TRANSGENIC CD4 T CELLS PROVIDE HELP FOR MEMORY B CELLS BUT DO NOT SURVIVE FOLLOWING ANTIGENIC CHALLENGE

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Table of Contents

Figures

Tables

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Abbreviations

ALN	auxillary lymph node
Ap	alum-precipitated
APCs	antigen presenting cells
BrdU	bromodeoxyuridine
CFA	complete Freund's adjuvant
CFSE	carboxyfluorescein diacetate succinimidyl ester
CLN	cervical lymph nodes
DC	dendritic cell
DNCB	dinitrochlorobenzene
DTH	delayed type hypersensitivity
ELISA	enzyme linked immunoadsorbant assay
GC	germinal centre
HEVs	high endothelial venules
HSA	heat stable antigen
ICAM	inter cellular adhesion molecule
IFA	incomplete Freund's adjuvant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILN	inguinal lymph node
I.P.	intra-peritoneally
I.V.	intravenously
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FDCs	follicular dendritic cells
LFA	lymphocyte function associated antigen
mAb	monoclonal antibody
MHC	major histocompatibility complex
MLN	mesenteric lymph node
NK cell	natural killer cell
OVA	ovalbumin
OVApep	ovalbumin peptide
PALS	periarterial lymphoid sheath
PBS	phosphate buffer solution
PBST	phosphate buffer solution tween
PE	phycoerythrin
Rpm	revs per minute
RT	room temperature
SA	streptavidin
SCID	severely compromised immune deficient
SD	standard deviation
Sol-OVA	soluble ovalbumin
TCL	T cell line
TCR	T cell receptor
Tg	transgenic
TNF	tumour necrosis factor
VLA	very late antigen

Abstract

Immunological memory has long been recognized and is described as a stronger and faster response upon a secondary infection. It is known that memory B cells produce antibody that is of a different isotype and of a higher affinity than that produced during a primary response. Both primary and secondary B cell responses are dependent on T cell help. However, it is not known whether T cells contribute to the accelerated memory response as specialised antigen experienced cells or by an increase in the number of antigen-specific cells that are available. In other words, is T cell memory a qualitative or a quantitative change?

To try and answer this question we used tg DO11.10 T cells which are specific for OVA peptide 323-339. We wanted to compare the ability of naïve, antigen primed, and long lived memory T cells to provide help for memory B cells. By priming mice with alum-precipitated OVA and then challenging with soluble OVA we could stimulate memory B cells but not naïve B cells. This allowed us to focus on the help that the memory B cells received. In establishing the adoptive transfer model we demonstrated how dependent the B cells were on T cell help, and also how the number of T cells determined the strength of the response. We also showed how a large proportion of the memory B cells resided in the IgM^- population.

When we compared naïve and primed KJ^+CD4^+ T cells in their ability to help primed B cells very little difference was observed. A significant difference ($p<0.05$) was observed on day 7 for both doses of T cells tested (3×10^5, 10^5), though by day 14 and beyond there was no difference. When KJ^+CD4^+ T cells were primed in BALB/cIgh recipients, which received primed B cells 8 weeks later, the response of tg T cell recipients was no different to controls. However when KJ^+CD4^+ T cells were primed in intermediate SCIDs and transferred to BALB/cIgh receipients they did not survive to provide help for a secondary response. Furthermore when primed KJ^+CD4^+ T cells were transferred to naïve SCID recipients they did not survive as well as naïve KJ^+CD4^+ T cells.

We attempted to alter the sequence of the OVA peptide in such a way as to reduce the affinity of the binding between the peptide and MHC. We hoped that this would reduce the strength of the signal via the TCR and thereby prevent the KJ^+CD4^+ T cells from undergoing apoptosis. However we discovered that amino acids 327 and 328 of the OVA peptide were required to induce in vivo proliferation of the KJ^+CD4^+ T cells.

Chapter 1 Introduction

Immunological Memory

Ever since Edward Jenner's pioneering experiment in 1798, when he inoculated an eight-year-old boy with fluid from a cowpox pustule, which resulted in the boy's protection from smallpox, we have been aware of the power of immunological memory. Nearly one hundred years later his work was followed by Louis Pasteur, who developed the first human vaccine in 1885. However, despite the length of time since these early experiments and the infinite number of vaccines administered worldwide annually, our understanding of some of the basic principles of immunological memory is still widely debated.

Although a secondary immune response is characterised by a stronger and faster immune reaction, it is not known whether this is due to an increase in the number of the responding antigen-specific cells or whether it is because the responding cells have been functionally altered by their encounter with antigen. It is also not known what role persisting antigen plays in the formation of long term memory. Debate still occurs as to whether "true" memory cells really exist or whether immunological memory is merely a result of a continued primary response. There is also no undisputed phenotypic marker for "memory" cells, which has hampered our understanding of these cells. Some researchers have even called into question the value of acquiring immunological memory {Zinkernagel 2002}, by arguing that if an individual can recover from a primary infection what need is there for a specialized response upon a secondary infection. However as with numerous biological functions nothing makes sense except when viewed in the light of evolution. As pampered as we are by modern society we forget the selective forces that existed when our genetic make up was shaped. The advantages of a speedy recovery from infection in a world where predators roamed and food had to be collected or hunted are obvious.

Apart from the basic scientific need to fully understand this important biological function there are numerous practical reasons for its study. Since its early beginnings with Louis Pasteur, vaccination has been successful in dramatically reducing the number of cases of measles, mumps, pertussis, rubella, poliomyelitis, tetanus, and smallpox. However despite its great success the potential of vaccines can be exploited further. With greater understanding new diseases may be eradicated and current

vaccines may be made more efficient and cost effective. Two possible strategies in vaccine design exist depending on how we view the persistence of memory. As was said, we still do not know whether persisting antigen is crucial for maintaining memory or whether memory cells originate following the primary proliferation stage. This means that vaccines should be designed either to ensure maximal clonal expansion by providing an optimal dose of antigen and appropriate adjuvant, or vectors should be chosen which would ensure long persistence of antigen. Also, if memory is more a result of a quantitative change rather than qualitative, then repeated administration of the vaccination agent may help prolong protection by continually increasing the number of memory cells. Determining which of these strategies is most protective is important for future vaccine design.

The vast array of viruses, bacteria, protozoans, and parasitic worms that can cause infection have contributed to our uncertainties about immunological memory. As these pathogens cause infection in a variety of ways they also induce various immune responses. Therefore the response that will protect against a gut dwelling nematode will not protect against a blood borne virus. This heterogeneity within the immune system, while protecting us against a multitude of infections, hampers our understanding of what is important for protection.

One result of the complex nature of the immune system is that it often has to be studied *in vivo*. As lymphocytes interact with a wide range of cells, as well as factors such as cytokines and chemokines, it is very difficult to recreate this environment *in vitro*. Therefore numerous experimental models for a variety of diseases have been developed. However it can be difficult also to create an experimental model that fully represents the infection in question.

As our main interest is on memory and not a specific disease we are studying the immune response to ovalbumin (OVA). We used this protein as our model antigen because of the availability of tg mice whose T cells are specific for Ova peptide 323-339. As these tg cells can be stained by a monoclonal antibody (mAb) we could track them *in vivo*. By comparing the ability of naïve and memory tg T cells to help OVA-experienced memory B cells, we hoped to determine whether memory T cells are truly different from naïve T cells. By measuring a functional element, i.e. the ability to help B cells produce antibody, we should be able to detect possible differences that encounter with antigen will cause. In doing so we hoped to show whether T cell memory is a result of "true" memory cells or is merely caused by an increase in the

number of antigen-specific T cells present in the host after the primary response. The answer to this question will have implications for vaccine development as well as numerous other areas of immunological interest.

This thesis is divided into four main chapters. Chapter 3 introduces the reasons for studying tg T cells, and describes previous studies which have used the tg mouse strain DO11.10. The results from studies examining the migration of tg T cells, in the absence and presence of antigen, are shown and discussed. Chapter 4 introduces the subject of B cell memory and shows the results obtained while developing an adoptive transfer model of primed B and T cells. Chapter 5 explains the current ideas on T cell memory and shows the results from comparing naïve and primed tg T cells. It also discusses the survival of tg T cells following antigen stimulation. Chapter 6 introduces the idea of antigen affinity affecting the fate of the T cell and shows the results from studies with shortened peptides. Chapter 7 discusses the implications of all of the results.

Aims

The major aim of this project was to examine differences between naïve and memory $CD4^+$ T cells. A tg mouse model was used to compare the ability of naïve and memory $CD4^+$ T cells in their ability to provide help for a secondary immune response. As tg cells were used it was necessary to examine whether they would behave like non tg cells following adoptive transfer. The effect of antigen presence on their migration was also to be examined.

In order to compare naïve and memory T cells on a functional basis an adoptive transfer model was developed. A system was required that would allow a quantative measurement of the secondary immune response. It was also necessary to demonstrate that the response was antigen specific and did not involve a primary response. A further criterion that was to be examined was that an excess of T or B cells was not transferred. Characterisation of the phenotype of the memory B cells was also to be undertaken.

Once our model was established the ability of naïve and antigen primed tg T cells to provide help for memory B cells would be compared. In order to compare them with naïve $CD4^+$ T cells, it was necessary to create memory T cells. This was to be attempted in both euthymic BALB/cIgh and lymphopoenic SCID mice.

T cell receptor antigen affinity has been implicated as a key factor in the formation of memory cells. To explore this concept the OVA323-339 peptide was manipulated in such a way as to alter the affinity of the tg TCR for this antigen. By reducing the affinity of the peptide it was hoped that it would prevent apoptosis of the tg $CD4^+$ T cells and lead to the creation of memory cells.

Chapter 2

Materials and Methods

2.1 Animals

Animals were bred in specific pathogen-free (SPF) conditions and maintained after weaning under conventional husbandry in the Biological Services Unit of the University of Manchester. Breeding pairs of DO11.10, DO11.10SCID and BALB/cIgH mice were obtained from Prof. Paul Garside (University of Glasgow). The SCID and DO11.10SCID mice were housed in isolated air filtered cages.

DO11.10 mice are a transgenic (tg) strain expressed on a BALB/c (H-2^d) background, in which the germline DNA has been rearranged so that the T Cell Receptors (TCRs) of these mice are specific for Ovalbumin peptide 323 (OVA-pep) (discussed more in Chapter 3). The DO11.10 transgene was bred onto a Severely Compromised Immune Deficiency (SCID) background to avoid possible interference from endogenously rearranged TCRs. BALB/cIgH mice are a congenic mouse strain which express the IgM^b allotype.

To obtain a homozygous DO11.10SCID strain, heterozygous pairs were mated and the offspring tested for the presence of the transgene. This was done by taking approximately 20µl of blood from the tail vein, lysing the red blood cells with Tris buffer, and testing the remaining cells for the presence of tg $CD4^+$ T cells as described in Section 2.22.

DO11.10/SCID or normal DO11.10 mice were used for donor T cells. BALB/cIgH mice were used for donor B cells. Either SCID or BALB/cIgH mice were used as recipient animals.

2.2 Antibodies

Table 2.1 gives a summary of the antibodies used for flow cytometry and ELISA.
The KJ1-26 antibody was produced from a cell hybridoma line held by Scottish Antibody Production Unit (SAPU) and was purified using Prosep affinity chromatography.

Antibody	Clone	Isotype	Usage	Source
Anti-mouse CD45RB	16A	Rat IgG2a, κ	1/100	Pharmingen
Anti-mouse CD19	1D3	Rat (Lewis) IgG2a, κ	1/50	Pharmingen
Anti-mouse CD8a (Ly-2)	53-6.7	Rat (LOU/Wsl/M) IgG2a, κ	1/50	Pharmingen
Biotin KJ1-26			1/100	SAPU
Biotin anti-mouse CD4 (L3T4)	GK1.5	Rat (Lewis) IgG2b, κ	1/1000	Pharmingen
Biotin anti-mouse CD8a (Ly-2)	53-6.7	Rat (LOU/Ws1/M) IgG2a	1/1000	Pharmingen
Biotin anti-mouse CD90.2 (Thy1.2)	30-H12	Rat (LOU/Ws1/M) Ig2b, κ	1/250	Pharmingen
Biotin anti-mouse IgM	R6-60.2	Rat (LOU) Ig2a, κ	1/500(staining) 1/100 (purifying)	Pharmingen
Biotin anti-mouse F4/80	CI:A3-1	Rat IgG2b	1/50	Caltag laboratories
FITC anti-mouse CD45RB	16A	Rat IgG2a, κ	1/100	Pharmingen
FITC anti-mouse CD4 (L3T4)	GK1.5	Rat (Lewis) IgG2b, κ	1/200	Pharmingen
FITC anti-mouse IgD	11-26c.2a	Rat IgG2a κ	1/500	Pharmingen
PE anti-mouse CD4	Rm4-5	Rat (DA) IgG2a, κ	1/100	Pharmingen
PE anti-mouse CD45R/B220	RA3-6B2	Rat IgG2a, κ	1/500	Pharmingen
PE anti-mouse CD44 (Ly-24)	IM7	Rat IgG2b, κ	1/500	Pharmingen
PE anti-mouse CD62L (L-selectin, LECAM-1, Ly-22)	MEL-14	Rat (Fischer) IgG2a, κ	1/500	Pharmingen

Table 2.1. Antibodies used.

List of antibodies used for flow cytometry including the antibody clones, isotype, dilution used, and source.

Antibody	Clone	Isotype	Usage	Source
PE anti-mouse Fas Ligand (FasL, CD95 Ligand)	MFL3	Armenian Hamster IgG, group 1, κ	1/500	Pharmingen
PE anti-mouse CD69	H1.2F3	Armenian Hamster IgG, group 1, κ	1/100	Pharmingen
PE anti-mouse CD49d (Integrin α_4 chain)	R1-2	Rat (Fischer) IgG2b, κ	1/100	Pharmingen
PE anti-mouse CD25 (IL-2 receptor α chain)	PC61	Rat (Outbred OFA) IgG1, κ	1/100	Pharmingen
Streptavidin-PE			1/500	Sigma
Streptavidin Tri-colour			1/200	Caltag laboratories
Rat Anti-mouse CD16/CD32 (FcγIII/II Receptor) "Fc Block"	2.4G2	Rat (Sprague-Dawley) IgG2b, κ	use at 1μl	Pharmingen
F (ab)$_2$ Goat Anti Rat IgG:FITC			1/200	Serotec
Alkaline phosphatase-conjugated goat anti-mouse IgG	A-9316	Goat	1/1000	Sigma

Table 1 (continued). Antibodies used.

List of antibodies used for flow cytometry including the antibody clones, isotype, dilution used, and source.

2.21 Biotinylation of the KJ1-26 Monoclonal antibody (mAb)

1. 1.57mg (peak 2) of purified KJ1-26 antibody was concentrated in a centricon-30 concentrator (Amicon) by centrifuging at 2800rpm (g=5) at 4°C for 30 min. The antibody was centrifuged until it was concentrated in a volume of 100µl.
2. Sodium bicarbonate buffer (0.05M; pH 8.5) was added to the top of the centricon and it was centrifuged as described in step 1. This was repeated again in order to concentrate the antibody in sodium bicarbonate.
3. A 10mM solution of sulf-NHS-biotin (Pierce) in sodium bicarbonate was made up, of which 57µl was added to the concentrated antibody. The volume was brought up to 1ml with sodium bicarbonate. This was incubated at RT for 1.5 hours.
4. The antibody/biotin cocktail was transferred to a centricon, filled to the top with sodium phosphate (0.1M; pH 7) and centrifuged as described in step 1. This was repeated twice.
5. The final biotinylated antibody was resuspended to 2ml in sodium phosphate. 30µl (0.01%) sodium azide was added to the bio-KJ1-26 and stored at 4°C in 1ml aliquots.

2.22 Antibody Staining Procedure for Flow Cytometric Analysis

For flow cytometric analysis 10^6 cells were taken from each cell population. For determining the percentage of KJ^+ cells, they were firstly stained with 0.5µg rat anti-mouse CD16/CD32 (FcIII/II Receptor) " Fc Block" for 10 mins on ice, followed by 10pg of CD45RB (FITC), 4pg CD4 (PE), and 0.157µg KJ-126 (bio) (concentrations in table 1) for 30 mins on ice. Cells were washed with PBSFCS (2% sodium azide) (see Appendix) and stained with Tri-SA for 30 mins on ice. Cells were washed again with PBSFCS (1% sodium azide), and examined on a BD flow cytometer as described in section 2.9. For staining of other cell populations (B cells) 10^6 cells were stained for 30 mins on ice with the appropriate antibody.

(a)

Antigen type	Used for:
ap-OVA	Priming naïve B cells or naïve tg KJ^+ T cells
ap-OVA peptide 323-339	Priming naïve KJ^+ T cells
sol-OVA	Secondary challenge

(b)

Peptide	Sequence	Molecular Weight
323 OVA	ISQAVHAAHAEINEAGR	1774
327 OVA	VHAAHAEINEAG	1218
328 OVA	HAAHAEINEAG	1119

Table 2.2. The different forms of antigen used.

A summary of the type of antigen used and its purpose (a) and the amino acid sequence of the OVA peptides (b). (ap = alum-precipitated, sol = soluble).

Peptide	Concentration	Molarity	Amount per 100µl	µmoles injected
323 OVA	1774 g/l	1M	N/A	N/A
323 OVA	1.774 mg/ml	1mM	N/A	N/A
323 OVA	1.0 mg/ml	0.56mM	100µg	0.056
323 OVA	0.887 mg/ml	0.5mM	88.7µg	0.05
327 OVA	0.609 mg/ml	0.5mM	60.9µg	0.05
328 OVA	0.559 mg/ml	0.5mM	55.9µg	0.05

Table 2.3. Antigen concentrations.

The concentration, molarity, and amount of OVA peptide injections.

2.3 Antigen

Chicken ovalbumin (OVA), (Grade V, Sigma, Poole) was the antigen upon which all experiments were based. However, different forms were used as shown in Table 2. Antigen was always injected intraperitonealy (i.p.). For primary immunization 100μg of antigen was injected, for secondary challenge 10μg was injected. The OVA full length peptide 323-339 (323 OVA) and the shortened peptides were synthesised by Sigma Genosys. The amino acid sequences for these peptides are in Table 2(b).
For studies with the shortened peptides the dose was based on molarity instead of weight. The relationship between molarity and dose is shown in Table 3. For convenience we opted to inject 0.5mM as the standard dose.

2.3.1 Alum-precipitation of peptides.

Intact ovalbumin or the OVA peptides were alum-precipitated as follows.
To 100μl of 10mg/ml OVA, 450ul 1M sodium bicarbonate was added. To this, 1ml of 0.2M aluminium potassium sulphate was added with constant stirring. This was left for 15 minutes at RT and washed thrice in PBS (centrifuge). The pellet was resuspended to1ml (1mg/ml) to give the desired concentration for injection.

2.4 ELISAs

To detect the amount of anti-OVA antibodies in the serum of challenged mice an enzyme linked immunoadsorbant assay (ELISA) was performed on each sample as follows:

96 well flat bottom microtitre flexible assay plates (Dynex) were coated for 1 hr with 50μl OVA binding solution (OVA diluted at 100μg/ml in 0.05M carbonate/bicarbonate buffer at pH 9.6 with 0.02% sodium azide) per well at room temperature. Plates were washed x 5 with Phosphate buffer solution containing 0.02% Tween (PBST). Plates were incubated for 1 hr with 100μl blocking buffer per well (Megablock from Bionostics, 1/500 in PBST). Plates were washed x 5 with PBST. 25μl of sample, standard, and control serum was added to 50μl of blocking buffer per well. The first two columns on each plate contained normal mouse serum, the third and fourth column contained the standard serum (described in Section 2.5) and the remainder of the plate contained experimental samples. All samples were diluted threefold starting from 1/90 and run in duplicate. After 1hr incubation at RT plates were

washed with PBST, incubated with 50µl of alkaline phosphatase-conjugated rabbit anti-mouse IgG (1/1000 in PBST) per well, and washed again x 5 with PBST. Plates were incubated with 50µl of p-nitrophenyl phosphate substrate (5mg tablet, from Sigma dissolved in 5ml of diethanolamine buffer, 1 mg/ml, pH 9.8) per well in the dark for between 15 and 20 mins. The reaction was stopped with 100µl of 3M NaOH per well, and the OD read at 405 nm using a Dynex MRX II ELISA reader. The results were analysed using Dynex Revelation 4.21.

2.5 Preparation of ELISA standard.

In order to measure the test serum accurately a standard serum of known anti OVA concentrations had to be created. This was done, by immunizing a group of normal BALB/cIgH mice with 100µg of ap-OVA, followed by a secondary challenge of 10µg soluble OVA 8 weeks later. Two weeks following the secondary challenge the mice were killed and serum was collected, from which the desired antibodies were affinity purified as described in section 2.5.1.

2.51. Preparation of CNBr activated Sepharose 4B beads.

1g freeze-dried CNBr activated Sepharose 4B beads (obtained from Pharmacia) was suspended in 1mM HCl (the beads then swelled to 3.5ml). This was washed on a sintered glass filter (connected to a suction pump) with 200mls 1mM HCl. 20mg OVA was dissolved in 100µl of coupling buffer (0.1M $NaHCO_3$, pH 9.3 containing NaCl). The 3.5mls CNBr beads and the 100µl OVA and coupling buffer were mixed and rotated end over end overnight at 4°C. The excess ligand was washed away with 20mls of coupling buffer. Any remaining active groups were blocked with Tris-HCl buffer (0.1M, pH 8) for 2 hours at room temperature. The product was then washed with three cycles of alternating pH. Each cycle consisted of a wash with 15mls of acetate buffer (0.1M, pH4) containing NaCl (0.5M) followed by a wash with Tris buffer (0.1M, pH 8) containing NaCl (0.5M). The product was then stored at 4-8°C.

2.52. Affinity Elution.

A glass column was set up with a pump, which was connected to an ultra violet spectrometer for measuring protein concentration. The column was filled with a 1:1 mixture of binding buffer, (pH 7) and the CNBr Sepharose 4B (OVA) beads. With the

pump at speed 8 this column was allowed to settle and washed with binding buffer (0.2M sodium phosphate pH7) for 1 hour. 2mls of serum from the immunized mice were diluted to 4mls with PBS, (in retrospect the serum should have been diluted with binding buffer) and was pumped over the column. The protein concentration of the run off solution was measured. Elution buffer (Glycine-HCl acid pH 2.7) was then washed over the column to allow release of the anti-OVA antibodies from the column. This was collected into 70µl of neutralizing buffer (1M Tris-HCl pH9). 20 drops (1ml) were collected at a time, each of which was read with the u.v. spectrometer at 280nm. The samples corresponding to the peak (obtained by the u.v. spectrometer) were retained and pooled as these contained the desired antibody. This solution was dialysed in 4 litres of PBS overnight at 4°C. The PBS solution was replaced twice the following day. After 22 hours in dialysis solution the volume remaining was found to be 1.8218mls. The protein concentration was determined photometrically at 280nm (extinction coefficient 1.35) which gave a value of 0.2962mg/ml of anti-OVA antibody.

This affinity-purified antibody was tested by ELISA concurrently with unpurified anti-OVA serum. By diluting the affinity purified anti-OVA protein, an OD reading could be directly linked to the relative concentration of specific antibody. This in turn was used to determine the absolute concentration of anti-OVA antibody in the original pool of anti-OVA sera. The concentration of the original pool was calculated using the mean of values obtained from dilution of 1/810 to 1/21870 (Figure 2.1) and resulted in a value of 6674µg/ml. The pooled anti-OVA serum was subsequently used as the standard on each ELISA plate to produce the "standard curve" (Figure 2.2).

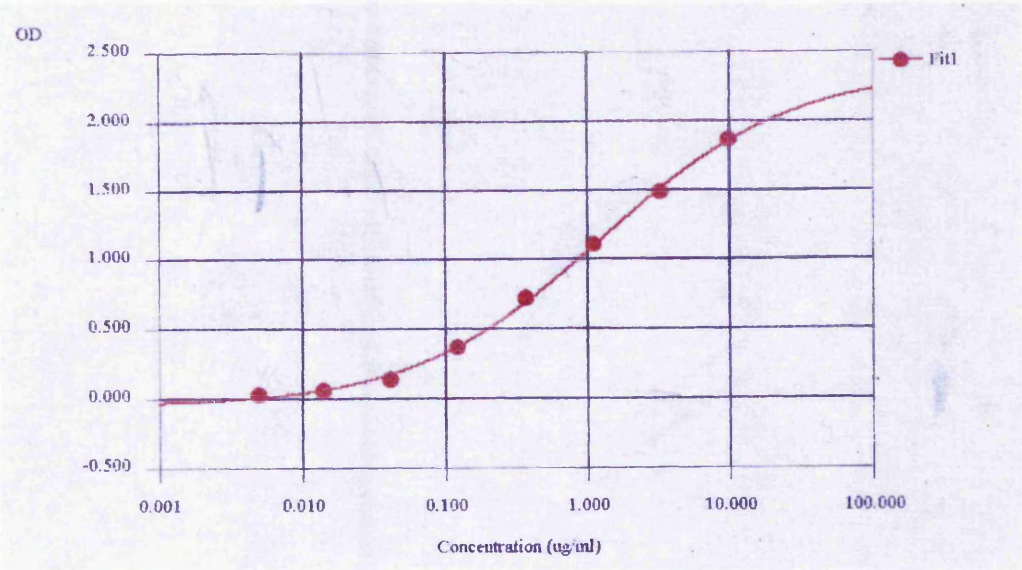


Figure 2.1. Dilution curve of purified anti OVA antibody.

Dilution curve of purified anti OVA antibody used to calculate absolute concentration of specific antibody

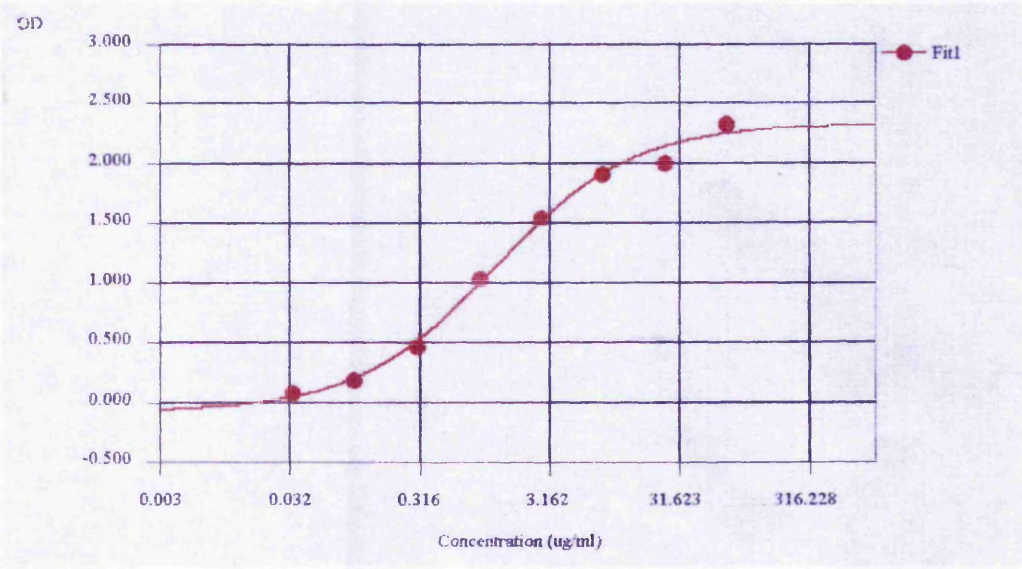


Figure 2.2 Dilution curve of serum from OVA primed mice.

Dilution curve of serum from OVA primed mice used as a standard curve in ELISAs.

2.6 B Cell Purification.

To obtain primed B cells, normal BALB/cIgH mice were injected i.p. with 100μg/ml of ap-OVA. 8 weeks later the mice were killed and spleen, MLN, ILN, CLN, and ALN were collected. Single cell suspensions were made, by teasing apart the tissues with forceps and passing through a monofilament filter. The red blood cells of the spleen were lysed, by resuspending in Boyles solution (1ml per spleen, see Appendix) at 37°C for 5 minutes. The cells were washed with PBSFCS and pooled. Viable cells were automatically counted using a Scharfe system CASY1 counter. A sample of 10^6 cells was removed and later stained with PE-B220 to assess starting percentage of B cells. The total cell population was stained with bio-CD8 (12pg per 10^8 cells), bio-CD4 (24pg per 10^8 cells), bio-Thy1.2 (96pg per 10^8 cells), and bio-F4/80 (96pg per 10^8 cells) for 30 minutes on ice. For purification of IgM^- B cells, bio-IgM was added at 2.4μg per 10^8 cells. A sample of 10^6 cells was removed and later stained with SA-PE to assess the percentage of cells, which had been stained with the biotin antibodies. Cells were washed twice with PBSFCS. In preparation for separation Biomag® Streptavidin beads (Metachem Diagnostics Ltd) were washed x 4 with PBSFCS. The cells were incubated with 50μl of beads per 10^8 cells, for 30 minutes on ice. The cell/bead suspension was placed on a strong magnet and allowed to adhere. The ferrous beads with cells attached stuck to the magnet while the cells remaining in suspension were poured off. The cells were washed twice with PBSFCS. A sample of 10^6 cells was stained with SA-PE to assess the purity of the separation. The cells were depleted again using 200μl of beads per 10^8 cells for 30 minutes on ice. The cells adhering to the beads were removed on a magnet while the cells remaining in suspension were poured off and washed twice. A sample of 10^6 cells was removed and later stained with SA-PE to assess the purity of the second separation. Finally the cells were counted and a sample stained with PE-B220 to assess the percentage of B cells in the final population. All samples were examined by flow cytometry as described in section 2.9. The B cells which were injected were between 80% and 90% $B220^+$ (Figure 2d, Chapter 4), however the percentage of T cells in the population was less than 0.05% (Figure 2c, Chapter 4).

2.7 T cell purification.

To purify a population of T cells for the migration studies DO11.10 mice were killed and MLN, ILN, ALN, and CLN removed. Single cell suspensions were made, by teasing apart the tissues with forceps and passing the subsequent suspension through a monofilament filter. The cells were washed twice in PBSFCS and counted using a Scharfe system CASY1 to determine a viable cell number. A sample was stained to determine the number of transgenic cells. The cells were stained with anti-mouse CD8 and anti-mouse CD19 for 30 minutes on ice. The cells were washed with PBSFCS and the labelled cells were removed by 2 rounds Biomag® goat anti-rat IgG beads (Metachem Diagnostics Ltd) as previously described in Section 2.6 for Biomag® Streptavidin beads. 20µl of beads were used per 10^6 cells. The remaining population of cells were stained as described in section 2.22 and examined on a BD flow cytometer to determine purity and number of cells for transfer. Of the cells that were injected ~55% were KJ^+CD4^+, ~25% KJ^-CD4^+, and the remaining cells were B cells which were not removed by the purification (Figure 2b, c).

2.8 Preparation of naïve donor T cells.

DO11.10SCID mice were killed and MLN, ILN, ALN, and CLN removed. A single cell suspension was prepared by teasing apart these tissues with forceps and passing through a monofilament filter. The cells were washed x 2 in PBSFCS and counted using a Scharfe system CASY1 to determine a viable cell number. Cells were stained as described in Section 2.22 and examined on a BD flow cytometer as described in Section 2.11. Based on the number of KJ^+CD4^+ T cells in the population the cells were resuspended in PBSFCS for injection into donor mice.

2.9 Preparation of primed $CD45RB^{lo}$ donor T cells.

Naïve KJ^+CD4^+ cells were prepared as described in Section 2.7. Either 1 x 10^6 or 5 x 10^6 KJ^+CD4^+ cells were injected i.v. to SCID recipients and primed i.p. with 100µg ap-OVA peptide. 7 to 14 days later recipient mice were killed and spleen, MLN, ILN, ALN, and CLN were removed to prepare single cell suspensions. The cells were counted and a sample stained to determine the number of donor transgenic cells. The remaining cells were stained with anti-mouse CD45RB (0.5µg per 10^8 cells) and

depleted of $CD45RB^+$ cells using 2 rounds of Biomag® goat anti-rat IgG beads (Metachem Diagnostics Ltd) as previously descried in Section 2.6 for Biomag® Streptavidin beads. 20µl of beads were used per 10^6 cells. The remaining population of cells was stained as described in section 2.22 and examined on a BD flow cytometer to determine purity and number of cells for transfer. The percentage of KJ^+CD4^+ T cells was between 5% and 10% of which 95% were $CD45RB^{lo}$ cells (Figure 5.2 (e) (f), Chapter 5).

2.10 CFSE labelling.

A suspension of LN cells from DO11.10 mice was washed twice in PBS, resuspended to 10 x 10^6/ml in PBS and warmed for 5 min at 37°C. CFSE was added to the cells at a concentration of 1pmole/10^7 cells and incubated for 15 min at 37°C. FCS was added to the cell suspension to achieve a final concentration of 10%. The cells were mixed, centrifuged for 10 mins (g = 5), washed x 2 in cold PBSFCS and resuspended at the desired concentration for injection into recipients.

2.11 Flow Cytometry.

All stained populations were analyzed using CellQuest software in conjunction with a FACScan cytometer (Becton Dickinson, Cowley, UK). All flow cytometry target populations were electrically gated by forward and side scatter for the characteristics of lymphocytes and the exclusion of dead and red blood cells. Transgenic T cells were identified, by the double positive staining of KJ1-26 and Tri-SA in FL3 and PE-CD4 in FL2. FITC-labelled antibodies and CFSE were examined in FL1.

2.12 Serum Preparation.

For obtaining blood samples mice were placed in a restrainer and the end of the tail sniped with a pair of scissors. 25µl of blood was removed and diluted 1/10 in PBS20 (PBS + 20 units/ml heparin to prevent clotting). This solution was centrifuged for 10 minutes (g = 5), the supernatant was drawn off and frozen at 20°C until required for ELISA.

For exsangunination mice were anaethesized and blood removed by cardiac puncture with a syringe and a needle. The blood was allowed to clot at RT for 1 hour.

The clot was removed, the blood centrifuged and the serum recovered and stored at 20°C until further use.

2.13 Calculation of movement of donor T cells.

The movement of donor T cells was calculated using the following equations:
Percentage $CFSE^+$ cells per tissue x total cell count = number of donor cells per tissue.
Number of donor cells in spleen + MLN + ILN + CLN = total number of donor cells per recipient.
Number of donor cells per tissue/Total number of donor cells = relative percentage donor cells per tissue.

2.14 Statistics.

Statistical differences between experimental groups were determined using the student T test. Differences were considered significant if $p < 0.05$.

Chapter 3

Use of DO11.10 tg mice

3.1 Introduction

3.1.1 Tracking antigen-specific cells *in vivo*.

Much of our knowledge on the behaviour of antigen-specific T cells has come from indirect means. For example T cell activation has been measured by analysing thymidine incorporation or cytokine production by antigen stimulated cultures of lymph node cells. Obvious flaws with this approach are that it does not distinguish between the antigen-specific cells or the cells in the culture that they stimulate. Also, studying cells *in vitro* precludes the complex factors that are present within the secondary lymphoid tissues. The isolation of antigen-specific T cell clones led to a better understanding of antigen recognition by the T cell receptor (TCR), as well as the biochemical signals which occurred during T cell activation (Chan *et al.*, 1994) (Mondino & Jenkins, 1994), and produced evidence that optimal stimulation of T cells required co-stimulatory molecules such as CD28 (Linsley & Ledbetter, 1993) (Mueller *et al.*, 1989). Nonetheless, T cell clones are limited as they become pre-programmed to a particular differentiation pathway and do not always share the flexibility of naïve T cells. They must also be generated *in vitro* which is essentially an artificial environment. Tracking naive antigen-specific cells *in vivo* is prevented by the low frequencies of these cells. Limiting dilution studies have suggested that the population of T cells specific for any given antigenic peptide could be less than 1 per 100,000 T cells in unimmunized, and less than 1 per 10,000 T cells in immunized individuals (Tse *et al.*, 1980). These low frequencies would be undetectable by flow cytometry and immunohistochemistry, even if antibodies were available for each antigen-specific cell. Recently with the creation of MHC class I tetramers, tracking of $CD8^+$ T cells *in vivo* has become possible (Altman *et al.*, 1996). However despite the success of MHC class I tetramers, MHC class II tetramers have proved more difficult to create. This is because the peptide in question must be covalently attached to the class II MHC molecule (Kozono *et al.*, 1994). Therefore tracking of native $CD4^+$ T cells *in vivo* remains elusive.

3.1.2 TCR transgenic cells.

Most of these obstacles were overcome with the creation of the TCR transgenic DO11.10 mice that expressed a TCR that was specific for a defined peptide/MHC complex (Murphy *et al.*, 1990). The germline DNA of these mice contains rearranged TCR-Vα and TCR-Vβ genes that encode a TCR specific for chicken ovalbumin peptide 323-339 (OVA-pep) bound to I-A^d class II MHC molecules (Murphy *et al.*, 1990). The tg TCR can be detected with the clonotypic monoclonal antibody KJ1-26 that binds only to this particular heterodimer (Haskins *et al.*, 1983). The transgene inhibits rearrangement of endogenous TCR genes and the tg TCR is MHC class II restricted, so a large proportion of the T cells in these mice are positively selected in the thymus and survive as KJ1-26 $(KJ)^+CD4^+$ T cells (Murphy *et al.*, 1990). A small but significant population of KJ^-CD4^+ T cells also survive and can be detected in the periphery. These cells probably express TCRs which contain the tg TCR-Vβ chain paired with an endogenous TCR-Vα chain, however they are not present in DO11.10/RAG KO mice which cannot undergo endogenous TCR gene rearrangements (Lee *et al.*, 1996). In order to avoid possible non-specific interferences from endogenous rearrangement, the transgene has been bred onto a BALB/c-SCID background where α chain rearrangement cannot occur. The majority (90%) of the KJ^+CD4^+ T cells from DO11.10 mice have a phenotype associated with a naïve cell, $CD45RB^{hi}CD62L^{hi}$ as would be expected from mice that have never been exposed to OVA (Lee *et al.*, 1996) (personal observation). The remaining KJ^+CD4^+ T cells have an activated phenotype $CD45RB^{lo}CD62L^{lo}$, which may represent recent thymic emigrants (Yang & Bell, 1994), or it may have been acquired because of expression of a second TCR containing an endogenous TCR-Vα (Lee *et al.*, 1996). This second TCR probably responds to environmental antigens, as $CD45RB^{lo}$ T cells are not found in normal unprimed BALB/c mice raised in a germ-free environment (Lee *et al.*, 1990). Nor are they found in DO11.10/RAG KO or DO11.10/SCID mice (Lee *et al.*, 1996) {personal observation}, which cannot undergo endogenous TCR gene rearrangement. Curiously, immunizing the tg mice directly with OVApep fails to induce responses that were characteristic of primary and secondary responses {E. Bell personal communication} (Kearney *et al.*, 1994). Why this occured is not known, though it may be caused by clonal competition between the artificially abundant TCR-transgenic T cells for peptide/MHC-bearing APCs which results in inefficient T cell activation (Pape *et al.*,

1997a). However by adoptively transferring tg T cells to normal mice where they were no longer the dominant population, the tg T cells behaved normally and could be primed in a conventional manner following immunization (Kearney *et al.*, 1994). This situation also allowed the tracking of a small population of antigen-specific $CD4^+$ T cells *in vivo* which previously was not possible.

It must be said that the first publication that uses the DO11.10 tg mice (Murphy *et al.*, 1990) does not describe the creation of this tg strain. For this, Murphy *et al* referred to a publication "in preparation". However despite the widespread use of this tg model I could not find any evidence of the reference in question. Therefore the exact details of how this mouse was created are not in the public domain.

3.1.3 Behaviour of KJ^+CD4^+ T cells following adoptive transfer.

In experiments where 2.5 x 10^6 KJ^+CD4^+ T cells were injected intravenously (i.v.) into BALB/c mice, the lymph nodes of these mice contained ~0.5% KJ^+CD4^+ T cells 24 hours later (Pape *et al.*, 1997a). Over the next two weeks the tg T cell numbers declined and a month later were undetectable (Pape *et al.*, 1997a). The T cells did not appear to be proliferating as judged by their lack of bromodeoxyuridine (BrdU) incorporation and were found in the blood, the T cell rich paracortical regions of the lymph nodes, and the periarterial lymphoid sheath (PALS) of the spleen (Pape *et al.*, 1997a). The T cells could not be detected in the B-cell rich follicles, the peritoneal cavity, or the parenchymal components of the liver and lungs (Pape *et al.*, 1997a). As expected these tg cells expressed cell surface phenotypes associated with naïve T cells, $CD45RB^{hi}CD62L^{hi}LFA\text{-}1^{lo}$ (Pape *et al.*, 1997a).

3.1.4 Behaviour of KJ^+CD4^+ T cells following adoptive transfer and antigen challenge.

Injection of soluble OVA or the OVA peptide in the absence of adjuvant resulted in the rapid activation of the transferred KJ^+CD4^+ T cells *in vivo* (Pape *et al.*, 1997a). As soon as 3 hours after i.v. injection of the OVA peptide, some of the T cells were observed to be producing IL-2 as assessed by intracellular lymphokine staining (Pape *et al.*, 1997a). Two days after OVA injection the number of cells in the lymph nodes and spleen had increased significantly and by day 3 the numbers had peaked at a level 7 times greater than the starting number (Pape *et al.*, 1997a). All of the KJ^+CD4^+

T cells appeared to be dividing as assessed by BrdU incorporation and had acquired an activated phenotype, $CD45RB^{lo}CD62L^{lo}LFA\text{-}1^{hi}$ (Pape *et al.*, 1997a). As well as being present in the lymph node paracortex and the PALS of the spleen, the $KJ^{+}CD4^{+}$ T cells also appeared in the liver and lung parenchyma (Pape *et al.*, 1997a). However they did not enter the B cell follicles after soluble antigen injection (Pape *et al.*, 1997a). After day 3 the number of $KJ^{+}CD4^{+}$ T cells fell dramatically and by day 7 few cells were detectable. Interestingly the few remaining $KJ^{+}CD4^{+}$ T cells on day 14 responded poorly to *in vitro* restimulation compared with naïve cells and failed to accumulate in the draining lymph nodes following a second *in vivo* injection with OVA. Injection of soluble OVA also failed to produce a significant anti-ovalbumin antibody response (Pape *et al.*, 1997a). It should not be surprising that stimulation with soluble antigen alone was ineffective at producing a persistent, expanded population of hyper-responsive memory T cells. It has long since been demonstrated that soluble foreign peptides are incapable of eliciting primary antibody production unless administered together with an adjuvant (Dresser, 1961) (Freund *et al.*, 1937). Injection of soluble antigen alone fails to induce a primary immune response and, in contrast, may induce a state of antigen-specific T cell tolerance. Therefore for T cells to become activated, additional signals normally stimulated by bacterial molecules, and mimicked by adjuvants, are required.

3.1.5 Effect of OVA plus adjuvant on transferred $KJ^{+}CD4^{+}$ T cells.

Injection of OVA peptide emulsified in incomplete (IFA) or complete Freund's adjuvant (CFA) resulted in a significantly higher level of accumulation of $KJ^{+}CD4^{+}$ T cells in the draining lymph nodes compared with injection of soluble OVA peptide (Pape *et al.*, 1997a). The majority of these cells were dividing, as assessed by BrdU incorporation (Pape *et al.*, 1997a). In contrast with soluble OVA injection where the $KJ^{+}CD4^{+}$ T cell numbers peaked on day 3, when Freunds adjuvant was used the $KJ^{+}CD4^{+}$ T cells peaked on day 5 and at a higher level (3 times as many) (Pape *et al.*, 1997a). However there was also a dramatic decline in cell numbers after this time point, and between day 8 and day 18 a stable level was reached which was much lower than the peak but greater than the starting level (Pape *et al.*, 1997a). This was in contrast to the injection of soluble OVA peptide when few $KJ^{+}CD4^{+}$ T cells were observed after day 5 (Pape *et al.*, 1997a). However the presence of $KJ^{+}CD4^{+}$ T cells

after day 18 was not examined in either situation. As with the injection of soluble OVA, injection of OVA plus CFA lead to the KJ^+CD4^+ T cells acquiring an activated phenotype, $CD45RB^{lo}CD62L^{lo}LFA\text{-}1^{hi}$, and to appear in the liver and lung parenchyma (Pape *et al.*, 1997a). Injection of OVA plus CFA also resulted in the appearance of KJ^+CD4^+ T cells in the B cell follicles by day 3 (Kearney *et al.*, 1994). The KJ^+CD4^+ T cells that were found weeks after OVA plus CFA injection were able to proliferate in response to lower doses of peptide *in vitro* than naïve KJ^+CD4^+ T cells and produced more IFN-γ but less IL-2 (Kearney *et al.*, 1994).

LPS has adjuvant properties on KJ^+CD4^+ T cells when injected with OVA peptide, resulting in an increased accumulation of cells in the lymph node follicles (Pape *et al.*, 1997b). Its effects can be mimicked by injection of either recombinant TNF-α or IL-1, suggesting that its effect was inflammatory (Pape *et al.*, 1997b). Injection of OVA plus LPS or TNF-α resulted in greater anti-ovalbumin antibodies than injection of soluble OVA. However LPS induced IgG1 and IgG2a production, where as TNF-α only resulted in IgG1 production (Pape *et al.*, 1997b). When IL-12 was injected with OVA peptide and TNF-α both IgG1 and IgG2a were produced (Pape *et al.*, 1997b). Mice which received OVA plus LPS had much lower anti-OVA IgG2a antibodies than mice which also received KJ^+CD4^+ T cells, suggesting that these T cells helped the host B cells to switch their Ig production (Pape *et al.*, 1997b).

In summary, these studies have shown us that following injection, the KJ^+CD4^+ T cells could be detected in the blood, the T cell-rich paracortical regions of the lymph nodes, and the PALS of the spleen. With no antigenic stimulus they were soon undetectable. If OVA peptide was injected alone, the T cells acquired an activated phenotype, increased in numbers in the T cell regions of the lymph nodes and spleen, and also appeared in the liver and lung parenchyma. By day 7 they were also soon undetectable. However if OVA peptide was injected with IFA or CFA the KJ^+CD4^+ T cells also acquired an activated phenotype, increased greatly in cell numbers in the lymph nodes and spleen, and appeared in the liver and lung parenchyma. In contrast they also appeared in the B cell follicles. The T cell clonal expansion in both instances appeared to be dependent on the CD28/B7 interactions (Pape *et al.*, 1997a). However the presence of an adjuvant provided an additional signal for the KJ^+CD4^+ T cells to migrate into the B cell regions.

3.1.6 *In vitro* studies with KJ^+CD4^+ T cells.

Tg T cells can be used to study the response of antigen-specific T cells to different forms of antigen presentation. They can also be used to show the changes that occur in cell division and phenotype following activation. This was shown in an *in vitro* study that utilized the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) (Lee & Pelletier, 1998). CFSE binds to tubulin in the cytoplasm and is transferred in equal amounts to daughter cells after mitosis (Lyons & Parish, 1994). Therefore there is a stepwise halving of CFSE fluorescence during successive cell divisions (Lyons & Parish, 1994). Daughter cells from each division appear as identifiable peaks of decreasing fluorescence during flow cytometric analyses.

CFSE labelled KJ^+CD4^+ T cells were stimulated *in vitro* with OVA peptide and showed characteristic signs of cell division as soon as 48 hours later (Lee & Pelletier, 1998). A correlation of the peak times of cell division (between 60 and 72 hours) as assessed by CFSE staining and [^{3}H]TdR incorporation was observed (Lee & Pelletier, 1998). In culture the OVA peptide stimulated the KJ^+CD4^+ T cells exclusively, demonstrating the antigen-specific nature of this tg model. Although not all of the cells underwent cell division, 94.6% of the KJ^+CD4^+ T cells had divided by the end of the culture (Lee & Pelletier, 1998). The expression levels of the TCR (DO11.10 clonotype) were not altered with either activation or cell division (Lee & Pelletier, 1998). However expression of CD4 increased prior to cell division and appeared to be an early marker of activation, as only cells which upregulated this molecule went on to divide (Lee & Pelletier, 1998). As expected some of the cells became $CD45RB^{lo}$ (discussed in Chapter 5), but not until after 3 rounds of cell division (Lee & Pelletier, 1998). In contrast a portion of the cells became $CD62L^{lo}$ before undergoing cell division, although by the end of the culture the majority of the cells were $CD62L^{lo}$ (Lee & Pelletier, 1998). The discordance observed between decreased CD45RB and CD62L expression was probably due to the way that these molecules are lost. CD62L is actively shed from the cell surface and is not replaced (Chao *et al.*, 1997) (Jung *et al.*, 1988), where as expression of CD45RB changes from that of the high molecular weight isoform to the low molecular weight isoform (Dianzani *et al.*, 1990). As loss of $CD45RB^{hi}$ expression appeared to occur gradually, it may have been due to the dilution of surface molecules (Lee & Pelletier, 1998). CD44 expression appeared similar to that

of CD4, in that stimulated cells upregulated CD44 prior to the onset of cell division and high expression levels were maintained throughout the culture (Lee & Pelletier, 1998).

3.1.7 $CD45RB^{lo}KJ^{+}CD4^{+}$ effector/memory T cells from OVA-naïve mice.

In a previous study the authors had identified a population of $CD45RB^{lo}KJ^{+}CD4^{+}$ T cells within unimmunized DO11.10 mice (Lee *et al.*, 1996). As previously indicated, this phenotype was probably acquired because of the expression of a second TCR containing an endogenous TCR-Vα which responded to environmental antigens (Lee *et al.*, 1996). However these cells displayed certain "memory characteristics". For example, when the purified $CD45RB^{lo}KJ^{+}CD4^{+}$ T cells were compared with $CD45RB^{hi}$ T cells following *in vitro* peptide stimulation, the $CD45RB^{hi}$ T cells produced high levels of IL-2 but no other cytokines, while the $CD45RB^{lo}$ T cells produced low levels of IL-2, and significant levels of IL-5, IL-10, and IFN-γ (Lee *et al.*, 1990). More interestingly the authors reported that when these T cells were co-cultured with hapten (TNP)-specific B cells and stimulated by TNP-OVA (whole molecule), Ig (specificity unknown) was only detected in the cultures containing $CD45RB^{lo}KJ^{+}CD4^{+}$ T cells (Lee *et al.*, 1990). This was despite the fact that both sets of T cells proliferated under these conditions (Lee *et al.*, 1990). These observations lead the authors to propose that these $CD45RB^{lo}KJ^{+}CD4^{+}$ T cells from unimmunized mice were resting memory cells, which had developed through a second TCR comprised of the Tg β-chain paired with an endogenous α-chain (Lee *et al.*, 1990). When these effector/memory $CD45RB^{lo}KJ^{+}CD4^{+}$ T cells were labelled with CFSE and stimulated *in vitro* they responded similarly to naïve $CD45RB^{hi}KJ^{+}CD4^{+}$, although they exhibited a more diffuse CFSE staining pattern (Lee & Pelletier, 1998). This may be because they had already differentiated and therefore did not respond in the uniform manner of naïve cells. However by the end of the culture period most of the cells exhibited forward and side light scatter properties of apoptotic cells (Lee & Pelletier, 1998). In a separate study $CD45RB^{lo}KJ^{+}CD4^{+}$ T cells were observed to represent the majority of T cells in the small intestinal lamina propria of OVA-naïve DO11.10 mice (Hurst *et al.*, 1997). When the cells from these mice were examined for cell turnover (by measuring BrdU incorporation), the T cells from the lamina propria and Peyer's patch had a turnover rate 3 to 5 fold greater than that of spleen T cells (both KJ^{+} and KJ^{-} T cells) (Saparov *et al.*, 1999). The incorporation of BrdU by

KJ^+CD4^+ T cells correlated with expression of $CD45RB^{lo}$ in each tissue (Saparov *et al.*, 1999). When the cells were isolated and stimulated *in vitro* with OVA peptide, the $CD45RB^{hi}KJ^+CD4^+$ T cells were found to only produce IL-2 while the $CD45RB^{lo}KJ^+CD4^+$ T cells produced primarily IFN-γ and IL-10 and a little IL-2 (Saparov *et al.*, 1999). This was in agreement with a previous study (Lee *et al.*, 1990). In contrast when DO11.10/RAG-$2^{-/-}$ and DO11.10/SCID mice were examined, the lamina propria and spleens of these mice consisted of $CD45RB^{hi}KJ^+CD4^+$ T cells which were undergoing significantly less cell turnover than cells from DO11.10 mice, and produced primarily IL-2 when stimulated *in vitro* (Saparov *et al.*, 1999). All of these observations suggested that DO11.10 T cells were recognizing enteric lumenal antigens through an endogenous, non-clonotypic TCR which caused them to develop effector/memory characteristics. To test this hypothesis KJ^+CD4^+ T cells (from DO11.10 and DO11.10/SCID mice) were cultured with cecal bacterial antigen (a naturally occurring enteric antigen) pulsed splenic adherent cells (Saparov *et al.*, 1999). Interestingly 8% of the DO11.10 KJ^+CD4^+ T cells progressed through cell cycle compared with none of the DO11.10/SCID KJ^+CD4^+ T cells, supporting the idea of dual TCR cells recognizing both OVA and an enteric antigen (Saparov *et al.*, 1999). As dual TCR-bearing cells have been previously identified in normal mice and humans (Padovan *et al.*, 1993) (Padovan *et al.*, 1995), it was proposed that memory may be maintained by recurrent stimulation from either the original priming antigen or an unrelated environmental antigen (Lee *et al.*, 1990). However whether this is a genuine phenomena or a tg artefact remains to be seen. As there is no way of controlling the pairing of TCRs, memory maintenance would be a random event based on an unrelated antigen, which seems unlikely.

3.1.8 Other studies involving DO11.10 mice.

As they allow the identification of antigen-specific T cells *in vivo* as well as giving control over the induction of antigen-specific responses, DO11.10 mice have been used for numerous studies which have furthered our understanding of T cell biology. A variety of problems have been addressed using this tg model, from changes that occur within the T cell to changes on a population level. For example using DO11.10 T cells it was shown that homeostatic expansion occurred independently of the costimulatory molecules CD28, CD40, and 4-BBL, which are required for antigen-

driven expansion (Prlic *et al.*, 2001b). The redistribution of the TCR on naïve antigen-specific $CD4^+$ T cells following antigen stimulation *in vivo* has also been shown using DO11.10 mice (Reichert *et al.*, 2001). This had previously only been demonstrated *in vitro* (Kupfer *et al.*, 1994). The peak time of TCR redistribution was observed to coincide with the peak of IL-2 production, and the area in which IL-2 was concentrated was also the area of the highest TCR concentration (Reichert *et al.*, 2001). It was also elegantly demonstrated that CD28 was not required for TCR redistribution but was required for IL-2 production (Reichert *et al.*, 2001). In a separate study using DO11.10 T cells, it was shown that CD28 co-stimulation was mediated through up-regulation of IL-2 transcription and through enhancement of mRNA stability (Abraham & Miller, 2001). It was also shown that engagement of LFA-1 (T cell accessory molecule) through its ligand ICAM-1 also enhanced IL-2 transcription but had no effect on IL-2 mRNA stability (Abraham & Miller, 2001). In a similar study using the tg T cells the *in vivo* location of dendritic cell (DC) antigen presentation to KJ^+CD4^+ T cells was shown to occur in the T cell-rich region of the lymph node (Ingulli *et al.*, 1997). The interactions between antigen-specific $CD4^+$ T and B cells *in vivo* following immunization was illustrated with DO11.10 tg T cells together with tg B cells (Chapter 4) (Garside *et al.*, 1998).

DO11.10 T cells have been used to demonstrate the complexity of thymic selection. It was shown that the introduction of the DO11.10 transgene to γ_c-deficient mice (γ_c is shared by receptors for IL-2, IL-4, IL-7, IL-9, and IL-15) increased the number of thymocytes sixfold on a $H\text{-}2^{d/d}$ background (Nakajima & Leonard, 1999). In contrast the number of $CD4^+$ splenic T cells (i.e. outside the thymus) was diminished in DO11.10 $\gamma_c^-H\text{-}2^{d/d}$ mice as compared with nontransgenic γc-deficient wildtype mice (Nakajima & Leonard, 1999). The DO11.10 TCR was shown to exhibit higher affinity for $I\text{-}A^b$ than $I\text{-}A^d$ (Liu *et al.*, 1996), and therefore more thmocytes and splenocytes were observed in DO11.10 $\gamma_c^+H\text{-}2^{d/d}$ than DO11.10 $\gamma_c^+H\text{-}2^{d/b}$ mice (Nakajima & Leonard, 1999). This was also the case for γ_c deficient $H\text{-}2^{b/b}$ mice (Nakajima & Leonard, 1999). Thus the increase in thymic cellularity seen in γ_c-deficient mice expressing the DO11.10 transgene in the $H\text{-}2^{d/d}$ background resulted from bypassing the defect in TCR rearrangement in γ_c-deficient mice and was influenced by the MHC background (Nakajima & Leonard, 1999). As DO11.10 γ_c^+ $H\text{-}2^{d/b}$ mice have substantial numbers of KJ^+CD4^+ splenocytes compared to DO11.10 γ_c^- $H\text{-}2^{d/b}$ mice (which have substantial numbers of $CD4^+$ thmocytes), it was suggested that γ_c-dependent signals

were required for the survival/expansion of T cells expressing a TCR with a relatively high affinity to the MHC/peptide complex (Nakajima & Leonard, 1999). Interestingly enforced expression of Bcl-2 significantly increased the numbers of thymocytes and KJ^+CD4^+ splenocytes in both γ_c^+ and γ_c^- DO11.10 H-$2^{d/b}$ mice (Nakajima & Leonard, 1999). However the cell numbers of DO11.10 Bcl-2^+ γ_c^- mice were still diminished compared with wild type mice indicating that γ_c must have additional signals beyond TCR rearrangement and Bcl-2 induction for promoting thymic development (Nakajima & Leonard, 1999).

