Each point represents the average of three mice with error bars indicating SEM.

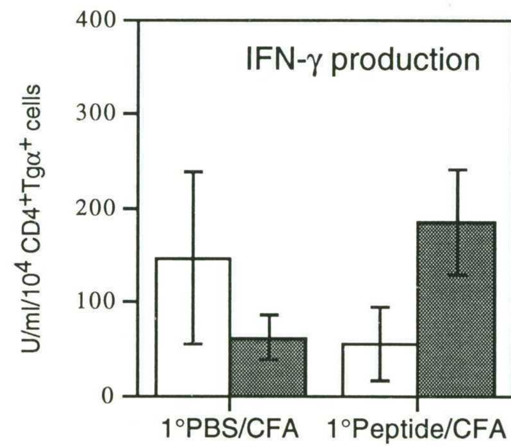
Figure 5.11. The recall response of subcutaneously immunised TCR transgenic mice upon subcutaneous rechallenge. Adult thymectomised -D TCR trangenic mice were immunised subcutaneously with PBS (1° PBS/CFA) or 10μg peptide in CFA (1° peptide/CFA). Six weeks later, each group was divided into two for subcutaneous re-challenge with PBS (2° PBS/CFA) or 10μg peptide emulsified in CFA (2° peptide/CFA). Draining lymph nodes (left panels), distant lymph nodes (middle panels), and spleen cells (right panels) were harvested three days after re-challenge and proliferation (upper panels) and IFN-γ production (lower panels) were measured *in vitro* after restimulation with 1μM peptide. Each point represents the average of three mice with error bars indicating SEM.

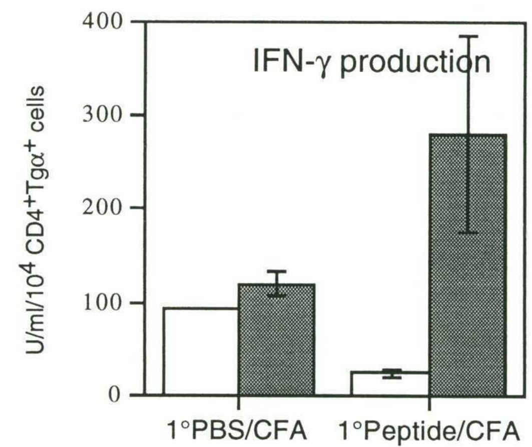

Draining lymph nodes
Proliferation
cpm/10^4 CD4+Tgα+ cells
1250000
1000000
750000
500000
250000
0
1°PBS/CFA
1°Peptide/CFA

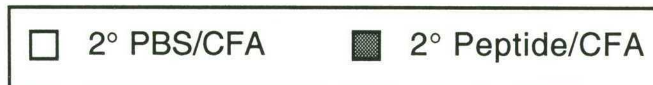

Distant lymph nodes
Proliferation
cpm/10^4 CD4+Tgα+ cells
600000
500000
400000
300000
200000
100000
0
1° PBS/CFA
1° Peptide/CFA


Spleen
Proliferation
cpm/10^4 CD4+Tgα+ cells
600000
500000
400000
300000
200000
100000
0
1°PBS/CFA
1°Peptide/CFA


IFN-γ production
U/ml/10^4 CD4+Tgα+ cells
3000
2000
1000
0
1°PBS/CFA
1°Peptide/CFA


IFN-γ production
U/ml/10^4 CD4+Tgα+ cells
400
300
200
100
0
1°PBS/CFA
1°Peptide/CFA


IFN-γ production
U/ml/10^4 CD4+Tgα+ cells
400
300
200
100
0
1°PBS/CFA
1°Peptide/CFA


2° PBS/CFA
2° Peptide/CFA

(Figure 5.11, lower middle and lower right panels, respectively). 1°peptide mice rechallenged with secondary peptide showed local increases in proliferation and IFN-γ production in the draining lymph nodes, although the increase in IFN-γ was not as marked as that seen in naive mice exposed to peptide/CFA. In contrast, IFN-γ recall responses in primed mice were significantly bigger in the distant lymph nodes and spleen than in naive mice. Thus peptide/CFA priming appeared to selectively increase distant IFN-γ responses while suppressing the local response to rechallenge.

The results presented earlier in this chapter (see Section 5.3) demonstrated that, at high peptide doses, *in vitro* proliferation of subcutaneously-primed $CD4^+Tg\alpha^+$ cells was equivalent to that of naive cells, while the response was exaggerated at lower peptide doses (see Figure 5.8). The recall experiment discussed above used a high dose of peptide (10μg) for secondary immunisation, so it was important to characterise the *in vivo* recall response of subcutaneously immunised animals to a low dose of peptide. Two groups of adult thymectomised -D transgenic mice were immunised subcutaneously with PBS or 10μg peptide in CFA. Six weeks later both groups were immunised subcutaneously (at the same site as 1° immunisation) with 1ng peptide in CFA. Three days later, the draining and distant lymph nodes were harvested and proliferation and cytokine production were measured after restimulation with peptide *in vitro*, as shown in Figure 5.12. As in the previous experiment, there was little difference in the proliferative response of draining and distant lymph node cells from 1°PBS and 1°peptide groups (Figure 5.12, upper panels). However, lymph node cells from the 1° peptide group produced more IFN-γ than naive T cells, and this effect was most marked in the distant lymph nodes (Figure 5.12, lower panels). Increased IFN-γ production was not due to an increase in the number of antigen-specific T cells *in vitro* or *in vivo*, since all results were normalised for input $CD4^+Tg\alpha^+$ T cell number.

