A transgenic mouse model approach to investigate the interactions between T cells during the course of an immune response

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Preface

The work described in this thesis was designed to investigate the interactions between T cells during an immune response through the use of a number of different strains of T cell receptor transgenic mice

All experiments were carried out by the author at the Centenary Institute of Cancer Medicine and Cell Biology between February 2001 and April 2006. Approval for animal experimentation was obtained from Institutional Ethics Committee at the University of Sydney. The work is entirely original and has not been presented previously for purpose of obtaining another degree.

Recombinant HELMCC-his protein used in all experiments described in Chapter 4 and in the experiments described in Figure 5.7.1 and Figure 5.7.2 was produced by Didrik Paus (B Cell Biology Group).

Publication arising from work presented in this thesis

Alexandra J Spencer, Didrik Paus, Adrian L. Smith, Robert Brink, Barbara Fazekas de St Groth (2006). Antigen-specific competitive inhibition of $CD4^+$ T cell recruitment into the primary immune response (submitted)

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Abstract

The experiments described in this thesis document the development of two *in vivo* models, to investigate the effect of competition for peptide-MHC and factors independent of MHC on T cell proliferation, differentiation, generation of memory cells and affinity maturation.

The first model made use of 3 strains of T cell receptor (TCR) transgenic (tg) mice of varying specificity for antigen-MHC class II. To determine the effect of antigen specific and non-specific competition on the early stages of the T cell response, the efficiency with which naïve antigen-specific $CD4^+$ T cells were recruited into an ongoing immune response was investigated. Recruitment into cell division and cytokine production was shown to decrease with an increasing time delay between two cell cohorts of the same specificity, leading to a significant drop in recruitment with a delay of only 24 hours. Injection of additional antigen could partially compensate for this decrease, suggesting that lack of available antigen limited recruitment of specific cells trafficking to the node after the initiation of the response. A role for antigen non-specific factors such as access to APCs, costimulatory signals or cytokines was ruled out by showing that the response to a second, independent antigen was unaffected by an ongoing response, even when the same APCs were presenting both antigens.

The second system modelled a situation in which a clone of uniformly high affinity T cells competed against a polyclonal population containing mixture of affinities. This situation would arise during a normal response to a single epitope, and would mimic the process of competition that drives affinity maturation of the $CD4^+$ T cell response. By substituting a high affinity response to a different antigen, a more complex reaction to multiple antigens, of different affinities was modelled. To avoid any possible effect of the two antigens competing for access to processing machinery, or binding to the same MHC class II allele, the two antigens were provided as synthetic peptides that bind to different MHC molecules. The data indicated that $CD4^+$ T cell competition for peptide-MHC is far more

potent than competition between $CD4^+$ T cell responses of different specificity. Antigen-specific competition reduced the level of T cell stimulation detected as early as day 3 of the response. In the face of high affinity antigen-specific competition, the representation of mixed affinity T cells within the effector and effector memory cells (T_{EM}) population declined progressively throughout the primary and secondary responses, suggesting that continued access to peptide-MHC is required to maintain maximum numbers of effector and T_{EM} cells. In contrast, the contribution of central memory (T_{CM}) was stable from day 7 onwards. Competition by $CD4^+$ cells of an unrelated antigenic specificity led to a minor reduction in peak cell number and cytokine production in the primary response, without altering the number or potency of memory cells.

Together these two models demonstrated a mechanism whereby the immune system exerts tight control over the size and kinetics of each individual antigen specific response without affecting the ability to respond to secondary infections or late-phase lytic antigens. Overall the results demonstrate a continued requirement for TCR stimulation for the generation of effector cells and the maintenance of a population of cytokine producing memory cells. However the generation of a stable population of central memory cells was unaffected by conditions of reduced T cell stimulation, ensuring that long-term memory can be maintained in the absence of antigen.

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Abbreviations

Ag	antigen
AICD	activation induced cell death
APC	antigen presenting cell
B7RP-1	B7 related protein
BrdU	5-bromo-2'deoxyuridine
BM	bone marrow
BSA	bovine serum albumin
BTLA	B and T lymphocyte attenuator
C	constant
Ca^{2+}	calcium
CD	cluster of differentiation
CDR	complementarity determining region
CHO	chinese hamster ovarian
CFA	complete Freuds adjuvant
CFSE	carboxyfluorescein diacetate succinimidyl ester
CRD	cysteine rich domain
CrmA	cytokine response modifier A
Cyt-C	Cytochrome C
CTLA-4	cytotoxic T lymphocyte antigen-4
D	diversity
DC	dendritic cell
DAPI	4',6'-diamidino-2-phenylinodole dihydrochloride
ELISA	enzyme-linked immunosorbent assay
FADD	Fas associated death domain
FCS	foetal calf serum
FLICE	Fas-associated death domain-like IL-1β converting enzyme
FLIP	FLICE inhibitory protein
FW	facswash
GM-CSF	granulocyte/macrophage colony stimulating factor
HEL	hen egg lysozyme

^{hi}	high
ICAM	intracellular adhesion molecule-1
ICOS	inducible costimulator
IFN	interferon
Ig	Immunoglobulin
IL	interleukin
^{int}	intermediate
i.p.	intraperitoneal
IRAK	IL-1R associated kinase
i.v.	intravenous
J	joining
KO	knockout
LB	liquid broth
LC	Langerhans cells
Lck	$p56^{lck}$
LCMV	lymphocytic choriomeningitis virus
LFA-1	lymphocyte function-associated antigen-1
LN	lymph node
^{lo}	low
LPS	lipopolysaccharide
LRR	leucine rich repeat
mAb	monoclonal antibody
MBP	myelin basic protein
MCC	tobacco hornworm moth cytochrome C
MHC	major histocompatibility complex
NPP	nitrophenyl phosphate
Ova	ovalbumin
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCC	pigeon cytochrome c
PCR	polymerase chain reaction
PD-1	programmed cell death

PFA	paraformaldehye
PI	propidium iodide
PLP	proteolipid protein
PRR	pattern recognition receptor
RPMI	Roswell Park Memorial Institute (medium)
s.c.	sub-cutaneous
SPF	specific pathogen free
TBS	tris buffered saline
Tc	cytotoxic T cell
T_{CM}	T central memory
TCR	T cell receptor
T_{EM}	T effector memory
Tg	transgenic
T_h	helper T cell
TIR	Toll/IL-1R
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
V	variable
μg	micrograms

Chapter 1: Literature Review

The immune system is divided into two arms that are intertwined and highly dependent on each other. The innate immune response has no recollection of previous encounter to a particular pathogen and the same response is mounted on each subsequent exposure. The adaptive immune system has the ability to remember a previous pathogen and, on second exposure, mounts a more rapid and specific response. The adaptive immune system is composed predominantly of T and B lymphocytes that express clonally unique receptors capable of recognising one specific antigen (Ag) (Burnet 1957). It is these clonally unique receptors that enable the adaptive immune response to remember specific antigens.

The Inate Immune System

The innate immune system is evolutionary conserved (Hoffmann et al. 1999) designed to discriminate between self and non-self antigens (Janeway 1992). This is achieved through a variety of pattern recognition receptors (PRRs) that recognise evolutionarily conserved constituents of pathogenic micro-organisms, termed pathogen-associated molecular patterns (PAMPs) (Janeway 1989). PRR include serum proteins produced by the liver during the early stage of the response, several macrophage surface receptors and a number of intracellular recognition systems (Fraser et al. 1998; Janeway and Medzhitov 2002). Included within the group of PRR is the Toll-like Receptor (TLR) superfamily containing receptors expressed on the cell surface or within the cell on the endosome membrane (Beutler 2003; Takeda and Akira 2005; Takeda et al. 2003) (Figure 1.1). Activation of the innate system by PRRs aids in opsonisation and subsequent phagocytosis of bacteria, and can induce macrophages to synthesise anti-microbial peptides or nitric oxide. It is also becoming apparent that the innate immune system plays a critical role in the activation of the adaptive immune response (Medzhitov and Janeway

1997; Medzhitov and Janeway 1998) and in controlling the type of responses generated (Fearon and Locksley 1996; Medzhitov and Janeway 1997; Romagnani 1992).

Toll-Like receptors

TLRs are the most important group of PRRs. To date 11 TLRs have been identified (Figure 1.1), although TLR 10 and TLR 11 are non-functional in mice (Takeda and Akira 2005) and humans (Zhang et al. 2004) respectively.

It was the similarities between the signalling pathway mediated via interleukin 1 receptor in a mammalian cell lines when compared to the anti-fungal Toll/Dorsal system described in *Drosophila* (Lemaitre et al. 1996) that first led to identification of a human homologue of Toll (Medzhitov et al. 1997). A year later, resistance of C3H/HeJ and C57BL/10ScCr mice to lipopolysaccharide (LPS)-induced septic shock was shown to be a result of mutations within the TLR4 gene (Poltorak et al. 1998).

TLRs are composed of a leucine rich repeat (LRR) sequence located in the extracellular domain (or within the endosome), together with a Toll/IL-1R (TIR) domain located in the cytoplasm (Figure 1.1). The TIR domain is involved in recruiting adapter protein MyD88 and IL-1R-associated kinase (IRAK) (Medzhitov et al. 1998; Muzio et al. 1997; Wesche et al. 1997), enabling signalling through the NFκB pathway and JNK pathways (Muzio et al. 1998).

The key to selective activation of the innate system upon stimulation with pathogenic but not commensal microorganisms is due to the expression patterns of TLRs. For example, TLR5 recognises the flagellin protein present both on commensal and pathogenic strains of bacteria. However since TLR5 is predominantly expressed on the basolateral surface of endothelial cells, an inflammatory response is initiated only when endothelial cell invasion occurs, a characteristic of pathogenic bacteria (Gewirtz et al. 2001). TLR9 provides another example of the selective control over the immune system mediated by distinct expression patterns. It is expressed intracellularly and will only recognise CpG-DNA motifs when they are exposed following internalisation and bacterial lysis within the endosome (Ahmad-Nejad et al. 2002).

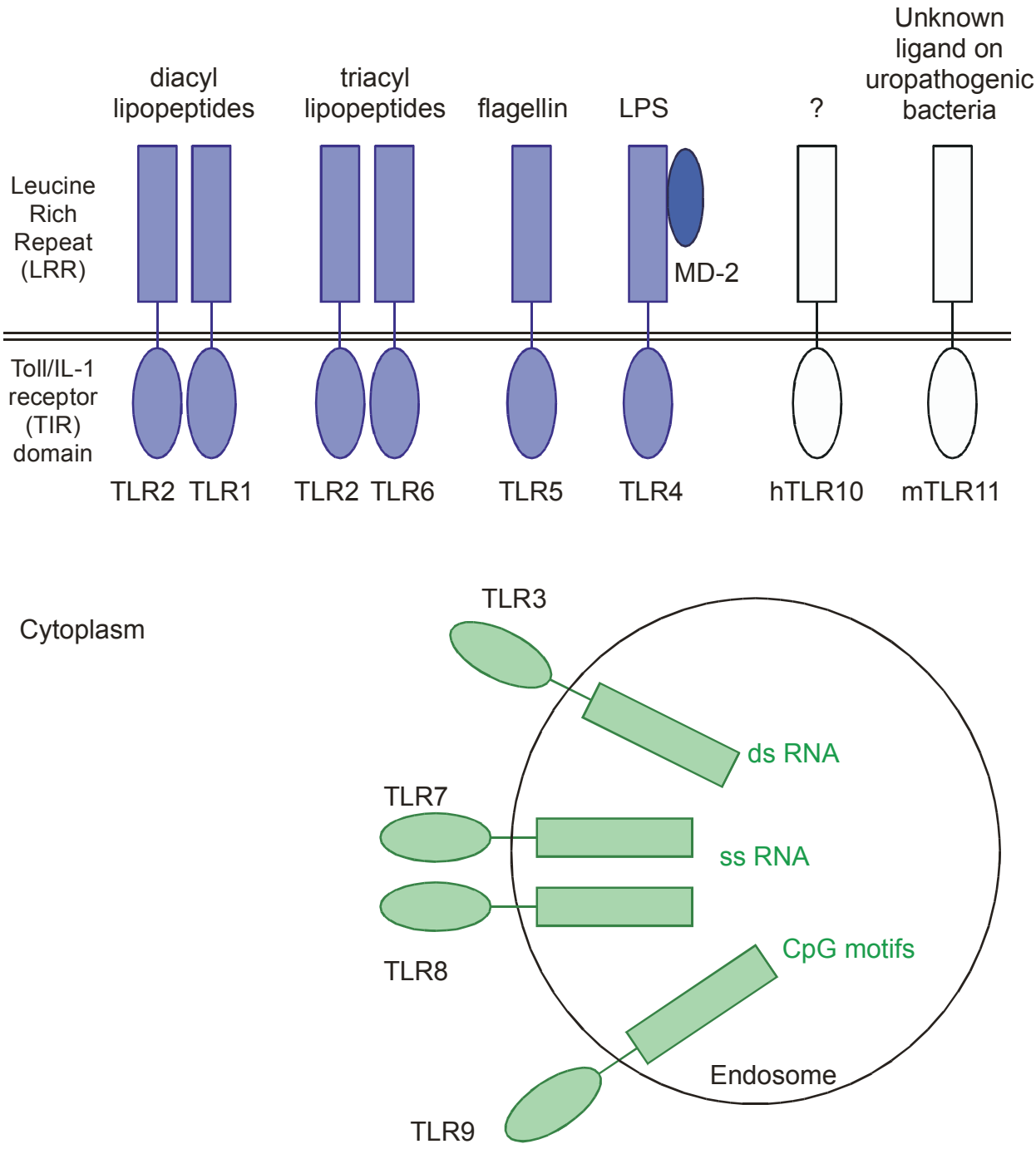


Figure 1.1 Toll Like Receptors (TLR) of the Innate Immune System
TLR recognize a variety of bacterial and viral components. Within the groups are TLR expressed on the cell surface or located within the cytoplasm and expressed on the wall of endosomes.

TLRs also provide a link between the innate and adaptive immune systems. Dendritic cells (DCs) express a number of TLRs (Muzio et al. 2000), and stimulation of DCs through TLR2 and TLR4 has been shown to induce DC maturation, as measured by decreased endocytic activity and increased expression of costimulatory molecules (Hertz et al. 2001; Michelsen et al. 2001). Recently it was demonstrated that the reduced ability of liver DCs to respond to LPS correlated with reduced expression of TLR4 (De Creus et al. 2005). Activation of TLRs also appears to play a critical role in determining the type of adaptive immune response generated. In MyD88 deficient mice, T helper type 2 but not T helper type 1 responses could be elicited following administration of antigen in complete Freunds adjuvant (CFA), a microbial rich emulsion (Schnare et al. 2001).

The Adaptive Immune System

The two predominate cells of the adaptive immune response are B and T lymphocytes. Both of these cell types express clonally unique receptors, but while B lymphocytes recognise intact protein antigens, T lymphocytes only recognise cognate antigen when presented as peptide loaded onto self major histocompatibility complex (MHC) molecules (Zinkernagel and Doherty 1974).

Despite this difference in antigen recognition, both cells undergo similar rearrangement of the genes encoding their antigen receptors (Tonegawa 1983). Under normal conditions, lymphocytes respond to foreign but not self-antigen, to which they are tolerant (Bretscher and Cohn 1970). Upon recognition of cognate antigen, both B and T cells undergo activation, proliferation and differentiation into effector and memory cells. It is these memory cells which mediate the more rapid response on second exposure to a particular antigen.

display the same antigen specificity (Fazekas de St. Groth et al. 1992; Ho et al. 1994; Jorgensen et al. 1992; Seder et al. 1992).

The genes encoding the α chain are located on chromosome 14 in both mice (Dembic et al. 1985) and humans (Caccia et al. 1985). The locus consists of multiple exons which are responsible for encoding the variable (V) and joining (J) regions (Arden et al. 1985; Becker et al. 1985; Chien et al. 1984; Hannum et al. 1984; Hayday et al. 1985; Saito et al. 1984; Winoto et al. 1985; Yoshikai et al. 1985) but only one constant region (Hayday et al. 1985; Yoshikai et al. 1985) (Figure 1.2). The genes encoding the β chain are located on mouse chromosome 6 and human chromosome 7 (Caccia et al. 1984). Similar to the α chain, multiple exons encode the V and J regions (Barth et al. 1985; Behlke et al. 1985; Gascoigne et al. 1984; Hedrick et al. 1984; Siu et al. 1984; Yanagi et al. 1984) but there are only two exons encoding a diversity (D) element (Clark et al. 1984; Siu et al. 1984) and two constant (C) regions (Gascoigne et al. 1984) (Figure 1.2). During TCR generation in the thymus, V, D and J elements of both the α and β chains undergo recombination events (Hedrick et al. 1984) such that one V exon will recombine with one J exon and a C single exon, or in the case of the β chain, a previously rearranged combination of D and J, and a C region (Snodgrass et al. 1985). The β chain genes are initially rearranged and expressed on the T cell surface, and upon the expression of a functional B chain, the α chain genes are subsequently rearranged (Chien et al. 1984; Raulet et al. 1985).

The highest region of TCR diversity is at the junction of V(D)J elements and this area corresponds to the third Ig hypervariable loop, termed CDR3 (Barth et al. 1985; Behlke et al. 1985; Fink et al. 1986) (Figure 1.2). The 2 other regions corresponding to Ig hypervariable loops, CDR1 and CDR2, are encoded within the V region and therefore only differ between α or β chains encoded by different selections of V regions. Initial work demonstrated that T cell clones specific for the same antigen all contained identical amino acid sequences in the region corresponding to CDR3 (Hedrick et al. 1988). Subsequently site-directed mutagenesis of this amino acids region was shown to abolish antigen specificity (Engel and Hedrick 1988; Kasibhatla et al. 1993; Nalefski et al. 1992) while

T lymphocytes

T lymphocytes are derived from a common haemopoietic precursor that migrates from the bone marrow (BM) to the thymus where cells undergo maturation and selection (reviewed by (Miller and Mitchell 1969). They are subdivided into two lineages depending on whether they express αβ or γδ T Cell Receptors (TCR). The predominant αβ TCR T cells express either cluster of differentiation (CD) molecules $CD4^+$ or $CD8^+$ (Spits et al. 1982; Swain 1981; Swain et al. 1984; Wilde et al. 1983). $CD8^+$ cytotoxic or killer T cells (Tc) are involved in cell-mediated immunity and direct killing of virally infected cells whereas $CD4^+$ helper T cells (Th) predominantly play a cytokine mediated helper role in activation of $CD8^+$ T cells or B cells . Both CD4 and CD8 T cells undergo the same maturation, TCR generation and selection events in the thymus but recognise peptides complexed to MHC Class II and Class I respectively.

αβ T Cell Receptor

The antigen specific TCR is a heterodimeric surface protein comprised of 2 glycosylated chains linked by disulphide bonds (Acuto et al. 1983; Allison et al. 1982; Haskins et al. 1983; Kappler et al. 1983; McIntyre and Allison 1983; Meuer et al. 1983; Meuer et al. 1983; Reinherz et al. 1983; Samelson et al. 1983). The TCR has both structural and sequence similarities to B cell immunoglobulin (Ig) (Chothia et al. 1988). The two chains contain both variable and constant regions (Kappler et al. 1983; McIntyre and Allison 1983) and are organised into Ig-like domains containing antiparallel β sheets (Novotny et al. 1986) with regions similar to the hypervariable loops of Ig (Arden et al. 1985; Barth et al. 1985; Becker et al. 1985; Patten et al. 1993). These domains are termed complementarity determining regions (CDR) (Davis and Bjorkman 1988). The majority of TCRs are composed of an acidic α and a basic β chain, while a minority consist of a combination of γ and δ chains (Davis and Bjorkman 1988). Antigen specificity was shown to reside in the genes encoding the α and β chains (Dembic et al. 1986; Saito et al. 1987), and this led the way for development of TCR transgenic mice in which most of the T cells

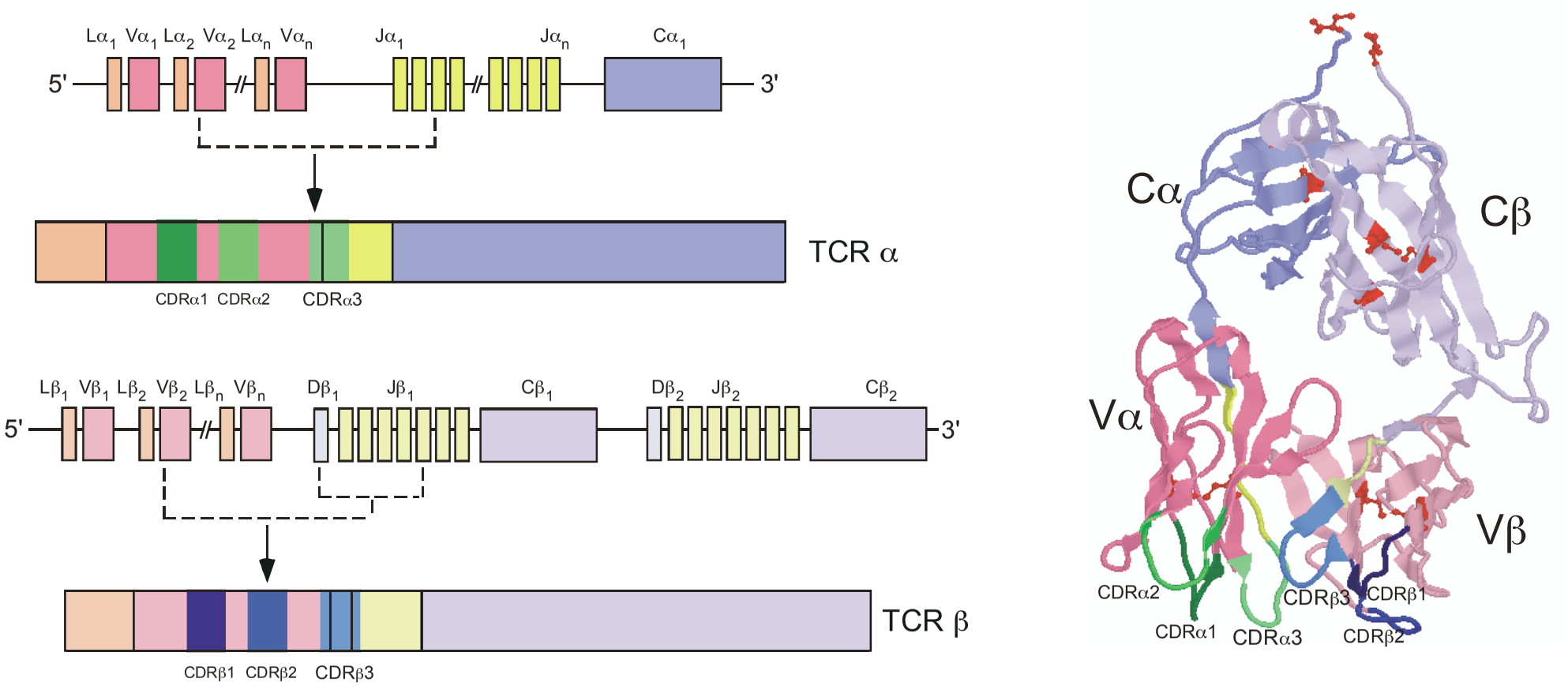


Figure 1.2: Gene segments of the TCRα and TCRβ chains and the crystal structure of a αβ TCR.
Both the α and β chains of the T Cell Receptor are encoded by a large number of Leader (L) and Variable (V) exons that recombine randomly with an exon of the Diversity (D) (only in the case of the β chain) and a Joining (J) regions, together with the Constant (C) region during T cell maturation in the thymus. After recombination of V(D)J segments the α and β chain are expressed together on the surface of the T cell and within each chain there are 3 Complementarity-determining regions (CDRs) which align over the peptide-MHC complex. CDR1 and CDR2 are encoded within the variable segment, while CDR3 is the region of highest diversity and is created by V(D)J recombination. Image of the TCR Crystal structure was adapted from Garcia et. al. (1996).

altering the amino-acid composition of the antigen resulted in corresponding selection of T cells with equivalent changes in amino acids within the CDR3 region (Jorgensen et al. 1992). All three CDRs are important in antigen recognition as only clones containing the same TCR backbone (Katayama et al. 1995) together with identical CDR3 regions will be specific for the same antigen-MHC complex (Patten et al. 1993).

Over the past decade, the roles of the different CDR elements in TCR binding to peptide-MHC has started to emerge. CDR1 and CDR2 are believed to be important in guiding the TCR into binding with MHC (Wu et al. 2002) and stabilising this interaction (Borg et al. 2005), while CDR3 is important for binding of the two α and β chains to peptide held within the binding grooves of MHC. Even prior to the crystallisation of the α chain (Fields et al. 1995), and β chain (Bentley et al. 1995) or a complete $\alpha\beta$ TCR complexed to MHC (Garcia et al. 1996), mutational studies suggested that the CDR3α chain bound to the amino terminal of the peptide, CDR3β bound to the carboxyl-terminal while chains bound to the central portion of the peptide (Jorgensen et al. 1992; Sant'Angelo et al. 1996). Finally, VDJ recombination, together with N-region diversification (Siu et al. 1984; Winoto et al. 1985) and α and β chain combinations (Fink et al. 1986) enables the generation a large number and diverse range of T cells with unique TCRs of varying specificity and affinity.

TCR associated molecules, CD3 and CD4 or CD8

CD3

The CD3 complex is a collection of polypeptides that are intimately associated with the TCR (Borst et al. 1983; Meuer et al. 1983; Oettgen et al. 1984; Weiss and Stobo 1984). The four chains of the CD3 complex include 25kDa γ, 21kDa δ, 26kDa ε and 16kDa ξ (Samelson et al. 1985). These polypeptides are expressed together as a heterodimer ($\delta\varepsilon$ and $\gamma\varepsilon$) or homodimer (ξ chains).

In early studies with CD3, a low concentrations of a monoclonal antibody against the CD3 complex was shown to stimulate T cells (Van Wauwe et al. 1980) but to have an inhibitory effect when administered at higher concentrations (Chang et al. 1981; Reinherz et al. 1983). Following on from these, original studies CD3 was subsequently shown to play a critical role in antigen recognition and signal transduction. Upon antigen recognition by the TCR, the 20kDa δ and 26kDa ε chains are phosphorylated by a protein kinase p56 ^{lck} (Lck) (Cantrell et al. 1985; Oettgen et al. 1986; Samelson et al. 1985; Samelson et al. 1985; Samelson et al. 1986) which subsequently leads to signal transduction and the transcription of growth factor IL-2.

CD4

CD4 is an integral membrane protein (Maddon et al. 1985) expressed on the surface of MHC Class II restricted helper T cells (Allison and Lanier 1985; Dialynas et al. 1983; Swain et al. 1984). CD4 contains 4 extracellular Ig domains and associates with Lck in the cytoplasmic domain. The outer-most extracellular Ig domain (D1) binds to the cleft created between the α_2 and β_2 domains of MHC Class II (Cammarota et al. 1992; Konig et al. 1992; Wang et al. 2001).