The hypothesis that the generation of functional effector/memory T cells requires stimulation by adjuvant as well as specific peptide was demonstrated *in vivo* using DO11.10 mice (Chen & Jenkins, 1998). This was done by tracking adoptively transferred KJ^+CD4^+ T cells in BALB/c mice infected subcutaneously (s.c.) with *Escherichia coli* expressing a MalE-OVA fusion protein (Chen & Jenkins, 1998). After infection the KJ^+CD4^+ T cells accumulated in the T cell-rich paracortical regions of the draining lymph nodes, proliferated there for a few days, and then moved into the B cell-rich follicles before slowly disappearing from the lymph nodes (Chen & Jenkins, 1998). The KJ^+CD4^+ T cells also accumulated in the s.c. infection site, a day later than in the draining lymph node (Chen & Jenkins, 1998). However if the MalE-OVA fusion protein was injected alone, the KJ^+CD4^+ T cells failed to enter the follicles, the mice did not produce anti-OVA antibodies, and when the T cells were recovered and re-stimulated *in vitro* they failed to produce IL-2 and IFN-γ (Chen & Jenkins, 1998). These results suggested that antigen-specific T cells were first activated in secondary lymphoid organs following primary bacterial infection and then migrated to the infection site, which was dependent on bacterial components in addition to the antigen (Chen & Jenkins, 1998). Following on from this idea it was shown that exposure to soluble OVA peptide *in vivo* resulted in the induction of clonal anergy that limited the expansion of KJ^+CD4^+ T cells and their ability to produce IL-2 (Malvey *et al.*, 1998). However, it did not prevent the KJ^+CD4^+ T cells from helping an IgG2a anti-hapten antibody response or partaking in delayed-type hypersensitivity reactions (Malvey *et al.*, 1998).

3.1.9 Limitations with the use of transgenic T cells.

Much of our recent knowledge on T cell biology has come from studies with tg T cells. Hence it is important to consider the inherent artificiality of these cells. Firstly,

even though small numbers of T cells are usually transferred in these studies, the resulting frequencies of antigen-specific T cells is several orders of magnitude higher than normal. The effects of this elevated frequency are unknown but may result in a faster immune response than is usually seen. It may also mean that not all the antigen-specific cells will respond as there may be abnormal competition for antigen. However as discussed earlier, until our methods for detecting naive antigen-specific cells *in vivo* improves, this remains the only alternative for detecting cells prior to clonal expansion.

A further flaw in any tg system is that in a normal immune response to any given peptide, l*et al*one pathogen, a variety of T cell clones respond, all with different affinities for their respective antigens. In contrast, a tg T cell population consists of cells which all share exactly the same antigen binding properties. This may not be a problem when examining cell-cell interactions such as the co-stimulatory molecules required for optimal stimulation. However, if examining cells on a population scale, such as the conditions required for memory cell generation, it may become a serious limitation. It is likely that memory cells develop from a population of cells expressing a variety of TCRs and not from a monoclonal population. Nonetheless evidence for this remains elusive.

There is also the question of how representative of all foreign antigens the OVA peptide 323-339 is. Although it elicits T cell responses in normal mice, it does not stimulate specific B cells to produce antibody (personal observation). Like any peptide, it must be administered with an adjuvant to generate a full T cell response. The inflammatory conditions stimulated by these adjuvants are also inherently artificial.

Despite these flaws tg models currently represent our best experimental systems for studying T cell at the clonal level. They have and continue to further our understanding of these complicated cells. However all results deduced from their study must be constrained by their artificial nature.

The purpose of this chapter was firstly to examine the migratory behaviour of KJ^+CD4^+ T cells immediately after adoptive transfer and to compare it with KJ^-CD4^+ T cells. There was no reason to expect differences between the two cells, as the TCR is not involved in migration but it was necessary to examine if these cells were to be used in adoptive transfer experiments. The second aim was to see what effect the presence of OVA peptide would have on the early migration of KJ^+CD4^+ T cells after adoptive transfer. We also wanted to examine how early cell division occurred and whether KJ^-CD4^+ T cells would also divide in the presence of OVA peptide.

Chapter 3

3.2 Results

3.2.1 Migration of KJ^+CD4^+ and KJ^-CD4^+ T cells.

Investigations by Ford and colleagues (Smith & Ford, 1983) showed that lymphocytes, upon adoptive transfer by intravenous (i.v.) injection, initially locate in the spleen, as this is where they are taken by the blood. By 22 hours many of the transferred cells have become redistributed to the lymph nodes, such as the mesenteric and inguinal lymph nodes.

The purpose of the present experiments was to examine the movement of KJ^+CD4^+ T cells immediately after adoptive transfer and to compare them with non-tg KJ^-CD4^+ T cells. It was important to establish whether the tg T cells re-circulated differently to non-tg T cells if they were to be used in adoptive transfer experiments. Previous experiments have examined the migration of KJ^+CD4^+ T cells after 24 hours but not before that time point.

Donor cells were obtained from DO11.10 mice and purified by CD8 and CD19 depletion (Figure 3.1b). The purified cells which contained both tg and non-tg $CD4^+$ T cells were labelled with CFSE (Fig 3.1d) which allowed tracking of the transferred cells by flow cytometric analysis. 30 x 10^6 $CFSE^+CD4^+$ T cells were injected intravenously into BALB/cIgH mice. Of the injected cells, between 55% and 65% were KJ^+ as shown in Figure 3b.

At 2 hours (Figure 3.2) and 22 hours (Figure 3.3) after transfer the spleens, MLNs, ILNs, and CLNs of the recipient mice were examined for donor cells. The total number of cells in each tissue was also counted. Using these two values the total number of donor cells per tissue was calculated. The donor population was gated and analysed for the percentage of KJ^+CD4^+ T cells. The total number of donor cells per animal was obtained by summing the number of donor T cells localized to each of the 4 tissues. By dividing the number of donor cells per tissue by the total number of donor cells per recipient the relative percentage of donor cells per tissue was calculated. This value was used to compare the migration of tg versus non-tg T cells at the two different time points. This relative percentage value was used instead of absolute cell numbers or percentage donor cells in order to allow for any variation between experiments in the

number of cells initially injected into each animal, and for any difference in cell survival between 2 and 22 hours. Three or four animals were examined at each time point and the results of 2 separate experiments were pooled. Mean values for each tissue at each time point were plotted on graphs to provide a visual comparison of the movement of KJ^+ and KJ^- donor T cells. These can be seen for the spleen in Figure 3.4, for the MLN in Figure 3.5, for the ILN in Figure 3.6, and for the CLN in Figure 3.7.

2 hours after injection a majority of donor cells (KJ^+ and KJ^- cells) were located in the spleen. Unsurprisingly relatively few donor T cells were found in the lymph nodes examined at 2 hours. At 22 hours donor T cell numbers decreased in the spleen and were found to have increased in the lymph nodes (LNs), particularly the mesenteric lymph node. In all LNs examined, KJ^+ T cells appeared to increase more than the KJ^- T cells between 2 and 22 hours. However this was due to the lower numbers of KJ^- T cells. When the data was compared on the basis of rate of increase there was no significant difference observed ($p<0.05$) between the KJ^+ and KJ^- T cells. There was no evidence of cell division as assessed by the loss of CFSE staining (Figures 3.2 & 3.3).

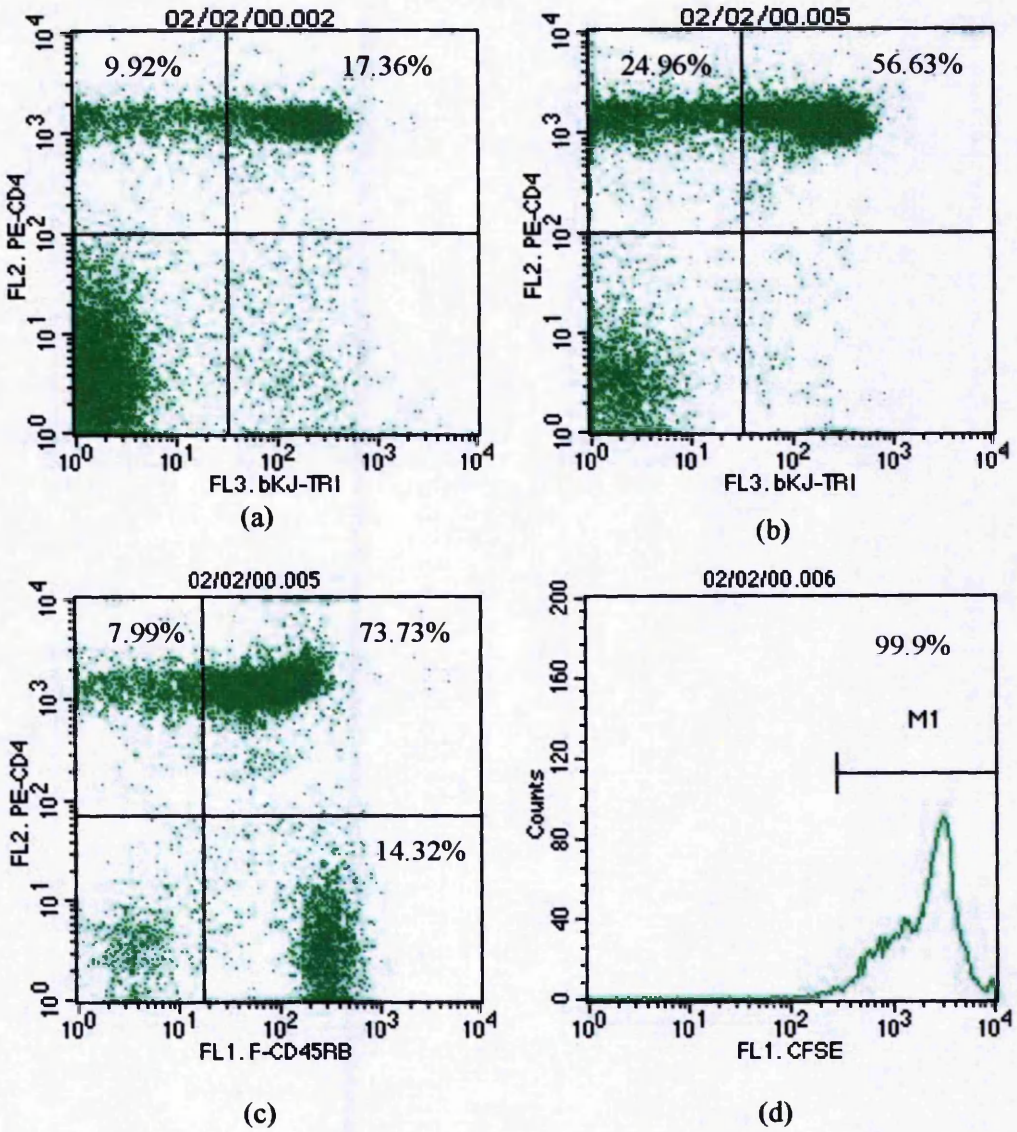


Figure 3.1. Flow cytometric analysis of tg donor T cells prior to transfer.

A representative flow cytometric analysis of tg donor cells prior to transfer into BALB/cIgH recipients before $CD8^+$ T cell and B cell depletion (a), following depletion (b) and (c), and after labelling with CFSE (d). The percentages indicate the proportion of cells within the quadrant or under the marker.

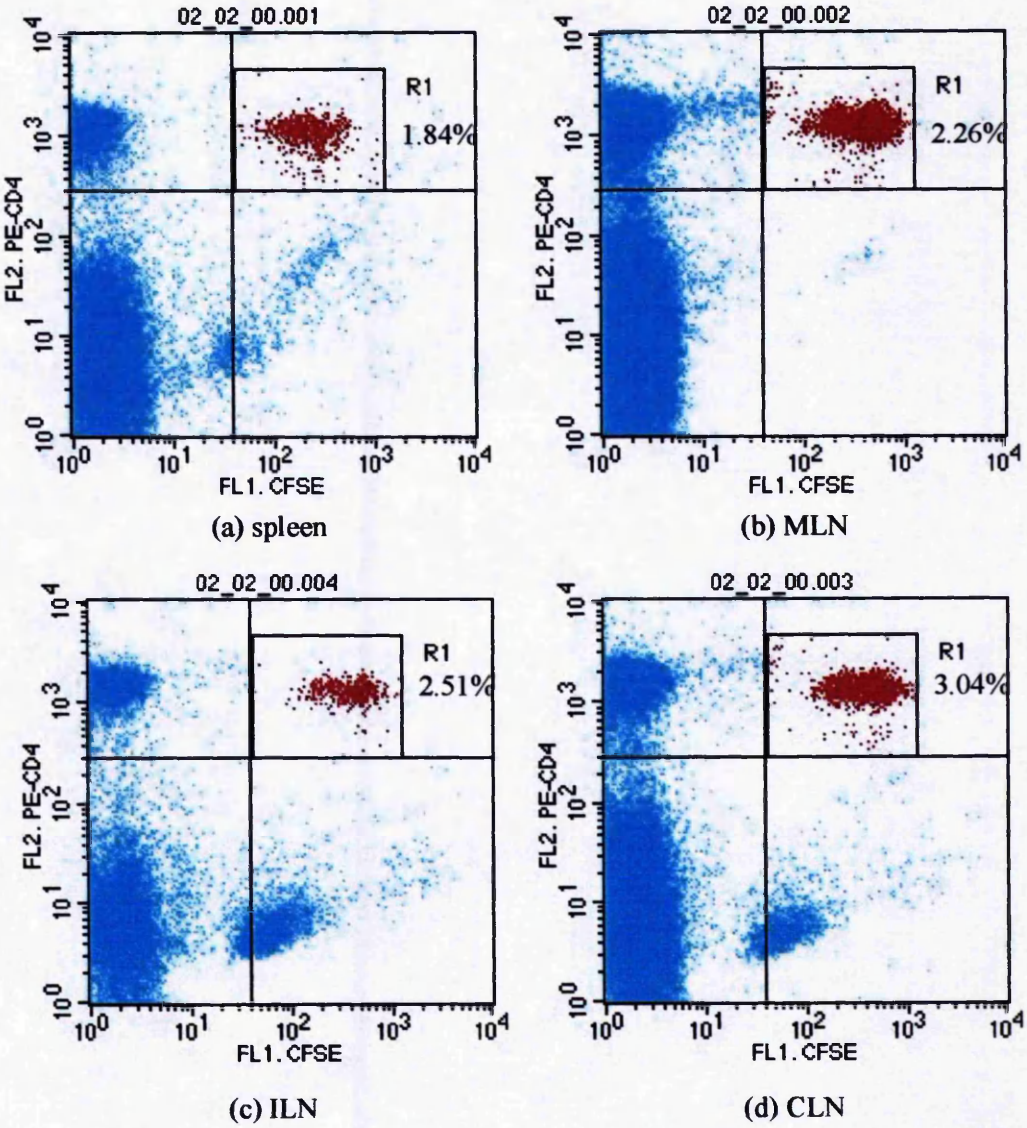


Figure 3.2. Flow cytometric analysis of tg donor T cells 2 hours after transfer.

Flow cytometric analysis of mice 2 hours after adoptive transfer of 30 x 10^6 $CFSE^+CD4^+$ T cells in the spleen (a), in the MLN (b), in the ILN (c), and in the CLN (d). The percentage of $CFSE^+$ donor T cells is shown in top right quadrant of each figure.

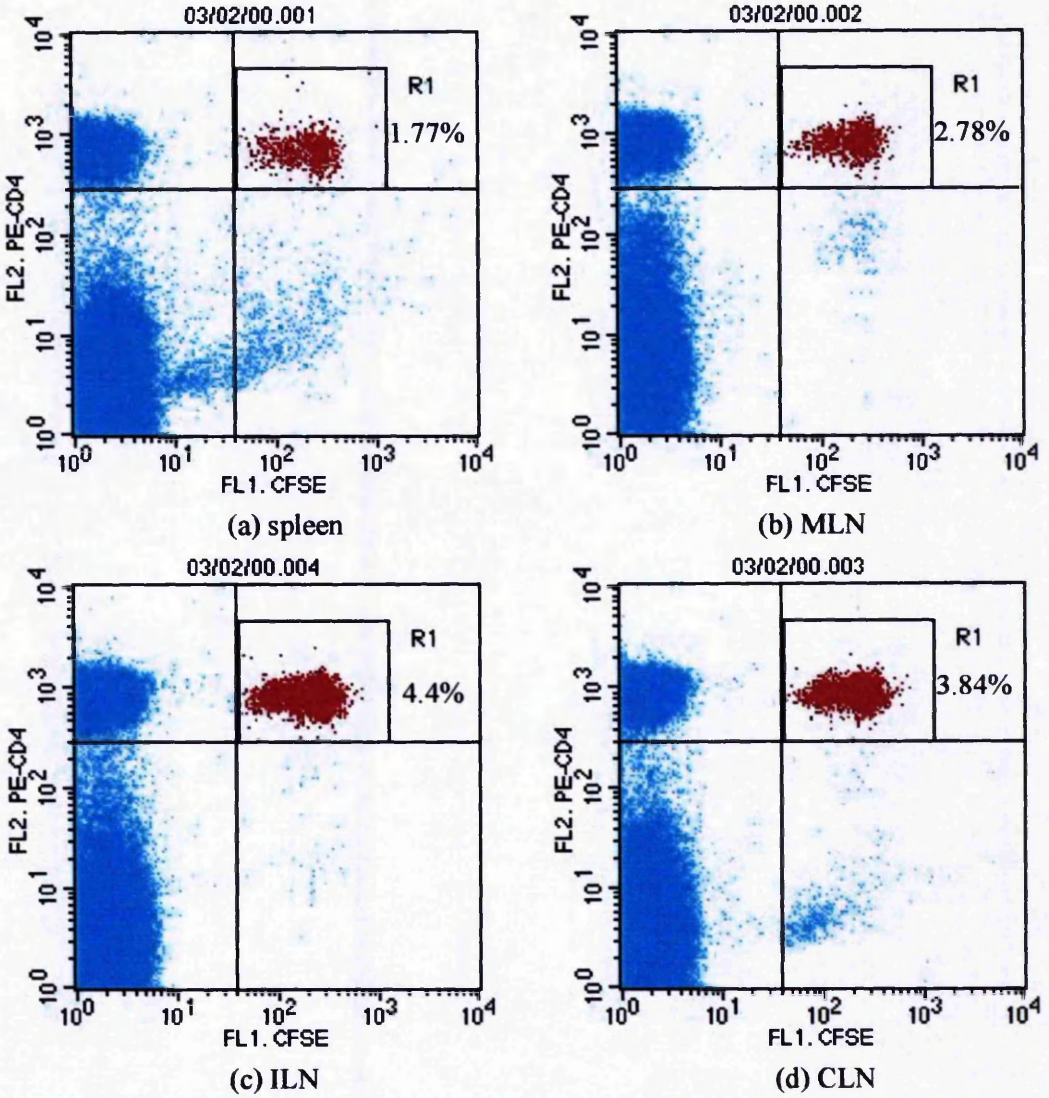


Figure 3.3. Flow cytometric analysis of tg donor T cells 22 hours after transfer.

Flow cytometric analysis of mice 22 hours after adoptive transfer of 30 x 10^6 $CFSE^+CD4^+$ T cells in the spleen (a), in the MLN (b), in the ILN (c), and in the CLN (d). The percentage of $CFSE^+$ donor T cells is shown in top right quadrant of each figure.

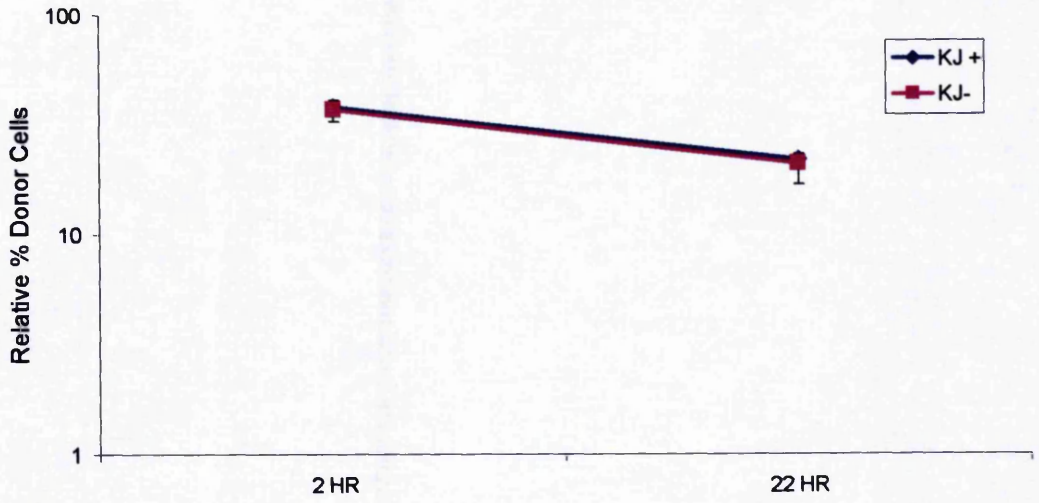


Figure 3.4. Comparison of KJ^+ and KJ^- T cells in the spleens of recipient mice.

Relative percentages of donor KJ^+CD4^+ and KJ^-CD4^+ T cells in the spleen at 2 hours and 22 hours after the adoptive transfer of 30 x 10^6 $CFSE^+CD4^+$ T cells from DO11.10 mice into BALB/cIgH mice. The mean values of 4 recipients per group are shown +/- SD.

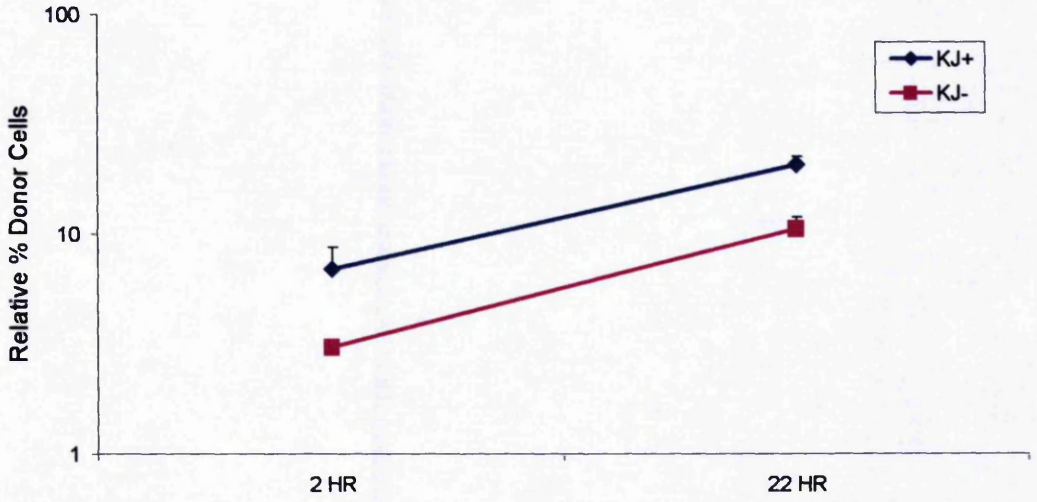


Figure 3.5. Comparison of KJ^+ and KJ^- T cells in the MLNs of recipient mice.

Relative percentages of donor KJ^+CD4^+ and KJ^-CD4^+ T cells in the MLN at 2 hours and 22 hours after the adoptive transfer of 30 x 10^6 $CFSE^+CD4^+$ T cells from DO11.10 mice into BALB/cIgH mice. The mean values of 4 recipients per group are shown + SD.

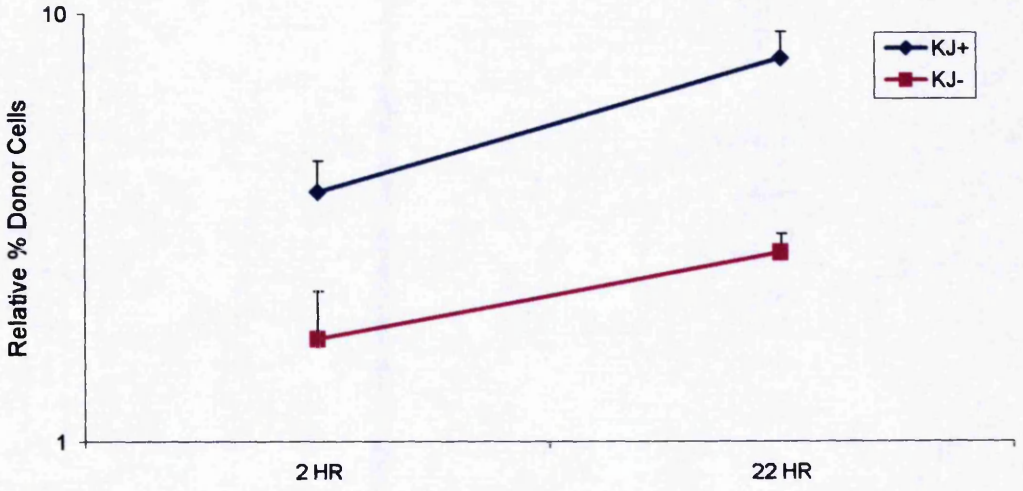


Figure 3.6. Comparison of KJ^+ and KJ^- T cells in the ILNs of recipient mice.

Relative percentages of donor KJ^+CD4^+ and KJ^-CD4^+ T cells in the ILN at 2 hours and 22 hours after the adoptive transfer of 30 x 10^6 $CFSE^+CD4^+$ T cells from DO11.10 mice into BALB/cIgH mice. The mean values of 4 recipients per group are shown + SD.

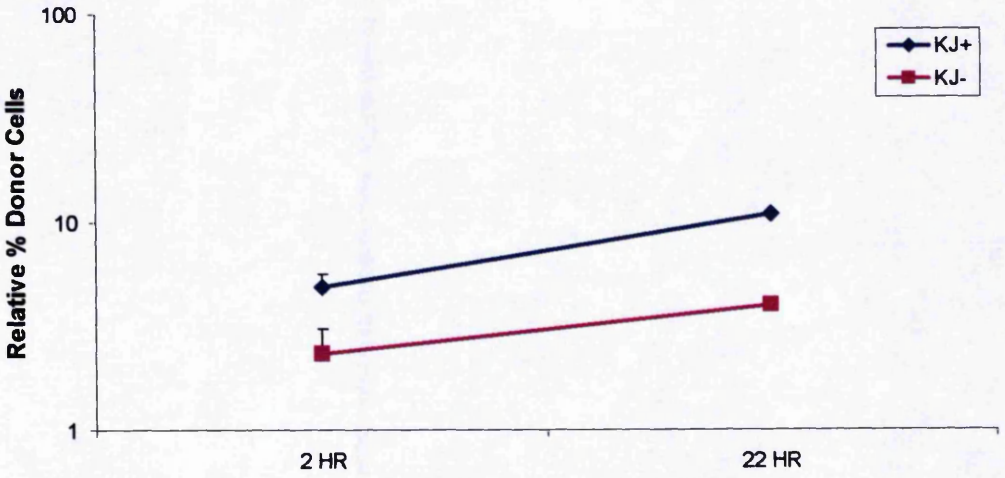


Figure 3.7. Comparison of KJ^+ and KJ^- T cells in the CLNs of recipient mice.

Relative percentages of donor KJ^+CD4^+ and KJ^-CD4^+ T cells in the CLN at 2 hours and 22 hours after the adoptive transfer of 30 x 10^6 $CFSE^+CD4^+$ T cells from DO11.10 mice into BALB/cIgH mice. The mean values of 4 recipients per group are shown + SD.

3.2.2. Migration of KJ^+CD4^+ T cells and KJ^-CD4^+ T cells in the presence of OVA peptide.

The purpose of these experiments was to determine whether the presence of specific antigen had an effect on the early migration of adoptively transferred KJ^+ T cells. It is known that tg T cells will bind to OVA peptide 323-339 presented by MHC class II. This protein was alum precipitated and 100μg was injected intraperitoneally (i.p.) into BALB/cIgH mice 3 days prior to injection of cells. The antigen was injected 3 days prior to the injection of cells to allow time for the antigen presenting cells to receive and present the antigen before the arrival of KJ^+CD4^+ T cells. Following i.p. injection of particulate (ap-OVA peptide) antigen the spleen and MLN are the first tissues to receive the antigen (E. Bell personal communication). In the ILN and CLN, the antigen arrives later. Therefore, the aim of these experiments was to determine whether an i.p. injection of OVA peptide could result in retention of KJ^+ T cells in the spleen and MLN and prevent their redistribution to the other lymph nodes.

Donor cells were obtained from DO11.10 mice, and purified by CD8 and CD19 depletion (Fig 3.8a). The purified cells were labelled with CFSE (Fig 3.8b) and 5×10^6 T cells were injected i.v. into BALB/cIgH mice. Half of the recipient mice were preimmunized with ap-OVA peptide, the other mice were used as a control group. The spleens, MLNs, ILNs, and CLNs, of the recipient mice were examined for the presence of donor cells (as judged by the CFSE fluorescence) at 2 hours (Fig 3.9), 22 hours (Fig 3.10), and 44 hours (Fig 3.11, 3.12, & 3.13) after cell transfer. The relative percentage of donor cells per tissue was calculated as described in Section 2.13 using the percentage of donor cells present and the tissue cell count. Mean values were calculated for each time point and are shown in Figures 3.14-3.17 for the spleen, MLN, ILN, and CLN respectively. Two or three animals were examined in each group (OVA recipients and controls) at each time point and the results of 2 separate experiments were pooled.

Examining the relative percentage of donor cells per tissue at 2 hours in the spleen, there was significantly ($p<0.05$) more donor T cells, both KJ^+ and KJ^- T cells in the animals which received ap-OVApep than in the controls. There was no difference between the KJ^+ and KJ^- T cells in these animals at this time point. In the MLN and CLN no difference was observed, but in the CLN there was significantly less ($p<0.05$) donor cells (both KJ^+ and KJ^-) in animals which received OVA peptide.

By 22 hours, the relative percentages of donor T cells in the spleen had decreased in both groups of animals, although the only significant decrease was in animals which received OVA peptide. At this time point the number of donor T cells in the MLN had increased in both groups. Although the increase of KJ^+ T cells in OVApep recipients was greater than in control animals, the difference was not significant. In both ILN and CLN at 22 hours, all groups of donor T cells increased, though there was no significant difference in the rates of increase between groups.

By 44 hours the KJ^+ T cells in mice which received OVApep had begun to proliferate as shown by loss of CFSE fluorescence (Figure 3.12 & 3.13). This cell division was observed in all the tissues examined. As expected KJ^+ T cells in mice which were not immunized with OVApep, did not proliferate and appeared to follow a re-circulation pattern similar to the KJ^- T cells.

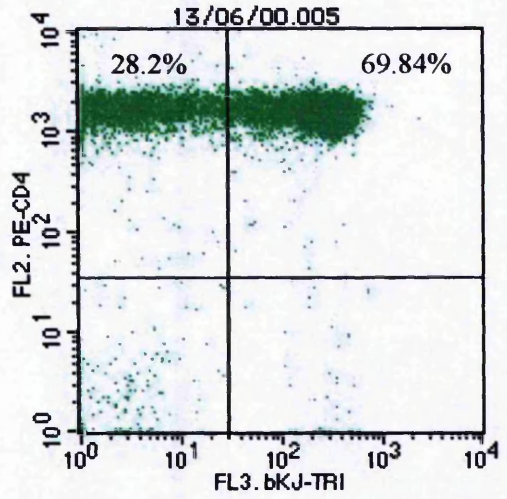
However when examining the actual number of donor cells per tissue at 44 hours a different picture was observed. Although KJ^+CD4^+ T cells in OVA immunized mice had begun to divide, there were more KJ^+CD4^+ T cells in the spleens, ILNs, and CLNs of control mice (Figures 3.18, 3.20, and 3.21). The difference was only significant in the spleen ($p<0.05$) (Fig 3.18). In the MLN there were greater numbers of KJ^+CD4^+ T cells in the OVA immunized recipients though the difference was insignificant.

At 2 hours as expected there were greater numbers of KJ^+CD4^+ T cells in the spleen ($p<0.05$) and the MLN (Figures 3.18 & 3.19) of OVA immunized mice. At 22 hours there were comparable numbers of donor cells in these tissues (Figure 3.18 & 3.19) as well as in the ILNs and CLNs at 2 hours and 22 hours (Figure 3.20 & 3.21).

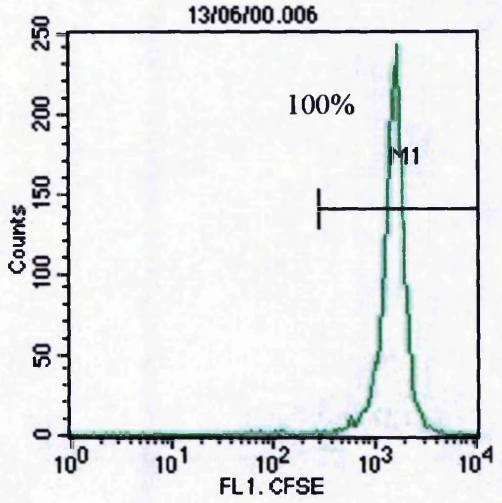
44 hours after injection, despite approximately 50% of the KJ^+CD4^+ T cells in OVA immunized recipients dividing (this should double the number of cells), there were more KJ^+CD4^+ T cells in the control group. Whether this was a genuine increase or due to experimental variation would require further investigation. Paradoxically the largest decline in cell number observed was in the spleen, which was also the site of the greatest amount of cell division (3 rounds, Figure 3.12a). Possible explanations for this were that once the cells began to divide they may have migrated out of the spleen (and the LNs). It was also possible that as soon as the cells began to divide they also started to die. This concept will be discussed more in Chapter 7.

There was no clear understanding of the changes in the distribution of cells that was observed. We had expected that the earlier presence of antigen within the spleen

would disrupt the normal migration of the KJ^+, but not the KJ^- T cells. It is possible that the relatively large number of KJ^+ (OVA-specific) T cells vastly exceeded the capacity of antigen present within the spleen to trap and hold the KJ^+ T cells within the spleen. Another complicating factor was the fact that by day 3 after the antigen injection (i.e. the delay before cell transfer) OVA would have reached the peripheral LNs and been able to affect their migration. In retrospect, it might have been better to have transferred the cells one day rather than 3 days after antigen injection.



(a)



(b)

Figure 3.8. Flow cytometric analysis of donor T cells prior to transfer.

Flow cytometric analysis of DO11.10 LN cells following depletion of $CD8^+$ T cells and B cells (a) and following labelling with CFSE (b). The percentage of cells is shown in each figure.

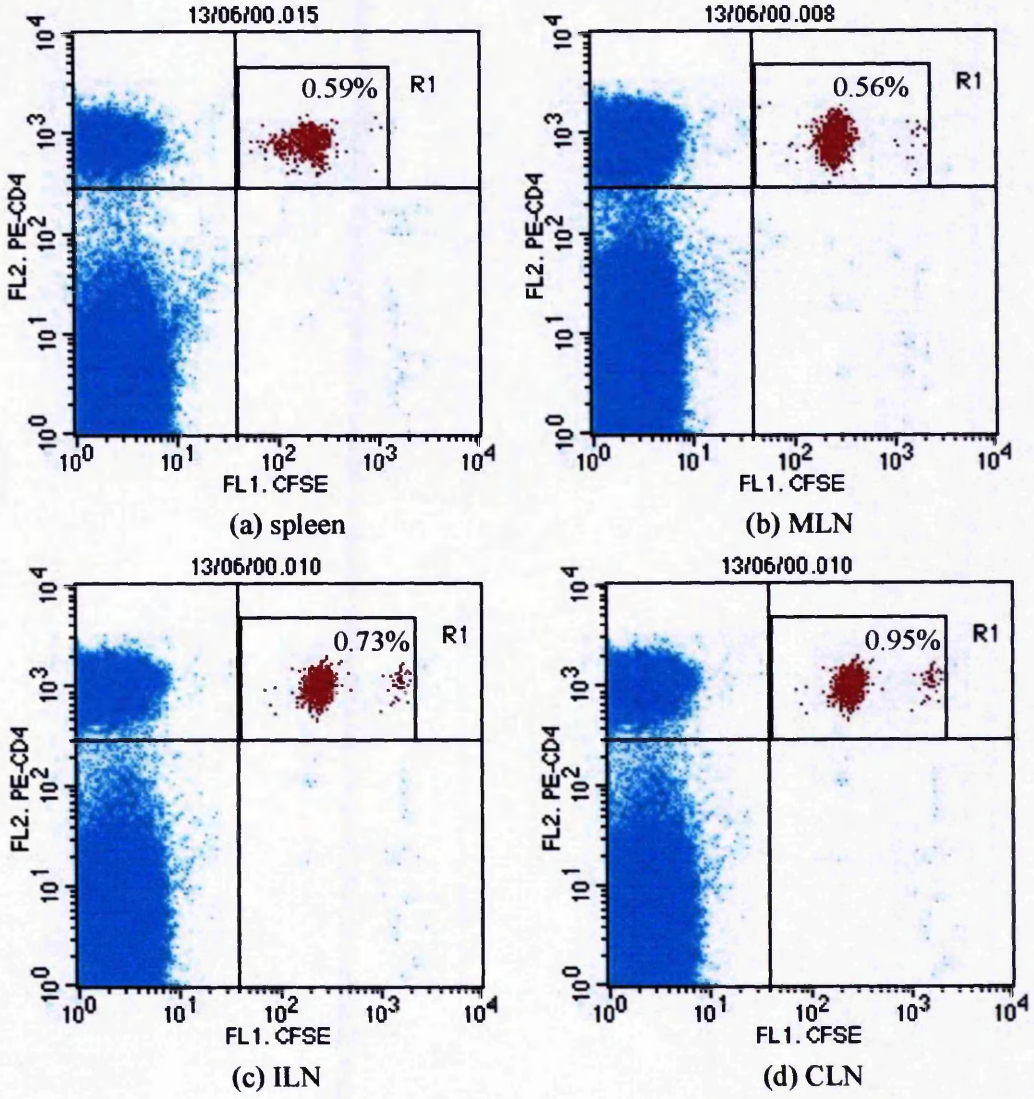


Figure 3.9. Flow cytometric analysis of tg donor T cells 2 hours after transfer to immunized recipients.

Representative flow cytometric analysis of OVApep pre-immunized BALB/cIgH mice, 2 hours after the adoptive transfer of 5 x 10^6 $CFSE^+CD4^+$ T cells in the spleen (a), in the MLN (b), in the ILN (c), and in the CLN (d). The percentage of $CFSE^+$ donor T cells is shown in top right quadrant of each figure.

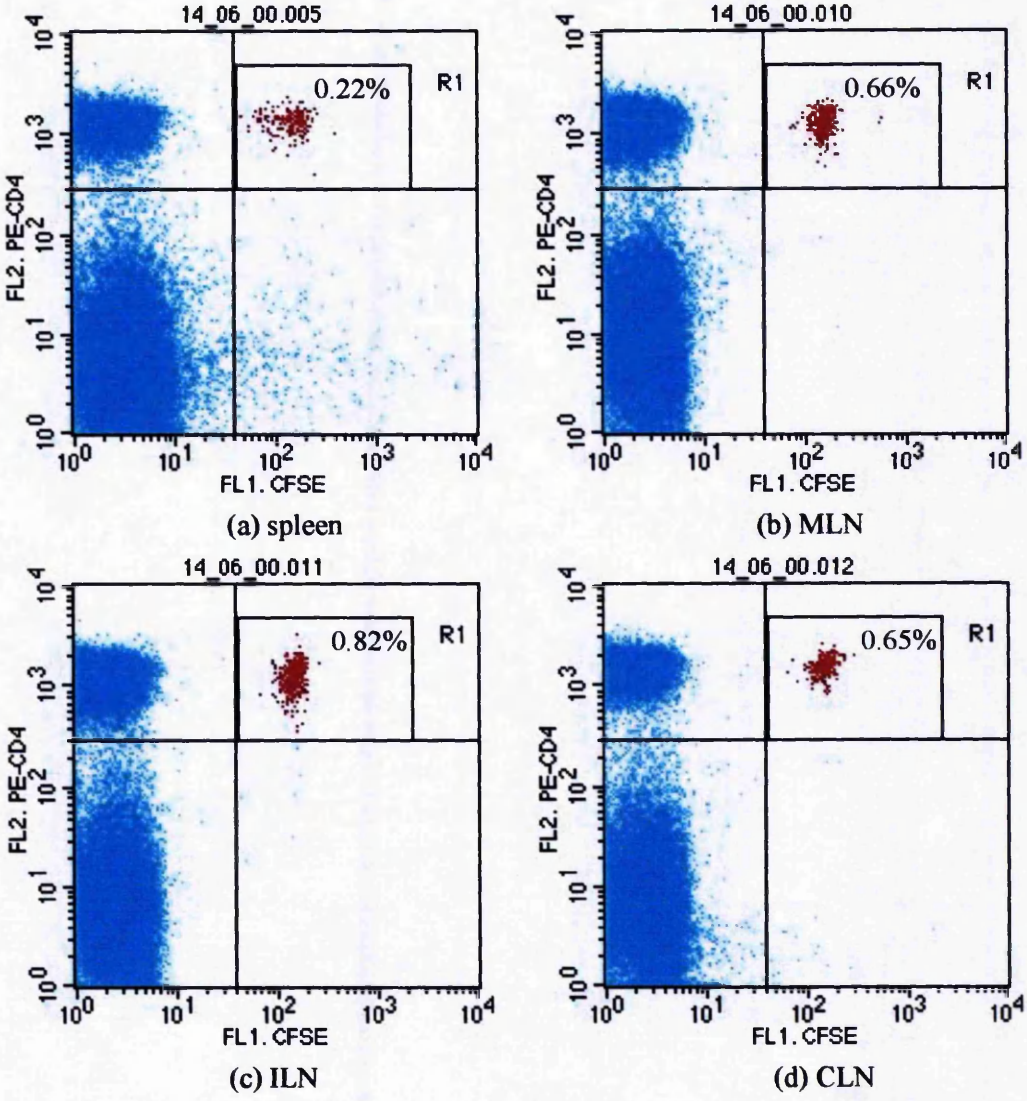


Figure 3.10. Flow cytometric analysis of tg donor T cells 22 hours after transfer to immunized recipients.

Representative flow cytometric analysis of OVApep pre-immunized BALB/cIgH mice, 22 hours after the adoptive transfer of 5 x 10^6 $CFSE^+CD4^+$ T cells in the spleen (a), in the MLN (b), in the ILN (c), and in the CLN (d). The percentage of $CFSE^+$ donor T cells is shown in the top right quadrant of each figure.

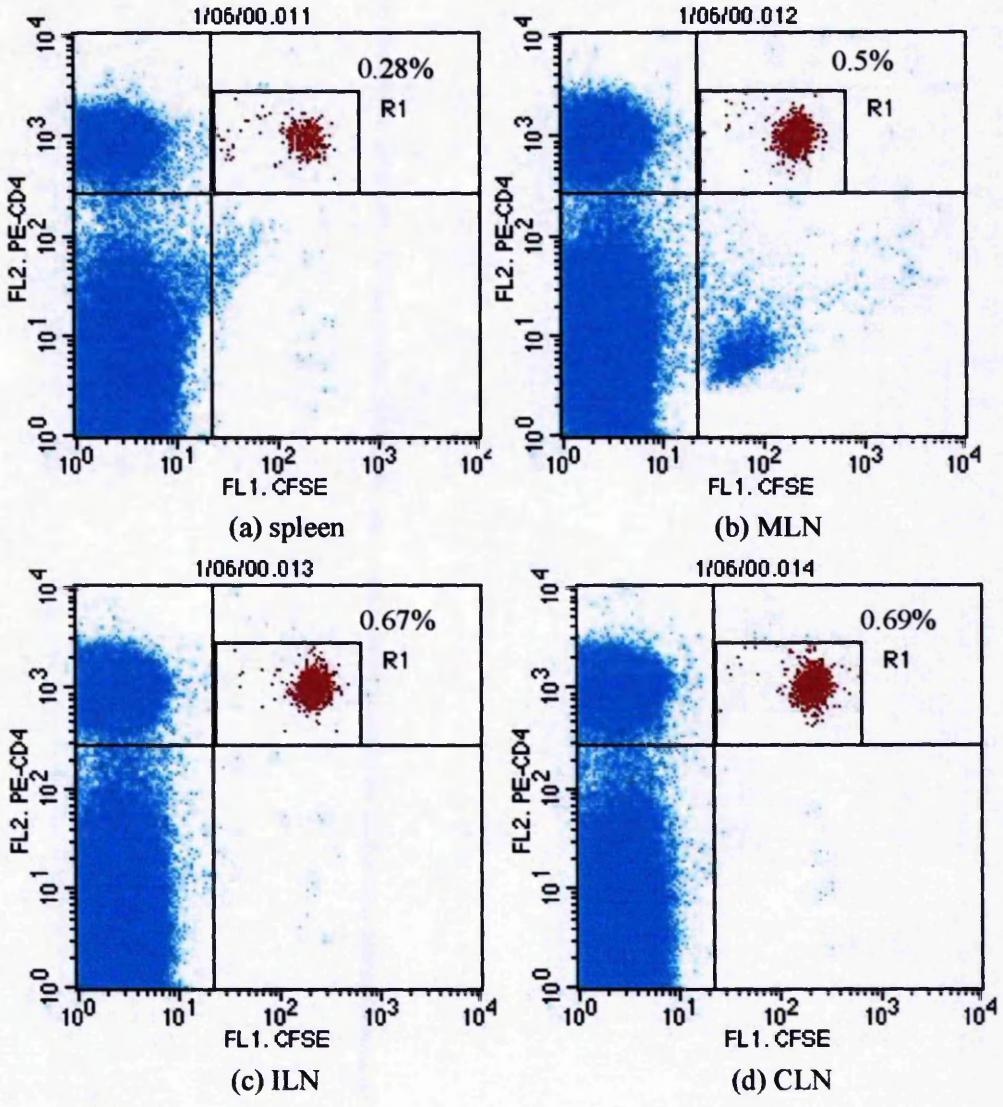


Figure 3.11. Flow cytometric analysis of tg donor T cells 44 hours after transfer to non-immunized recipients.

Representative flow cytometric analysis of control BALB/cIgH mice, 44 hours after the adoptive transfer of 5 x 10^6 $CFSE^+CD4^+$ T cells in the spleen (a), in the MLN (b), in the ILN (c), and in the CLN (d). The percentage of $CFSE^+$ donor T cells is shown in the top right quadrant of each figure.

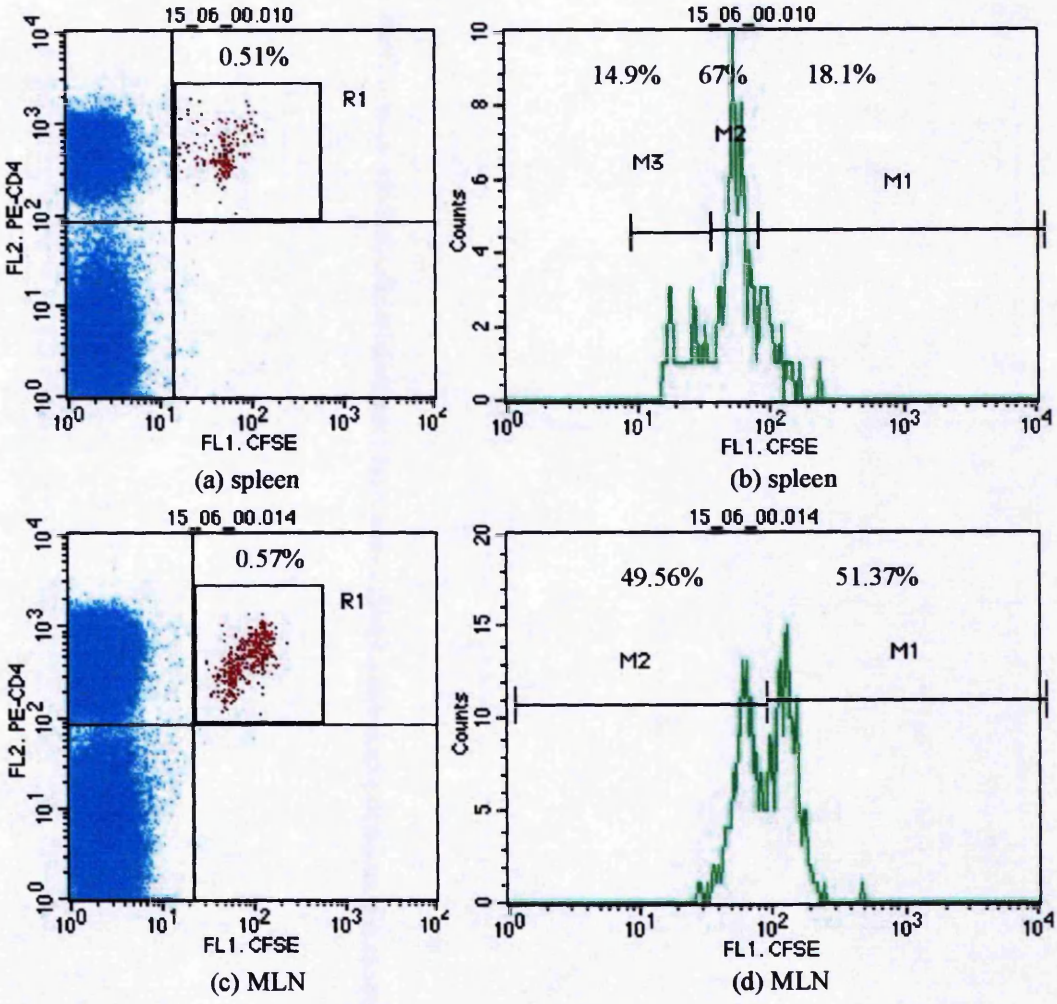


Figure 3.12. Flow cytometric analysis of tg donor T cells 44 hours after transfer to immunized recipients.

Representative flow cytometric analysis of OVApep pre-immunized BALB/cIgH mice 44 hours after injection of 5 x 10^6 $CFSE^+CD4^+$ T cells in the spleen (a) and the MLN (c). Donor T cells in both tissues had begun to show signs of cell division as evidenced by loss of CFSE. The donor T cells were gated (R1) and their CFSE fluorescence profile was examined more closely showing 3 separate peaks, M1, M2, and M3, for the cells in the spleen (b), and two separate peaks, M1 and M2, for the cells in the MLN (d). The percentage of $CFSE^+$ donor T cells is shown in top right quadrant of (a) and (c) and the percentage of cells for each CFSE peak is also shown in (b) and (d).

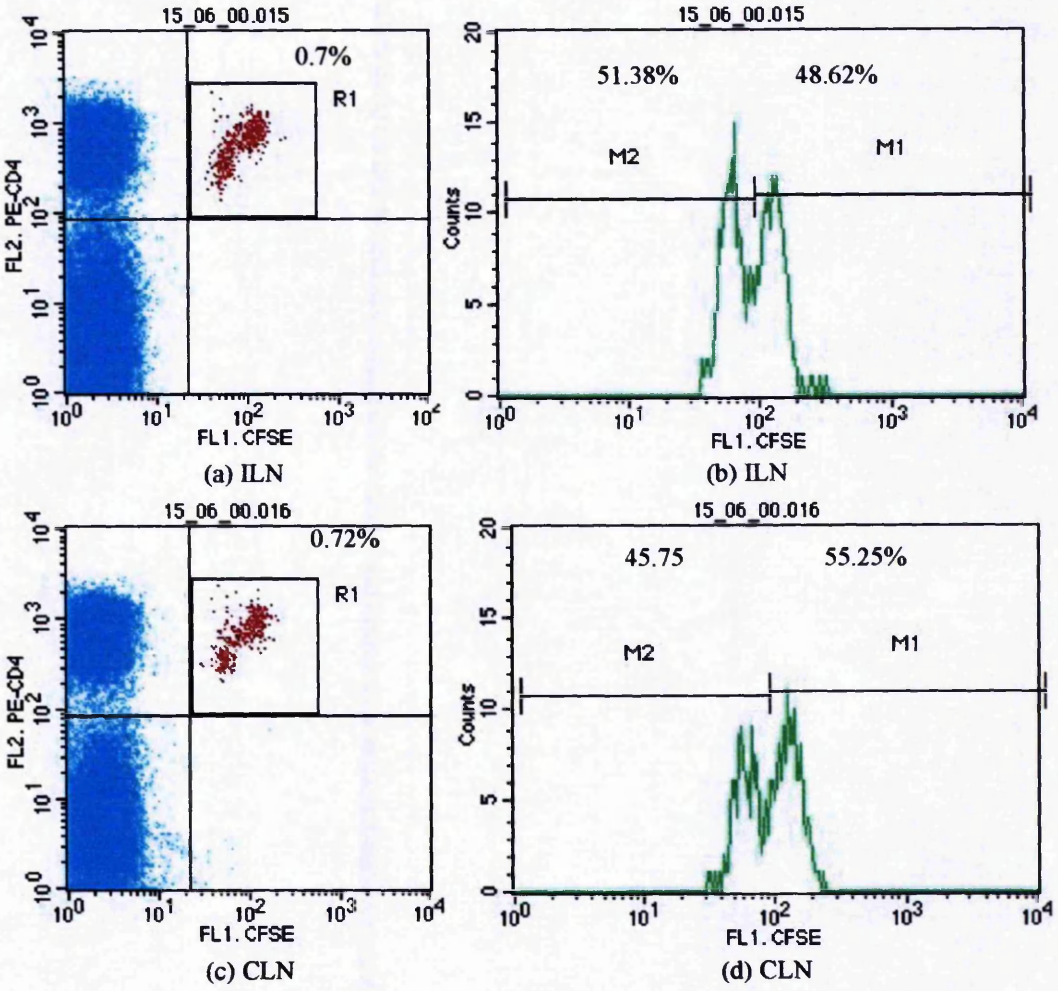
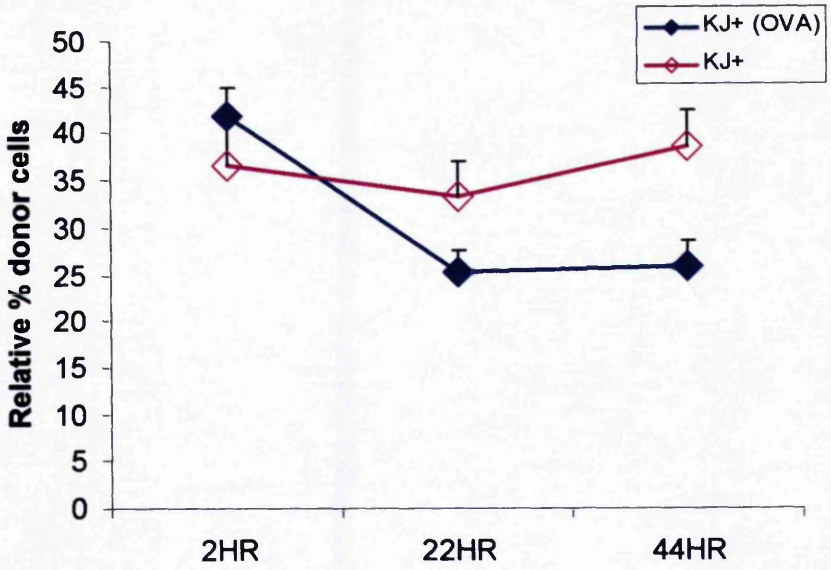
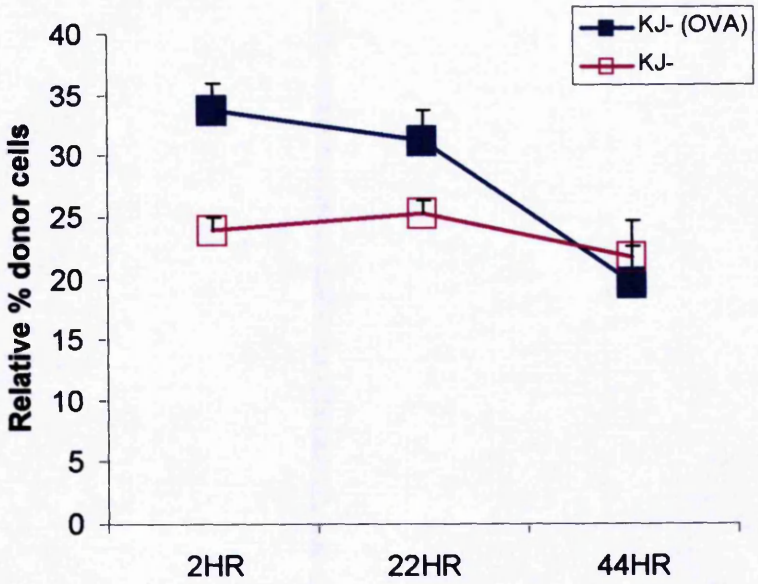


Figure 3.13. Flow cytometric analysis of tg donor T cells 44 hours after transfer to immunized recipients.

Representative flow cytometric analysis of OVApep pre-immunized BALB/cIgH mice 44 hours after injection of 5 x 10^6 $CFSE^+CD4^+$ T cells in the ILN (a) and the CLN (c). Donor T cells in both tissues had begun to show signs of cell division as evidenced by loss of CFSE. The donor T cells were gated (R1) and their CFSE fluorescence profile was examined more closely showing 2 separate peaks, M1, M2, in both ILN (b) and CLN (d). The percentage of $CFSE^+$ donor T cells is shown in top right quadrant of (a) and (c) and the percentage of cells for each CFSE peak is also shown in (b) and (d).