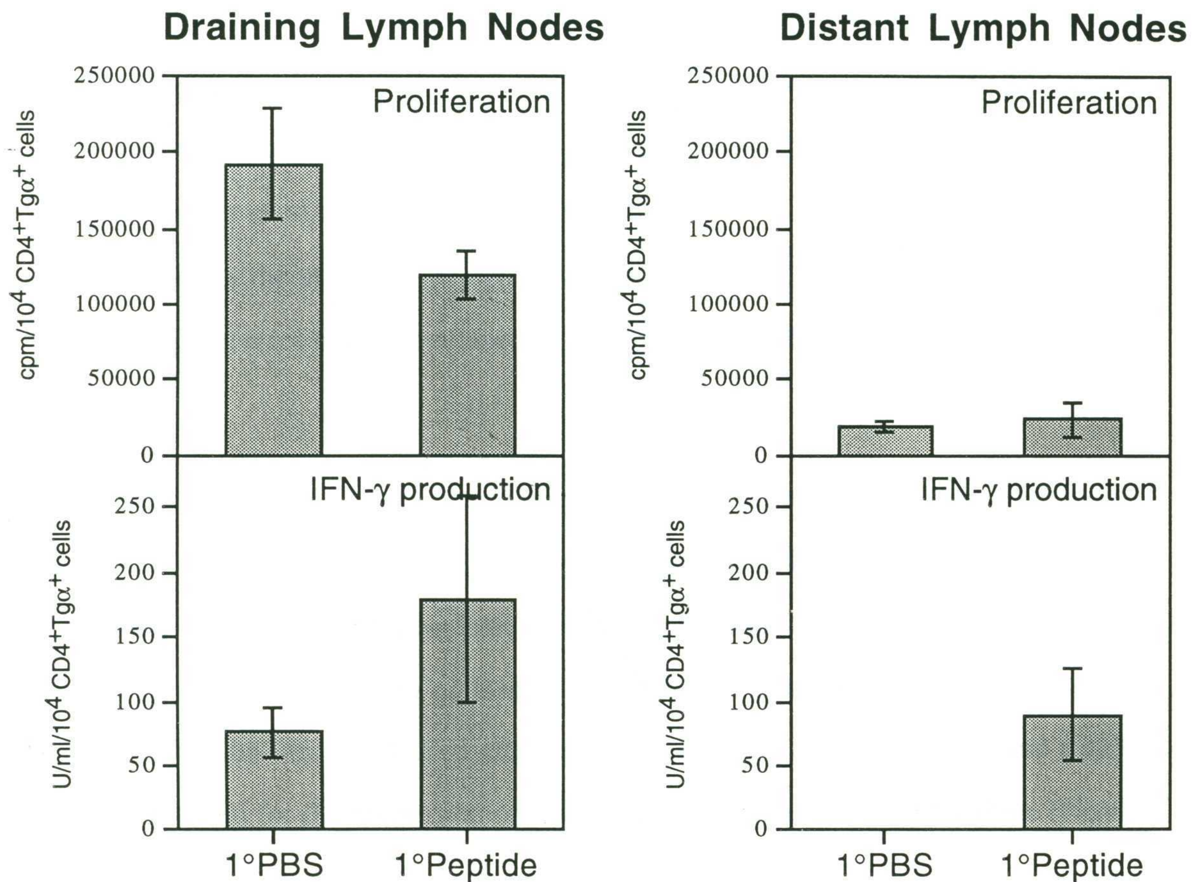


Figure 5.12. Subcutaneously immunised TCR transgenic mice exhibit enhanced IFN-γ production when a low dose of peptide is used to elicit the recall response. Adult thymectomised -D TCR trangenic mice were immunised subcutaneously with PBS (1°PBS) or 10μg peptide in CFA (1°Peptide). Six weeks later, both groups were re-challenged subcutaneously with 1ng peptide emulsified in CFA. Draining lymph nodes (left panels) and distant lymph nodes (right panels) were harvested three days after re-challenge and restimulated with peptide *in vitro* to measure proliferation (upper panels) and IFN-γ production (lower panels). Each point represents the average of three mice with error bars indicating SEM.

5.6. The dose of peptide administered subcutaneously influences the profile of cytokines produced on subsequent *in vitro* rechallenge.

The phenomenon of immune deviation was initially characterised *in vivo* as a function of antigen dose and immunogenicity (Parish 1971; Parish 1972; Parish and Liew, 1972) and has been correlated with differential development of Th1 and Th2 subsets *in vivo* (Bretscher et al., 1992) and *in vitro* (Hosken et al., 1995). The influence of antigen dose on the spectrum of cytokines produced by Th cells was investigated in TCR transgenic mice after subcutaneous immunisation with different doses of peptide in CFA.

In an initial experiment, adult euthymic -D TCR transgenic mice were immunised subcutaneously with PBS or 15ng, 1.5μg, or 150μg peptide in CFA. Draining lymph nodes were harvested three days later. Pooled lymph nodes from an untreated group of transgenic mice served as an additional control. Proliferation, IL-2, IL-3, IL-4 and IFN-γ production were measured after restimulation with peptide *in vitro* and results were normalised for the proportion of $CD4^{+}Tg\alpha^{+}$ cells present in each culture (Figure 5.13).

The response of draining lymph node cells from mice immunised with peptide was elevated in comparison with both untreated mice and mice that had received PBS/CFA. Proliferation and production of IL-2, IL-3, and IFN-γ peaked at 1.5μg and declined at the higher dose of 150μg. In contrast, IL-4 production continued to rise with increasing antigen dose. These results indicate that antigen dose has an influence on the ratio of Th2 to Th1 cytokines, Th2 cytokines being preferentially stimulated by higher antigen doses, whilst Th1 cytokines were depressed (possibly as a result of IL-4 feedback).

To further explore the effect of antigen dose on cytokine profile, the experiment was repeated with a greater range of peptide doses. Adult euthymic -D TCR transgenic mice were immunised subcutaneously with PBS, or dose range of 1ng to 1mg peptide in

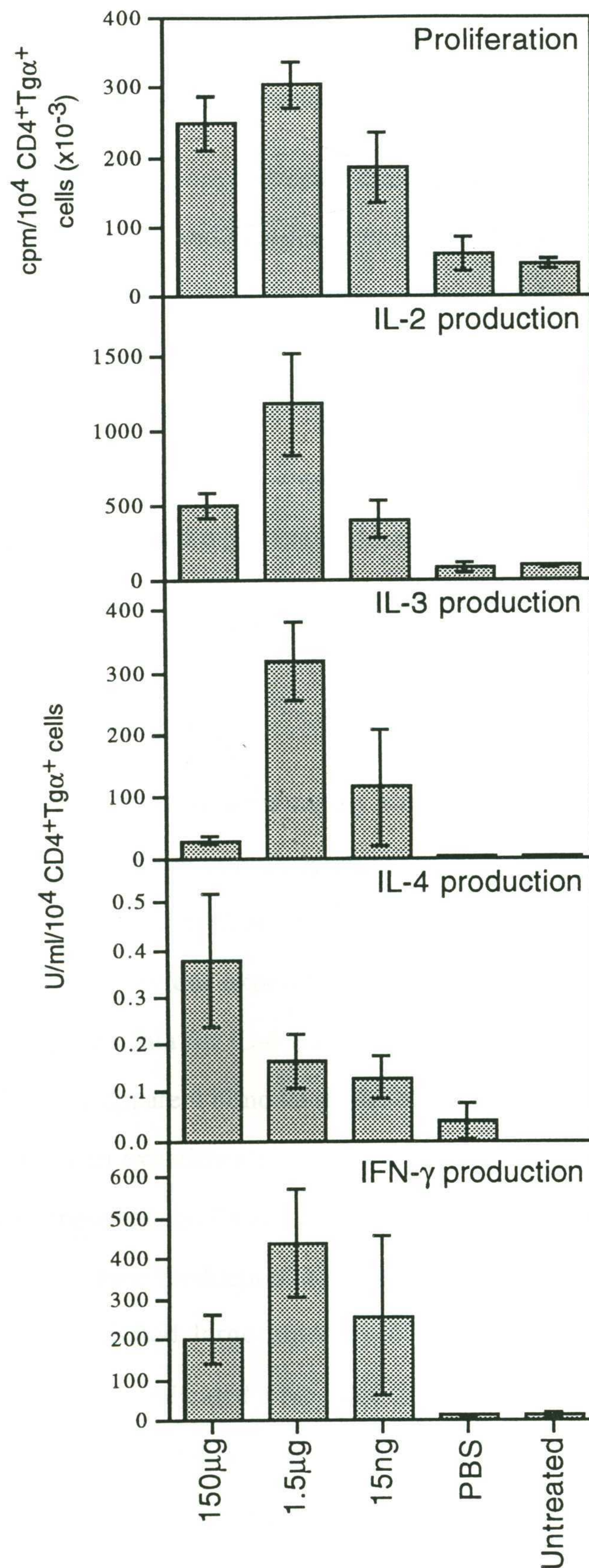


Figure 5.13. The dose of peptide administered subcutaneously influences the spectrum of cytokines produced by cells from the draining lymph nodes. Adult non-thymectomised -D TCR transgenic mice were immunised subcutaneously with 15ng, 1.5µg, or 150µg peptide emulsified in CFA, or PBS in CFA. Draining lymph nodes were harvested three days later, along with pooled lymph nodes from untreated mice, and restimulated with peptide *in vitro*. Proliferation, IL-3 production, IL-4 production, and IFN-γ production were measured after 72 hours culture. IL-2 production was measured after 24 hours culture. Each point represents the average of 3-4 mice with error bars indicating SEM.