Initially it was believed that binding of CD4 to MHC II mediated cell adhesion and stabilised the interaction of the TCR to MHC (Doyle and Strominger 1987). More recently binding kinetics studies have shown that the relative binding of CD4 to MHC II is of low affinity and does not alter the strength of the interaction between TCR and MHC (Xiong et al. 2001). Hence the primary role of CD4 is to recruit Lck into the peptide-MHC TCR complex after initial antigen recognition by the TCR (Xiong et al. 2001) thereby enabling the initiation of signal transduction pathways.

CD8

CD8 was shown to play a role in T cell function after a monoclonal antibody was found to block T cell cytotoxic activity (Swain 1981). The CD8 molecule is composed of 2 disulphide linked chains which are expressed in humans as a homodimer of two 34kd

CD8 α chain (Snow and Terhorst 1983). By contrast, mice express CD8 as either a heterodimer consisting of a 38kd α and a 30kd β chain or a 34kd α' and a 30kd β chain (Ledbetter et al. 1981; Reilly et al. 1980). CD8 binds to the α3 domain of MHC Class I (Gao et al. 1997; Giblin et al. 1994; Salter et al. 1990) which was initially thought to play a role in the initiation of cell-cell interations (Norment et al. 1988) and stabilisation of the TCR-MHC complex (Garcia et al. 1996). However recent biacore studies have demonstrated that CD8 binds to MHC without any effect on the strength of TCR binding to peptide-MHC, thereby excluding a role for the molecule in the initiation of cell adhesion (Wyer et al. 1999). Therefore, like the role of CD4 in T cell activation, CD8 is believed to act primarily by recruiting Lck into the TCR peptide-MHC complex only after TCR recognition of specific peptide-MHC complexes.

Positive and Negative Selection

Although the predicted possible number of different V(D)J and αβ chain combinations is around 10^{15} (Davis and Bjorkman 1988), elimination of non-functional and self-reactive clones by negative (and positive) selection leads to contraction of the peripheral T cell repertoire. Recent studies estimate that the number of different clones present in a mouse spleen is approximately 2×10^6 (Casrouge et al. 2000) compared to 2.5×10^7 different clones in human peripheral blood (Arstila et al. 1999). Positive and negative selection of T lymphocytes is a critical process, ensuring peripheral T cells will be able to recognise foreign antigens when presented on self-MHC while eliminating potentially self-reactive clones.

Positive selection is the process by which T lymphocytes become educated to self-MHC molecules (Fink and Bevan 1978; Zinkernagel et al. 1978). Blocking CD8 binding to MHC Class I either through administration of an anti-MHC Class I monoclonal (Marusic-Galesic et al. 1988) or through targeted deletion of light chain β2-microglobulin (Koller et al. 1985; Zijlstra et al. 1990) leads to a deficiency in the number and frequency of $CD4^-CD8^+$ cells. Conversely administration of anti-MHC Class II antibodies (Kruisbeek et al. 1985) or

targeted deletion of MHC Class II (Grusby et al. 1991) leads to a deficiency in $CD4^+CD8^-$ cells.

Negative selection refers to the process by which T cells that are specific for self-antigens are deleted during maturation in the thymus (Kappler et al. 1987). Negative selection requires the expression of self-antigens on thymic epithelia interstitial DCs (Brocker et al. 1997) or medullary epithelia cells (Anderson and Shaw 2005) and is a process that occurs continuously throughout thymic development (Baldwin et al. 1999). Overall negative selection accounts for the 50-60% loss of T cells that have already survived positive selection (van Meerwijk et al. 1997).

As a result of selective events during T cell maturation in the thymus, the T cell repertoire is purged of non-functional cells incapable of recognising cognate antigen when bound to self-MHC molecules while also eliminating potentially harmful self-reactive clones.

Antigen Presenting Cells

From the midsomite stage of embryonic development, all somatic cells have the capacity to express MHC Class I when stimulated in response to inflammatory signals such as interferon (IFN) (Heron et al. 1978). As a consequence, all cells are capable of presenting antigen to $CD8^+$ T cells via MHC Class I. However the term “antigen presenting cells” (APCs) is reserved for a group of cells that constitutively express a basal level of MHC Class II, which can be upregulated to enhance antigen presentation to $CD4^+$ T cells. B cells, macrophages and DCs are all referred to as APCs, but while B cells and macrophages interact with primed T cells to elicit helper cell functions, DCs are believed to be the most important subgroup of APCs in activating naïve $CD4^+$ T cells. Adoptively transferred antigen primed DCs are able to stimulate strong T cell responses *in vivo* (Inaba et al. 1990) and act as the main mediators of antigen-presentation after *in vivo* administration of antigen (Crowley et al. 1990; Guery et al. 1996).

DC Morphology and Distribution

In the 1860s, cells with “dendrite” morphology in the skin were identified and termed Langerhans cells (LCs). It wasn’t until many years’ later that a similar cell was described in secondary lymphoid tissues. Using preparations of glass adherent mouse splenic cells Steinman (1973) identified DCs on the basis of their distinct morphology (large cytoplasm and nucleus and pseudopods of varying length, width, form and number). The name “dendritic cells” was coined because these cells had the ability to assume a variety of branching forms containing fine processes that were noted to constantly extend and retract. In a subsequent paper (Steinman et al. 1974), DCs were shown to be morphologically and functionally distinct from lymphocytes and macrophages and able to undergo substantial turnover in the spleen despite no evidence of proliferation.

DCs are found in a number of non-lymphoid as well as lymphoid tissues (reviewed by Steinman (1991) where antigen presenting roles differ depending on their location. For example, interstitial DCs in the thymus play a critical role in negative selection due to their capacity to present self antigens during T cell maturation (Brocker et al. 1997).

A considerable amount of work in the past 20 years has attempted to sub-divide DCs into different functional subsets based on the expression of surface proteins CD11c, CD11b, DEC205 (CD205), MHC Class II, CD4, CD8, Langerin (CD207) (reviewed by (Liu 2001; Pulendran et al. 2001; Shortman and Liu 2002). CD11c is expressed on all sub-types of DCs but not on B cells or macrophages (Metlay et al. 1990). Given that the expression of the other molecules mentioned above can change as a consequence of activation or even location (Vremec and Shortman 1997), classifying DCs on the basis of CD markers has proved unsatisfactory.

A better way of classifying DCs has come from the discovery of two distinct lineages, lymphoid and myeloid. Myeloid DCs arise from a common BM precursor (Inaba et al. 1993) present in peripheral blood that can differentiate into granulocytes, macrophages or DCs following the addition of tumour necrosis factor (TNF) and granulocyte/macrophage colony-stimulating factor (GM-CSF) (Inaba et al. 1992; Inaba et al. 1992; Reid et al. 1990; Reid et al. 1992). On the other hand lymphoid DCs are derived from a $CD34^+$ thymic

precursor that can also give rise to T, B and NK cells (Ardavin et al. 1993; Galy et al. 1995; Marquez et al. 1998). Based on the location in which lymphoid and myeloid DCs predominantly reside, a functional differentiation between these cells has been proposed (Fazekas de St. Groth 1998). Myeloid DCs (including LCs and interstitial DCs) reside in peripheral tissues and upon activation via microbial signals migrate to draining lymph nodes where they stimulate immunogenic T cell responses. Lymphoid DCs predominantly reside within secondary lymphoid tissues where they present self-antigen and induce T cell tolerance in the absence of microbial signals.

More recently it has been proposed that DCs should be sub-divided based on their activation state (Wilson and Villadangos 2004). The original DC paradigm suggests that DCs in peripheral tissues are immature until encounter with pathogenic signals, following which they mature and migrate to secondary lymphoid tissue and present peripheral antigen to T cells (Banchereau and Steinman 1998). Conversely, secondary lymphoid tissues are thought to predominantly contain DCs with an immature phenotype that have high antigen uptake but express lower levels of MHC Class II expression (Wilson et al. 2003). Immature DCs are believed to induce toleragenic T cell responses due to the low level expression of costimulatory molecules and antigen-MHC complexes. However, upon microbial signals these immature DCs are believed to differentiate into mature cells capable of inducing immunogenic responses (Wilson et al. 2004).

Antigen Uptake and Processing

While it was established in the 1970s that T cells could only recognise antigen in association with self-MHC (Zinkernagel and Doherty 1974), it took several different lines of investigation to show that APCs must take up and degrade soluble proteins before T cell stimulation could be induced (reviewed by Schwartz (1985). These initial studies demonstrated a time-delay between the addition of antigen and the ability of APCs to activate T cells (Kleijmeer et al. 1995; Ziegler and Unanue 1981). Moreover agents that raised the pH of lysosomes led to a reduction in antigen processing (Stossel et al. 1990) and abolished the ability of APCs to stimulate $CD4^+$ T cells (Ziegler and Unanue 1982).

Finally T cells were shown to be stimulated by both denatured and globular proteins or even small peptide fragments (Matis et al. 1982; Shimonkevitz et al. 1984).

Typically, endogenous antigens such as viral proteins and tumour antigens, are presented to $CD8^+$ T cells via the MHC Class I processing pathway, while $CD4^+$ T cells recognise exogenous antigens. However some level of cross presentation occurs enabling the presentation of exogenous antigens to $CD8^+$ T cells (Reis e Sousa and Germain 1995; Svensson et al. 1997).

There are 3 main mechanisms of antigen uptake; macropinocytosis, phagocytosis and receptor mediated endocytosis (reviewed by (Austyn 1992; Lanzavecchia 1996; Watts 1997).

Macropinocytosis refers to the endocytosis of bulk-fluid mediated by membrane ruffling and the formation of large vesicles (Sallusto et al. 1995). This process enables DCs to take up large volumes of fluid and concentrate antigen.

Phagocytosis of microbes by macrophages is not only a critical step in the initiation of the innate immune responses (as discussed earlier) but is also important in the clearance of apoptotic cells from sites of tissue damage or infection (reviewed by Aderem (1999). Phagocytosis is an important means by which LCs and DC precursors take up antigen such as whole bacteria, viruses, yeast cell wall proteins and even latex beads (Inaba et al. 1993; Reis e Sousa et al. 1993; Svensson et al. 1997).

Finally, receptor mediated endocytosis is a common mechanism of antigen uptake by all subtypes of APCs. This process can utilise a number of cell surface receptors, for example the binding of yeast cell wall protein by the mannose receptor has been shown to initiate endocytosis by macrophages and DCs (Sallusto et al. 1995). B cells take up antigen that has initially bound to their BCR, while both B cells and DCs internalise immune complexes bound to surface Fc receptors (Sallusto and Lanzavecchia 1994). The c-type lectin DEC-205 has also been shown to play an important role in the uptake of glycosylated proteins by DCs (Jiang et al. 1995).

Once taken up by APCs, proteins are degraded by catalytic enzymes proteasomes and broken down into small amino-acid sequences (reviewed by (Monaco and Nandi 1995).

Newly synthesised MHC Class II molecules enter the endosome where they become bound to peptide to MHC complexes prior to transportation to the cell surface. In contrast, endogenous proteins are digested by proteasomes in the cytoplasm and transported into the endoplasmic reticulum where they become bound to MHC Class I molecules.

DC Maturation and Migration

Naïve T and B lymphocytes are only capable of circulating between blood and secondary lymphoid organs, therefore for T cell antigen recognition to occur, antigen must be transported to secondary lymphoid organs from the site of entry infection.

A characteristic feature of immature DCs and LCs is high endocytic and antigen processing capacity (Nijman et al. 1995). Although immature DCs express low levels of surface MHC Class II they synthesise a large amount of MHC Class II (Kampgen et al. 1991; Pure et al. 1990), concentrated at the site of antigen processing (Nijman et al. 1995; Pierre et al. 1997). Upon culturing *ex vivo*, maturation is associated with a decrease in endocytic activity and MHC Class II synthesis. However an increase in the expression of surface MHC Class II expression leads to an enhanced capacity to stimulate T cells (Kampgen et al. 1991; Nijman et al. 1995; Pure et al. 1990; Reis e Sousa et al. 1993; Romani et al. 1989; Schuler and Steinman 1985; Streilein and Grammer 1989). *In vitro* DC maturation can also be induced with the addition of GM-CSF, TNF or CD40L (Sallusto and Lanzavecchia 1994), or through the stimulation of DCs via TLRs (Hertz et al. 2001; Michelsen et al. 2001; Muzio et al. 2000). These inflammatory signals initiate the binding of antigen to MHC Class II (Inaba et al. 2000) and increase the half-life of MHC recycling from 10 hours to over 100 hours, leading to an accumulation of peptide loaded MHC Class II complexes on the surface of DCs thereby enhancing the ability of DCs to stimulate T cells (Cella et al. 1997).

Maturation of DCs *in vivo* has been shown to accompany migration of LCs from dermal layers of the skin into draining lymph nodes (Larsen et al. 1990). Original migration studies demonstrated migration of LCs into draining lymph nodes as early as 30 minutes after skin painting with FITC (Macatonia et al. 1987) or sub-cutaneous injection into the

foot-pad (Kupiec-Weglinski et al. 1988). Migrating DCs localise to the T-dependent areas of the node (Austyn et al. 1988) with migration plateauing around 24 hours-although T cell stimulatory capacity is retained for up to 3 days (Macatonia et al. 1987). Migration of LCs migration can be enhanced under conditions of local inflammatory responses (Larsen et al. 1990; Lukas et al. 1996) while systemic administration of LPS, TNF and IL-1 can also induce rapid migration of interstitial DC from peripheral organs (Roake et al. 1995).
The process of DC maturation and migration enables transportation of peripheral antigen from the site of an infection to the draining lymph node where naïve T cells are then able to scan a repertoire of presented antigens.

T cell Activation

TCR binding to peptide-MHC

The critical step in the initiation of a $CD4^+$ T cell response is binding of the TCR to the specific antigen-MHC complex. TCR antigen recognition leads to phosphorylation of the CD3 complex by tyrosine kinase Lck after which a signal transduction pathway is initiated. After a series of phosphorylation events, calcium (Ca^{2+}) is released from intracellular stores and transcription of IL-2 is initiated (Gardner 1989; Nisbet-Brown et al. 1985; Shapiro et al. 1985).
Ca^{2+} release can be achieved following the interaction of the TCR with only one peptide-MHC complex (Irvine et al. 2002), however prolonged signalling through the TCR is required for full activation and production of IL-2 (Iezzi et al. 1998). Initial estimates suggested that naïve cells require at least 20 hours of stimulation to commit to proliferation and differentiation (Iezzi et al. 1998). The length of antigenic stimulation can have a substantial influence on the quality of the response generated. Reducing the period of antigen presentation can not only reduce the level of proliferation but also the ability of cells to secrete IFN-γ (Obst et al. 2005). Prolonged signalling not only commits cells into proliferation and differentiation but also leads to enhanced survival due to

increased expression of pro-survival molecules such as Bcl-2 and Bcl-x_L (Gett et al. 2003).

Recent reports suggest that prolonged signalling may not be mediated through the association of the TCR with a single peptide-MHC complex but may be achieved through the summation of signals received through multiple encounters with peptide-MHC complexes (Depoil et al. 2005; Faroudi et al. 2003; Gunzer et al. 2000; Huppa et al. 2003; Mempel et al. 2004). Following on from work demonstrating that T cell-DC interactions were short-lived, Faroudi and colleagues demonstrated that $CD4^+$ T cell proliferation could be achieved through multiple intermittent signalling events (Faroudi et al. 2003). More recently $CD8^+$ T cells were also shown to be stimulated by multiple TCR-peptide-MHC encounters (Stone and Stern 2006). The ability of T cells to be stimulated by multiple intermittent signalling events may explain how a small number of peptide-MHC complexes can activate a large number of T cells (Valitutti et al. 1995). Direct visualisation studies suggest that T cell activation occurs in 3 phases, an initial period of short DC-T cell interactions, followed by a period of longer interactions and finally a period of cell division and high motility (Mempel et al. 2004).

Despite an ability to be stimulated through multiple different TCR-MHC encounters, recent estimates suggest that at least 10 hours of TCR signalling is required for full effector function (Huppa et al. 2003). Prolonging the time of TCR peptide-MHC association is achieved through cytoskeleton rearrangement and the formation of an immunological synapse (Grakoui et al. 1999; Monks et al. 1998; Schwartz 1990; Valitutti et al. 1995; Wulfing et al. 1998).

Immunological Synapse

Formation of an immunological synapse (Figure 1.3) serves to enhance T cell activation by stabilising interactions between TCR and peptide-MHC complexes and prolonging TCR peptide-MHC association time. After antigen recognition, TCR bound to peptide-MHC accumulates in the centre of the complex and becomes surrounded by lymphocyte

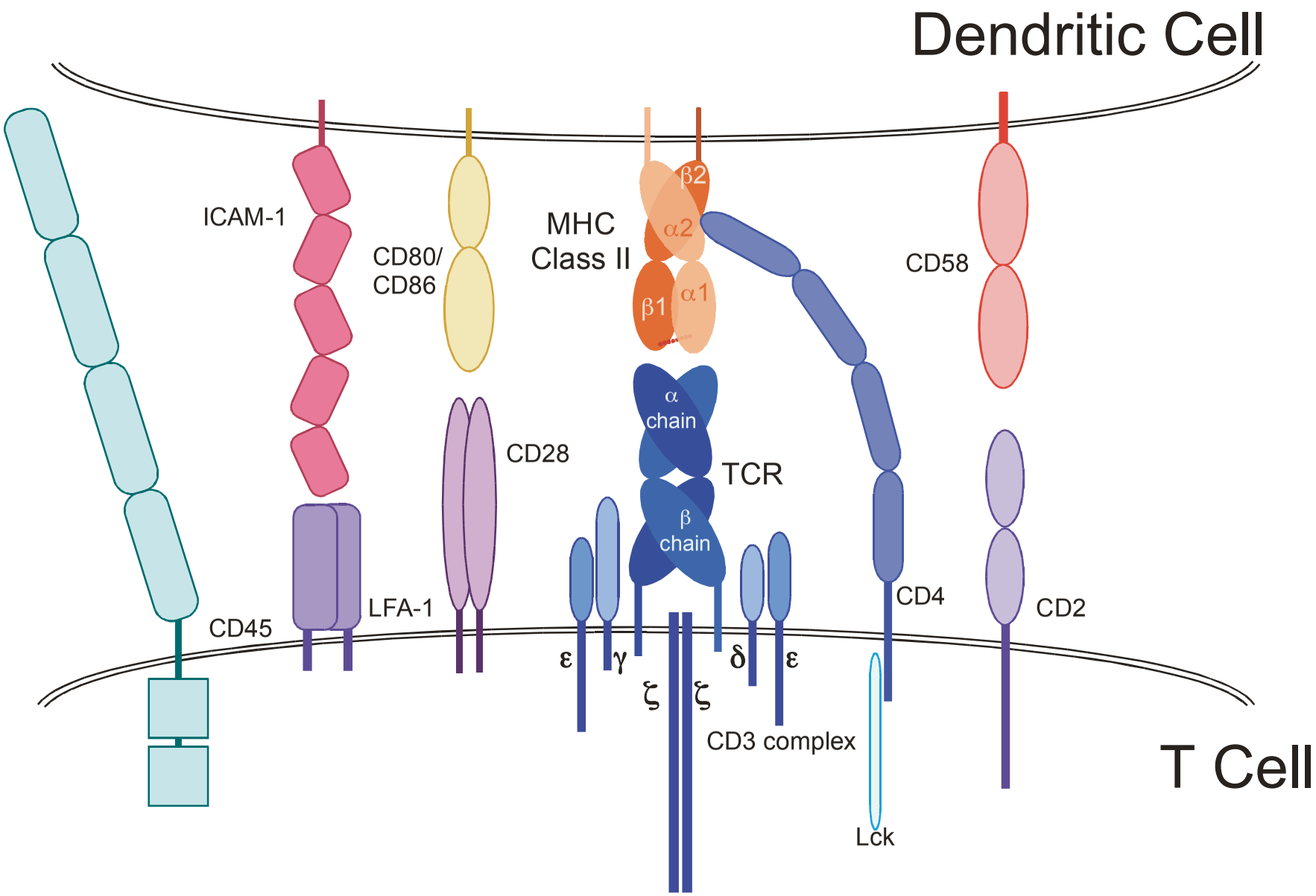


Figure 1.3: A schematic diagram of the immunological synapse

function-associated antigen-1 (LFA) (T cell surface) that is bound to the intracellular adhesion molecule-1 (ICAM) (present on APCs) (Monks et al. 1998). In so doing, molecules involved in T cell activation, eg protein kinase C-θ (Monks et al. 1997) CD3ζ (Krummel et al. 2000) and CD4 or CD8 (Potter et al. 2001), become concentrated into a small space that promotes continuous intracellular signalling. Immunological synapses will not form in the absence of antigen (Monks et al. 1998) or with antagonist peptides (Viola et al. 1997), and can be dissolved if TCR peptide-MHC interactions are disrupted (Faroudi et al. 2003; Huppa et al. 2003; Valitutti et al. 1995). CD2 is another adhesion molecules that plays a role in synapse formation and can therefore decrease the threshold for T cell activation (Bachmann et al. 1999). Binding of CD2 to its ligand CD48 (in rodents, but CD58 in humans) initiates a process of clustering and cytoskeletal rearrangement (Dustin et al. 1998). While LFA-1 binding to ICAM and CD2 binding to CD48 are independent events, together they are able to shift the dose of antigen required for T cell activation by around a factor of 100 (Bachmann et al. 1999). Preventing immunological synapse formation through blocking antibodies (Davignon et al. 1981) or through the absence of LFA (Kandula and Abraham 2004) does not prevent T cell activation but merely reduces the level of T cell stimulation, leading to reduced proliferation and cytokine production.

Costimulatory Molecules

In a series of papers by Jenkins and Schwartz, stimulation of T cells through the TCR without a second signal led to a state of T cell unresponsiveness, termed “anergy” (Jenkins et al. 1988; Jenkins et al. 1987). DCs express a basal level of some of these costimulatory ligands (secondary signals) and upon stimulation by TLR or other innate receptors, the level of surface expression is increased. The two signal model provides a mechanism to maintain tolerance to peripheral self-antigen under steady-state conditions while enhancing antigen specific responses after stimulation of the immune system by pathogenic signals. Since the early experiments of Jenkins and Schwartz, a large number of costimulatory molecules have been identified and include molecules that can induce either positive or negative signals. Costimulatory molecules can be sub-divided into 2

major groups, namely the B7 superfamily and the tumor necrosis factor receptor (TNFR) superfamily (Figure 1.4).

B7 superfamily

CD28/CTLA-4 and CD80/CD86

CD28 is an 80kDa (mouse) or 90kDa (human) homodimeric protein expressed constitutively on the surface of T cells (Gross et al. 1990; Martin et al. 1986; Weiss et al. 1986). Stimulation with anti-CD28 antibodies alone cannot induce T cell activation, instead CD28 signalling synergises with TCR signalling to enhance Ca^{2+} release and IL-2 production (Harding et al. 1992; Martin et al. 1986; Weiss et al. 1986). In the absence of CD28, T cells can still be activated with peptide, however a higher dose of peptide is required to reach the same degree of proliferation with cells producing a reduced amount of IL-2 (Lucas et al. 1995). Overall this would support the notion that CD28 tunes the threshold of T cell activation through the induction of IL-2 (Sharpe and Freeman 2002).
Cytotoxic T Lymphocyte Antigen 4 (CTLA-4) was the second member of the B7 family to be identified (Brunet et al. 1986). The majority of CTLA-4 is present in the cytoplasm, while surface expression is cell cycle dependent and only increases after TCR activation and cell division (Alegre et al. 1996). CTLA-4 acts as a negative regulator of T cell proliferation (Walunas et al. 1994) controlling the size (Chambers et al. 1999; Doyle et al. 2001; Kuhns et al. 2000) and repertoire of the $CD4^+$ response (Kuhns et al. 2000). Blocking studies provided the first indication that CTLA-4 induced a negative signal (Walunas et al. 1996). This was further supported by the generation of a knockout (KO) mouse line, characterised by development of a severe lymphoproliferative disorder leading to death within the first 4 weeks after birth (Tivol et al. 1995). As eluded to earlier, CTLA-4 regulation is antigen activation dependent, since TCR-tg mice on a CTLA-4 KO background do not develop lymphoproliferative disease (Chambers et al. 1999).
Two ligands for CD28 and CTLA-4 have been identified, CD80 (B7.1) (Freeman et al. 1989; Freeman et al. 1991; Yokochi et al. 1982) and CD86 (B7.2) (Azuma et al. 1993; Freeman et al. 1993; Hathcock et al. 1993). The extracellular domain of both CD80 and

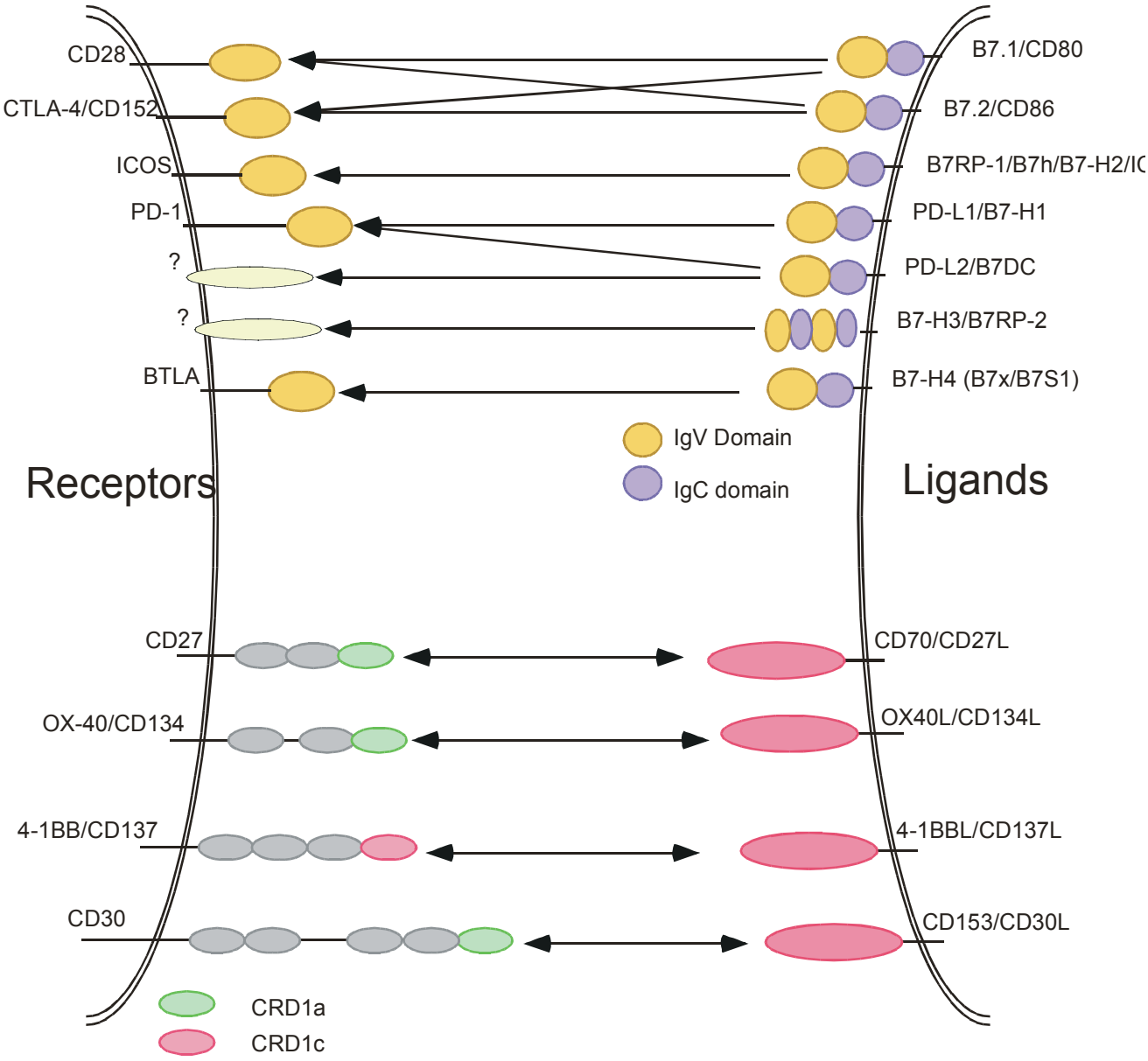


Figure 1.4: Receptor Ligand Interactions
The top panel illustrates the interaction between receptors and ligands of the B7 superfamily of proteins (adapted from Sharpe and Freeman 2002) with their common IgC and IgV like domains.
The bottom panel illustrates the interactions between members of the TNF superfamily (adpated from Locksley et. al. 2001). Common cysteine rich domains (CRD) are indicated.