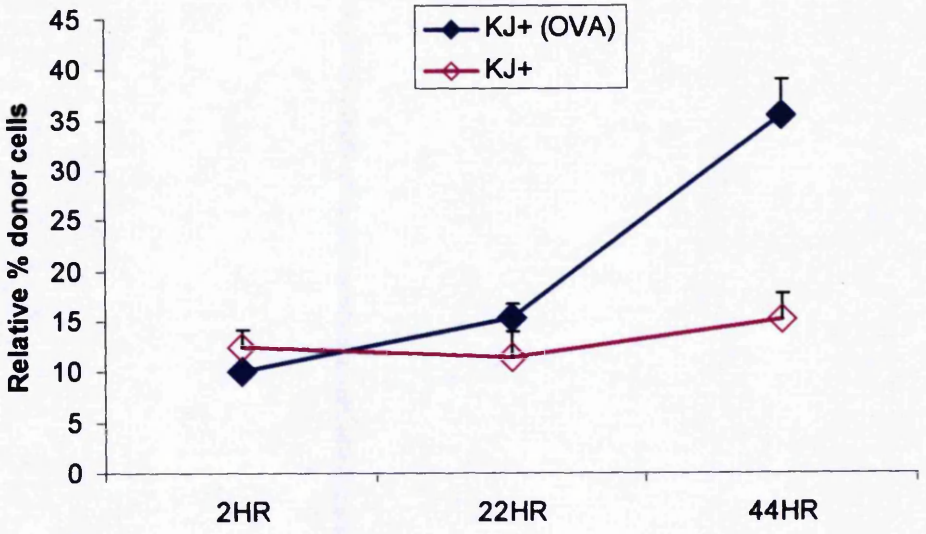
(a) KJ^+



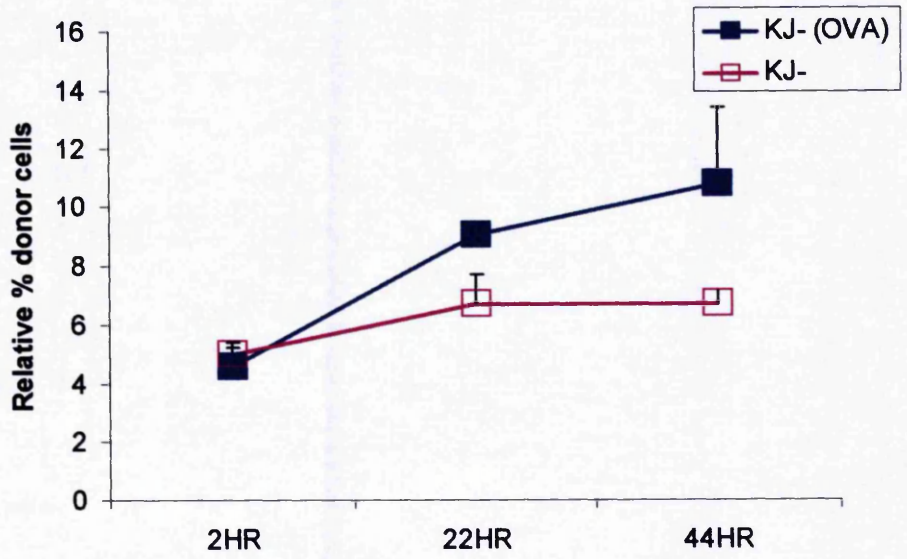
(b) KJ^-

Figure 3.14. Comparison of KJ^+ and KJ^- T cells in the spleens of immunized and non-immunized recipients.

Relative percentages of donor KJ^+CD4^+ (a) and KJ^-CD4^+ (b) T cells in the spleen at 2 hours, 22 hours and 44 hours after the adoptive transfer of 5 x 10^6 donor T cells into apOVA pre-immunized and control BALB/cIgH mice. The mean values of 4-6 recipients per group are shown + SD.



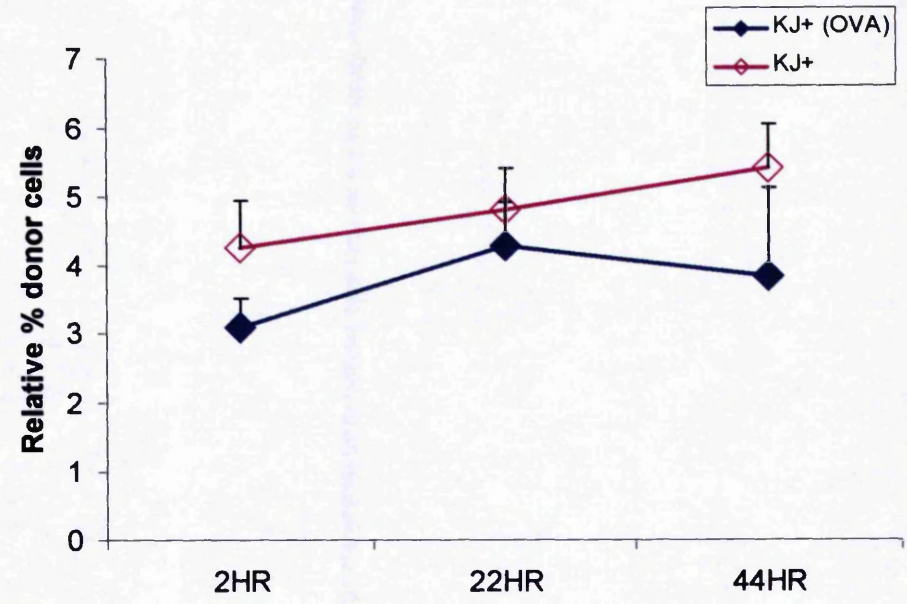
(a) KJ^+



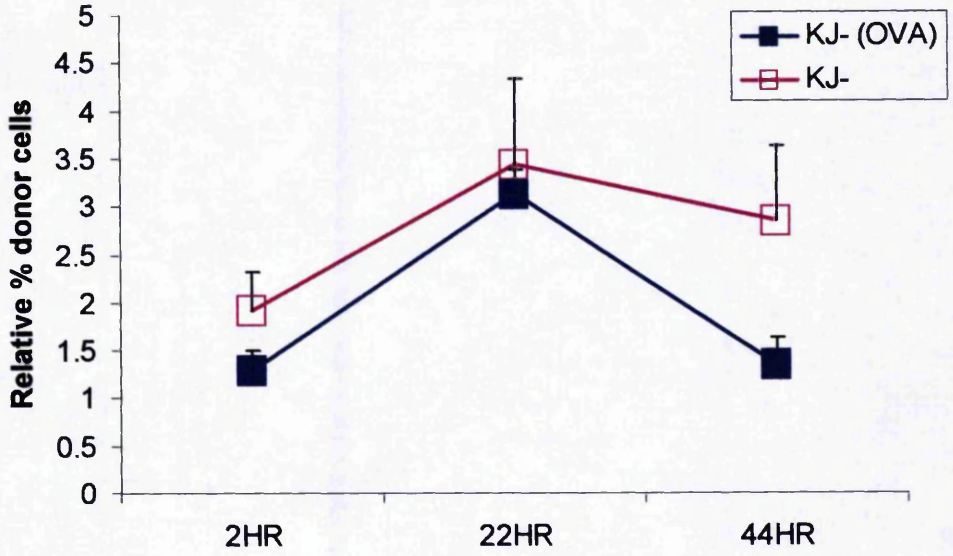
(b) KJ^-

Figure 3.15. Comparison of KJ^+ and KJ^- T cells in the MLNs of immunized and non-immunized recipients.

Relative percentages of donor KJ^+CD4^+ and KJ^-CD4^+ T cells in the MLN at 2 hours, 22 hours and 44 hours after the adoptive transfer of 5 x 10^6 donor T cells into apOVA pre-immunized and control BALB/cIgH mice. The mean values of 4-6 recipients per group are shown + SD.



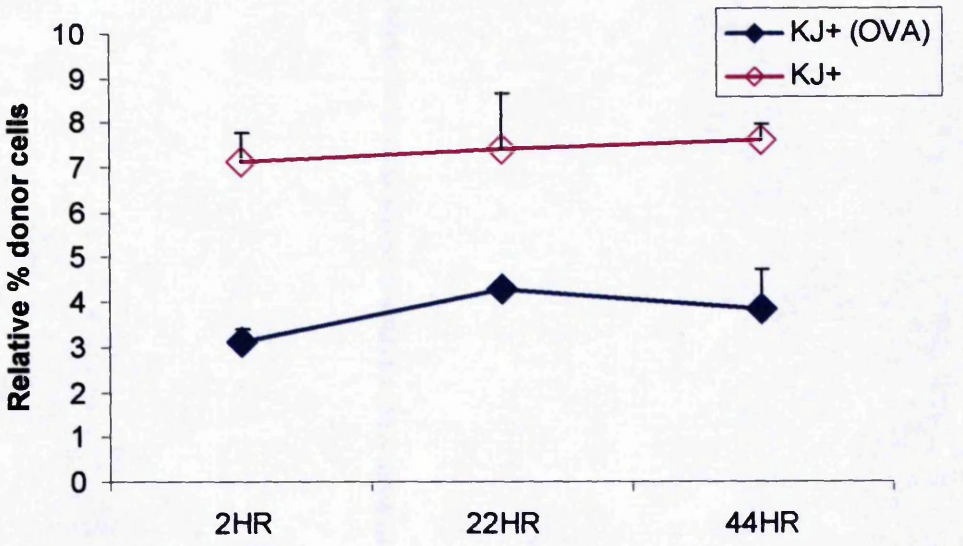
(a) KJ^+



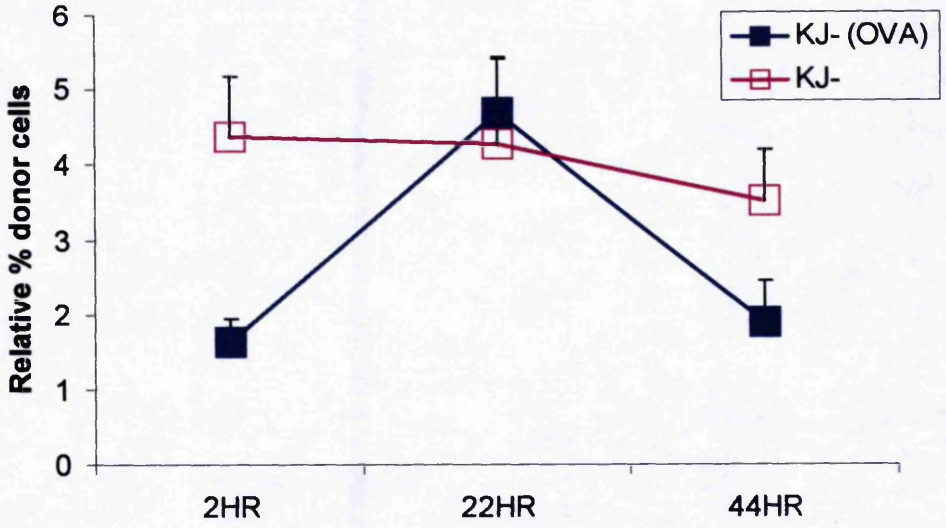
(b) KJ^-

Figure 3.16. Comparison of KJ^+ and KJ^- T cells in the ILNs of immunized and non-immunized recipients.

Relative percentages of donor KJ^+CD4^+ and KJ^-CD4^+ T cells in the ILN at 2 hours, 22 hours and 44 hours after the adoptive transfer of 5 x 10^6 donor T cells into apOVA pre-immunized and control BALB/cIgH mice. The mean values of 4-6 recipients per group are shown + SD.



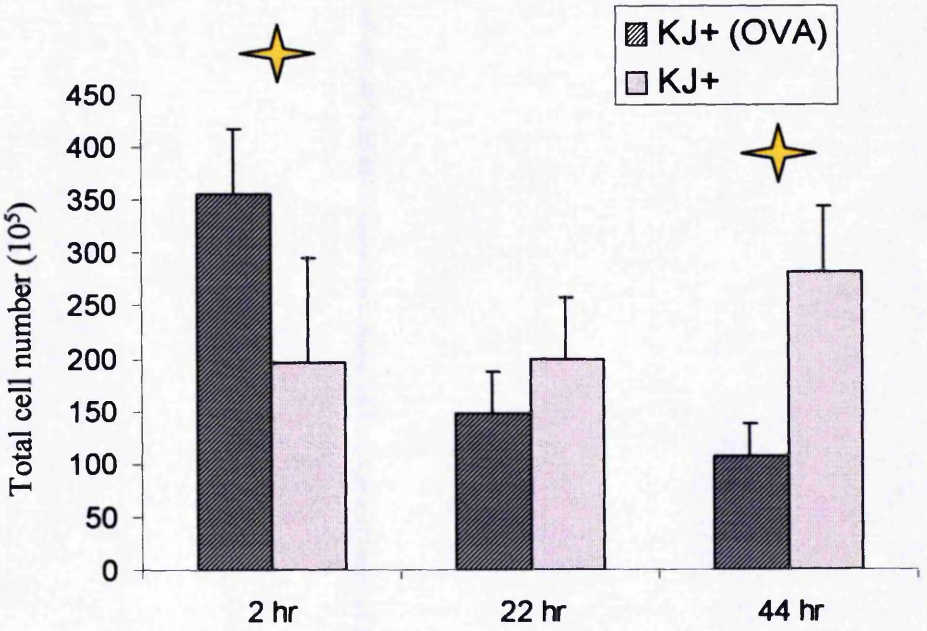
(a) KJ^+



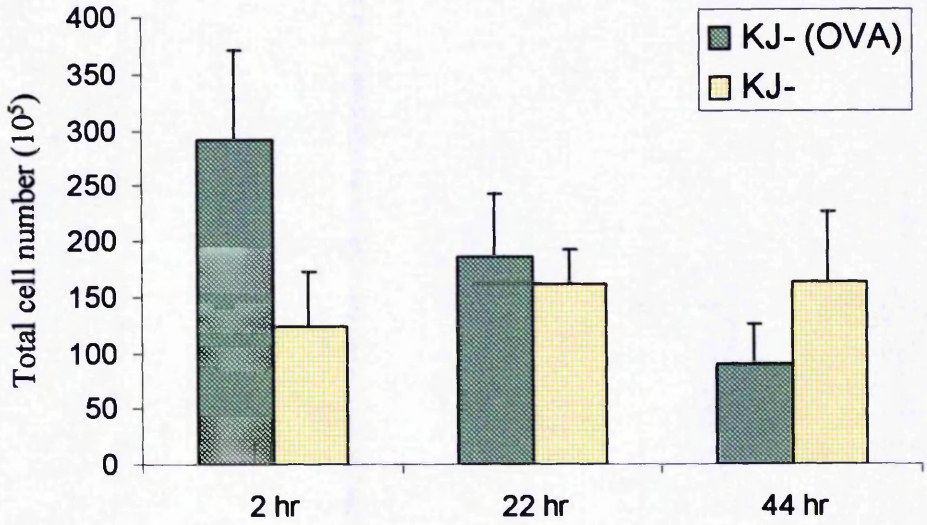
(b) KJ^-

Figure 3.17. Comparison of KJ^+ and KJ^- T cells in the CLNs of immunized and non-immunized recipients.

Relative percentages of donor KJ^+CD4^+ and KJ^-CD4^+ T cells in the CLN at 2 hours, 22 hours and 44 hours after the adoptive transfer of 5 x 10^6 donor T cells into apOVA pre-immunized and control BALB/cIgH mice. The mean values of 4-6 recipients per group are shown + SD.



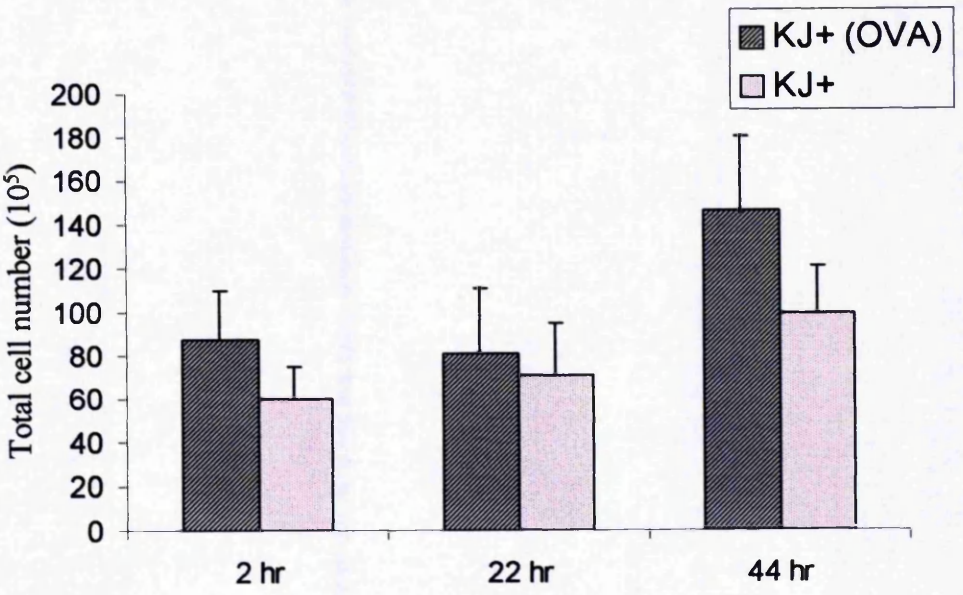
(a) KJ^+



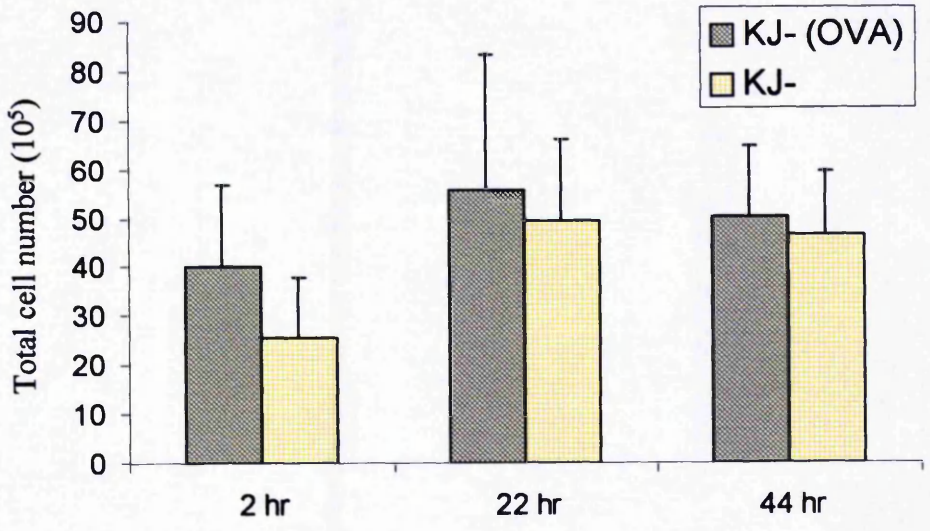
(b) KJ^-

Figure 3.18. Comparison of the number of KJ^+ and KJ^- T cells in the spleens of immunized and non-immunized recipients.

Total number of donor cells, KJ^+ (a) and KJ^- (b) in the spleens of OVA immunized and control BALB/cIgh recipients 2 hrs, 22 hrs, and 44 hrs after the adoptive transfer of 5 x 10^6 $CFSE^+CD4^+$ T cells from DO11.10 mice. The mean values of 4-6 recipients per group are shown + SD. A star represents a significant difference ($p<0.05$).



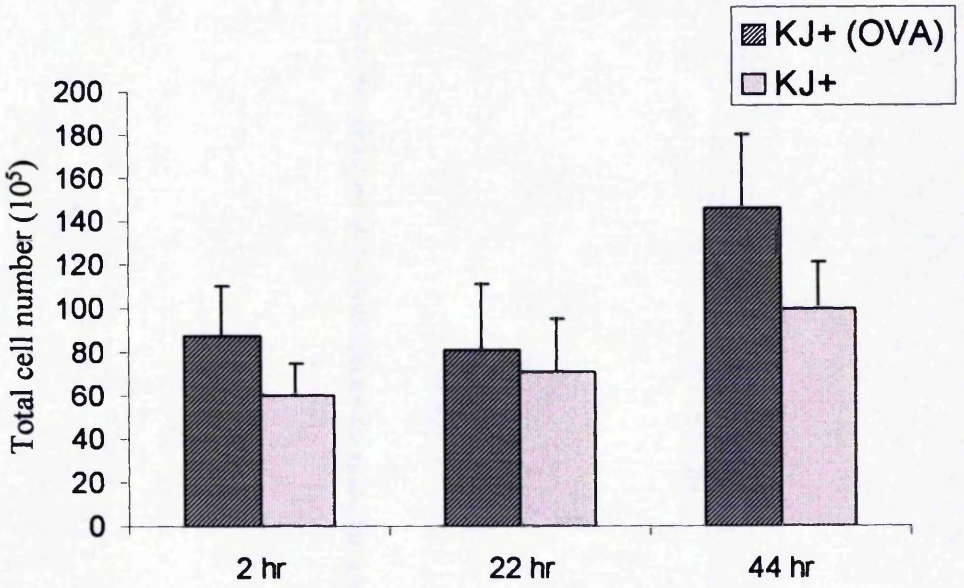
(a) KJ^+



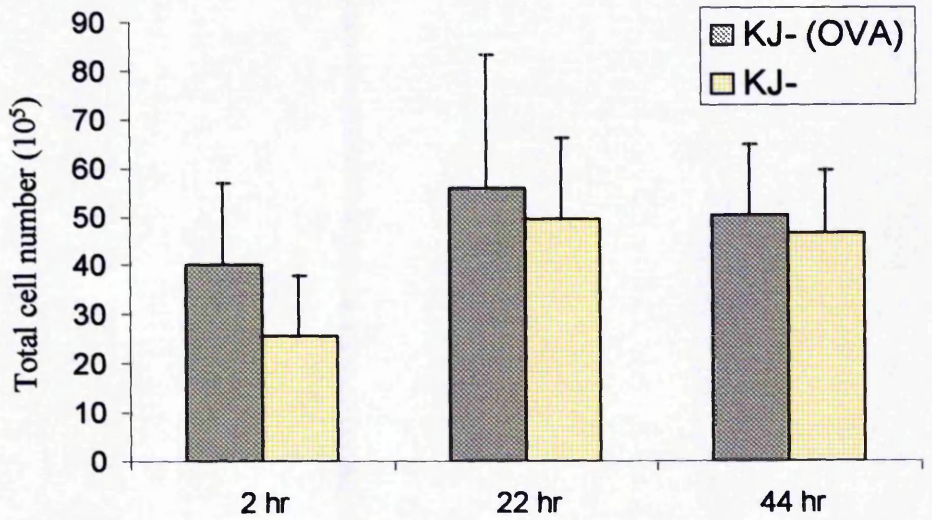
(b) KJ^-

Figure 3.19. Comparison of the number of KJ^+ and KJ^- T cells in the MLNs of immunized and non-immunized recipients.

Total number of donor cells, KJ^+ (a) and KJ^- (b) in the MLNs of OVA immunized and control BALB/cIgh recipients 2 hrs, 22 hrs, and 44 hrs after the adoptive transfer of 5 x 10^6 $CFSE^+CD4^+$ T cells from DO11.10 mice. The mean values of 4-6 recipients per group are shown + SD.



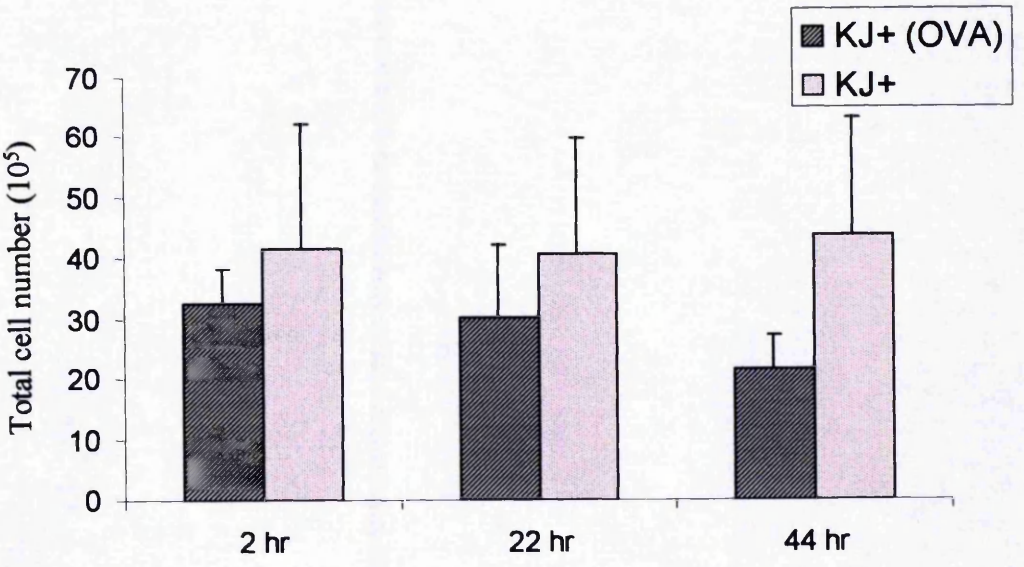
(a) KJ^+



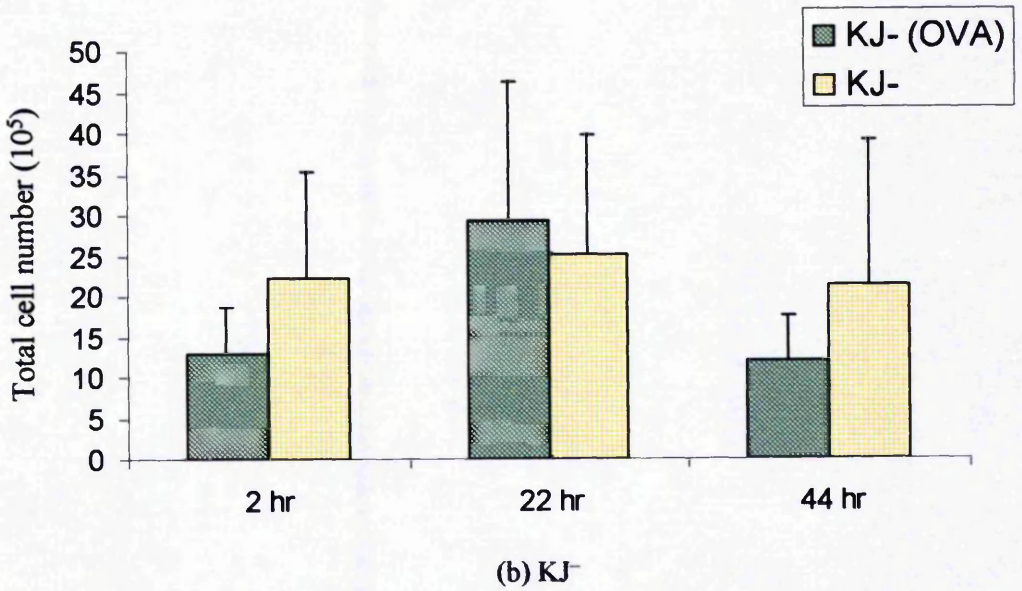
(b) KJ^-

Figure 3.20. Comparison of the number of KJ^+ and KJ^- T cells in the ILNs of immunized and non-immunized recipients.

Total number of donor cells, KJ^+ (a) and KJ^- (b) in the ILNs of OVA immunized and control BALB/cIgh recipients 2 hrs, 22 hrs, and 44 hrs after the adoptive transfer of 5 x 10^6 $CFSE^+CD4^+$ T cells from DO11.10 mice. The mean values of 4-6 recipients per group are shown + SD.



(a) KJ^+



(b) KJ^-

Figure 3.21. Comparison of the number of KJ^+ and KJ^- T cells in the CLNs of immunized and non-immunized recipients.

Total number of donor cells, KJ^+ (a) and KJ^- (b) in the CLNs of OVA immunized and control BALB/cIgh recipients 2 hrs, 22 hrs, and 44 hrs after the adoptive transfer of 5 x 10^6 $CFSE^+CD4^+$ T cells from DO11.10 mice. The mean values of 4-6 recipients per group are shown + SD.

3.2.3. Analysis of Proliferating KJ^+CD4^+ T cells.

To determine whether the donor T cells were dividing CFSE dye was used. CFSE is a fluorescent dye, which was described in 3.1.6 Daughter cells from each cell division appeared as identifiable peaks of decreasing fluorescence during flow cytometric analysis.

Mice were injected i.p. with 100µg apOVApep 3 days prior to i.v. injection of 5 x 10^6 $CFSE^+CD4^+$ T cells (both KJ^+ and KJ^-). 44 hours after the adoptive transfer of cells, the KJ^+CD4^+ T cells in the spleens and LNs of OVApep recipients were observed to be dividing as shown in Figures 3.12 & 3.13. The donor KJ^-CD4^+ T cells in OVApep injected mice (Fig 3.22b), and the donor KJ^+CD4^+ and KJ^-CD4^+ in control mice (Fig 3.23) showed no evidence of cell division. This indicated that KJ^+CD4^+ T cells were proliferating in response to OVApep, and that non tg T cells did not respond to this antigen. This confirms the important antigen-specific nature of this tg model.

Figure 3.22a showed that dividing KJ^+CD4^+ T cells could be separated into 4 distinct populations as identified by 4 separate peaks of CFSE flourescence. M1 represents the initial parent population which have yet to divide as well as the undivided KJ^-CD4^+ T cells, M2 identifies the first daughter population, M3 the second daughter population, and M4 contains cells that have undergone 3 or more cell divisions. It was reported that CD4 expression was up-regulated prior to cell division and was therefore an early marker of T cell activation (Lee & Pelletier, 1998). The KJ^+CD4^+ T cells which had not undergone cell division (R6, Fig 3.22b), and the KJ^+CD4^+ T cells which had undergone one or more cell divisions (R5, Fig 3.22b), were gated and assessed individually for levels of CD4 expression as labelled by PE-CD4. CD4 expression level was assessed in two separate experiments (Figure 3.24). The profile of the undivided populations (blue) was overlaid on the profile of divided T cells (green) for comparison. In one experiment CD4 appeared to be slightly up-regulated on cells which had divided (Fig 3.24a), but when this experiment was repeated the opposite was observed (Fig 3.24b). Therefore up-regulation of CD4 expression does not appear to be a reliable marker for early T cell activation.

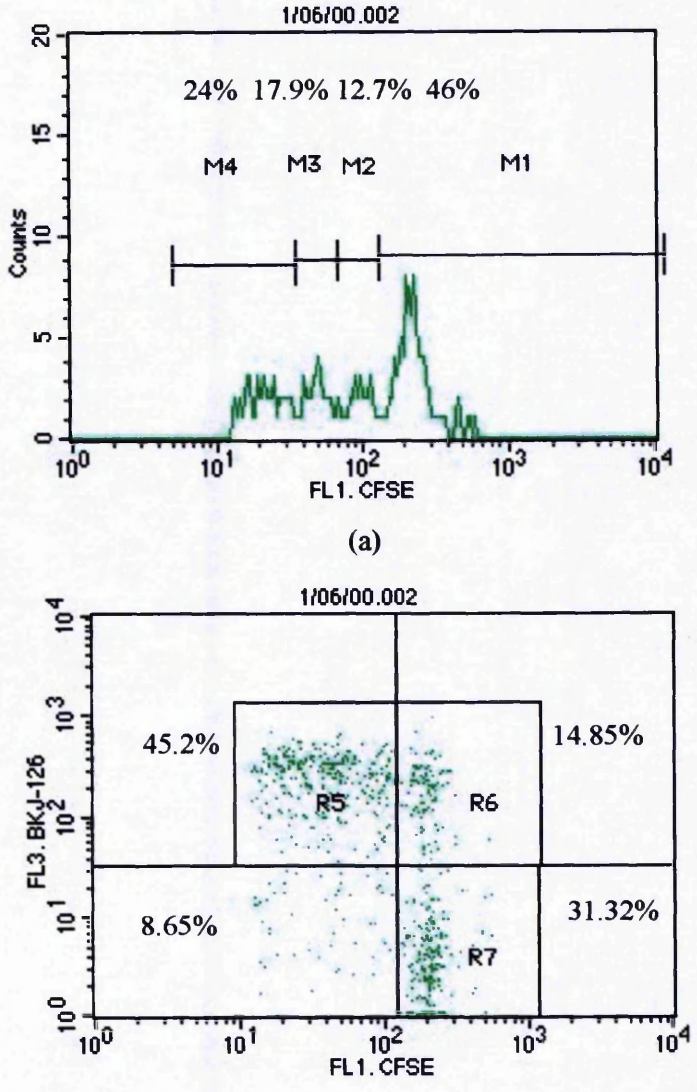


Figure 3.22. Flow cytometric analysis of KJ^+ and KJ^- T cells after transfer to immunized recipients.

Flow cytometric analysis of MLN from OVApep pre-immunized BALB/cIgH 44 hours after the adoptive transfer of 5 x 10^6 $CFSE^+CD4^+$ T cells from DO11.10 mice. In this tissue 4 peaks of CFSE fluorescence can be seen (a). When KJ expression was examined alongside CFSE (b), it is clear that only the KJ^+ T cells have divided (R5, R6) where as KJ^- cells had not (R7). The percentage of cells in each peak or gate is shown.

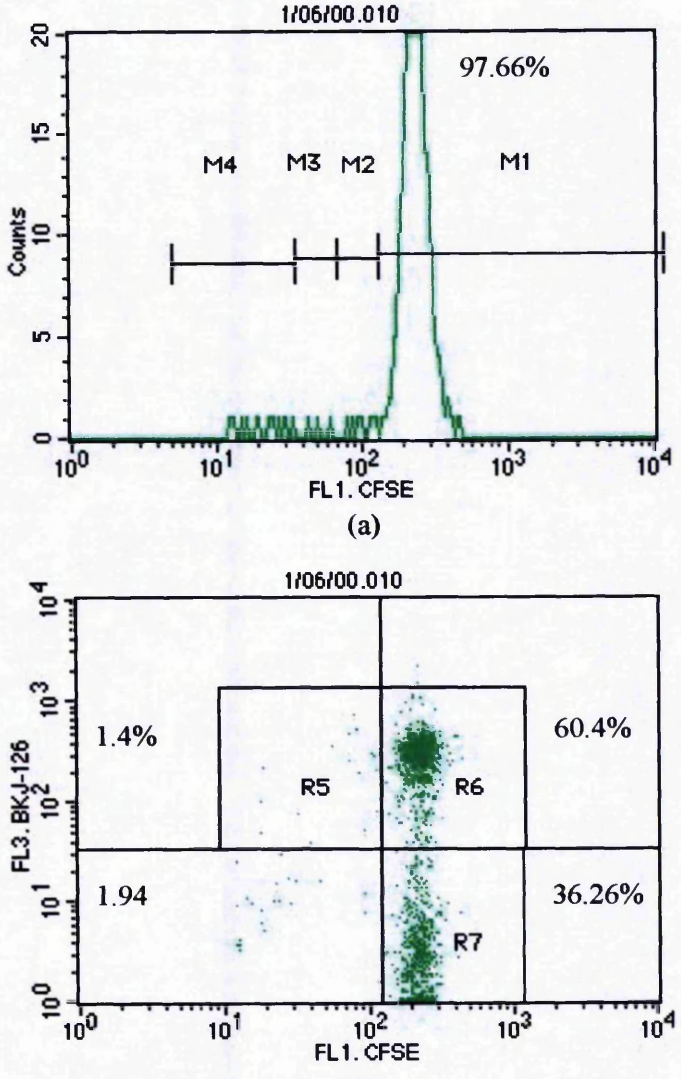
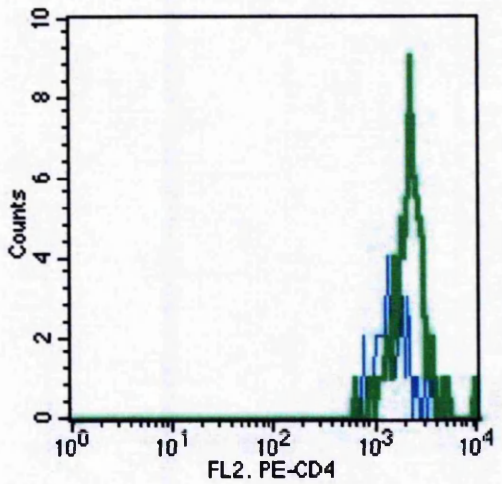
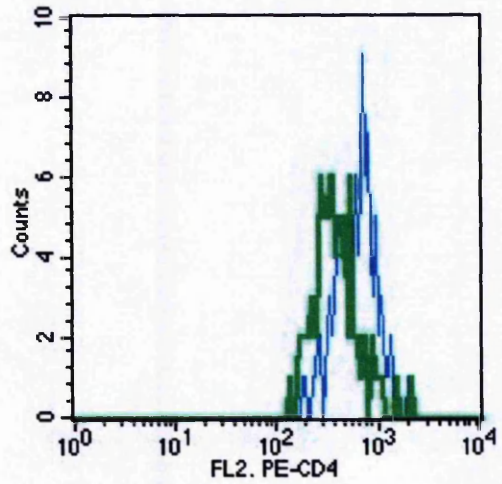


Figure 3.23. Flow cytometric analysis of KJ^+ and KJ^- T cells after transfer to non-immunized recipients.

Flow cytometric analysis of MLN from control BALB/cIgH mice 44 hours after the adoptive transfer of 5 x 10^6 $CFSE^+CD4^+$ T cells from DO11.10 mice. In these unimmunized mice a single peak of CFSE fluorescence was observed in both the KJ^+CD4^+ T cells and the KJ^-CD4^+ T cells (a) (b).



(a)



(b)

Figure 3.24. CD4 expression of KJ^+ T cells before and after cell division.

Contrasting CD4 expression of KJ^+CD4^+ T cells 44 hours after adoptive transfer into OVApep pre-immunized BALB/cIgH mice. The blue profile represents KJ^+CD4^+ T cells which have not undergone cell divsion and the green profile are KJ^+CD4^+ T cells which have divided. Two separate experiments are shown (a, b) illustrating contradicting findings.

Chapter 4

Establishing Adoptive Transfer System.

4.1 Introduction

4.1.1 B Cells require T cell help

For the initiation of a humoral immune response, either primary or secondary, $CD4^+$ T cell help is required. This occurs when antigen-binding receptors on $CD4^+$T cells link to antigenic peptide-class II MHC complexes on antigen-presenting cells. This interaction, combined with the necessary co-stimulatory signal, leads to activation and proliferation of the T cells. The clonally expanded population of antigen-specific $CD4^+$ T cells can then help the activation of the antigen-specific B cells.

A mature antigen-committed B lymphocyte may act as the antigen-presenting cell. For this to occur the antigen cross-links membrane-bound antibody molecules on the B cell (Morris *et al.*, 1994). Some of the bound antigen is internalised by receptor-mediated endocytosis (Morris *et al.*, 1994). The antigen is processed within the cell and its antigenic peptides are presented within class II MHC molecules, on the cell surface (Morris *et al.*, 1994). Antigen binding also initiates signalling through the B cell receptor (BCR), which causes the cell to up-regulate a number of cell-membrane molecules, one of which is the co-stimulatory ligand B7 (Han *et al.*, 1997). When a $CD4^+$ T cell recognises the antigen, surrounded by the necessary molecules it immediately binds forming a T-B conjugate. It must be said that B cells only become important antigen presenting cells to naïve $CD4^+$ T cells at low levels of antigen (Rivera *et al.*, 2001). This is because of the way they specifically recognise antigen through their membrane Ig. This allows B cells to present antigen at concentrations 100 to 10 000 times lower than that required for presentation by macrophages or dendritic cells, which are the major antigen presenting cells at higher concentrations (Rivera *et al.*, 2001).

The formation of the T-B conjugate leads to the up-regulation of CD40L on the $CD4^+$ T cell (Foy *et al.*, 1994). CD40L (CD154) is a member of the tumour necrosis factor receptor (TNFR) family and interacts with CD40 on the B cell. This results in the activation of a number of intracellular signal pathways, which ultimately lead to changes in gene expression. The combination of the CD40-CD40L interaction and the

antigen cross linkage of membrane Ig pushes the B cell into G_1 (Han *et al.*, 1997). At this stage the B cells are able to proliferate, but without cytokines they fail to differentiate. They begin to express membrane receptors for a variety of cytokines, such as IL-2, IL-4, and IL-5, which are produced by the interacting $CD4^+$ T cell (Stepmann *et al.*, 1996). These cytokines allow the B cell to differentiate and form antibody secreting plasma cells and memory B cells. It has been shown using antigen-specific T and B lymphocytes that both Th1 and Th2 $CD4^+$ T cells support B cell responses to a similar degree *in vivo* (Smith *et al.*, 2000). Interestingly, while the kinetics of T cell clonal expansion were similar between the two subsets, Th2 cells expanded significantly less than Th1 cells (Smith *et al.*, 2000). Despite this both cell types were able to support a similar level of B cell clonal expansion and antibody production (Smith *et al.*, 2000) This suggested that Th1 and Th2 $CD4^+$ T cells may provide help in different ways; Th1 cells may provide poorer help on a cell-cell basis but overcome this by expanding to provide more cells, whereas Th2 cells provide more help per cell and therefore do not have to expand as much (Smith *et al.*, 2000). However, both Th1 and Th2 cells provided help via the CD154-CD40 interaction (Smith *et al.*, 2000). It is likely that the difference between the two is a result of the cytokines that they produce.

4.1.2 Help from naïve and effector/memory T cells.

Using a variety of T cell clones with restricted cytokine profiles, it has been shown that naïve Th1 and Th2 T cells induced antibody synthesis in primed and unprimed B cells *in vitro* (DeKruff *et al.*, 1989). It was also shown that the induction of IgG1 synthesis in secondary responses could occur via several pathways, one involving IL-4, IL-5, and the other involving IL-2 (DeKruff *et al.*, 1993). IgE and Ig2a synthesis required specific cytokines (Ig2a requiring IFN-γ and IL-2) (DeKruff *et al.*, 1993). Croft and Swain showed that the combination of IL-2, IL-4, and IL-6 was essential for Ig secretion of all isotypes from naïve B cells in response to help from naïve, effector, and memory T cells (Croft & Swain, 1991). However they did report that 4 day primed "effector" T cells provided significantly better help than resting T cells (Croft & Swain, 1991). These observations were supported by further *in vitro* studies in which human primed ($CD45RB^{dim}$) $CD4^+$ T cells provided greater *in vitro* help for B cell antibody production than naïve ($CD45RB^{bright}$) $CD4^+$ T cells (Tortorella *et al.*, 1994). In these studies the cells were stimulated with anti-CD3 mAb rather than specific antigen

(Tortorella *et al.*, 1994). A similar *in vitro* study showed that although naïve $CD45RB^{hi}$ T cells interacted effectively with B cells to induce an activation signal, in the absence of IL-4, plus IL-5 or IL-6 they did not provide help for an antibody response (Lee & Vitetta, 1991). In order to secrete these cytokines they firstly had to differentiate into $CD45RB^{lo}$ T cells (Lee & Vitetta, 1991). However all of these studies were conducted *in vitro* and did not examine antigen-specific cells. More recently using an adoptive transfer model in the rat it was shown that naïve T cells as well as primed and "revertant" memory T cells could provide help for memory B cells in an antigen-specific manner. A direct comparison of the help provided by the naïve and memory T cells could not be made due to the lack of a marker for the antigen-specific cells. This can be overcome with the use of tg T cells which all share the same antigen-specificity. One study which used tg T cells specific for pigeon cytochrome C, showed that culturing naïve $CD4^+$ T cells with primed resting B cells did not lead to Ig production (Croft & Swain, 1995). However, if the T cells were activated first by either DCs or activated B cells they were able to induce IgM production (Croft & Swain, 1995). Fibroblast cell lines expressing the B7-1 costimulatory molecule alone, or with ICAM-1 (both strong costimulatory molecules) were also able to activate naïve tg T cells to help B cell antibody production, as did culture with anti-CD28 and anti-CD3 (Croft & Swain, 1995). Interestingly, helper activity was markedly reduced in the presence of anti-IL-4, suggesting a crucial role for this cytokine in T cell help for B cells (Croft & Swain, 1995). The ability of tg T cells to help B cells *in vivo* has yet to be fully explored.

4.1.3 Development of the Primary Response.

It was originally believed that initial contact between naïve $CD4^+$ T and B cells occurs either in the periarteriolar lymphoid sheath (PALS) of the spleen or the paracortex of the lymph nodes (Tarlinton & Smith, 2000). Using antigen-specific T and B lymphocytes it has been shown that T cells firstly become activated in the T cell area (paracortex), and then migrate to the edges of the follicles where they interact with B cells (Garside *et al.*, 1998). As proliferation continues the B cells migrate deep into the primary follicles of the spleen or the cortex of the lymph nodes (Tarlinton & Smith, 2000). Here the cells can form germinal centres, where rapidly dividing B cells (centroblasts), which are characterised by their large size, expanded cytoplasm, diffuse

chromatin, and lack of membrane Ig give rise to smaller cells called centrocytes (Fliedner *et al.*, 1964) (Tarlinton & Smith, 2000). These are non-dividing cells, which express membrane Ig and come into contact with antigen bearing follicular dendritic cells (FDCs). They have already undergone somatic hypermutation, which generates a few cells with receptors of higher affinity and many cells with receptors of unchanged, lower, or no affinity (Tarlinton & Smith, 2000). These cells must then compete for antigen displayed by the FDCs. Because this antigen is limited, only centrocytes with receptors of high affinities receive a signal, which allows them to survive. The centrocytes must also receive signals from an interacting $CD4^+$ T cell, some of which induce class switching of the B cell's antibodies. By allowing the isotype of the antibodies heavy chain to change, while keeping the specificity unaltered, it enables the antibody to participate in a variety of effector functions. The new class of Ig is determined by cytokines produced by the $CD4^+$ T cell and is dependent upon the type of antigen presented. In addition the microenvironment influences the class of plasma cell that emerges. For example, plasma cells leaving the follicles of Peyer's patches are committed to IgA production, while those, which originate in the spleen or peripheral lymph nodes are mainly IgG.

4.1.4 Generation of Plasma Cells and Memory B Cells.

When a centrocyte has undergone hypermutation and expresses high affinity membrane Ig for the antigen presented by FDCs, it may differentiate into plasma cells or memory B cells. This occurs in the apical light zone and is determined by the type of signal it receives. A study of affinity maturation in antibody-secreting versus memory cells suggested that expression of high-affinity antibody may favour plasma cell rather than memory cell development (Smith *et al.*, 1997). In a separate *in vitro* study of B cell differentiation, it was observed that in the presence of IL-2 and particularly IL-10, and the absence of CD40L, plasma cell differentiation occurred (Zhang *et al.*, 2001). A novel growth factor, produced by FDCs called FDC_SM_8D6 was also discovered (Zhang *et al.*, 2001). When this protein was blocked in culture by the mAb 8D6, it inhibited the differentiation of GC-B cells into Ig-secreting plasma cells but did not affect memory B cell proliferation (Zhang *et al.*, 2001). Differentiation was only inhibited when the blocking antibody 8D6 was added at the beginning of the culture (Zhang *et al.*, 2001). Interestingly the addition of the mAb 8D6 inhibited proliferation

and Ig secretion in cultures of $CD27^+$ B cells but not $CD27^-$ B cells (Zhang *et al.*, 2001).

Upon plasma cell differentiation, there was an immediate increase in Ig production, which was mainly of the secreted form. These changes were observed intracellularly as there was an increased cytoplasmic to nuclear ratio, and prominent rough endoplasmic reticulum and secretory vacuoles (Calame, 2001). As well as surface Ig other B cell-specific surface proteins were downregulated. These included MHC class II, B220, CD19, CD21, and CD22 (Calame, 2001). Expression of the chemokine receptors CXCR5 and CCR7 were decreased, which reduced responsiveness to the B and T cell zone chemokines CXCL13, CCL19 and CCL21 (Calame, 2001). These changes together with the maintenance of CXCR4 expression, allowed the plasma cells to move from the follicles to other locations such as the bone marrow. The proteoglycan syndecan-1 (recognises extracellular matrix) and the integrin very late antigen (VLA-4) (mediates cell-cell adhesion) were both induced on antibody-producing cells (Calame, 2001).

For differentiation to a memory B cell, a centrocyte must receive the necessary survival signals from a $CD4^+$ T cell. The CD40L-CD40 interaction is important for this. This has been shown in experiments, in which the action of CD40 was blocked and the B cells in the GC failed to enter the memory pool (Siepmann *et al.*, 2001). Those B cells that failed to bind, internalise and present antigen to the $CD4^+$ T cell did not elicit CD40L expression and underwent apoptosis. Blocking of CD40 *in vivo* only prevented memory B cell formation if the antibody was administered in the first five days following immunization (Gray *et al.*, 1994). Therefore it was not the germinal centre that was affected. It was also observed that B cells from the GC of CD40-Ig treated mice could not be rescued from apoptosis by the addition of anti-CD40 *in vitro*, unlike GC B cells from normal mice (Gray *et al.*, 1997). This suggested that the delivery of the CD40 rescue signal to the GC B cell is dependent upon previous CD40 ligation during the early T-B cell interaction (Gray *et al.*, 1997). One way that CD40L may operate is by up-regulation of the transcriptional factor BSAP on B cells (Liu & Banchereau, 1997). Recent work has also shown that expression of SAP by $CD4^+$ T cells is essential for generating long-lived plasma cells and memory B cells (Crotty *et al.*, 2003). It is not required for initial B cell help or isotype switching suggesting a crucial role in memory cell formation (Crotty *et al.*, 2003).

In the *in vitro* system, which showed the importance of IL-10 for plasma cell differentiation, it was also shown that IL-4 helps the generation of memory B cells (Zhang *et al.*, 2001). Though lacking a distinctive membrane marker, there are phenotypic differences between naïve B cells and memory B cells. While naïve B cells express only IgM and IgD, memory B cells express additional isotypes such as IgG, IgA, and IgE, while often expressing little or no IgM or IgD (McHeyzer-Williams *et al.*, 2000) (Schittek & Rajewsky, 1990). In fact it has been shown, using a murine transgenic system in which all of the B cells expressed transgenic IgD as well as endogenous IgD, that the presence of transgenic IgD on memory B cells severely affected the secondary response (Yuan *et al.*, 2001). B cell development and the primary response were not affected, and the depressed secondary response can be overcome by prior aggregration of the transgenic IgD with allotype-specific anti-IgD antibodies (Yuan *et al.*, 2001). Therefore loss of IgD following activation may be necessary for optimal stimulation of memory B cells. Recently CD27 has been proposed as a possible marker for human memory B cells. Reasons for this are that CD27 expression increase with age, $CD27^+$ B cells produce high levels of Ig in the presence of stimuli such as IL-10, SAC plus IL-2 and IL-4 plus CD40 signalling (Agematsu *et al.*, 2000). $CD27^+$ B cells differentiate easily into plasma cells compared with $CD27^-$ B cells, and more importantly, they possess identifiable mutations in their Ig V region (Agematsu *et al.*, 2000).

Three models have been proposed to explain the formation of plasma cells and memory cells. The first is that memory B cells and plasma cells develop from an unequal division of a common precursor. The second proposes that cytokines or other signals within the GC influence the differentiation of a common precursor cell into both cell types. One such model proposes that when Ig secreted by plasma cells has reached a sufficiently high concentration in the GC, B cells interacting with antigen will have a high probability of having their FcγRII receptors engaged (Tarlinton & Smith, 2000). This inhibitory signal may be the key to inducing formation of the memory B cell compartment (Tarlinton & Smith, 2000). The third model suggests that the cells arise from different lineages, each of which clonally expands following primary antigen exposure. This latter model has received evidence from studies which showed that these two different precursor cells could be distinguished by different levels of the heat-stable antigen (HSA) marker (Sprent, 1994). Following adoptive transfer, HSA^+ B cells elicited strong primary responses to antigen, but fail to generate memory cells (Sprent,

1994). In contrast $HSA^{lo/-}$ B cells gave poor primary responses, but functioned well as precursors of memory cells (Sprent, 1994). Another B cell lineage marker that has been proposed is J11d. It was shown in mice that B cells that express high levels of J11D mainly give rise to plasma cells, while B cells that express low levels of J11D give rise to memory B cells after initial immunisation (Liu & Banchereau, 1997).

4.1.5 Secondary Immune Responses.

Upon re-encounter with the same antigen a memory response occurs. This is characterised by a stronger and more rapid response. In the humoral arm of the immune system, as well as being faster and stronger, the memory response is also different in quality. Because of the affinity maturation and class switching that occurs within the B cell population during the primary response, the antibodies produced in the secondary response are of a higher affinity and of different isotypes, predominantly IgG. The memory response will also be more rapid, partly because the immunized animal will have an increased number of B cells specific for the antigen, as well as an increased number of antigen-specific T cells. It has recently been suggested that the faster response is helped by the ability of IgG^+ memory B cells to proliferate rapidly upon antigen encounter (Martin & Goodnow, 2002). The unique membrane tail of the IgG molecule has been implicated as the sole determinant for this (Martin & Goodnow, 2002). This was observed by comparing the *in vivo* antibody response from homogenous Ig-tg B cells, which differed only within the constant region segments (IgM versus IgG) or only in the COOH-terminal membrane tail (IgMG), in which the exons of the secreted product were from IgM but the membrane tail exons were from IgG (Martin & Goodnow, 2002). The presence of the IgG membrane tail resulted in a greatly increased clonal burst during the extrafollicular antibody response (Martin & Goodnow, 2002). However less is known about the secondary response than the primary response. It is not known whether the formation of germinal centres is required, or where secondary stimulation of B cells occurs (Ochsenbein *et al.*, 2000). One site that has been proposed is within the bone marrow (Ochsenbein *et al.*, 2000). Although germinal centre formation may not be required $CD4^+$ T cell help is essential for a secondary humoral response.

As with T cell memory, the mechanisms for the maintenance of B cell memory are not fully understood. However, following a successful primary immune response,

the majority of the responding cells will undergo apoptosis. This is a vital element of lymphocyte homeostasis and ensures that the animal retains a balance of naïve and memory cells. Despite the death of the majority of the antibody secreting cells, antibody levels in the circulation may persist at high levels for years after the initial infection (Slifka & Ahmed, 1998). This has often been explained by the suggestion that the immune system is stimulated repeatedly by either low grade chronic infection or intermittent re-exposure to the pathogen. However there are numerous cases where long-term humoral immunity has occurred in the absence of re-exposure to the pathogen. The most famous case is perhaps Panum's study of measles infection on the Faroe Islands (Panum, 1847). In this instance the islanders were protected against a second epidemic 50 years following the first, despite the absence of re-exposure to measles during the intervening years (Panum, 1847).

Similar to this hypothesis of pathogen re-exposure maintaining immunological memory is the idea that antigen-antibody complexes on the surface of follicular dendritic cells continually stimulate memory B cells to produce antibody. Evidence for this has come from experiments where transfer of primed lymphocytes in the absence of antigen has resulted in loss of memory response, and conversely cotransfer with antigen leads to maintenance of memory (Gray & Skarvall, 1988). However more recent experiments have produced results that dispute this claim. By using a genetic switch mediated by Cre recombinase, it was shown that memory B cells switching their antibody specificity away from the immunizing antigen were maintained in the animal over long periods of time, similar to cells retaining their original antigen-binding specificity (Maruyama *et al.*, 2000). In another study it was shown that transfer of $B220^+$ B cells only resulted in antibody secretion if antigen was cotransferred (Manz *et al.*, 1998). In contrast transfer of $B220^-$ plasma cells resulted in persistent serum antibody levels which was independent of antigen (Manz *et al.*, 1998).

The major problem in the argument that antigen is required for the persistence of B cell memory, is how and where this antigen is stored within the lymphoid tissue for prolonged periods. Evidence does exist that antigen may be sequestered on the surface of follicular dendritic cells in germinal centres for months, and possibly even years (Tew *et al.*, 1990). However, studies have also shown that the immune complexes decline with an average half-life of eight weeks (Tew & Mandel, 1979). There is also the question of why the antigen is not consumed during the restimulation of memory B cells. It is highly probable that antigen is retained by FDCs, which may help to

maintain short term immunity but it seems unlikely that this is the cause of long term immunological memory. Stimulation of memory B cells by other means such as cross-reactivity to self or environmental antigens has also been proposed as a means of prolonging antibody production. However it is not clear what the mechanisms are that would control this situation.

4.1.6. Long-lived Plasma Cells.

One theory which would explain the persisting levels of circulating antibody without the need for continual stimulation is the existence of long lived plasma cells. The B cells that exit the germinal centre and enter the memory pool appear to consist of two general sub-types, one that is able to secrete antibody (i.e. a plasma cell) and the other a non-secreting precursor to the memory response (McHeyzer-Williams & Ahmed, 1999). Early studies on plasma cells showed that during the initial immune response these cells were short lived and had a half life of only a few days (Schooley, 1961). However, during this early stage of the immune response, B cells were undergoing continual proliferation, selection, differentiation, and apoptosis. When their numbers were examined several months following a vaccination a different pattern was observed (Miller, 1964). As well as observing rapidly disappearing ^{3}H labelled plasma cells from the draining lymph nodes in the first few weeks following vaccination, the authors also saw at later time points, the numbers of plasma cells declining more slowly with a substantial number of cells remaining at 6 months after vaccination (Miller, 1964).

More recent experiments have given better evidence that plasma cells can survive for several months in the absence of repopulation by memory B cells. The first of these experiments monitored the persistence of lymphocytic choriomeningitis virus (LCMV) specific antibody levels as well as plasma cell numbers in mice that were depleted of memory cells by irradiation (Slifka *et al.*, 1998). Following irradiation, the mice were reconstituted with naïve allotypic B cells (whose antibodies could be distinguished from the host cell's due to the presence of a different allotype, IgH^{b}, where the host cells were IgH^{a}) (Slifka *et al.*, 1998). Despite becoming fully reconstituted no donor-derived virus-specific antibody was detected, and donor cells were not stimulated into becoming memory B cells (Slifka *et al.*, 1998). Using mathematical modelling plasma cell longevity was examined and the rate at which

serum antibody declined after memory B cell depletion indicated that murine plasma cells had a half-life of 138 days (Slifka *et al.*, 1998). There was little difference observed between IgG1 and IgG2a secreting plasma cells (Slifka *et al.*, 1998). Virus specific plasma cells were also adoptively transferred into naïve mice and antibody levels were observed over four months indicating that these plasma cells were long lived and maintained a prolonged antibody response (Slifka *et al.*, 1998). Using an ELISPOT assay the number of plasma cells in the spleen and bone marrow was examined, which showed that plasma cells in both tissues were equally long-lived which contradicted what was previously thought (Slifka *et al.*, 1998). In another experiment, which demonstrated evidence for long-lived plasma cells, ovalbumin-specific plasma cells were labelled with bromodeoxyuridine during vaccination and the number of plasma cells was examined for 120 days (Manz *et al.*, 1997). It was observed that over 60% of ovalbumin-specific plasma cells in the bone marrow survived for at least 90 days without undergoing cell division (Manz *et al.*, 1997). Labelling with bromodeoxyuridine between 19 and 120 days after vaccination indicated that very few antigen-specific plasma cells originated 60 days after vaccination (Manz *et al.*, 1997). This supported the idea that long lived plasma cells in the bone marrow were not a result of continuous proliferation of memory B cells.