CFA. Draining lymph nodes were harvested three days later, expression of CD69 was detected by flow cytometry, and proliferation and IL-4 and IFN-γ production were measured after restimulation with peptide *in vitro*. Figure 5.14 shows that although no change in the level of expression of CD69 by $CD4^{+}Tg\alpha^{-}$ cells was seen (data not shown), expression of CD69 by $CD4^{+}Tg\alpha^{+}$ cells in the draining lymph nodes was increased by all doses of peptide in comparison with the PBS control. The number of cells expressing CD69 reached a plateau at doses between 10ng and 10μg of peptide and decreased at higher doses. Proliferation was highest at a dose of 10μg, although there was considerable variability between individual mice for doses between 10ng and 100μg.

Both IL-4 and IFN-γ production were highly variable in this experiment for reasons that remain unclear. Production of both cytokines were primed by peptide doses above 1ng, but the dose response curves appeared to have two peaks. The curve for IFN-γ was consistent with the previous experiment (Figure 5.13) if outliers at 1mg and 1μg were to be removed. The data for IL-4 was too variable to distinguish outliers. Thus it is not clear whether the apparent bimodal nature of the dose response curve, with peaks at 100ng and 1mg, is an experimental artefact or not. Nonetheless, some conclusions can be drawn from these results. Firstly, there was a correlation between the extent of T cell activation and cytokine production at low doses. Secondly, although high doses of peptide consistently elicited large amounts of IL-4 production, IL-4 and IFN-γ production at lower doses indicated a far more complex relationship between antigen dose, T cell activation and cytokine priming *in vivo*. Thirdly, there was a paradoxical decrease in the number of $CD69^{+}$ cells seen at day three following immunisation with very high doses of antigen.

5.7. The influence of intravenous peptide on the subcutaneous response.

The results presented in this and the earlier chapter demonstrated that intravenous administration of peptide induced peripheral tolerance, whereas subcutaneous

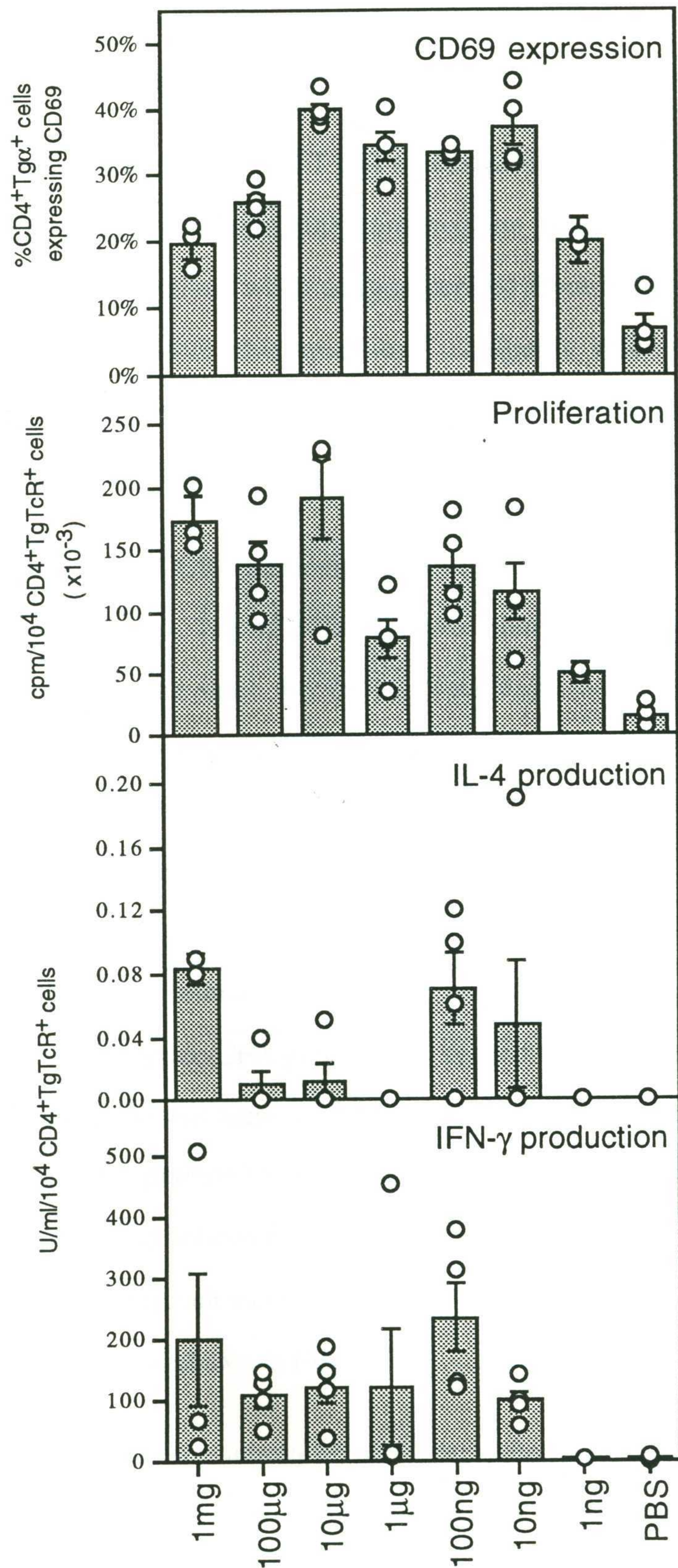


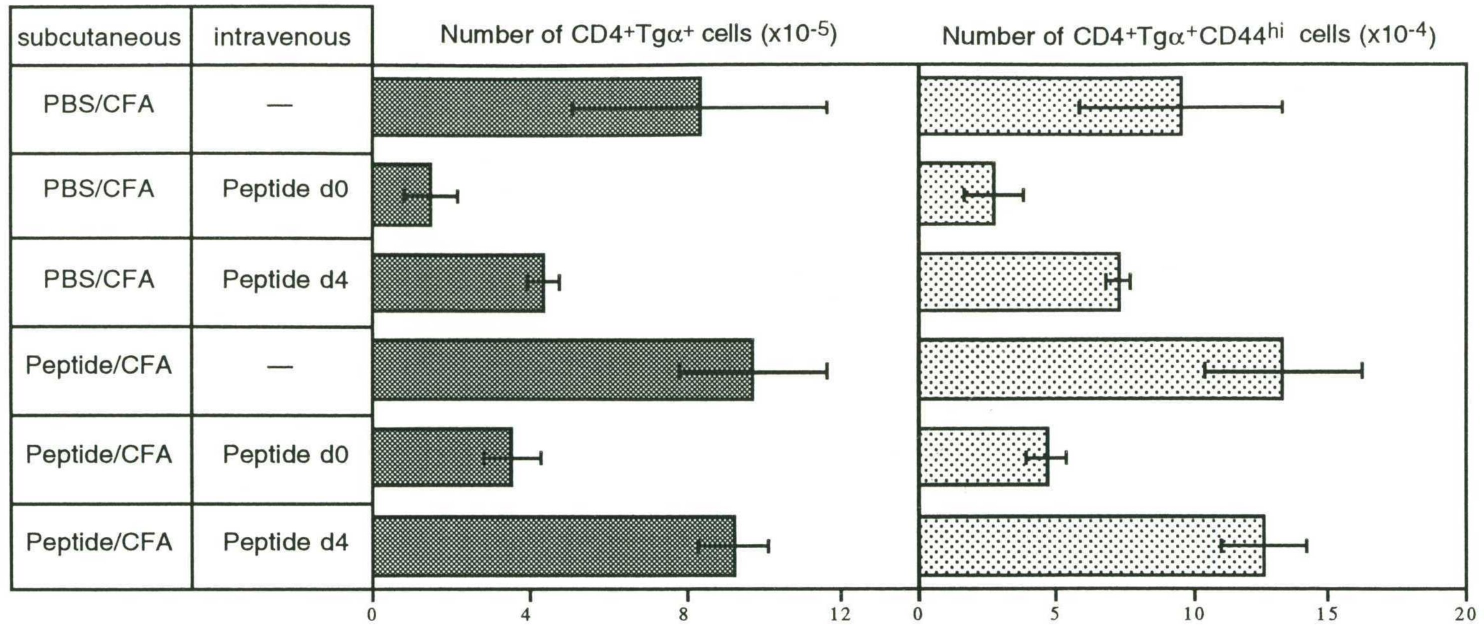
Figure 5.14. Relationship between the dose of peptide used for subcutaneous immunisation and T cell activation, proliferation and cytokine production by cells from the draining lymph nodes. Adult non-thymectomised -D TCR transgenic mice were immunised subcutaneously with PBS or 1ng, 10ng, 100ng, 1µg, 10µg, 100µg or 1mg peptide emulsified in CFA. Draining lymph nodes were harvested three days later. CD69 expression is shown as a percentage of $CD4^{+}Tg\alpha^{+}$ cells. Proliferation, IL-4 production, and IFN-γ production were measured *in vitro* after restimulation with peptide. The results for each mouse in every group are shown in addition to the average with error bars indicating SEM.