CD86 consists of two Ig-like domains (Azuma et al. 1993; Freeman et al. 1989; Freeman et al. 1993). DCs and B cells express a basal level of CD80 and CD86 expression that is increased upon cell activation. CTLA-4 binds to CD80/CD86 with higher affinity (van der Merwe et al. 1997) but since it is not expressed until after T cell activation, T cells can still receive positive signals through CD28 during the induction stage of the response.

Other B7 members

Inducible costimulator (ICOS) and B7 related protein (B7RP-1) (also termed B7h and ICOS-L) are another stimulatory receptor ligand pair of the B7 family. ICOS is a homodimeric protein whose expression, as its name would suggest, is induced on T cells following activation. T cell stimulation via ICOS does not enhance IL-2 production but superinduces the production of IL-10 (Hutloff et al. 1999; Yoshinaga et al. 2000). Its ligand, B7RP-1, is a type-1 transmembrane protein with around 20% homology to CD80 and CD86 (Swallow et al. 1999; Yoshinaga et al. 2000). TNF-α enhances B7RP-1 expression on B cells and monocytes while inhibiting expression on DCs (Yoshinaga et al. 2000). Unlike the other members of the B7 family, TNF-α can induce the expression of ICOS-L on non-lymphoid tissues such as kidney, liver, peritoneal cavity and testes (Swallow et al. 1999). Stimulation via ICOS-B7RP-1 interactions is dependent on initial T cell activation via CD28 (Riley et al. 2001; Yoshinaga et al. 2000) and appears to play a predominant role in T-dependent B cell responses and isotype switching. Thus ICOS KO mice produced reduced amounts of antigen specific IgE and IgG1 upon immunisation with whole protein (Dong et al. 2001) or infection with *Leishmania mexicana* (Greenwald et al. 2002).

Programmed cell death (PD-1) is another receptor of the B7 superfamily originally identified by subtractive hybridisation of a T cell hybridoma undergoing apoptosis (Ishida et al. 1992). It is expressed on the surface of activated T and B cells (Agata et al. 1996; Ishida et al. 1992) and has been shown to induce decreased proliferation and cytokine production. PD-1 is also believed to play a role in T cell exhaustion, since blocking PD-1 can enable the resolution of chronic infections (Barber et al. 2006). Two ligands for PD-1

have been identified, PD-L1 (also termed B7-H3) and PD-L2 (also termed B7DC). In light of data demonstrating stimulatory (Dong et al. 1999; Tseng et al. 2001) and inhibitory (Freeman et al. 2000; Latchman et al. 2001) effects of both PD-L1 and PD-L2 mediated stimulation, it is now proposed that there is a second currently unidentified receptor expressed on T cells that mediates positive signalling via these two ligands.

B and T lymphocyte attenuator (BTLA) is the latest inhibitory receptor of the B7 family to be identified (Watanabe et al. 2003). Like the other inhibitory receptors, expression is limited to activated T and B cells and is associated with reduced cytokine production and lymphocyte proliferation (Prasad et al. 2003; Sica et al. 2003; Watanabe et al. 2003). The ligand for BTLA (B7-H4/B7S1/B7x) is expressed on a variety of peripheral tissues and surface expression has been shown to be induced on T cells, B cells, monocytes and DCs by *in vitro* activation (Sica et al. 2003).

B7-H3 is the sixth member of the B7 family, displaying 20-30% homology to other ligand members, and binds to a currently unidentified receptor on activated T cells (Chapoval et al. 2001; Sun et al. 2002) that is distinct from CD28, CTLA-4, ICOS and PD-1 (Chapoval et al. 2001). Similar to ICOS-L, it is expressed on lymphoid and non-lymphoid tissues, and treatment with LPS can upregulate surface expression (Prasad et al. 2004). There are conflicting data on the stimulatory ability of B7-H3. In humans, B7-H3 fusion proteins enhanced T cell proliferation and IFN-γ production (Chapoval et al. 2001) while in mice, B7-H3 may have a dual role, both as a regulator of Th1 responses (Prasad et al. 2004; Suh et al. 2003) and as an enhancer of tumour clearance (Luo et al. 2004).

In summary, the B7 superfamily of costimulatory receptor ligand pairs can induce both stimulatory and inhibitory signals. CD28 is the only receptor to be constitutively expressed on T cells while the other receptors require antigen recognition prior to surface expression. Therefore stimulation of T cells via TCR and CD28 is not only critical in the initial production of IL-2, but induces the expression of other stimulatory and inhibitory members of the B7 family. Currently, it appears that there is a level of redundancy, as many receptor ligand pairs have overlapping functions. It is therefore likely that each receptor ligand pair plays an important role in different situations and environments.

TNFR superfamily

A number of costimulatory receptor ligand pairs within the TNFR superfamily have been identified (Figure 1.4). Each receptor contains several cysteine rich domains (CRD) and ligands all contain internal aromatic residues involved in trimer formation (reviewed by Locksley (2001).

CD27 and its ligand, CD70, are both members of the TNFR superfamily (Bowman et al. 1994; Camerini et al. 1991; Goodwin et al. 1993; Oshima et al. 1998; Tesselaar et al. 1997). CD27-CD70 interactions are thought to primarily play a role in T-B cell interactions as both receptor and ligand can be expressed on either T or B cells (Gravestein et al. 1995), and lymphocyte activation leads to increased surface expression (Bigler et al. 1988; Hartwig et al. 1997).

CD30 and its ligand CD153 represents another receptor ligand pair believed to play a role in T-B interactions (Alzona et al. 1994; Cerutti et al. 2000). CD30 expression is regulated via cytokine production (Gilfillan et al. 1998; Nakamura et al. 1997). IL-4 enhances CD30 expression, whereas IFN-γ has the opposite effect (Nakamura et al. 1997). CD30 is thought to play a role in T cell differentiation as stimulation of naïve T cells via CD30 leads to the development of Th2 cells (Del Prete et al. 1995), which in turn results in further IL-4 production and CD30 expression.

Ox-40/Ox-40L (CD134 and CD134L) is another receptor ligand pair of the TNFR superfamily (Arch and Thompson 1998) that mediates positive signals both through the receptor and ligand. Ox-40 surface expression is induced upon T cell activation (Calderhead et al. 1993) and peaks around days 2 to 3 of the response (Gramaglia et al. 1998). T cell stimulation via Ox-40 is not critical since Ox-40 KO mice mount similar humoral, anti-viral and anti-bacterial responses when compared to wild-type controls (Pippig et al. 1999). Ox-40 signalling has been shown to synergise with other signals to enhance T cell proliferation and generation of memory cells (Gaspal et al. 2005; Gramaglia et al. 2000; Gramaglia et al. 1998; Weatherill et al. 2001). Ox-40L is expressed at a low level on some B cells and DCs, but expression is substantially increased upon activation with LPS or CD40L engagement (Brocker et al. 1999; Calderhead et al. 1993;

Ohshima et al. 1997). Like some other members of the TNFR superfamily, signalling via Ox-40/Ox-40L is bi-directional; where crosslinking of Ox-40L on B cells has been shown to enhance B cell proliferation and Ig secretion (Stuber et al. 1995; Stuber and Strober 1996) and constitutive expression leads to the accumulation of follicular CD4 T cells in the B cell follicle (Brocker et al. 1999).

The final member of the TNFR superfamily shown to play a stimulatory role in T cell responses is 4-1BB (CD137) along with its ligand 4-1BBL (CD137L) (Arch and Thompson 1998). 4-1BB is another receptor whose surface expression is induced upon T cell activation (Pollock et al. 1995; Pollock et al. 1993). In the absence of 4-1BB/4-1BBL signalling mice still mount humoral responses in response to viral challenge, albeit reduced compared to wild-type mice (DeBenedette et al. 1999). Activation of T cells via 4-1BB leads to enhanced proliferation and cytokine production, sustained cell survival and generation of memory cells (Bertram et al. 2002; Cannons et al. 2001; Diehl et al. 2002; Pollock et al. 1993). 4-1BB expression is not restricted to activated T cells, as stimulation of DCs via 4-1BB has been shown to enhance secretion of IL-6 and IL-12. Interactions between 4-1BB on DCs and 4-1BBL on preactivated B cells augments Ig secretion and is important in B cell affinity maturation (Pauly et al. 2002), while bidirectional signalling has also been demonstrated to play an important role in monocyte adhesion (Langstein et al. 1998).

CD27/CD70, CD30/CD153, Ox-40/Ox-40L and 4-1BB/4-1BBL are all members of the TNFR superfamily shown to play a role in T cell responses. The complete absence of any of these receptor does not ablate T cell responses, however the costimulatory pathways they initiate serve to enhance early TCR and CD28 signalling while also playing a role in effector function (eg T cell B cell interactions).

Effector T cells

Naïve T cells circulation is restricted to blood and secondary lymphoid tissue due to expression of a variety of homing receptors. Upon activation and differentiation into

effector cells, the expression of homing receptors changes, enabling activated T cells to migrate into extra lymphoid sites of infection (reviewed by Butcher (1996). While CD8 T cells mediate killing through the release of granzymes and perforin, CD4 T cells mediate their effector function through the release of interleukin molecules, collectively termed cytokines. Based on the combination of cytokines released, T helper cells can be sub-divided into Th_1 and Th_2 cells (Cherwinski et al. 1987; Mosmann et al. 1986). Th_1 cells secrete IL-2 and IFN-γ, which in turn induces B cells to secrete IgG_{2a} (Snapper and Paul 1987; Stevens et al. 1988). Th_2 cells secrete IL-4 which promotes B cell isotype switching to IgG_1 and IgE (Coffman et al. 1986; Isakson et al. 1982; Stevens et al. 1988) and IL-5 which leads to secretion of IgA (Coffman et al. 1987). It is important to note that while IFN-γ and IL-4 are important for the differentiation into either Th_1 or Th_2 cells, these are not the only cytokines to be produced by these cells.

Differentiation into either Th_1 or Th_2 was originally shown to be a consequence of the cytokines present at the time of T cell stimulation. The addition of IL-4 into cultures during T cell activation induced differentiation into Th_2 cell type (Hsieh et al. 1992) while the IL-12 was shown to induce differentiation into a Th_1 phenotype (Hsieh et al. 1993). Following T cell activation, differentiation of Th_2 cells could be diverted into a Th_1 phenotype both *in vitro* and *in vivo* by the addition of IL-4 (Seder et al. 1992) or blocking IFN-γ (Scott 1991). Conversely, blocking IL-4 has been shown to induce Th_1 differentiation (Chatelain et al. 1992).

The strength of TCR signalling appears to be another factor influencing T cell differentiation. Using TCR tg cells it was noted that immunisation with a low dose of peptide (Constant et al. 1995), peptide with low affinity for MHC (Kumar et al. 1995) or peptide with low affinity for TCR (Tao et al. 1997; Tao et al. 1997) led to differentiation of cells into a Th_2 phenotype. Low affinity generation of Th_2 cells has also been shown to rely on costimulation; thus blocking signalling via CD28-CD80/CD86 decreased differentiation into a Th_2 response (Corry et al. 1994; Shahinian et al. 1993; Tao et al. 1997).

In vivo DCs are believed to drive the differentiation of naïve T cells into the different Th subsets. DCs secrete IL-12 in response to signals via TLR or IFN-γ released by activated

natural killer cells or natural killer T cells. IL-12 secreted by DCs drives the differentiation of naïve cells into IFN-γ producing cells (Macatonia et al. 1995) while IFN-γ secreted by T cells acts in a feedback loop to stimulate further secretion of IL-12 by DCs. Although basophils can produce IL-4 (Seder et al. 1991), T cells appear to be the predominate source of IL-4 *in vivo*. Stimulation of naïve T cells via CD30 (Del Prete et al. 1995) or Ox-40 (Flynn et al. 1998; Ohshima et al. 1998) have both been suggested to induce further secretion of IL-4 again leading to a feedback loop and further differentiation of T cells along the Th_2 pathway.

In addition to the classic Th_1 and Th_2 cells, a recent Th_{17} subset has been defined based on the ability of these cells to secrete IL-17 (Harrington et al. 2005; Mangan et al. 2006). Differentiation into a Th_{17} phenotype is dependent on the presence of TGF-β, suggesting a role for these cells in immune regulation (Weaver et al. 2006).

In summary, it appears that the strength of TCR and costimulatory signals leads to the initial secretion of particular cytokines, which then further drives the differentiation of $CD4^+$ T cells into either the different Th phenotype (Rogers and Croft 1999; Rogers and Croft 2000). Irrespective of the different combinations of signals required for differentiation into either Th_1, Th_2 or Th_{17} cells, the process of T cell differentiation is critical for effector functions of $CD4^+$ T cells.

Cell Death

Cell death is an important regulatory process in the immune response leading to the deletion of autoreactive T and B lymphocytes, destruction of virus infected cells and elimination of effector cells that are no longer required. Two types of cell death have been described, necrosis and apoptosis (Kerr 1972).

Necrosis describes the process whereby cells swell and the cell nucleus, organelles and plasma membrane rupture which leads to the complete break up of the cell and release of intracellular components into the extracellular milieu (reviewed by Wyllie (1980). These

cellular components are subsequently phagocytosed by macrophages resulting in macrophage activation and induction of an inflammatory response. Necrosis of virus infected cells is often the initial triggering event responsible for the induction of an immune response and can lead to death of many neighbouring cells as a consequence of the ensuing inflammatory response.

Apoptosis describes the process of controlled cell death of individual cells. As a consequence of apoptosis, dying cells separate from adjacent cells. This is followed by nuclear and membrane condensation and the formation of apoptotic bodies that are subsequently absorbed by adjacent cells (reviewed by Wyllie (1980). Apoptosis does not cause an inflammatory response and often the only evidence that cell death has occurred is a small amount of non-digestible material. There are two distinct pathways that lead to the initiation of apoptosis in lymphocytes, an intrinsic or mitochondrial induced pathway and an extrinsic or receptor induced pathway (Strasser et al. 1995) (Figure 1.5). Although the initial triggering signals differ, both pathways induce apoptosis through a common pathway of effector caspases (reviewed by Hengartner (2000).

Intrinsic or Mitochondrial Pathway

A balance between pro-apoptotic and anti-apoptotic members of the BH3-only family controls the intrinsic apoptosis pathway (reviewed by Strasser (2005). Expression of pro-apoptotic members due to DNA damage, decreased stimulation via the TCR or removal of survival cytokines, leads to disruption of mitochondrial integrity and the release of cytochrome-C (Cyt-C) (Figure 1.5). The release of Cyt-C further induces a cascade of effector caspases eventually culminating in cell death (Figure 1.5). The intrinsic pathway is regulated by the balance between expression of pro-apoptotic and anti-apoptotic molecules. Overexpression of anti-apoptotic molecules can prevent the initiation of apoptosis. The first such molecule to be described was Bcl-2 (Vaux et al. 1988) which mediates its protective effects by stabilising mitochondrial membrane potential and preventing the release of Cyt-C (Vander Heiden and Thompson 1999). Due to the presence of these anti-apoptotic molecules, cell death mediated via the intrinsic pathway

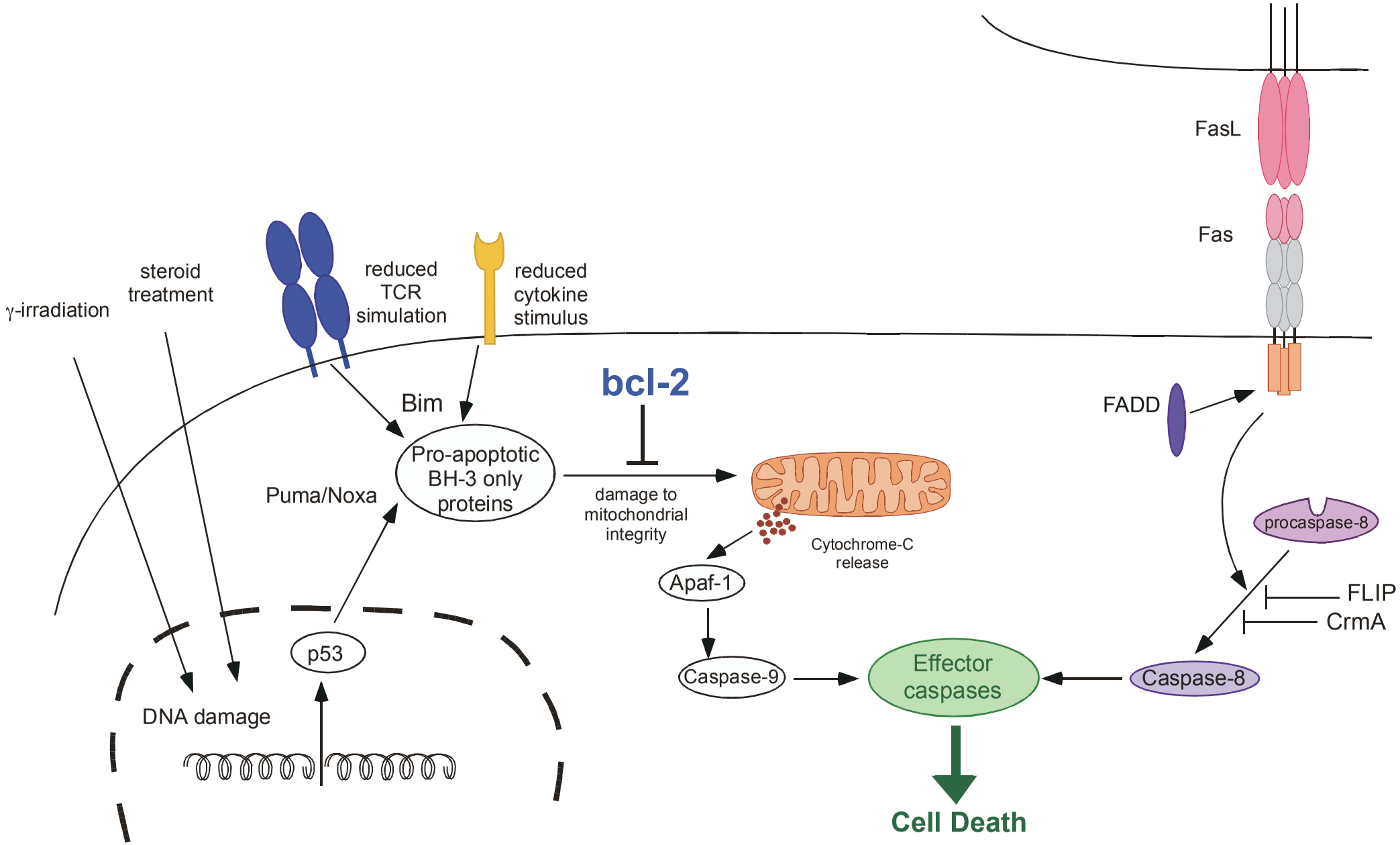


Figure 1.5: Two distinct apoptosis pathways

can be prevented by overexpression of Bcl-2 or knocking out pro-apoptotic molecules such as Bim (Bouillet et al. 1999; Strasser et al. 1991).

One way by which the contraction of T cell responses is thought to occur is through the balance in the expression of pro- and anti-apoptotic molecules of the BH3 family. TCR stimulation leads to increased expression of anti-apoptotic molecules Bcl-2 and Bcl-x_L, with the level of expression related to the level of TCR stimulation such that less Bcl-x_L is expressed with lower levels of T cell stimulation (Gett et al. 2003). Decreased levels of survival cytokines such as IL-2 and IL-7 have also been shown to decrease expression of Bcl-x_L (Broome et al. 1995) while increasing that of Bim (Bouillet et al. 1999; Hildeman et al. 2002; Pellegrini et al. 2003). Consequently disruption to the balance between pro and anti-apoptotic molecule expression could either protect a cell from apoptosis (as occurs during the expansion phase) or render it susceptible (during the contraction phase).

Extrinsic or Receptor Mediated Pathway

The extrinsic pathway is initiated by signalling through TNFR family member Fas (CD95) and relies on caspase-8 (also known as Fas-associated death domain-like IL-1β converting enzyme (FLICE)) and adapter protein fas-associated death domain (FADD) for signal transduction (Varfolomeev et al. 1998; Yeh et al. 1998; Zhang et al. 1998). Binding of FasL to Fas induces the formation of a trimeric complex that in turn induces the recruitment of FADD and the conversion of procaspase-8 into active caspase-8 (Figure 1.5) (reviewed by Hengartner (2000). Mutations in FasL or Fas, seen naturally in *gld* mice (Takahashi et al. 1994) or in *lpr* mice respectively (Watanabe-Fukunaga et al. 1992), can prevent cell death mediated via this pathway. Consequently these strains of mice develop a syndrome characterised by systemic autoimmunity and lymphoproliferative disease (reviewed by Cohen (1991). Just as in the case of the intrinsic pathway, there are also anti-apoptotic molecules that can regulate cell death via the extrinsic pathway. FLIP (FLICE inhibitory protein) binds to procapase-8 and blocks the binding with FADD, preventing conversion into its active form (Irmler et al. 1997). FLIP is expressed early

during T cell activation and as expression declines, cells become more susceptible to apoptosis (Irmler et al. 1997). Higher expression of FLIP in primed/memory cells compared to naïve cells is thought to account for the increased resistance of memory cells to Fas induced cell death (Inaba et al. 1999).

Although not a naturally occurring gene in lymphocytes, insertion of cowpox virus encoded protease protein, cytokine response modifier A (CrmA), has also been shown to inhibit Fas mediated apoptosis, both *in vitro* (Li et al. 2000; Ray and Pickup 1996) and *in vivo* (Smith et al. 1996; Tewari and Dixit 1995; Tewari et al. 1995). CrmA blocks Fas induced apoptosis by binding to caspase-8 and inhibiting its protease activity (Smith et al. 1996; Tewari and Dixit 1995).

Another means by which contraction of the immune response is thought to occur is via induction of activation-induced cell death (AICD) (Brunner et al. 1995; Dhein et al. 1995; Ju et al. 1995). Following T cell activation Fas and FasL are upregulated (Brunner et al. 1995; Strasser et al. 1995). It is the increased expression of Fas that renders activated cells susceptible to apoptosis via the extrinsic pathway.

Regardless of the means by which apoptosis is initiated, cell death is an important component of the immune response, designed to ensure self-reactive clones are eliminated, and that there is a contraction of the number of effector lymphocytes once antigen is no longer present.

Memory T cells

Following the expansion and contraction phases of the T cell response, a small pool of long-lived memory cells remains. This pool consists largely of resting cells which undergo some degree of turnover (Tough and Sprent 1994). In comparison to naïve T cells, memory cells respond more rapidly to antigen, require reduced levels of TCR stimulation

(Demotz et al. 1990; Harding and Unanue 1990) and are less dependent on costimulatory signals (Croft et al. 1994). Overall, memory cells respond more rapidly than naïve T cells to antigen in terms of proliferation (Bruno et al. 1995; Cho et al. 1999; Veiga-Fernandes et al. 2000), cytokine production (Bachmann et al. 1999; Cho et al. 1999; Panus et al. 2000; Veiga-Fernandes et al. 2000), lytic activity (Bachmann et al. 1999; Kedl and Mescher 1998) and migration to the site of infection (Kedl and Mescher 1998).

Currently there are no clear markers to distinguish memory from activated T cells. Although CD44 expression is increased and remains high after antigen recognition (Budd et al. 1987), it does not differentiate between cells that will die during the contraction phase and those cells that will form the stable pool of memory cells. CD62L has also been proposed as a marker of memory cells status due to the rapid down-regulation of CD62L on activated T cells (Bradley et al. 1992). However a population of $CD62L^{hi}$ cells that display a memory phenotype has been identified, thus alone CD62L cannot be used to distinguish between memory and naïve or activated T cells (Dobber et al. 1994).

To explain the heterogeneity within the human memory T cell pool, Sallusto and colleagues (Sallusto et al. 1999) proposed a sub-division of memory cells into either central memory (T_{CM}) or effector memory cells (T_{EM}) based on the differential expression of the chemokine receptor CCR7. T_{CM} cells constitutively express CCR7 and CD62L and are the predominate memory cell found within secondary lymphoid tissues. T_{EM} cells predominantly reside in peripheral tissues and have lost constitutive expression of both CCR7 and CD62L-enabling migration of these cells through peripheral tissues. T_{EM} cells rapidly produce a variety of effector cytokines within several hours of antigen restimulation, compared to T_{CM} that require a number of divisions before the production of IFN-γ or IL-4 can be detected (reviewed by Sallusto (2004). In mice, it also appears as though memory cells can be sub-divided based on their peripheral locations and cytokine production (Reinhardt et al. 2001). Although sub-dividing memory T cells into either T_{CM} or T_{EM} cells appears to represent a useful means of distinguishing cells on the basis of functional differences, heterogeneity still exist within these subsets in terms of costimulatory and adhesion molecule expression as well as cytokine profiles. At present

there is therefore no combination of CD markers available that will identify from the pool of effector cells those that will become long-lived memory cells.