There are obvious advantages to having a population of long-lived plasma cells able to maintain a humoral response. Firstly, as they are selected on the basis of affinity they provide an immediate source of highly specific antibody without the delay of the primary humoral response. Also, if antibody responses were sustained merely by short-lived plasma cells, then numerous memory B cells and $CD4^+$ T cells would be required. Long-lived plasma cells do not require as many accessory cells as short-lived plasma cells (Slifka *et al.*, 1998). It has been suggested that they do not need $CD4^+$ T cell help at all, as they down-regulate MHC Class II molecules (Slifka *et al.*, 1998). However more work is required to confirm these ideas. Much is still not known about long-lived plasma cells, such as what mechanisms maintains their longevity. It is possible that it may be controlled by the microenvironment of the bone marrow, as mucosal antibody responses are short-lived compared to humoral responses (Sifka & Ahmed, 1996). Other issues which remain unresolved are whether there are phenotypic differences between short-lived plasma cells and long-lived plasma cells, possible differences are the lack of surface immunoglobulin and MHC class II molecules (Slifka *et al.*, 1998). Another important question is what conditions are necessary for the generation of long-

lived plasma cells. All these questions must be answered to further our understanding of immunological memory.

4.1.7 Heterogeneity of Memory B Cells.

Of the cells that leave the GC reaction and go on to provide the memory response two general types of cells exist. There is the antibody secreting long-lived plasma cell as previously described and there is the memory response precursor cell (McHeyzer-Williams *et al.*, 2000). Memory response precursors are generally described as $IgM^-IgD^-B220^+$ Ag-binding cells that do not secrete antibody until rechallenge with antigen (McHeyzer-Williams *et al.*, 2000). In a recent study examining Ag specific murine B cells in an immune response to the hapten (4-hydoxy-3-nitrophenyl) acetyl (NP), three distinct subtypes of memory responders were identified (McHeyzer-Williams *et al.*, 2000). Their model consisted of $B220^+$ memory B cells, which upon antigen rechallenge rapidly produced $B220^-$ B cells. These $B220^-$ B cells could self-replenish upon antigen rechallenge and did not produce antibody, but gave rise to $CD138^+$ antibody secreting cells (McHeyzer-Williams *et al.*, 2000). Therefore this $B220^-$ B cell acts as an intermediate between the quiescent memory B cell and the antibody secreting cells. It was further subdivided into two subsets, $CD11b^{++}IgG^+$ and $CD11b^+IgE^+$, which were both distinguishable from the other two memory subtypes, which were $CD11b^-$ (McHeyzer-Williams *et al.*, 2000). CD11b may provide an alternative coreceptor for the BCR. IgG expression was dominant in the $B220^+$, $CD138^+$ and $CD11b^{++}$ subsets while sIgE was only found in the $B220^-CD11b^+$ subset, although no serum IgE was detected (McHeyzer-Williams *et al.*, 2000).

In a follow up study, using the same experimental system, it was shown that the $B220^-$ B cells emerge as a distinct cellular consequence of the primary immune response (Driver *et al.*, 2001). In comparison to the $CD138^+$ plasma cells, which reach peak numbers by day 5, and the $B220^+$ GC B cells, which reach peak numbers by day 7, the $B220^-$ B cells emerge more gradually to peak levels by day 21 (Driver *et al.*, 2001). These $B220^-$ B cells have undergone somatic hypermutation, and loss of B220 appears to be a post-GC event (Driver *et al.*, 2001). Both $B220^+$ B cells and $B220^-$ B cells persist in the spleen at similar numbers for at least 8 weeks post priming (Driver *et al.*, 2001). The $B220^-$ B cells localize to the red pulp of the spleen, from where they migrate to the bone marrow where they may account for nearly 1% of total bone

marrow 21 days after priming (Driver *et al.*, 2001). It is not clear why they migrate in such large numbers to the bone marrow, though it may contain the necessary growth factors for their long-term survival. In a study by a different group it was observed that adoptive transfer of $B220^+$ B cells only resulted in antibody secretion if antigen was cotransferred. In contrast, transfer of $B220^-$ bone marrow cells resulted in persistent serum antibody production which was independent of cotransferred antigen (Manz *et al.*, 1998).

In a study on human B cells, a novel population of phenotypically and functionally distinct memory B cells was identified, which has parallels with the murine study (Bar-Or *et al.*, 2001). These cells were identified as $CD19^+CD27^+CD80^+$ (Bar-Or *et al.*, 2001). Compared with $CD27^+CD80^-$ memory B cells these cells had a lower threshold for activation but did not proliferate to the same extent upon antigenic challenge (Bar-Or *et al.*, 2001). They also produced large amounts of Ig in contrast with the $CD27^+CD80^-$ population, and were more effective at presenting antigen to $CD4^+$ T cells (Bar-Or *et al.*, 2001). However, although it is not suggested that that these human and murine B cell subsets represent equivalent memory B cell subpopulations, both studies show the phenotypic and functional heterogeneity within the memory B cell compartment. This is illustrated in Figure 4.1.

4.1.8 Maintenance of B Cell Memory.

In principle there must be a mechanism to prevent complete depletion of the non-secreting memory B cells. As these cells act as a precursor to the rapid antibody secreting cells, which arise following secondary challenge, they must either be preserved or replenished after antigen encounter, in order to maintain the memory pool. However this is the area of immunological memory about which little is known. One way it may occur is that during antigen-dependent lymphocyte development, there may be a phase in which terminal differentiation is prevented and from which effector cells can be generated indefinitely. This phase could be the memory lymphocyte. It has been suggested that this occurs by the active suppression of terminal differentiation in these cells, giving them a self-renewing capacity similar to that of stem cells in other organ systems (Fearon *et al.*, 2001). Evidence to support this came from studies examining antigen-driven B cell differentiation. As B cells divide numerous times within the germinal centre while undergoing somatic mutation, they cannot predict how many

times they must mutate before acquiring the necessary high affinity Ig receptors that ensure their survival. Therefore it was proposed that a mechanism must exist that suspends terminal differentiation until this stage is reached (Fearon *et al.*, 2001). Bcl-6 is a transcriptional repressor that is expressed by germinal centre B cells, but not by naïve B cells or plasma cells, and is required for the germinal centre reaction (Fearon *et al.*, 2001). It suppresses terminal differentiation of the B cell by preventing the expression of Blimp-1 (B-lymphocyte-induced maturation protein 1), a transcription factor that drives the development of plasma cells. Therefore while expressing Bcl-6 the germinal centre B cell can continually replenish itself. Memory B cells also express Bcl-6, and it has been proposed that this is how they prevent complete depletion following additional antigen challenges (Fearon *et al.*, 2001). Finding similar evidence for the self-renewal capacity of T lymphocytes has been more difficult, partly because the terminally differentiated T cell is not as distinct as the plasma cell, and it may also be able to revert to earlier stages of differentiation (Fearon *et al.*, 2001). However there is some evidence suggesting a similar model for T cell memory though this will be discussed in the next Chapter.

So, in summary, we know that a primary encounter with a foreign antigen may trigger the humoral immune response. For this to occur B cells require help from $CD4^+$ T cells, which results in the formation of germinal centres. These are specialised areas that form within the spleen and lymph nodes, and it is here that affinity maturation and class switching can occur. Upon receiving the necessary signals the B cell then differentiates into plasma cells and memory B cells. In the event of a secondary antigenic challenge, the humoral response replies faster, with an increased production of high affinity immunoglobulin compared with the primary response. The secondary response also requires $CD4^+$ T cell help, although it has been shown experimentally that naïve T cells as well as memory T cells can provide this help. Nonetheless it is easy to distinguish between the primary and secondary responses.

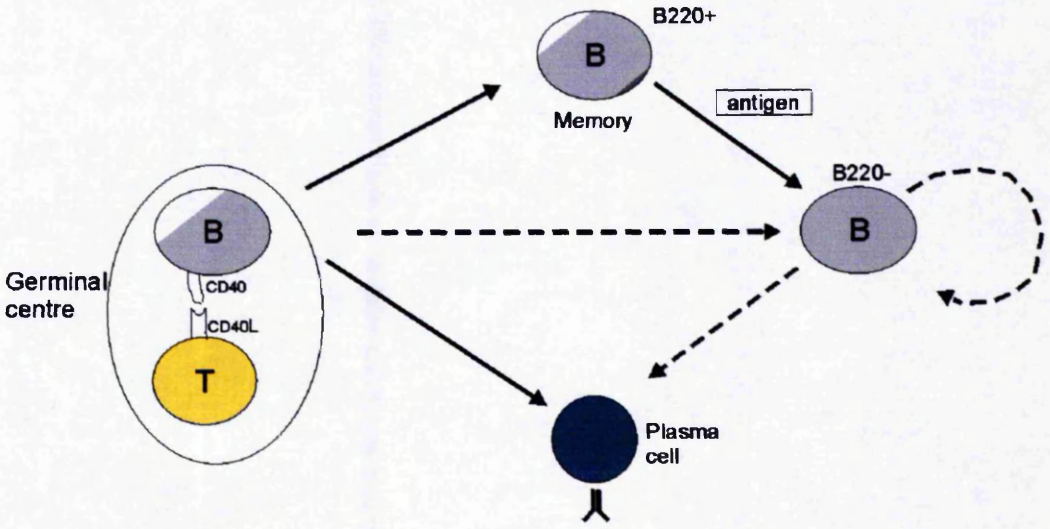


Figure 4.1. Summary of memory B cell formation.

For a primary response to occur a naïve B cell must receive help from a CD4 T cell. This occurs in the germinal centre. Upon receiving the necessary signals the B cell may differentiate into plasma cells which secrete antibody and memory B cells. Upon secondary antigen encounter the memory B cells differentiate into $B220^{-}$ intermediaries which may become plasma cells. The $B220^{-}$ B cells are also thought to be cabale of self-renewal. (Dotted lines represent unknown or uncertain pathways).

Chapter 4

4.2 Results

4.2.1 Sol-OVA challenge does not stimulate Naïve B Cells.

For the main purposes of this study, B cells were primarily used as a tool for studying tg T cell function. As we were interested in secondary immune responses, we generated a population of memory B cells by immunizing normal BALB/cIgh mice with alum-precipitated OVA (ap-OVA). By precipitating OVA with alum OVA acquires adjuvant properties including a slower release of the antigen from the injection site. The aggregate nature of antigen increases its likelihood of phagocytosis. Alum-precipitated antigen also tends to polarize the immune response to a Th2 type. In contrast with the alum-precipitated form of antigen, many soluble proteins fail to stimulate a primary response and instead induce tolerance (Mitchison, 1968). However following priming with ap-antigen a secondary response can be induced by challenge with soluble protein (Bell *et al.*, 2001). In the presence of $CD4^+$ T cells previous studies indicated that sol-OVA could induce primed B cells, but not naïve B cells to produce antibody (Bell *et al.*, 2001). It was important to determine whether transgenic $CD4^+$ T cells behaved in a similar way following sol-OVA challenge. To test this, primed B cells and naïve B cells were adoptively transferred to SCID mice with tg T cells and challenged with 10μg sol-OVA. Anti-OVA antibody responses were measured, shown in Figure 4.2. Naïve B cells in the presence of naïve KJ^+ T cells failed to produce a significant antibody response to sol-OVA. However, sol-OVA challenge induced a strong antibody response by primed B cells, provided that $CD4^+$ T cell help was also available. In the absence of T cells primed B cells failed to respond. The experiment also demonstrates that tg KJ^+ T cells were successful in providing help.

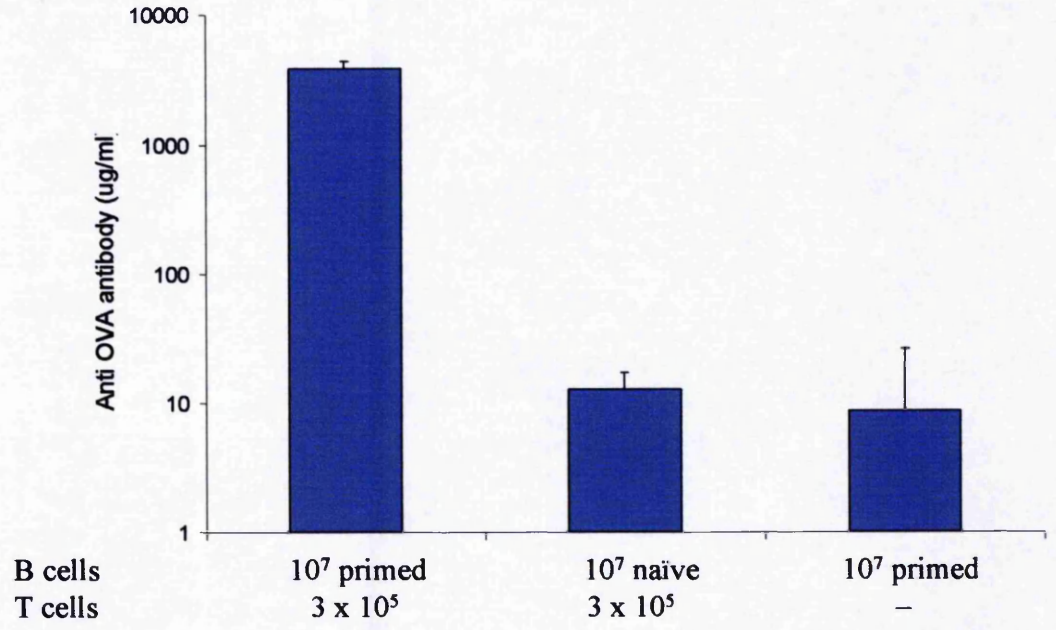


Figure 4.2. Sol OVA challenge stimulates primed B cells but not naïve B cells.

Anti OVA antibody levels of SCID mice that received either 10^7 primed B cells with 3 x 10^5 naïve KJ^+ T cells, or 10^7 naïve B cells with 3 x 10^5 naïve KJ^+ T cells. 10^7 primed B cells were transferred on their own as a control. Mice were challenged with 10μg sol OVA injected i.p. immediately after transfer of cells and blood samples were taken on day 10. The values shown represent the means + SD of 6 recipients per group.

4.10 Preparation of B cells from Primed Mice.

Following primary immunisation, mice were left for at least eight weeks to allow sufficient time for the memory B cells to develop. After this time the mice were killed and a lymphocyte population was prepared from spleen, MLN, ILN, and ALN as described in section2.6. As there is no definitive marker for memory B cells, the cells were purified by depleting the suspension of $CD4^+$ and $CD8^+$ T cells. When the depleted population was adoptively transferred to SCID mice (which have no lymphocytes of their own) any antibody production that was induced would be dependent on the co-transferred $CD4^+$ T cells. The flow cytometric analysis of a representative B cell purification is shown in Figure 28. The starting population was found to contain 24.6% T cells (Figure 4.3b) and 66.5% $B220^+$ B cells (Figure 4.3a). Following two rounds of depletion on SA-coated Biomag particles, the number of cells stained for T cell markers was reduced to 0.05%. (Figure 4.3c). The final suspension used for injection was found to contain 85.5% $B220^+$ B cells (Figure 4.3d), which would include the memory B cells. There is a significant population of non-B lymphocytes in the transferred population, though these cells which would include NK cells and macrophages should not interfere with the experiments. To ensure that all antibody production was a direct result of the help from the transferred tg $CD4^+$ T cells, in each experiment a population of purified B cells were transferred on their own as a control. Without exception these cells produced little or no antibody.

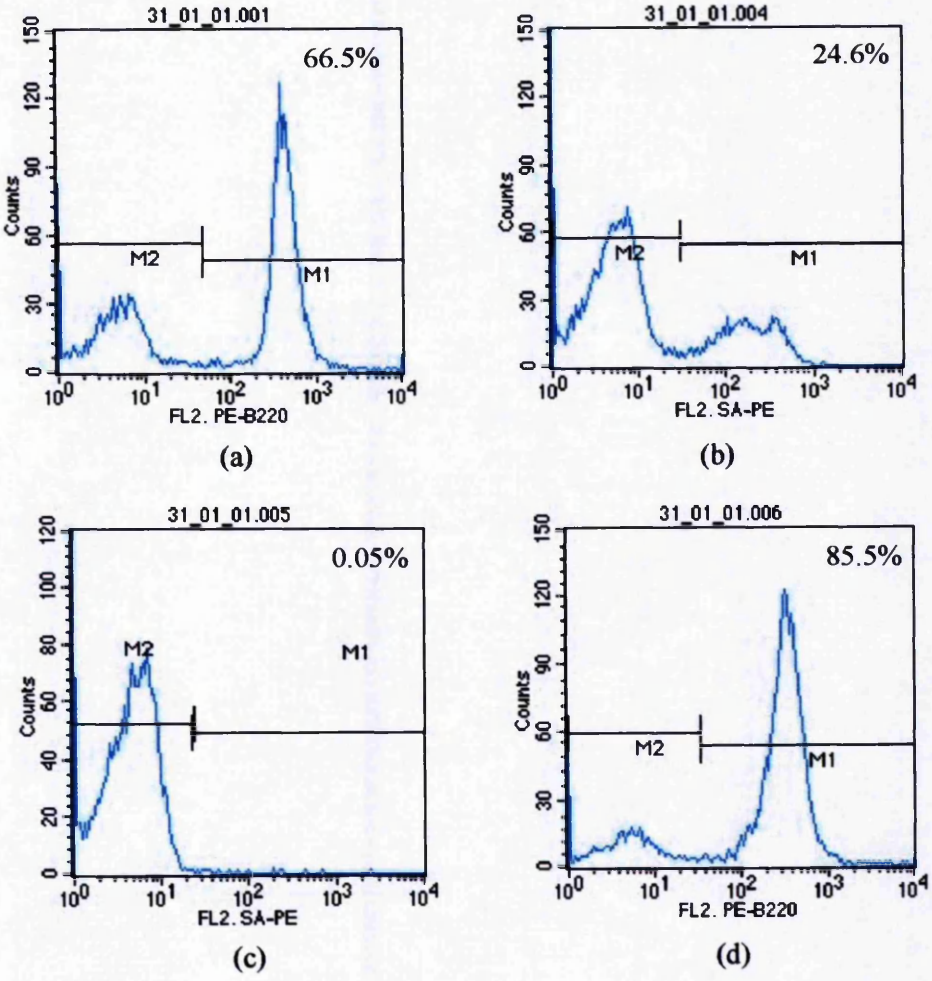


Figure 4.3. Purification of primed B cells before transfer.

A representative purification of B cells from OVA primed mice. Spleen and LN cell suspensions were stained with PE-B220 (a) or b-CD4, b-CD8, b-Thy1/SA-PE (b) before depletion. Following two rounds of depletion on SA-coated Biomag particles, cells were stained with SA-PE (c) to assess the effect of T cell depletion and with PE-B220 (d) to determine the percentage of B cells in the final suspension used for injection. The percent of positive cells in gate M1 is shown in the upper right corner of each histogram.

4.2.3 Titration of Primed B cells.

In order to compare help from tg $CD4^+$ T cells for antigen-experienced B cells, we wanted to establish a dose of T cells that could be used in an adoptive transfer to stimulate a measurable antibody response. Since we were not purifying the antigen-specific or memory B cells, it was assumed that a relatively large number of primed B cells would have to be transferred, as the OVA specific memory B cells would represent only a small fraction of the total B cell population. To ensure that the response would not be limited by the amount of T cell help, all recipients received an excess of $CD4^+KJ^+$ T cells. Figure 4.4 shows the results from the transfer of graded doses of primed B cells (3×10^7, 10^7, 10^6) together with 3×10^6 KJ^+CD4^+ T cells into SCID recipients followed by challenge with 10μg sol-OVA. There was little difference in the antibody response between the two higher doses of B cells. However when the dose was reduced to 10^6 primed B cells, the antibody response was diminished significantly. To ensure there were sufficient memory B cells present in the suspension, we elected to transfer 10^7 primed B cells in subsequent experiments.

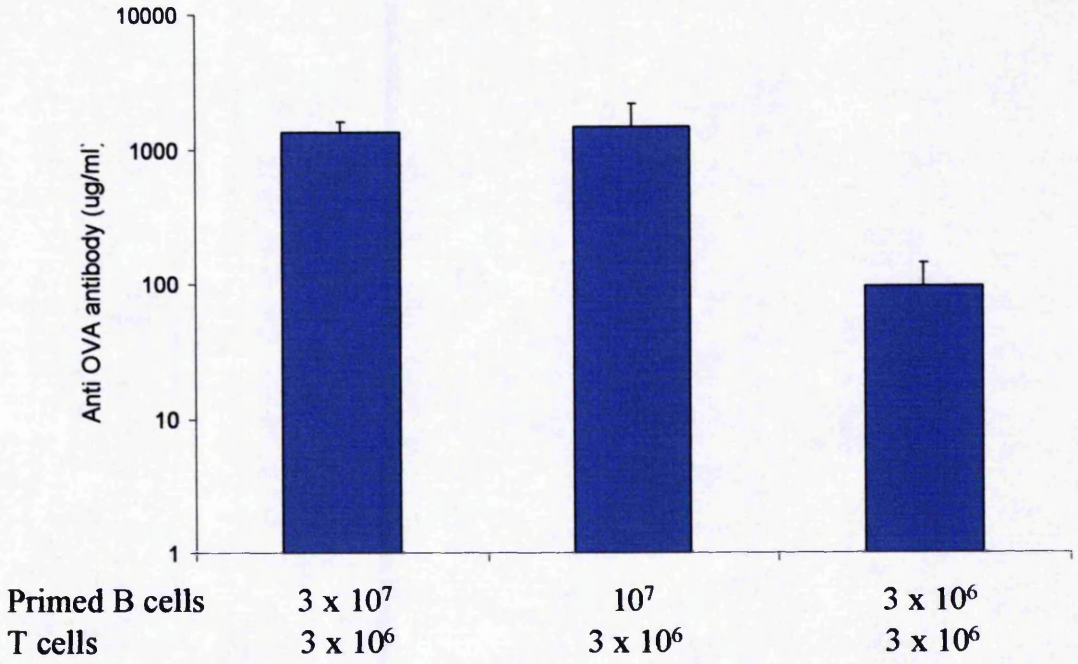


Figure 4.4. Titration of primed B cells.

Anti OVA antibody levels of SCID mice 10 days after adoptive transfer of either 3 x 10^7, 10^7, or 10^6 primed B cells with 3 x 10^6 KJ^+CD4^+ T cells. Mice were challenged with 10μg sol OVA injected i.p. immediately after transfer of cells. The values shown represent the means + SD of 6 recipients per group.

4.2.4 Titration of KJ^+CD4^+ T cells needed to help Memory B cells.

Because the transferred KJ^+CD4^+ T cells were OVA specific, less of these cells had to be transferred compared to the primed B cells. Also, as we wanted to compare the ability of naïve, primed or memory $CD4^+$ T cells to help memory B cells, it was important that the number of T cells that was transferred was not in excess. If an abundance of T cells (compared to the number of memory B cells) was transferred, any qualitative difference between the T cell subsets may be reduced, as the number of B cells became the limiting factor. Therefore the titration of transferred KJ^+CD4^+ T cells was more extensive than that for primed B cells. 3×10^6, 3×10^5, 10^5, 3×10^4, or 10^4 SCID-derived KJ^+CD4^+ T cells were transferred together with 10^7 primed B cells into SCID recipients and challenged with 10µg sol-OVA. A clear linear decrease in antibody production was seen with the decreasing number of transferred T cells (Figure 4.5). The lowest dose of T cells transferred, 10^4 cells, resulted in minimal antibody production. Mice receiving 3×10^4 T cells produced a significant amount of antibody, but the results were highly variable within the group. Therefore it was decided to select 10^5 or 3×10^5 KJ^+CD4^+ T cells for future experiments. This was a dose that produced a substantial amount of antibody production but ensured that the response was limited by the population of $CD4^+$ T cells transferred.

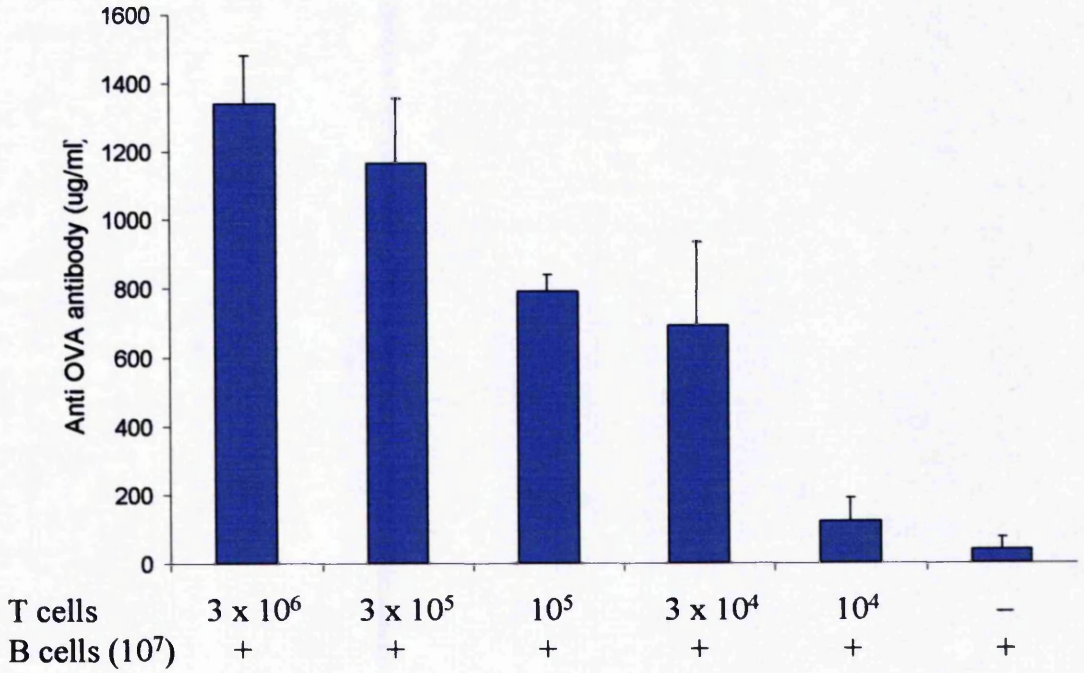


Figure 4.5. Titration of naïve KJ^+CD4^+ T cells.

Anti OVA antibody levels of SCID mice 10 days after adoptive transfer of 10^7 primed B cells together with graded doses of KJ^+CD4^+ T cells (3 x 10^6, 3 x10^5, 10^5, 3 x 10^4, 10^4). Mice were challenged i.p. with 10µg sol OVA immediately after transfer of cells. The values shown represent the means + SD of 4-6 recipients per group.

4.2.5 Effect of increasing Antigen on KJ^+CD4^+ T cell proliferation.

For initiating secondary antibody responses 10μg sol-OVA injected i.p. was sufficient to stimulate a memory B cell response. This dose was based on similar studies in the rat (Bell *et al.*, 2001), and was used for the majority of experiments. The effect of increasing the amount of antigen was not examined until later. To determine the effect of antigen dose, 5 x 10^6 KJ^+CD4^+ T cells were transferred into 4 groups of SCID recipients and challenged immediately with no antigen, 10μg, 30μg, or 100μg sol-OVA. Phenotypic analysis of KJ^+CD4^+ T cells 7 days post challenge indicated that the greatest difference in donor cell proliferation was in the ILN, where the highest dose of OVA resulted in nearly 8 times as many KJ^+CD4^+ T cells compared to the standard dose of 10μg sol-OVA, although there was considerable variance in this group (Figure 4.6). In the MLN there was 3 times as many KJ^+CD4^+ T cells with the highest dose of stimulation and in the spleen there was 5 times as many KJ^+CD4^+ T cells. The results show that following the transfer of 5 x 10^6 KJ^+CD4^+ T cells the dose of sol-OVA antigen was the limiting factor. When the sol-OVA challenge was increased from 10μg to 100μg more KJ^+CD4^+ T cells were able to respond. Curiously the greatest difference was observed in the inguinal lymph nodes. This may be due to more KJ^+ T cells localizing to the ILN, as occurred in the mice which received no antigen. Unlike ap-OVA which was retained within the peritoneal cavity and so primarily affected the spleen and MLN, sol-OVA diffused quickly throughout the mouse and was available in equal amounts to the ILN and MLN.

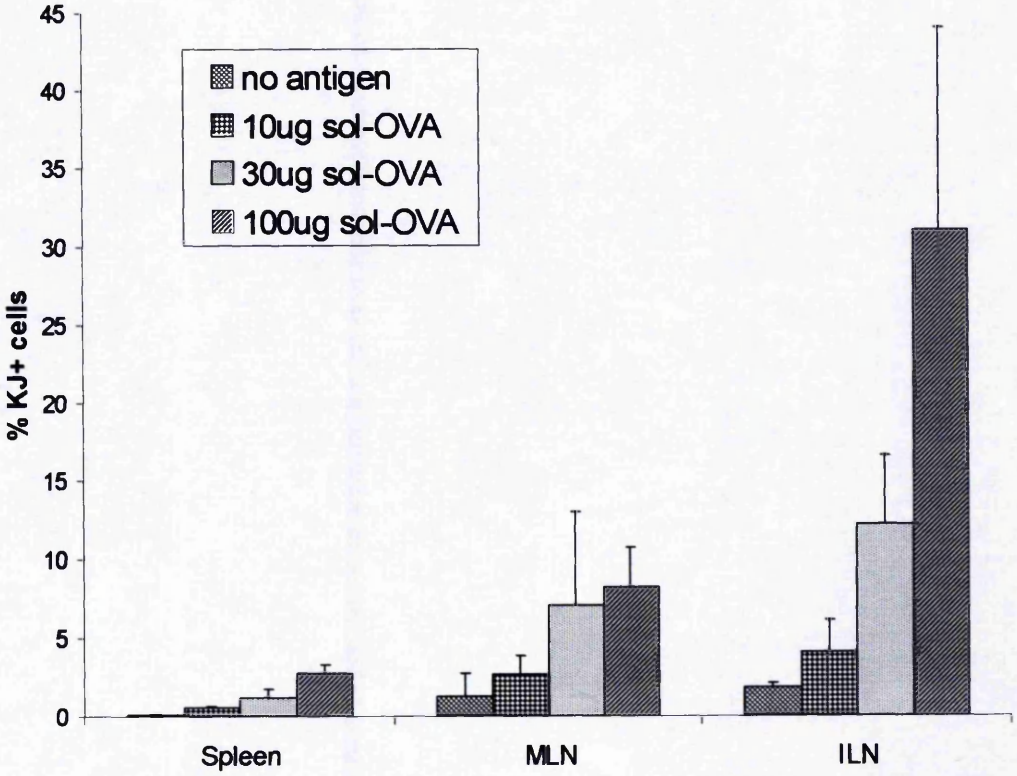


Figure 4.6. Effect of increasing antigen on KJ^+ T cells in SCID recipients.

The percentage of KJ^+CD4^+ T cells in the spleen, MLN, and ILN of SCID mice on day 7 following the adoptive transfer of 5 x 10^6 KJ^+CD4^+ T cells. Mice were challenged i.p. with 10μg, 30μg, or 100μg sol-OVA. A control group received no OVA. The values shown represent the means + SD of 4 recipients per group.

4.2.6 Effect of increasing Antigen on Antibody Production by Primed B cells.

In view of the significant increase in KJ^+CD4^+ T cell proliferation related to the dose of antigen challenge, it was important to determine what effect this would have on antibody production by memory B cells. To test this, SCID mice received 3 x 10^5 KJ^+CD4^+ T cells together with 10^7 primed B cells and were challenged with 10μg versus 100μg sol-OVA. On day 7 mice which received 100μg sol-OVA had antibody levels nearly 3 times higher than those which received 10μg sol-OVA (Figure 4.7). By day 14 the difference between the two groups was greater than 5 fold, and by day 21 it was 4 fold. Superficially it would appear that the 3-5 fold increase in antibody production following challenge with the higher dose of 100μg sol-OVA was equally linked to the 3-5 fold increase in KJ^+CD4^+ T cells in the spleen and MLN (Figure 4.6). However when examined in conjunction with the results from the titration of KJ^+CD4^+ T cells (Figure 4.5), this link is not as clear. In those experiments it was observed that a 10-fold increase of KJ^+CD4^+ T cells from 3 x 10^5 to 3 x 10^6 resulted in a mere 15% increase in anti-OVA antibody (Figure 4.5). Merely increasing the number of available KJ^+CD4^+ T cells, was therefore unlikely to be the explanation for the antibody increase observed. What may be significant is that with a greater availability of antigen more KJ^+CD4^+ T cells are likely to become activated, up regulate stimulatory molecules, and release cytokines which will result in greater B cell activation. Equally important is the fact that the higher concentration of OVA will recruit more memory B cells into the response, so that there would be more of them activated and ready for signals from the KJ^+CD4^+ T cells. These experiments clearly show that the antibody produced by the standard protocol was only limited by the number of antigen-specific KJ^+CD4^+ T cells. This satisfies a major objective of the investigation to compare functional differences between naïve, primed, and "revertant" $CD4^+$ T cells.

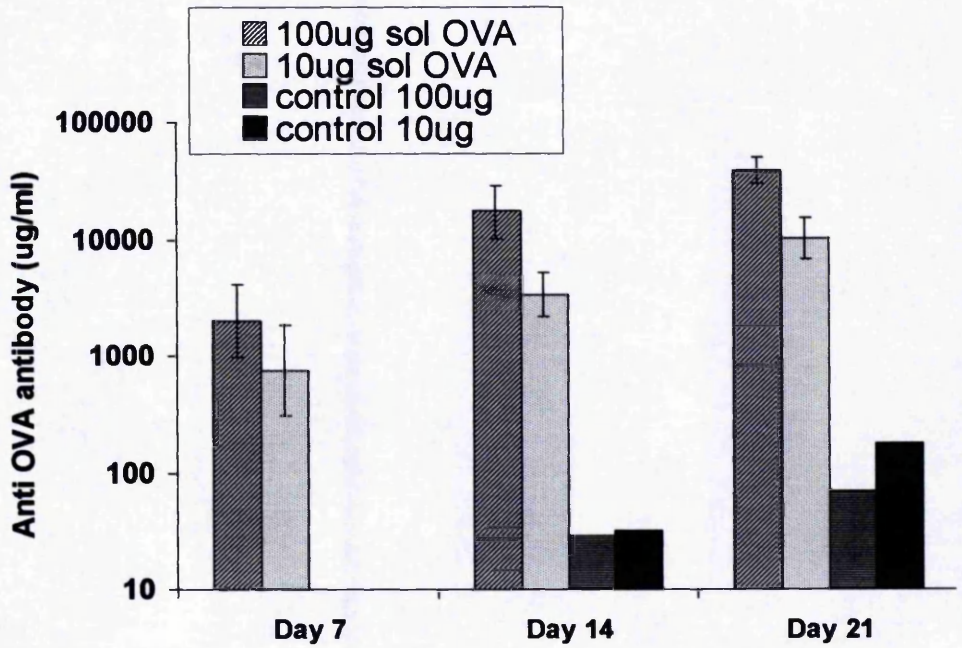


Figure 4.7. Effect of increasing antigen on antibody production by primed B cells.

The effect of antigen dose on antibody production by primed B cells. SCID recipients received 10^7 primed B cells alone (control) or together with 3 x 10^5 naïve KJ^+ cells, and were challenged i.p. with 100μg or 10μg sol OVA on the day of transfer. The values shown represent the means +/- SD of 5 recipients per group. (2 per control group).

4.2.7 Characterising Memory B cells.

Spleen and LN cells from OVA-primed mice were depleted by magnetic adherence of T cells ($CD4^+$, $CD8^+$, $Thy1^+$) and stained for B220 (PE), IgD (FITC) and IgM (Tricolour) (Figure 4.8 b, c, d). The cells analysed were defined on forward and side scatter parameters by gate R1 (Figure 4.8a). A majority of the $B220^+$ cells also expressed IgD (85%) (Figure 4.9b). Less than half (48.9%) of the $B220^+$ cells were IgM^+ (Figure 4.9c). Approximately half of the IgD^+ cells were also IgM^+ (Figure 4.9a).

Work by others (Stavnezer, 1996) (Hodgkin *et al.*, 1996) suggested that the memory B cell population could be found within the IgM^- subset. To test this, spleen and LNs from OVA-primed donors were depleted of (i) $CD4^+$, $CD8^+$, $Thy\text{-}1^+$, $F4\text{-}80^+$, IgM^+ cells (IgM^-) or (ii) $CD4^+$, $CD8^+$, $Thy\text{-}1^+$, $F4\text{-}80^+$ ($IgM^{+/-}$). Cells stained with these two different cocktails of biotinylated mAbs were analysed before magnetic depletion (Figure 4.10a, b) and after (Figure 4.10c, d). The IgM^- and $IgM^{+/-}$ subsets were 99.5% and 97% pure respectively. Further analysis following staining with B220 and b-IgM/Tricolour showed unexpectantly the presence of IgM^+ cells (25%) in the IgM^- subset after depletion (Figure 4.11a). We were unable to reduce the residual IgM^+ population by further rounds of magnetic depletion. The $IgM^{+/-}$ subset contained a similar number of IgM^+ B cells (30%) (Figure 4.11b). The IgM^- B cells were compared with the $IgM^{+/-}$ B cells by adoptively transferring each subset into SCID recipients with 3×10^5 KJ^+CD4^+ T cells and challenging with the higher dose (100µg) sol-OVA (Figure 4.12). The results showed that a large proportion of the memory cells were found in the IgM^- population, as 2×10^5 IgM^- B cells produced a very significant secondary response that was comparable with 50 times as many $IgM^{+/-}$ B cells. However, there was little difference in the responses between the high and low doses of IgM^- B cells suggesting that the results may be influenced by a limiting number of KJ^+CD4^+ T cells. Further work will be necessary to clarify the situation.

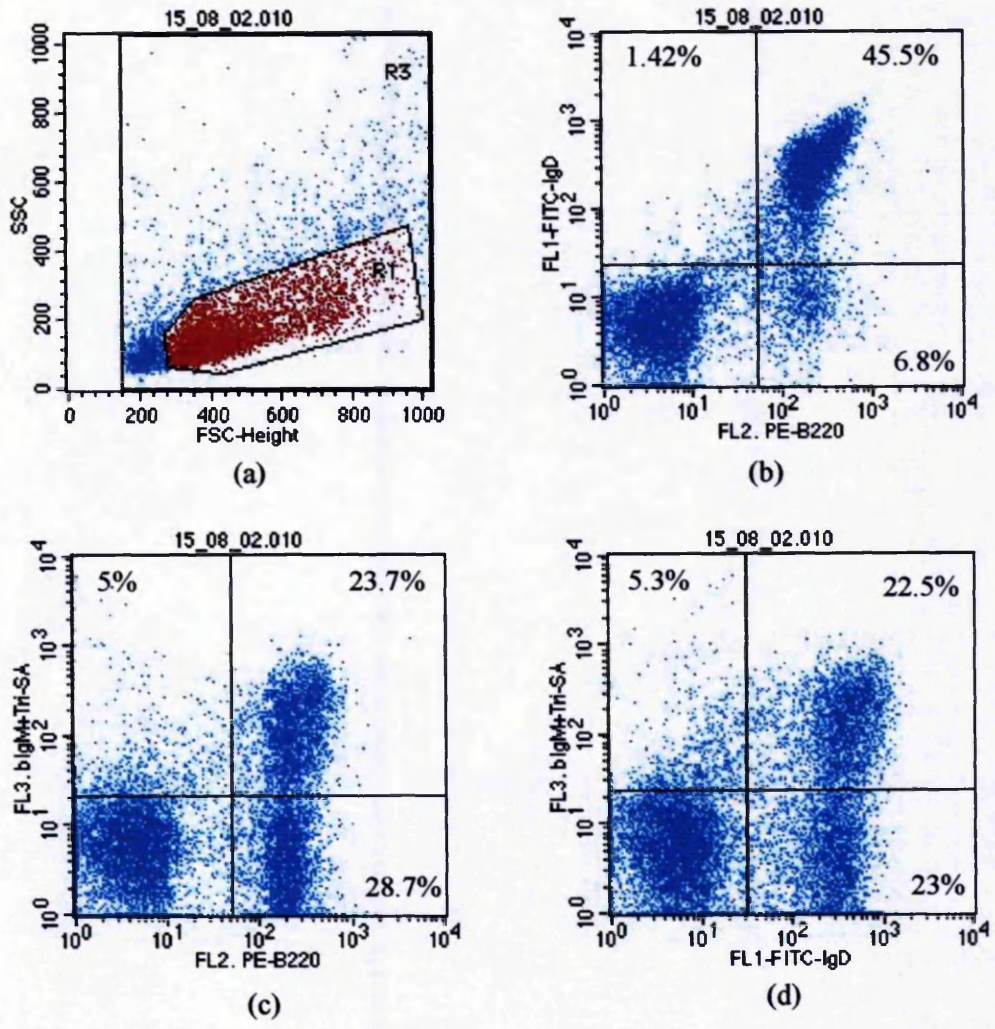


Figure 4.8. Flow cytometric analysis of primed B cells before transfer.

Flow cytometric analysis of spleen and LN cells from OVA primed mice following depletion of T cells by magnetic coated particles. The cells were assessed by forward and side scatter (a) and the lymphocyte population gated (R1). This gated population was stained with PE-B220, FITC-IgD, and b-IgM + Tricolour and the subsequent populations are shown in (b), (c), and (d). The percentage of cells in each quadrant is shown.

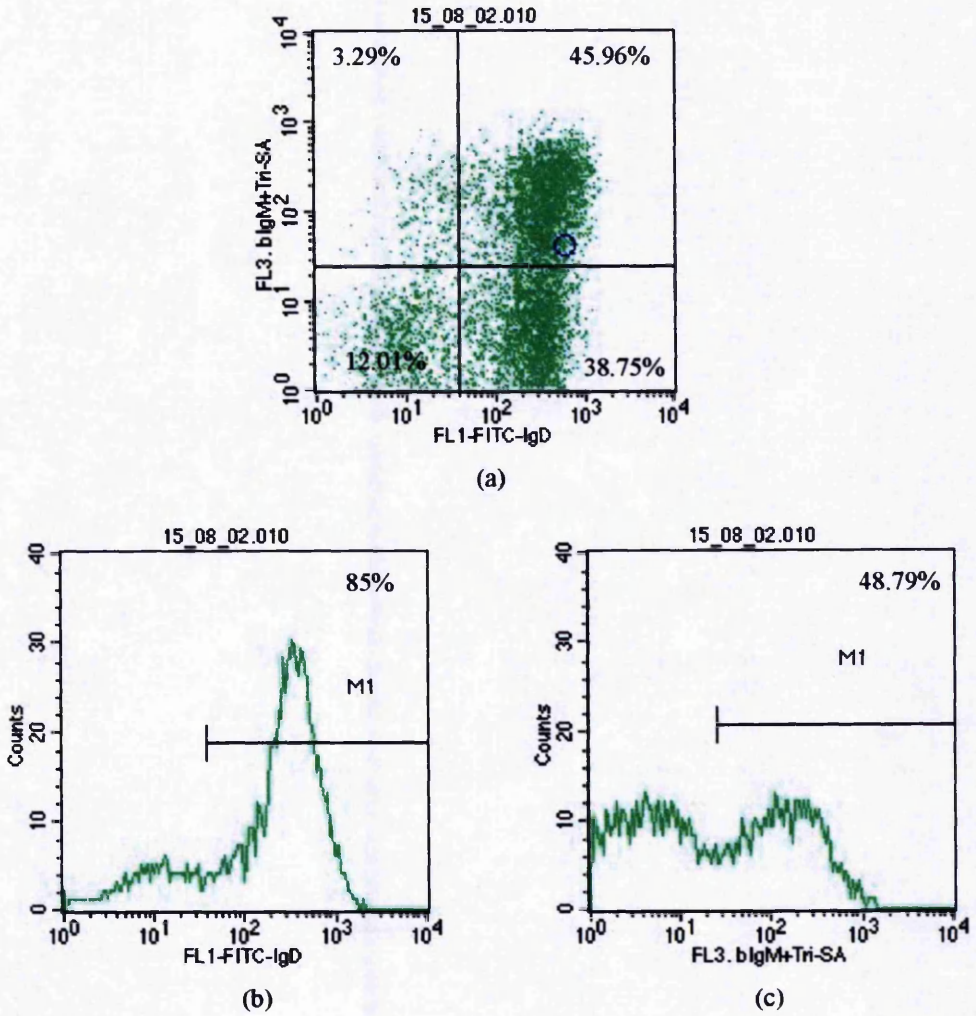


Figure 4.9. Flow cytometric analysis of B220$^+$ B cells before transfer.

Flow cytometric analysis of B220$^+$ cells from 7(c). (a) shows the cells expression of IgD and IgM with the percentages of each quadrant shown in the corner. (b) shows the percentage that were IgD$^+$ (85%) and (c) shows the percentage that were IgM$^+$ (48.79%).

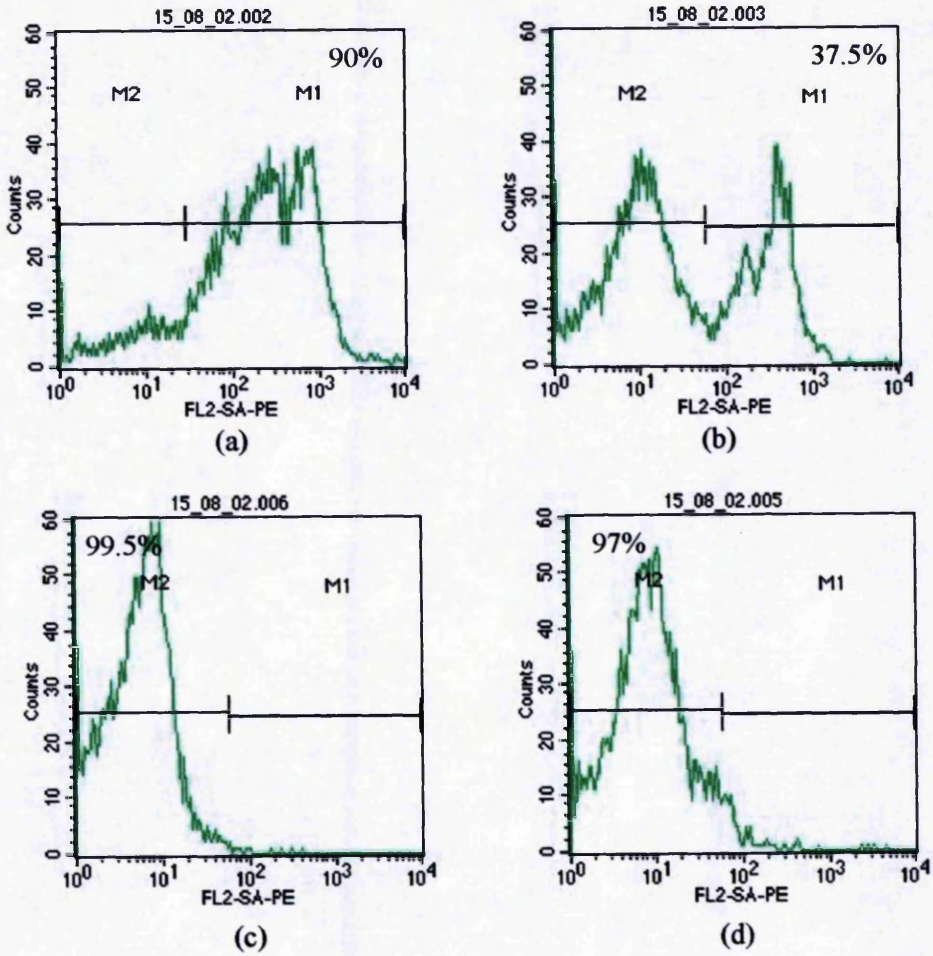
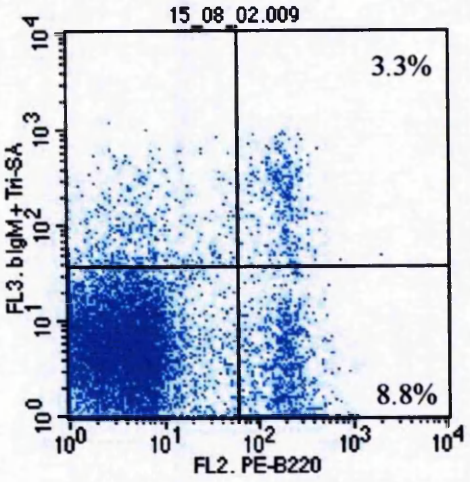
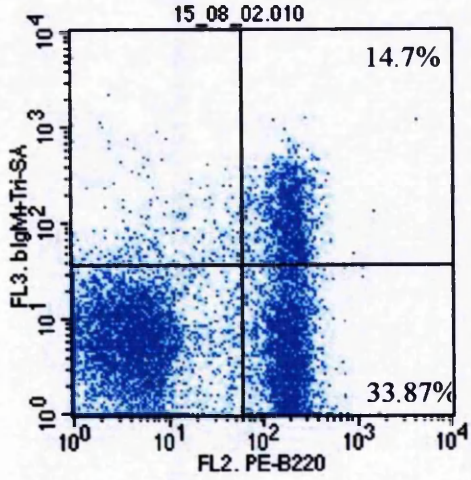


Figure 4.10. Purification of IgM$^-$ and IgM$^{+/-}$ B cells from OVA primed mice.

Spleen and LN cells were divided into two groups and stained before depletion with b-CD4, b-CD8, b-Thy-1, b-F4-80, b-IgM/SA-PE (a) or with b-CD4, b-CD8, b-Thy-1, b-F4-80/SA-PE (b). Following two rounds of depletion on SA-coated Biomag particles, cells were stained with SA-PE, (c) and (d), to assess the effectiveness of cell depletion. The percentage of positive cells in gate M1 or M2 is shown in the upper right or left corner of each histogram.



(a)



(b)

Figure 4.11. Flow cytometric analysis of purified IgM^- and $IgM^{+/-}$ B cells.

Following purification of IgM^- (a) and $IgM^{+/-}$ (b) B cells, the cells were stained with PE-B220 and b-IgM/Tricolour. The percentage of $B220^+$ cells is shown in the right-hand quadrants.

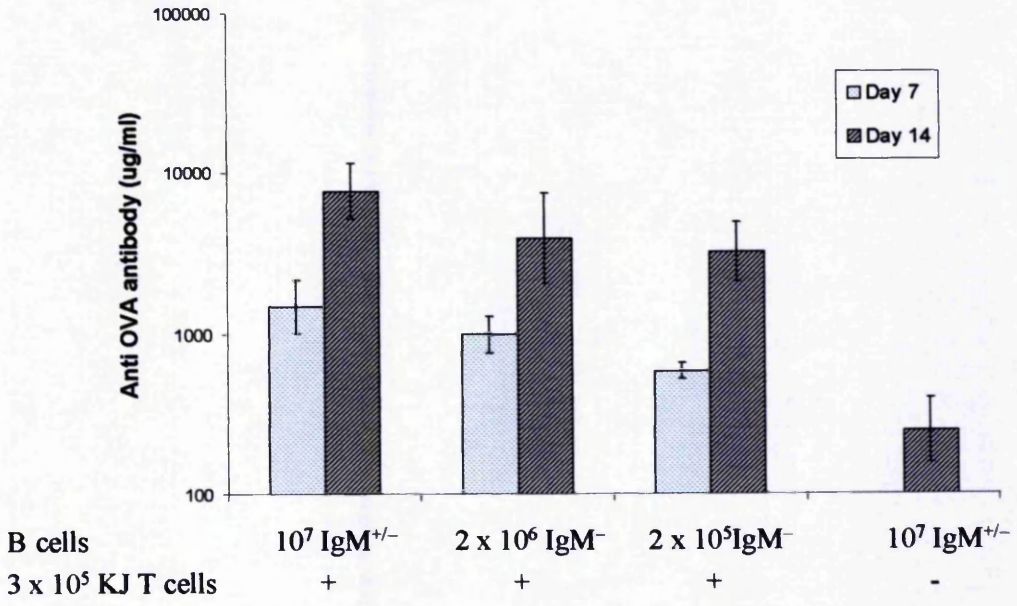


Figure 4.12. Comparison of anti-OVA antibody produced by IgM^- and $IgM^{+/-}$ B cells from OVA primed mice.

Anti-OVA antibody levels from SCID mice which received either 10^7 $IgM^{+/-}$ B cells, 2 x 10^6 IgM^- B cells, or 2 x 10^5 IgM^- B cells (from primed mice) together with 3 x 10^5 naïve KJ^+CD4^+ T cells. Control mice received 10^7 $IgM^{+/-}$ B cells alone. Mice were challenged i.p. with 100μg sol-OVA immediately after transfer of cells. Values shown are mean +/- SD of 4 recipients in each group (2 recipients per control group).

Chapter 5

Comparison of naïve and primed KJ^+CD4^+ T cells

5.1 Introduction

As was stated and shown in the previous chapter $CD4^+$ T cells are essential for an effective humoral immune response, be it primary or secondary. Through both cell mediated signals and the release of cytokines $CD4^+$ T cells act as the conductor of the immune orchestra. The importance of immunological memory in combating infection and disease has been discussed earlier (Chapter 1) and $CD4^+$ memory T cells are a crucial part of this response. However it is still not clear whether memory $CD4^+$ T cells are qualitatively different from naïve $CD4^+$ T cells or whether the memory response is merely due to an increase in the frequency of the responding antigen-specific cells. To try and answer this we must first consider the concept of naïve $CD4^+$ T cells and then compare them in phenotype and function with memory $CD4^+$ T cells.

5.1.1 Entry of naïve $CD4^+$ T cells into lymph nodes and spleen.

Truly naïve cells can only be defined as cells that have not encountered antigen. These cells congregate in secondary lymphoid tissues and continuously migrate from one lymphoid organ to another via blood and lymph (Von Andrian & Mackay, 2000). The reasons they do this is to seek out antigen from infectious agents. As the number of cells whose T-cell receptors (TCRs) recognize any individual antigen is very limited, these few cells must in theory be able to detect antigen throughout the whole body. However the lymphatic system has evolved as a system which concentrates foreign (and host) antigen into a compact space therefore minimising the area the T lymphocytes must cover. In fact naïve T cells are often incapable of entering sites of initial infection (Sprent & Surh, 2002). The ability to migrate across tissue boundaries is governed by the expression of a variety of cell surface adhesion molecules. These molecules act as mechanical anchors in the intense pressure of high endothelial venules (HEVs) from which T lymphocytes enter into lymph nodes (Von Andrian & Mackay, 2000). As well as mediating the physical aspect of migration, these adhesion molecules function as tissue-specific recognition markers (Von Andrian & Mackay, 2000). For example the HEVs in lymph nodes express the peripheral-node addressin, where as the

HEVs in Peyer's patches express mucosal addressin-cell adhesion molecule I (Von Andrian & Mackay, 2000). L-selectin (CD62L), which is expressed on all naïve T cells binds both of these addressins. However for entry into Peyer's patches binding of $\alpha_4\beta_7$ integrin is also required (Von Andrian & Mackay, 2000). While selectins are constitutively active, integrins must be activated to mediate adhesion. This occurs when a cell receives signals from chemokines on endothelial surfaces. Chemokines bind to specific surface receptors which can be up-regulated or lost as cells differentiate, which allows the cell to coordinate its migratory route with its function.

5.1.2 Antigen Presentation to naïve $CD4^+$ T cells by Dendritic Cells.

The entry of lymphocytes into lymphoid organs is predominantly with the purpose of encountering antigen. For this dendritic cells are essential. Immature dendritic cells are found in most tissues where they engulf microorganisms, dead cells, and cellular debris. Upon exposure to inflammatory signals, they enter local lymph nodes where they undergo further maturation. They lose their receptors for inflammatory chemokines and upregulate receptors for chemokines which allow them to enter into the T-cell areas of the lymph node. As the dendritic cell now expresses high levels of adhesion and costimulatory molecules, such as B7-1 and B7-2 (Banchereau *et al.*, 2000) as well high levels of MHC class II, it is the perfect cell for presenting antigen to a naïve $CD4^+$ T cell. It has been shown both *in vitro* and *in vivo* that bone marrow-derived dendritic cells are the most effective antigen presenting cells for $CD4^+$ T cells (Steinman, 1991). However, resting dendritic cells are poor stimulators of $CD4^+$ T cells and will often induce tolerance (Jenkins *et al.*, 2001). As soon as the antigen-carrying dendritic cells enter the T cell zone, specifically reactive T cells recognize the peptide/MHC class II complex and form a tight synapse with the antigen presenting cell. This has been demonstrated *in vivo* using dye labelled DC and naïve tg $CD4^+$ T cells specific for OVA peptide (Ingulli *et al.*, 1997). Synapse formation results in triggering of the TCR/CD3 complex by peptide/MHC on the DC. This is aided by CD4 receptors and by a large number of costimulatory/adhesion molecules such as CD28, LFA-1, CD40L, ICOS, OX40, CD2, CD27, and 41BB which all bind to complementary molecules on the DC (Chambers & Allison, 1999) (Watts & DeBenedette, 1999) (Coyle & Gutierrez-Ramos, 2001). These costimulatory/adhesion molecules work in a variety of ways; some may provide essential secondary signals for

T cell activation, while others may enhance triggering of the TCR. Others such as CD28 are important for cytokine induction while CD40L is important for maintaining activation of DCs (June *et al.*, 1987) as well as modulating T-B cell interactions (Gray *et al.*, 1994). Although DCs are essential for activating $CD4^+$ T cells through costimulatory molecules and cytokines, the net effect on the fate of the T cell may be dependent upon the stage of the immune response. During the early phase of the response when antigen is likely to be plentiful, large numbers of activated DCs enter the T cell zones. The DCs activate T cells which proliferate rapidly, release a variety of cytokines and differentiate into effector $CD4^+$ T cells as well as cytotoxic T cells (for $CD8^+$ cells). As these cells mount an immune response the infection may be cleared leading to a dramatic reduction in the availability of antigen for DCs. As a result fewer activated DCs will enter the T cell zones and in these conditions the DC will still activate antigen-specific $CD4^+$ T cells but production of stimulatory cytokines is diminished. It may be that these conditions are important in the formation of memory cells.