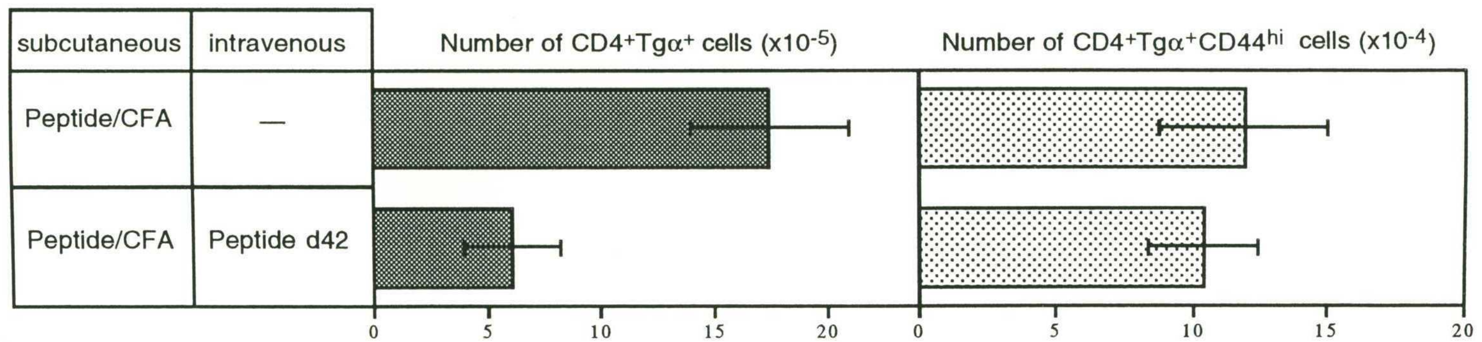
immunisation induced immunity and T cell memory. Since two distinct responses to peptide could be elicited by altering the immunisation protocol, it was of interest to determine possible interactions when antigen derived from both routes was present simultaneously. To explore this question, transgenic mice were immunised subcutaneously, and then administered intravenous peptide to determine if peripheral deletion could still be induced.

Adult thymectomised -D TCR transgenic mice were immunised subcutaneously with PBS or 1μg peptide in CFA. The mice then received either no further treatment, or 1μg intravenous peptide on the same day (d0) or four days (d4) later. The number of $CD4^{+}Tg\alpha^{+}$ cells in the draining lymph nodes was measured six weeks later. Figure 5.15A (left panel) shows no significant difference in the number of $CD4^{+}Tg\alpha^{+}$ cells six weeks after subcutaneous immunisation with PBS/CFA or peptide/CFA, confirming the data in Figure 5.2. However, intravenous peptide administered on the same day as subcutaneous immunisation caused more than half of the $CD4^{+}Tg\alpha^{+}$ cells in both PBS/CFA and peptide/CFA groups to be deleted. Partial deletion was also seen when intravenous peptide was administered four days after subcutaneous immunisation with PBS/CFA but not peptide/CFA. The number of $CD4^{+}Tg\alpha^{+}$ cells expressing a $CD44^{hi}$ phenotype closely followed the total number of $CD4^{+}Tg\alpha^{+}$ cells. Surprisingly, PBS/CFA induced a significant increase in $CD44^{hi}$ cells in this particular experiment, in contrast to the pooled data shown in Figure 5.6.

When intravenous peptide was administered 42 days after subcutaneous immunisation, deletion was again seen (Figure 5.15B, left panel), although $CD44^{hi}$ cells were selectively retained (Figure 5.15B, right panel). There were no significant changes in the number of $CD4^{+}Tg\alpha^{-}$ cells throughout these experiments, indicating that the effects described were antigen-specific (data not shown). Similar observations were made in the distant lymph nodes and spleen, although the differences were not always as marked as they were in the draining lymph nodes (data not shown). Thus it appears that

Figure 5.15. Interaction between responses to intravenous and subcutaneous peptide. A. Adult thymectomised -D TCR transgenic mice were immunised subcutaneously with PBS or 1μg peptide emulsified in CFA. Each group was divided into three, and one third were left untreated, one third received 1μg peptide intravenously on the same day as subcutaneous immunisation, and the other third received 1μg peptide intravenously four days after subcutaneous immunisation. Six weeks later, the number of $CD4^{+}Tg\alpha^{+}$ and $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells was calculated in the draining lymph nodes after immunostaining and flow cytometry. Each point represents the average of three mice with error bars indicating SEM. **B.** Adult thymectomised -D TCR transgenic mice were immunised subcutaneously with 1μg peptide emulsified in CFA. Six weeks later, half of the mice received 1μg of peptide intravenously. A further six weeks later, the number of $CD4^{+}Tg\alpha^{+}$ (left panel) and $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells (right panel) was determined in the draining lymph nodes. Each point represents the average of 2-3 mice with error bars indicating SEM.


A.
Immunisation route
subcutaneous
intravenous
Number of CD4+Tgα+ cells (x10-5)
Number of CD4+Tgα+CD44hi cells (x10-4)
PBS/CFA
—
PBS/CFA
Peptide d0
PBS/CFA
Peptide d4
Peptide/CFA
—
Peptide/CFA
Peptide d0
Peptide/CFA
Peptide d4
0
4
8
12
0
5
10
15
20


B.
Immunisation route
subcutaneous
intravenous
Number of CD4+Tgα+ cells (x10-5)
Number of CD4+Tgα+CD44hi cells (x10-4)
Peptide/CFA
—
Peptide/CFA
Peptide d42
0
5
10
15
20
0
5
10
15
20

activated and memory T cells generated by subcutaneous antigen administration are resistant to peripheral deletion.

5.8. Discussion.

It has long been known that subcutaneous administration of antigen emulsified in adjuvant induces T cell immunity and long-lived memory (Dresser, 1961). The success of subcutaneous immunisation protocols has been attributed mainly to the presence of an adjuvant that is derived from (or mimics) microbial products capable of encouraging local inflammation and stimulating APC function (Audibat and Lise, 1993). In doing so, adjuvants create a potent environment for antigen presentation and T cell activation. The cellular events that take place during the induction of protective T cell immunity involve the selective activation and differentiation of antigen-specific lymphocytes, as first defined by Burnet over three decades ago (Burnet, 1959). In the time since then, experimental data have confirmed Burnet's paradigm, but it is only recently that the processes occurring during the induction of immunity have been visualised at the cellular level (Kearney et al., 1994; McHeyzer-Williams and Davis, 1995). The results presented in this section add further detail to the cellular events first hypothesised by Burnet.