Generation and Maintenance of Memory T Cells

From some early studies it was suggested that persistent antigen was required for the generation and maintenance of the T cell memory pool (Gray and Matzinger 1991; Oehen et al. 1992). While it is clear that both $CD4^+$ and $CD8^+$ T cells require an initial period of antigen exposure, transfer of activated cells into an antigen free environment (Bruno et al. 1995; Kaech and Ahmed 2001; Swain 1994; van Stipdonk et al. 2001; Wong and Pamer 2001) was shown not to effect the long term survival of either CD4 or CD8 memory cells. Survival of antigen experienced cells in MHC deficient mice (Hou et al. 1994; Murali-Krishna et al. 1999; Swain et al. 1999) provides strong evidence against the requirement of TCR stimulation for memory cell longevity. However, other reports would suggest that maintenance of activated cells in the absence of continued TCR stimulation reduces the recall capacity of these cells (Gray and Matzinger 1991; Kassiotis et al. 2002; Kundig et al. 1996; Markiewicz et al. 1998; Tanchot et al. 1997).

It is becoming increasingly apparent that different cytokines play an important role in the differentiation and maintenance of memory cells. IL-15 has been shown to synergise with a number of different cytokines to enhance T cell proliferation, although it appears to be more critical in the maintenance of $CD8^+$ rather than $CD4^+$ memory T cells (Judge et al. 2002; Kennedy et al. 2000; Zhang et al. 1998). IL-15 has been shown to enhance $CD44^{hi}CD8^+$ T cell turnover and survival (Judge et al. 2002; Zhang et al. 1998) while IL-$15^{-/-}$ mice have reduced numbers of $CD44^{hi}CD8^+$ memory cells that can be overcome with injection of IL-15 (Kennedy et al. 2000).

IL-7 is another cytokine that is believed to play a role in the generation and maintenance of memory cells. Selective expression of IL-7R on $CD8^+$ T cells has been proposed as a marker of memory cell progenitors, since IL-$7R^{hi}$ cells have enhanced survival and recall abilities when compared to IL-$7R^{lo}$ cells (Huster et al. 2004; Kaech et al. 2003). IL-7 has

also been shown to promote $CD4^+$ T cell proliferation and survival (Li et al. 2003; Seddon et al. 2003).

While these cytokines do appear to be important in the development of different populations of memory cells, the role of different cytokines and cytokine combinations in the maintenance of these different subsets of memory has yet to be clarified.

Repertoire Restriction and Affinity Maturation

T cell responses are often characterised by conserved usage of specific Vα and Vβ chains not only between different individuals (Chen et al. 1997; Deckhut et al. 1993; Rodewald et al. 1989) but also between antigen specific clones (Casanova et al. 1992; Cose et al. 1995; Lehner et al. 1995). Typically, there is high conservation in the region of the TCR involved in antigen recognition, namely CDR3. Repertoire restriction has been shown to parallel an increase in the overall strength of the response to antigen, a process termed affinity maturation.

Unlike B cell affinity maturation that occurs through a regulated process of somatic hypermutation of immunoglobulin genes (Berek et al. 1991; Jacob et al. 1991), T cell affinity maturation is believed to occur through a competition dependent mechanism, whereby cells of highest affinity dominate the response. This idea is supported by data that demonstrated that stimulation of cells with a sub-dominant or low dose of antigen leads to a population of higher affinity clones when compared to cells stimulated in conditions of reduced competition, ie with a high dose or dominant antigen (Alexander-Miller et al. 1996; Campos-Lima et al. 1997; Rees et al. 1999).

Work by McHeyzer-William demonstrated that, in response to pigeon cytochrome-C (PCC), there was selection for T cells with conserved lengths of the CDR3 region (McHeyzer-Williams and Davis 1995). This was further shown to correlate with antigen driven selection of higher affinity PCC specific T cells (McHeyzer-Williams et al. 1999). Simultaneously Busch and Pamer demonstrated a restriction in the Vβ repertoire

paralleled by an increase in overall affinity of *L.monocytogenes* specific T cell clones with repeated antigen exposure (Busch and Pamer 1999).

Several studies of the $CD4^+$ T cell response have shown a narrowing of the TCR repertoire during transition from the primary to memory response, which is paralleled by an increase in the affinity of the overall response (Fasso et al. 2000; Malherbe et al. 2004; McHeyzer-Williams et al. 1999; McHeyzer-Williams and Davis 1995; Savage et al. 1999). This process has also been demonstrated for $CD8^+$ T cells (Bachmann et al. 1997; Campos-Lima et al. 1997; Maryanski et al. 1999). However the timing by which restriction of either the CD4 or CD8 repertoire occurs is unclear.

Some studies suggest restriction solely occurs during the primary response (Blattman et al. 2000; Casanova et al. 1991; Cole et al. 1994; Malherbe et al. 2004; Maryanski et al. 1996; Sourdive et al. 1998; Vijh and Pamer 1997) while other groups suggest that selection is continuous (McHeyzer-Williams and Davis 1995). In a recent report by Malherbe (2004), the timing of affinity maturation was investigated through the use of two strains of PCC specific cells with different affinities. It was concluded that selection occurred in two distinct stages. An initial selection event occured to recruit cells above a particular threshold into division, and this was followed by a second selection event within the first 3 to 7 days of the response for cells above a second affinity threshold. From this point onwards, there were no further selective events, and proliferation continued independently of a cells affinity for antigen.

Despite this discrepancy as to the timing of repertoire restriction, it is clear competition aids in increasing the overall strength of the response through the selective expansion of higher affinity cells (McHeyzer-Williams et al. 1999) and/or the loss of lower affinity clones (Malherbe et al. 2004; Savage et al. 1999).

T cell competition

Over the course of a response, T cells have been suggested to compete for antigen-MHC complexes, costimulatory molecules and/or APC derived factors. Antigen-specific competition refers to competition observed between cells recognising the same antigen-

MHC complex. Antigen non-specific competition refers to competition between cells that recognise different antigen-MHC complexes and therefore, if it occurs, cell compete for factors independent of antigen. While the first evidence that a response to one protein could suppress the overall response to an independent protein was reported in the early 1900's (Michaelis 1902)(reviewed by Pross (1974) it wasn't until the creation of peptide-MHC tetramers and TCR tg mice, that competition between cells recognising the same antigen-MHC complexes was first demonstrated (Butz and Bevan 1998). Since this original study there have been two more studies in which transfer of a large number of TCR tg cells resulted in suppression of the endogenous response to the same antigen (Kedl et al. 2000; Probst et al. 2002). In all of these studies, no effect on the endogenous response to other antigens, either within the same protein or derived from different proteins was observed, ie antigen non-specific competition. However there have been two other studies in which a large number of OT1 cells (specific for ovalbumin (Ova) $Ova_{257-264}$) was shown to suppress the endogenous response to the minor Ova epitope (Ova_{55-62}) (Kedl et al. 2000). In this model, both antigen-specific and antigen non-specific competition could be overcome with additional antigen, suggesting access to peptide-MHC as the primary source of T cell competition (Kedl et al. 2000; Probst et al. 2002). Similarly Grufman and colleagues have demonstrated that immunodominace to BALBc major and minor epitopes can be overcome by injection of more antigen into recipient mice (Grufman et al. 1999).

Antigen specific competition between CD4 cells has also been demonstrated *in vivo* models. Reduced proliferation of 5C.C7 TCR tg cells was observed when 5C.C7 cells were transferred into an intact 5C.C7 TCR tg mouse, although no effect was seen when these same cells were transferred into myelin basic protein (MBP) TCR tg mice (Smith et al. 2000). In another study, priming with higher affinity $MBP_{Ac1-9(4Y)}$ 7 days prior to immunisation with whole protein or peptide was shown to suppress the response to MBP_{Ac1-9} and MBP_{89-101} (Anderton and Wraith 1998), while priming with $MBP_{Ac1-9(4Y)}$ had no effect on the response to residues 139-151 of proteolipid protein (PLP).

The only evidence of antigen non-specific competition between T cells that can not be explained as the result of antigen processing (ie the epitopes are from the same protein or are presented on the same MHC complex) has come from some initial *in vitro* work which was followed up by one *in vivo* experiment (Hayball et al. 2004; Lake et al. 1999). In this study, introduction of a 24-hour interval between the transfer of the competing cohort and the readout cohort led to reduced recruitment of the second cohort into division, as measured by the percentage undivided cells remaining at day 3.

While it is not clear at this present time the primary source of competition between T cells, competition appears to be intimately associated with affinity maturation of the T cell response, generation of immunodominance hierarchies and could have important implications for the design of recombinant multi-epitope vaccines.

Project aims

T cells are believed to compete for both activation signals and survival signals and as a consequence this can results in a number of different outcomes for the response. Yet despite the intense interest in competition over the past 100 years, it is still not clear which aspects of T cell competition occur *in vivo*. By understanding the underlying process of T cell competition, insight can be gained into the normal regulatory mechanisms that function during the course of a T cell response. At the same time, studying T cell competition provides a means of analysing the relative contributions different signals make to the T cell response. For instance, by introducing a competing response for peptide-MHC, one can investigate the effect of reduced TCR stimulation. Alternatively, studying the effect of two simultaneous antigen independent responses can provide insight into the role of costimulatory or other survival signals.

In studies performed during my honours degree, an *in vitro* model of T cell competition was established to investigate the role of competition in affinity maturation. While an effect of both antigen-specific and antigen non-specific competition was observed, there was no

clear role for competition in affinity maturation as the effect was mediated by soluble factors-believed to be a decline in nutrients due to the presence of an ongoing response. It was, however, important to translate this system into an *in vivo* model in which competition for nutrients would be minimised, and where the effect of competition on the generation of effector and memory cells could be studied. This project initially aimed to determine whether competition between CD4 T cells of the same and different specificity occurred *in vivo* through the development of an *in vivo* model in which the effect of competition at different stages of a CD4 response could be measured.

The experiments described in this thesis document the establishment of two separate *in vivo* model of T cell competition. The first model was used to study the role of antigen-dependent and antigen-independent signals on the early stages of the T cell response, such as recruitment into division and early T cell proliferation. This model was also used to investigate the effect of antigen processing and presentation on T cell competition. The second model of competition enabled the effect of competition on the generation of effector and memory cells to be investigated while also assessing the role of competition in T cell affinity maturation. In so doing the relative contributions of signalling via the TCR or costimulatory signals to the generation of effector and memory cells was investigated. Finally the changing phenotype of DCs during the course of an adjuvant boosted T cell response was investigated in an effort to determine the process mediating T cell competition.

Chapter 2: Materials and Methods

2.1 Mice

All mice were bred and housed under specific pathogen-free (SPF) conditions in the Centenary Institute Animal Facility. Mice were sacrificed by CO_2 asphyxiation or cervical dislocation prior to organ harvest. Approval for all animal experimentation was obtained from the Institutional Ethics Committee at the University of Sydney.

Inbred and congenic strains

B6.SJLPtprc^a, B10.BR and C57BL/6 mice were purchased from the Animal Resource Centre (Perth, Western Australia). All other inbred strains were bred at the Centenary Institute animal facility. Mice were maintained as inbred colonies and intercrossed to create F1 progeny for experimental use. Table 2.1 illustrates the breeding and intercrosses performed to generate mice of specific F1 backgrounds.

T Cell Transgenic mice

5C.C7 TCR tg mice (-I)

TCR tg mice specific for the COOH-terminal epitope of the tobacco hornworm moth cytochrome C (MCC) (residues 86-103) (Figure 2.1) were originally created using a rearranged Vα11.1 and Vβ3 genes isolated from the 5C.C7 T cell clone (Fink et al. 1986), co-integrated and expressed under the control of the endogenous 3' β chain enhancer (Fazekas de St. Groth et al. 1992; Girgis et al. 1999; Seder et al. 1992). CD4$^+$ 5C.C7 T cells respond to a number of peptide-MHC Class II complexes including MCC (residues 94-103) in association with IE^k, and MCC in association with IE$\alpha^k\beta^b$ or IE$\alpha^d\beta^b$.
The 5C.C7 line is maintained on a B10.BR background and was crossed with C57BL/6 or B6.SJLPtprc^a to generate offspring of the correct genotype for experimental use (Table 2.2).

Table 2.1 Recipient mice

Name	Parent 1	Parent 2	MHC Designation	Other Genes
KK	B10.BR	B10.BR	H-2^{kk}	
BK	B10.BR	C57BL/6	H-2^{bk}	
F1L	B10.BR	B6.SJLPtprc^{a}	H-2^{bk}	Ly5.1
KU	B10.BR	B10.PL	H-2^{ku}	

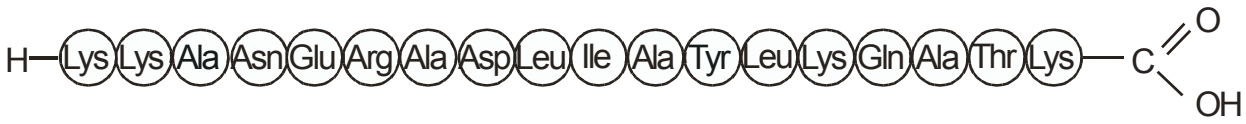
Table 2.2 TCR Tg Mice

Name	Parent 1	Parent 2	MHC Designation	TCR	Other genes
5C.C7 KK	5C.C7 B10.BR	5C.C7 B10.BR	H-2^{kk}	5C.C7	
5C.C7 BK	5C.C7 B10.BR	C57BL/6	H-2^{bk}	5C.C7	
5C.C7 F1L	5C.C7 B10.BR	B6.SJLPtprc^{a}	H-2^{bk} (Ly5.1)	5C.C7	Ly5.1
5C.C7 KU	5C.C7 B10.BR	B10.PL	H-2^{ku}	5C.C7	
5C.C7β BK	5C.C7β B10.BR	C57BL/6	H-2^{bk}	5C.C7β	
5C.C7β F1L	5C.C7β B10.BR	B6.SJLPtprc^{a}	H-2^{bk} (Ly5.1)	5C.C7β	Ly5.1
3A9 KK	3A9 B10.BR	3A9 B10.BR	H-2^{kk}	3A9	
3A9 BK	3A9 B10.BR	C57BL/6	H-2^{bk}	3A9	
3A9 F1L	3A9 B10.BR	B6.SJLPtprc^{a}	H-2^{bk} (Ly5.1)	3A9	Ly5.1
3A9 KU	3A9 B10.BR	B10.PL	H-2^{ku}	3A9	
MBP KU	MBP B10.PL	B10.BR	H-2^{ku}	19	
5C.C7β.Bcl-2 BK	Bcl-2 C57BL/6	5C.C7β B10.BR	H-2^{bk}	5C.C7β	Bcl-2
5C.C7β.CrmA	CrmA C57BL/6	5C.C7β B10.BR	H-2^{bk}	5C.C7β	CrmA

a. Hen Egg Lysozyme (HEL_{46-61})

Molecular Weight = 1759 Da

b. Moth Cytochrome C (MCC_{86-103})



Molecular Weight = 2061 Da

c. Myelin Basic Protein (MBP_{Ac1-11})

Molecular Weight = 1316 Da

Figure 2.1: Amino acid sequences and molecular weights of the different peptide antigens

5C.C7β TCR tg mice (-V)

The 5C.C7β TCR tg mouse strain expresses the Vβ3 chain only of the 5C.C7 TCR (Jorgensen et al. 1992). TCRs are composed of Vβ3 rearranged with an endogenous Vα chain, leading to a mixed variety of TCR with varying affinities for MCC. The 5C.C7β line is maintained on a B10.BR background and intercrossed to generate various strains of experimental mice (Table 2.2).

3A9 TCR tg mice (-E)

Transgenic mice expressing $CD4^+$ T cells specific for hen egg lysozyme (HEL) were originally made by inserting the 3A9 VJα and VDJβ fragments into a modified genomic shuttle vector, resulting in $CD4^+$ T cells expressing rearranged Vα3.2 and Vβ8.2 chains (Ho et al. 1994). 3A9 $CD4^+$ T cells recognise HEL residues 46-61 when expressed on IA^k MHC Class II molecules (Figure 2.1). Mice are maintained on a B10.BR background and intercrossed with C57BL/6, B6.SJLPtprc^a or B10.PL to produce F1 progeny for experimental use (Table 2.2).

MBP TCR tg mice (-Q)

MBP TCR tg mice were originally generated from the myelin basic protein (MBP) specific $CD4^+$ Vβ8.2^+ Clone 19 (Hardardottir et al. 1995). $CD4^+$ T lymphocytes express an $I\text{-}A^u$ restricted TCR with low affinity for the acetylated N-terminal 16 amino acid fragment of MBP (Figure 2.1) (Baron et al. 1993). Mice were maintained on a B10.PL background and intercrossed with B10.BR mice to create F1 experimental progeny (Table 2.2).

Bcl-2 tg (P-)

bcl-2-25 transgenic mice constitutively overexpress the anti-apoptotic Bcl-2 transgene (Strasser et al. 1991). Bcl-2 is a member of the BH3 only proteins involved in apoptosis via the mitochondrial mediated pathway (see Chapter 1). Overexpression of Bcl-2 was originally shown *in vitro* to prevent cell death initiated by steroid treatment, gamma

irradiation or cytokine withdrawal. Bcl-2 tg mice are maintained on a C57BL/6 background and were intercrossed with 5C.C7β TCR tg mice to generate F1 double transgenic progeny for experimental use (Table 2.2).

CrmA tg (V-)

CD2-crmA tg mice (Smith et al. 1996) have an insertion of the of cowpox virus encoded protease transgene, cytokine response modifier A (CrmA), under the control of the human CD2 enhancer, with the greatest level of CrmA mRNA and protein levels in the T cell lineage. CrmA has been shown to prevent cell death mediated through the Fas-FasL apoptotic pathway by binding to caspase 8 (see Chapter 1). CrmA tg mice are maintained on a C57BL/6 background and were intercrossed with 5C.C7β TCR tg mice to generate double 5C.C7βCrmA^{+} tg mice (Table 2.2).

2.2 Cellular Biology

Cell Preparations

Adoptive Transfers

Peripheral (inguinal, brachial, sub-scapular, auricular and cervical), mesenteric and para-aortic lymph nodes (LNs) were harvested into cold RPMI 1640 (JRH Biosciences, Lenexa, KS, USA) supplemented with 10% heat inactivated foetal calf serum (FCS) (Trace MultiSer Biosciences, Australia). Single cell suspensions were prepared by teasing lymph nodes through 80 gauge metal sieves prior to washing twice in RPMI-10% FCS. Cells were layered over FCS to remove any clumps of debris before viable cell counting in 0.1% Trypan Blue (Koch Light Laboratories, Colnbrook, England) and intravenous (i.v.) injections.

CFSE labelling

Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA) is an intracellular dye primarily used to track cell division. Each time a CFSE-labelled cell divides, the amount of CFSE is equally distributed into each of the two daughter cells. Thus as the cells progress through each division, the fluorescence intensity is halved. By measuring the amount of intracellular CFSE by flow cytometry, distinct population bands corresponding to each division are visible (Lyons and Parish 1994) (Figure 2.3a).

After preparation of a single cell suspension, cells were washed once in cold RPMI with no FCS (as the protein interferes with CFSE labelling) and resuspended in pre-warmed RPMI (37^{o}C) at a concentration of 5 x 10^{7} cells/ml. Cells were then incubated for 10 minutes (at 37^{o}C) with 5 μM CFSE and gently mixed every 2 to 3 minutes to ensure homogeneous CFSE labelling. The reaction was stopped after 10 minutes by washing cells twice with cold RPMI/10% FSC.

Antigens

Cytochrome C

A synthetic peptide comprising residues 86-103 of MCC (MCC_{86-103}) (Figure 2.1b) was purchased from Auspep Pty. Ltd. (Parkville, Australia). Pigeon cytochrome C (PCC) protein (Sigma) has one extra amino acid at the C-termini, but is still capable of initiating an antigen specific 5C.C7 response. Both peptide and protein were dissolved in phosphate buffered saline (PBS) (8g/L NaCl, 0.2g/L KCl, 1.15g/L Na_2HPO_4 pH 7.2), MCC peptide was stored in aliquots at –70^{o}C while PCC protein was made fresh prior to use.

Hen Egg Lysozyme (HEL)

A synthetic peptide HEL 46-61 (HEL_{46-61}) (Figure 2.1a) was synthesised by Auspep Pty. Ltd.. Whole HEL protein, used for immunisation and protein standards, was purchased from Sigma Aldrich. Both peptide and protein were dissolved in PBS and while HEL

peptide was stored in aliquots at –70°C, fresh solutions of HEL protein were made prior to use.

Myelin Basic Protein Peptide (MBP)

MBP Ac1-9 ($MBP_{Ac\ 1-11}$) (Figure 2.1c) was synthesised by Auspep Pty. Ltd., dissolved in PBS and stored as small aliquots at –70°C.

Immunisation

For subcutaneous injections, peptides were diluted in PBS and mixed with Complete Freund's Adjuvant (CFA) (Sigma Aldrich) to a final ratio of 1:1 (Peptide:CFA). CFA is a mineral solution containing 1mg of heat killed *Mycobacterium tuberculosis* and was originally shown to induce inflammation and enhance the production of immunoglobulins (Freund and McDermott 1942). Each mouse received a sub-cutaneous (s.c.) injection containing a total of 200μl with 50μl injected in each footpad and 100μl at the base of the tail. The dose of total peptide refers to the total amount each mouse received distributed between the injection sites.

Organ Preparation for Flow Cytometry

Draining (inguinal, popliteal, para-arotic), non-draining (cervical) LNs and spleens were harvested into Facswash (FW) (5% FCS, 5mM Sodium Azide in PBS). Single cell suspension were prepared by teasing organs through 80 gauge metal sieves prior to washing once and counting on Sysmex KX-21 automated cell counter (Toa Medical Electronic Company, Kobe, Japan).

DC enrichment by collagenase digestion

An adaptation of the Vremec and Shortman (Vremec and Shortman 1997) protocol was used to enrich dendritic cells for flow cytometric analysis. Organs were harvested into

RPMI-10% FCS then finely sliced and incubated at room temperature in medium containing 0.3mg/mL of collagenase/dispase (Boehringer Mannheim, Indianapolis Indiana, USA) and 0.02mg/mL of DNAse I (Sigma Aldrich, St. Louis, Missouri, USA). To further disrupt DC-T cell clusters, 200μl of 0.1M EDTA (pH7.2) was added after 30 minutes and samples were incubated for a further 5 minutes. Samples were then passaged through 80 gauge metal sieves to prepare single cell suspensions, prior to washing in FW.

2.3 Flow Cytometry

Surface Staining

1-2x10^6 cells were aliquoted into a 96 well round bottom flexible PVC plates (ICN, Costa Mesa, CA, USA) and pelleted by centrifugation (1500rpm at 4^{o}C for 3 minutes). Staining was performed in 50μl of FW containing pre-titred monoclonal antibodies (mAb) or secondary fluorescence conjugated antibodies. All layers of staining were performed at 4^{o}C in the dark for a minimum of 30 minutes and cells were washed with 150μl of FW between layers. Propidium iodide (PI) and 4',6'-Diamidino-2-phenylindole dihydrochloride (DAPI) are two intracellular dyes impermeable to the intact cell membrane of live cells. Either PI or DAPI was added to samples at a final concentration of 1-2μg/ml at least 5 minutes prior to running samples on the flow cytometer. PI positive cells were excited by 488nm laser and detected at a wavelength above 630nm on the FACSCalibar, FACSCanto and FACSStarPlus, DAPI positive cells were excited with the ultra-violet on the FACS Vantage and FACS LSRII and detected around a wavelength of 440nm.
In some experiments, anti-rat Ig secondary antibodies were used to detect unconjugated primary rat mAb. In order to prevent cross-reactivity with subsequent rat antibodies, after labelling with the primary unconjugated rat mAb, samples were blocked with 1% normal rat serum for at least 10 minutes.

Table 2.3: Monoclonal Antibodies

Ligand	Flurochrome	Clone	Species	Source
αBrdU	PE			Pharmingen
αCyt-C	pure	7H8.C12	mouse IgG1	CI
αhamster	FITC		goat	Caltag
αhamster	PE		rabbit	Southern Biotechnology
αhamster	Texas Red		goat	Caltag
αHEL	pure	HyHEL9	mouse IgG1	CI
αHEL	biotin	HyHEL10	mouse IgG1	CI
αm3A9 TCR	biotin	1G12	mouse IgG1	CI
αmB7RP-1	pure	HK5.3	rat IgG2aκ	CI
αmCD4	Alexa 647	RM4-5	rat IgG2aκ	Ebiosciences
αmCD4	APC	RM4-5	rat IgG2aκ	Pharmingen
αmCD4	APCCy7	GK1.5	rat IgG2bκ	Pharmingen
αmCD4	FITC	GK1.5	rat IgG2bκ	Pharmingen
αmCD4	PE	RM4-5	rat IgG2aκ	Pharmingen
αmCD4	PerCP	GK1.5	rat IgG2bκ	Pharmingen
αmCD4	PerCPCy5.5	RM4-5	rat IgG2aκ	Pharmingen
αmCD5	biotin	53-7.3	rat IgG2aκ	Pharmingen
αmCD11c	PE	HL3	hamster IgG1κ	Pharmingen
αmCD25	biotin	7D4	Rat IgMκ	Pharmingen
αmCD25	PE	PC61	rat IgG1λ	Pharmingen
αmCD27	biotin	LG.3A10	hamster IgG1κ	Pharmingen
αmCD30	biotin	2SH12-5F-2D	hamster IgG1κ	Pharmingen
αmCD44	APC	IM78.1	rat IgG2bκ	CI
αmCD44	biotin	IM78.1	rat IgG2bκ	CI
αmCD45 (Ly5.1)	biotin	A20	mouse IgG2aκ	CI
αmCD45 (Ly5.1)	Alexa 594	A20	mouse IgG2aκ	CI
αmCD45 (Ly5.1)	Alexa 647	A20	mouse IgG2aκ	Ebiosciences
αmCD45 (Ly5.1)	APC	A20	mouse IgG2aκ	CI
αmCD45 (Ly5.1)	Pacific Blue	A20	mouse IgG2aκ	CI
αmCD45R (B220)	FITC	RA3-6B2	rat IgG2aκ	Pharmingen
αmCD45R (B220)	PE	RA3-6B2	rat IgG2aκ	Pharmingen
αmCD45R (B220)	PerCP	RA3-6B2	rat IgG2aκ	Pharmingen
αmCD62L	biotin	MEL14	rat IgG2aκ	Pharmingen
αmCD62L	pure	MEL14	rat IgG2aκ	CI
αmCD69	PE	H1.2F3	hamster IgG1λ	Pharmingen
αmCD70	pure	FR70	rat IgG2b	CI
αmCD80	biotin	16-10A1	hamster IgG1κ	Pharmingen
αmCD86	biotin	GL1	rat IgG2aκ	Pharmingen

αmCD122 (IL-2R α chain)	PE	TM-b1	rat IgG2aκ	Pharmingen
αmCD127 (IL-7R α chain)	biotin	A7R34	rat IgG2aκ	Ebiosciences
αmCD132 (common γ chain)	biotin	TUGM2	rat IgG2bκ	Pharmingen
αmCD134 (Ox-40)	biotin	Ox-86	rat IgG1	Pharmingen
αmCD134L (Ox-40L)	pure	RM134L	rat IgG2b	CI
αmCD137	biotin	1AH2	rat IgG1	Pharmingen
αmCD137L (4-1BBL)	pure	TKS-1	rat IgG2a	CI
αmCD152 (CTLA-4)	biotin	4F10	hamster	CI
αmCD153 (CD30L)	pure	RM153	rat IgG2b	CI
αmI-A/I-E	biotin	M5/114	rat IgG2bκ	CI
αmI-A/I-E	APC	M5/114	rat IgG2bκ	CI
αmI-Ek	biotin	14.4.4S	mouse IgG2aκ	CI
αmIFN-γ	PE	XMG1.2	rat IgG1	Pharmingen
αmIL-2	PE	JESS6-5H4	rat IgG2b	Pharmingen
αmIL-4	PE	11B11	rat IgG1	Pharmingen
αmIL-10	PE	JESS5-16ES	rat IgG1	Pharmingen
αmMBP TCR	biotin	19G11.2	Mouse IgG1	CI
αmIgG	Alkine Phosphatase			Sigma
αmVα11	supernatant	RR8	rat	CI
αmVα11	biotin	RR8	rat	CI
αmVβ3	supernatant	KJ25	hamster IgG2κ	CI
αmVβ3	biotin	KJ25	hamster IgG2κ	CI
αmVβ8.2	biotin	F23.2	mouse IgG2aκ	CI
αrat	Alexa 647			Molecular Probes
αrat	FITC		goat	Caltag
αrat	Texas red		goat	Caltag
streptavidin	Alexa 594			Molecular Probes
streptavidin	Alexa 488			Molecular Probes
streptavidin	Alexa 700			Molecular Probes
streptavidin	AlkPhos			Sigma
streptavidin	APC			Molecular Probes
streptavidin	FITC			Molecular Probes
streptavidin	Pacific Blue			Molecular Probes
streptavidin	PerCP			Pharmingen
streptavidin	PE			Pharmingen
streptavidin	Quantum red			Sigma

APC – allophycocyanin
Cy – cyanine
FITC – fluorescein isothiocyanate
PE – R-phycoerythrin
PerCP – peridinin chlorophyll-a protein

In experiments staining for DCs, where possible unlabelled rat anti-FcγRII (clone 2.4G2) was used to block Fc receptors before labelling with successive surface markers. In preparations whereby using an anti-rat conjugated secondary antibody was used, 1% normal mouse serum was used to block Fc receptors for at least 10 minutes prior to staining with mAbs.