5.1.3 T Cell Effector Functions.

As antigen-specific $CD4^+$ T cells are likely to be low in frequency at the time of initial infection, upon antigen encounter these cells must rapidly proliferate in order to mount an effective immune response. This is largely mediated by an increase in the production of IL-2 and an increase in the expression of the IL-2 receptor by the activated T cells. The responding cells may divide 2-3 times per day for 4-5 days which generates a large population of cells to carry out effector functions. As well as undergoing rapid cell division the $CD4^+$ T cells change expression of a variety of cell surface molecules and begin to secrete a wide range of cytokines. $CD4^+$ T cells have long been classified on the basis of cytokine secretion into two subsets, Th1 and Th2 (Mosmann *et al.*, 1986). Th1 cells were defined as those that produced IL-2, interferon (IFN)-γ, and tumour necrosis factor (TNF)-β, while Th2 cells were defined as those that produced IL-4, IL-5, IL-6, IL-10 and IL-13 (Mosmann *et al.*, 1986). Following this classification, studies occurred which further distinguished the functional differences between these two subsets. It was proposed that Th1 cells mediated DTH responses (Mosmann & Coffman, 1989), and that Th2 cells provided help for the humoral response (Bottomly, 1988). Furthermore, it was shown that as well as producing

cytokines which acted as self autocrine growth factors (Lichtman *et al.*, 1987), each set of polarized cells secreted cytokines which cross-regulated the other's development and activity. For example IFN-γ produced by Th1 cells amplifies Th1 development and inhibits proliferation of Th2 cells (Fitch *et al.*, 1993), whereas IL-10 produced by Th2 cells prevents activation of Th1 cells (Fiorentino *et al.*, 1989). However in recent years with the improved ability to stain individual cells for cytokine production, it has become apparent that not all $CD4^+$ T cells can be defined on the basis of these two subsets. Individual cells may possess complex patterns of cytokine production with various combinations of IL-2, IL-4, IL-5, and IFN-γ and often produce cytokines such as IL-10 and TGF-β, which are not characteristic of either subset yet serve important functions. Doubt has also been cast on defining each subset as mediators of particular responses. For example Th2 $CD4^+$ T cells have long been characterised as the cells which provide help for the humoral reponse (Bottomly, 1988), but recent work using OVA-specific TCR tg T cells polarized *in vitro*, has shown that both Th1 and Th2 cells support B cell responses to a similar extent *in vivo* (Smith *et al.*, 2000). They both achieved this by migrating into B cell follicles to promote CD154-dependent B cell clonal expansion and antibody production (Smith *et al.*, 2000). Despite this there are cases where a clear polarization of the T cell response does occur, such as persistent infections with microbes such as Leishmania, Listeria, mycobacteria, and helminths, and in these instances resistance and susceptibility can be attributed to specific Th1 and Th2 responses (Sher & Coffman, 1992). What all of these studies should tell us though, is that trying to define such a heterogeneous group of cells such as T lymphocytes into just two groups is an oversimplification of the real picture.

The fate of the T cell may be determined in the first few hours of activation, and may be largely dependent upon the manner and environment in which stimulation occurs. For example T cells stimulated in the presence of IL-12, produced by DCs or macrophages are more likely to become polarized to a Th1 type response. Many microbial products such as endotoxin, and intracellular bacteria such as Listeria stimulate macrophages IL-12 production (Gazzinelli *et al.*, 1993). The amount of antigen present is also an important factor in influencing the fate of the T cell, as low antigen concentrations mostly favour a Th1 response where as high antigen concentrations induce Th2 responses (Bretscher *et al.*, 1992) (Hosken *et al.*, 1995). This may be due to the involvement of different antigen-presenting cells or it may be that with higher antigen concentrations T cells are repeatedly stimulated leading to IL-4

production and Th2 polarization. As well as determining the cytokine profile of a responding T cell, antigen presentation may be important in determining whether the cell becomes an effector T cell or a memory T cell. However there is little evidence for this.

5.1.4 Phenotypic Changes that occur with Activation; CD45R.

The cell surface changes that occur upon T cell activation are often used as phenotypic markers of effector and memory cells. Some of these changes are a result of rearrangement of the TCR and others are primarily functional such as the changes that occur in expression of cell surface addressins and integrins. CD45, also known as the common leukocyte antigen, is a transmembrane tyrosine phosphatase found on all leukocytes comprising 10% of the cell surface (Towbridge & Thomas, 1994). It plays a major role in T cell development and thymocyte differentiation, possibly for the transition from double-positive to single-positive thymocytes (Kishihara *et al.*, 1993). Purification of thymocyte CD45 has revealed it to be one of the largest known lymphocyte surface molecules, with its three exons (ABC) extending away from the cell in a rod like structure (Woolett *et al.*, 1985) (McCall *et al.*, 1992), as well as revealing certain structural similarities with cell adhesion molecules, such as fibronectin (Bork & Doolittle, 1993). Several isoforms of the CD45 molecule may be expressed depending upon the activation state of the T cell. Because of this it was originally used as a marker of memory T cells (Akbar *et al.*, 1998). Resting $CD4^+$ T cells express the high molecular weight isoform, $CD45R^{hi}$, but following antigenic challenge they switch to expression of the low molecular weight isoform, $CD45R^{lo}$. The resting cells can be identified by mAbs and in humans are identified as $CD45RA^+$, in mice as $CD45RB^+$, and in rats as $CD45RC^+$ (Towbridge & Thomas, 1994). The reciprocal phenotype, which occurs when exon products A, B, and C are spliced out, $CD45R^{lo}$, can be identified by loss of mAb staining ($CD45RA^-$, RB^-, RC^-). In humans the low molecular weight isoform can be specifically stained by the anti-CD45RO mAb UCHL-1, which detects an epitope created by the juxtaposition of carbohydrates at the splice junction site (Pulido *et al.*, 1994). It has been argued that conflicting data, which occurs between species, may be due partly to the use of different exon-specific antibodies and the differences in isoform expression. However this problem can be overcome by examining the mRNA that encodes the CD45R isoform splice variants.

This has been elegantly demonstrated by Hargreaves and Bell (1997) who showed that antigen experienced CD45RC$^-$ T cells only produced message for the null isoform O and the single-exon B, while naïve CD45RC$^+$ T cells contained mRNA for ABC, AB, BC, B, C and O. This was paralleled by work in the mouse which showed that CD45RB^{lo} T cells only had message for B and O, while CD45RB^{hi} T cells expressed message for ABC and AB (Rogers *et al.*, 1992). In humans the picture was similar with CD45R^{hi} T cells expressing message for ABC, AB, BC, B and O, and CD45R^{lo} T cells expressing high levels of message for O and B as well as variable expression of double exons AB and BC (Rogers *et al.*, 1992). Therefore, there appears to be a high level of concordance across the species.

One suggested role for the different CD45 isoforms on resting and activated T cells may be to differentially regulate the interaction and activation of these cells with APCs. The interaction of CD45 isoforms with other cell surface molecules may alter the phosphatase activity of CD45 or its accessibility to substrates, which could in turn modify the activation signal required through the TCR (Luomann & Bottomly, 1992). Therefore cells expressing the low molecular weight isoform may require less stringent signals from their APCs. Antibodies used against CD45 *in vitro* have produced somewhat conflicting results, either inhibiting or enhancing a variety of cellular function (Towbridge & Thomas, 1994). These differences were probably due to the experimental system used but may reflect the wide role of CD45. Experiments with a CD45$^-$ cell line showed that the CD45 molecule was essential for activation by antigen or anti-CD3 antibody *in vitro* (Pingel & Thomas, 1989). It was later shown that it was the cytoplasmic domain and not the extracellular exons that were required for TCR-mediated signalling (Volarevic *et al.*, 1993). However, more recent work has shown that the smallest isoform CD45RO homodimerizes more quickly than the larger isoforms (which have a strong negative charge) (Zheng & Weiss, 2002). This results in decreasing the total CD45 activity in the cell and may act as a negative feedback signal to the TCR, thus acting to down-regulate the primary immune response (Zheng & Weiss, 2002). CD45 has also been shown to be required for the transduction of a signal through the B-cell antigen receptor and so may play a similar role in B cells (they express the highest molecular weight isoform, B220 (CD45RABC)), though less is known about its role in this cell (Justement *et al.*, 1991).

In addition to the changes that occurred with CD45R, cells were observed to undergo other molecular changes following antigenic stimulation. The CD45R^{lo} T cells

also expressed increased levels of adhesion molecules, such as CD2, lymphocyte function associated antigen 1 (LFA-1), LFA-3, CD44, intercellular adhesion molecule 1 (ICAM-1) and very late antigens 4, 5 and 6 (VLA-4, -5, -6) as well as reduced levels of L-selectin (Sanders *et al.*, 1988) (Mackay, 1991). A memory phenotype was attributed to the $CD45R^{lo}$ T cells mainly because of the accumulation of *in vitro* evidence. For example it was observed that antigen-induced recall responses were largest in the $CD45R^{lo}$ subset (Merkenschlager *et al.*, 1988), and that after a prolonged culture T cells remained $CD45R^{lo}$, which suggested an irreversible switch (Akbar *et al.*, 1998). $CD45R^{lo}$ T cells can also be stimulated by much lower amounts of antigen or anti-CD3 mAbs (Byrne *et al.*, 1988), and produce a much wider range of cytokines (Akbar *et al.*, 1991), compared with $CD45R^{hi}$ T cells. The finding that the majority of T cells in the afferent lymph of sheep were $CD45R^{lo}$, lead to the proposal by Mackay that naive and memory T cells migrated by different routes, with naïve T cells entering lymph nodes by crossing HEV, and memory T cells entering lymph nodes through afferent lymphatics (Mackay *et al.*, 1990). This theory seemed to concur nicely with the changes that occurred in the expression of cell surface adhesion molecules following antigenic stimulation and $CD45R^{lo}$ expression.

However the concept of the $CD45R^{lo}$ isoform as a reliable marker of memory $CD4^+$ T cells was put into doubt when it was discovered that rat $CD4^+$ T cells expressing the $CD45R^{lo}$ memory phenotype could re-express the high molecular weight isoform $CD45R^{hi}$ (Bell & Sparshott, 1990). This was observed following the adoptive transfer of $CD45R^{hi}$ and $CD45R^{lo}$ allotype-marked $CD4^+$ T cells into athymic nude rats (Bell & Sparshott, 1990). Initially the donor cells expanded and became $CD45R^{lo}$, but with time $CD45R^{hi}$ cells appeared and by seven and a half months after transfer they constituted one third of donor-derived cells (Bell & Sparshott, 1990). The reversion to the "naïve" $CD45R^{hi}$ phenotype appeared to correlate with function as judged by the "revertant" $CD45R^{hi}$ cell's ability to give strong graft-versus-host responses in a popliteal assay (Bell & Sparshott, 1990). When similar studies were carried out in euthymic rats the switch back to the $CD45R^{hi}$ isoform was much more rapid, occurring within 6 hours for some cells, and within a week for the entire cohort of transferred cells (Sparshott & Bell, 1994). Subsequent studies in humans revealed a similar switch in T cells that were CD45RO, which became CD45RA over time (Michie *et al.*, 1992), as did various *in vitro* studies which showed that $CD4^+$ T cells switched isoforms in both directions (Rothstein *et al.*, 1991) (Warren & Skipsey, 1991) (Sarawar *et al.*,

1993). This switch was also observed to occur during the natural development of rat $CD4^+$ T cells, as recent thymic emigrants exiting the thymus bore the low molecular weight isoform but within in a week became $CD45R^+$ (Yang & Bell, 1992). Further evidence accumulated which raised serious questions about the validity of $CD45R^{lo}$ isoforms as markers for memory cells. Most noticeably when it was shown in humans, rats, and mice, that it was the $CD45R^{hi}$ subset, and not the "memory" $CD45R^{lo}$ cells that were slow cycling and long lived (Michie *et al.*, 1992) (Tough & Sprent, 1994) (Sparshott & Bell, 1994).

The studies on isoform switching from the $CD45R^{lo}$ activated phenotype back to the $CD45R^{hi}$ resting state also raised important questions about the role of persisting antigen in maintaining immunological memory. The idea that memory was dependent on continual antigenic stimulation arose following the observation that primed B cells, $CD8^+$ T cells, and $CD4^+$ T cells appeared to die within months of transfer unless the recipients were challenged with specific antigen (Gray & Skarvall, 1988) (Gray & Matzinger, 1991). However this concept has long since been challenged. In a study by Bunce and Bell using a delayed type hypersensitivity (DTH) model where contact sensitivity to dinitrochlorobenzene (DNCB) was transferred to athymic nude rats by recirculating $CD4^+$ T cells, the role of antigen in maintaining memory was clearly shown (Bunce & Bell, 1997). As expected $CD45R^{hi}$, and not $CD45R^{lo}$, $CD4^+$ T cells from unprimed rats transferred a DNCB-specific DTH response (Bunce & Bell, 1997). Four days after sensitisation the response was transferred in the $CD45R^{lo}$ $CD4^+$ T cells and not the $CD45R^{hi}$ $CD4^+$ T cells (Bunce & Bell, 1997). However when cells were transferred two months following priming both subsets transferred the response (Bunce & Bell, 1997). More interestingly, when $CD45R^{lo}$ $CD4^+$ T cells were parked in intermediate nude recipients, the DNCB-specific activity was found wholly in the revertant $CD45R^{hi}$ population 2 months later (Bunce & Bell, 1997). Importantly this did not occur if the cells were parked in rats which contained persisting antigen (Bunce & Bell, 1997). Therefore it appeared that in the absence of antigen the revertant $CD45R^{hi}$ $CD4^+$ T cells carried the long term "memory" response, although when antigen remained the emergence of the revertant memory population was prevented (Bunce & Bell, 1997).

Reversion of OVA primed $CD4^+$ T cells was also observed when these cells were transferred to antigen free recipients. Before immunization help was provided for memory B cells by the $CD45R^{hi}$ $CD4^+$ T cells, though the antibody response was weak

and slow to respond (Bell *et al*, 2001). As expected 7 days after priming with ap-OVA, help for primed B cells was provided almost exclusively by the $CD45R^{lo}$ population while the $CD45R^{hi}$ cells provided very little help (Bell *et al*, 2001). Two months after priming the majority of help for primed B cells was still found in the $CD45R^{lo}$ subset, although a substantial amount of help was given by the $CD45R^{hi}$ subset. By 6 months after priming at least as much help was found in the $CD45^{hi}$ subset as the $CD45^{lo}$ subset (Bell *et al*, 2001). The possibility that newly emigrated $CD45R^{hi}$ T cells were contributing to this response was removed by the thymectomy of the rats. To test directly whether antigen primed $CD45R^{lo}$ $CD4^+$ T cells were reverting to a naïve $CD45R^{hi}$ phenotype OVA primed $CD4^+$ T cell subsets were parked in intermediate nude recipients but not challenged with antigen (Bell *et al*, 2001). Nine weeks later these cells were recovered and retransferred with primed B cells. $CD4^+$ T cells from $CD45R^{hi}$ injected intermediates were unable to provide help for primed B cells, indicating that this population was devoid of OVA-specific $CD4^+$ T cells (Bell *et al*, 2001). The $CD45R^{lo}$ $CD4^+$ T cells upon injection into nude recipients proliferated and generated both $CD45R^{hi}$ and $CD45R^{lo}$ progeny (Bell *et al*, 2001). Both subsets were purified and retransferred with primed B cells to secondary nude recipients. On this occasion the majority of help was found in the $CD45R^{hi}$ revertant subset, with very little help in the $CD45R^{lo}$ subset (Bell *et al*, 2001). The parking of primed $CD45R^{lo}$ subsets in intermediate nudes was repeated only this time the rats were immunized 4 weeks earlier with ap-OVA. This was to examine the effect of residual antigen on reversion of $CD45R^{lo}$ $CD4^+$ T cells. When the progeny of these cells were recovered 9 weeks later and retransferred with primed B cells the help was now contained within the $CD45R^{lo}$ subset with relatively little activity in the $CD45R^{hi}$ revertant subset (Bell *et al*, 2001). This confirmed the hypothesis that in the absence of antigen primed $CD45R^{lo}$ $CD4^+$ T cells reverted to a naïve $CD45^{hi}$ phenotype and provided a memory response.

More recent evidence for this idea of reversion has come from studies using the tg DO11.10 strain (Merica *et al.*, 2000). Merica and colleagues (2000) saw that following *in vivo* antigen priming of the tg $CD4^+$ T cells and subsequent adoptive transfer to antigen free hosts, the cells reverted to a naïve phenotype ($CD45RB^{hi}LFA\text{-}1^{lo}$). Despite reversion, the T cells retained the capacity for rapid IL-2 production, but regained the clonal expansion potential of naïve T cells (Merica *et al.*, 2000). Further evidence that T cells may revert to a naïve phenotype was provided by a murine study

examining protective immunity to *Mycobacterium tuberculosis* (Andersen & Smedegaard, 2000). Mice were rendered immune by a primary infection followed by antibiotic treatment and rest. The $CD4^+$ T cells from these mice were purified based on CD45RB expression, and adoptively transferred to nude mice which were challenged with *M. tuberculosis* (Andersen & Smedegaard, 2000). Significantly higher levels of resistance were transferred by the $CD45RB^{hi}$ T cells compared with $CD45RB^{lo}$ T cells from immunized mice (Andersen & Smedegaard, 2000). $CD45RB^{hi}$ T cells from control naïve mice transferred much lower levels of resistance (Andersen & Smedegaard, 2000). This experiment provided clear evidence that antigen experienced $CD4^+$ memory T cells were contained within the $CD45RB^{hi}$ subset (Andersen & Smedegaard, 2000).

5.1.5 A Model for Immunological Memory.

These and other observations lead to the proposal by Bell and colleagues that the characteristics of CD4 T cell memory, speed, size, and persistence is carried by two populations of "memory" cells (Bell *et al.*, 1998). These are recent antigen experienced $CD45R^{lo}$ $CD4^+$ T cells and "revertant" $CD45R^{hi}$ $CD4^+$ T cells. The $CD45R^{lo}$ cells are the product of clonal expansion following initial antigen contact. They are metabolically upregulated, more sensitive to low antigen concentrations (Leitenberg *et al.*, 1996), express activated adhesion molecules (Mackay, 1991), and respond with faster kinetics (Moskophidis *et al.*, 1993). As described earlier this $CD45R^{lo}$ phenotype is dependent on re-exposure to antigen (Bunce & Bell, 1997). Continual retention of antigen after clearance of infection is a controversial issue but as residual antigen is likely to be unequally distributed, re-encounter with specific antigen would be a random event. This situation would favour a reversion to $CD45R^{hi}$ expression. The speed at which this reversion may occur is still debated, with evidence from the rat favouring a rapid switch back (Bell & Sparshott, 1990), but evidence from humans suggesting a slower process (McLean & Michie, 1995). However the persisting antigen may also result in further clonal expansion and increase the number of $CD45R^{lo}$ T cells. Many of these cells may differentiate to effector cells and ultimately undergo apoptosis, although how and where this occurs is unclear. Upon complete disappearance of antigen, memory is not lost. In fact long-term survival of antigen-experienced memory T cells may depend upon the clonally-expanded T cells downregulating adhesion

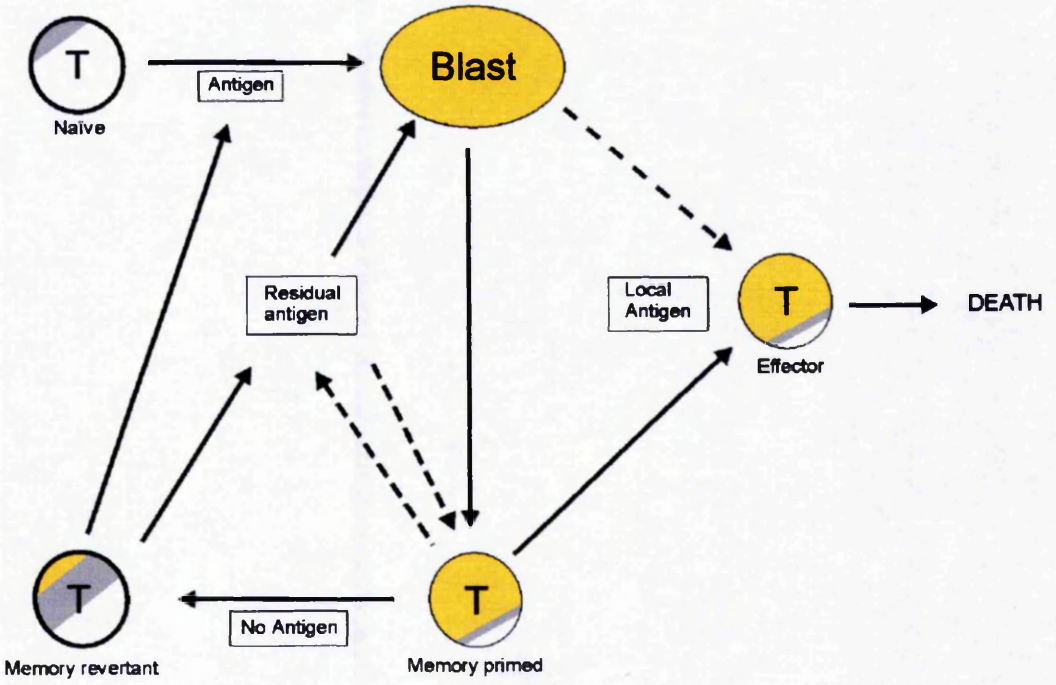


Figure 5.1 A proposed model for T cell memory.

Naïve $CD45R^{hi}$ $CD4^{+}$ T cells proliferate in response to antigen, undergo blast transformation and express a $CD45R^{lo}$ phenotype. These memory primed cells can respond rapidly to antigen and include effector T cells that are induced to differentiate terminally, to release cytokines and die by apoptosis. In the absence of antigen $CD45R^{lo}$ cells revert to expression of $CD45R^{hi}$ isoform. These revertant memory T cells resemble naïve T cells but upon antigen re-ecounter become $CD45R^{lo}$ T cells. Dashed lines represent alternative or uncertain pathways. Adapted from (Bell *et al.*, 1998).

molecules, disengaging cytokine production, uncoupling apoptotic pathways, and re-expressing the high molecular weight isoform of CD45R (Bell *et al.*, 1998). These revertant memory T cells may be phenotypically identical to naïve T cells as well as appearing to be functionally similar, as judged by their DTH and graft-versus-host responses (Bell & Sparshott, 1990) (Sarawar *et al.*, 1993) (Sparshott & Bell, 1994).

5.1.6 Immunological Memory: qualitative versus quantitative change.

If revertant memory cells are functionally the same as naïve cells, then the revertant cells must provide memory through an increase in the number of antigen-specific cells available to respond to a secondary infection. To test whether these revertant T cells are truly identical to naïve T cells, or whether encounter with antigen has functionally altered them, it is necessary to compare antigen-specific cells of both subsets in a functional assay. One such way is to use tg T cells, and assess their ability to help memory B cells, as physiologically revertant memory T cells are more likely to provide help in a secondary rather than a primary response. It has been shown in rats that revertant $CD4^+$ T cells, as well as naïve $CD4^+$ T cells, help memory B cells, although the difference in help could not be measured as the number of antigen-specific cells in each population was unknown (Bell *et al.*, 2001). There is some evidence that resting memory T cells are qualitatively different from naïve T cells that share the same antigen specifity. Using an adoptive transfer model of *in vitro* stimulated AND tg $CD4^+$ T cells, naïve, effector and resting memory cells (as defined by antigen-experienced, small, resting $CD44^{high}CD62L^{+/-}CD25^-LFA\text{-}1^{high}$ cells) were compared (Rogers *et al.*, 2000). Memory cells were able to synthesize a variety of polarized cytokines (IL-2, IL-4, and IFN-γ) with very rapid kinetics, while naïve $CD4^+$ T cells exhibited a lag period before production of just IL-2 (Rogers *et al.*, 2000). In addition, the memory cells were able to proliferate in response to APCs at lower antigen dose and to peptides, which resulted in lower affinity interactions (Rogers *et al.*, 2000). However, to induce cytokine production, memory and naïve cells had equivalent requirements for peptide concentration, which were much higher than those of the corresponding effector cells (at least a 10 fold difference) (Rogers *et al.*, 2000). Using peptide analogues, with both a higher affinity (QASA) and a lower affinity (K99A), memory cells gave detectable responses, at lower doses compared with naïve cells and also incorporated more radiolabel, suggesting a higher rate of prolieration (Rogers *et al.*, 2000). Interestingly,

the overall level of production of cytokines by memory cells was much lower than that of effector cells, even though the kinetics of the response were similar (Rogers *et al.*, 2000). A possible explanation for the fast kinetics of both cell populations is that differentiation of T cells is accompanied by demethylation of the cytokine promoter region. This characteristic may remain in memory cells and thus allow rapid cytokine synthesis (Fitzpatrick *et al.*, 1998) (Agarwal & Rao, 1998). So differences between naïve and memory $CD4^+$ T cells of the same antigen specificity were observed, although these properties were all observed *in vitro*.

5.1.7 Characterisation of Memory $CD4^+$ T cells by other cell surface markers.

A variety of studies have attempted to define naïve, effector, and memory $CD4^+$ T cells based on the expression of certain cell surface molecules. As described earlier the expression of the low molecular isoform of CD45R was used as a marker of memory $CD4^+$ T cells until it was shown that cells could revert to the reciprocal naïve phenotype in the absence of antigen. What this and similar studies have shown is that the memory $CD4^+$ T cell pool is largely heterogenous consisting of persisting effectors and resting memory T cells. One such study divided human memory T cells into two functionally distinct subsets based on expression of CCR7 (Sallusto *et al.*, 1999). CCR7 is a chemokine receptor involved in adhesion and transmigration of lymphocytes into the lymph nodes (Campbell *et al.*, 1998). It was noticed that staining of peripheral human blood T cells with antibodies to CD45RA and CCR7 revealed three subsets of $CD4^+$ T cells, a $CD45RA^+CCR7^+$, a $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ (Sallusto *et al.*, 1999). Both subsets of cells which expressed CCR7 also expressed high levels of CD62L, while the $CD45RA^-CCR7^+$ cells also expressed CCR4, CCR6, CXCR3 and intermediate levels of β1 and β2 integrins (required for homing to inflamed tissue (Baron *et al.*, 1993)) (Sallusto *et al.*, 1999). In contrast $CCR7^-$ T cells expressed high levels of β1 and β2 integrins, CD103 and CLA (tissue-specific homing receptors), and receptors for inflammatory cytokines such as CCR1, CCR3, and CCR5 (Sallusto *et al.*, 1999). The different subsets were then sorted and compared for their capacity to produce cytokines and upregulate CD40L following stimulation. Both groups of $CCR7^+$ T cells produced IL-2 only, while the $CCR7^-$ T cells produced high levels of IL-4, IL-5, and IFN-γ and reduced levels of IL-2 (Sallusto *et al.*, 1999). Following activation CD40L was upregulated to a similar degree in both effector/memory $CD45RA^-$ subsets

which was higher than that in the $CD45RA^+$ subset (Sallusto *et al.*, 1999). Another difference observed was that $CD45RA^-$ cells ($CCR7^-$ cells were consistently more responsive than $CCR7^+$) had increased sensitivity to stimulation by anti-CD3, both in the presence and absence of costimulation, compared with $CD45RA^+$ cells (Sallusto *et al.*, 1999). Antigen specifity for tetanus toxoid was examined and found to be in the two $CD45RA^-$ subsets, both of which increased their proliferative responses following a booster injection (Sallusto *et al.*, 1999). However IFN-γ production was only found in the $CCR7^-$ cells (Sallusto *et al.*, 1999). When the cells were sorted and stimulated *in vitro*, it was observed that $CD45RA^-CCR7^+$ T cells lost CCR7 expression and acquired the capacity to produce effector cytokines (Sallusto *et al.*, 1999). In contrast following stimulation $CD45RA^-CCR7^-$ retained their $CCR7^-$ phenotype and effector function (Sallusto *et al.*, 1999). This suggested a stepwise differentiation from naïve T cells to $CCR7^+$ T cells to $CCR7^-$ T cells and was supported by analysis of telomere length which decreases as a function of cell division (Weng *et al.*, 1998). Taken together these observations lead to the proposal by the authors that T cell memory is divided into two subsets; lymph node homing central memory T cells (T_{CM}) which lack inflammatory and cytotoxic function, and tissue homing effector memory T cells (T_{EM}) which possess various effector functions (Sallusto *et al.*, 1999). The T_{EM} cells represent a readily available pool of antigen primed cells which can enter peripheral tissues to mediate inflammatory reactions or cytotoxicity (Sallusto *et al.*, 1999). The T_{CM} cells represent a clonally expanded antigen primed population which express CCR7 and CD62L and can circulate through the lymphatics, and upon secondary challenge can provide help for B cells and produce more effector cells (Sallusto *et al.*, 1999). This model for immunological memory mirrors that proposed by Bell and colleagues (1998) (as described in section 5.1.5), in suggesting that memory is provided by two populations of cells: a population of activated effector cells and a population of resting memory cells. However, in the Bell model the effector cells were maintained only by continual antigen presence, which Sallusto *et al* (1999) were not able to test in the human system. The expression of CCR7 by the rat effector and memory T cells could not be examined due to the lack of a suitable antibody. A more recent study has produced results which, on first impressions, seem to agree with the general model of a heterogenous population of memory T cells (Ahmadzadeh *et al.*, 2001). Using a tg model and an activation profile to different types of TCR-mediated stimuli, two memory subsets were observed, a $CD62L^{lo}$ effector $CD4^+$ T cell population and a $CD62L^{hi}$ memory $CD4^+$ T cell

population. These two subsets differed in their overall proliferative capacity, kinetics of activation, activation profile, and spontaneous proliferation to MHC class II (Ahmadzadeh *et al.*, 2001). However ignoring several previous studies (Bell & Sparshott, 1990) (Bell *et al.*, 1998) (Akbar *et al.*, 1991), they used the $CD45RB^{lo}$ subset of splenic $CD4^+$ T cells as their standard for resting memory $CD4^+$ T cells (Ahmadzadeh *et al.*, 2001). Therefore the $CD62L^{hi}CD45RB^{lo}CD4^+$ T cells, which they propose as resting memory cells, may represent one of several types of cell population. They may represent a recently activated population of cells which has not yet lost or regained expression of CD62L, they may represent an intermediate population between effector and memory cell, or they may represent a tg artefact, as in some activation profiles these "memory" cells performed worse than naïve cells (Ahmadzadeh *et al.*, 2001).

5.1.8 Role of antigen in maintenance of memory.

In the model of immunological memory as proposed by Bell and colleagues (1998), a role for antigen is proposed, but only in the maintenance of short-lived $CD45R^{lo}$ memory primed $CD4^+$ T cells. They proposed that following antigen clearance long lived memory is retained by the $CD45^{hi}$ revertant $CD4^+$ T cells (Bell *et al.*, 1998). The original idea that memory was dependent on antigen came from early adoptive transfer experiments (Gray & Skarvall, 1988). It was observed that a memory response was only transferred when antigen was also present (Gray & Skarvall, 1988). However recent experiments have produced evidence which supports the idea that T cell memory is independent of persisting antigen. These experiments have focused on the adoptive transfer of antigen primed T cells into MHC deficient hosts, so that antigen presentation cannot occur. $CD4^+$ T cells from AND TCR tg mice were stimulated *in vitro* and adoptively transferred to MHC class II KO recipients (Swain *et al.*, 1999). The transfer of effector cells resulted in the development of long-term CD4 memory despite the fact that no further antigen was introduced (Swain *et al.*, 1999). A linear relationship between the number of transferred cells and the size of the memory population recovered was observed (Swain *et al.*, 1999). The memory cells recovered were small and resting, and expressed high levels of CD44 (Swain *et al.*, 1999). In contrast when naïve $CD4^+$ T cells were transferred to MHC class II deficient hosts they exhibited a shortened lifespan (Swain *et al.*, 1999). Similar studies with $CD8^+$ T cells

produced similar results. In these latter experiments lymphocytic choriomeningitis virus (LCMV) specific $CD8^+$ T cells were adoptively transferred into both MHC class I-deficient ($\beta_2M^{-/-}$) mice and MHC class I-positive mice (Murali-Krishna *et al.*, 1999). Although the initial expansion of the transferred cells was less in the class I deficient mice, by day 22 and day 50 the numbers of $CD8^+$ T cells was at similar levels in both groups (Murali-Krishna *et al.*, 1999). Although the total $CD8^+$ T cell population transferred consisted of $CD44^{lo}$ (naïve) and $CD44^{hi}$ (effector/memory) cells, by day 50 after transfer nearly all of the donor $CD8^+$ T cells were $CD44^{hi}$ (Murali-Krishna *et al.*, 1999). However this could reflect the proliferation of these cells following transfer rather than preferential survival as suggested by the authors (Murali-Krishna *et al.*, 1999). In contrast with the $CD4^+$ T cells which were non-proliferating after transfer to MHC class II deficient hosts, the $CD8^+$ T cells in class I deficient hosts were continually proliferating (Murali-Krishna *et al.*, 1999). However, similarly to the naïve $CD4^+$ T cells, naïve $CD8^+$ T cells did not survive in MHC deficient mice. The $CD8^+$ T cells from class I deficient hosts also exhibited functional characteristics of memory cells by secreting IFN-γ rapidly after ex vivo peptide stimulation. (Murali-Krishna *et al.*, 1999). Therefore these two experiments showed that MHC independence is a property of both CD4 and CD8 memory.

However, there were flaws in the *in vitro* tests used to assess the functional characteristics of memory T cells as demonstrated by more recent work by Kassiotis *et al* (2002). They used an experimental system in which two different populations of monoclonal TCR-tg memory $CD4^+$ T cells were generated and maintained either in the absence of MHC class II antigens (TCR triggering could not occur) or in the presence of allogeneic MHC class II antigens (Kassiotis *et al.*, 2002). They discovered, in agreement with the previous studies (Swain *et al.*, 1999) (Murali-Krishna *et al.*, 1999), that the absence of MHC contact did not affect the survival of the cells *in vivo* or their ability to proliferate or express intracytoplasmic cytokines after *in vitro* stimulation by peptide-pulsed dendritic cells (Kassiotis *et al.*, 2002). However when the behaviour of these cells was examined *in vivo* they did not appear to possess "memory" characteristics. The memory $CD4^+$ T cells that were maintained in MHC class II deficient mice were unable to help naïve B cells produce IgG antibodies (Kassiotis *et al.*, 2002). They were also unable to reject skin transplants expressing the antigen (Kassiotis *et al.*, 2002). Rejection of the graft requires processing and presentation of skin antigens by the graft Langerhans cells, which indicates that memory cells

maintained in the absence of MHC contact are unable to respond to antigens presented by dendritic cells *in vivo* (Kassiotis *et al.*, 2002). Another difference observed between the cells maintained in the presence or absence of MHC was the decreased survival of $CD4^+$ T cells in MHC deficient hosts, when cotransferred with a competing cell population (equal numbers of OT-I $CD8^+$ T cells) (Kassiotis *et al.*, 2002). This suggested a direct role for MHC class II-derived signals in memory $CD4^+$ T cell homeostasis. Furthermore following *in vivo* antigen (host-expressed C5) presentation by syngenic B cells, although both cell populations became activated (as indicated by increased cell size), only cells from MHC sufficient hosts expanded (Kassiotis *et al.*, 2002). Comparative analysis of different cell surface molecules showed an increase in TCR and CD3 expression on memory T cells in the absence of MHC contact (Kassiotis *et al.*, 2002). They also exhibited fourfold up-regulation of CD5 compared with T cells from MHC sufficient hosts (Kassiotis *et al.*, 2002). However it is not clear whether these phenotypic differences reflected changes due to the lack of MHC contact or if they were characteristic of the specific TCR-tg T cells. In summary, these experiments demonstrated that although memory $CD4^+$ T cells may survive in the absence of stimulation, they may lose the functional properties of memory cells. They also highlighted the dangers of drawing conclusions on memory from *in vitro* experiments and demonstrated the importance of testing memory cells under more physiological conditions.

5.1.9 Generation of T memory cells.

While the role of continual stimulation maintaining functional memory cells is still debated, even less is known about what factors are necessary to generate memory T cells. To consider this we must remember that the vast majority of T lymphocytes participating in the primary immune response are short-lived cells which are rapidly eliminated at the end of the response (Sprent, 1993). These cells die once the pathogen has been eliminated presumably because they are no longer required. If they all survived, the repertoire of the remaining lymphocyte population would be unbalanced towards that particular pathogen and the primary response to other pathogens would be compromised. However, in order for memory to occur, a small proportion of the responding cells must not be eliminated. How these memory cells avoid death is still unclear though it has been proposed to be caused by the up-regulation of anti-apoptotic

molecules such as Bcl-2, in combination with the down-regulation of apoptotic molecules such as Fas (Akbar, 1993) (Boise, 1995) (Cory, 1995). However, little is known about the factors which control the regulation of these molecules on T cells which are either programmed to die or selected for survival and memory maintenance (Sprent *et al.*, 1997).

As suggested earlier it may be the timing of the encounter with antigen which influences the selection of memory cells. During the initial stages of an immune response the concentration of antigen in the draining lymph nodes will be high and the available antigen-specific T cells become activated, proliferate and differentiate into effector cells. These cells leave the lymph nodes and through a variety of effector mechanisms destroy the invading pathogen. As this occurs the concentration of antigen on the dendritic cells rapidly decreases, as does T cell proliferation, differentiation and cytokine production. It is under these conditions that the effector cells rapidly die after mediating their effector functions. Death may be caused by loss of contact with antigen and cytokines which results in the down regulation of anti-apoptotic molecules. However it has been proposed that it is these conditions which may favour memory cell formation (Sprent *et al.*, 1997). As antigen is limiting only cells of high affinity would be able to maintain antigen contact and thus receive the necessary signals to survive (Sprent *et al.*, 1997). These so called "straggling" cells (as they enter the lymph node during the terminal stage of the immune response) would therefore go on to provide the memory response (Sprent *et al.*, 1997). This idea is still largely hypothetical, although it is favoured by some as it parallels the affinity maturation that occurs with memory B cells (Tarlinton & Smith, 2000). In agreement with this model Hou *et al* (1994) observed that the size of memory T cell populations correlated with T cell burst size, suggesting that the processes that drove *in vivo* T cell expansion also influenced memory T cell development. However, in a study which examined the effect of inflammatory signals on $CD8^+$ T cells in the absence of antigen, it was observed that early reinfection (before the primary infection could be cleared) induced further expansion of antigen-specific T cells that kinetically approximated the typical memory response that was induced by reinfection months after the resolution of a primary infection (Busch *et al.*, 2000). This suggested that the ability to mount a memory T cell response was established before the completion of the expansionary phase of the primary response and was contained within the initial effector population (Busch *et al.*, 2000). Although inflammation did promote *in vivo* T cell expansion, antigen

presentation was required for memory cell generation (Busch *et al.*, 2000). In a more recent study using AND tg mice (T cells specific for pigeon cytochrome c), it was shown that effector $CD4^+$ T cells gave rise to resting memory cells without further cell division and differentiation, following adoptive transfer to MHC deficient hosts (Hu *et al.*, 2001). The authors suggested that this was evidence for a linear pathway from effector to memory cells and that the properties of memory cells were predetermined during effector cell generation (Hu *et al.*, 2001). However, the effector cells were generated *in vitro*, and the functionality of these "memory" cells was only tested for the ability to produce cytokines *in vitro*. Furthermore when the effector cells were transferred to hosts with resident $CD4^+$ T cells, the memory population was significantly reduced (Hu *et al.*, 2001). It has also been proposed that memory cells may simply be a subset of low affinity cells (Sprent, 1994). Because of their low affinity these cells proliferate less extensively in the primary response and are therefore less susceptible to exhaustion and elimination. The extra avidity acquired through upregulation of various adhesion molecules may compensate for their reduced TCR affinity. There is some recent evidence which supports this concept. The authors used a variety of peptide analogues (the acetylated NH_2-terminal nonameric peptide (Acl-9) of myelin basic protein (MBP)) which varied in their affinity for MHC class II, and therefore antigenic strength (Anderton *et al.*, 2001). They found that immunization *in vivo* with the peptide of strongest MHC binding led to the elimination by apoptosis of the T cells bearing high affinity T cell receptors (Anderton *et al.*, 2001). In contrast, immunization with the weakest binding MHC peptide led to expansion of T cells which induced encephalomyelitis (Anderton *et al.*, 2001). Whether memory cells are selected based on their affinity for antigen requires further study. This concept of affinity and cell survival will be discussed more in Chapter 6.

5.1.10 Homeostasis of Naïve and Memory T cells.

Following the clearance of infection a percentage of the antigen-specific cells must survive in order to provide a memory response for that animal. However, mechanisms must exist which prevent too many of the cells surviving as this would compromise the normal T cell repertoire, as the total number of cells in any animal is under strict regulation. Mechanisms may also exist which prevent the newly acquired memory cells totally replacing previously existing memory cells. However what these

mechanisms are is still not clear. Studies with naïve lymphocytes have demonstrated that these cells require some kind of signal in order to survive. Evidence has accumulated that naïve cells require contact with self-MHC/peptide and various cytokines for long term survival (Boursalian & Bottomly, 1999b) (Kirberg *et al.*, 1997) (Takeda *et al.*, 1996). Cytokines which have been implicated in cell survival are IL-2, IL-7, IL-9 and IL-15 (Marrack *et al.*, 2000) (Nakajima *et al.*, 2000), with IL-7 appearing to have the dominant role (Tan *et al.*, 2000). These cytokines bind to receptors containing the common γ-chain (Geignat *et al.*, 2001), and can induce changes in the expression or trafficking of pro- and anti- apoptotic Bcl-2 family proteins (von Freeden-Jeefy *et al.*, 1997) (Khaled *et al.*, 1999). However other factors are involved as Bcl-2 tg T cell populations still require IL-7 to undergo homeostatic expansion (Tan *et al.*, 2000). Also, continual exposure to IL-7 maintains metabolic activity through the activation of phosphatidylinositol 3-kinase, rather than the JAK-STAT pathway that is involved in the upregulation of Bcl-2 expression (Rathmell *et al.*, 2001). Cytokines that do not use the γc receptors may also influence T cell survival and homeostatic expansion. Evidence exists which suggests that transforming growth factor-β (TGF-β) may play a role in restraining the size of the T cell compartment (Gorelik & Flavell, 2000) (Lucas *et al.*, 2000). IL-6 has also been shown to rescue T cells from apoptosis *in vitro* (Teague *et al.*, 1997), although it does not appear to be required *in vivo* (Viven *et al.*, 2001).

This combination of self-MHC/peptide and cytokines can also result in T cell proliferation in the absence of exogenous stimulation, known as homeostatic proliferation. This may occur physiologically following lymphoid ablation of normal animals. It is also observed in experimental systems following adoptive transfer of T cells into lymphopenic hosts (Bell *et al.*, 1987) (Kieper & Jameson, 1999). As the same factors that have been implicated in naïve T cell survival are also crucial for homeostatic proliferation, the question of how a T cell chooses to survive rather than proliferate must be considered. The host T cell compartment must play a crucial role in this, as the proliferation of T cells in a T cell depleted environment can be reduced by the co-transfer of competitor T cells (Lucas *et al.*, 2000). Therefore it may occur by a T cell-T cell interaction or may be a result of competition for limiting resources. These resources may be cytokines such as IL-7 or access to APCs which present self-MHC ligands, although there is little direct evidence for this. The cotransfer of an excess of $CD8^+$ T cells with $CD4^+$ T cells can inhibit the homeostatic expansion of both cell

types. This suggests that homeostasis is not a result of competition for self-MHC, as these cells recognize different MHC classes (Ernst *et al.*, 1999). Interestingly, $CD8^+$ T cells are better competitors than $CD4^+$ T cells in these assays. This may due to self-MHC presentation to $CD8^+$ T cells by non-professional APCs. This has been supported by experiments which showed that homeostatic expansion of $CD8^+$ T cells, but not $CD4^+$ T cells, can occur outside of normal secondary lymphoid organs (Dai & Lakkis, 2001). This would suggest a role for self-MHC recognition in homeostatic expansion, as $CD4^+$ T cells would have to seek out MHC class II molecules, most of which are in lymphoid tissue. In comparison to APC presentation of foreign antigens, the APC interactions which occur during homeostatic proliferation can occur in the absence of co-stimulatory molecules such as CD28 and CD40 (Prlic *et al.*, 2001a). Interestingly cytotoxic T-lymphocyte antigen 4 (CTLA-4) deficiency was observed to induce polyclonal T-cell proliferation, which has indicated a role for this molecule in T cell homeostasis (Egen *et al.*, 2002). Homeostatic proliferation has often given rise to both $CD4^+$ and $CD8^+$ T cells which express certain memory phenotypes (Goldrath *et al.*, 2000) (Gudmundsdottir & Turka, 2001). In some instances the acquisition of memory functionality has also been observed despite the absence of antigenic challenge, although this has not been vigorously tested (Gudmundsdottir & Turka, 2001). This has lead to the controversial proposal that specific foreign antigen is not required for the formation of memory T cells, although what these cells retain a "memory" for has not been described by the authors (Gudmundsdottir & Turka, 2001). Therefore when considering the studies of memory T cells generated in lymphopenic animals all of these complex factors must be considered.

However, whether the same factors responsible for homeostasis of naïve T cells are also required for memory T cell survival is still unknown. Evidence exists which suggests that the sizes of the naïve and memory pools are regulated independently, which indicates that the T cell populations do not compete for the same limiting resources (Freitas & Rocha, 2000) (Tanchot & Rocha, 1995). Recent work with tg $CD8^+$ T cells which showed that memory cells are more resistant to apoptosis than naïve cells, produced evidence which supports this (Grayson *et al.*, 2002). The same pattern of a more rapid expansion and diminished contraction of memory cells compared with naïve cells, following antigen re-exposure, was observed when the cells were transferred together or into separate mice (Grayson *et al.*, 2002). This suggested

that previous antigen experience rather than nonautonomous factors were responsible for the preferential survival of memory cells (Grayson *et al.*, 2002).

The question of continual antigen/MHC stimulation has already been discussed (Section 5.1.8), with current evidence favouring memory cells surviving independently of antigen, although functional responses possibly require intermittent TCR triggering. IL-15 has been shown to play an important role in $CD8^+$ memory T cell homeostasis in normal animals (Zhang *et al.*, 1998). IL-15 can also, along with IL-7, drive homeostatic proliferation of memory $CD8^+$ T cells in lymphopenic animals (Tan *et al.*, 2002). IL-2 has been proposed to oppose memory $CD8^+$ T cell survival possibly by causing the death of these cells (Ku *et al.*, 2000). In contrast $CD4^+$ memory T cells do not appear to be affected by the presence or absence of γc cytokines (Tan *et al.*, 2002) (Lantz *et al.*, 2000). In fact $CD4^+$ memory T cells have been observed to survive and proliferate in the absence of IL-4, IL-7, and IL-15 (Tan *et al.*, 2002). Therefore finding the factors that control and regulate $CD4^+$ memory T cell homeostasis remains an interesting challenge. An important factor to consider may be thymic atrophy which occurs with old age and results in an almost complete cessation of naïve T cell production (Scollay *et al.*, 1980). This corresponds with a gradual accumulation of memory phenotype cells with age (Sprent, 1994). This makes evolutionary sense as the chances of an individual encountering a "new" pathogen in their latter years is less than that of re-exposure to a pathogen to which they have been previously exposed. Therefore in old age it is better to have a T cell population that has been skewed by a life of acquired immunity at the expense of a diverse repertoire. Whether memory T cells survive because there is more "space" or because of reduced competition from lower numbers of naïve T cells is not known. However the factors involved in this preferential survival of memory cells may have important therapeutic value.

So it is clear that immunization results in the formation of a memory response that can be observed many years later. However, the problems in distinguishing effector $CD4^+$ T cells from memory $CD4^+$ T cells has hampered our understanding of this response. For example we do not know how memory T cells originate, how they are maintained or even if they are truly "memory" cells and not a continually stimulated effector population. Also unknown, is whether a memory response is a result of an increase in the number of antigen-specific cells that are available to respond, or whether a qualitative change is required to establish the memory cell. The availability of tg T cell clones should allow us to follow antigen-specific cells before and after antigen

stimulation and therefore answer these questions. To examine whether antigen-experienced T cells are qualitatively different from naïve T cells we planned to compare them functionally in an *in vivo* response. The response we tested was their ability to provide help for antigen-experienced B cells. The subsets of T cells we wished to compare were naïve $CD4^+$ T cells, antigen primed "effector" $CD4^+$ T cells, and antigen experienced "revertant" memory $CD4^+$ T cells. Any differences observed between these tg cells would be a result of their encounter with antigen as they will all share the same antigen-specific TCRs. We also wished to examine the role of persisting antigen on memory cell formation as well as examining possible phenotypic differences between the T cell subsets.

Chapter 5

5.2 Results

5.2.1 OVA priming of KJ^+CD4^+ T cells.

To assess the phenotypic changes that occur following antigen stimulation we adoptively transferred 5 x 10^6 KJ^+CD4^+ T cells (Figure 5.2a) into BALB/cIgh recipients and challenged i.p. with ap-OVA peptide. 7 days following transfer the mice were examined for the presence of tg cells (Figure 5.2b). The cell surface expression of a variety of phenotypic activation markers was also examined, and compared with expression by naive cells before transfer. Figure 5.3a shows the expression of the common leukocyte antigen, CD45RB. Prior to transfer 95% (black) of the T cells were $CD45RB^{hi}$. In contrast following antigenic challenge 35% (green) of the T cells were $CD45RB^{hi}$. Figure 39b shows the changes that occur in CD44 expression, another molecule often used as a marker for effector/memory cells. Antigen challenge resulted in a change from 74.2% $CD44^{med}$ before antigen challenge to 14.4% $CD44^{med}$ after antigen challenge. Figure 5.3c shows the change in expression of L-selectin, the lymphocyte homing receptor. 94.7% of the naive tg cells were $CD62L^{hi}$, but following antigen challenge 27% of the cells remained $CD62L^{hi}$. CD69 expression is often used as a marker of early T cell activation. This was confirmed in Figure 5.3d, which shows that before transfer of the tg cells, 91.75% were $CD69^{lo}$, but following antigen challenge 47% were $CD69^{lo}$. CD25, the IL-2αR, is another marker of early T cell activation, although only 29.9% of the KJ^+CD4^+ T cells increased their expression of this molecule following antigen challenge (Figure 5.3e). A similar increase (27%) in expression of CD49b ($\alpha_4\beta_7$ integrin) was also observed following *in vivo* peptide stimulation (Figure 5.3f).

It was clear that the changes which occured in the expression of these activation markers were quite variable (summarized in Table 5.1). Over 60% of the KJ^+CD4^+ T cells changed expression of CD45RB, CD44, and CD62L in comparison with less than 30% of cells upregulating CD25 and CD49b expression following challenge with OVA peptide. The reason for these differences was not clear but reflect the complex nature of T cell activation and the heterogeneity found within an activated T cell population. Based on these observations and results from the literature (section 5.1.4) expression of

the low molecular weight isoform of CD45RB ($CD45RB^{lo}$) was used as the marker for antigen activated T cells.

To generate a population of antigen primed tg $CD45RB^{lo}$ T cells, 5 x 10^6 $KJ^+CD4^+CD45RB^{hi}$ T cells (from DO11.10SCID mice) (Figure 5.4a, b), were adoptively transferred into SCID mice and challenged i.p. with 100μg ap-OVA peptide. 10 days following transfer the mice were killed, the spleens and LNs removed and prepared for flow cytometric analysis. An example of the antigen activated cell population is shown in Figure 5.4 c, d shows that 69.12% of the KJ^+CD4^+ T cells were $CD45RB^{lo}$. The $CD45RB^{lo}$ T cells were purified by depletion of the $CD45RB^{hi}$ cells as described in Section 2.9. Following depletion 97.6% of the KJ^+CD4^+ T cells were $CD45RB^{lo}$ (Figure 5.4 e, f). These cells were used as OVA primed KJ^+CD4^+ T cells for further experiments.

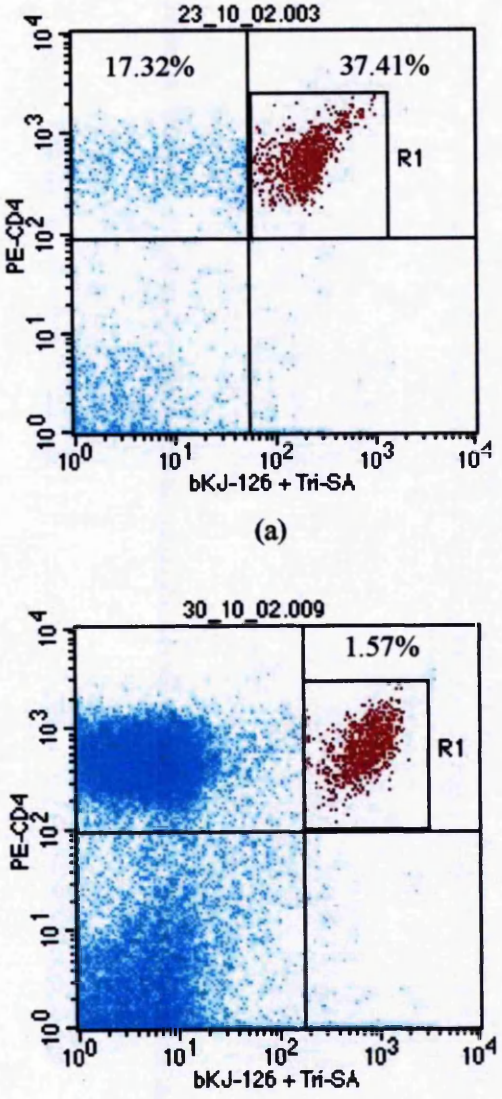


Figure 5.2. Flow cytometric analysis of KJ^+CD4^+ T cells.

Flow cytometric analysis of KJ^+CD4^+ T cells (from DO11.10 mice) prior to injection (a) and after injection into BALB/cIgH mice and OVApep immunization. The percentage of tg in R1 is shown in the upper right quadrant.

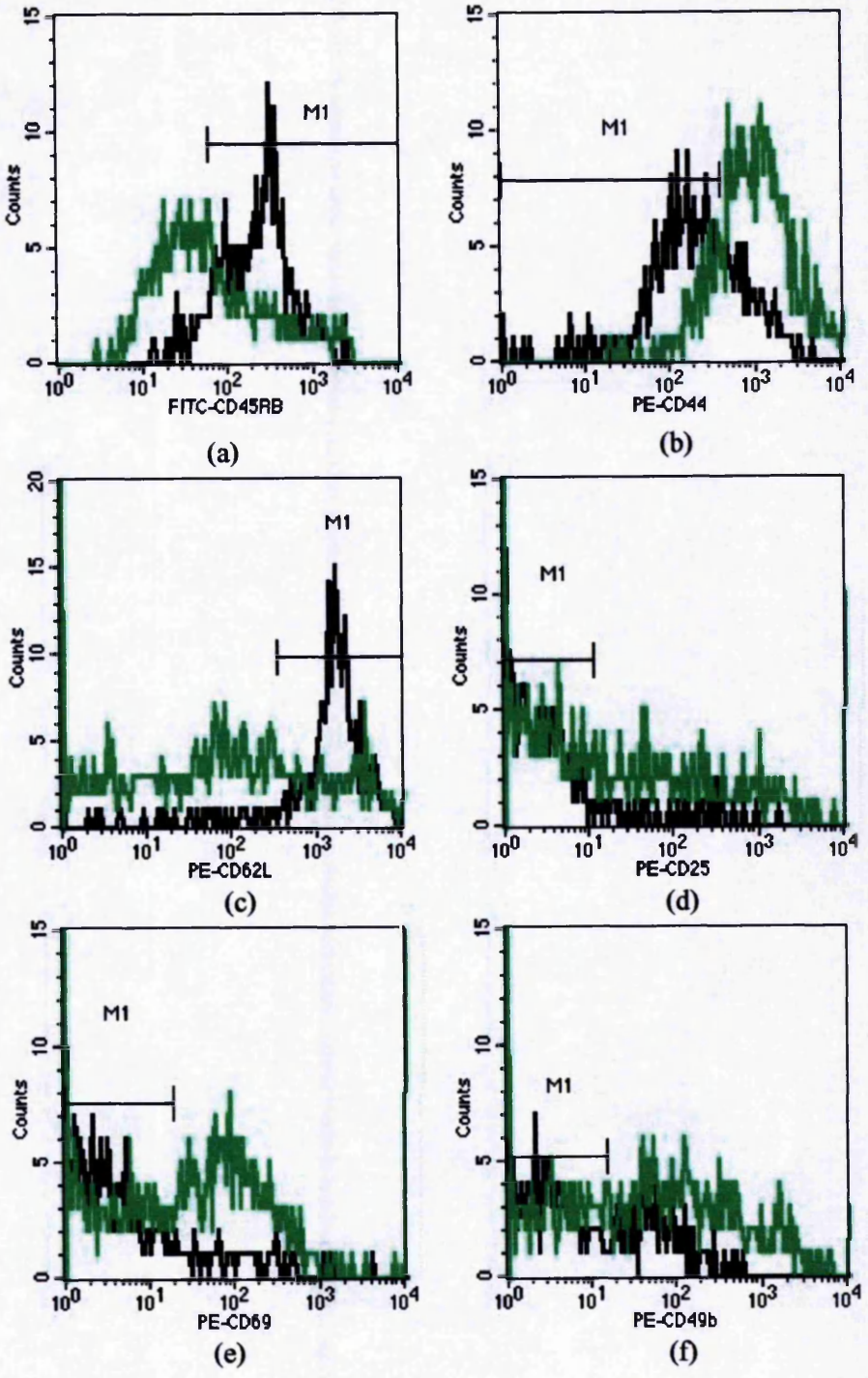


Figure 5.3. Flow cytometric analysis of KJ^+CD4^+ T cells before and after transfer.