Subcutaneous immunisation of TCR transgenic mice with peptide emulsified in CFA elicited a T cell response that was characterised by its localisation to the draining lymph nodes. Immunisation induced expression of CD69 on a small percentage of $CD4^{+}Tg\alpha^{+}$ cells in the draining lymph nodes within 24 hours, and by day four, 50% of all $CD4^{+}Tg\alpha^{+}$ cells in the draining lymph nodes expressed CD69 (Figure 5.5). Activated $CD4^{+}Tg\alpha^{+}$ cells exhibited increased cytokine production on days 3-4, producing large amounts of IL-3, IFN-γ and detectable quantities of IL-4 in response to restimulation with peptide (Figure 5.3). Interestingly, proliferation and IL-2 production were not significantly increased with respect to precursor cells, indicating that significant differentiation to secondary cytokine production had occurred. T cell activation in the draining lymph nodes was followed by clonal expansion, increasing the number of $CD4^{+}Tg\alpha^{+}$ cells by nine-fold within the first seven days (Figure 5.1). There was no

significant increase in the number of CD4$^+$Tgα^+ cells in the distant lymph nodes and spleen during the course of the response, indicating that clonal expansion was localised to the draining lymph nodes. After day seven a slow decline in the total number of CD4$^+$Tgα^+ cells was seen (Figure 5.1). The net result was a return to baseline levels of CD4$^+$Tgα^+ cells in the draining lymph nodes by six weeks, although the number and proportion of cells exhibiting an activation/memory phenotype remained elevated (see below).

The number of CD4$^+$Tgα^+ cells expressing high levels of CD44 increased in the draining lymph nodes until day eight and then declined. While there was a 7-9-fold increase in the number of CD4$^+$Tgα^+ cells at the peak of the response (Figure 5.1), the number of CD4$^+$Tgα^+CD44^{hi} cells increased 35-100-fold. Subsequently, the number of CD4$^+$Tgα^+CD44^{hi} cells declined but did not return to baseline levels, remaining elevated three-fold in the draining lymph nodes on day 43 (Figure 5.5). A large proportion of these CD44^{hi} cells expressed high levels of Tgα, compared to a smaller proportion in the PBS control, suggesting that they were truly peptide-specific. In contrast, the CD44^{hi} cells present six weeks after intravenous immunisation expressed low levels of Tgα (Figure 4.17) and probably represented a distinct population of cells reactive to environmental antigens. Size analysis showed that a proportion of the CD4$^+$Tgα^+CD44^{hi} cells remaining in the draining lymph nodes after subcutaneous immunisation were large (Figure 5.7), consistent with the presence of a small number of CD69$^+$ cells at this time. This phenotype can be attributed to a continuing low grade response to a persistent antigen/CFA depot. However, the majority of CD4$^+$Tgα^+CD44^{hi} cells at day 43 were small CD69$^-$ cells, suggesting that subcutaneous immunisation generated a long-term increase in antigen-specific T cells with a resting memory phenotype. Moreover, the increase in the proportion of CD44^{hi} cells expressing high levels of Tgα lends further support to the notion that the T cells with a memory phenotype were generated specifically by subcutaneous peptide.

Subcutaneous immunisation also exerted measurable effects at distant sites. The early appearance of $CD4^+Tg\alpha^+CD69^+$ cells in the distant lymph nodes and spleen, followed on day eight by $CD44^{hi}$ cells (Figure 5.5), suggested migration of peptide-loaded APC and/or activated T cells. Moreover, the presence of significant numbers of small $CD4^+Tg\alpha^+CD44^{hi}$ cells in the distant lymph nodes and spleen at late time points (Figure 5.5) indicated the generation of a recirculating population of cells with a memory phenotype. Interestingly, immunisation with peptide/CFA resulted in a decrease in the total number of both $CD4^+Tg\alpha^+$ and $CD4^+Tg\alpha^-$ cells distant from the site of immunisation (Figure 5.2). It is likely that cell loss was in fact generalised, but was masked in the draining lymph nodes by the low level of continuing local proliferation which was maintained for at least six weeks after immunisation. This conclusion is supported by significantly higher levels of CD69 expression and increased forward scatter in $CD4^+Tg\alpha^+CD69^+$ cells in the draining lymph nodes relative to other lymph nodes six weeks after immunisation (Figure 5.7).

The ten-fold decline in $CD4^+Tg\alpha^+CD44^{hi}$ cells between days eight and 43 presumably represents attrition of effector cells, leaving a residual population of long-lived memory cells, as characterised by Sprent and Miller two decades ago (Sprent 1976; Sprent and Miller 1976a; Sprent and Miller, 1976b). Bruno et al. (1995) observed a ten-fold reduction in cell number during the three weeks after transfer of activated (large, $CD44^{hi}$) male antigen-specific T cells immunised ten days previously. Loss of activated ($CD44^{hi}$) cells was also seen by a number of other investigators (Kearney et al., 1994; McHeyzer-Williams and Davis, 1995; Figure 5.5), suggesting that it represents a physiological characteristic of normal immune responses.

The results for intact -D TCR transgenic mice closely resemble published results of Kearney et al. (1994) and McHeyzer-Williams and Davis (1995) obtained in models with a lower frequency of antigen-specific T cells. Kearney et al. (1994) employed adoptive transfer of T cells from TCR transgenic mice into non-transgenic hosts and showed that subcutaneous immunisation induced clonal expansion of transgenic T cells

in the draining lymph nodes but not at distal sites. The number of transgenic T cells rapidly increased 15-fold in the draining lymph nodes, peaking on days 3-5, and then declined, but was still elevated three-fold above the control at the conclusion of the experiment on day 18. Immunohistochemical staining showed that the increase in transgenic T cells in the draining lymph nodes coincided with their accumulation in the lymph node follicles, with a peak on day five, while a few transgenic T cells entered the follicles in distant lymph nodes.

McHeyzer-Williams and Davis (1995) characterised the T cell response to subcutaneous immunisation with PCC in unmanipulated B10.BR mice. This response uses a highly restricted TCR repertoire of which the vast majority are $V_{\alpha}11V_{\beta}3$. Careful FACS analysis of the tiny $V_{\alpha}11V_{\beta}3$ population demonstrated a significant increase in $CD44^{hi}$ and L-selectin^{lo} cells in response to antigen. The number of antigen-activated T cells peaked in the draining lymph nodes six days after immunisation, with a 27-fold increase over baseline levels. By eight weeks, numbers were still elevated 1-2-fold over baseline. The difficulty with this model is that not all $V_{\alpha}11V_{\beta}3$ cells are PCC-reactive and the true baseline level of PCC-reactive cells cannot be estimated. The baseline calculated by McHeyzer-Williams and Davis represents the number of activated/memory cells for all antigens to which $V_{\alpha}11V_{\beta}3$-bearing cells can react.

McHeyzer-Williams et al. (1996) have reported faster kinetics for the subcutaneous response in intact TCR transgenic mice of the -I line (Seder et al., 1992), created using the same constructs as -D. They compared the appearance of activated (large $CD44^{hi}$ or L-selectin^{lo}) cells in the draining lymph nodes of transgenic animals expressing both chains of the transgenic TCR, or only a single chain (α or β), after subcutaneous immunisation with nitrophenylated PCC in adjuvant. Activated T cells appeared rapidly in the draining lymph nodes of TCR$\alpha\beta$ and TCRβ transgenic mice and peaked on day two, with most of the activated cells disappearing by day seven. In contrast, the subcutaneous response peaked on day five in TCRα transgenic mice, closely resembling the response of non-transgenic mice (see above). There were ten-fold fewer

antigen-specific T cells in TCRα transgenic mice than TCRβ mice, leading the authors to suggest that the rapid kinetics seen TCRβ mice were a function of the high frequency of responder T cells (McHeyzer-Williams et al., 1996). This is inconsistent with the data presented in Figure 5.5, which shows that the kinetics of CD44 upregulation on $CD4^+Tg\alpha^+$ cells in -D TCR transgenic closely resembled those observed by McHeyzer-Williams et al. (1996) in non-transgenic mice, peaking between days four and eight, rather than on day two. These data suggest that a rapid downregulation of the response is not produced simply as a result of a high frequency of responder T cells.