Antigenic Restimulation and Intracellular cytokine staining

Lymph node and spleens were harvested into TCM prior to preparations of single cell suspensions. Samples were set-up in quadruplicate with approximately 1 x 10^7 cells plated per well of a 24 well Tissue culture plate (BD Falcon, Frankiln Lakes, N.J., USA) and MCC_{86-103} at a final concentration of 10μM. Samples were restimulated for a total of 6 hours with 5μg/ml Brefeldin A (Sigma Aldrich) added for the final 4 hours.

In experiments analysing cytokine production by resting memory cells, samples were restimulated *ex vivo* for a total of 16 hours with the addition of Brefeldin A for the final 12 hours.

After 6 hours of antigen restimulation, replicate wells were pooled and cells washed once in Facswash. Surface marker staining was performed as per the standard protocol described above prior to cell fixation in 4% paraformaldehyde (PFA) (Sigma Aldrich) for 20 minutes in the dark at 4^{o}C.

Cells were stained for intracellular cytokines using a modification of the method of Sander (1991). Cells were permeablised by washing in saponin BSA solution, 0.5% saponin (Sigma Aldrich) and 0.1% bovine serum albumin (BSA) (USB, Cleveland, Ohio, USA). All staining steps were performed for 30 minutes with antibody diluted in saponin BSA. Cells were washed 3 times between layers before a final 2 washes in FW.

BrdU pulsation and detection of BrdU uptake by flow cytometry

5-bromo-2'-deoxyuridine (BrdU) (Sigma Aldrich), a thymidine analogue, is incorporated into the DNA of dividing cells during the S phase of cell. It can be used to analyse the

proliferative capacity of dividing cells by flow cytometry as BrdU incorporation can be detected by staining with an anti-BrdU mAb.

BrdU was dissolved in PBS to a final concentration of 20mg/ml and mice were injected intraperitoneally (i.p.) with 100μl of BrdU (2mg total) or 100μl of PBS 6 hours prior to sacrifice. LNs and spleens were harvested and single cell suspensions prepared and processed as described by Hasbold (1998).

Cell samples were stained for surface markers (Ly5.1 and Vβ3) prior to fixation of cells in 2% paraformaldehyde (20 minutes at room temperature). Cells were permeablised overnight in 0.1% Tween-20, 0.5% paraformadehyde solution in PBS. Digestion with DNAse I (Sigma Aldrich) at a final concentration of 50μg/ml in BrdU buffer was performed for 1 hour at 37^{o}C (20mM Tris, 10mM NaCl, 1mM $MgCl_2$). Intracellular BrdU was subsequently detected with a PE conjugated anti-BrdU monoclonal antibody (BD Pharmingen) with staining performed in FACSwash.

Acquisition

A number of flow cytometers (all Becton Dickinson, Franklin Lakes, NJ) were available for data acquisition. Samples stained with up to 4-colours were analysed on a FACSCalibur, up to 5-colours on a FACSStar Plus or FACS Canto and up to six colours analysed on a FACSVantage or FACSLSRII. Data was analysed using FlowJo software (Tree Star Inc. San Carlos, CA, USA). Acquisition performed on the FACS Canto, FACSLSRII or FACSVantage was collected in uncompensated digital format, as opposed to precompensated analogue format on either the FACSCalibar or FACSStarPlus. For analysis of digital data, a software compensation matrix was calculated in FlowJo and applied to each sample.

General Gating Strategy

A general gating strategy was employed for the analysis of flow cytometry data (Figure 2.2). A size gate (Forward Scatter versus Side Scatter) was applied to all samples to

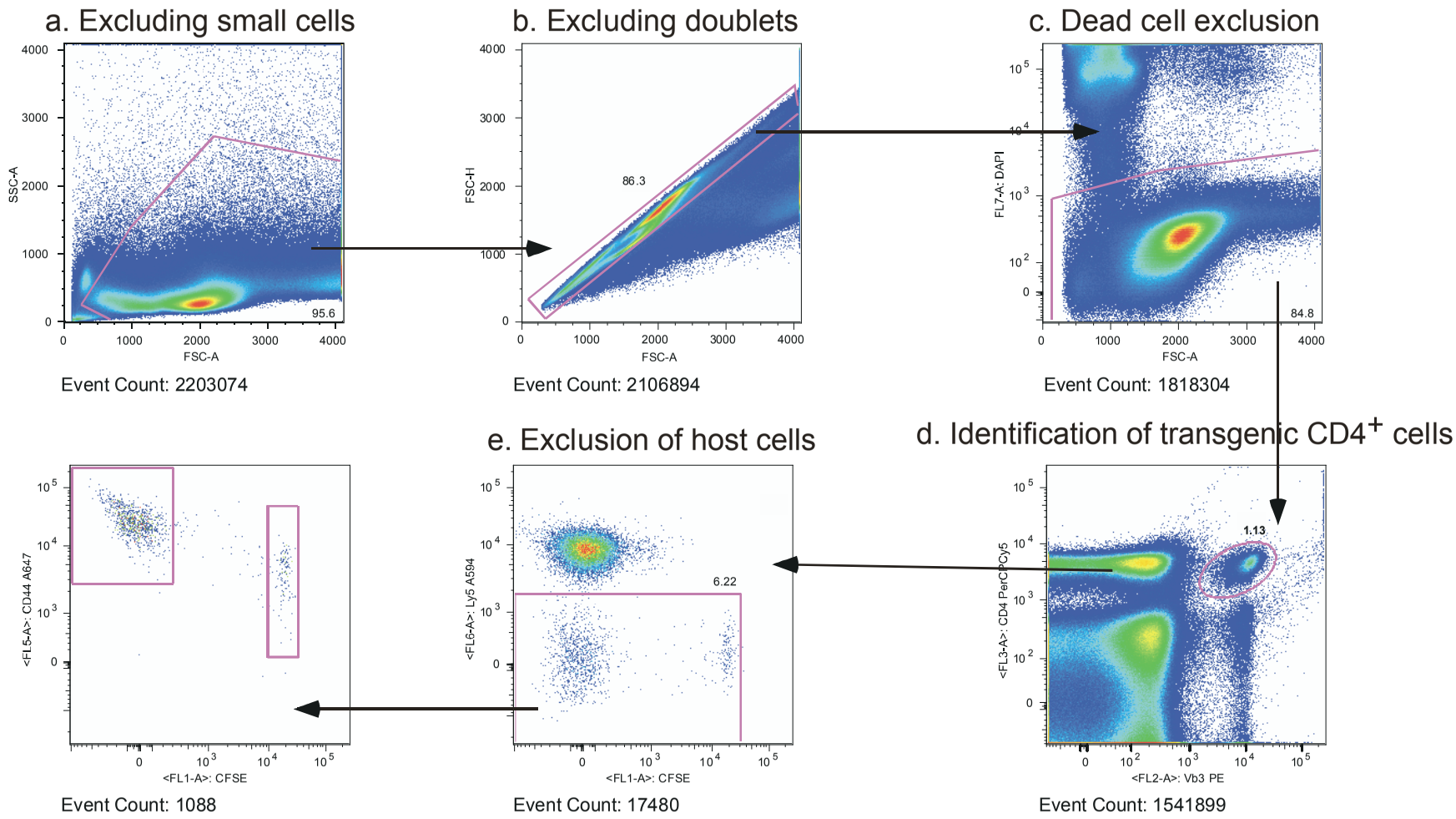


Figure 2.2 General gating strategy for Flow Cytometry analysis
(**a**.) An initial size gate was included to exclude small and dead cells followed by an exclusion of doublets by gating on FSC-Area versus FSC-Height (**b.**) (only available for data acquired on the FACS Vantage/FACS Canto). (**c.**) Dead cells were excluded by gating for DAPI- or PI- cells followed by identification of transgenic cells through positive expression of CD4 and expression of specific α or β chain (**d.**). (**e.**) In some experiments, donor cells were distinguished from host cells by the differential expression of Ly5.1 (CD45.1).

exclude small debris, followed by a FSC-Area versus Height gate to exclude any doublets for further analysis (this was only possible for data acquired with the digital software). Any further remaining dead cells were removed from the analysis by gating for either DAPI or PI negative cells (both DAPI and PI are excluded by live cells). TCR transgenic cells were identified by positive staining for CD4 and expression of the relevant α or β chain of the TCR. In experiments where the expression of Ly5.1 was used to distinguish donor cells from host or competing cells, a further Ly5.1 gate was applied to each sample. In experiments determining the percentage of cytokine positive cells, an initial gate was applied and then compared to an isotype control was ensure the correct positioning of the cytokine positive gate.

Calculations involving CFSE

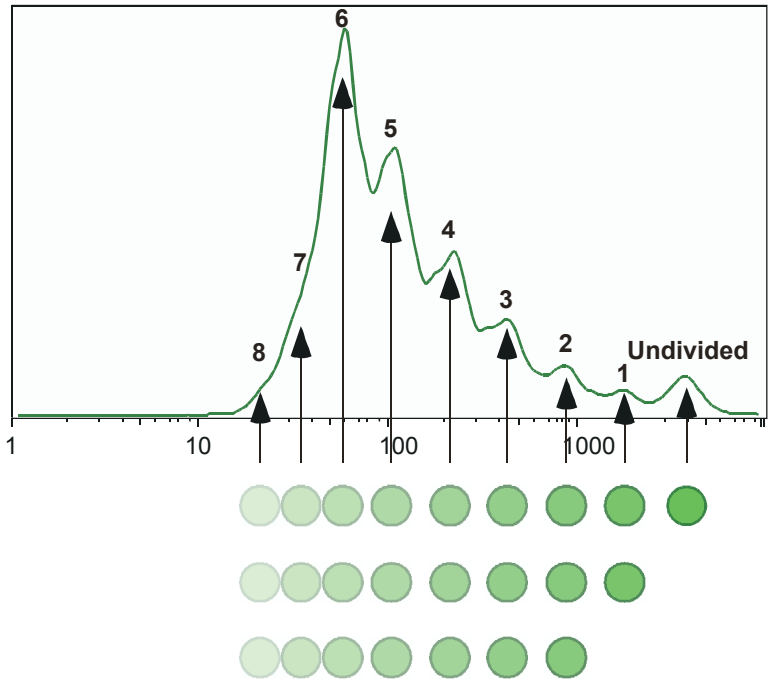
To calculate the number of donor cells recruited into division, the number of cells in each division must be adjusted for the doubling in cell number with each round of cell division. Using this rationale, a simple formula (Figure 2.3b) can be used to determined the number of cells from the original population that have undergone at least one round of cell division (Fazekas de St. Groth et al. 1999). Since the level of CFSE declines towards background staining with the increase in division number, this calculation is only accurate for populations in which no cells have divided more than 8 times.

The percentage of dividing cells per division provides some insight into the progression of dividing cells through cell division and enables the statistical analysis of division profiles between different groups. Using equation 4 (Figure 2.3c), the percentage of dividing cells per division is calculated by dividing the number of cells in each separate division by the total number of dividing cells.

Statistical Analysis

All graphical and statistical analysis was performed using Prism 4 software (Graphpad). Student t-tests were used to analyse pair-wise comparison, while mutli-group analysis

a.



b.

By gating on each division, equation (**1**) can be used to calculate the number of donor cells that have gone through **i** rounds of cell division

$$(1)\ x_i = \frac{n_i}{2^i}$$

where **n** = number of cells within each division

Equation (**2**) can then be used to calculate the total number of donor cells that entered cell division

$$(2)\ T = \sum x_i$$

Equation (**3**) is used to calculate the percentage of donor cells recruited into division

P = [1-(undivided/total number of cells)] x 100%

$$(3)\ P = \left[1-\left(\frac{x_0}{\sum \frac{n_i}{2^i}}\right)\right] \times 100\%$$

c. The percentage of divided cells per division provides an insight into the progression of donor cells through cell division and is calculated with equation (4).

D = (number of cells in division n / total number of dividing cells) x 100%

$$(4)\ D = \left[\frac{n_i}{\left(\sum n_i\right) - n_0}\right] \times 100\%$$

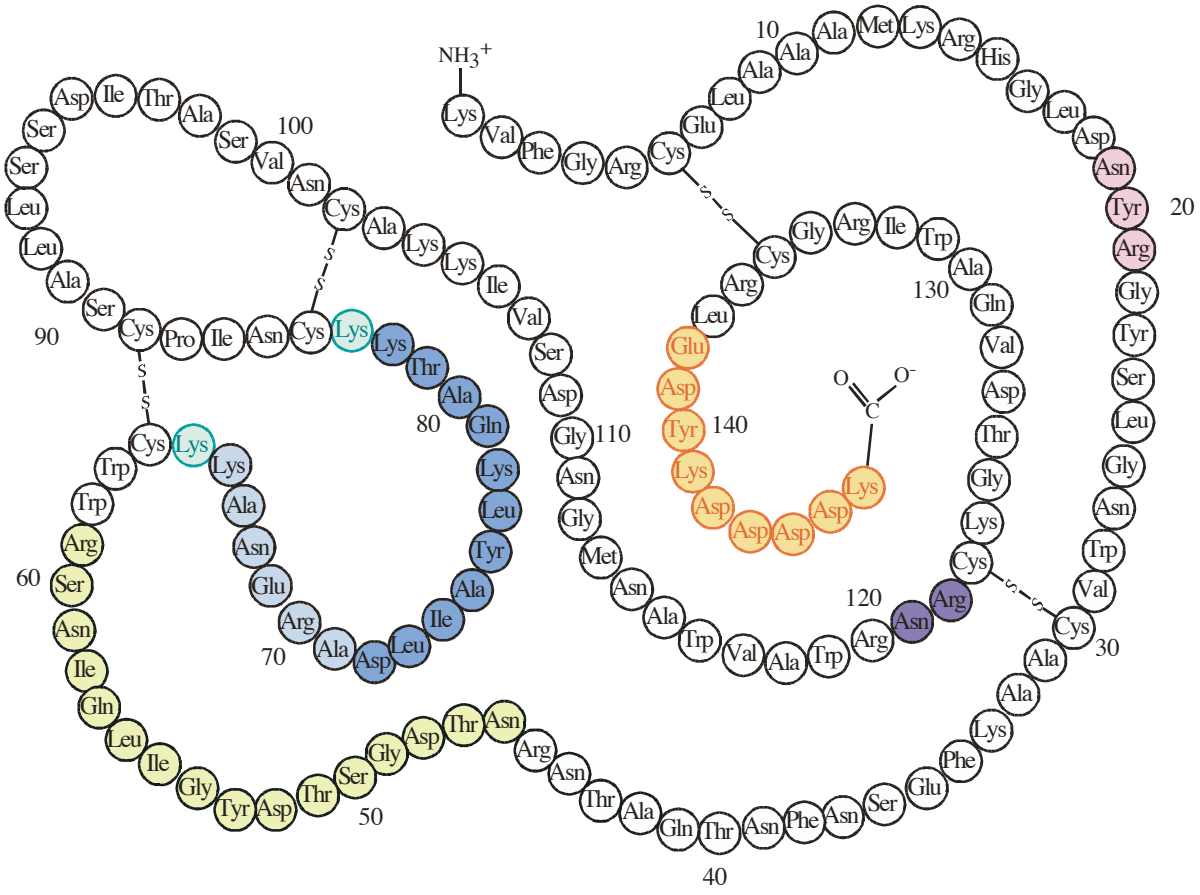
Figure 2.3: Calculations involving CFSE
The intracellular dye CFSE, is halved equally between each daughter cell with each division. This leads to serial dilution of dye and consequently each division becomes visible as a single band of fluorescence (**a.**). By gating on each division number the above equations can be used to calculate the percentage of donor cells recruited into division (**b.**) and the percentage of divided cells within each division (**c.**).

was performed with one-way ANOVA. Two-way ANOVA was used to analyse mutli-group and mutli-parameter.

2.4 Molecular Biology - Recombinant HELMCC

Recombinant HELMCC protein was originally cloned by Adrian Smith (T Cell Biology Group, Centenary Institute) and is a mutagenised form of HEL in which a hydrophilic loop at residues 83-93 was replaced with the MCC epitope (residues 87-103) flanked by cathepsin B sites (double lysine, KK) (Figure 2.4). Initially the C-terminal MCC epitope was fused to the C-terminal end of whole HEL protein. However published studies have indicated that when the C-terminal MCC epitope was fused to the C-terminal end of glutathione-S-transferase, the MCC epitope could be presented to transgenic T cells without a requirement for processing, suggesting lack of secondary structure (Colledge et al. 2001). Therefore to ensure that the presentation of both HEL and MCC antigens required processing, a modification of the system of Verma (1995) was adopted. Molecular modelling of a number of lysozyme proteins derived from different bird species was used to identify a hypervariable loop flanked by conserved cysteine residues that was unlikely to be required for protein folding and stability. In the HEL protein, this loop corresponded to the 11 amino acids between cysteine residues 64 and 76 of the mature protein (residues 82 and 94 when the numbering begins at the start of the signal sequence). To make the HELMCC fusion protein, these 11 aa were replaced by a total of 19 aa encoding the C-terminal sequence of MCC (residues 87-103) flanked by an additional lysine at either end. This created two cathepsin cleavage sites on either side of the MCC sequence, previously shown to increase the efficiency of MCC processing and presentation (Verma et al. 1995).

The original construct contained a frame shift mutation that was corrected by Robert Brink (B Cell Biology Group, Centenary Institute) who also added a Flag Tag for protein purification purposes. This new construct was cloned into the pcDNA3 vector but later into



RECOMBINANT HELMCC-FLAG
146aa, 16.4 kD

Figure 2.4: Amino Acid sequence of HELMCC
Blue residues indicate the inserted MCC amino acid (MCC_{87-103}) sequence flanked by lysine linkers (aqua). The double lysines flanking the MCC sequence created cathepsin B cleavage sites to enable enhanced presentation of MCC. Orange residues indicate the FLAG epitopes. The binding site of HyHEL9 is identified by pink residue, HyHEL 10 by purple residues. The yellow amino acids correspond to the 3A9 TCR reactive epitopes while the dark blue amino acids illustrate the location of 7H8.2C12 (anti-MCC) binding which corresponding to the 5C.C7 TCR reactive epitopes.

the pIRES vector (Figure 2.5) to enable higher yields of recombinant protein. This expression system yielded minimal amounts of protein (a total yield of less than 1mg) and therefore a yeast expression system was adopted (this was all performed by Didrik Paus and Robert Brink (B Cell Biology Group, Centenary Insitute of Cancer Medicine and Cell Biology). In the following section, the transfection of chinese hamster ovarian (CHO) cells with plasmid containing HELMCC-Flag and the subsequent purification and concentration of recombinant HELMCC-Flag will be described. For all the experiments described in Chapter 4 and Figure 5.7.1 and Figure 5.7.2, HELMCC-his (made by Didrik Paus) was used.

Amplification, Purification and Storage of Plasmid DNA

DH5α *E.coli* transformed with pcDNA3-HELMCC-Flag or pIRES-HELMCC-Flag (courtesy of Robert Brink) were cultured overnight in liquid broth (LB) (5g/L yeast extract, 10g/L tryptone and 10g/L NaCl) medium containing 100μg/ml ampicillin. Plasmids were purified using GenElute Plasmid Miniprep Kit (Sigma Aldrich). Miniprep DNA was quantified using mass spectrophotometry.

Stocks of bacteria containing plasmid were prepared and stored at –70^{o}C. After overnight culturing, transformed clones were grown in LB containing ampicillin for 6 hours, 1ml of 65% glycerol stock solution was added to 2ml of medium containing bacteria.

Polymerase Chain Reaction

DNA sequences encoding HELMCC were amplified using polymerase chain reaction (PCR). PCR was performed in a final volume of 10μl containing 0.24μl of 5U/μL Red*Taq* DNA polymerase, 1μl of 10X PCR buffer, 0.3μl of 50mM Mg^{2+} buffer, 0.08μl of 10mM dNTPs (Boheringer Manneheim/Roche Manneheim, Germany), 0.6μl of 50pmol/μl 3' primer and 0.6μl 50pmol/μl 5' primer and 2μl of template DNA. Five minutes of initial denaturation at 95^{o}C was followed by 30 cycles of denaturation at 95^{o}C for one minute,

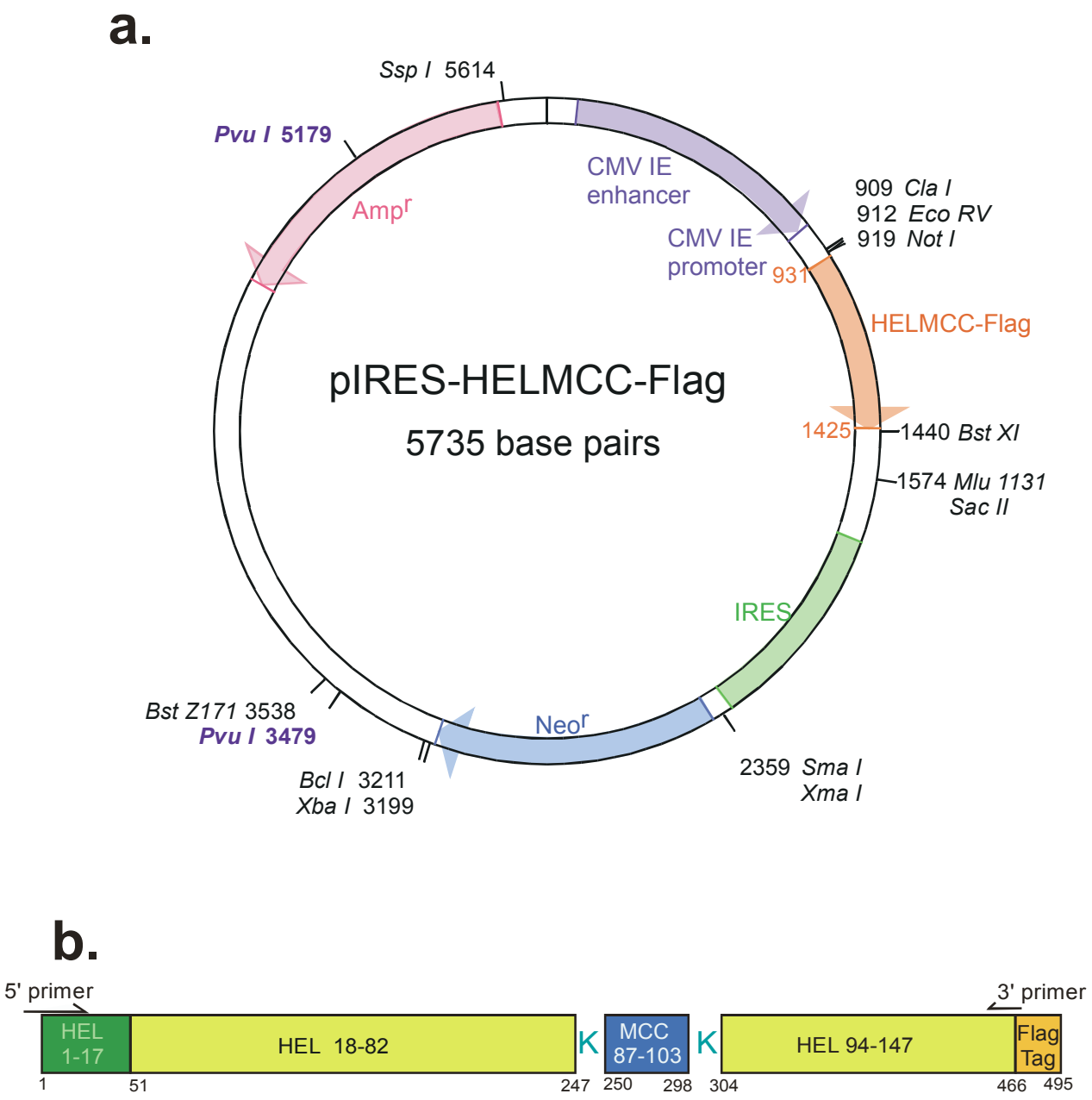


Figure 2.5: pIRES-HELMCC-Flag sequence

a. Map or pIRES vector containing HELMCC-Flag

The plasmid is 5735 bp in length containing distinct regions encoding the CMV promoter and enhancer (purple), recombinant HELMCC protein (orange), repeat sequence (IRES) (green). The construct contains a gene encoding ampicillin resistance to enable selection of transformed *E.coli* (pink) and the gene encoding for neomycin resistance (light blue) for the selection of transfected mammalian cells with G418. Enzyme restriction site are indicated on the exterior of the construct.

b. A schematic representation of the insert containing the signal sequence of HELMCC-Flag. The leader sequence of HEL is encoded by the first 18 amino acids. The binding sites of the 5' and 3' primers are shown and screening by PCR was expected to yield a product of approximately 470bp.

annealing at 56°C for two minutes and elongation at 72°C for one minute and a final 15 minutes of extension at 72°C.