Flow cytometric analysis of KJ^+CD4^+ T cells before (black) and 7 days after (green) OVApep stimulation. The percentage changes in expression of CD45RB (a), CD44 (b), CD62L (c), CD25 (d), CD69 (e), and CD49b (f) are summarized in Table 4.

Phenotype	Before	After	% Change
$CD45RB^{hi}$	95%	35%	60%
$CD44^{hi}$	25.8%	85.6%	59.8%
$CD62L^{hi}$	94.7%	27%	67.7%
$CD69^{hi}$	8.25%	47%	38.75%
$CD25^{hi}$	2.1%	32.2%	30.1%
$CD49b^{hi}$	25.3%	47.7%	22.4%

Table 5.1. Phenotypic changes which occurred following priming.

Summary of the phenotypic changes that occurred on $KJ^{+}CD4^{+}$ T cells 1 week after adoptive transfer to BALB/cIgH recipients and immunization with OVApep.

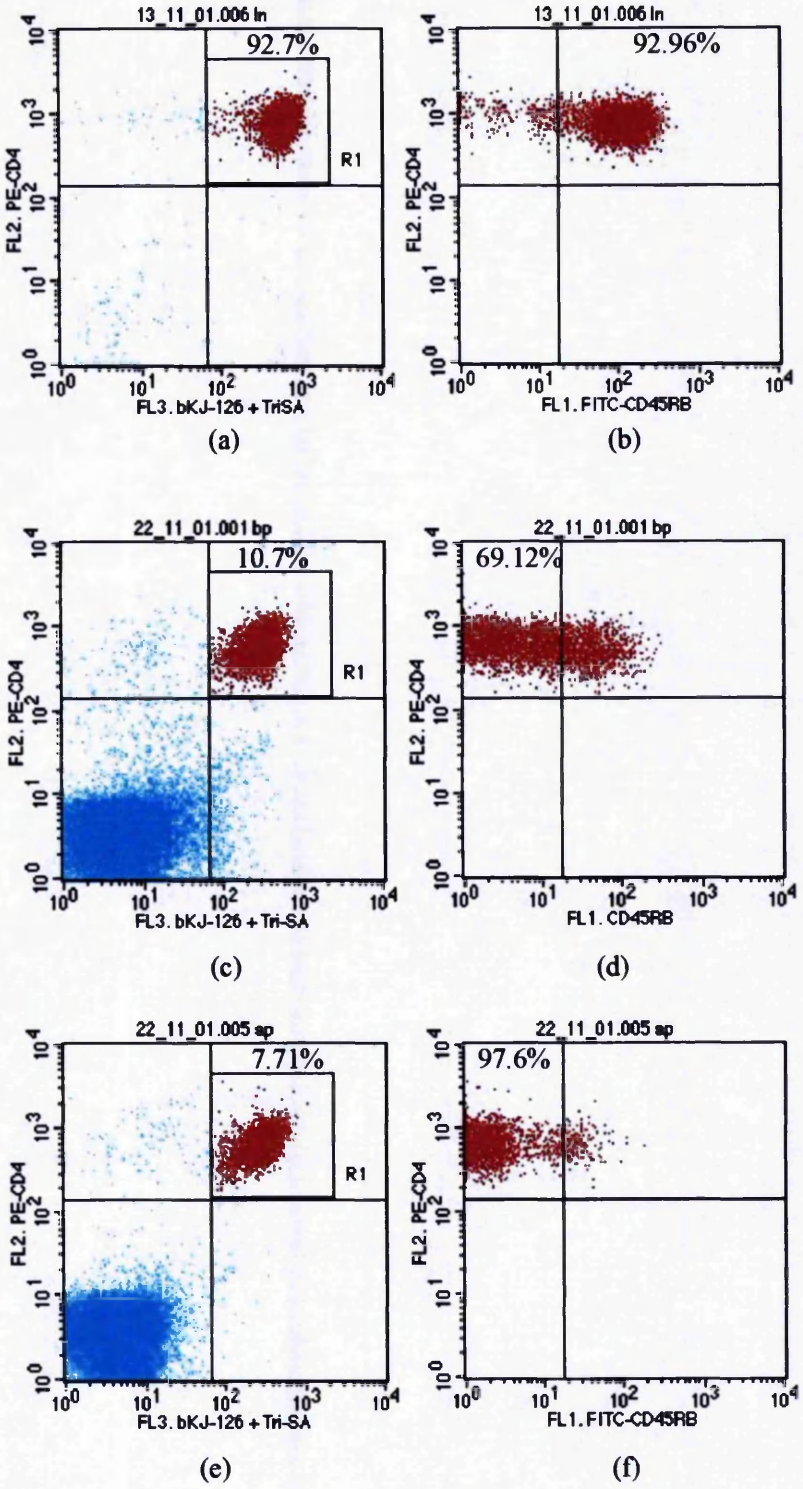


Figure 5.4. Purification of primed CD45RB^{lo}KJ^{+}CD4^{+} T cells.

Flow cytometric analysis of DO11.10SCID LN cells before adoptive transfer (a, b). The cells were transferred to SCID recipients, challenged with ap-OVApep and collected on day 10 for analysis (c, d) and purification (e, f). The KJ^{+}CD4^{+} T cells in the R1 gate were analysed for expression of CD45RB (b, d, f). The 10-day primed KJ^{+}CD4^{+} T cells were depleted of CD45RB^{hi} cells (e, f).

5.2.2 Comparison of help from naïve versus primed KJ^+CD4^+ T cells for OVA primed B cells.

To compare the functional differences between naïve and primed KJ^+CD4^+ T cells we tested their ability to provide help for antigen-experienced B cells. To do this we used the adoptive transfer system as described in Chapter 4. 10^7 purified B cells from OVA primed mice were adoptively transferred either with a population of naïve $CD45RB^{hi}KJ^+CD4^+$ T cells (Figure 5.4a, b) or a population of OVA peptide primed $CD45RB^{lo}KJ^+CD4^+$ T cells (Figure 5.4e, f) into SCID recipients and challenged with 10μg sol-OVA. A population of B cells without any T cells was also transferred as a control. The recipient mice were bled on days 7, 14, and 21 and examined for anti-OVA antibodies as described in Section 2.4. The results of the transfer of 3×10^5 KJ^+CD4^+ T cells are shown in Figure 5.5. The mice which received OVA primed KJ^+CD4^+ T cells produced significantly more antibody (2587μg/ml) than those which received naïve KJ^+CD4^+ T cells (851μg/ml) on day 7 ($p>0.01$). By days 14 and 21 any difference observed was insignificant. Control mice which received B cells alone produced a minimal amount of antibody, showing that antibody production was dependent on help from the transferred KJ^+CD4^+ T cells.

Although a difference was observed in the help given by naïve compared to primed KJ^+CD4^+ T cells, the difference observed was not as great as expected. To test whether antigen priming might have a greater effect when numbers of T cells were more limiting we repeated the experiment with a lower dose (10^5) of KJ^+CD4^+ T cells. This number of T cells should be enough to provide help for primed B cells (Figure 5.6), although the antibody produced should be less than when 3×10^5 T cells were transferred. Mice were also bled on day 5 to determine whether primed T cells conferred an advantage observed by an earlier response than that induced by naïve T cells. As shown in Figure 5.6, a small amount of antibody was detectable in both groups of recipients on day 5. Although the mice which received primed T cells produced slightly more anti OVA antibody at this time point, the difference was not statistically significant. Similar to the transfer of 3×10^5 KJ^+CD4^+ T cells (Figure 5.5), when 10^5 KJ^+CD4^+ T cells were transferred, the only significant difference observed was on day 7 ($p<0.05$). By day 14 and 21(data not shown) there was no significant difference between the groups.

Both of these experiments have important implications in answering the question of immunological memory. By comparing T cells which share the same antigen-specificity, we were able to evaluate the effect of antigen priming on the ability of T cells to help memory B cells. Unexpectedly priming the T cells with antigen before transferring them with B cells did not result in either a faster or a stronger response (the difference on day 7 was modest and barely significant ($p<0.05$), when compared with mice which received naïve T cells. The crucial stages in any immune response are firstly the detection and uptake of antigen by APCs, followed by processing and presentation to T cells. This stage will have taken a similar amount of time for both groups. The T cell once activated upregulates a variety of cell surface molecules, which allows it to enter the B cell areas of lymphoid tissue and provide help. As the antigen primed $KJ^{+}CD4^{+}$ will already be in an upregulated state, it suggests three possibilities. Firstly, that this activation of the T cell or entry into the B cell areas may not be crucial for a secondary B cell response. Secondly, perhaps activation of the T cell occurs very quickly. Or thirdly, that memory B cells, following activation by T cells, require further differentiation to produce antibody that is independent of T cells. Whatever the reason the experiments suggest that the characteristics of a humoral memory response are primarily determined by the properties of the memory B cell. The component of memory that resides with the $CD4^{+}$ T cell compartment may only be quantitative - an increase in the number of antigen-specific cells available to provide help.

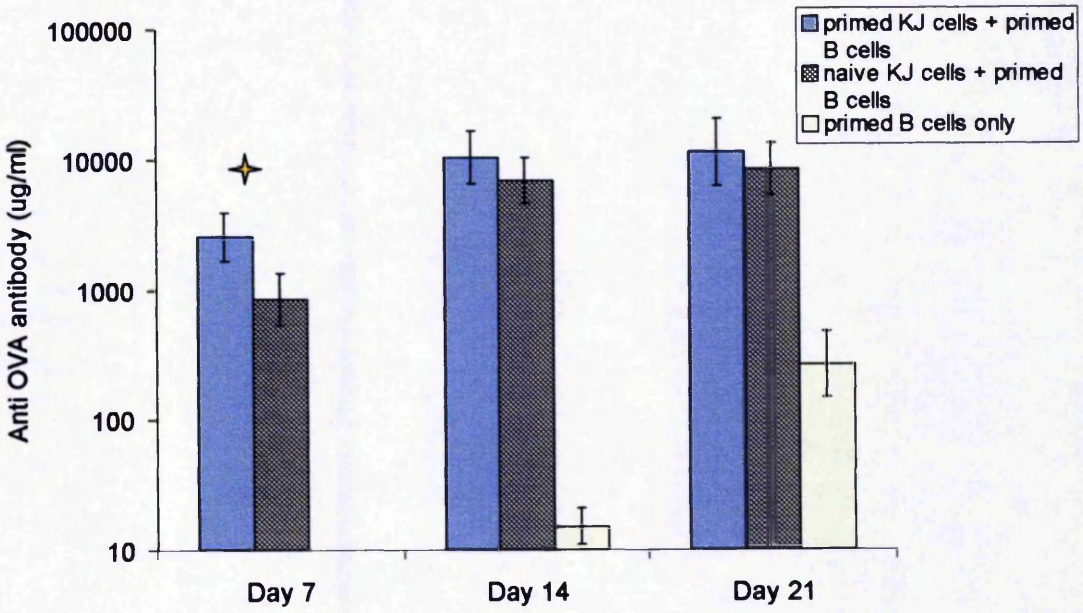


Figure 5.5. Comparison of help for OVA primed B cells between naïve and primed KJ^+CD4^+ T cells.

Anti OVA antibody levels from SCID recipients which received either 3 x 10^5 naïve $CD45RB^{hi}KJ^+CD4^+$ T cells, or 3 x 10^5 OVA primed $CD45RB^{lo}KJ^+CD4^+$ T cells, transferred with 10^7 B cells and challenged i.p. with 10μg sol-OVA. 10^7 B cells were transferred on their own as a control. A significant difference was observed between the naïve and primed T cells on day 7 ($p<0.05$). The mean values of 6 recipients per group are shown +/- SD.

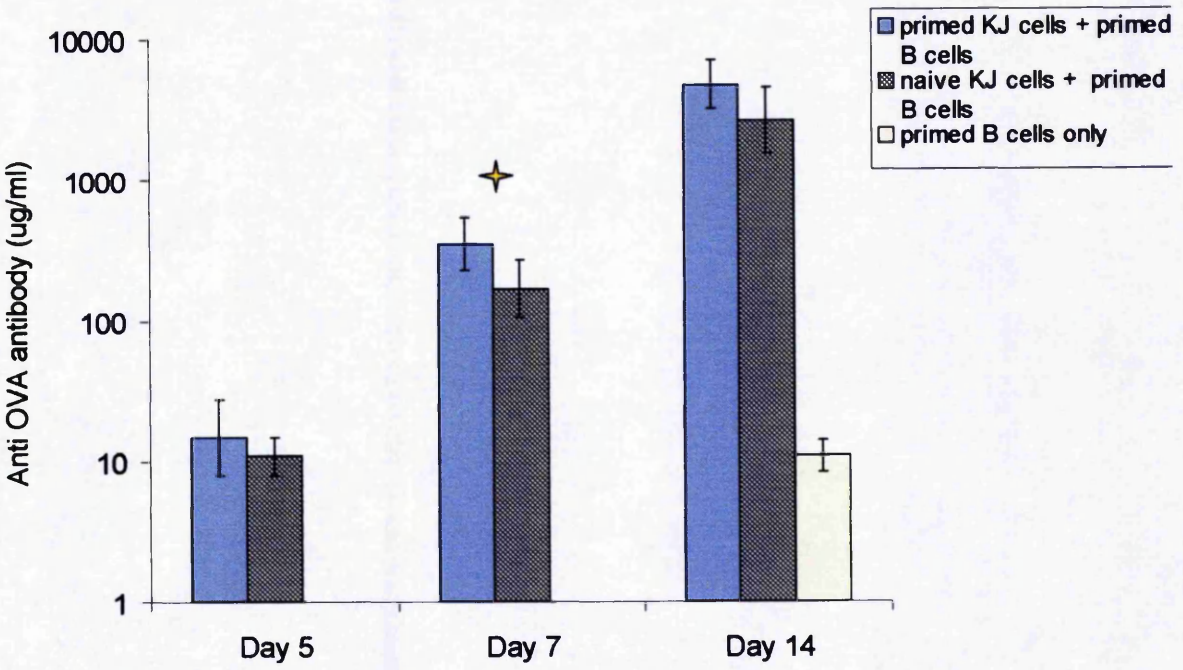


Figure 5.6. Comparison of help for OVA primed B cells between naïve and primed KJ^+CD4^+ T cells.

Anti OVA antibody levels from SCID recipients which received either 10^5 naïve $CD45RB^{hi}KJ^+CD4^+$ T cells or 10^5 OVA primed $CD45RB^{lo}KJ^+CD4^+$ T cells, transferred with 10^7 B cells and challenged i.p. with 10μg sol-OVA. 10^7 B cells were transferred on their own as a control. A significant difference was observed between the naïve and primed T cells on day 7 ($p<0.01$). The mean values of 6 recipients per group are shown +/- SD.

5.2.3 Behaviour of KJ^+CD4^+ T cells in a "normal" BALB/cIgh environment.

As there are inherent flaws in studying memory cells in lymphopenic animals (section 5.1.10) such as SCIDs, we wanted to examine whether KJ^+CD4^+ T cells would behave like memory cells when stimulated *in vivo* in immune competent mice. The design for this experiment is shown in Figure 5.7. This experiment consisted of 4 groups of 6 BALB/cIgh mice, 2 of which received 5 x 10^6 KJ^+CD4^+ T cells by i.v. injection. One of the groups which received the cells also received 100μg ap-OVA peptide by i.p. injection (group 1), as did one of the control groups (group 3). The other two groups received PBS (groups 2 & 4). The mice were left for 8 weeks, and after this time period received 10^7 OVA primed B cells and were challenged with 10 μg sol-OVA. They were tail-bled 7 and 14 days after this secondary challenge and the serum measured for anti-OVA antibody as described in Section 2.4.

The idea behind this experiment was to determine whether priming KJ^+CD4^+ T cells *in vivo* would generate a long lived population of KJ^+CD4^+ memory T cells. The presence of memory T cells was tested by the transfer of OVA primed B cells and challenge with sol-OVA. As the OVA peptide is not recognised by B cells, immunizing with the peptide (ap-OVApep) will prime KJ^+CD4^+ T cells without generating memory B cells within the recipient mice. Although the ap-OVApep might also prime $CD4^+$ T cells of the host, the transfer of OVA specific KJ^+CD4^+ T cells should at least increase the frequency of the antigen-specific memory T cells.

The antibody results from this experiment are shown in Figure 5.8. On day 5 and 7 both groups immunized with ap-OVA peptide produced an antibody response, with group 1 producing more (122μg/ml and 448μg/ml) than group 3 (83μg/ml and 251μg/ml). By day 14 antibody synthesis increased for both groups (group 1 1282μg/ml) (group 3 744μg/ml). However none of these differences were significant. By day 14 groups 2 and 4, which were unimmunized, also began to produce an anti OVA antibody response, (27μg/ml, and 81μg/ml respectively).

Therefore immunizing with ap-OVA peptide stimulated memory T cell production, although surprisingly, the level of T cell help found in control mice was comparable to that in mice that received KJ^+CD4^+ T cells. We did not anticipate that the OVA peptide of 17 amino acids would be so strong antigenically to stimulate the host T cell repertoire. The lack of a significant difference in antibody responses between the two groups that were immunized with ap-OVA peptide, suggests that

primed KJ^+CD4^+ T cells (potential memory T cells) did not survive to contribute to the secondary response. However this was not the case as shown by FACS analysis of the MLN of these mice (Figure 5.9). FACS analysis of the MLNs of 3 recipients 14 days after the transfer of primed B cells and sol-OVA challenge clearly shows the presence of a small population of KJ^+CD4^+ T cells. Based on the percentage of cells in the total lymphocyte population (0.36%-0.4%) this corresponds to about 10^5 KJ^+CD4^+ T cells in the MLNs. The expression of CD45RB (Figure 5.9 b, d, f) indicated that the majority of the KJ^+CD4^+ T cells were $CD45RB^{lo}$ (90%). In contrast, mice which received KJ^+CD4^+ T cells but were not immunized with ap-OVA pep did not contain a convincing population of tg cells (Figure 5.10).

Initially the full implications of this experiment were not clear, and raised further questions. We wanted to determine whether immunizing KJ^+CD4^+ T cells within an immune competent environment (normal mouse) would result in the production of a long lived memory T cell population. We did find a small population of KJ^+CD4^+ T cells 10 weeks after transfer (Figure 5.9) that were not present in the absence of immunization (Figure 5.10). It was not clear whether the tg T cells detected in the MLNs was a result of a small number of surviving T cells proliferating due to the secondary challenge or whether they represented a stable population of "memory" type cells. However the presence of these cells did not confer an advantage on the recipient in providing help for a secondary response, control mice which were immunized with peptide but received no KJ^+CD4^+ T cells produced a comparable secondary response (Figure 5.8). Therefore immunization with the OVA peptide generated a population of host derived memory cells which were able to help a secondary response by day 5. In contrast control mice which were not immunized did not respond till day 14. The antibody detected on day 14 in these mice was most likely a primary response elicited by the sol OVA challenge.

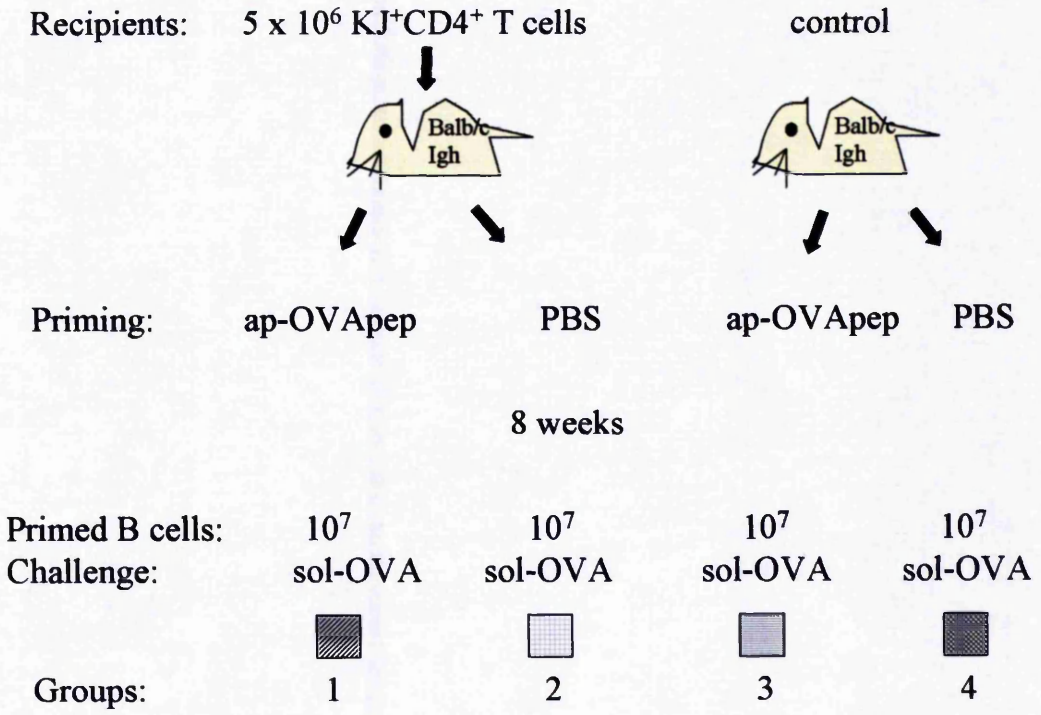


Figure 5.7. Experimental protocol to assess whether priming of KJ^+CD4^+ T cells in BALB/cIgH recipients would result in a long lived "memory" population.

Mice received 100μg ap-OVApep and 5 x 10^6 KJ^+CD4^+ T cells (Group 1), PBS and 5 x 10^6 KJ^+CD4^+ (Group 2), 100μg ap-OVApep (Group 3), and PBS (Group 4). Mice were left for 8 weeks before receiving 10^7 OVA primed B cells and challenge i.p. with 10μg sol-OVA.

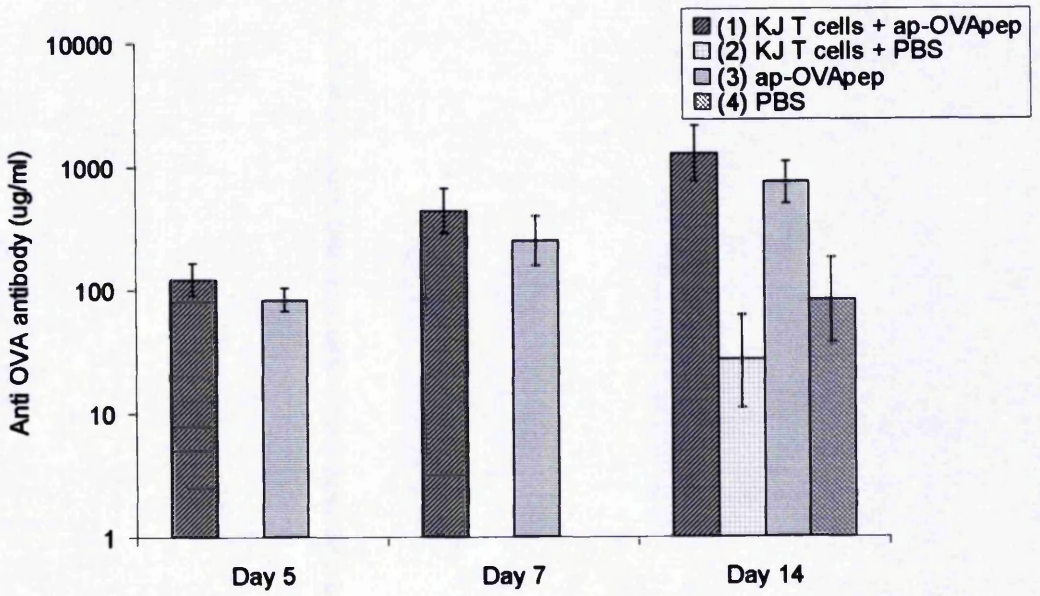


Figure 5.8. Results from priming KJ^+CD4^+ T cells in BALB/cIgh recipients.

Anti OVA antibody levels from mice that received 100µg ap-OVApep and 5 x 10^6 KJ^+CD4^+ T cells (Group 1), PBS and 5 x 10^6 KJ^+CD4^+ (Group 2), 100µg ap-OVApep (Group 3), and PBS (Group 4). Mice were left for 8 weeks before receiving 10^7 OVA primed B cells and challenge i.p. with 10µg sol-OVA. The mean values of 6 recipients per group are shown +/- SD.

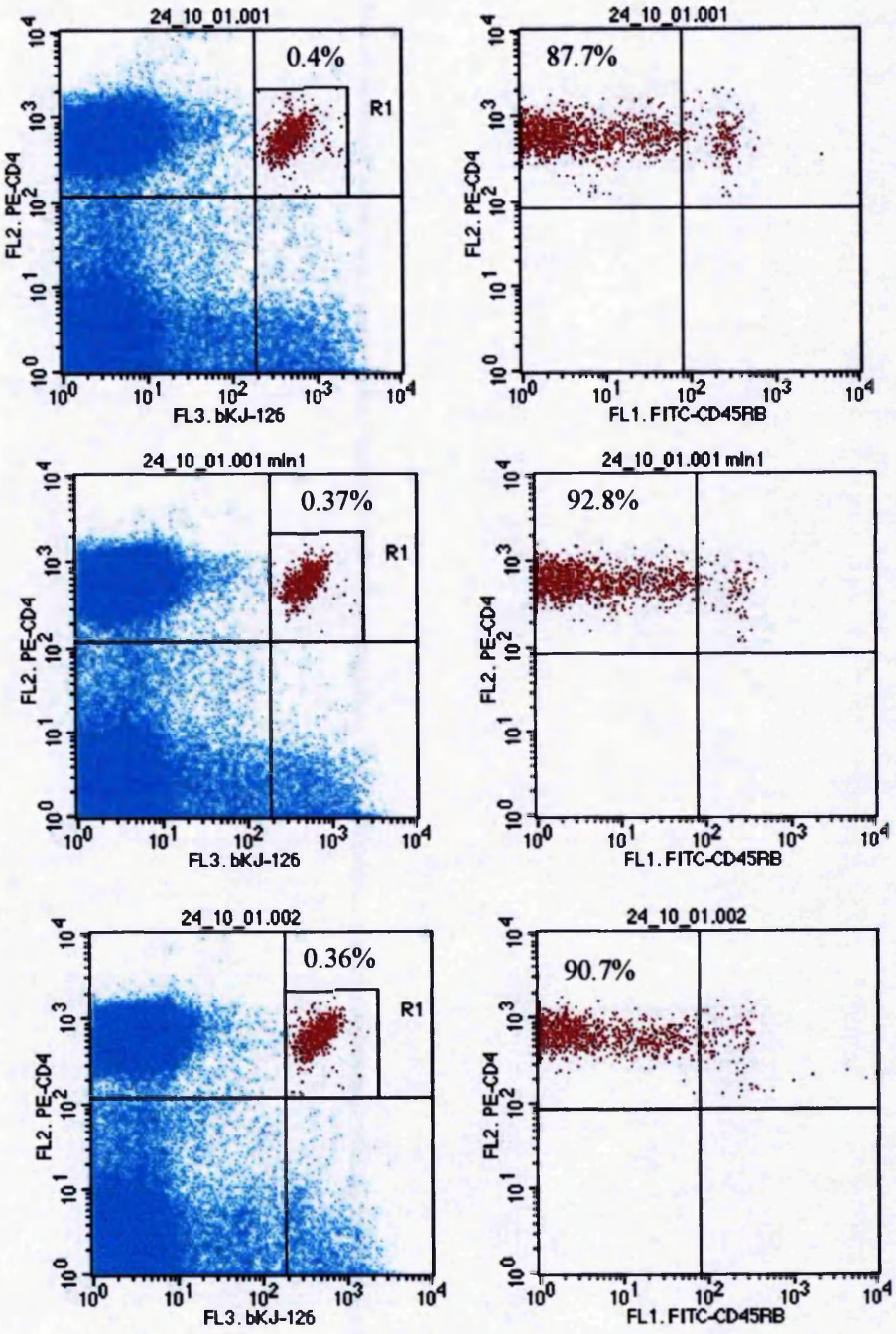


Figure 5.9. Flow cytometric analysis of KJ^+CD4^+ T cells in immunized BALB/cIgh recipients.

Flow cytometric analysis of MLNs from 3 mice which received 5 x 10^6 KJ^+CD4^+ T cells and 100μg apOVA-pep i.p., followed 8 weeks later by 10^7 OVA primed B cells and 10μg sol-OVA i.p. Mice were killed for analysis 14 days after sol-OVA challenge.

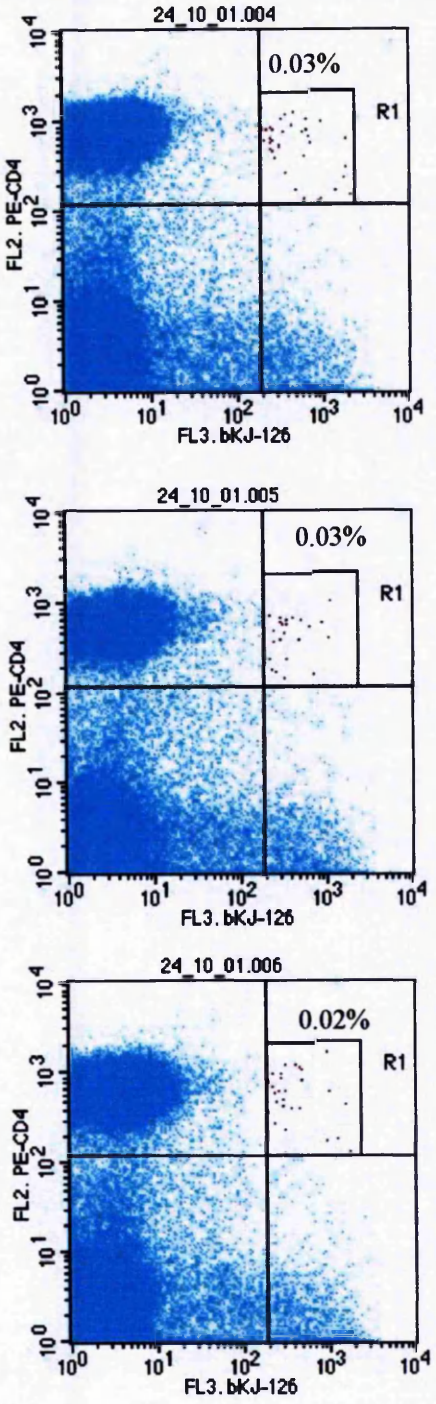


Figure 5.10. Flow cytometric analysis of KJ^+CD4^+ T cells in non-immunized BALB/cIgh recipients.

Flow cytometric analysis of MLNs from 3 mice which received 5 x 10^6 KJ^+CD4^+ T cells and PBS, followed 8 weeks later by 10^7 OVA primed B cells and 10μg sol-OVA i.p. Mice were killed for analysis 14 days after sol-OVA challenge.

5.2.4 The effect of antigen on survival and functionality of primed KJ^+CD4^+ T cells.

As KJ^+CD4^+ T cells only survived in mice that had been immunized with OVA peptide, we wanted to examine further whether it was the antigen priming which created a population of long lived cells, or whether it was a continual stimulation by residual antigen which maintained the population of tg T cells. To try and answer this question we changed the experimental protocol as shown in Figure 46. In this experiment the KJ^+CD4^+ T cells were firstly primed in, and recovered from, intermediate SCID recipients. These cells were then purified for OVA peptide primed $CD45RB^{lo}$ cells as described in Section 2.9 and shown in figure 5.4e, f. 10^6 $CD45RB^{lo}KJ^+CD4^+$ T cells were adoptively transferred into 2 groups of BALB/cIgh mice. One of these groups had been pre-immunized i.p. 2 weeks previously with 100μg ap-OVApep. 2 control groups were also set up which did not receive KJ^+CD4^+ T cells, one of which was pre-immunized with ap-OVApep. As we know that immunization with OVA peptide will stimulate the host T cells, this group would test whether primed tg T cells survived and provided an enhanced secondary response. The mice were left for 6 weeks before transferring OVA primed B cells and challenging with sol-OVA. The mice were bled on day 7 and 14 and the serum measured for anti OVA antibody.

The results of the antibody levels are shown in Figure 5.12. As expected the mice which were immunized with ap-OVApep (Group 1, 3) produced an early day 7 secondary anti OVA antibody response. Group 3 (which did not receive $CD45RB^{lo}KJ^+CD4^+$ T cells) had similar antibody levels as group 1 which received tg T cells (1148μg/ml and 777μg/ml respectively). Group 2 recipients, which received primed $CD45RB^{lo}KJ^+CD4^+$ T cells but were not immunized failed to produce a secondary memory response suggesting that none of the donor cells survived. Like the control group 4 it produced a minimal amount of antibody that did not appear until day 14.

When mice were examined for the presence of KJ^+CD4^+ T cells in MLNs none were detected (Figure 5.13). Spleen and ILN were also examined (data not shown) but no evidence of tg T cells was discovered. Apparently in either the absence or presence of antigen the primed KJ^+CD4^+ T cells did not survive. How memory T cells are generated if antigen priming results in complete cell death raises difficult questions. It would be reasonable to assume that even if undetectable numbers of KJ^+CD4^+ T cells

were present they would have proliferated upon the secondary challenge and therefore been identified, but this was not the case. Since KJ^+CD4^+ T cells were identified at a similar time point in the previous experiment (Figure 5.9), it must be considered what conditions favoured the survival of these cells. The crucial difference with the second experiment was that only OVA primed $CD45RB^{lo}$ T cells were transferred to the BALB/cIgh mice. In the first experiment a naïve population of cells was transferred and then immunized in situ. For whatever reason all of the KJ^+CD4^+ T cells may not have been stimulated as evidenced in Figure 5.4 d, which showed that following OVApep priming 35% of the KJ^+CD4^+ T cells remained $CD45RB^{hi}$. It may have been these unstimulated cells which survived and then became activated upon the secondary challenge. This would not however, explain why the naïve unstimulated KJ^+CD4^+ T cells did not survive in the mice which were not immunized (Figure 5.10).

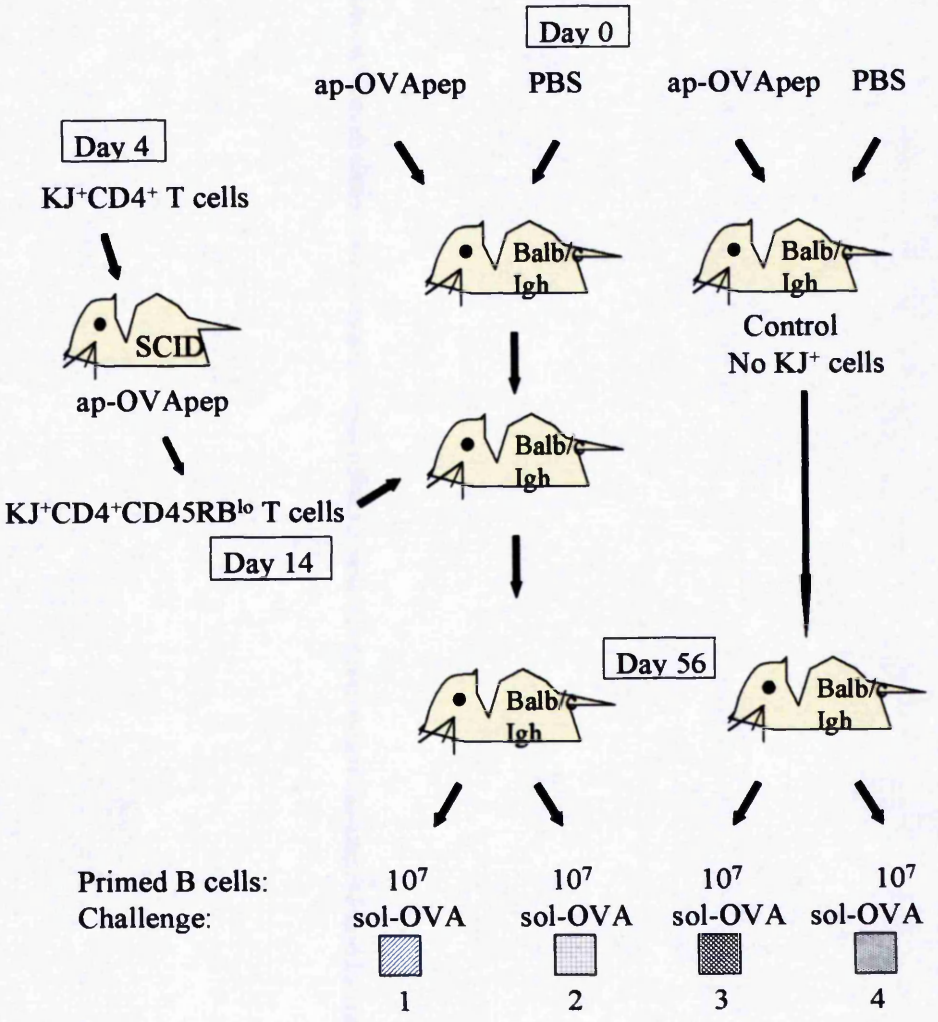


Figure 5.11. Experimental protocol to assess the effect of priming and persisting antigen on survival of KJ^+CD4^+ T cells.

Mice received 10^6 $CD45RB^{lo}KJ^+CD4^+$ T cells and 100µg ap-OVApep, 100µg ap-OVApep, 10^6 $CD45RB^{lo}KJ^+CD4^+$ T cells and PBS, and PBS. Mice were left for 6 weeks before adoptive transfer of 10^7 OVA primed B cells and challenge i.p. with 10µg sol-OVA

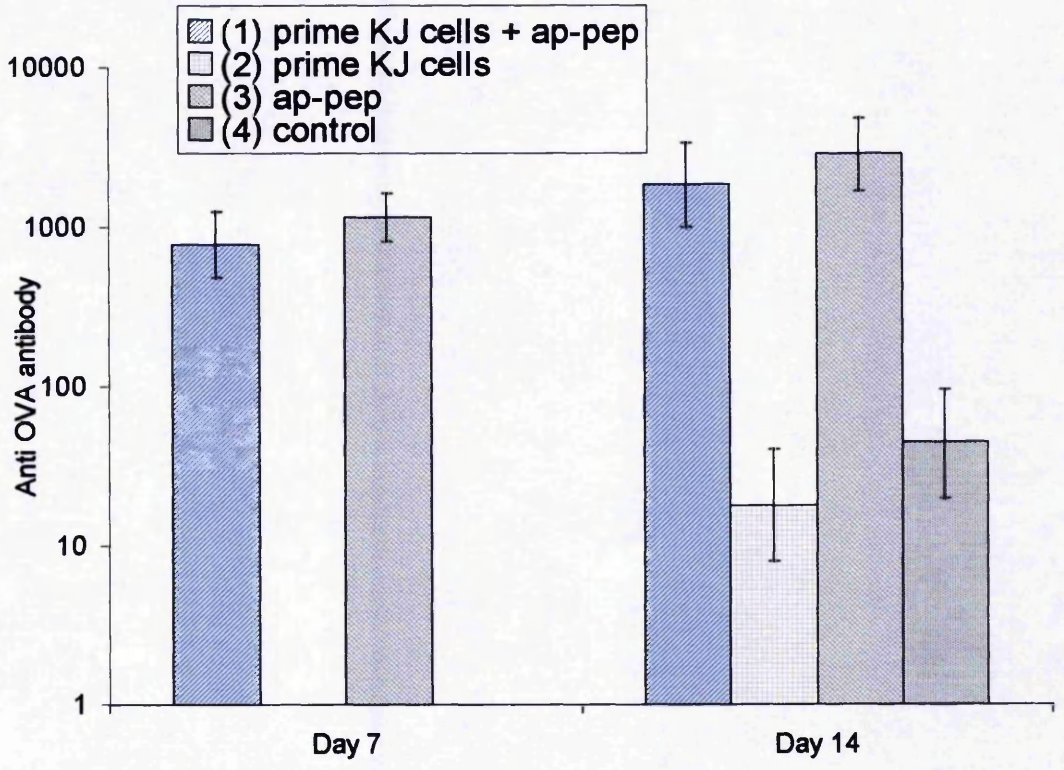
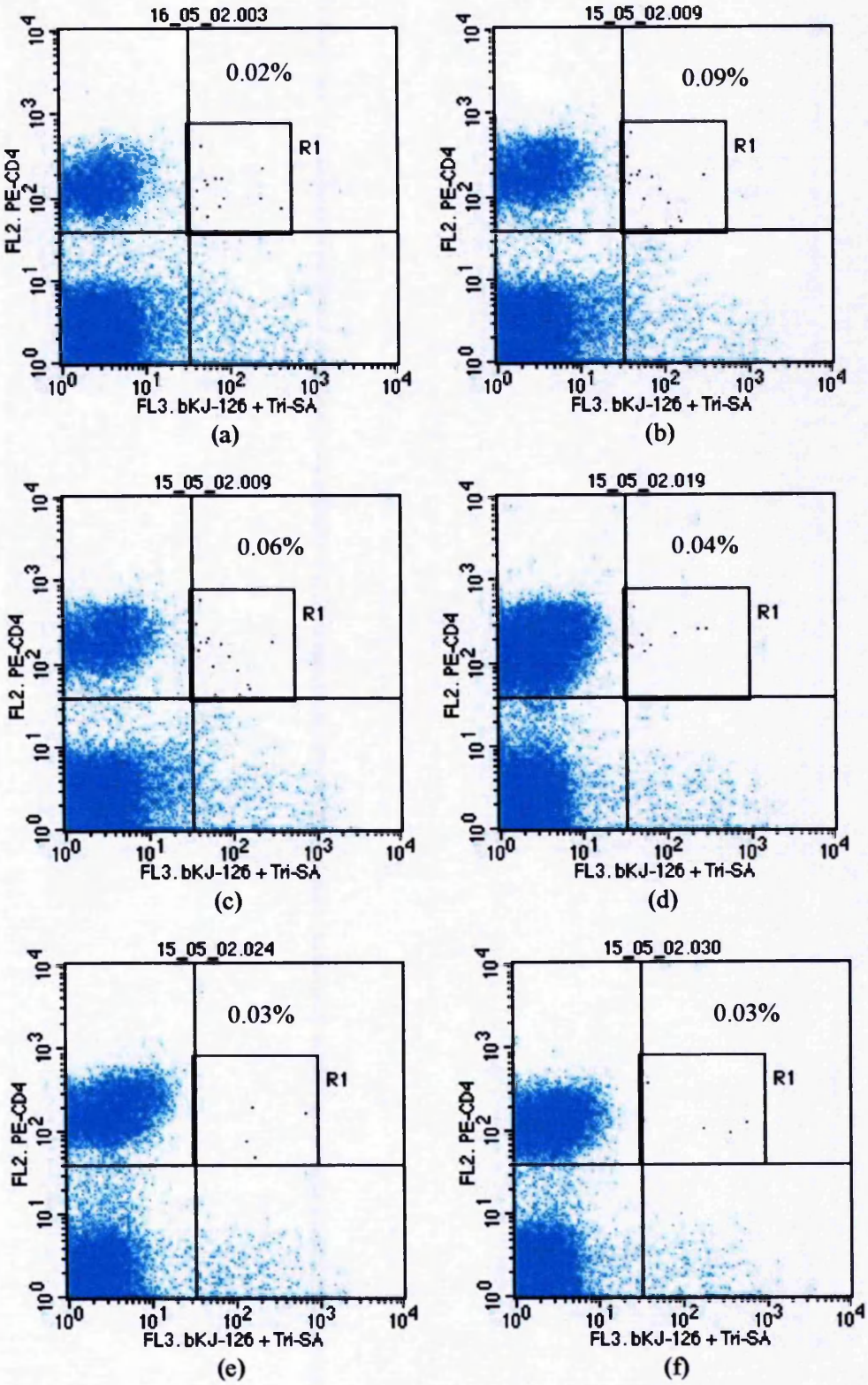


Figure 5.12. Results from transfer of primed KJ^+CD4^+ T cells to immunized and non-immunized recipients.

Anti OVA antibody levels from mice that received 10^6 $CD45RB^{lo}KJ^+CD4^+$ T cells and 100μg ap-OVApep, 100μg ap-OVApep, 10^6 $CD45RB^{lo}KJ^+CD4^+$ T cells and PBS, and PBS. Mice were left for 6 weeks before adoptive transfer of 10^7 OVA primed B cells and challenge i.p. with 10μg sol-OVA. The mean values of 6 recipients per group are shown +/- SD.

Figure 5.13. Primed KJ^+CD4^+ T cells did not survive in immunized or non-immunized Balb/cIgh recipients.

Flow cytometric analysis of MLNs from mice which received 100μg apOVA-pep (a, c, e) or 100μl PBS (b, d, f) by i.p. plus 10^6 $CD45RB^{lo}KJ^+CD4^+$ T cells, followed 6 weeks later by 10^7 OVA primed B cells and 10μg solOVA i.p.


16_05_02.003
0.02%
R1
FL2. PE-CD4
FL3. bKJ-126 + Tri-SA
(a)
15_05_02.009
0.09%
R1
FL2. PE-CD4
FL3. bKJ-126 + Tri-SA
(b)
15_05_02.009
0.06%
R1
FL2. PE-CD4
FL3. bKJ-126 + Tri-SA
(c)
15_05_02.019
0.04%
R1
FL2. PE-CD4
FL3. bKJ-126 + Tri-SA
(d)
15_05_02.024
0.03%
R1
FL2. PE-CD4
FL3. bKJ-126 + Tri-SA
(e)
15_05_02.030
0.03%
R1
FL2. PE-CD4
FL3. bKJ-126 + Tri-SA
(f)

5.2.5 Survival and phenotype of $CD45RB^{lo}KJ^{+}CD4^{+}$ T cells in SCID recipients in the absence of antigen.

The main aim of this project was to compare naïve T cells with long lived memory T cells that had previously experienced antigen and returned to a resting $CD45RB^{hi}$ state. This information would in turn help to assess whether frequency of antigen-specific cells was more important than previous antigen experience. Therefore, we needed to generate a population of antigen-experienced $KJ^{+}CD4^{+}CD45RB^{hi}$ memory T cells. Since the previous experiment demonstrated that antigen primed tg cells in normal BALB/cIgh mice failed to survive, we wanted to examine their behaviour in the lymphopenic environment of SCID mice. It was possible that the $KJ^{+}CD4^{+}$ T cells did not survive in the BALB/cIgh mice as they were outcompeted by the host T cells for survival signals such as cytokines. As SCID mice do not possess their own lymphocytes, competition for limited resources should not be a problem. Furthermore, as discussed in Section 5.10, lymphocytes will undergo homeostatic proliferation when transferred to a lymphopenic host. In practical terms this approach might generate a long lived population of antigen experienced T cells which could be tested for memory characteristics.

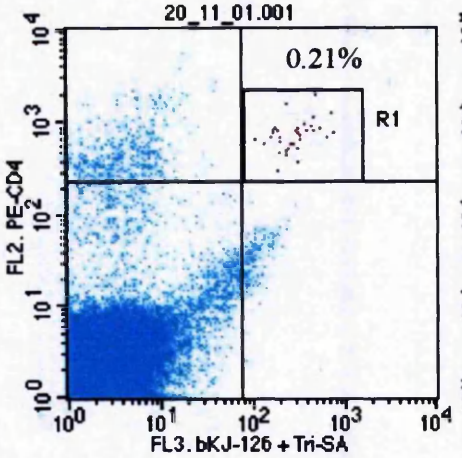
In the following experiments 8 x 10^6 $KJ^{+}CD4^{+}$ T cells (from DO11.10/SCID donors) were adoptively transferred to SCID recipients and immunized with 100μg ap-OVApep. These primary intermediate mice were left for 10 days, their spleens and LNs removed and a single cell population prepared. The resultant cell population was purified for $CD45RB^{lo}$ cells by $CD45RB^{hi}$ depletion, and 8 x 10^6 $CD45RB^{lo}KJ^{+}CD4^{+}$ T cells (as shown in Figure 5.4 e, f) adoptively transferred to secondary SCID recipients without antigen to allow $CD45RB^{lo}$ cells to revert to $CD45RB^{hi}$. Unexpectedly, when spleens, MLNs, and ILNs of these mice were examined 1 and 3 weeks later for the presence of $KJ^{+}CD4^{+}$ T cells (Figure 5.14 and 5.15) very few tg cells were detected at either of the time points. The percentages shown represent very few cells as the total cell counts of these SCID tissues is of the order of 10^6 for spleen and 10^5 for lymph nodes. Furthermore, there was no evidence that the primed $CD45RB^{lo}KJ^{+}CD4^{+}$ T cells had reverted to $CD45RB^{hi}$.

The experiment was repeated and included a control group of mice which received naïve $KJ^{+}CD4^{+}$ T cells. In this experiment the auxiliary lymph node (ALN) was examined instead of the ILN as it contained a slightly larger cell population. Figure

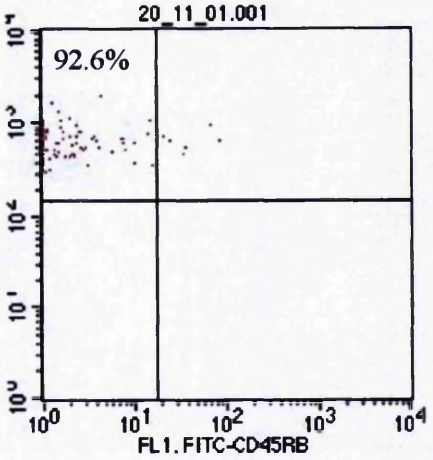
5.16 shows the spleen, MLN, and ALN from week 1 following transfer. A population of KJ^+CD4^+ T cells was clearly visible in the mice which received naïve cells (Figure 5.16 a, c, d), in contrast to mice which received primed cells where only a small population of tg cells was visible in the spleen (Figure 5.16 b, d, f). By 4 weeks after transfer, the naïve KJ^+CD4^+ T cells had increased slightly while the primed KJ^+CD4^+ T cells were still at minimal levels (Figure 5.17). Although the MLN and ALN contained a significant percentage of primed tg cells (1.68% and 2.35% respectively) the LNs were tiny. Since the absolute numbers of tg T cells in SCID lymph nodes was calculated to be 10^4 and 10^5, these percentages represent only a few hundred or thousand cells at the very most as summarized in Table 5.2. Figures 5.18 shows the mice from week 8 and at this time point very few KJ^+CD4^+ T cells were evident in recipients which received primed $CD45RB^{lo}$ T cells. At each time point there was only one mouse in each group, therefore it was difficult to draw any conclusions about the kinetics of the loss of cells.

Figure 5.14. Primed KJ^+CD4^+ T cells did not survive 1 week following transfer to SCID recipients.

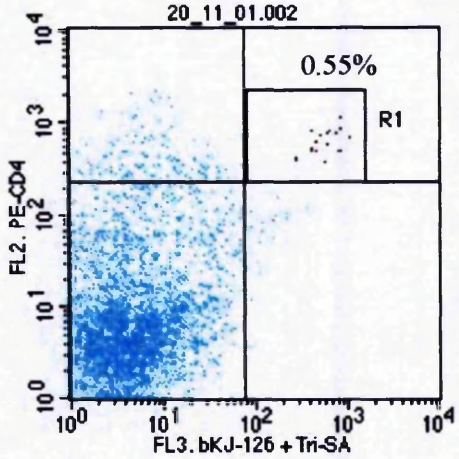
Representative flow cytometric analysis of SCID spleen (a), MLN (e), and ILN (c) 1 week after adoptive transfer of 8 x 10^6 $CD45RB^{lo}KJ^+CD4^+$ T cells. The tg cells are gated in R1 and their CD45RB expression is shown in (b) spleen, (d) MLN, and (f) ILN. The percentages shown indicate the proportion of cells within the quadrant.



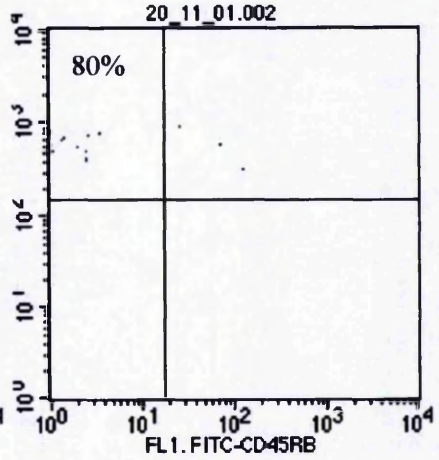
(a) spleen



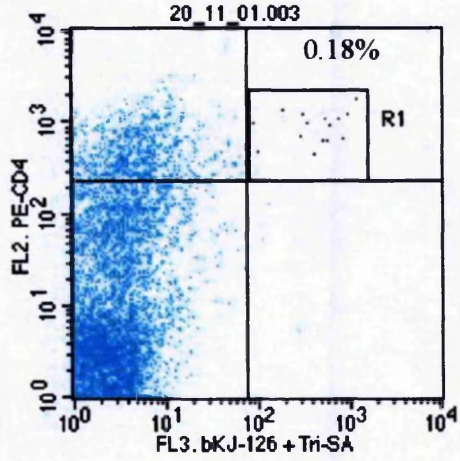
(b) spleen



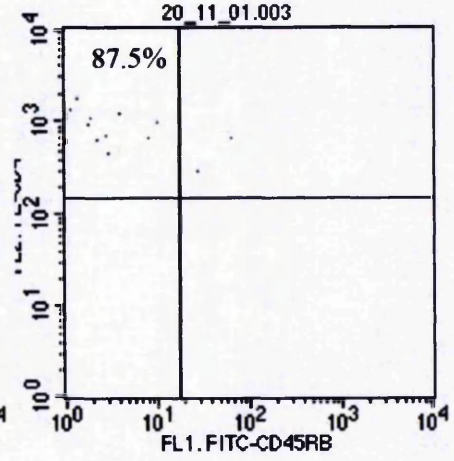
(c) MLN



(d) MLN



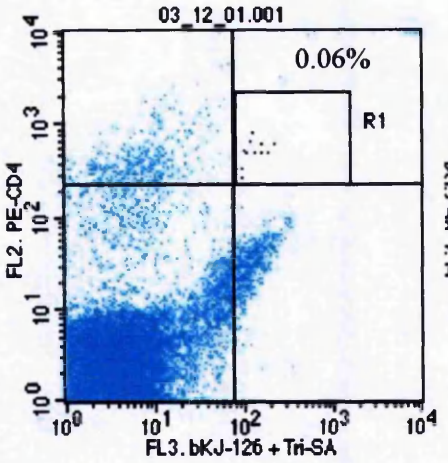
(e) ILN



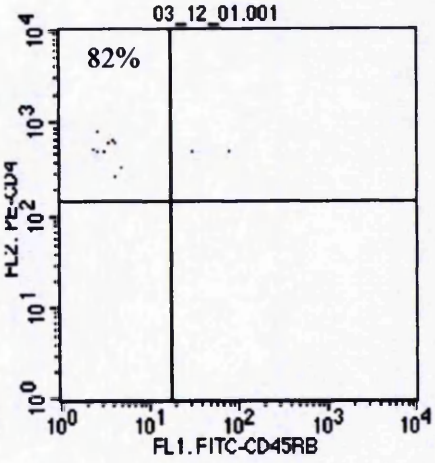
(f) ILN

Figure 5.15 Primed KJ^+CD4^+ T cells did not survive 3 weeks following transfer to SCID recipients.

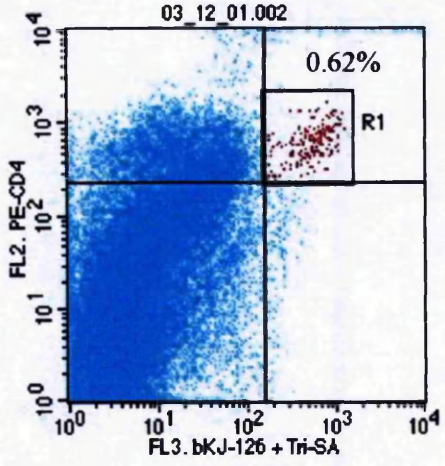
Representative flow cytometric analysis of SCID spleen (a), MLN (e), and ILN (c) 3 weeks after adoptive transfer of 8 x 10^6 $CD45RB^{lo}KJ^+CD4^+$ T cells. The tg cells are gated in R1 and their CD45RB expression is shown in (b) spleen, (d) MLN, and (f) ILN. The percentages shown indicate the proportion of cells within the quadrant.



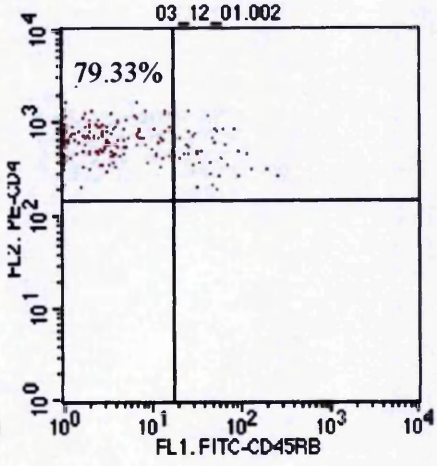
(a) spleen



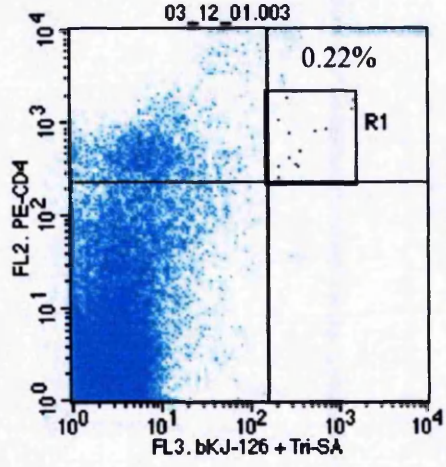
(b) spleen



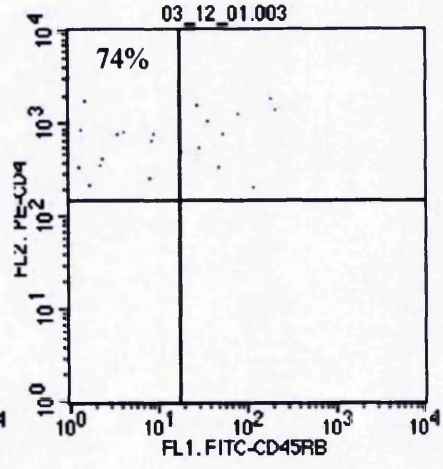
(c) MLN



(d) MLN



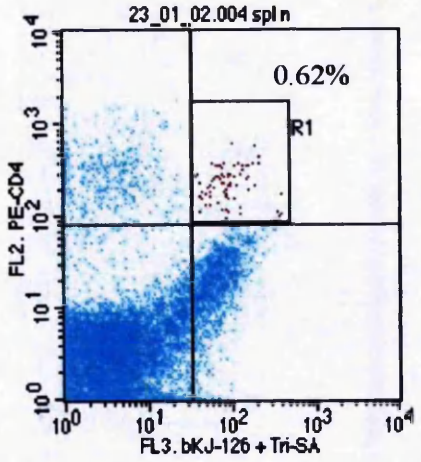
(e) ILN



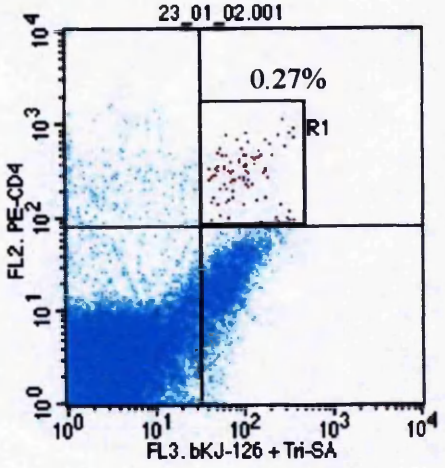
(f) ILN

Figure 5.16. Comparison of survival of naïve and primed KJ^+CD4^+ T cells 1 week following transfer to SCID recipients.