A three-fold increase in the number of $CD4^+Tg\alpha^-$ cells was seen in the draining lymph nodes of mice subcutaneously immunised with peptide in CFA (Figure 5.1). Two explanations are likely. Firstly, $CD4^+Tg\alpha^-$ cells could have been responding to the mycobacteria in the adjuvant. Although a similar increase was not seen in control mice immunised with PBS in CFA, it is possible that the powerful simultaneous response of $CD4^+Tg\alpha^+$ cells to peptide assisted the $CD4^+Tg\alpha^-$ response by providing IL-2 for clonal expansion as part of a bystander effect. Alternatively, the increase in the number of $CD4^+Tg\alpha^-$ cells could have been due to TCR downregulation by activated $CD4^+Tg\alpha^+$ cells, producing an artefact in cell gating during FACS analysis. Further experiments in -D mice on a homozygous Rag-1-deficient background are required to differentiate between these possibilities.

Despite the clear tolerogenic effect of soluble peptide administered intravenously (see Section 4), soluble peptide administered by the subcutaneous route induced an increase in both the numbers and percentage of $CD4^+Tg\alpha^+CD44^{hi}$ cells remaining in the lymph nodes and the spleen six weeks after immunisation, though not to the same extent as peptide/CFA (Figure 5.9). The generation of long-lived $CD4^+Tg\alpha^+CD44^{hi}$ cells was accompanied by a small decrease in the total number of $CD4^+Tg\alpha^+$ cells in the distant lymph nodes and spleen, also seen following subcutaneous immunisation with peptide/CFA (Figure 5.9). Thus, it appears the subcutaneous route of antigen administration is responsible for producing a bias in the T cell response towards

memory and immunity, and that adjuvant simply augments this response. It will be of interest to follow the local response to soluble peptide at the level of proliferation and cytokine production, and to determine the responsiveness of the $CD4^{+}Tg\alpha^{+}$ cells six weeks after immunisation.

As discussed earlier, the population of $CD4^{+}Tg\alpha^{+}$ cells remaining after subcutaneous immunisation contained an increased number and percentage of cells with a memory phenotype, and re-challenge experiments *in vitro* and *in vivo* indicated memory function. Draining lymph node cells harvested six weeks after subcutaneous peptide immunisation were more sensitive to peptide *in vitro*, displaying an average of 3-4-fold greater proliferation per cell at low doses of peptide than cells from PBS-treated mice. However, at higher peptide doses there was no functional difference between peptide- and PBS-treated cells *in vitro*. This is in agreement with the data published by Croft et al. (1994) who reported that Tm and naive T cells isolated from TCR transgenic mice were functionally equivalent when restimulated with antigen *in vitro* under optimal conditions; under suboptimal conditions, such as low antigen concentration, Tm responded better than naive cells and were less dependent on costimulation.

Further evidence of memory function in subcutaneously immunised TCR transgenic mice was obtained from *in vivo* experiments. Subcutaneous re-challenge with a large dose of peptide induced systemic expression of CD69 equal to the levels seen in the draining lymph nodes, whereas CD69 expression was confined to the draining lymph nodes of mice exposed to peptide/CFA for the first time (Figure 5.10). This was consistent with the *in vitro* lymphokine responses generated by subcutaneous re-challenge. Spleen and lymph nodes distant from the site of injection showed greater production of the secondary lymphokine IFN-γ in primed mice, whereas there was no increase in IFN-γ production by cells from the draining lymph nodes (Figure 5.11). In fact, peptide re-challenge elicited two-fold-greater IFN-γ production per draining lymph node cell in mice previously immunised with PBS/CFA compared with peptide/CFA (Figure 5.11). $CD4^{+}Tg\alpha^{+}$ cells from the draining lymph nodes of primed mice made

more IFN-γ only when a very small dose of peptide was used for subcutaneous re-challenge (Figure 5.12), as predicted by the *in vitro* study (Figure 5.8). Further work is required to characterise the functional capacity of memory $CD4^{+}Tg\alpha^{+}$ cells. For example, it would be of interest to measure the secondary response to peptide administered subcutaneously at a different site from the primary immunisation, with and without adjuvant. Bruno et al. (1995) demonstrated that memory cells expanded more vigorously than naive cells and sustained a longer secondary response when re-challenged *in vivo*. McHeyzer-Williams and Davis (1995) observed the same phenomenon in normal mice.

The influence of persistent antigen on the maintenance of T cell memory is controversial. There is convincing evidence for the existence of long-lived resting memory cells *in vivo* (Tough and Sprent, 1994; Mullbacher, 1994; Bruno et al., 1995) but also evidence that T cells with an activation/memory phenotype have a rapid turnover (Mackay et al., 1990; Michie et al., 1992; Tough and Sprent, 1994). Gray and Matzinger (1991) were the first to present evidence that antigen was important for the maintenance of memory, but others have found that T cell memory persists in the absence of antigen (Mullbacher, 1994; Bruno et al., 1995). Zinkernagel et al. (1996) found that a low level of continual priming by persistent antigen was essential for maintaining host protection to some viruses but whether this is the only valid definition of memory is controversial. The results from -D TCR transgenic mice show that the $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells persisting after subcutaneous immunisation comprise a majority of small resting cells, with a small number of large cells, presumably recently activated by antigen (Figure 5.7). It seems to be a matter of definition whether the population of large activated $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells are regarded as memory cells poised to respond to antigen re-challenge or remnants of the primary response. A definitive answer to the question of which cells are responsible for *in vivo* memory in the -D model would require sorting and adoptive transfer of the small and large $CD44^{hi}$ cells.

The dose of peptide administered subcutaneously influenced the spectrum of cytokines produced by draining lymph node T cells. The initial dose-response experiment suggested that proliferation and IL-2, IL-3 and IFN-γ production had similar dose response curves, particularly in terms of the decline at large doses of antigen (Figure 5.13). In contrast, IL-4 production continued to increase as a function of antigen dose (Figure 5.13). These results are consistent with a model of positive feedback regulation of IL-4 production and dominant Th2 subset development at high peptide doses first suggested by Seder et al. (1992) and later supported by the *in vitro* data of Hosken et al. (1995). In the second dose-response experiment, results were far more variable. At very low doses, T cell activation, IL-4 and IFN-γ production increased in tandem with the peptide dose; at intermediate doses there was significant proliferation but little priming of either cytokine; and at high doses significant IL-4 production was seen, little change in IFN-γ production and a paradoxical decline in T cell activation (Figure 5.14) which may actually reflect faster kinetics of CD69 downregulation. Further experiments (including a time-course study) are required to clarify the effects of antigen dose on the cytokine profile of activated T cells. In addition, it would be of interest to study the influence of peptide dose on the generation of memory cells capable of producing IL-4 and IFN-γ.