PCR Primers:

5' Primer: 5' HEL F4 - TAG GGA TCC ATG AGG TCT TTG CTA ATC TTG GTG

3' Primer: 3 HEL - TAG CTC GAG TCA CAG CCG GCA GCC TCT GAT CC

Agarose Gel Electrophoresis

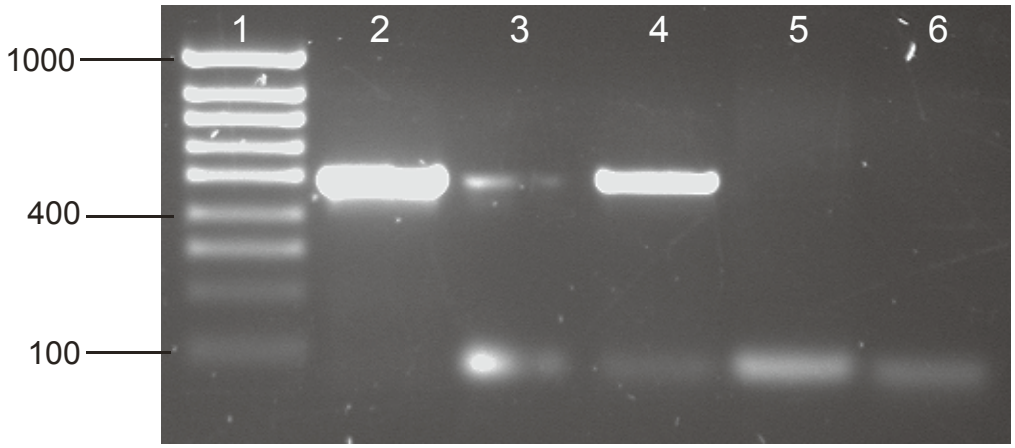
DNA fragments were separated according to size on agarose gels in TAE buffer (2 M Tris, 0.57% acetic acid, 0.05 M EDTA), pH 8). Agarose concentrations varied between 1% and 1.5% depending on the estimated size of the DNA fragments. Fragments sizes were estimated using a DNA molecular weight markers VIII (19bp-1.11 kb) or (100-1000bp). For visualisation of DNA fragments, 0.6μg/mL Ethidium Bromide was added to the gel prior to electrophoresis and gels examined under UV light.

Transformation of HELMCC into eukaryotic cells

DNA digestion by restriction endonuclease

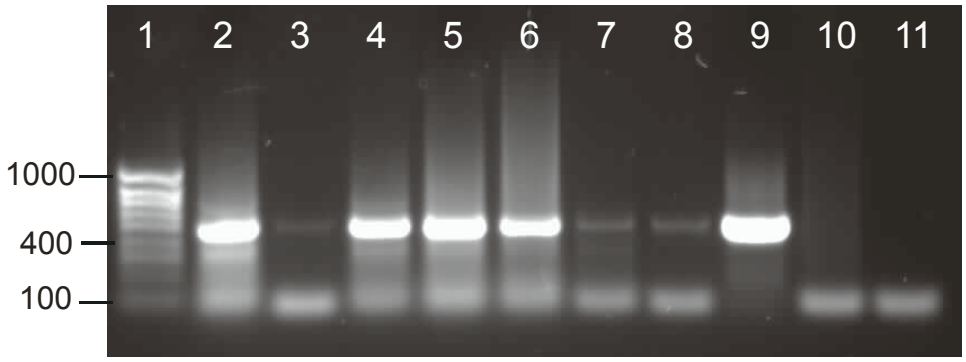
Digests of DNA contained 0.1-5μg of purified plasmid DNA with Buffer M (Boehringer Mannheim/Roche, Manneheim, Germany) to yield a final concentration of 1X and restriction endonuclease BglII at 5U per μg of DNA. Samples were incubated at 37°C for at least 2 hours prior to incubation at 60°C for 15 minutes to inactivate any excess enzyme. Digestion of pIRES-HELMCC-Flag with BglII yielded up to 4 bands when run on a 0.5% agarose gel. These bands corresponded to uncut plasmid DNA, single cut plasmid DNA running slightly faster than uncut plasmid, a 4000bp fragment containing the HELMCC-Flag insert and a smaller fragment of around 1700bp. The single cut plasmid and 4000bp bands were excised and extracted from the gel with QIAEX II DNA gel extraction kit. Linearised plasmid DNA was screened by PCR to ensure the correct bands had been excised and extracted from the gel (Figure 2.6a).

a. PCR Screen of purified plasmid DNA and linearized plasmid



1. 100bp ladder
2. pIRES-HELMCC-Flag plasmid
3. linearised pIRES-HELMCC-Flag
4. pcDNA3 HELMCC-Flag
5. pcDNA3 empty plasmid
6. TDW

b. PCR Screen of pIRES-HELMCC-Flag transfected CHO Cells



1. 100bp ladder
2. HELMCC Clone D5A3
3. HELMCC Clone D5A5
4. HELMCC Clone D5A10
5. HELMCC Clone D5D12
6. HELMCC Clone D5F8
7. HELMCC Clone D5F12
8. pIRES-neo HELMCC-Flag
9. pcDNA3 HELMCC-Flag
10. pcDNA3
11. water

Figure 2.6 PCR screen of purified plasmid DNA and genomic DNA from transfected CHO cells
Purified plasmid DNA (**a.**) and DNA isolated from transfected CHO cells (**b.**) was screened for the HELMCC-Flag insert by PCR.

Electroporation

CHO cells were grown in RPMI supplemented medium to 80% confluencey and single cell suspensions prepared. $2x10^6$ CHO cells and up to 10μg of linearised DNA were cooled on ice for 10 minutes prior to transfer into a 0.4cm Bio-Rad Gene Pulser Cuvette. Cells were electroporated on a Bio-Rad Gene Pulser at a voltage of 200V and a capacitance of 960μFd, resulting in a time constant ranging between 30 and 40. Cells were rested on ice for 10 minutes after electroporation and then plated in a 24 well Tissue Culture Plate (Baxter). After 2 days of culture, cells were washed with PBS and fresh RPMI supplemented medium containing 1mg/ml G418 selective medium was added to each well. Transfected cells were grown in selective medium for 2 weeks before screening by enzyme-linked immunosorbent assay (ELISA) (see below) to measure the level of HELMCC secretion. Transfected CHO cells with the highest level of HELMCC-Flag secretion (clones C1, B1, D4 and D5) were further sub-cloned by limiting dilution. For each clone, two 96 wells were seeded to contain either 1 cell per well or 10 cells per well. Clone D5 was chosen for further screening (Figure 2.7) and selection as it had the least number of positive wells and therefore had the highest probability that each positive well was originally derived from a single clone (Poisonian distribution).

HELMCC capture ELISA

The supernatant of HELMCC transfected clones was screened by HEL capture ELISA (Goodnow et al. 1988). Unless otherwise stated, all incubations were performed at 37^{o}C for one hour, followed by 3 washes with 0.05% Tween in PBS.

Each well of a 96-well flat bottom plate (Greiner Bio-one) was incubated with 60μl of HyHEL10 at 25μg/ml in PBS. Wells were then blocked with 300μl of 1% BSA in PBS. 50μl of CHO cell supernatant was added per well in duplicate, whole HEL protein standards of 1ηg to 100ηg/mL (in RPMI supplemented medium) were added in duplicate to control wells and RPMI supplemented medium was used as a negative control. Wells were subsequently incubated with 50μl of HyHEL9-bi (1 in 3000 dilution of stock solution)

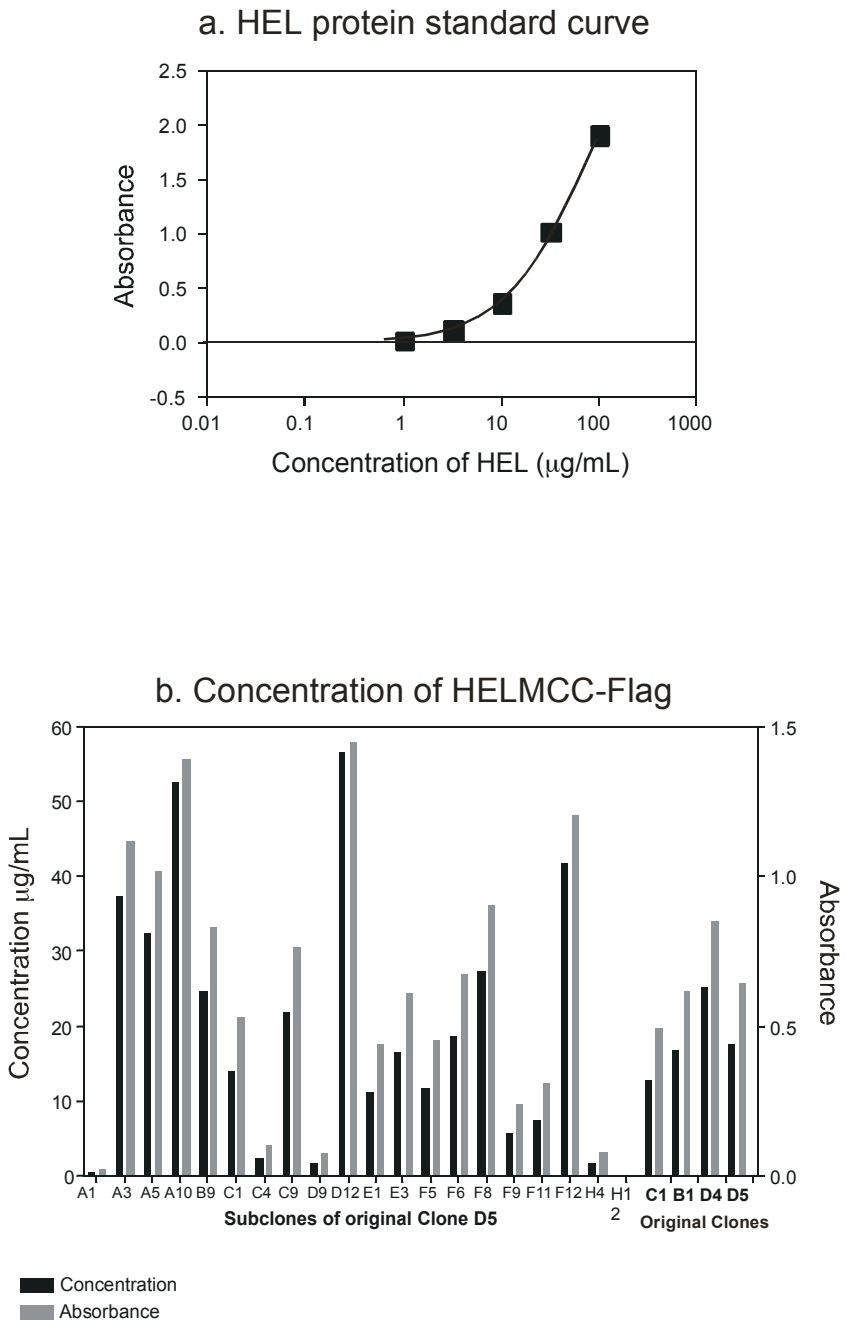


Figure 2.7: ELISA screen to determine the concentration of HELMCC-FLag

followed by an incubation with 50μl of strepavidin conjugated alkaline phosphatase (Sigma Aldrich) diluted in 0.1% BSA. The assays were developed with 100μl of p-nitrophenyl phosphate (NPP) (ICN) at a concentration of 1mg/ml in NPP buffer (10mM Na_2CO_3, 35mM $NaHCO_3$, 0.1 mM $MgCl_2$, 0.02% NaN_3). Absorbance was measured at 405nm on a Multiskan Multisoft plate reader. Standard curves were calculated with Prism 4 Software (GraphPad) (Figure 2.7a).

Screening of CHO transfecetd clones by PCR

To ensure CHO cells had been stably transfected with HELMCC-Flag, genomic DNA was purified and screened for the HELMCC insert by PCR. Genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, WI, USA) and used as template DNA in the PCR described above. pcDNA-HELMCC-Flag and pcIRES-Flag served as positive controls while pcDNA empty vector and water both served as negative controls. A band of around 470bp was seen in PCR products from all CHO transfected clones and in the positive controls (plasmid DNA) but was not visible in PCR products from the two negative controls (Figure 2.6b)

Purification of HELMCC

Recombinant HELMCC was purified with an anti-FLAG M2 Affinity gel (Sigma), in which agarose beads are covalently linked to anti-FLAG antibody that binds FLAG tagged proteins as the supernatant is passaged over the column. HELMCC-Flag supernatant was passaged over anti-FLAG column and then eluted under low pH by the addition of 1M Glycine.HCl pH3.5. After elution from the column, HELMCC-Flag protein was dialysed in cutoff dialysis tubing (Pierce) against PBS to remove any excess salt prior to concentration with a Macrosep Centrifugal Device (PALL Life Sciences, Ann Arbor USA) with a molecular weight cut off of 3kDa.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

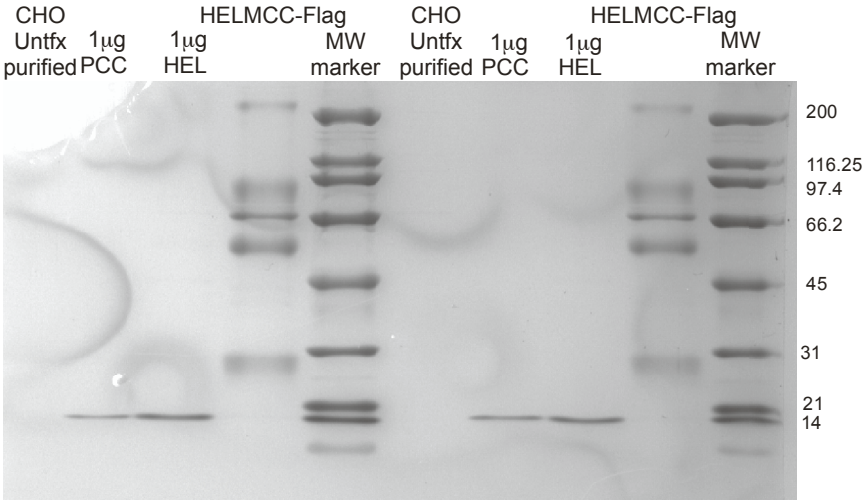
Glycine SDS-PAGE enables analysis of high-molecular weight proteins and relies on discontinuous gel system consisting of a resolving gel (12-15% acrylamide) and a stacking gel (4% acrylamide) or a single 12% resolving gel (Gradipore). Samples were added to 2X sample reducing buffer (0.025 M Tris-HCl, 10% glycerol, 5% SDS, 0.025% Bromophenol Blue, 2% β-mercaptoethanol) and boiled for 5 minutes. Relative molecular weights were determined by comparison to molecular weight standards, either BioRad broad range (6.5-200kDa) for Coomassie staining or Kaleidoscope Prestained Standards (7.5-200kDa) for Western Blotting. Electrophoresis was performed using a mini-Protein II cell (Bio-Rad) at a voltage of 150-200V (3.02g/L Tris, 14.4g/L Glycine, 1g/L SDS, pH 8.3) until the dye front reached the bottom of the gel. Proteins were visualised by staining with 0.05% Coomassie brilliant blue (in 40% methanol and 10% acetic acid) for at least 1 hour, followed by destaining in 40% methanol and 10% acetic acid.

Purified HELMCC-Flag from pIRES transfected CHO cells showed several high molecular weight bands between 70 and 200kDa (Figure 2.8), while the smallest size protein was close to 31kDa, almost twice the expected size of HELMCC-Flag, suggesting purified HELMCC-Flag protein was contaminated with high molecular weight proteins or HELMCC-Flag had formed dimers of varying sizes. HELMCC-His from Didrik Paus displayed only one band of around 14kDa. For the experiments using recombinant HELMCC, since the absolute concentration of HELMCC was to be determined by comparison to *in vivo* dose response curves for MCC and HEL peptide alone, contamination with high molecular weight proteins or the formation of dimers/trimers did not pose a large problem as only the presence of both the 3A9 and 5C.C7 epitopes on one protein was desired. Therefore the ability of HELMCC to induce both 5C.C7 and 3A9 responses *in vivo* was tested without too much concern for the purity.

Detection of HELMCC by Western Blot

Proteins were transferred into poly-vinyl membrane by electroblotting at a constant current of 300mAmps for 70 minutes and on ice to prevent overheating (25mM Tris, 192mM

a.



b.

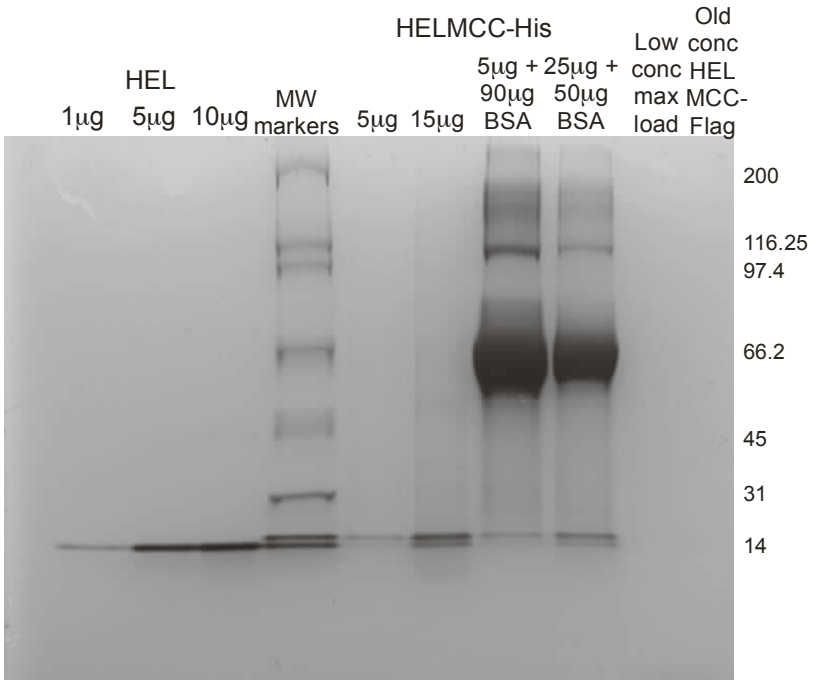


Figure 2.8 SDS-PAGE analysis of recombinant HELMCC
Samples were boiled for 5 minutes before electrophoresis on a **a.** 15% gel or a **b.** 12% prepoured gel (Gradipore) and stained with Coomassis Brilliant Blue.
a. HELMCC-FLAG produced through the transfection of CHO cells with pIRES-HELMCC-his was analysed by SDS-PAGE gel. No clear band was seen at the expected weight of 16kd.
b. High and Low concentration HELMCC-his, produced by Didrik Paus through the transfection of yeast cells, was analysed by SDS-PAGE gel and compared to a known concentration of HEL protein. BSA was added to enhance the visualization of single bands of recombinant protein.

glycine). Membranes were blocked overnight in 4% BSA solution to reduce non-specific binding. After 3 washes in tris-buffered saline Tween (TBS-Tween) (10mM Tris, 150mM NaCl, 0.05% Tween-20, 0.5g/L $MgCl_2$), membranes were incubated with primary antibody 7H8.C12 (mouse anti-Cytochrome C diluted 1 in 5000 in 3% BSA TBST) for 3 hours. Membranes were washed three times in TBST, followed by incubation with secondary antibody anti-mouse alkaline phosphatase (diluted 1 in 4000 in 3% BSA in TBS-Tween) for a further 2 hours. Blots were developed using Chemiluminescence (Pierce) and exposure to X-ray film (Kodak).

Although PCC was detected with the monoclonal against the C-termini of cytochrome C (7H8.C12), bands corresponding to either HELMCC-Flag or HELMCC-His were not detected with this antibody (Figure 2.9). Since MCC is contained within the HEL sequence and the C-terminal epitope of MCC recognised by 7H8.C12 not exposed, it is possible that the MCC insert cannot be detected by immunoblotting with this antibody.

Testing HELMCC *in vivo*

Although a band corresponding to the correct molecular weight of HELMCC-Flag was not visible by SDS-PAGE analysis, nor were the MCC portions of HELMCC-Flag or HELMCC-His detectable by Immunoblotting, we went on to test the ability of recombinant HELMCC to induce proliferation of 5C.C7 and 3A9 TCR tg cells *in vivo*. In the first experiment, CFSE labelled 5C.C7 or 3A9 cells were adoptively transferred into host mice followed by immunisation with a total dose (10μg or 1μg) of either HELMCC-Flag (protein concentration was estimated by BCA protein kit (Pierce)) or HEL protein emulsified in CFA. Draining and non-draining lymph nodes were harvested 3 days later and cell proliferation analysed by flow cytometry. Only a small amount of proliferation by 5C.C7 was detected when stimulated with 1μg HELMCC (Figure 2.10a) with no proliferation seen in the non-draining node. Proliferation was enhanced with an increased dose of HELMCC (10μg dose). Proliferation of 3A9 cells was detected at both doses of HEL protein, but no proliferation was seen with either dose of HELMCC-Flag, suggesting the actual concentration of HELMCC-Flag was much lower than predicted and the high molecular

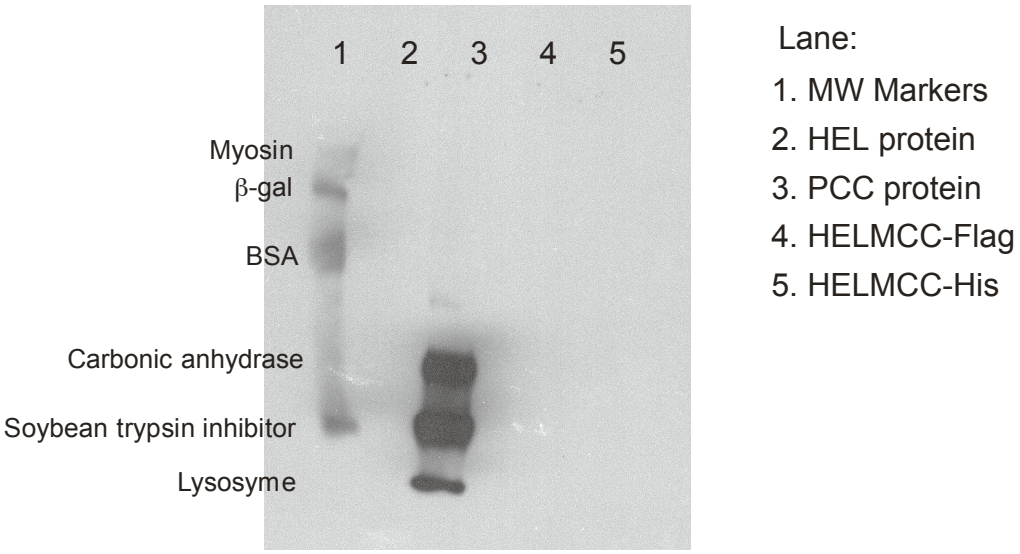


Figure 2.9 Western Blot of purified HELMCC protein
Samples were electrophoresed on a 12% SDS-Page gel, then transferred onto nitrocellulose membrane and probed with anti-Cytochrome C (7H8. 2C12) monoclonal antibody followed by anti-mouse conjugated to alkaline phosphatase. Membranes were developed with chemiluminescence and exposed to X-ray film for 30 seconds.

weight bands visible by SDS-PAGE and coomassie staining were in fact contaminating proteins.

In the second experiment, host mice received CFSE labelled 5C.C7 or 3A9 cells and were immunised with a mixture containing 25μg PCC and 25μg HEL proteins emulsified together in CFA or 25μg HELMCC-His in CFA. Draining and non-draining lymph nodes were harvested 3 days later and proliferation measured by flow cytometry. Proliferation of 5C.C7 was seen when mice were immunised with either HELMCC-His or PCC and HEL. Similarly proliferation of 3A9 cells was also detected with the either PCC/CFA or HELMCC-his/CFA (Figure 2.10b). The data demonstrate recombinant HELMCC-His contains both the MCC_{86-103} and HEL_{46-61} and is capable of stimulating 5C.C7 and 3A9 TCR tg cells when emulsified in CFA and administered to mice via a sub-cutaneous injection.

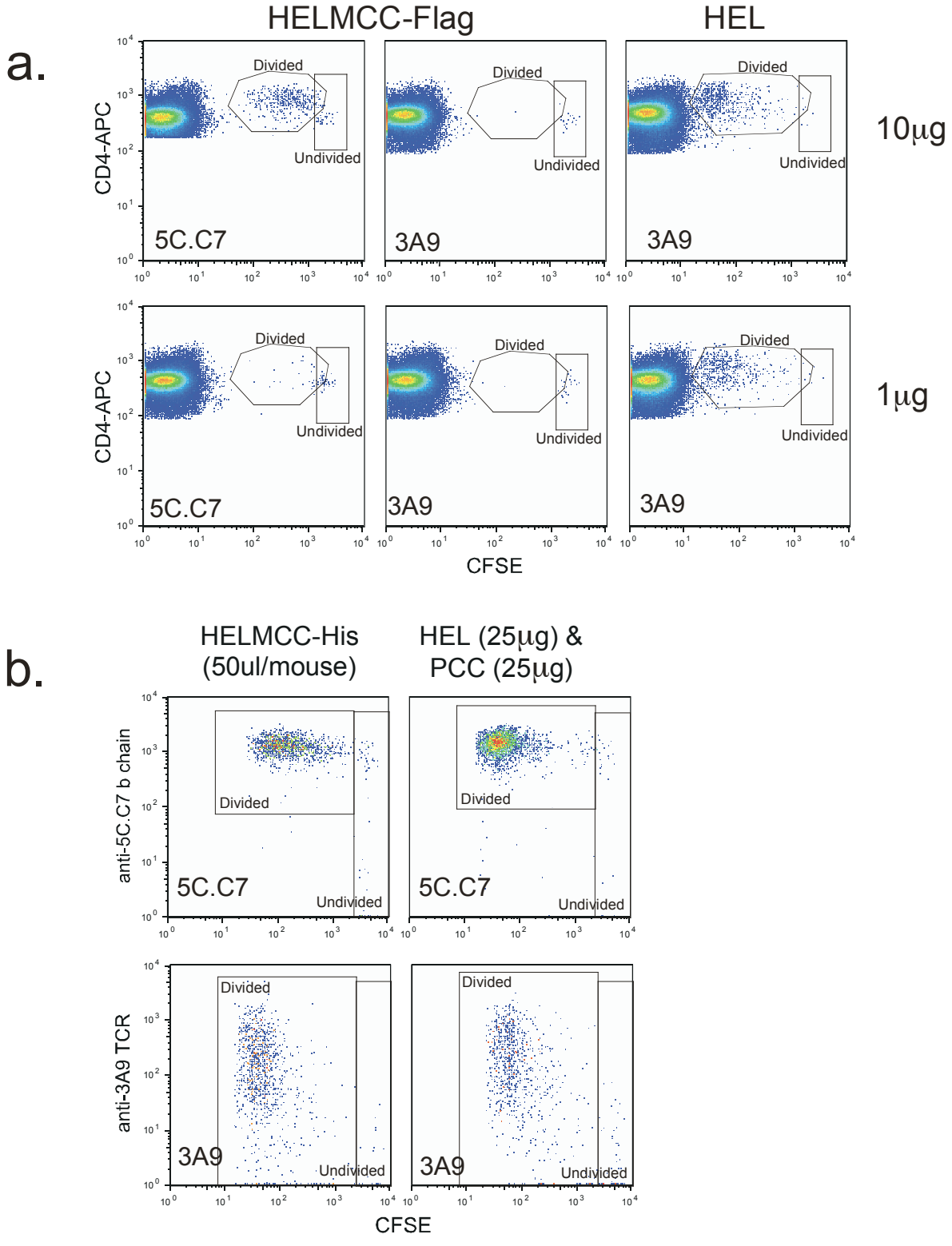


Figure 2.10: Response of 5C.C7 and 3A9 cells to recombinant HELMCC *in vivo*

a. 1x10^6 CFSE labelled 5C.C7 or 3A9 cells were adoptively transferred into recipient mice followed by immunisation with approximately 10μg or 1μg of HELMCC-Flag or HEL protein. 3 days later recipient mice were sacrificed and the proliferation of 5C.C7 and 3A9 cells was determined by flow cytometry. Samples were gated for $CD4^+$, PI^- cells.

b. Recipient mice received 4x10^6 CFSE labelled 5C.C7 or 3A9 cells and were immunised with either 25μg HEL protein, 25μg PCC protein or 25μg of HELMCC-his (purified from yeast cells). Proliferation of 5C.C7 or 3A9 cells was measured 3 days later by flow cytometry. Samples were gated for $CD4^+$, $CFSE^+$, PI^- cells.