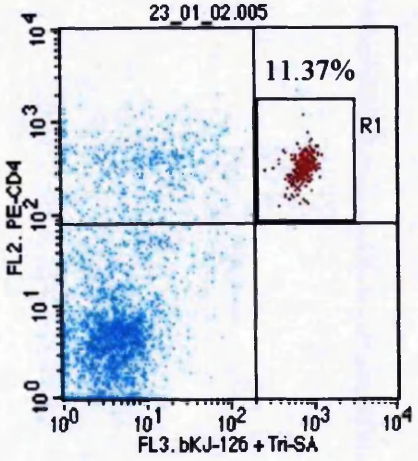
Flow cytometric analysis of spleen, MLN, and ALN from SCID recipients which received 5 x 10^6 naïve $CD45RB^{hi}KJ^+CD4^+$ (a, c, e) or OVApep primed $CD45RB^{lo}KJ^+CD4^+$ (b, d, f) T cells 1 week previously. Percentage of cells is shown in upper right quadrant.



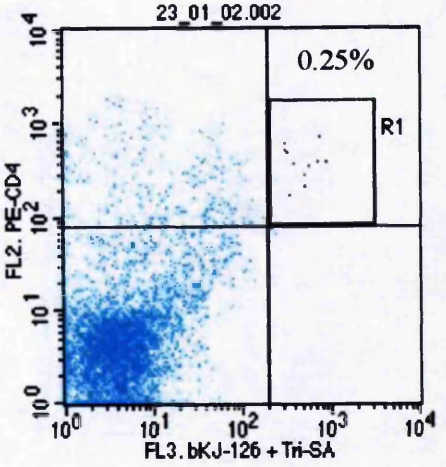
(a) spleen



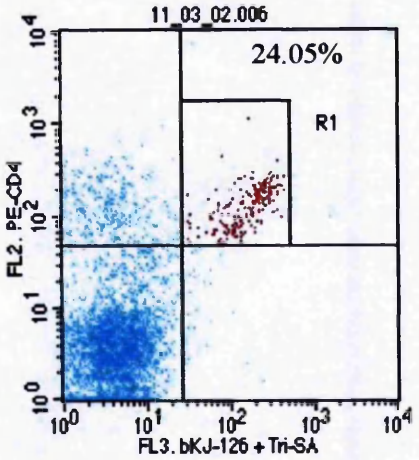
(b) spleen



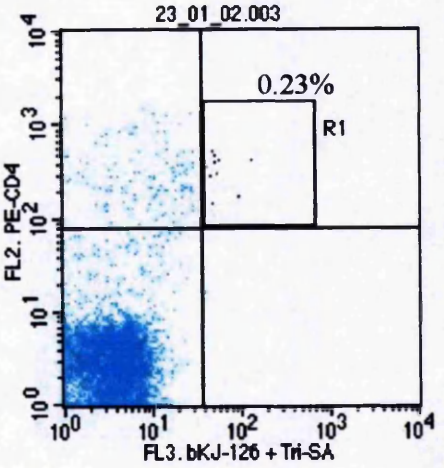
(c) MLN



(d) MLN



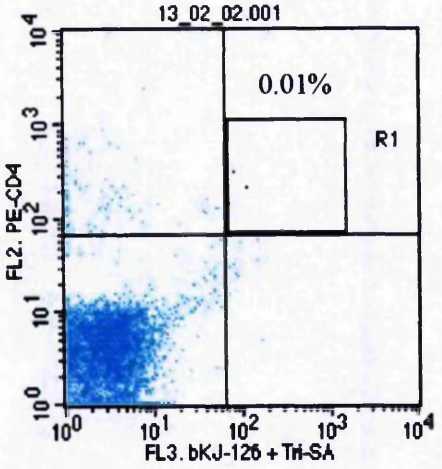
(e) ALN



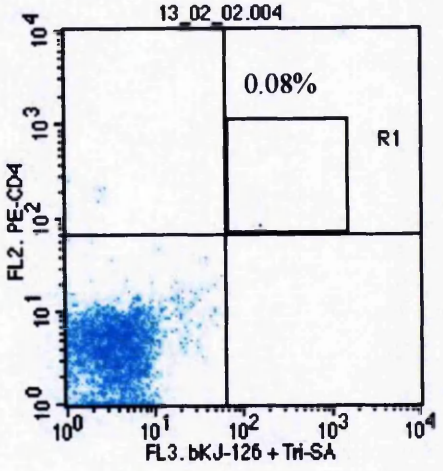
(f) ALN

Figure 5.17. Comparison of survival of naïve and primed KJ^+CD4^+ T cells 4 weeks following transfer to SCID recipients.

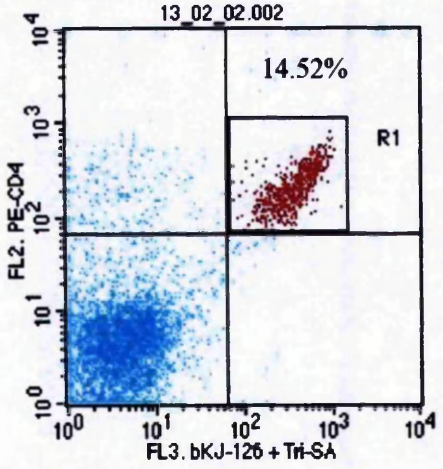
Flow cytometric analysis of spleen, MLN, and ALN from SCID recipients which received 5 x 10^6 naïve $CD45RB^{hi}KJ^+CD4^+$ (a, c, e) or OVApep primed $CD45RB^{lo}KJ^+CD4^+$ (b, d, f) T cells 4 weeks previously. Percentage of cells is shown in upper right quadrant.



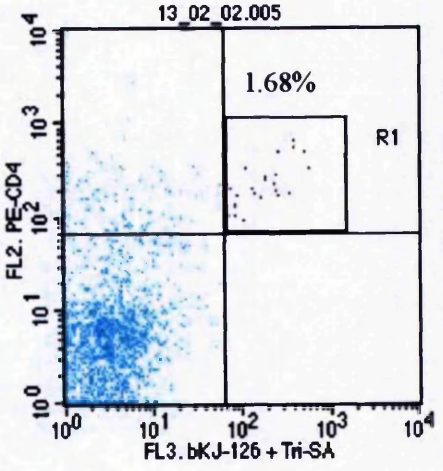
(a) spleen



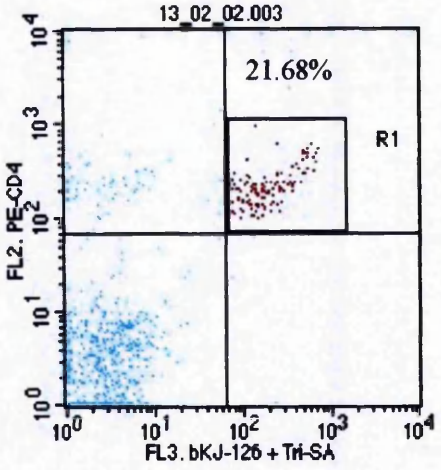
(b) spleen



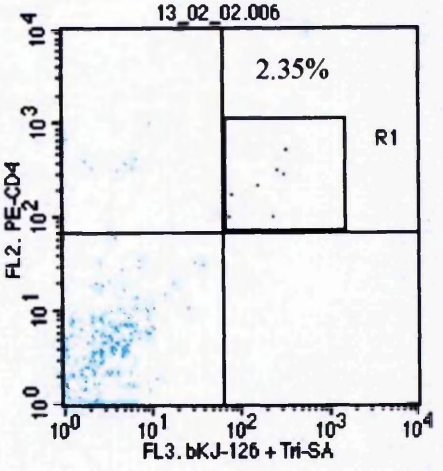
(c) MLN



(d) MLN



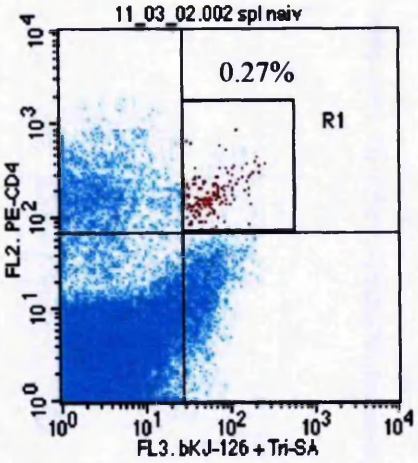
(e) ALN



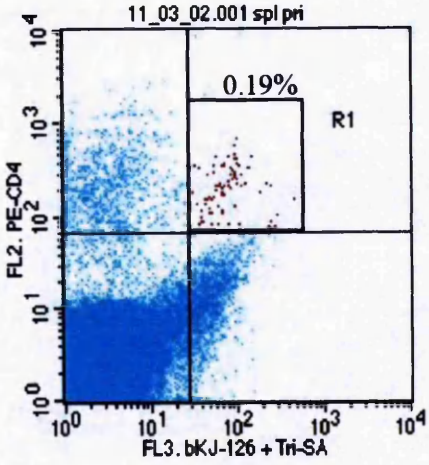
(f) ALN

Figure 5.18 Comparison of survival of naïve and primed KJ^+CD4^+ T cells 8 weeks following transfer to SCID recipients.

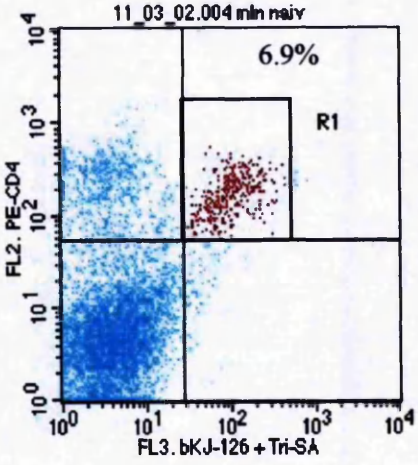
Flow cytometric analysis of spleen, MLN, and ILN from SCID recipients which received 5 x 10^6 naïve $CD45RB^{hi}KJ^+CD4^+$ (a, c, e) or OVApep primed $CD45RB^{lo}KJ^+CD4^+$ (b, d, f) T cells 8 weeks previously. Percentage of cells is shown in upper right quadrant.



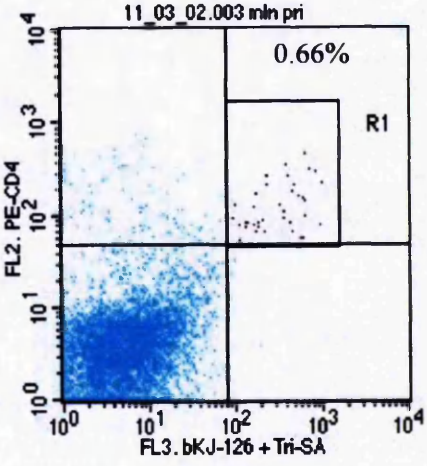
(a) spleen



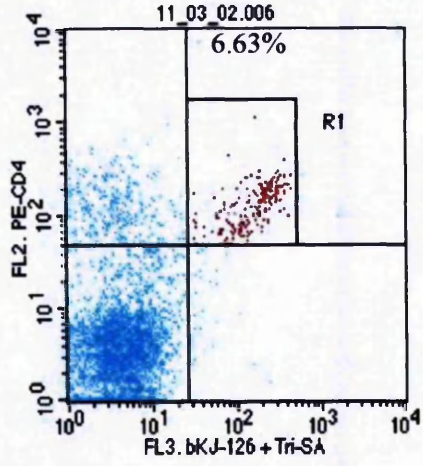
(a) Spleen



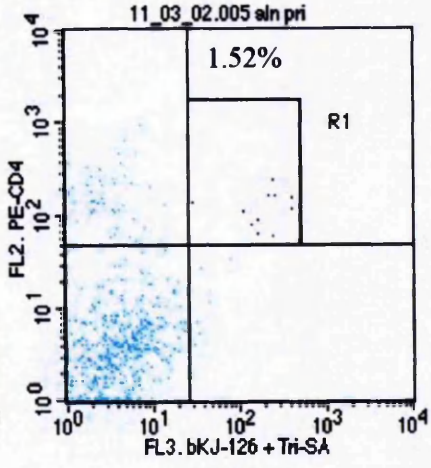
(c) MLN



(d) MLN



(e) ALN



(f) ALN

Time	Group	Tissue	Cell count	%KJ^+CD4^+ cells	Total number KJ^+CD4^+ cells
Week 1	Control	Spleen	1.34×10^7	0.62%	8.308×10^4
		MLN	2.44×10^5	11.37%	2.774×10^4
		ALN	2.7×10^5	24.05%	6.4935×10^4
	Primed	Spleen	1.84×10^7	0.27%	5.049×10^4
		MLN	5.54×10^5	0.25%	1.385×10^3
		ALN	1.84×10^5	0.23%	4.232×10^2
Week 4	Control	Spleen	1.54×10^7	0.01%	1.54×10^3
		MLN	7.34×10^5	14.52%	1.0657×10^5
		ALN	2.54×10^5	21.68%	5.506×10^4
	Primed	Spleen	1.21×10^7	0.08%	9.68×10^3
		MLN	6.32×10^5	1.68%	1.061×10^4
		ALN	3.1×10^5	2.35%	7.285×10^3
Week 8	Control	Spleen	1.49×10^7	0.27%	4.023×10^4
		MLN	2.4×10^5	6.9%	1.656×10^4
		ALN	2.14×10^5	6.63%	1.418×10^4
	Primed	Spleen	1.04×10^7	0.19%	1.976×10^4
		MLN	3.84×10^5	0.66%	2.534×10^3
		ALN	2.24×10^5	1.52%	3.408×10^3

Table 5.2 Survival of naïve and primed KJ^+CD4^+ T cells.

Summary of the number and percentage of tg cells in SCID recipients after the transfer of either 5×10^6 $CD45RB^{hi}KJ^+CD4^+$ T cells (Control) or 5×10^6 $CD45RB^{lo}KJ^+CD4^+$ T cells (Primed).

Chapter 6

Study of shortened OVA peptides.

6.1 Introduction

In the previous chapter it was shown that $KJ^{+}CD4^{+}$ T cells following stimulation with OVA peptide 323-339 failed to give rise to a population of long lived memory T cells in either lymphopenic or normal hosts. It has also been observed that a proportion of the tg T cells stain positively for early signs of apoptosis as soon as they begin to divide after peptide challenge (E. Bell personal communication). The reasons for this abnormal behaviour are not clear. One possibility is that the tg T cells possess high affinity for the OVA peptide and that this results in a failure of the cell to either switch an anti-apoptotic pathway on or an apoptotic pathway off. This idea will be investigated in this Chapter.

6.1.1 Shaping the T cell repertoire.

During its life, a T cell is constantly subjected to checks and controls. Beginning in the thymus, developing T cells (thymocytes) firstly undergo positive selection, which only allows the survival of T cells whose TCRs recognize self-MHC molecules. This is followed by negative selection that eliminates T cells which react too strongly with self-MHC or with self-MHC plus self-peptides. These processes generate a primary T cell repertoire that is self-tolerant. As thymic output continues for much of early life and can average 1% of the peripheral pool each day (Scollay *et al.*, 1980), mechanisms must exist that control the number of peripheral T cells. This occurs through complex homeostatic mechanisms which cause T cell death either actively or passively. T cell death may occur passively through loss of contact with peptide/MHC (Kirberg *et al.*, 1997) (Takeda *et al.*, 1996) or loss of survival signals by certain γ-chain cytokines (Marrack *et al.*, 2000). Active apoptosis requires TCR stimulation, and involves death molecules and cytokines such as FasL and TNF (Dhein *et al.*, 1995) (Zheng *et al.*, 1995). As a result of these two types of apoptosis, T cells are eliminated following an immune response. However, a fraction of cycling T cells may escape both active and passive apoptosis and become a long-lived pool of memory T cells. As discussed earlier the mechanisms underlying this survival are poorly understood.

As the fate of a developing T cell is determined by its sensitivity for peptide antigens encountered in the thymus, it stands to reason that this form of selection may continue in the periphery. A single antigenic epitope has the potential to stimulate a heterogeneous population of T cells that express diverse TCRs and respond with a wide range of antigen sensitivities. However it has not been established how the affinity of the TCR for its specific peptide influences the fate of the T cell. Nevertheless a recent study has provided evidence for an avidity-based model of peripheral T cell clonal expansion in response to antigenic challenge (Anderton *et al.*, 2001). The encephalitogenic, H-2 A^u-restricted, acetylated NH_2-terminal nonameric peptide (Ac1-9) epitope from myelin basic protein was used as the model antigen (Anderton *et al.*, 2001). This peptide forms highly unstable complexes with A^u so that a binding affinity could not be determined (Mason *et al.*, 1995) (Fugger *et al.*, 1996). However immunization with the Ac1-9 peptide or intact myelin activates Ac1-9 reactive T cells and induces experimental autoimmune encephalomyelitis (EAE) (Zanvil *et al.*, 1986) (Wraith *et al.*, 1989). It was noticed that the Ac1-9 peptide binds A^u through its interaction of residues 4Lys and 5Arg with the peptide binding groove (Wraith *et al.*, 1989) (Wraith *et al.*, 1992) (Gautam *et al.*, 1994), and that altering 4Lys to a more hydrophobic residue greatly increased the affinity for A^u (Fugger *et al.*, 1996) (Wraith *et al.*, 1989) (Pearson *et al.*, 1999). Using this knowledge they created a series of peptide analogues which displayed a hierarchy of affinities for A^u: Ac1-9 (4Tyr) > Ac1-9 (4Val) > Ac1-9 (4Ala) >> Ac1-9 (4Lys) (Anderton *et al.*, 2001) (Anderton *et al.*, 1998).

6.1.2 Different responses from Ac1-9 peptide analogues.

Competitive binding assays showed that the 4Tyr peptide bound to A^u MHC class II molecules at a strength 5 log higher than the wild type 4Lys peptide (Anderton *et al.*, 2001). The 4Val peptide showed slightly weaker binding than 4Tyr, where as the 4Ala peptide showed intermediate binding (Anderton *et al.*, 2001). These differences in MHC binding translated directly into antigenic activity *in vitro* (Anderton *et al.*, 2001). When cultured with three different T cell lines (TCLs) the three analogue peptides all showed "superagonist" activity inducing responses at femtomolar concentrations (compared with nanomolar for the wild type peptide) (Anderton *et al.*, 2001). Because of these results it was expected that these superagonist peptides would be powerful

inducers of EAE *in vivo*. However this was not the case. Although immunization with the wild-type (wt) 4Lys peptide induced EAE in 60% of mice, immunization with the intermediate 4Ala analogue rarely induced disease (10%), whereas the 4Val and 4Tyr superagonists did not induce EAE at all (Anderton *et al.*, 2001). This suggested that increasing antigenic strength *in vivo* reduced the imunogenicity of the peptides which correlated with a reduced activation of pathogenic T cells (Anderton *et al.*, 2001).

In further experiments where TCLs specific for each peptide were created, it was observed that TCLs primed against superagonists were far less sensitive to stimulation with the wild-type peptide (Anderton *et al.*, 2001). This suggested that the superagonist peptides failed to induce EAE *in vivo* because they were unable to expand T cells of sufficient sensitivity to respond to wild type Ac1-9 in the central nervous system. A suggested explanation for the insensitivity of superagonist-primed T cells to the wt 4Lys peptide was that they recognized distinct amino acids as TCR contact residues. However, the results from measuring the response profiles of TCLs to panels of peptide analogues containing Ala substitutions at individual peptide residues, indicated that there was no significant qualitative deviation of TCR recognition caused by alterations at position 4 of the peptide (all TCLs tested focused on position 3 and 6 as TCR contacts) (Anderton *et al.*, 2001). Evidence of tuning of the cell lines *in vivo* was also observed. This appeared to be a result of the selective expansion of distinct repertoires of T cells in response to the different peptides, rather than biochemical detuning of the signalling machinery within a homogeneous Ac1-9-specific repertoire (Anderton *et al.*, 2001). This was deduced from the observation that no differences in antigen receptor expression levels between the different panels of hybridomas occurred (Anderton *et al.*, 2001). Also T cells which respond to wt Ac1-9 have been shown to predominantly express TCRs using Vβ8 variable region gene products (Acha-Orbea *et al.*, 1998). In contrast, the superagonist-primed hybridomas did not use Vβ8 (Anderton *et al.*, 2001).

6.1.3 An avidity model of T cell clonal expansion.

Based on the above results, the authors (Anderton *et al.*, 2001) proposed an avidity model of T cell clonal expansion in which those cells with sensitivity to antigenic challenge above a certain threshold are purged from the productive immune response. Irrespective of the signal given by the priming antigen, the resulting immune

response will be tuned to fall within a window of sensitivity by selecting distinct repertoires of T cells for expansion (Anderton *et al.*, 2001). To test this theory the fate of highly sensitive Ac1-9 reactive T cells primed with the high affinity 4Tyr analogue was examined *in vivo*. The T cells were labelled with CFSE, transferred to nontransgenic recipients, and immunized with either 4Lys peptide, 4Tyr peptide, or adjuvant (CFA) alone (Anderton *et al.*, 2001). In recipients which did not receive antigen $CFSE^+$ T cells were not detected (Anderton *et al.*, 2001). In contrast, in recipients primed with either peptide plus CFA, $CFSE^+$ T cells were identified in the draining popliteal and inguinal lymph nodes (Anderton *et al.*, 2001). The level of CFSE fluorescence was low indicating that three to five rounds of cell division had occurred (Anderton *et al.*, 2001). The superagonist 4Tyr peptide did not appear to cause a more rapid division of the transferred cells (Anderton *et al.*, 2001). However when the cells were stained with annexin V and 7-AAD, greater than 70% of the $CFSE^+$ cells from 4Tyr peptide primed mice showed signs of early apoptosis compared with less than 10% after priming with the wt 4Lys peptide (Anderton *et al.*, 2001). This showed that Ac1-9 sensitive T cells were deleted after exposure to the 4Tyr superagonist *in vivo* (Anderton *et al.*, 2001).

The authors next examined the affinity of cells following priming with either the 4Lys or 4Tyr peptide. This was made possible by the creation of soluble MHC class II-peptide complexes (Savage *et al.*, 1999) (Crawford *et al.*, 1998) (Rees *et al.*, 1999). TCLs generated against 4Lys or 4Tyr peptide and stained with tetrameric complexes of A^u-Ac1-11(4Tyr) (Radu *et al.*, 2000) revealed 4Lys TCL with a broad staining pattern indicating a heterogeneous population using a range of TCRs with varying affinities for the tetrameric complex (Anderton *et al.*, 2001). In contrast the 4Tyr TCL stained weakly in a single peak, indicating a more homogenous population expressing only TCRs of low affinity (Anderton *et al.*, 2001). T cells from the tg mouse Tg4 (tg for a Vα4/Vβ8.2 TCR specific for the Ac1-9 peptide) (Liu *et al.*, 1995) exhibited a homogenous population of intermediate affinity when stained with the tetramer (Anderton *et al.*, 2001). The 4Tyr TCL also showed a more rapid loss of tetramer staining than either 4Lys TCL or Tg4 TCL (Anderton *et al.*, 2001).

6.1.4 A potential model for DO11.10 T cells.

It has been shown that altering the strength of the antigenic signal by manipulation of the peptide sequence, can vary the affinity for MHC class II and hence immunogenicity (Anderton *et al.*, 2001). When T cells bearing high affinity TCRs received a powerful antigenic stimulus *in vivo* they rapidly progressed to the contraction phase of the response and were removed by apoptosis (Anderton *et al.*, 2001). In contrast, the T cells which expanded after priming with superagonist expressed lower affinity TCRs (Anderton *et al.*, 2001). When tetrameric 4Tyr-A^u complexes were used to assess TCR affinity following immunization with the wt 4Lys peptide, a wide range of affinities from low to high was observed in the responding T cells (Anderton *et al.*, 2001). Interestingly the transgenic Tg4.TCL revealed a homogenous population with a fixed affinity of intermediate strength (Anderton *et al.*, 2001). However this Tg4.TCL was not examined for early signs of apoptosis following antigen priming.

So, based on this avidity model, if a tg cell population shares the same affinity for a specific peptide, and the affinity is of a high strength, following priming all of the cells will progress to apoptosis. If this occurs, it would prevent the development of a long-lived antigen-experienced population. Does this reflect what was observed with the DO11.10 T cells? Taking this model a step forward, if the T cell affinity is determined by the strength of binding between MHC class II molecules and the peptide, then altering the peptide sequence to reduce the strength of binding should reduce the affinity of the T cell for the peptide. Will this in turn prevent all of the cells from undergoing apoptosis? In this chapter our aim is to alter the sequence of the OVA peptide 323-339, so that the affinity of the KJ^+CD4^+ T cells for this peptide is reduced to determine whether this will promote the survival of a population of long-lived antigen experienced cells.

6.1.5 Sequence of OVA peptide 323-339.

The OVA peptide 323-339 was first characterised in a study by Shimonkevitz *et al* (Shimonkevitz *et al.*, 1984), in which they examined antigen recognition by H-2 restricted T cells. Following high pressure liquid chromatography (HPLC) of trypsin-digested whole chicken Ovalbumin, the biological activity (as defined by the ability to stimulate IL-2 production by the T hybridoma 3DO-54.8) was restricted to a single

region corresponding to one of the OD_{214}-absorbing peptide peaks (Shimonkevitz *et al.*, 1984). The digested peptide was purified and subjected to amino acid composition analysis, and compared with the predicted amino acid sequence based on the published cDNA nucleotide sequence for cOVA (McReynolds *et al.*, 1978). This revealed a sequence corresponding to residues 323-339 of the published cOVA sequence (Shimonkevitz *et al.*, 1984). To test this sequence, an identical peptide was synthesised with the addition of a single tyrosyl residue attached to the C-terminal arginine, and it showed the same activity and dose response as the HPLC-purified tryptic peptide when used to stimulate 3DO-54.8 T cells (Shimonkevitz *et al.*, 1984). It was observed that the OVA 323-339 peptide was responsible for 25%-35% of the T cell response in BALB/c mice immunized with whole Ovalbumin (Shimonkevitz *et al.*, 1984). It was also shown that the OVA peptide was only recognized in association with $I\text{-}A^d$ (Shimonkevitz *et al.*, 1984).

6.1.6 Modification of the OVA peptide.

The authors modified the peptide and examined its ability to stimulate OVA specific TCLs (Shimonkevitz *et al.*, 1984). Using a staphylococcal V8 protease digest which caused a cleavage at glutamyl residues, two shortened peptides were created, a 14 residue peptide cleaved at position 336 (P323-336), and an 11 residue peptide cleaved at position 333 (P323-333) (Shimonkevitz *et al.*, 1984). Of the 4 T cell hybridomas tested with these peptides none were stimulated by the P323-333 peptide, while 3 of the four hybrids were stimulated by the P323-336. The DO11.10 cell line showed no reactivity to P323-336 at any dose (Shimonkevitz *et al.*, 1984). To test whether the P323-336 peptide formed a stable interaction with Ia molecules, B lymphoma cells were cultured with excess P323-336 peptide before been added to DO11.10 T cells and P323-339 peptide (Shimonkevitz *et al.*, 1984). 24 hours later IL-2 was detected in the culture indicating that the P323-339 peptide was presented to the T cells despite the competition from excess P323-336 peptide (Shimonkevitz *et al.*, 1984). This suggested that if the P323-336 peptide did bind to $I\text{-}A^d$, it was an unstable binding and of low affinity, or that stable binding could not occur without engagement of the T cell receptor (Shimonkevitz *et al.*, 1984).

6.1.7 Crystal Structure of I-A^d-OVA complex.

Since its initial characterisation, the OVA peptide 323-339 has been used in a variety of studies including the creation of the DO11.10 tg mouse strain (Murphy *et al.*, 1990). More recently the crystal structure of the OVA peptide covalently linked to an I-A^d molecule has been determined (Scott *et al.*, 1998). From this study two interesting features of the binding of the OVA peptide to the MHC molecule were revealed. Firstly the OVA peptide does not use large side-chain residues to anchor itself into the peptide-binding groove (Scott *et al.*, 1998). The key interaction appears to be the insertion of a valine side chain into the central P4 pocket of the MHC molecule (Scott *et al.*, 1998). Secondly, with one exception, all of the hydrogen bonds between the I-A^d and the peptide are to the peptide backbone, revealing an essentially sequence-independent H bond network (Scott *et al.*, 1998). Unlike I-E^k, the peptide-binding groove of I-A^d has only one moderately large pocket, P1, between the main-chain atoms of the peptide and the peptide-binding groove surface (Scott *et al.*, 1998). In addition it contains three smaller cavities corresponding to the P4, P6, and P9 pockets (Scott *et al.*, 1998). In the I-A^d-OVA complex, the large P1 pocket was revealed to be occupied only partially by Ser324, while the third largest pocket, the P9 pocket, was filled only partially by Ala332 (Scott *et al.*, 1998). Val327 sits firmly in the P4 pocket anchoring the peptide register in a strong hydrophobic interaction (Scott *et al.*, 1998). The Val327 and Ala332 residues were the two residues also shown to be the most important for I-A^d binding in a separate study by Grey and coworkers (Sette *et al.*, 1987). However full occupancy of the P4 pocket does not appear to be essential for peptide binding as mutation of the OVA residue Val327 to alanine did not significantly reduce the binding affinity for I-A^d (Sette *et al.*, 1989).

The last four residues of the $OVA_{323-339}$ peptide were not visible in the electron density map which suggested that they do not make any well defined contact with I-A^d (Scott *et al.*, 1998). This was supported by a study which showed that $OVA_{323-335}$ binds to I-A^d with an affinity equal to that of $OVA_{323-339}$ (Sette *et al.*, 1987). As these 4 residues appear not to be required for binding, the OVA peptide may be able to interact with I-A^d in a variety of ways. Therefore the register observed in the I-A^d-OVA complex should represent the most energetically favoured register adopted by the OVA peptide. However truncation of the OVA peptide at the N-terminal end has a more dramatic effect. $OVA_{327-339}$ binds with approximately one third of the affinity of

$OVA_{323-339}$ (Scott *et al.*, 1998). This causes either Val327 to bind in the P4 pocket leaving a large proportion of the binding groove empty, or $OVA_{327-339}$ to bind I-A^d in alternative registers by placing suboptimal residues in the P4 and P9 pockets (Scott *et al.*, 1998). Which of these is the case remains to be seen.

6.1.8 Identification of the epitope recognized by DO11.10 T cells.

It appeared that multiple epitopes within $OVA_{323-339}$ could be presented by I-A^d. To assess this idea a more specific approach was adopted where by amino- and carboxy-terminal truncations of $OVA_{323-339}$ were used to locate the approximate ends of the epitopes recognized by DO11.10 T cells, and single amino acid substitutions were used to identify critical TCR contact residues of the $OVA_{323-339}$ peptide (Robertson *et al.*, 2000). The removal of amino acids from the N-terminal end of the OVA peptide had little or no effect on the DO11.10 response until amino acids 327 and 328 were deleted (Robertson *et al.*, 2000). When these amino acids were removed a substantial decrease in DO11.10 proliferation was observed, which indicated that these amino acids are situated on the N-terminal edge of the DO11.10 T cell epitope (Robertson *et al.*, 2000). The response of OT-II T cells, another tg T cell specific for $OVA_{323-339}$, was similar to that of DO11.10 T cells (Robertson *et al.*, 2000). When truncations occurred on the C-terminal side of $OVA_{323-339}$ it was observed that amino acids 338 and 339 were not important for DO11.10 T cell proliferation, but amino acids 337 and 336 were required (Robertson *et al.*, 2000).

To determine the specific residues of $OVA_{323-339}$ responsible for DO11.10 T cell proliferation, a series of analogue peptides with an alanine (or serine if the original amino acid was an alanine) substituted at each position of $OVA_{323-339}$ was created and used to stimulate DO11.10 T cells *in vitro* (Robertson *et al.*, 2000). This approach revealed that proliferation of DO11.10 T cells was dependent on the conservation of amino acids 331, 333, and 335 (for OT-II T cells it was 331, 333, and 336) (Robertson *et al.*, 2000). Using this knowledge the authors then identified the primary TCR residue as defined functionally as that residue which will accept the fewest amino acid substitutions (Robertson *et al.*, 2000). Peptide analogues were created with each amino acid at positions 331, 333, and 335 and used to stimulate DO11.10 T cells (Robertson *et al.*, 2000). Only one very conservative substitution at 333 was accepted, 5 substitutions were accepted at amino acid 331, and the majority of substitutions were accepted at

residue 335 (Robertson *et al.*, 2000). The response for OT-II T cells was similar (Robertson *et al.*, 2000). These results suggested that the primary TCR contact residue for both DO11.10 and OT-II T cells is amino acid 333 with the core 9-aa epitope spanning peptide residues 329-337 (Robertson *et al.*, 2000).

However defining the epitope of $OVA_{323-339}$ did not appear to be so simple. When T cell clones from OVA primed BALB/c mice were generated, and the epitope which they recognized was examined by truncations and substitutions of the $OVA_{323-339}$ peptide, a different response was observed than that of DO11.10 or OT-II T cells (Robertson *et al.*, 2000). The results indicated that the $OVA_{323-339}$ peptide could bind using three different epitopes (Robertson *et al.*, 2000).

Peptide:MHC class II crystal structures, including the $OVA_{323-339}$:I-A^d structure, have shown MHC anchor residues to be at positions 1, 4, 6, and 9 of the core 9-aa epitope (Scott *et al.*, 1998) (Fremont *et al.*, 1996). As amino acid substitutions revealed residue 333 as the primary contact residues for the epitope recognized by DO11.10 T cells, it was predicted that amino acids 329, 332, 334, and 337 (positions 1, 4, 6, and 9) were the MHC anchor residues for the epitope recognized by DO11.10 T cells (Robertson *et al.*, 2000). As $OVA_{323-339}$ has been shown to possess three distinct T cell epitopes, peptide binding assays could not be used to analyze the position of MHC anchor residues. Instead all four proposed anchor residues were replaced with peptides whose affinities were known to have high or low MHC affinities. Peptides were made in which amino acids 329, 332, 334, and 337 were replaced with the corresponding anchor residues from the high affinity sperm whale myoglobulin (SWM) peptide 106-118 (England *et al.*, 1995) or the lower affinity MHC class II-associated invariant chain peptide (CLIP) 85-99 (Liang *et al.*, 1995). As expected the peptide containing the high-affinity SWM anchor residue did not affect DO11.10 T cell proliferation, where as the weaker affinity CLIP anchor resulted in 10-100 fold less T cell proliferation (Robertson *et al.*, 2000). While not conclusive proof, this does suggest that positions 1, 4, 6 and 9 corresponding to amino acids 329, 332, 334, 337 are the MHC anchor residues for the epitope recognized by DO11.10 T cells (Robertson *et al.*, 2000). However the importance of the residues outside of the core 9-aa epitope was also demonstrated. When position 327 was substituted with a biotinylated lysine residue, this resulted in a 10 fold decrease in DO11.10 T cell proliferation (Robertson *et al.*, 2000). The presence of a biotinylated amino acid blocked MHC binding. In contrast, the substitution of position 324 with a biotinylated amino acid resulted in a 10 fold increase in T cell

sensitivity as well as a stronger peak response (Robertson *et al.*, 2000). The reasons for this are not clear but it demonstrates that an alteration outside of the core 9-aa epitope and its flanking residues can affect the T cells response when multiple epitopes may be presented (Robertson *et al.*, 2000).

In summary it appeared that $OVA_{323-339}$ contains at least three distinct epitopes that may be presented to the T cell repertoire. The evidence suggested that DO11.10 T cells recognize the C-terminal epitope spanning amino acids 329-337. Residue 333 has been identified as the primary TCR contact with positions 329, 332, 334, and 337 acting as the MHC anchor residues. The importance of secondary TCR contact residues as well as peptide flanking residues is unclear. However it is clear that manipulation of either the peptide length or sequence can have a dramatic affect on the T cell response, potentially increasing or decreasing proliferation. The effect on T cell survival or apoptosis has yet to be examined.

Our aim in this Chapter was to alter the affinity of the DO11.10 T cells for the OVA peptide to determine if we could alter the fate of the T cell following peptide induced proliferation. As was demonstrated by Anderton *et al* (Anderton *et al.*, 2001), strongly antigenic peptides can induce cell death while those cells with lower affinity may survive. We hoped that by substituting residues in the epitope recognized by DO11.10 T cells with amino acids which allowed the peptide to bind more weakly, we could prolong the survival of the tg T cells. After all, CD4 T cell memory depends in some way on the perpetuation of antigen experienced T cells.

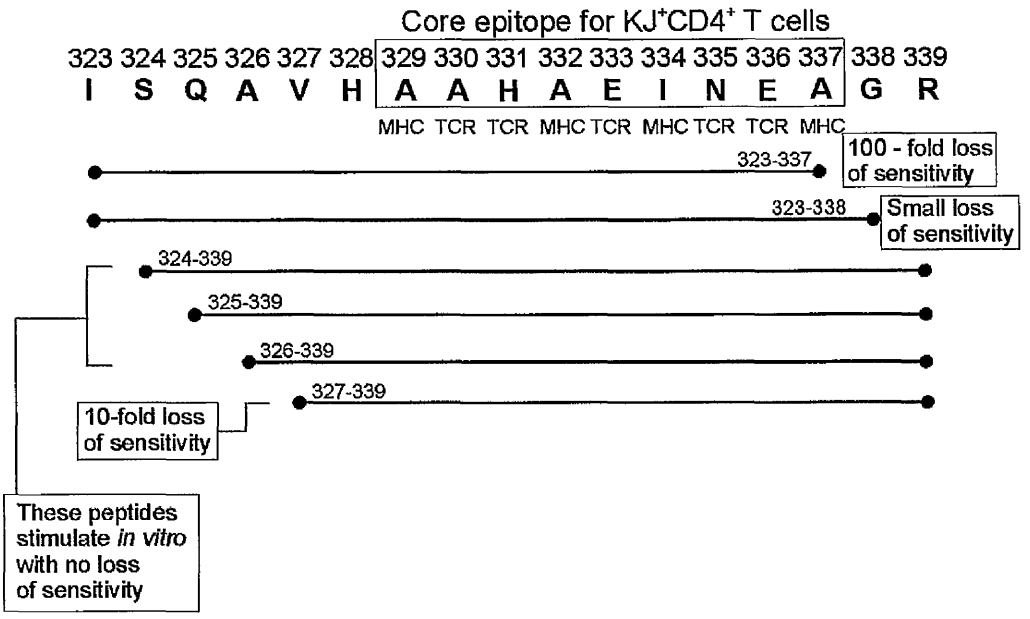


Figure 6.1. Summary of 323-339 OVA peptide.

The amino acid sequence of the OVA peptide recognized by $KJ^{+}CD4^{+}$ T cells including the core epitope, 329-337 and the effect of removing N or C terminal amino acids.

Chapter 6

6.2 Results

6.2.1 Use of $OVA_{327-338}$.

According to the study by Robertson (2000), the core epitope recognized by DO11.10 T cells was sited between position 329 and 337 of the OVA peptide. As our aim was to alter the affinity of the OVA peptide for DO11.10 T cells for the OVA peptide, we wanted to shorten it so that there were fewer amino acids for a potential substitution. Therefore we synthesised two peptides with sequences: $OVA_{327-338}$ (OVA327) and $OVA_{328-338}$ (OVA328). Removal of the R at position 339 should have no effect, as it does not appear to be important for MHC or TCR binding (Robertson *et al.*, 2000). Removal of amino acids at positions 327 and 328 begins to cause a decrease in T cell proliferation *in vitro*, indicating that these residues are located on the edge of the epitope that is recognised (Robertson *et al.*, 2000). However, these peptides were shown to induce significant proliferation *in vitro*, unlike $OVA_{329-339}$, which indicated that position 328 was an important flanking residue (Robertson *et al.*, 2000). The results of the removal of these amino acids is illustrated in Figure 6.1.

Before considering possible amino acid substitutions that would alter the TCR affinity for MHC-peptide, we first wanted to examine how well the shortened peptides would stimulate DO11.10 T cells *in vivo* in comparison to full length OVA323. To do this we adoptively transferred 5 x 10^6 KJ^+CD4^+ T cells (from DO11.10/SCID mice) into BALB/cIgh recipients and challenged the mice i.p. with 0.05μmoles of either ap-OVA323, sol-OVA323, ap-OVA327, or sol-OVA327. We used μmoles as the peptide concentration unit to allow a direct comparison between an equivalent number of molecules of the two different peptides.

Mice were examined on days 3, 7, 14 and 21 for the presence of KJ^+CD4^+ T cells in the spleen, MLN, and ILN. In the spleen (Figure 6.2) the response of KJ^+CD4^+ T cells to apOVA323 appeared to peak between day 3 and 7, and then decline rapidly such that by day 21 very few cells were detected. Sol-OVA323 did not induce as strong a response as was expected. However, unexpectedly both apOVA327 and sol-OVA327 stimulated the KJ^+CD4^+ T cells poorly. By day 21 (in the spleens of all recipients examined) very few KJ^+CD4^+ T cells were detected.

In the MLN (Figure 6.3) a similar picture was observed. ApOVA323 stimulated KJ^+CD4^+ T cells such that the peak response was observed on day 7. However, challenge with sol-OVA323, apOVA327, or sol-OVA327 did not result in any proliferation of KJ^+CD4^+ T cells. By day 21 very few donor cells were detected.

In the ILN (Figure 6.4) the peak response to apOVA323 also appeared to be between day 3 and 7. In this tissue sol-OVA323 appeared to induce more proliferation than observed in the spleen and MLN. This was observed previously and may reflect a high amount of soluble antigen reaching this tissue. There were significantly more ($p < 0.05$) KJ^+CD4^+ T cells on day 7 in the ILN of mice injected with apOVA327 compared with those that received sol-OVA327. This seems to reflect the adjuvant effect of alum-precipitation. The number of KJ^+CD4^+ T cells stimulated by apOVA327 was still much lower than in those challenged with the full-length peptide. By day 21 no donor cells were detected in any of the groups.

The expression of CD45RB by the KJ^+CD4^+ T cells was also examined. Expression of the low molecular weight isoform of CD45RB is associated with activated/memory T cells as discussed in section 5.1.4. The percentage of donor KJ^+CD4^+ T cells that were $CD45RB^{lo}$ is shown in Figure 6.5 for the spleen, Figure 6.6 for the MLN, and Figure 6.7 for ILN. A similar pattern was observed for all tissues examined. As expected the greatest number of $CD45RB^{lo}$ donor cells were those challenged with apOVA323. A large number of KJ^+ T cells stimulated with sol-OVA323 (60-65%) were also $CD45RB^{lo}$ on day 7, but by day 14 this number had decreased unlike those challenged with adjuvant. Stimulating KJ^+ T cells with OVA327 compared with OVA323 induced far fewer $CD45RB^{lo}$ T cells. There was also a difference in CD45RB expression between cells challenged with either apOVA327 compared with soluble OVA327 but the difference was not statistically significant. The pattern appeared to mirror that of the ap versus sol OVA323 challenge.

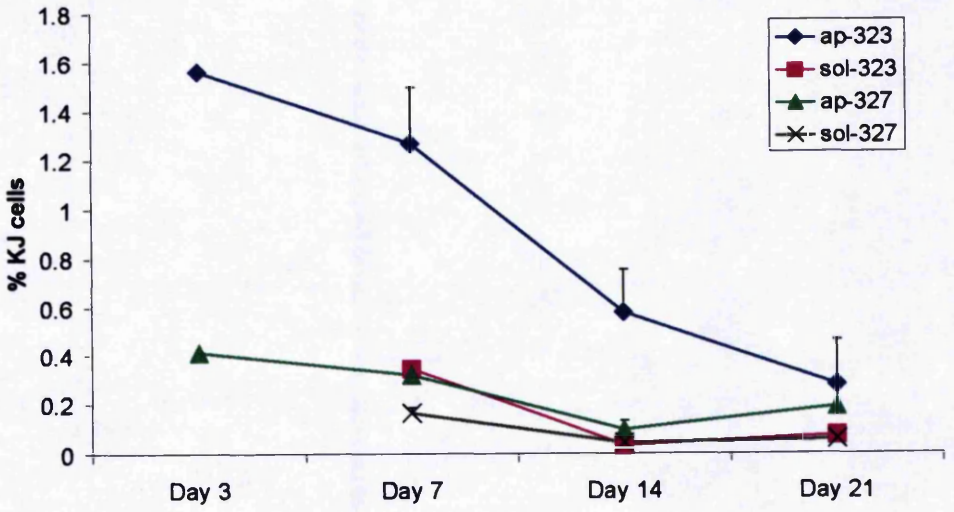


Figure 6.2. Percentage of KJ^+CD4^+ T cells in the spleen following challenge with OVA323 or OVA327.

The percentage of KJ^+CD4^+ T cells in the spleens of BALB/cIgh recipients 3, 7, 14, and 21 days after the adoptive transfer of 5 x 10^6 KJ^+CD4^+ i.v., followed by i.p. challenge with 0.05μmoles of apOVA323, sol-OVA323, apOVA327, or sol OVA327. Values shown are means + SDs of 3 recipients per group.

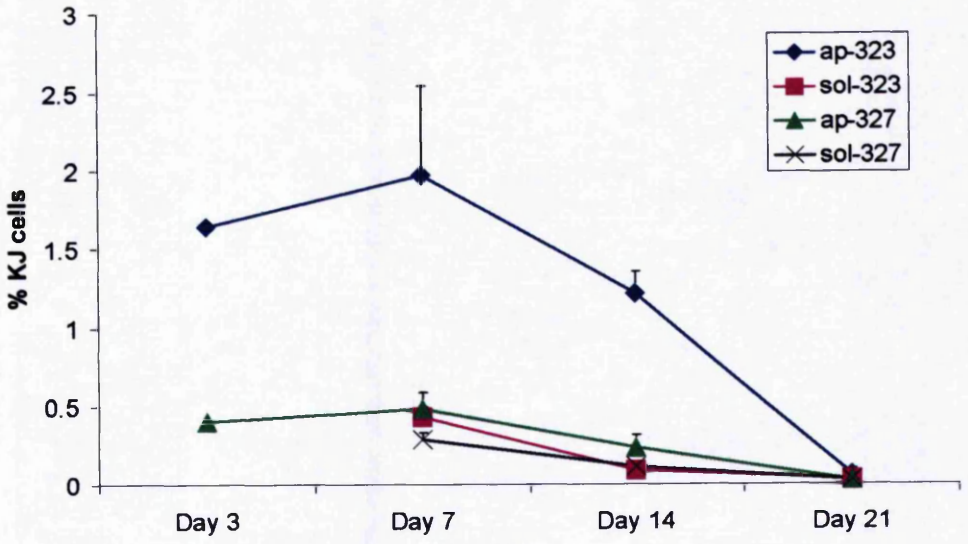


Figure 6.3. Percentage of KJ^+CD4^+ T cells in the MLN following challenge with OVA323 or OVA327.

The percentage of KJ^+CD4^+ T cells in the MLNs of BALB/cIgh recipients 3, 7, 14, and 21 days after the adoptive transfer of 5 x 10^6 KJ^+CD4^+ i.v., followed by i.p. challenge with 0.05μmoles of apOVA323, sol-OVA323, apOVA327, or sol OVA327. Values shown are means + SDs of 3 recipients per group.

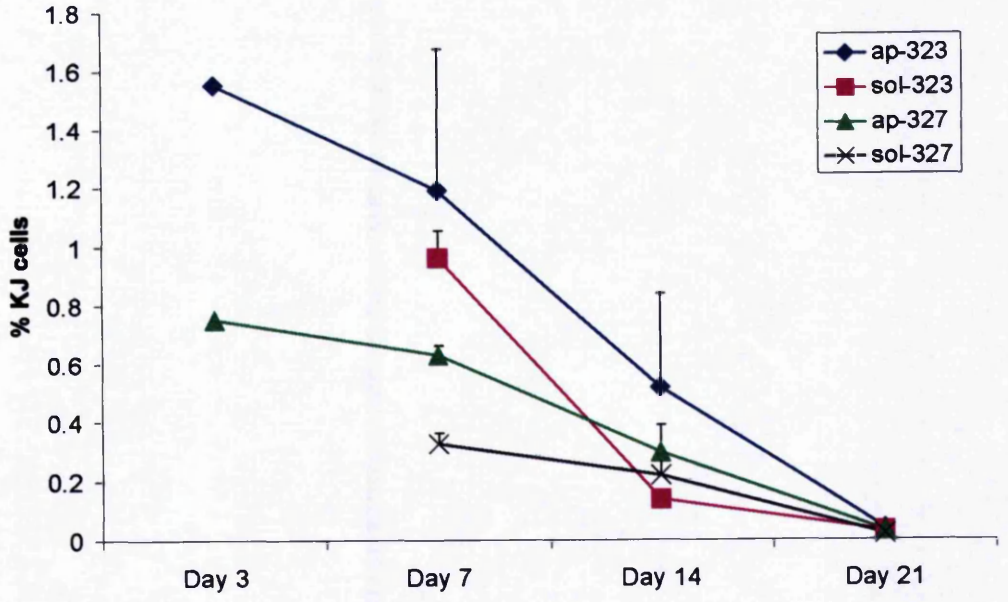


Figure 6.4. Percentage of KJ^+CD4^+ T cells in the ILN following challenge with OVA323 or OVA327.

The percentage of KJ^+CD4^+ T cells in the ILNs of BALB/cIgh recipients 3, 7, 14, and 21 days after the adoptive transfer of 5 x 10^6 KJ^+CD4^+ i.v., followed by i.p. challenge with 0.05μmoles of apOVA323, sol-OVA323, apOVA327, or sol OVA327. Values shown are means + SDs of 3 recipients per group.

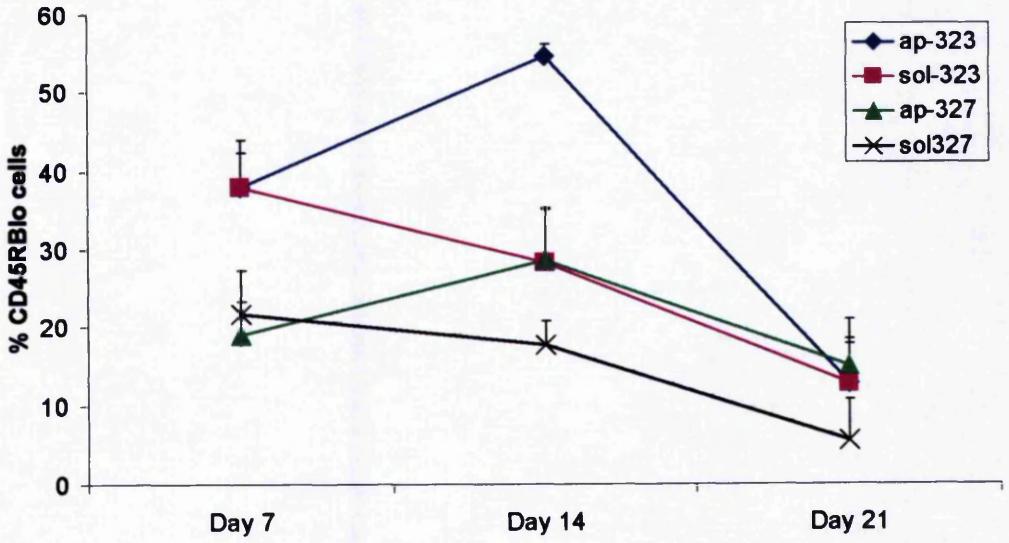


Figure 6.5. CD45RB expression of KJ^+CD4^+ T cells in the spleen following challenge with OVA323 or OVA327.

The percentage of KJ^+CD4^+ T cells which were $CD45RB^{lo}$ in the spleens of BALB/cIgh recipients 7, 14, and 21 days after the adoptive transfer of 5×10^6 KJ^+CD4^+ i.v., followed by i.p. challenge with 0.05μmoles of apOVA323, sol-OVA323, apOVA327, or sol OVA327. Values shown are means + SDs of 3 recipients per group.

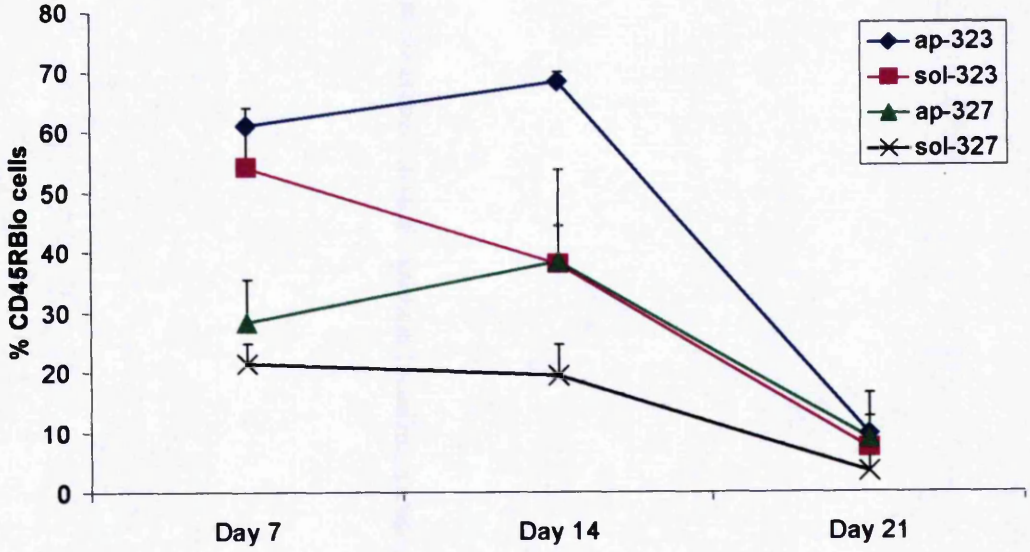


Figure 6.6. CD45RB expression of KJ^+CD4^+ T cells in the MLN following challenge with OVA323 or OVA327.

The percentage of KJ^+CD4^+ T cells which were $CD45RB^{lo}$ in the MLNs of BALB/cIgh recipients 7, 14, and 21 days after the adoptive transfer of 5 x 10^6 KJ^+CD4^+ i.v., followed by i.p. challenge with 0.05µmoles of apOVA323, sol-OVA323, apOVA327, or sol OVA327. Values shown are means + SDs of 3 recipients per group.

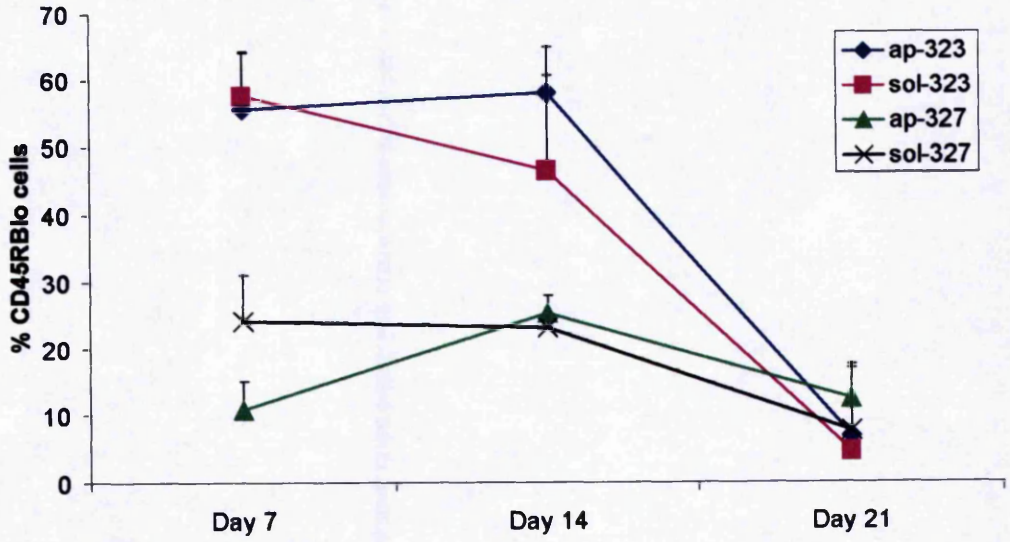


Figure 6.7. CD45RB expression of KJ^+CD4^+ T cells in the ILN following challenge with OVA323 or OVA327.

The percentage of KJ^+CD4^+ T cells which were $CD45RB^{lo}$ in the ILNs of BALB/cIgh recipients 7, 14, and 21 days after the adoptive transfer of 5 x 10^6 KJ^+CD4^+ i.v., followed by i.p. challenge with 0.05µmoles of apOVA323, sol-OVA323, apOVA327, or sol OVA327. Values shown are means + SDs of 3 recipients per group.