Simultaneous subcutaneous and intravenous immunisation produced a mixed response, neither protocol truly dominating the other. The number of $CD4^+Tg\alpha^+$ cells in the draining lymph nodes six weeks after intravenous peptide was increased by simultaneous subcutaneous immunisation with PBS or peptide in CFA (Figure 5.15). At the same time, the number of $CD4^+Tg\alpha^+CD44^{hi}$ cells generated by subcutaneous peptide was reduced by intravenous peptide, presumably leading to a reduction in T cell memory. When intravenous immunisation was delayed four days, the number of $CD4^+Tg\alpha^+$ cells and the size of the $CD4^+Tg\alpha^+CD44^{hi}$ population in the draining lymph nodes six weeks later was unaffected. Administration of intravenous peptide six weeks after subcutaneous peptide/CFA deleted some $CD4^+Tg\alpha^+$ cells, but there

appeared to be no effect on the number of $CD4^+Tg\alpha^+CD44^{hi}$ cells either within the draining lymph nodes or systemically. Taken together, it appears that activated $CD4^+Tg\alpha^+$ cells acquire resistance to deletion by intravenous peptide by day four after subcutaneous immunisation, and that $CD4^+Tg\alpha^+CD44^{hi}$ cells generated by subcutaneous immunisation are resistant to deletion even at late time points. However, it is not clear from these results whether the deletion-resistance of activated $CD4^+Tg\alpha^+$ cells and $CD4^+Tg\alpha^+CD44^{hi}$ cells is a qualitative property or a quantitative effect of coincident T cell responses possibly affecting overlapping but not identical subsets of T cells. It will be of interest to further characterise the effects of simultaneous intravenous and subcutaneous immunisation by measuring the kinetics of the local and systemic responses and assessing T cell memory at late time points.

Section 6. General Discussion.

The studies presented here provide a detailed map of the cellular events that occur during the induction of peripheral tolerance and immunity in TCR transgenic mice. Importantly, tolerance and immunity can be induced to the same peptide antigen by using different routes of administration: tolerance by the intravenous route and immunity by the subcutaneous route. By comparing and contrasting the features of the subcutaneous and intravenous responses, some conclusions can be made about the key events that take place.

The subcutaneous and intravenous responses share three features. Firstly, antigen administration stimulates T cell activation characterised by early expression of CD69, increased cytokine production upon restimulation *in vitro* and upregulation of CD44 expression. Secondly, T cell activation precedes clonal expansion of antigen-specific T cells *in vivo*, and finally, the vast majority of cells produced by clonal expansion disappear from the periphery. Despite these common features, the subcutaneous and intravenous responses differ in their kinetics, localisation, degree of T cell deletion, generation of long-lived T cells with a memory phenotype and responsiveness of the T cells remaining at the resolution of the response. Intravenous peptide induces rapid T cell activation peaking within hours of administration and resolving by day seven (Figure 4.5). Subcutaneous peptide induces local T cell activation peaking several days after immunisation but maintained at a low level for at least six weeks (Figure 5.5), reflecting the long-term persistence of the antigen/adjuvant depot. Acute T cell responses to subcutaneous peptide are mainly localised in the draining lymph nodes, in contrast to the systemic response to intravenous peptide. Intravenous immunisation is characterised by the precipitous decline in the number of antigen-specific cells between days three and ten (Figure 4.1). In contrast, the number of antigen-specific cells declines slowly after the peak of clonal expansion in the draining lymph nodes (Figure 5.1). Both routes of immunisation induce upregulation of CD44 on divided cells, but

the total number of CD4$^+$Tgα^+CD44^{hi} cells remaining six weeks after immunisation is elevated only in subcutaneously immunised mice (Figures 4.5 and 5.5). In addition, the residual CD44^{hi} population is quite distinct in the two responses. The CD44^{hi} cells present both before and after intravenous immunisation do not contain a distinct Tgα^+ subpopulation. Those few cells within the Tgα^+ gate probably represent cells primed by environmental antigens as a result of co-expression of endogenous TCR α chains. In contrast, the population of long-lived CD44^{hi} cells generated by subcutaneous immunisation expresses a high level of Tgα (Figure 5.8B) consistent with a true anti-cytochrome memory phenotype. Although total CD4$^+$Tgα^+ cell number drops below the starting number in both responses, the final phenotype of the response is quite different. Whereas intravenously immunised mice respond less vigorously than naive mice to re-challenge with intravenous peptide *in vitro* and *in vivo* (Figure 4.11), the response of subcutaneously immunised manifests the features of memory, namely sensitivity to low doses of antigen *in vitro* and *in vivo* (Figures 5.8 and 5.12), earlier systemic activation (Figure 5.10) and a bias towards secretion of secondary cytokines such as IFN-γ and IL-4. Thus subcutaneous immunisation induces T cell memory, whereas intravenous immunisation renders the mice tolerant.

As mentioned previously (see Section 1.2), all available evidence indicates that protein antigen is presented to T cells as a peptide bound to the cleft of MHC, such that the route of administration and form of antigen would have no effect on the peptide/MHC complex bound by the TCR. The differential effects of subcutaneous and intravenous immunisation may therefore be mediated via APC, which are well-placed to regulate T cell responses and have been shown to exert differential effects *in vivo* (see Section 1.3.2). Furthermore, recent characterisation of a variety of non-MHC molecules on the surface of APC, such as costimulatory molecules, has indicated that APCs can directly influence T cell activation and cytokine production (see Section 1.3.1). This premise forms the basis of a hierarchical model for the regulation of tolerance and immunity in the periphery (Fazekas de St. Groth, 1995). The hierarchical model places APCs, T

cells and B cells in a hierarchy of control (Figure 6.1). T cell activity is under the control of APCs, and B cell activity is under the control of T cells. Thus the outcome of an encounter with antigen is determined by the activity of APCs. In the absence of effective antigen-presentation, T cells and B cells remain in a resting state and functional tolerance is maintained. By placing APC at the top of the hierarchy, the characteristics of the antigen and the context in which it is encountered have a dominant effect on the outcome.

The results presented in this thesis are consistent with a hierarchical model of T cell regulation in which APC are responsible for determining the phenotype of the T cell response. The crucial factor determining the outcome of immunisation of TCR transgenic mice with soluble peptide is the route of administration. The simplest explanation is that the intravenous and subcutaneous routes deliver antigen to functionally different APC. In this model, subcutaneous peptide induces immunity rather than tolerance because it delivers it to immunogenic rather than tolerogenic APC. This hypothesis is consistent with what is already known about the subcutaneous route of immunisation. Subcutaneous antigen is captured in the skin by resident Langerhans cells (LC) and transported to the draining lymph nodes. LC mature into potent APC as they migrate to the lymph node and enter the T cell areas of the node to present antigen to T cells (Szakal et al., 1983, Moll et al., 1993). The effect of adjuvant on the subcutaneous response is consistent with the hierarchical model since adjuvants exert their effects principally by activating APCs (Audibat and Lise, 1993).