Chapter 3: Antigen-specific competitive inhibition of $CD4^+$ T cell recruitment into the primary immune response

To induce full activation and differentiation, T cells require stimulation via TCR by binding to specific Ag-MHC complexes and a second signal via costimulatory receptors, such as CD28 (Jenkins et al. 1991). However the relative contributions of each of these signalling pathways to the early stages of the response remain unclear. The experiments described in this chapter were designed to study these questions by means of introducing a cohort of high affinity T cells that would compete for either Ag-MHC, or factors independent of MHC (ie costimulatory molecules and survival signals).

The first evidence that responses to two independent antigens could suppress each other was documented in the early 1900s when immunising mice with a mixture of horse and bovine serum albumins together elicited less antibody production than immunisation with either one alone (Michaelis 1902) (reviewed by Pross (1974). It was not until after the discovery of two subsets of lymphocytes that reduced interactions between T and B cells were shown to mediate reduced production of immunoglobulin to the second antigen (Albright et al. 1970; Kerbel and Eidinger 1971). In the last 5 years, an increasing number of studies have tracked the effect of either antigen-specific and/or antigen non-specific competition on the overall response of antigen specific cells. At the commencement of this work, only two papers studying the effect of competition on the early events of T cell activation had been published. In an *in vitro* system, Lake (1999) demonstrated reduced proliferation of CD4 T cells as early as 72 hours after TCR activation when the stimulating APCs were preexposed to a competing cohort of CD4 cells. The degree of inhibition was related to the affinity of T cells for cognate antigen, such that higher affinity HA specific T cells could suppress a subsequent response by lower affinity OVA specific cells but not vice versa. In previous work by other members of the T cell group (Smith et al. 2000), the

proliferation of CFSE labelled 5C.C7 cells was shown to be reduced 3-fold by day 3 after transfer into an intact 5C.C7 tg mouse rather than a wild-type control. Yet despite a 45 fold increase in the number of competing cells, transferring MBP specific cells into intact 5C.C7 TCR tg mice did not alter division profiles and there was only a minor reduction in total MBP numbers at day 3. Therefore, to enable the detection of competition at an early timepoint in the response, competing cells would require an advantage in terms of total precursor numbers or early access to APCs.

The experiments described in this chapter were designed to investigate the effect of competition for peptide-MHC or costimulatory signals on the early stages of T cell activation, based on the results described above. Three different strains of TCR tg mice were available, (5C.C7, 3A9 and MBP), each expressing a TCR of different MHC Class II-restricted antigen specificity. Based on antigen dose response curves (Figure 3.0), 5C.C7 cells were considered of highest affinity for antigen (MCC), followed by 3A9 cells that responded to HEL with medium affinity. Finally MBP cells were considered of lowest affinity due to the increased dose of antigen that was required to elicit a half maximal response. To control for the variability between the number of T cells expressing the TCR tg in the different strains of mice, the percentage of $CD4^+$ cells was determined by flow cytometry prior to cell transfer and the total number of LN cells transferred adjusted, such that each recipient mouse received a total of $2.5x10^6$ $CD4^+$ cells. In all experiments described in this chapter, a time delay between competing and readout cells was introduced (described in the next section). To ensure consistency in the time intervals between experiments, recipient mice were immunised with peptides emulsified together in CFA and 12 hours was allowed for the passage of antigen to the node (Ingulli et al. 1997; Norbury et al. 2002) before the first cohort of antigen specific T cells was adoptively transferred into recipient mice. Recruitment and proliferation of CFSE labelled cells was measured 2.5 days after transfer, when cells proliferating at a maximal rate were still distinguishable from host cells.

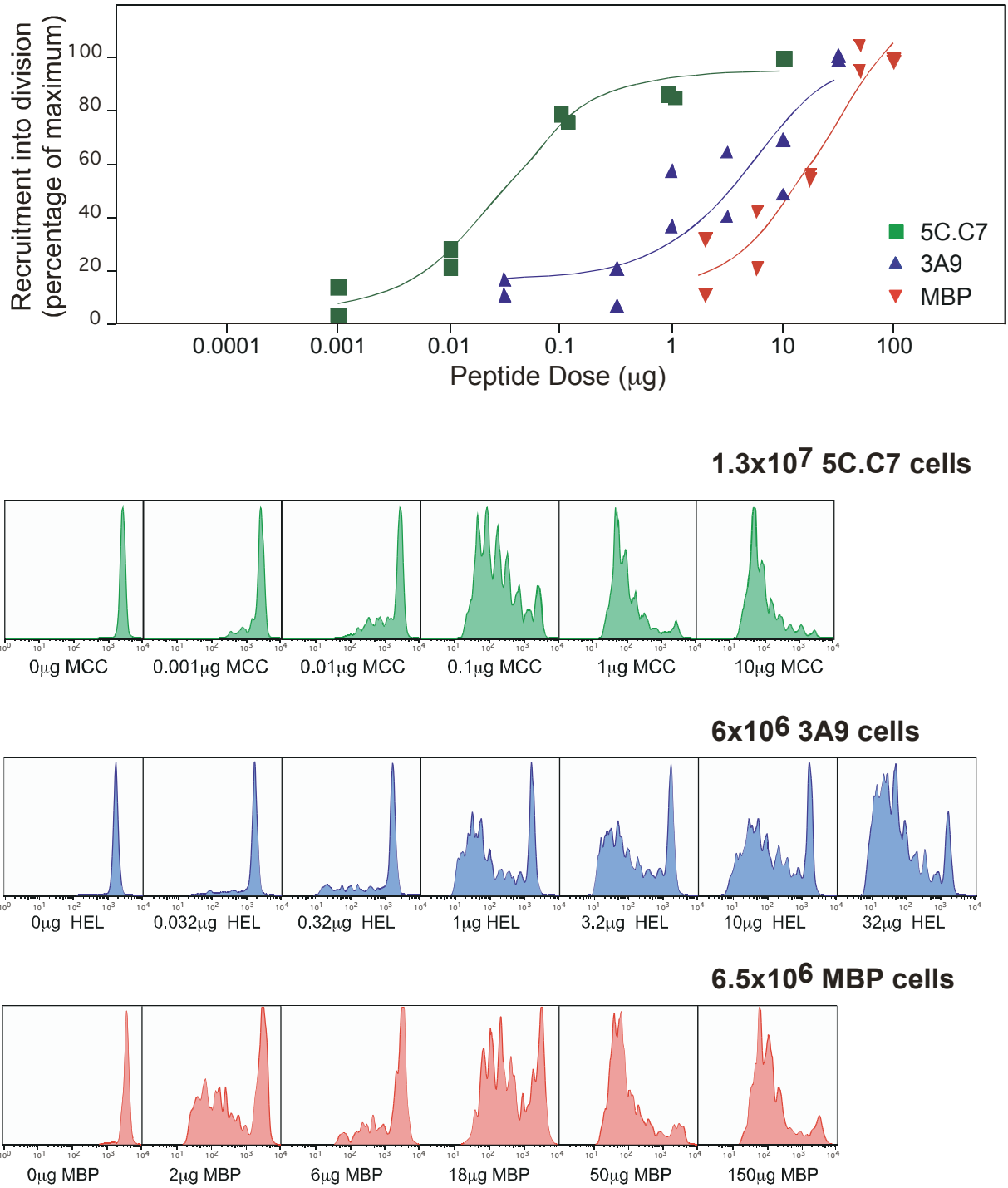


Figure 3.0 Dose response curves of TCR tg cells to peptide

The response TCR tg $CD4^+$ T cells to graded doses of specific peptide was measured. CFSE labelled cells from TCR tg mice were adoptively transferred into syngenic recipient mice 2 days prior to immunization with a range of peptide doses emulsified in CFA. 3 days later draining nodes were harvested and analyzed by flow cytometry. TCR tg cells were identified by staining with monclonal antibodies against CD4 and the relevant TCR α or β chains.

The graph represents the percentage of TCR tg cells recruited into division expressed as a percentage of the maximum response (ie the response to the highest dose of antigen). The curves were fitted to the mean of 2 mice per dose with each mouse shown as a single point.

3.1 Sequential stimulation of T cell cohorts is required to visualise an effect of competition by day 2.5

As mentioned above, competition between $CD4^+$ T cells was previously documented on day 3 of the response only when the competing cohort had a significant advantage in terms of total cell number (Smith et al. 2000) or earlier access to antigen (Lake et al. 1999). During my honours thesis, I developed an *in vitro* model of T cell competition in which cells of different antigen specificity were shown to reduce the proliferation of a second cohort of T cells only when the competing response was stimulated 24 hours in advance. Therefore in the first *in vivo* experiments described here, the necessity of a time interval between the 2 cell cohorts was investigated.

The treatment of the 5 experimental groups is detailed in Figure 3.1.a. Syngenic recipient mice were immunised with a mixture of HEL and MCC peptides emulsified together in CFA on day –1.5 (36 hours prior to the transfer of CFSE labelled readout cells) and 12 hours later (day –1), two groups of mice received either $1x10^7$ unlabelled 5C.C7 or $2x10^7$ unlabelled 3A9 cells (twice the number of 3A9 cells were injected as 5C.C7 TCR tg cells contain around twice the proportion of CD4 cells). A further 24 hours later (day 0), all recipient mice received $1x10^7$ CFSE labelled 5C.C7 cells while two of the five groups also received either $1x10^7$ unlabelled 5C.C7 cells or $2x10^7$ 3A9 cells. At day 2.5, draining LNs were harvested and analysed by flow cytometry.

When 5C.C7 cells were activated in the presence of a 3A9 response, initiated either simultaneously or 1 day earlier, there was no change in the CFSE division profile (Figure 3.1b) or recruitment of cells into division (Figure 3.1c), and divided cells progressed through an equal number of divisions by day 2.5 (Figure 3.1d). Competition by a 5C.C7 response initiated 1 day earlier led to a 25% reduction in the number of CFSE labelled 5C.C7 cells recruited into division (Figure 3.1c) and divided cells progressed through fewer rounds of division by day 2.5 (Figure 3.1d). Co-transferring an equal number of unlabelled 5C.C7 cells at the same time did not alter recruitment into division or the number of division by day 2.5. Therefore to detect an effect of competition between $CD4^+$

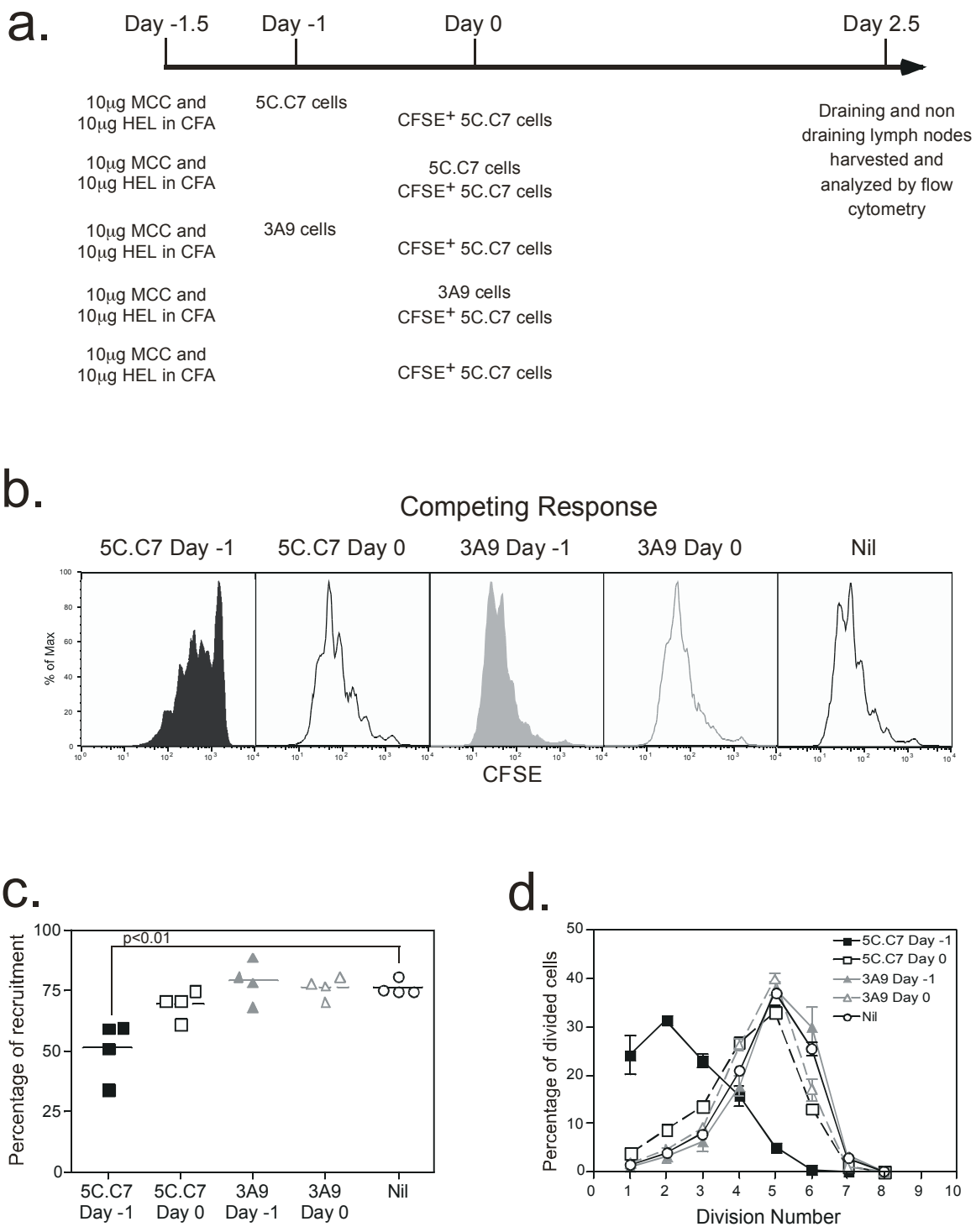


Figure 3.1: Competition between sequential versus simultaneous T cell responses
a. On day -1.5, five groups of KK recipient mice were immunised with 10μg MCC and 10μg HEL emulsified together in CFA, 12 hours later two of the groups received either $1x10^7$ 5C.C7 KK or $2x10^7$ 3A9 KK cells. On day 0, CFSE labelled 5C.C7 KK cells were adoptively transferred into recipient mice. Mice were sacrificed 2.5 days later and draining nodes analyzed by flow cytometry. (**b.**) The proliferation of CFSE labelled 5C.C7 T cells in the presence of a competing 5C.C7 or 3A9 response transferred 24 hours of at the same time as the CFSE labelled 5C.C7 cells. (**c.**) The percentage of 5C.C7 cells recruited into division was calculated as described in Figure 2.3 equation 4. (**d.**) The percentage of divided cells in each division was calculated by dividing the number of cells in each division by the total number of divided cells.

T cells on day 2.5, a 24 hour time interval between the adoptive transfer of the competing and readout cells was introduced.

3.2 Competition between $CD4^+$ T cells of different antigen specificities

To determine the effect of antigen-specific and antigen non-specific competition on the early stages of $CD4^+$ T cell responses, an experiment in which each TCR tg specificity was tested against all 3 individuals specificities was designed (3 way matrix). In three separate experiments, the recruitment and proliferation of CFSE labelled 5C.C7, 3A9 or MBP cells under varying competitive environments was measured (Figure 3.2a).

In the absence of a competing response, 60% of CFSE labelled 5C.C7 cells were recruited into division (Figure 3.2c left panel), and divided cells progressed through 4 to 6 divisions by day 2.5 (Figure 3.2d left panel). Recruitment into division was reduced to 35% when CFSE labelled 5C.C7 cells were transferred 24 hours into an ongoing 5C.C7 response and divided cells progressed through fewer divisions by day 2.5 (Figure 3.2d left panel). Transferring CFSE-labelled 5C.C7 cells 24 hours into an ongoing 3A9 or MBP response had no effect on either recruitment into division (Figure 3.2c left panel) or proliferation by day 2.5 (Figure 3.2d left panel).

In a separate experiment, recruitment of 3A9 cells into division decreased from 25% to less than 10% when cells were transferred 24 hours into an ongoing 3A9 response (Figure 3.2c middle), and divided cells progressed through fewer rounds of division by day 2.5 (Figure 3.2d middle). Consistent with the previous experiment, competition by cells of different antigen specificity did not alter recruitment of 3A9 cells into division or proliferation by day 2.5 (Figure 3.2d middle).

In the third part of this experimental series, the effect of antigen specific and antigen non-specific competition on the recruitment and proliferation of MBP cells was investigated. In contrast to the previous two experiments, antigen non-specific competition (ie transferring

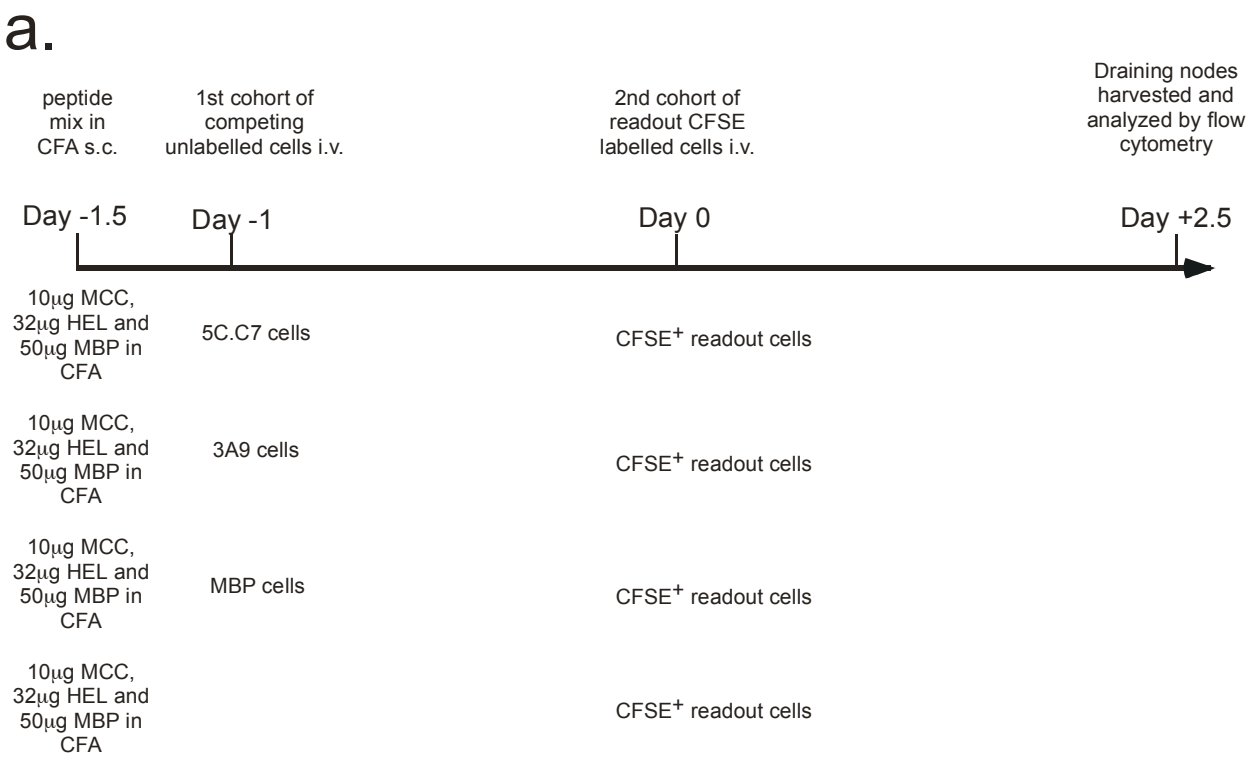
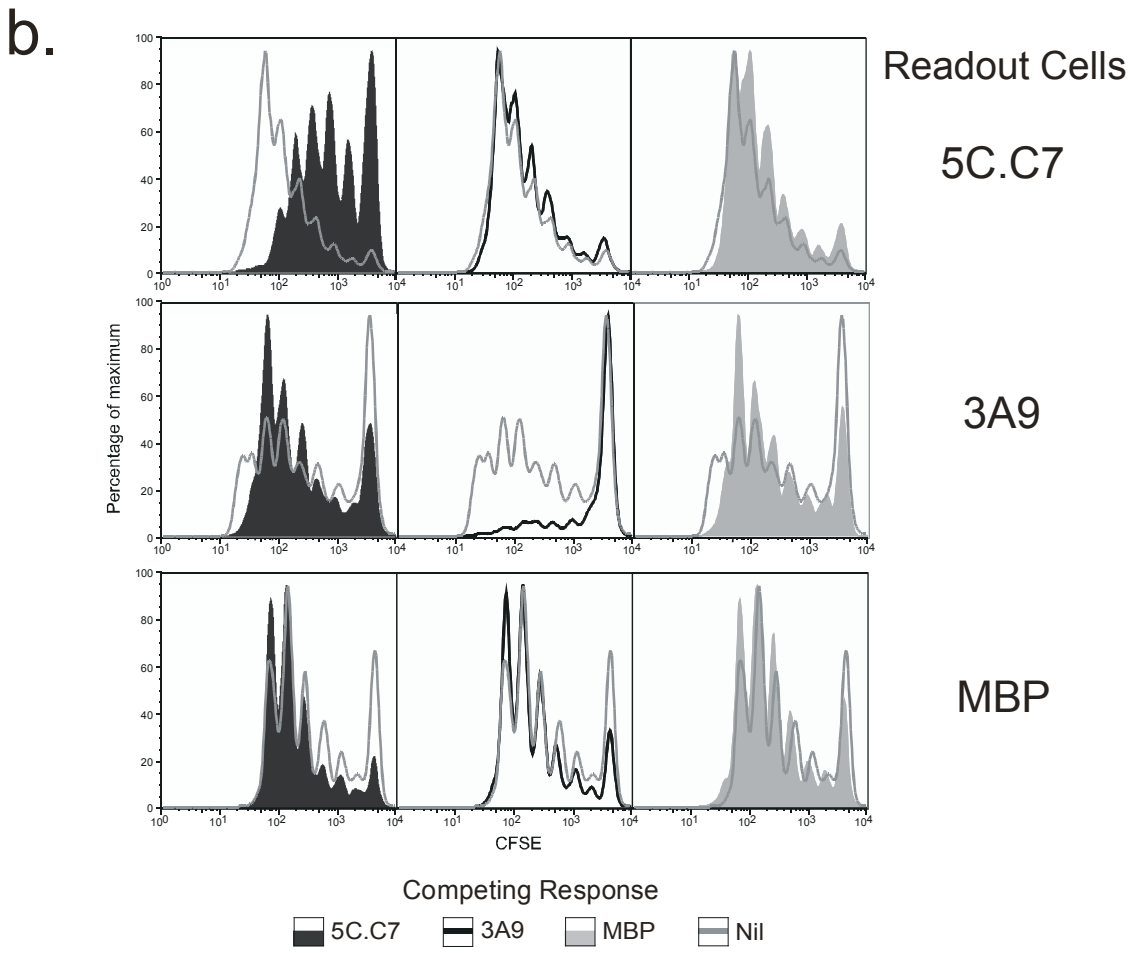
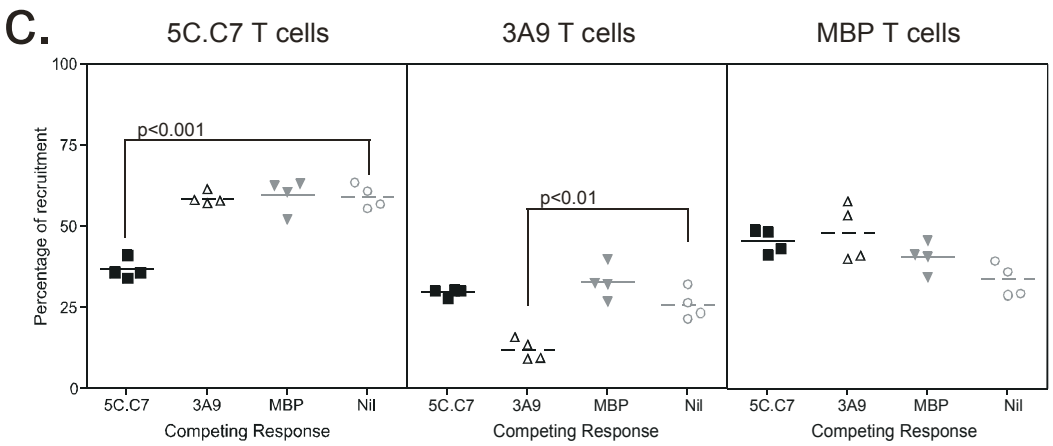
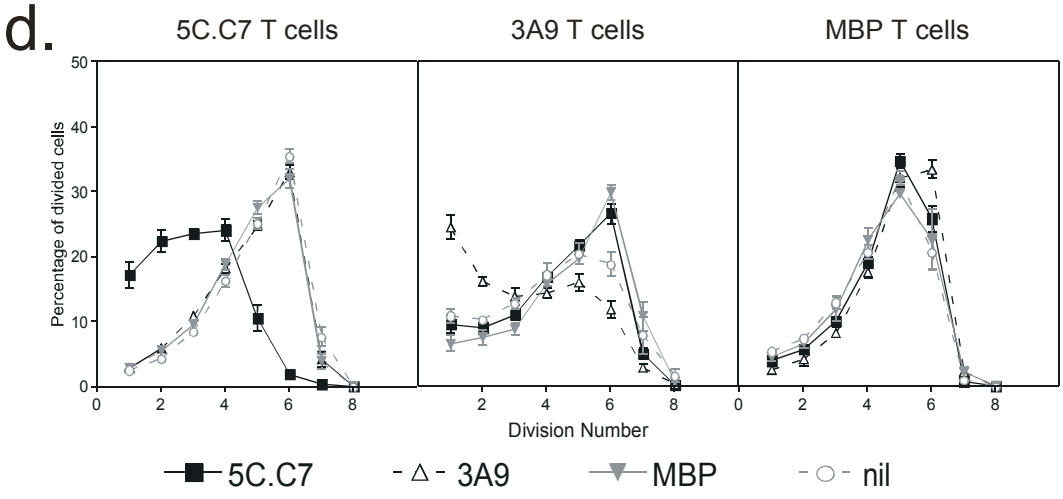


Figure 3.2: Specificity of high affinity competition
a. On day -1.5, KU recipient mice were immunised with 10μg MCC, 32μg HEL and 50μg MBP emulsified together in CFA 12 hours prior to the transfer of 5C.C7 KU, 3A9 KU or MBP KU competing cells. On day 0, CFSE labelled readout cells were transferred with the draining lymph nodes harvested 2.5 days later for analysis by flow cytometry.
b. CFSE profiles of readout 5C.C7 (top panel), 3A9 (middle panel) or MBP (bottom panel) cells when competing 5C.C7 (black filled), 3A9 (black open), MBP (grey filled) or no competing cells (grey open) were transferred into recipient mice 1 day earlier. Plots are one representative mouse per group.
c. Percentage of 5C.C7 (left panel) , 3A9 (middle panel) or MBP (right panel) cells recruited into division when competing 5C.C7 (black square), 3A9 (black triangle), MBP (grey triangle) or no cells (grey circle) were transferred into recipient mice 1 day earlier. Lines represent the mean of four mice per group with individual mice displayed as a single point.
d. The percentage of divided 5C.C7 (left panel), 3A9 (middle panel) or MBP (right panel) cells per division in the presence of a competing 5C.C7, 3A9, MBP or no competing response. Lines represent the mean of four mice per group +/- SEM.


b.
Readout Cells
5C.C7
3A9
MBP
Percentage of maximum
CFSE
Competing Response
5C.C7
3A9
MBP
Nil


c.
5C.C7 T cells
3A9 T cells
MBP T cells
Percentage of recruitment
p<0.001
p<0.01
5C.C7
3A9
MBP
Nil
Competing Response


d.
5C.C7 T cells
3A9 T cells
MBP T cells
Percentage of divided cells
Division Number
5C.C7
3A9
MBP
nil

MBP cells 24 hours into an ongoing 5C.C7 or 3A9 response) appeared to enhance the recruitment of CFSE labelled MBP cells into division, although the increase was only small (Figure 3.2d right panel). Despite the change in recruitment, divided MBP cells progressed through an equal number of divisions by day 2.5 (Figure 3.2d). Unlike the effect of antigen specific competition on the response of 5C.C7 and 3A9 cells, no effect on either the recruitment or proliferation was evident when CFSE labelled cells were transferred 24 hours into an ongoing MBP response.