6.2.2 Studies with OVA328.

To determine whether KJ^+CD4^+ T cells could be stimulated *in vivo* using an even shorter peptide, we explored the response induced with OVA328. Note that the 328 position is outside the core epitope (Figure 6.1) and that OVA328-339 induced proliferation of TCL *in vivo* (Robertson *et al.*, 2000), albeit poorly. 5 x 10^6 KJ^+CD4^+ T cells were adoptively transferred into BALB/cIgh mice and challenged with 0.05μmoles apOVA323, 0.05 μmoles apOVA328, or 0.5 μmoles apOVA328. Seven days after challenge the spleen, MLN, and ILN of recipient mice were examined as shown in Figure 6.8. As expected KJ^+CD4^+ T cells in the spleen and MLN of mice challenged with OVA323 increased significantly. The same dose or even a 10 fold larger dose of OVA328 failed to stimulate a response. This was confirmed with CD45RB staining (Figure 6.9), showing a large increase in $CD45RB^{lo}KJ^+CD4^+$ T cells following OVA323 injection, but no evidence of an increase in $CD45RB^{lo}$ staining following OVA328 injection. The staining pattern for the ILN in Figure 6.9 was not consistent with the analysis of the other tissues and cannot be interpreted with confidence. There was no reason to suspect a technical error, but this cannot be excluded. Clearly from Figure 6.9, OVA323 stimulated KJ^+CD4^+ T cells in the ILN, as evidenced by the high percentage of $CD45RB^{lo}$ staining. Overall there was no evidence that OVA328 was recognised by KJ^+CD4^+ T cells *in vivo*.

In summary, our attempts to manipulate the affinity of the KJ^+CD4^+ T cells for an altered OVA peptide requires further work. The shortened peptides that we created were ineffective at stimulating a T cell response in comparison to the full length OVA323 peptide as illustrated in figure6.10. However we did learn that position 328 is crucial for KJ^+CD4^+ T cell activation *in vivo* and that position 327 is also important, at least at viable peptide concentrations. Therefore our next strategy would be to examine amino acids 326-338 for possible substitutions, which will reduce the binding affinity of KJ^+CD4^+ T cells. We can then examine whether this will prolong their survival following proliferation.

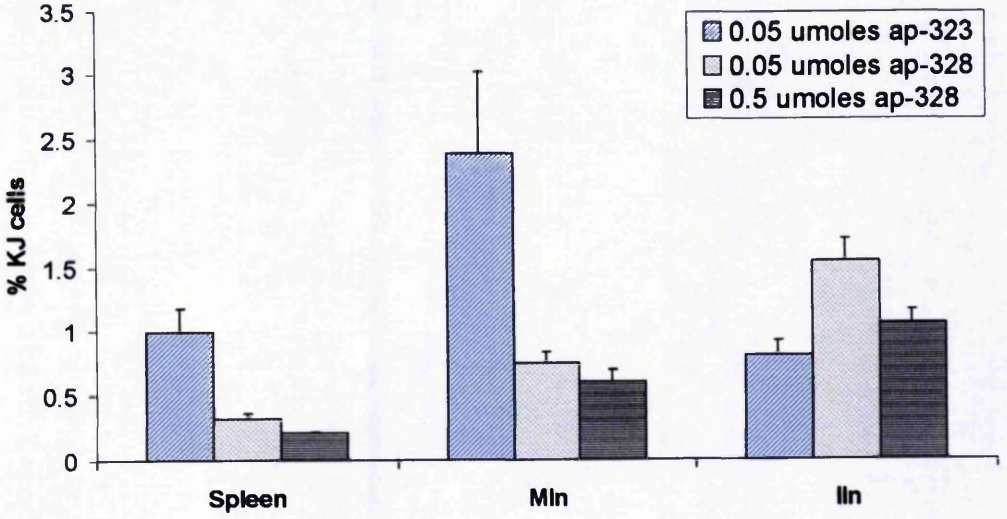


Figure 6.8. Percentage of KJ^+CD4^+ T cells following challenge with OVA323 or OVA328.

The percentage of KJ^+CD4^+ T cells in BALB/cIgh recipients 7 days after the adoptive transfer of 5 x 10^6 KJ^+CD4^+ T cells i.v., followed by i.p. challenge with 0.05μmoles apOVA323, 0.05μmoles apOVA328, or 0.5μmoles apOVA328. Values shown are means + SDs of 3 recipients per group.

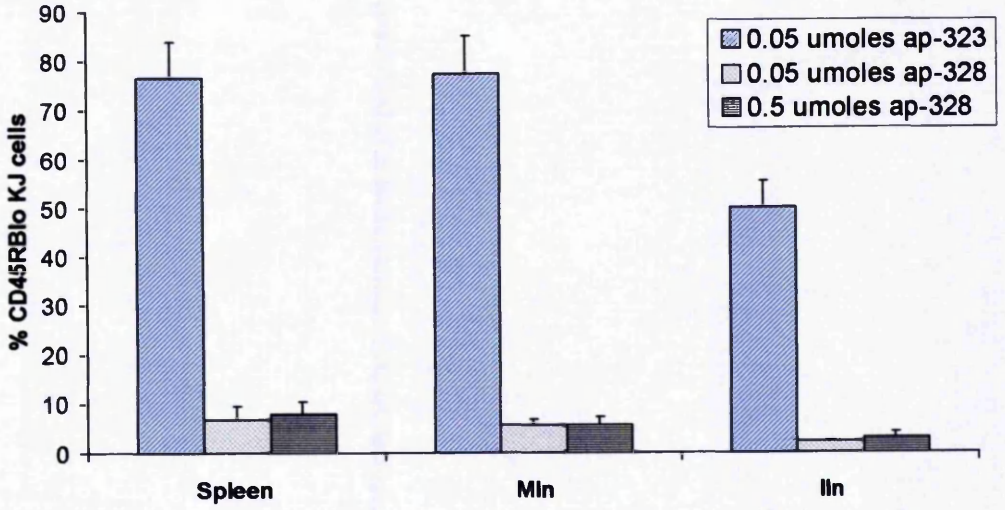


Figure 6.9. CD45RB expression of KJ^+CD4^+ T cells following challenge with OVA323 or OVA328.

Figure 8. The percentage of KJ^+CD4^+ T cells that are $CD45RB^{lo}$ in BALB/cIgh recipients 7 days after the adoptive transfer of 5 x 10^6 KJ^+CD4^+ T cells i.v., followed by i.p. challenge with 0.05μmoles apOVA323, 0.05μmoles apOVA328, or 0.5μmoles apOVA328. Values shown are means + SDs of 3 recipients per group

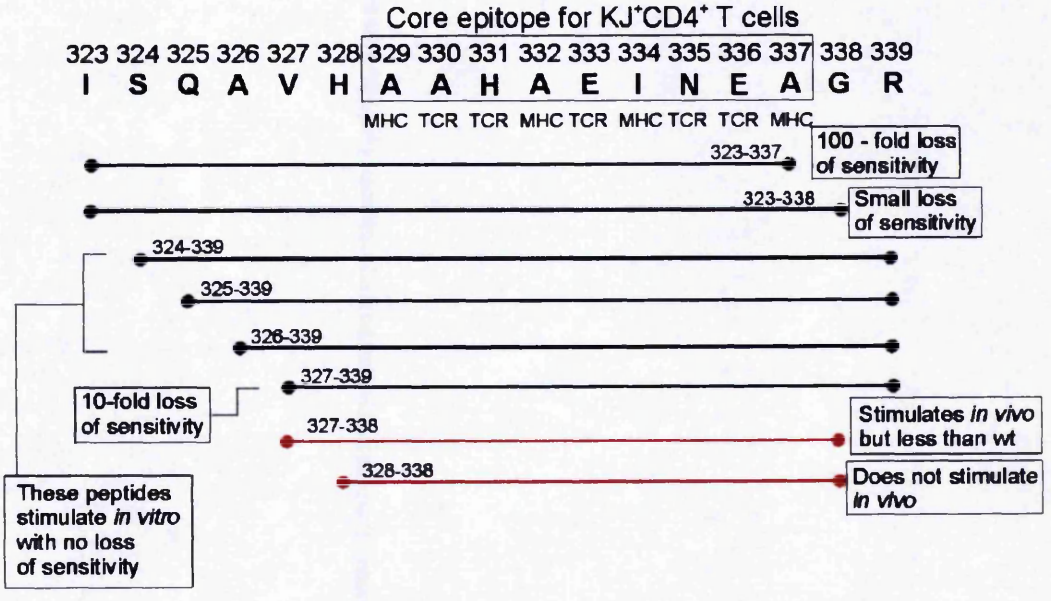


Figure 6.10. Summary of OVA peptides and results from shortened OVA327 and OVA328.

The amino acid sequence of the OVA peptide recognized by KJ^+CD4^+ T cells including the core epitope, 329-337, the effect of removing N or C terminal amino acids, and the results from challenging with OVA327 and OVA328.

Chapter 7
Discussion

7.1 Migration of adoptively transferred KJ^+CD4^+ T cells in the absence of antigen.

As KJ^+ T cells were to be used in adoptive transfer experiments we firstly wanted to examine if they would migrate in a similar pattern to non tg T cells immediately following transfer. To do this a population of cells, containing both KJ^+ and KJ^- T cells, were labelled with CFSE and transferred to normal BALB/cIgh mice. After 2 hours and 22 hours the spleen, MLN, ILN, and CLN of the recipient mice were examined for the presence of donor cells by FACS. At 2 hours after transfer donor T cells were detected in all tissues examined, although the majority were in the spleen. At 22 hours donor T cells were still present in all tissues examined, though by this time point less cells were observed in the spleen and more were in the lymph nodes. When the cells were examined as a relative percentage of the total donor cells per animal, it revealed that between the two time points, both KJ^+ and KJ^- T cells moved from the spleen to the lymph nodes at a similar rate. This showed us that in terms of early migration patterns after adoptive transfer, KJ^+ T cells were no different from KJ^- T cells. As expected in the absence of antigen, no evidence of cell division was observed.

The results observed were in general agreement with early migration studies by Smith & Ford (1983). The authors conducted a comprehensive study of lymphocyte re-circulation in rats, examining a range of time points from 1 minute to 24 hours after transfer (Smith & Ford, 1983). They observed that immediately after transfer (1-10 min) the injected cells were located in the blood, lungs, and liver (Smith & Ford, 1983). However after 30 minutes the cells began to accumulate in the spleen, with numbers peaking in this tissue after an hour, after which they began to fall (Smith & Ford, 1983). Although we only examined two time points, we did see an early accumulation of transferred cells (both KJ^+ and KJ^-) in the spleen at 2 hours after injection, and then a decline in cell numbers by 22 hours. In the rat study, in the first two hours after transfer, more cells were detected in the cervical and popliteal LNs than in the mesenteric LN (Smith & Ford, 1983). However by 2 hours more cells were located in the mesenteric LN than the other LNs examined (Smith & Ford, 1983). This was also

the case in our studies at 2 and 22 hours post transfer, as more cells (both KJ^+ and KJ^-) were located in the mesenteric LNs than the cervical or inguinal LNs.

In studies with DO11.10 tg T cells there is no published data on the behaviour of the cells in the first 24 hours after adoptive transfer. However following the transfer of 2.5 x 10^6 DO11.10 T cells, 24 hours later the lymph nodes of recipient mice were reported to contain ~0.5% KJ^+CD4^+ T cells (Pape *et al.*, 1997a). As was also observed in our studies, in the absence of antigen KJ^+CD4^+ T cells showed no signs of cell division and retained a naïve phenotype (Pape *et al.*, 1997a).

7.2 Migration of adoptively transferred KJ^+CD4^+ T cells in immunized recipients.

We next wanted to examine the effect of antigen on the early migration of KJ^+ T cells. To do this we adoptively transferred a population of CFSE labelled KJ^+ and KJ^- T cells, into either BALB/cIgh mice pre-immunized 3 days previously with 100μg ap-OVA peptide, or control BALB/cIgh mice. Following i.p. injection of particulate antigen (ap-OVA peptide) the spleen and MLN are the first tissues to receive the antigen (E. Bell personal communication). Therefore we expected that by pre-immunizing the mice in this way, KJ^+ T cells would remain in the spleen and MLN and begin proliferation, where as KJ^- T cells would re-circulate as normal. However, this is not what we observed and in hindsight the approach we took was flawed. For instance injecting the antigen 3 days prior to the cell transfer allowed antigen presenting cells to reach the peripheral lymph nodes and present antigen there. Also, considering that 5 x 10^6 KJ^+ T cells were injected, the amount of OVA peptide located to the spleen was probably insufficient to engage the entire antigen-specific population, and therefore unable to prevent the migration of these cells out of this tissue.

Between 2 and 22 hours little difference was observed in the relative percentages of KJ^+ T cells, in the absence or presence of OVA peptide. The same was true for KJ^- T cells. However at 2 hours there were significantly more donor T cells (both KJ^+ and KJ^-) in the spleens of immunized mice than in controls. As was observed when no antigen was present, in both groups of animals the overall pattern between 2 and 22 hours was a decrease in the relative percentage of cells in the spleen and an increase in the lymph nodes. When total cell numbers were examined there were greater numbers of KJ^+ T cells in the spleens of mice which were pre-immunized with

OVA peptide. This may have been a result of antigen "trapping" the cells and preventing their exit out of the spleen. However, there was no evidence for trapping when the mice were examined at 22 hours.

By 44 hours, KJ^+CD4^+ T cells in all tissues examined from immunized mice showed signs of cell division as evidenced by loss of CFSE. In contrast KJ^+CD4^+ T cells from mice that were not immunized showed no signs of cell division. In a study examining DO11.10 T cell proliferation *in vitro*, cell division was observed to occur 60 hours after peptide stimulation (Lee & Pelletier, 1998). The 12 hour discrepancy between the onset of cell division may have been caused by a delay in the antigen presenting *in vitro*. In our studies the OVA peptide was injected 3 days prior to the cell transfer, therefore allowing time for the peptide to be processed and presented, before the introduction of KJ^+CD4^+ T cells. When examined *in vivo*, KJ^+CD4^+ T cells were observed to undergo cell division in the spleen and all lymph nodes, as evidenced by BrdU uptake, 3 days after soluble OVA challenge (Pape *et al.*, 1997a). This was also the case when OVA was injected emulsified in CFA or IFA (Pape *et al.*, 1997a).

However, despite the clear evidence of cell division in mice that were immunized, there was no substantial increase in the total number of KJ^+CD4^+ T cells when compared to mice that were not immunized. In contrast, in the spleen there were significantly more KJ^+CD4^+ T cells in mice that did not receive OVA peptide. In the MLN and ILN, but not the CLN, there were more KJ^+CD4^+ T cells in immunized mice though the differences were not statistically significant. These results do not agree with previous studies which reported that the number of tg T cells was 7 times greater than the starting level 3 days after sol OVA challenge (Pape *et al.*, 1997a). When the peptide was injected emulsified in IFA or CFA the increase in KJ^+CD4^+ T cell numbers was even greater, 3 times that of sol-OVA challenge, but peaking on day 5 rather than day 3 (Pape *et al.*, 1997a). When KJ^+CD4^+ T cells were transferred and challenged with OVA peptide plus LPS subcutaneously, the tg cells proliferated with cell numbers peaking on day 3 and then declining (Pape *et al.*, 1997b). When LPS was not administered with the OVA peptide, the accumulation and persistence of KJ^+CD4^+ T cells in the draining lymph nodes was much reduced (Pape *et al.*, 1997b). The adjuvant properties of LPS could be mimicked by injection of TNF-α and IL-1 (Pape *et al.*, 1997b).

Three possible explanations exist for the apparent failure of KJ^+CD4^+ T cells to accumulate in significant numbers in our studies. One possibility is that the tg T cells upon activation exited the spleen and lymph nodes to enter non-lymphoid tissues. When

the presence of tg OT-II T cells (specific for OVA peptide also) was examined throughout the body of recipient mice immunized with OVA peptide plus LPS, the cells were detected in the interstitial spaces of the liver, lung, salivary gland, lamina propria of the gut, and the medulla of the thymus 3 days after transfer (Reinhardt *et al.*, 2001). However despite this, numbers of tg T cells also peaked in the spleen and lymph nodes on day 3 (Reinhardt *et al.*, 2001). So in this case despite the appearance of tg cells throughout the body, the number of cells peaked on day 3 in the spleen and lymph nodes (Reinhardt *et al.*, 2001). The other more intriguing possibility is that the KJ^+CD4^+ T cells upon activation and proliferation begin to die. It has been observed that a significant proportion of the KJ^+CD4^+ T cells that have undergone one round of cell division also stain positively for annexin, a marker for early apoptosis (E. Bell personal communication). This has also been observed *in vitro* when peritoneal exudate cells (PEC) were used as the APCs (Takeuchi *et al.*, 1999). It was reported that up to 28% of KJ^+CD4^+ T cells showed signs of apoptosis (Takeuchi *et al.*, 1999), as assessed by the TUNEL assay, which identifies cells with DNA breaks characteristic of programmed cell death. In comparison, OVA-primed T cells from C57BL/6 mice stimulated *in vitro* in a similar manner, showed apoptotic rates of 18% (Takeuchi *et al.*, 1999). Interestingly, the apoptotic rate could be reduced by treatment of the PECs with TGF-β_2, or by addition of anti-IL-12 to the cultures (Takeuchi *et al.*, 1999). The hypothesis that KJ^+CD4^+ T cells were dying prematurely was also supported by our studies examining the survival of primed KJ^+CD4^+ T cells in both BALB/cIgh and SCID mice which will be discussed in Section 7.6. The third, but unlikely, explanation for the observed results is that the KJ^+CD4^+ T cells down-regulated their tg TCR following activation, and therefore could not be stained by the mAb KJ-126. However there is little evidence for this in the literature, apart from the single report that DO11.10 T cells tolerized by challenge with sol-OVA expressed lower levels of TCR than naïve mice (Pape *et al.*, 1998).

By transferring a mixture of CFSE labelled KJ^+ and KJ^- T cells into normal recipients and challenging with ap-OVA peptide we were able to demonstrate the antigen-specific nature of this tg model. By 44 hours after transfer 3 rounds of cell division were observed among the KJ^+CD4^+ T cell population in the OVA immunized mice. In the same mice there was no evidence of cell division from the KJ^-CD4^+ T cells. This agrees with the *in vitro* studies which demonstrated that KJ^+ but not KJ^- T cells from DO11.10 mice were stimulated to divide by OVA peptide challenge (Lee &

Pelletier, 1998). We also showed, as expected, that neither KJ^+ nor KJ^- T cells underwent cell division in the absence of antigen challenge.

Following *in vitro* stimulation of KJ^+CD4^+ T cells it was observed that the cells up-regulate CD4 expression prior to cell division, and this was suggested as a possible marker to distinguish effector cells (Lee & Pelletier, 1998). The increased CD4 expression levels were reported to be stable throughout the culture (Lee & Pelletier, 1998). However, when we examined the levels of CD4 expression on KJ^+CD4^+ T cells which had divided, we saw contrasting results. On one occasion our results agreed with the *in vitro* study (Lee & Pelletier, 1998), but in another separate experiment we saw the opposite effect; daughter KJ^+CD4^+ T cells expressing lower CD4 levels than the parent population. Therefore it appeared that up-regulation of CD4 expression was not a reliable marker for early activation status *in vivo*.

7.3 Establishing the experimental model.

In order to study secondary immune responses we generated populations of antigen-primed B cells. To do this we immunized normal BALB/cIgh mice with alum-precipitated OVA (ap-OVA). By precipitating OVA with alum, OVA acquires adjuvant properties including a slower release of the antigen from the injection site. Also the aggregate nature of the antigen increases its likelihood of phagocytosis. Alum-precipitated antigen has also been reported to polarize the immune response to a Th2 type (Brewer *et al.*, 1999). In contrast with the alum-precipitated form of antigen, many soluble proteins fail to stimulate a primary response and instead induce tolerance (Mitchison, 1968). However, following priming with ap-antigen a secondary response can be induced by challenge with soluble protein. This was illustrated in a previous study in rats, where it was shown that in the presence of $CD4^+$ T cells, sol-OVA could induce primed B cells, but not naïve B cells to produce antibody (Bell *et al.*, 2001). It was important to establish whether KJ^+CD4^+ T cells behaved in a similar manner following sol-OVA challenge.

To test this, KJ^+CD4^+ T cells were adoptively transferred with either naïve or OVA-primed B cells to SCID recipients and challenged with 10μg sol-OVA. As was observed in the rat study (Bell *et al.*, 2001), naïve B cells failed to produce a significant antibody response following sol-OVA challenge, unlike primed B cells which produced a strong antibody response. This experiment also demonstrated that naïve KJ^+CD4^+ T

cells were able to provide sufficient help to primed B cells following a sol-OVA challenge. This is also in agreement with the rat study which demonstrated that naïve $CD4^+$ T cells could provide help for primed B cells, but not unprimed B cells, when challenged with sol-OVA (Bell *et al.*, 2001). Because sol-OVA only induced a response from primed B cells, we could be sure that we were examining secondary and not primary responses. This was an important criterion to establish for these studies.

As there is no unequivocal marker to distinguish antigen experienced B cells from naïve B cells, we purified the whole B cell population for adoptive transfer. Mice were immunized with 100μg ap-OVA at least 8 weeks prior to collection of cells to allow time for memory B cells to develop. The B cells were purified by magnetic depletion of $CD4^+$, $CD8^+$, and $Thy1.2^+$ cells. Although a significant population of non-B cells remained following purification, these cells were likely to be NK cells, DC cells, or macrophages which should not interfere with the response we were examining. To be certain that all T cell help was provided by the transferred KJ^+CD4^+ T cells, a control group of recipient mice received only primed B cells in each experiment. Without exception these control groups produced little or no antibody demonstrating that all antibody production was dependent on help from the KJ^+CD4^+ T cells. This was crucial if we were to examine potential differences between naïve and revertant memory T cells.

Another important objective in establishing the experimental model was to determine the ideal number of cells to transfer. If we transferred either too few or too many B or T cells, there was the possibility that the response would be limited by either subset. Therefore it was important to determine a physiological balance in the dose of cells transferred. Firstly we examined how many B cells would be necessary to produce a measurable antibody response. As we were not purifying antigen-specific B cells, but rather the whole population containing a small number of memory B cells in an excess of naïve cells, we assumed that a relatively large number of B cells from the primed donors would be required. The OVA-specific memory B cells would only represent a small fraction of this total B cell population. We transferred three doses of primed B cells, 3×10^7, 10^7, and 10^6 with an excess of KJ^+CD4^+ T cells (3×10^6) into SCID recipients, challenged with sol-OVA and measured the antibody response on day 10. There was little difference in the response observed between the two high doses but when the dose was reduced to 10^6 primed B cells, the antibody response was

diminished significantly. Therefore we elected to transfer 10^7 primed B cells in all experiments.

As the KJ^+CD4^+ T cells were OVA specific, determining the ideal number of T cells to transfer required a more detailed approach. Although we knew that 3 x 10^6 KJ^+CD4^+ T cells was sufficient to help 10^7 primed B cells, we surmised that this number was in excess. As we wanted to compare the ability of naïve, primed or memory $CD4^+$ T cells to help memory B cells, it was important that the number of T cells was not in excess, otherwise any qualitative differences between the T cell subsets would be obscured, as the number of B cells became the limiting factor. We adoptively transferred either 3 x 10^6, 3 x 10^5, 10^5, 3 x 10^4, or 10^4 SCID derived KJ^+CD4^+ T cells with 10^7 primed B cells to SCID recipients, challenged with sol-OVA and measured the antibody response on day 10. This experiment decisively demonstrated that B cell function was dependent on T cell help; there was a linear decrease in antibody production as the number of transferred T cells was decreased. The lowest dose of T cells transferred (10^4) produced a minimal amount of antibody that was comparable with the control group. Although 3 x 10^4 T cells induced a significant antibody response the quantity of antibody was highly variable. Therefore it was decided that 10^5 or 3 x 10^5 was the ideal dose of KJ^+CD4^+ T cells for transfer, as it provided sufficient help for 10^7 primed B cells but also ensured that the T cell population was the limiting factor in the response. A similar dose (10^5) was used by other investigators to mediate isotype switching by tg7 $CD4^+$ T cells (specific for a peptide derived from the glycoprotein of vesicular stomatitis virus (VSV-G)) in T cell deficient recipients (Maloy *et al.*, 1999).

KJ^+CD4^+ T cells have been shown to provide help for tg B cells (MD4) when challenged with OVA-HEL in CFA (Smith *et al.*, 2000) (Garside *et al.*, 1998). *In vitro* polarized (to Th1 and Th2) and non-polarized KJ^+CD4^+ T cells provided similar levels of help, although the antibody response from polarized cells peaked on day 5, while that from non-polarized cells peaked on day 7 (Smith *et al.*, 2000). 2 x 10^6 KJ^+CD4^+ T cells and 3-6 x 10^6 tg B cells were transferred in these experiments (Smith *et al.*, 2000). In a study examining the effect of adjuvants on the stimulation of KJ^+CD4^+ T cells, it was observed that injection of sol-OVA resulted in minimal anti-OVA antibody production (Pape *et al.*, 1997b). This was in agreement with our studies, although the authors concluded that this was due to a failure of sol-OVA to activate KJ^+CD4^+ T cells rather than an effect on B cells (Pape *et al.*, 1997b). Our studies showed that sol-OVA

alone was insufficient for stimulating naïve B cells, but induced a response from primed B cells in the presence of KJ^+CD4^+ T cells. In contrast, injection of OVA plus LPS resulted in the production of OVA specific IgG1 in both KJ^+CD4^+ T cell-injected recipients and in normal controls (Pape *et al.*, 1997b). IgG1 antibody levels were equivalent in both sets of mice, despite one group receiving 2.5 x 10^6 KJ^+CD4^+ T cells (Pape *et al.*, 1997b). Interestingly IgG2a was only detected in the KJ^+CD4^+ T cell-injected recipients (Pape *et al.*, 1997b). These results suggested that KJ^+CD4^+ T cells promoted a Th1 response following injection of OVA plus LPS (Pape *et al.*, 1997b). Our initial studies showed that the antibody produced by ap-OVA primed B cells was IgG1 (data not presented). This was in agreement with the studies which showed that ap-antigen induced a Th2 type response. The antibody levels induced by injection of OVA plus LPS could be replicated by injection of OVA plus TNF for IgG1, and by injection of OVA plus IL-12 for IgG2a, in mice which received KJ^+CD4^+ T cells (Pape *et al.*, 1997b).

Apart from these studies of the primary response, KJ^+CD4^+ T cells have not previously been shown to provide help for primed B cells. In fact, surprisingly little is known about specific T and B cell interactions in secondary immune responses *in vivo*. Some work has been conducted *in vitro* using T cell clones. One such study examining the secondary response showed that induction of IgG1 synthesis by primed B cells could occur via two separate pathways, one involving IL-2, and the other involving IL-4 and IL-5 (DeKruff *et al.*, 1993). However recent work by our group using IL-2 KO KJ^+CD4^+ T cells, revealed that although IL-2 was required for a normal primary antibody response, it did not seem to be essential for a secondary antibody response (personal observation). This is an area for potential future studies.

In the study by Bell *et al* (2001), 100μg sol-OVA was sufficient to induce a secondary response from primed B cells in the rat. For our studies in the mouse it was assumed that 10μg sol-OVA would be adequate to stimulate a response, and this proved to be the case. Nevertheless we still wished to examine the effects of increasing the amount of antigen on both the KJ^+CD4^+ T cell response and the antibody response. When SCID recipients containing 5 x 10^6 KJ^+CD4^+ T cells were challenged with increasing amounts of sol-OVA T cell numbers in the spleen, MLN, and ILN increased with increasing dose. Interestingly, the greatest difference observed between challenging with 10μg or 100μg sol-OVA, was in the inguinal lymph node; there were 8 times more KJ^+CD4^+ T cells found in the ILN of mice which received the high dose

of sol-OVA. Soluble antigen, unlike alum-precipitated antigen which is retained within the peritoneal cavity, is free to disseminate throughout the body. It could therefore reach the MLN and ILN in equal amounts. The greater increase of KJ^+CD4^+ T cells in the ILN compared to the MLN may reflect the fact that relatively more KJ^+CD4^+ T cells localized early to the ILN after transfer. Therefore, when antigen was abundant, more of the T cells were able to proliferate.

In view of the fact that increasing antigen dose had a significant effect on T cell proliferation, we wanted to examine what effect it would have on antibody production by primed B cells. 3×10^5 KJ^+CD4^+ T cells and 10^7 primed B cells were transferred to SCID recipients, challenged with either 10μg or 100μg sol-OVA, and examined for antibody production. The higher dose of antigen resulted in 3 times more antibody on day 7, 5 times more on day 14, and 4 times more on day 21. This 3-5 fold increase in antibody production appeared initially to reflect the 3-5 fold increase in KJ^+CD4^+ T cells in the spleen and MLN following challenge with the higher dose. However, the results from the titration of KJ^+CD4^+ T cells revealed that a 10 fold increase of T cells, (from 3×10^5 to 3×10^6), resulted in a mere 15% increase in anti-OVA antibody. Therefore, merely increasing the number of available T cells was unlikely to be the sole reason for the dramatic effect on antibody production. With an increase in antigen, more of the KJ^+CD4^+ T cells were likely to become activated, to up-regulate stimulatory molecules, and to produce cytokines. Also with an increase in the availability of antigen, more memory B cells were likely to be recruited into the response and be receptive to the cytokines produced by the greater number of proliferating T cells. Nevertheless, these experiments further demonstrate that by using the standard protocol the antibody response was always limited by the number of antigen-specific KJ^+CD4^+ T cells.

7.4 Characterization of Memory B cells.

As stated earlier, there is no definitive marker for memory B cells. Nevertheless, they have been characterised as having particular phenotypes such as IgD^-, IgM^-, IgG^+, and $B220^{+/-}$ (McHeyzer-Williams *et al.*, 2000) (Schittek & Rajewsky, 1990) (Black *et al.*, 1978) (Hayakawa *et al.*, 1987). We sought to examine our population of primed B cells on the basis of IgM expression. We purified primed B cells into $IgM^{+/-}$ and IgM^- populations and compared their ability to produce antibody following sol-OVA

challenge. Despite repeated efforts to deplete the population we were unable to obtain a pure IgM^- B cell population. Nevertheless, 2 x 10^5 purified IgM^- B cells produced a response that was comparable with 4 times as many non-depleted ($IgM^{+/-}$) primed B cells, indicating that large numbers of memory B cells resided within this this sub-population. Clearly, more work will be required to extend these preliminary experiments. This approach provides the potential for examining the phenotype of memory B cells, since we know that only primed B cells respond to sol-OVA challenge.

7.5 Comparison of naïve and primed KJ^+CD4^+ T cells.

Our interest in memory B cells primarily originated from a need to have a tool for studying T cells. The major aim of this project was to compare the ability of naïve and memory T cells to help memory B cells, as was examined by Bell *et al* (2001). However, in contrast to that study, we were able to use a defined population of antigen-specific cells. This would allow us to compare naïve, recently activated and memory cells on a cell per cell basis. The first step was to compare naïve and antigen primed T cells. Following stimulation with antigen in the presence of an adjuvant, T cells underwent characteristic changes. As well as the cell division that was reported in Chapter 3, T cells changed their expression of certain cell surface markers. Before adoptive transfer, KJ^+CD4^+ T cells were $CD45RB^{hi}$, $CD62L^{hi}$, $CD44^{med}$ and $CD25^{lo}$, a phenotype associated with naïve T cells. They were also $CD69^{lo}$ and $CD49b^{lo}$, markers associated with activated T cells. As expected following antigen challenge, and in agreement with *in vivo* (Pape *et al.*, 1998) and *in vitro* studies (Lee & Pelletier, 1998) (London *et al.*, 1999), a majority of the KJ^+CD4^+ T cells expressed the low molecular weight isoform of CD45RB. This marker has been used previously to identify primed T cells (Bell *et al.*, 2001) and was also the marker upon which we based our identification of such cells. The majority of KJ^+CD4^+ T cells also lost expression of CD62L, the lymph node homing receptor, and increased expression of CD44, both of which also occurred to KJ^+CD4^+ T cells following *in vitro* stimulation (Lee & Pelletier, 1998) (London *et al.*, 1999). These three molecules are commonly used to describe effector type cells, as they are evidence of previous antigen encounter. The less commonly used activation markers, CD25, CD49b, and CD69 were also examined. Although a significant proportion of the KJ^+CD4^+ T cells became $CD25^{hi}$, $CD49b^{hi}$, and $CD69^{hi}$,

the changes in their expression following antigen challenge were less than the other three markers examined. Expression of CD25, the IL-2αchainR, is a characteristic of cycling cells (London *et al.*, 1999). Only 32.2% of the KJ^+CD4^+ T cells appeared to be in cycle. CD69 has been reported to up-regulate rapidly following activation but returned to resting levels within six cell divisions (Oehan & Brduscha-Rien, 1998). This may explain why only 47% of the cells were $CD69^{hi}$ when examined.

In order to obtain a population of antigen-primed T cells, 5 x 10^6 KJ^+CD4^+ T cells were transferred to SCID recipients and challenged with ap-OVA peptide. 10 days after transfer the spleens and LNs of recipient mice were collected and purified for primed cells by removal of $CD45RB^{hi}$ cells. The population of purified $CD45RB^{lo}KJ^+CD4^+$ primed T cells were then transferred with OVA-primed B cells into secondary SCID recipients and challenged with sol-OVA. Naïve $CD45RB^{hi}KJ^+CD4^+$ were also transferred with primed B cells from the same population and challenged with sol-OVA so a comparison of the responses could be made. At two different doses of cells, 3 x10^5 or 10^5, primed KJ^+CD4^+ T cells provided significantly greater help ($p<0.05$) for primed B cells on day 7. There was no difference observed at any other time point. Control mice which received only B cells produced a minimal amount of antibody, showing that antibody production was dependent on help from the transferred KJ^+CD4^+ T cells. Although the difference between naïve and primed T cells was statistically significant ($p<0.05$), it was surprisingly small. It was expected that antigen priming of KJ^+CD4^+ T cells would confer upon them a distinct advantage in the timing or level of help that was provided to primed B cells. However as it did not, some interesting questions have been raised about immunological memory. Antigen experienced effector T cells were little better than naïve T cells, so perhaps the advantage conferred by immunological memory reside instead with the increased frequency of antigen-specific T cells. In addition, the two characteristic traits of immunological memory, speed and strength, may be more dependent on qualitative changes that are properties of the memory B cell and not the memory T cell. This idea is in agreement with a study that examined the memory B cell response to particular B and T cell epitopes, where it was revealed that a strong B cell memory response could be induced in the absence of primed T cells (Leclerc *et al.*, 1995).

It is also possible that some of the benefits of T cell priming were irrelevant in the type of response we examined. For example, one of the consequences of priming T cells with antigen plus adjuvant is that the T cells become able to enter the B cell rich

follicular regions of the lymphoid tissue (Pape *et al.*, 1997a). However, it was demonstrated that challenge of KJ^+CD4^+ T cells with sol-OVA does not induce the migration of KJ^+CD4^+ T cells to the B cell regions of the LNs (Pape *et al.*, 1997a). Despite this KJ^+CD4^+ T cells were able to provide sufficient help for primed B cells following sol-OVA challenge. This suggested the interaction between T cells and memory B cells may occur outside of the B cell region. This may also explain why primed T cells were no better than naïve T cells at providing help in this situation, as there was no requirement for migration into the follicles. It would be interesting to examine *in vivo* if primed KJ^+CD4^+ T cells were better than naïve KJ^+CD4^+ T cells at providing help to naïve B cells in a primary response. In this situation migration into the B cell follicles would be necessary, which may give primed T cells an advantage over naïve T cells. In an *in vitro* study where KJ^+CD4^+ T cells were co-cultured with naïve tg B cells that express an OVA specific Ig receptor, primed T cells provided between 10 and 100 fold more help than naïve T cells (Dustin *et al.*, 1995). Although this study was carried out *in vitro* (Dustin *et al.*, 1995), it does suggest that there may be a much greater difference between naïve and primed T cells when helping a primary response.

The observation that T cells from unprimed mice were capable of providing sufficient help to primed B cells was in agreement with a similar study by Bell and colleagues in the rat (Bell *et al.*, 2001). Although unable to make a direct comparison they showed that both naïve $CD45RC^+$ and primed $CD45RC^-$ T cells could provide help to primed B cells following sol-OVA challenge (Bell *et al.*, 2001). They also demonstrated that 7 days following priming the vast majority of cells that provided help were $CD45RC^-$ (Bell *et al.*, 2001). This merely reflected the fact that following priming the OVA specific T cells resided in the activated population (Bell *et al.*, 2001). This did not occur to the same extent in the tg system we used, as following priming only 55% of the KJ^+CD4^+ T cells were $CD45RB^{lo}$. Why this was is not clear, although considering the large numbers of antigen-specific cells (5×10^6) transferred, perhaps not all of them were stimulated. Although in our experiment naïve T cells appeared to be as effective as primed T cells, other studies have shown this not always to be the case. When tg7 $CD4^+$ T cells were transferred to normal recipients and challenged with a recombinant Vaccinia virus expressing the VSV-G peptide, as many as 10^7 transferred cells failed to provide protection (Maloy *et al.*, 1999). In contrast, when the tg7 $CD4^+$ T cells were primed *in vitro*, 10^6 transferred cells were sufficient to provide

protection (Maloy *et al.*, 1999). One of the crucial differences between the cells appeared to be the ability of primed T cells to produce high levels of IFN-γ (Maloy *et al.*, 1999). Therefore although naïve T cells did not share the same capabilities as primed T cells, when required to help primed B cells, they appeared relatively equal. However it must be noted that the immune response to a virus is different from that to a single peptide.

An important aim of this project was to generate a population of long-lived memory T cells. To examine if this was possible we set up an experiment as described in Figure5.7. 5 x 10^6 KJ^+CD4^+ T cells were transferred to BALB/cIgh recipients, half of which were immunized with ap-OVA peptide. As the OVA peptide will stimulate some of the host T cells we included two control groups, one which was immunized with ap-OVA peptide. The mice were left for 8 weeks to allow memory cells to develop and then received 10^7 OVA primed B cells and were challenged with sol-OVA. As B cells do not recognize the OVA peptide, the host B cell repertoire will not be stimulated by the initial immunization. When the recipients were bled and their antibody levels tested, there was no significant difference between the two immunized groups, one of which had received KJ^+CD4^+ T cells. As expected, mice which were not immunized did not produce any antibody until day 14 and even then it was of a very low level. Therefore, KJ^+CD4^+ T cells which were not stimulated with OVA peptide did not survive. However, it also appeared that KJ^+CD4^+ T cells that had been stimulated did not survive because there was no difference in antibody levels between immunized recipients which received KJ^+CD4^+ T cells and those which did not. Unexpectedly however, FACS analysis of the MLNs of these mice revealed the presence of a small population of $CD45RB^{lo}KJ^+CD4^+$ T cells. Whether these tg cells were a stable population of long lived memory cells, or were the result of a small number of surviving T cells proliferating due to the secondary challenge, could not be determined. However, despite their presence, they did not confer an advantage in helping the response of the primed B cells when compared with immunized mice that contained no tg T cells. It may be that in the presence of an abundant host-derived memory T cell population their presence was masked.

Following these observations we wanted to examine whether this long-lived, antigen-experienced cell population was dependent on the presence of antigen, as recent reports favoured the survival of memory cells in the absence of antigen (Swain *et al.*, 1999) (Murali-Krishna *et al.*, 1999). Using the protocol described in Figure5.11,

primed KJ^+CD4^+ T cells, which had been recovered from intermediate SCID recipients, were transferred to either OVA peptide-immunized, or non-immunized, BALB/cIgh mice. 6 weeks later recipients received 10^7 primed B cells and were challenged with sol-OVA. Again there was no significant difference between the two immunized groups, despite the transfer of KJ^+CD4^+ T cells. In contrast with the earlier experiments when the spleens and MLNs of all recipient mice were examined, there was no evidence of KJ^+CD4^+ T cells, either in the presence or absence of antigen. This suggested that primed KJ^+CD4^+ T cells did not survive following antigen stimulation. In the earlier experiment we detected a population of KJ^+CD4^+ T cells 10 weeks after the initial immunization. These could have been cells which were not stimulated by the initial immunization, and survived to clonally expand after the secondary immunization. This of course does not explain why KJ^+CD4^+ T cells did not survive in non-immunized hosts.

7.6 Survival of primed KJ^+CD4^+ T cells.

We tried to generate a population of long-lived, antigen-experienced T cells in SCID mice to enable comparisons with naïve T cells. It was possible that primed KJ^+CD4^+ T cells did not survive in BALB/cIgh mice as they had to compete with the host T cells for survival signals such as cytokines. As SCID mice have no lymphocytes of their own, competition for survival factors should not be a problem. Therefore KJ^+CD4^+ T cells were transferred to intermediate SCID recipients for antigen priming, purified for $CD45RB^{lo}$ cells, and re-transferred to naïve secondary SCID recipients. When the spleen and LNs of these mice were examined 1 and 3 weeks later for the presence of tg T cells very few were detected. Furthermore the KJ^+CD4^+ T cells showed no signs of reverting to $CD45RB^{hi}$ expression. The experiment was repeated and a control population of naïve KJ^+CD4^+ was also transferred for comparison. When these mice were examined 1, 4, and 8 weeks later very few primed KJ^+CD4^+ T cells were recovered, in contrast to the naïve KJ^+CD4^+ T cells which were clearly visible in the LNs examined. However it must be stated that primed T cells down-regulate CD62L and cannot enter lymph nodes unlike naïve T cells. Therefore a comparison of the survival of primed and naïve T cells must be made on their presence in the spleen and not the lymph nodes. Nevertheless, few cells, either naïve or primed, were found in the spleens of recipient mice. If primed KJ^+CD4^+ T cells were to behave like non-tg T

cells, then over time they would be expected to revert to a naïve pheotype. This would enable them to enter the lymph nodes. However this did not occur as very few primed KJ^+CD4^+ T cells were found in the LNs at any time points. Therefore it appeared that following antigen priming, KJ^+CD4^+ T cells did not survive and as a result did not give rise to a long-lived population of antigen experienced memory cells. This unexplained phenomenon prevented us from achieving our main aim, which was to compare the ability of naïve and memory T cells to help primed B cells.

The failure of KJ^+CD4^+ T cells to survive following antigen priming was difficult to explain. However when the survival characteristics of transgenic and polyclonal CD4 and CD8 T cells were examined, it was revealed that tg CD4 T cells had an intrinsically lower capacity for survival (Ferreira *et al.*, 2000). Following thymectomy A1 TCR and A18 TCR CD4 tg strains were shown to gradually lose their peripheral T cells over a period of 6 weeks (Ferreira *et al.*, 2000). This did not occur in the CD8 tg strains examined (Ferreira *et al.*, 2000). When these cells were examined for their susceptibility to apoptosis *in vitro* the tg CD4 T cells were observed to undergo apoptosis much more rapidly than their polyclonal counterparts (Ferreira *et al.*, 2000). In contrast the tg CD8 T cells examined did not differ in their susceptibility to apoptosis (Ferreira *et al.*, 2000).

Other studies using tg T cells have revealed curious results. For example 1.5 x 10^7 $CD4^+$ T cells from AND TCR tg mice (specific for pigeon cytochrome c) were transferred into thymectomized mice and their survival and phenotype were examined over time (Boursalian & Bottomly, 1999a). Of the starting population, 88-94% of the T cells expressed the Vα11/Vβ transgene and could respond to moth or pigeon cytochrome c (Boursalian & Bottomly, 1999a). The remaining cells expressed transgenic Vβ-chains paired with endogenous Vα-chains, and could therefore respond to environmental antigens (Lee *et al.*, 1996) (Linton *et al.*, 1996). However, by 3 weeks after transfer, the percentage of donor T cells which were $V\alpha11^+$ began to decline and by 15 weeks accounted for less than 20% of the transferred population (Boursalian & Bottomly, 1999a). At the same time the percentage of $V\alpha11^-$ and $V\alpha11^{int}$ T cells increased (Boursalian & Bottomly, 1999a). The total number of donor cells also declined up until about 6 weeks after transfer. At this point donor cells represented about half the starting level, and became a more stable population (Boursalian & Bottomly, 1999a). It appeared that the donor T cells which expressed the Vα11/Vβ transgene were not surviving, while the donor cells which expressed endogenous Vα

chains were proliferating due to the "empty space" in their new hosts. In support of this was the observation that the majority of Vα11$^-$ T cells had obtained an activated phenotype CD45RB^{lo}, CD62L^{lo} 3 weeks after transfer, while the majority of the Vα11$^+$ T cells retained a naïve phenotype (Boursalian & Bottomly, 1999a). Furthermore when the tg T cells were primed in intermediate hosts and transferred to secondary naïve recipients the tg cells could not be detected after 7 weeks (Boursalian & Bottomly, 1999a). The pattern of the AND TCR tg T cells appeared to reflect the poor survival pattern that we observed with DO11.10 T cells. In spite of these results, which were largely ignored, the authors chose instead to focus on a stable population of so called CD45RB^{lo} "memory" T cells which were most likely due to the proliferation of Vα11$^-$ T cells to environmental antigens (Boursalian & Bottomly, 1999a).

Despite these as well as our own observations, a study by Merica and colleagues (2000) had reported the survival and reversion to a naïve phenotype (CD45RB^{hi}, LFA-1^{lo}) of KJ$^+$CD4$^+$ T cells, 10 weeks after initial OVA/CFA injection. Following a secondary immunization the proliferation of the tg cells was much less than that following the primary immunization (Merica *et al.*, 2000). If these were true "memory" cells then this was not the expected behaviour. However, this defect was overcome when KJ$^+$CD4$^+$ T cells were primed with OVA/CFA in intermediate recipients and then transferred to a naïve environment. One week after transfer to the secondary recipients the KJ$^+$CD4$^+$ T cells reverted to a naïve phenotype and displayed the clonal expansion pattern of naïve cells (Merica *et al.*, 2000). Despite this they produced IL-2 more quickly than naïve cells and showed evidence of at least 4 cell divisions, proving that they were truly antigen-experienced cells (Merica *et al.*, 2000). However, no FACS data of the revertant cells were shown in the publication. London *et al* (1999) also reported the survival, and partial reversion to a naïve phenotype, of KJ$^+$CD4$^+$ T cells 10 weeks after initial immunization, although in this instance the cells were primed *in vitro*.

7.7 Antigen-specificity may determine T cell survival following priming.

Our observation that following antigen priming, KJ$^+$CD4$^+$ T cells initially proliferated but did not give rise to a population of long-lived antigen experienced cells, led us to hypothesise that the high affinity of the tg TCR for OVA peptide/MHC prevented the cells from surviving. The basis for this suggestion originated from the

study by Anderton *et al* (2001), who used the encephalitogenic, H-2 A^u-restricted, acetylated NH_2-terminal nonameric peptide (Acl-9) epitope from myelin basic protein to create a series of peptide analogues which displayed a hierarchy of affinities for A^u. Immunization of mice with the wild type antigen, the Ac1-9 peptide, activated the high affinity T cells which induced EAE (Anderton *et al.*, 2001). In contrast immunization with the strongly antigenic peptide (4Tyr) led to the activation and subsequent elimination of the high affinity T cells, thereby preventing disease (Anderton *et al.*, 2001). These authors showed that the strength of the antigenic signal could be altered by manipulating the peptide sequence which in turn varied the affinity of the peptide for MHC class II and hence immunogenicity (Anderton *et al.*, 2001). Using this model we proposed that if the signal to the T cell was determined by the strength of binding between MHC class II molecules and the peptide, then altering the peptide sequence should reduce the strength of the signal by reducing the affinity of the TCR for the peptide. By altering the sequence of the OVA peptide in such a way as to reduce the affinity of binding between peptide and MHC, we hoped to reduce the strength of the signal via the TCR and thereby prevent the KJ^+CD4^+ T cells from undergoing apoptosis.

Based on the crystal structure of the OVA peptide covalently linked to an I-A^d molecule (Scott *et al.*, 1998), as well as studies which determined the peptide epitopes recognized by DO11.10 T cells (Robertson *et al.*, 2000), it appeared that the core epitope for binding to the MHC was between position 329 and 337 of the OVA peptide. In order to alter the peptide we wanted as short a sequence as possible so that there were fewer potential residues for substitution. Therefore we synthesised two peptides with sequences: $OVA_{327-338}$ (OVA327) and $OVA_{328-338}$ (OVA328). Before considering possible amino acid substitutions that would alter the TCR affinity for MHC-peptide, we first wanted to examine how well the shortened peptides would stimulate DO11.10 T cells *in vivo* in comparison to full length OVA323. To do this we adoptively transferred 5 x 10^6 KJ^+CD4^+ T cells into BALB/cIgh recipients and challenged the mice i.p. with 0.05μmoles of either ap-OVA323, sol-OVA323, ap-OVA327, or sol-OVA327. The mice were examined for KJ^+CD4^+ T cells in the spleen, MLN, and ILN on days 3, 7, 14, and 21. Surprisingly, challenge with the OVA327 resulted in a much reduced proliferation of the KJ^+CD4^+ T cells compared to OVA323. However it did cause some stimulation as evidenced by the significant number of $CD45RB^{lo}KJ^+CD4^+$ T cells in all

tissues examined. When OVA328 was examined at 0.05μmoles and 0.5μmoles, it failed to induce any KJ^+CD4^+ T cells to become $CD45RB^{lo}$ or increase in number.

In the study by Robertson *et al* (2000), the T cell epitope recognised by KJ^+CD4^+ T cells was determined by creating a series of truncated peptides. The altered peptides were added to the tg cells *in vitro* and their ability to induce proliferation at various concentrations was measured. At the N-terminal end of the peptide little effect was seen until the removal of amino acid 327 (Robertson *et al.*, 2000). Significant proliferation to OVA327 did occur *in vitro*, although it was slightly reduced when compared with longer peptides (Robertson *et al.*, 2000). This was in contrast with our *in vivo* results, where the effect of OVA327 on KJ^+CD4^+ T cell proliferation was much reduced compared with OVA323. The reasons for this difference were not clear, although it could be due to the higher concentrations of the peptides *in vitro*. In the Robertson study OVA328 had a significantly impaired ability to cause proliferation of KJ^+CD4^+ T cells though it did stimulate T cells at high concentrations (1-100μM) (Robertson *et al.*, 2000). At the two concentrations we tested *in vivo* there was no evidence of KJ^+CD4^+ T cell activation or proliferation. We were also assuming that removal of the C-terminal position 339 had no effect, as removal of amino acids 339 and 338 had no effect *in vitro*. Nevertheless our studies showed it would be wrong to make the assumption that what was true *in vitro* was also reflected *in vivo*. We did not have a suitable control peptide with which to study the effect of the removal of amino acid 339.

Our first attempts to manipulate the affinity of the KJ^+CD4^+ T cells for an altered OVA peptide were inconclusive. The shortened peptides that we created were ineffective at stimulating a useful T cell response. However we did learn that position 328 was crucial for KJ^+CD4^+ T cell activation *in vivo* and that position 327 was also important, at least at the peptide concentrations we used. Our next strategy would be to examine amino acids 326-338 for possible substitutions, to reduce the binding affinity of OVA-peptide for MHC. We can then examine whether they will stimulate KJ^+CD4^+ T cells and prolong their survival.

7.8 Conclusions.

While there is no doubt regarding the importance of immunological memory in combating infection and disease, there are still some questions regarding how it is achieved. It is typically characterised as a faster and stronger response upon a secondary infection, but whether this is due to an increase in the number of cells that occur following the primary infection or whether it is due to specialized memory cells which arise out of that infection is still debated. There is clear evidence that at least for the humoral memory response there are memory B cells which are qualitatively different from naïve B cells (Tarlinton & Smith, 2000) (Liu & Banchereau, 1997). They produce Ig that is of a higher affinity and of different isotypes than the Ig produced in the primary response (Tarlinton & Smith, 2000). These changes occur in the germinal centre and are well documented (Han *et al.*, 1997) (Tarlinton & Smith, 2000). However, as T cell function is more difficult to measure than B cell antibody production, it is still not clear whether T cells undergo a similar change to B cells. Possible changes which may be beneficial to memory T cells are increased abilities to produce effector cytokines or to undergo cell division. Increased or decreased expression of certain adhesion molecules or chemokine receptors may also enhance the memory response. Alternatively T cell memory may merely be an increase in the number of antigen-specific cells that are available to provide help to the memory B cell.

This question of frequency over function can only be answered by comparing naïve and memory cells on a cell per cell basis. It is also vital that the cells share the same antigen-specificity. Therefore we aimed to use the DO11.10 tg model to compare the ability of naïve, primed, and memory $CD4^+$ T cells to help memory B cells. The form of the antigen was crucial in establishing an experimental model that precluded the T cell help required by naïve B cells during a primary response, from that needed by memory B cells for a secondary response. Numerous studies have shown that soluble antigen injection tolerizes the T cell response. However ourselves and others (Bell *et al.*, 2001), have shown that a soluble antigen challenge following an antigen-adjuvant immunization induces antibody synthesis from memory B cells, but not naïve T cells. This established that the help we measured was for a memory response.

As was illustrated, despite our best efforts we failed to generate a population of tg memory T cells to compare with naïve T cells. Nevertheless, our comparisons of naïve and primed T cells produced some interesting results. Surprisingly, little

difference was observed between naïve T cells and those that were primed in their ability to help memory B cells. The fact that antigen priming conferred little advantage suggested that frequency of the T cells may be the crucial factor in the memory response. This hypothesis was also supported by results from the titration of T cell help for memory B cells. These results clearly demonstrated that as the number of T cells was reduced so was the memory antibody response. Further evidence in favour of this, was the response of naïve BALB/cIgh mice when injected with primed B cells and challenged with sol-OVA. The observed response did not occur until 14 days after antigen challenge in contrast to 7 days for immunized mice. When naïve $KJ^{+}CD4^{+}$ T cells were transferred with primed B cells the response also occurred after 7 days. Therefore an adequate number of naïve antigen-specific T cells provided help as quickly as memory T cells from immunized mice. This is further evidence that memory is a result of increased frequency of antigen-specific T cells. However although these results demonstrate the importance of frequency for a memory response, qualitative differences between naïve and memory T cells cannot be ruled out. To test for these differences it is still necessary to compare the two cell types directly.

If an increase in the number of a particular antigen-specific cell is essential for immunological memory, then a relatively large percentage of effector cells will survive following the primary response. The signal that ensures this cell survival has yet to be determined and should form the basis of numerous future studies. However as the number of lymphocytes in a given animal is relatively fixed, if T cells of certain specificities survive preferentially, then they must take the place of other T cells. This may explain the reason for thymic involution. Shrinkage of the thymic lymphoid tissue begins after puberty and continues such that for humans at the age of 50 only 10% of the initial amount of thymic tissue is left (Romanyukha & Yashin, 2003). This means that production of new T cells is drastically reduced and is one reason why old people are more susceptible to new infections. Nonetheless, it makes perfect evolutionary sense. In the environment in which our immune systems evolved, encountering new diseases and infections later in life would have been a much rarer occurrence than in today's globalized world. In such a situation re-encounter with the same pathogens would be far more likely than encountering new ones. Therefore, it would have been evolutionarily advantageous to retain memory for diseases previously encountered at the expense of maintaining a diverse immune repertoire. Unfortunately we live in a world unlike our ancestors, where new diseases such as severe acute respiratory

syndrome (SARS) can occur in one corner of the globe and be on our doorstep in a matter of days.

The observation that although the number and functional activity of T cells declines with age, the population of B cells does not experience significant change (Romanyukha & Yashin, 2003), raises interesting ideas about memory. Unlike T cells it is known that functional changes occur with B cells following antigen encounter. Perhaps this tuning during the primary response alleviates the need for an increase in the frequency of the antigen-specific B cell. It was interesting that memory B cells could receive equal help from either naïve or primed T cells. Also of interest was the fact that memory B cells but not naïve B cells responded to sol-OVA. This may be due to the location of the response. As sol-OVA challenge does not result in the appearance of KJ^+CD4^+ T cells within the B cell follicles (Pape *et al.*, 1997a), then the secondary B-T cell conjugate must occur elsewhere. Deciphering this location requires further work. Also as T cells do not enter B cell follicles following sol-OVA challenge, stimulation of naïve B cells cannot occur.

It was disappointing that KJ^+CD4^+ T cells did not survive following priming to become long lived memory cells. This prevented us from asking whether memory is a result of a quantitative or qualitative change. Whether this unexplained phenomena will tell us more about the nature of T cell survival, or about inherent flaws in transgenic models remains to be seen. Nonetheless, our observations, as well as those reported by others (Boursalian & Bottomly, 1999a) (Ferreira *et al.*, 2000) raise questions about the suitability of tg models for studies on memory. The proposal by Anderton *et al* (2001) that the fate of the T cell is determined by antigen affinity was one we sought to explore using the tg model. However, further work is required before we can draw any conclusions on this.

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Appendix

Solutions for preparing cells and ELISAs.

PBSFCS
Phosphate Buffer Solution (?M; pH 7.2) containing 2% Fetal Calf Serum

Boyles Solution
450ml of 0.16M NH4Cl and 50ml of 0.17M Tris pH7.65
Adjust pH to 7.2 with HCl

PBS20
500ml PBS with 20 units/ml heparin

PBSTween
2.5L of PBS with 500ml of Tween

Blocking Buffer
MegaBlock from Bionostics, diluted 1/500 in PBSTween

Solutions for affinity purification of ELISA standard

Coupling Buffer
4.2g $NaHCO_3$ and 14.61g NaCl in 500ml H_20

Acetate Buffer
4.1g CH_3COONa + 500ml H_20
2.87ml CH_3COOH + 497.13ml H_20
Mix both to make up 1 litre

Elution Buffer
Glycine-hydrochloric acid buffer, pH 2.7. Titrate pH of 500ml of 0.2M glycine to 2.7 with 0.2 M HCl.

Ethanolamine
30.54ml $CH_2(OH)CH_2NH_2$ + 469.46ml H20

Neutralizing Buffer
1 M Tris-HCl pH9

Binding Buffer
0.2M Sodium Phosphate pH7

Amino Acids

A: Alanine
E: Glutamic acid
G: Glycine
H: Histidine
I: Isoleucine

N: Asparagine
Q: Glutamine
R: Arginine
S: Serine
V: Valine