The APCs that present intravenous peptide to T cells have yet to be identified but there is evidence that a population of DC are responsible (Fazekas de St. Groth et al., 1997). Since the response to intravenous peptide is extremely rapid and activates the vast majority of naive T cells (Figure 4.5A), it is likely that the APCs responsible for intravenous tolerance are closely associated with naive T cells in peripheral lymphoid tissue. The only APCs that fulfil this requirement are interdigitating DC. B cells are normally sequestered from T cells until they arrest in the T cell zones of the spleen and

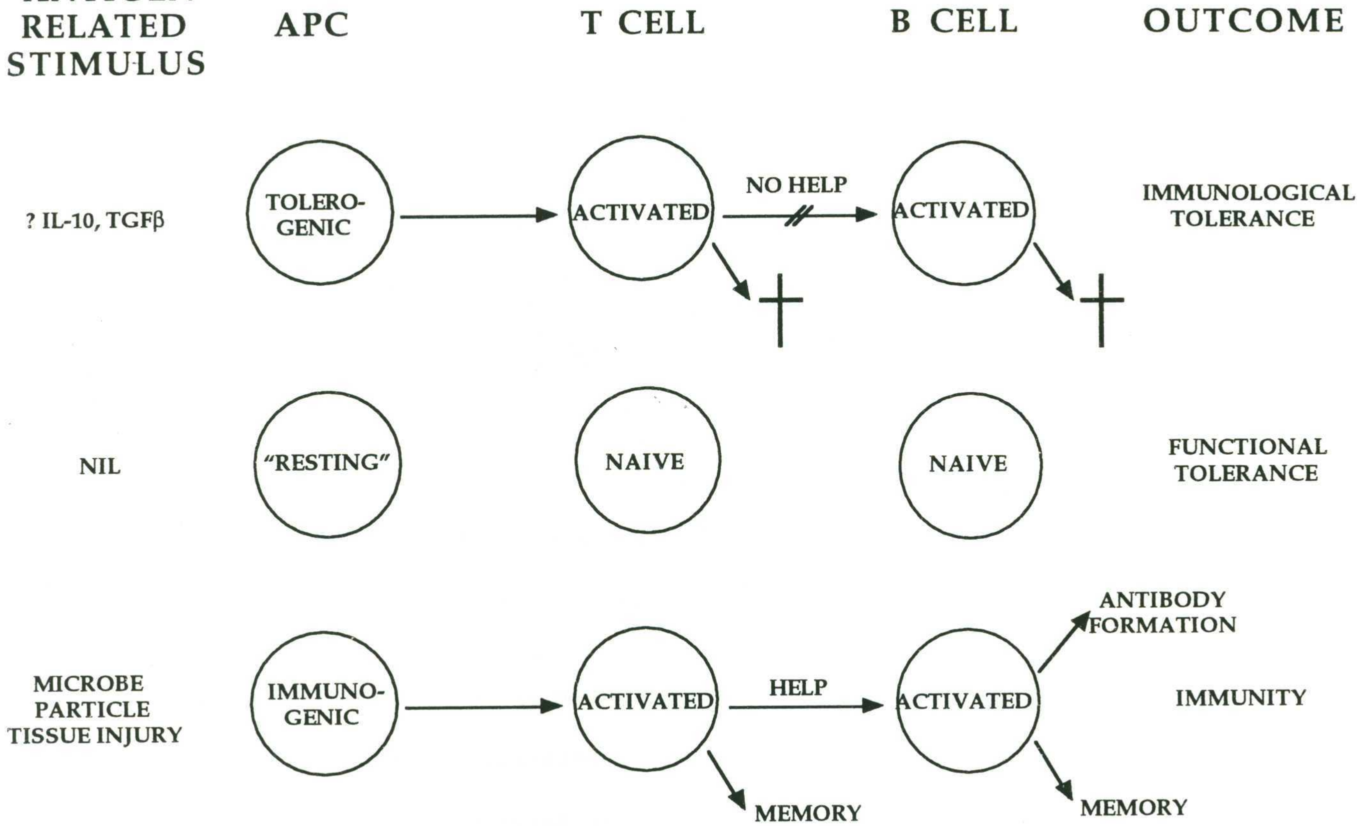


Figure 6.1. A heirarchical model of the regulation of tolerance and immunity. Adapted from Fazekas de St. Groth (1995) with permission.

lymph nodes (Fulcher et al., 1996). This hypothesis is supported by a recent study showing that T cell tolerance to peptide and superantigens can be induced in B-cell-deficient mice (Vella et al., 1996). Studies are under way to explore the role of DCs in tolerance and to test the immunogenicity and tolerogenicity of the myeloid and lymphoid lineages of DC (Fazekas de St. Groth et al., 1997).

Intravenous tolerance can be bypassed by intact antigen (Figure 4.15) suggesting either that tolerogenic APC have a defect in the internalisation or processing of intact antigen, or that antigen-processing alters the functional state of the tolerogenic APC. Analysis of the Tgα profile of CD44^{hi} cells after intravenous protein suggests that low level memory may be generated by this protocol (Figure 4.17). Immunodeficient hosts are also unable to induce tolerance (Figure 4.20-22). Two explanations are possible: that a dominant mechanism controls peripheral T cell number, or that immunodeficient mice lack particular tolerogenic APC populations. Further experiments are required to understand the exact APC-T cell interactions in these two responses.

The cytokine profiles induced by intravenous and subcutaneous peptide are consistently different. In contrast to subcutaneous peptide (Figure 5.3), intravenous peptide fails to prime IL-4 production. Freeman et al. (1995) have observed that APC expressing B7-2, but not B7-1, stimulate human peripheral blood T cells to produce IL-4 *in vitro*. DC and macrophages constitutively express B7-2 and low levels of B7-1, whilst resting B cells may express low levels of B7-1, but not B7-2 (reviewed by Bluestone, 1995). Thus, intravenous peptide may not prime IL-4 production because it is presented by an APC incapable of stimulating IL-4 production. Alternatively, the transient nature of T cell activation by intravenous peptide may be responsible, since IL-4 is induced late after activation of naive T cells (Tanaka et al., 1993).

A detailed investigation of the properties of APC *in vivo* remains a great challenge. The isolation and purification of APC is known to alter their properties (Pure et al., 1990; Inaba et al., 1994) as do cytokines (Doherty and Coffman, 1993) and other signalling

molecules (Hosoi et al., 1993). Such variability in the properties of APC accounts at least in part for the confusion and contradiction in the literature on the immunogenicity and tolerogenicity of various APC populations *in vitro* and *in vivo*. A greater understanding of the tolerogenic and immunogenic features of APC and the factors that modulate these properties is required to further our understanding of the role of APCs in regulating T cell immunity and tolerance.

T cell memory is generated by subcutaneous but not intravenous peptide. The mechanisms of the generation of T cell memory are poorly understood although it is accepted that memory cells are generated at some point during the response to antigen. Two possibilities exist: Tm are derived from a separate lineage of T cells, or they are an end-stage of T cell maturation. Sprent and Miller established early on that the majority of activated T cells are destined to die, with a minority persisting as Tm (Sprent, 1976; Sprent and Miller, 1976a; Sprent and Miller, 1976b). There has been some suggestion that there are specific requirements for the generation of Tm (Swain et al., 1990) but these have yet to be characterised with any certainty. The function of the $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells remaining at the resolution of the intravenous response needs to be formally tested before the possibility that intravenous peptide can generate memory T cells be ruled out. However it is clear from the response to intravenous administration of peptide that the generation of long-lived $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells is not an obligatory consequence of T cell activation. Subcutaneous administration of peptide without adjuvant increased the proportion of long-lived $CD4^{+}Tg\alpha^{+}CD44^{hi}$ cells but not to the extent seen with adjuvant (Figure 5.9), leaving open the possibility that APCs play a role in the generation of Tm.

In conclusion, these studies on peripheral T cell immunity in TCR transgenic mice have provided a thorough framework for the continued study of the mechanisms that regulate tolerance and immunity *in vivo*. Although the two routes of immunisation were used to administer purified foreign antigen, they serve as paradigms for the systemic immune responses to self- and foreign antigen. Subcutaneous administration is well accepted to

mimic exposure to pathogenic antigen via the skin. In contrast, self-antigens are presented in a non-inflammatory fashion which induces tolerance. Although intact foreign antigen administered intravenously could not induce tolerance, it is possible that it lacked "self" markers required for internalisation into tolerogenic APC (Fazekas de St. Groth et al., 1997), for example via the DEC 205 molecule (Jiang et al., 1995). Thus the response to intravenous peptide or superantigens may provide our first insight into the cellular mechanics of peripheral tolerance to self-antigens. The TCR transgenic model will continue to be an effective tool to answer such questions and to further our understanding of how the immune system works to protect the host from within and without.

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