In summary, for both MCC specific 5C.C7 cells and HEL specific 3A9 cells, an effect of competition on the early stages of the CD4 response was seen only between cells of the same antigen specificity. Transferring CFSE labelled cells 24 hours into an ongoing response reduced both the recruitment of cells into division and the proliferation of these cells by day 2.5. Surprisingly, antigen specific competition was not observed between MBP cells, considered to be of lowest affinity.

3.3 The effect of competition at sub-optimal antigen doses

In the previous experiments, stimulating TCR tg cells with maximal peptide doses may have reduced the requirement for costimulatory signals due to adequate signalling via the TCR. This may have masked a low grade effect of antigen non-specific competition. Therefore in a subsequent experiment, TCR tg cells were stimulated with half maximal doses of each peptide to determine whether antigen non-specific competition occurs when antigen is limiting (Figure 3.3). 3A9 T cells were chosen for analysis in the subsequent experiment, as these cells have an affinity for antigen between the relative affinities of 5C.C7 and MBP cells.

Recipient mice were immunised with a mixture containing 18μg MBP, 3.2μg HEL and 0.1μg MCC emulsified together in CFA, followed 12 hours later by a cohort of $2.5x10^6$ $CD4^+$ 5C.C7, 3A9, MBP or no competing cells (Figure 3.3a). 24 hour after the first cohort

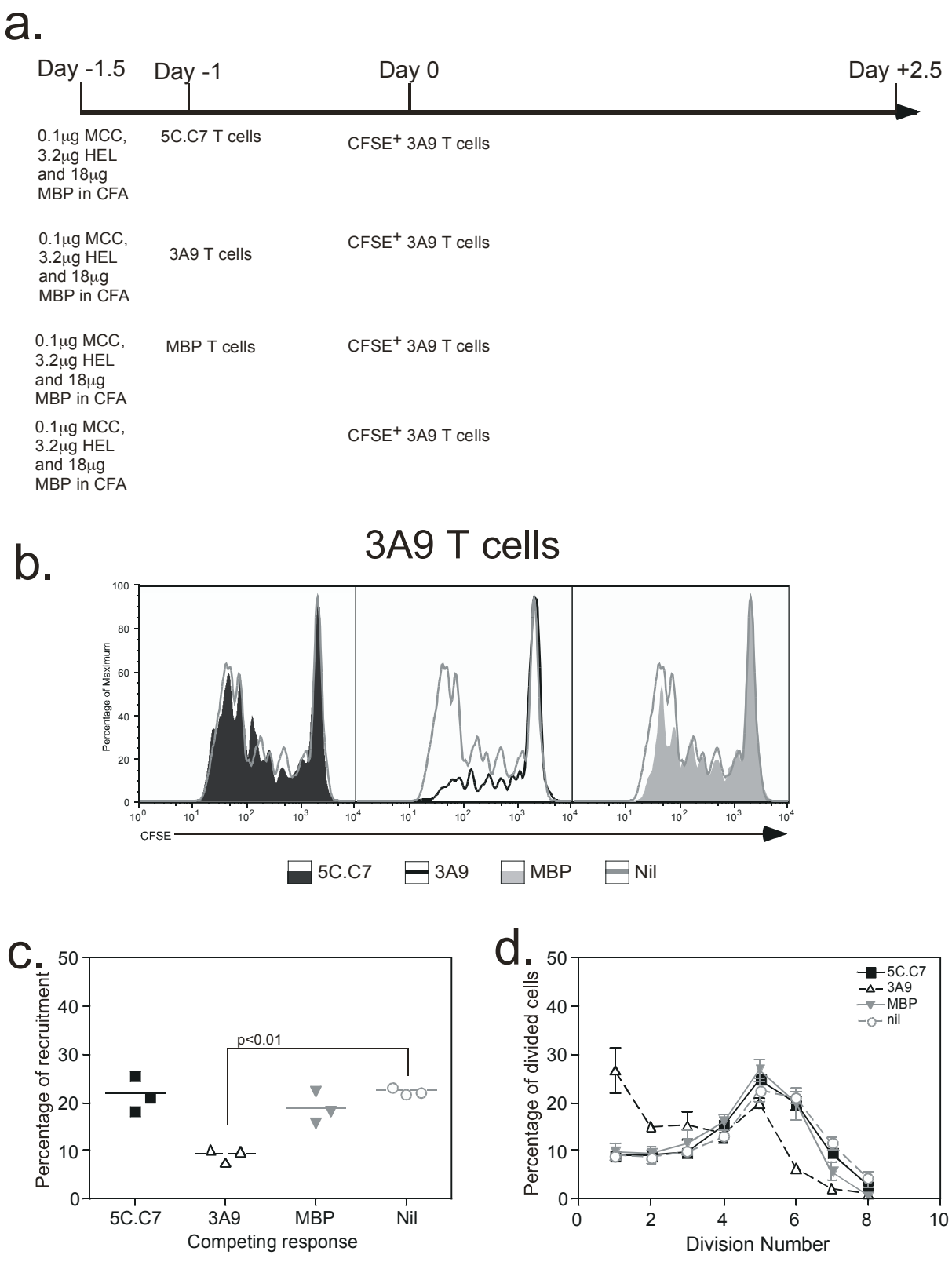


Figure 3.3: Specificity of high affinity competition at sub-optimal antigen doses
a. Four groups of KU recipient mice were immunised with 0.1μg MCC, 3.2μg HEL and 18μg MBP emulsified together in CFA 12 hours prior to the transfer of 5C.C7 KU, 3A9 KU or MBP KU competing cells. On day 0, readout CFSE labelled 3A9 KU cells were transferred and draining nodes harvested on day 2.5 for analysis by flow cytometry.
b. CFSE profiles of readout 3A9 cells transferred 1 day after 5C.C7 (black filled), 3A9 (black open) or MBP (grey filled) competing cells. Plots show one representative mouse per group.
c. Percentage of 3A9 T cells recruited into division when either a competing 5C.C7 (black square), 3A9 (black triangle), MBP (grey triangle) or no cells (grey circle) were transferred into host mice 1 day earlier. Line represent the mean of three mice per group with individual mice shown as single points.
d. The percentage of divided 3A9 cells per cell division in the presence of a competing 5C.C7, 3A9, MBP or no competing response. Lines represent the mean of three mice per group +/- SEM.

of cells, all recipient mice received 2.5x10^6 $CD4^+$ CFSE labelled 3A9 cells and draining LNs were harvested a further 2.5 days later for analysis by flow cytometry.
Consistent with the previous experiment, reduced recruitment of 3A9 cells into division (Figure 3.3c) was seen when cells were transferred 24 hours into an ongoing 3A9 response, and divided cells progressed through fewer rounds of cell division in 2.5 days (Figure 3.3d). Despite the reduction in antigen dose, no effect of antigen non-specific competition was seen when CFSE labelled 3A9 cells were transferred 24 hours into either an ongoing 5C.C7 or MBP response. An equal percentage of 3A9 cells were recruited into division (Figure 3.3c) and divided cells progressed through an equal number of divisions by day 2.5 (Figure 3.3d). Thus despite the predicted increased requirement for costimulatory signals due to a reduction in the level of TCR stimulation, no effect of antigen non-specific competition was seen in this experiment.

3.4 The effect of increasing the time interval between competing cohorts of cells

In the earliest studies of antigenic competition performed in the 1960s, an enhanced effect was demonstrated when the time between competing antigens was increased (Radovich and Talmage 1967). In these experiments, an effect could be visualised with a 24 hour interval between administration of competing antigens, but the greatest effect of antigenic competition was detected with a 4 day interval. Almost no effect was seen with a 10 day interval. The effect of increasing the time interval between competing cells on antigen non-specific competition was therefore investigated in the experimental system employed here (Figure 3.4).
All recipient mice were immunised with 10μg MCC, 32μg HEL and 50μg MBP peptides emulsified together in CFA and 12 hours later 2.5x10^6 $CD4^+$ competing 5C.C7, 3A9 or MBP cells were adoptively transferred into recipient mice (Figure 3.4a). 3 days later all recipient mice received 2.5x10^6 $CD4^+$ CFSE labelled 3A9 cells and the proliferation of

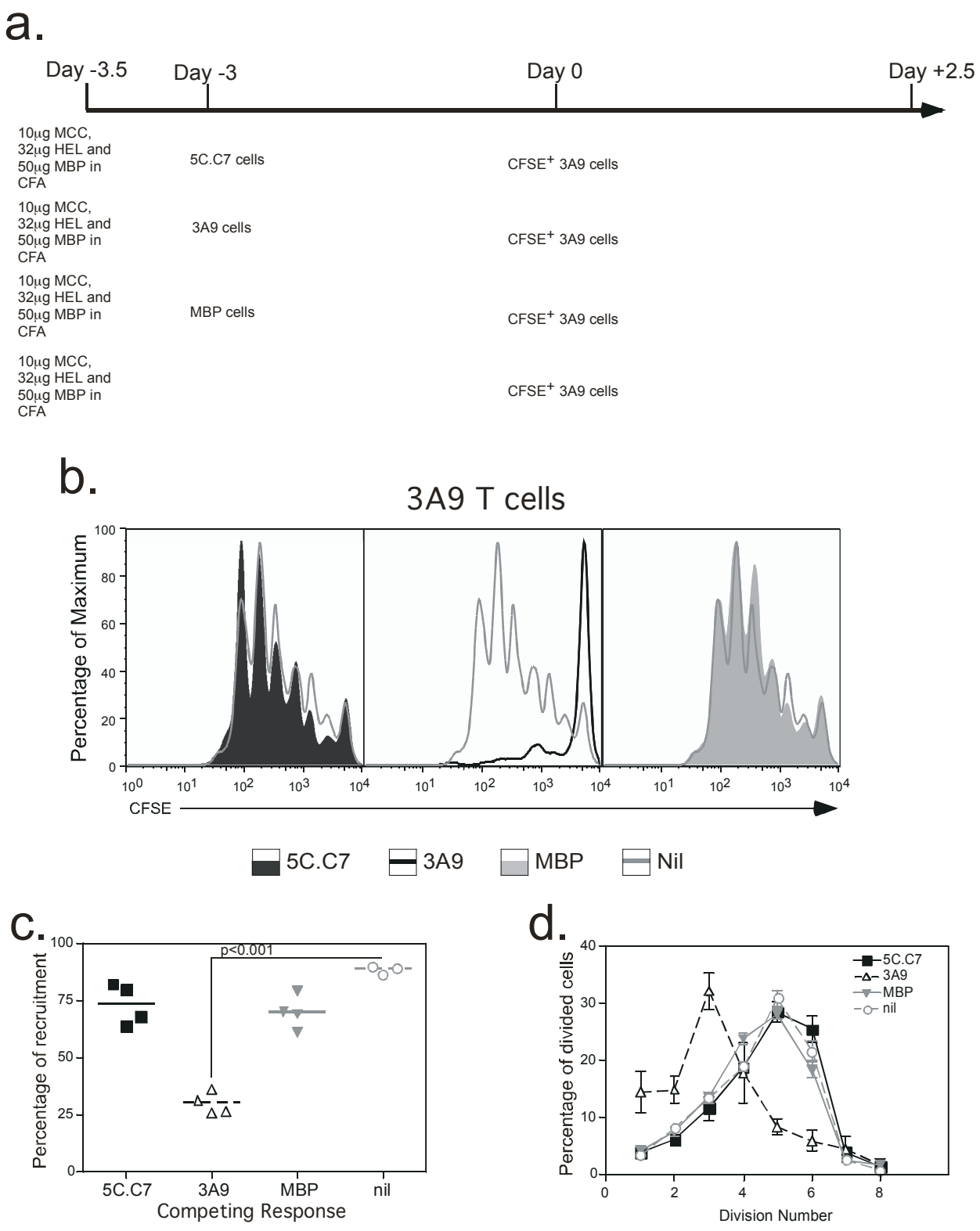


Figure 3.4: Response of CFSE labelled 3A9 cells transferred 3 days after competing cells
a. On day -3.5, KU recipient mice were immunised with 10μg MCC, 32μg HEL and 50μg MBP peptides emulsified in CFA with competing 5C.C7 KU, 3A9 KU or MBP KU cells adoptively transferred on day -3. Readout CFSE labelled 3A9 KU cells were transferred on day 0 and mice sacrificed 2.5 days later for the analysis of draining nodes by flow cytometry.
b. CFSE profiles of 3A9 T cells (grey open) transferred 3 days after a competing 5C.C7 (black filled), 3A9 (black open) or MBP (grey filled) response. Plots show one representative mouse per group.
c. Percentage of 3A9 cells recruited into division when either a competing 5C.C7 (black square), 3A9 (black triangle), MBP (grey triangle) were transferred into host mice 3 days earlier. Line represent the mean of four mice per group with individual mice shown as a single point.
d. The percentage of divided 3A9 cells in each cell division in the presence of a competing 5C.C7 (black square), 3A9 (black triangle), MBP (grey triangle) or no competing response (grey circle). Line represent the mean +/- SEM.

these cells was measured a further 2.5 days later. In this experiment, a monoclonal antibody against B220 was included to enable the exclusion of any contaminating B cells from the gating strategy. Consequently the percentage of 3A9 cells recruited into division was higher than in earlier experiments due to an exclusion of B cells from the undivided cell gate. Consistent with earlier observations, transferring CFSE labelled cells of the same specificity into an ongoing response reduced recruitment of cells into division, and divided cells progressed through fewer rounds of division by day 2.5 (Figure 3.4d). Transferring CFSE labelled 3A9 cells 3 days into an ongoing 5C.C7 or MBP response led to a slight but not statistically significant reduction in the percentage of 3A9 cells recruited into division (Figure 3.4c). However there was no difference in CFSE division profiles (Figure 3.4b) or the progression of divided 3A9 cells through subsequent divisions (Figure 3.4d).

3.5 The effect competition with an increased time interval and sub-optimal antigen

In the previous experiments, both increasing the time interval between competing cohorts, and decreasing the dose of antigen, increased the effect of antigen specific competition without any evidence of competition between cells of different antigen specificity. Therefore the effect of simultaneously increasing the time interval between competing cohorts together with reducing the dose of antigen was investigated (Figure 3.5).
In this experiment, Ly5.1 congenic marker expression was used to discriminate between readout CFSE labelled cells and competing or host cells. According to the schema in Figure 3.5a, both $Ly5.1^+$ and $Ly5.1^-$ recipient mice were immunised with 0.1μg MCC and 3.2μg HEL together in CFA. At this same time, $Ly5.1^+$ mice received $1x10^6$ $CD4^+$ unlabelled 3A9 $Ly5^+$ cells or no competing cells while $Ly5.1^-$ mice received $1x10^6$ $CD4^+$ 5C.C7 $Ly.1^-$ cells or no competing cells. 3 days later $Ly5.1^+$ recipient mice $1x10^6$ $CD4^+$ CFSE labelled $Ly5.1^-$ 5C.C7 cells while all $Ly5.1^-$ recipient mice received $1x10^6$ $CD4^+$

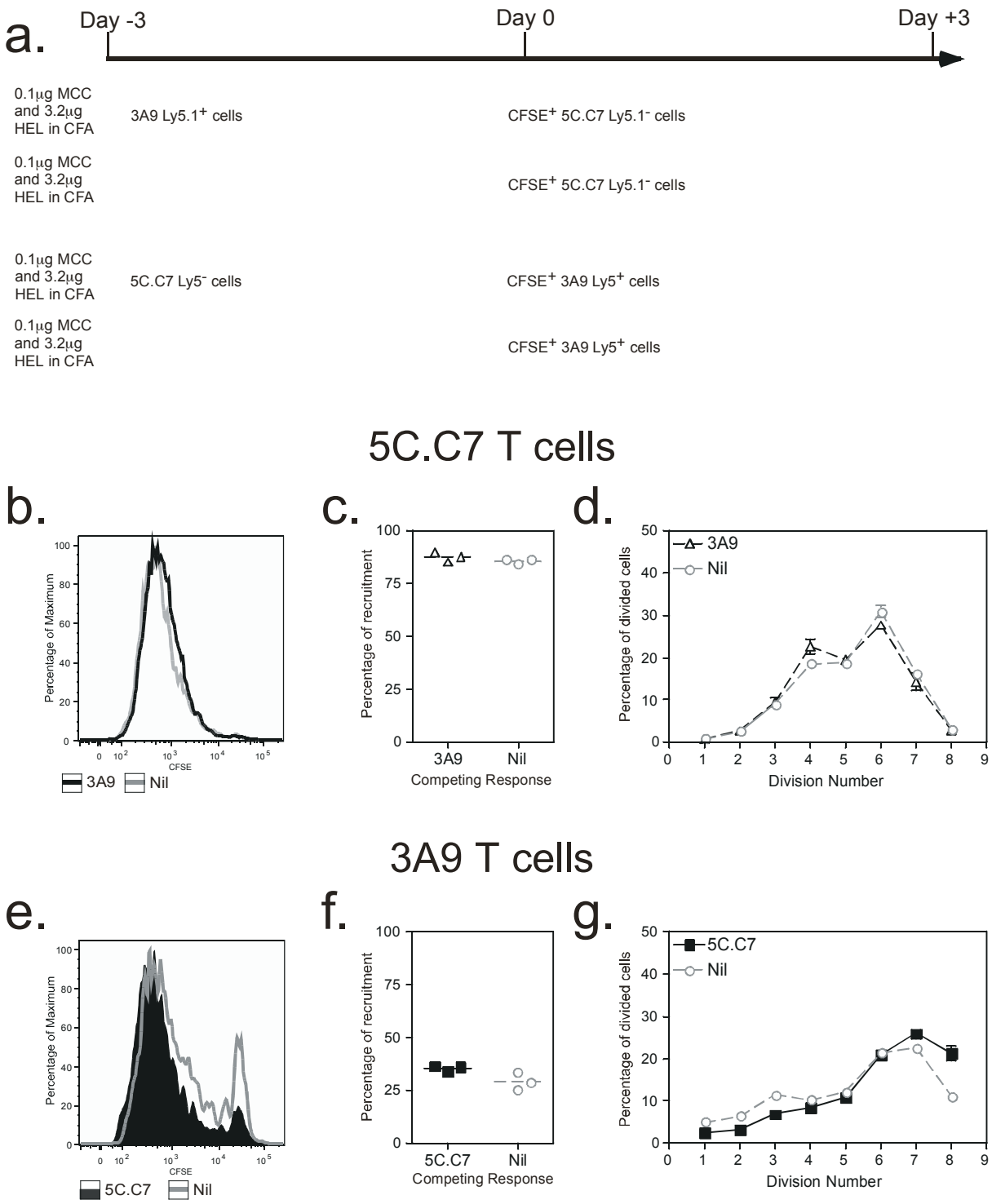


Figure 3.5: Increasing the time interval between competing cells when cells were stimulated with low peptide doses.

a. On day -3, all recipient mice (F1L or BK) were immunised with a mixture containing 0.1μg MCC and 3.2μg HEL peptides emulsified together in CFA. At the same time two groups received unlabeled 3A9 F1L or 5C.C7 BK competing cells. Readout CFSE labelled 5C.C7 F1L cells or CFSE labelled 3A9 BK cells were transferred on day 0 and mice sacrificed 3 days later.

CFSE profiles of 5C.C7 T cells (**b.**) or 3A9 cells (**e.**) transferred 3 days after a competing 3A9 (black open line) or 5C.C7 (black filled line) or no competing response (grey line). Plots show one representative mouse per group.

The percentage of 5C.C7 (**c.**) or 3A9 (**f.**) cells recruited into division in the presence of a competing 3A9 response (open triangle) or 5C.C7 (black squares) or no competing response (grey circles). The mean of is represented by the line with each mouse represented as an individual symbol.

The percentage of divided 5C.C7 cells (**d.**) or divided 3A9 cells (**g.**) in each division is represented by the mean of three mice per group +/- SEM.

CFSE labelled Ly5.1$^+$ 3A9 cells. The proliferation of CFSE labelled 3A9 and 5C.C7 cells was measured 3 days later by flow cytometry.

At sub-optimal doses of antigen, with a three day time interval between the competing 3A9 cells and readout CFSE labelled 5C.C7 cells, no effect of antigen non-specific competition was seen. Recruitment of 5C.C7 cells into division (Figure 3.5c) and progression of 5C.C7 cells through division was equivalent to the control (Figure 3.5d). A similar result was observed when CFSE labelled 3A9 cells were transferred 3 days into an ongoing 5C.C7 response. Recruitment of 3A9 cells into division (Figure 3.5f) and progression of cells through division (Figure 3.5g) was unaltered by the presence of a competing 5C.C7 response.

In summary, even in the presence of sub-optimal antigen stimulation with an increased time interval between competing cells, competition between relatively high affinity T cells specific for independent antigenic epitopes was not detected.

3.6 The effect of antigen specific competition with an increased time interval

By comparing the experiments described in Figures 3.2 and 3.4, it was apparent that increasing the time interval between competing 3A9 cells enhanced the effect of antigen-specific competition. To confirm this hypothesis, the effect of increasing the time interval on competition between 5C.C7 cells was tested directly (Figure 3.6).

Recipient mice were immunised with 10μg MCC peptide in CFA and 12 hours later $2.5x10^6$ CD4$^+$ unlabelled 5C.C7 cells were adoptively transferred into one group of recipient mice (Figure 3.6a). 2 days later a separate group of mice received $2.5x10^6$ CD4$^+$ unlabelled 5C.C7 cells and a further 24 hours later all mice received CFSE labelled 5C.C7 cells. The effect of antigen specific competition on proliferation and cytokine production by 5C.C7 cells was measured 2.5 days after the transfer of CFSE labelled 5C.C7 cells.

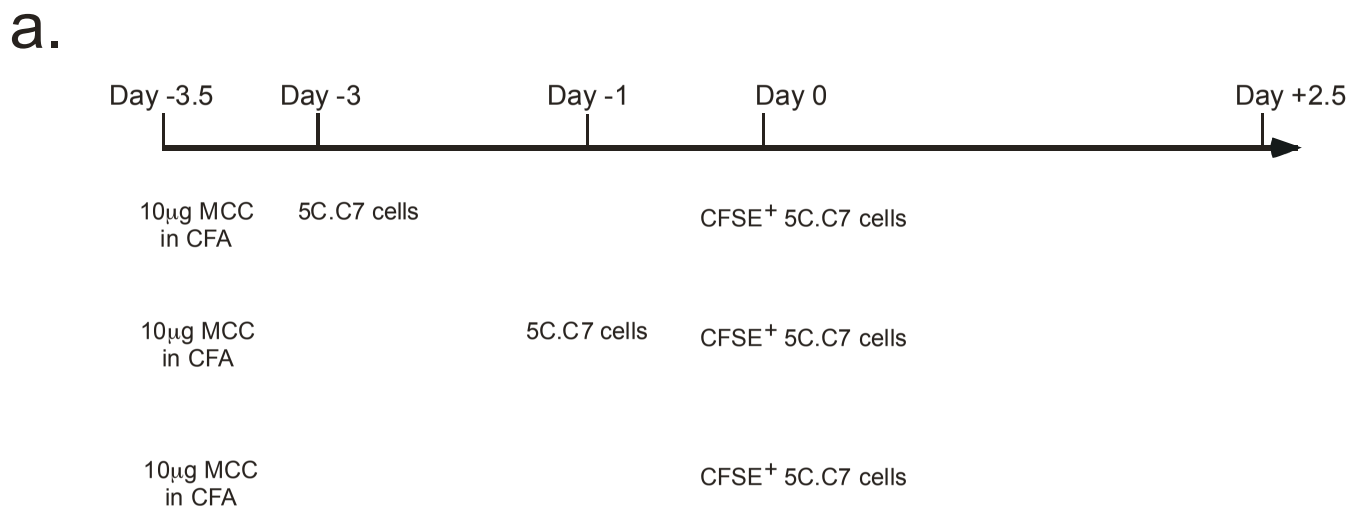
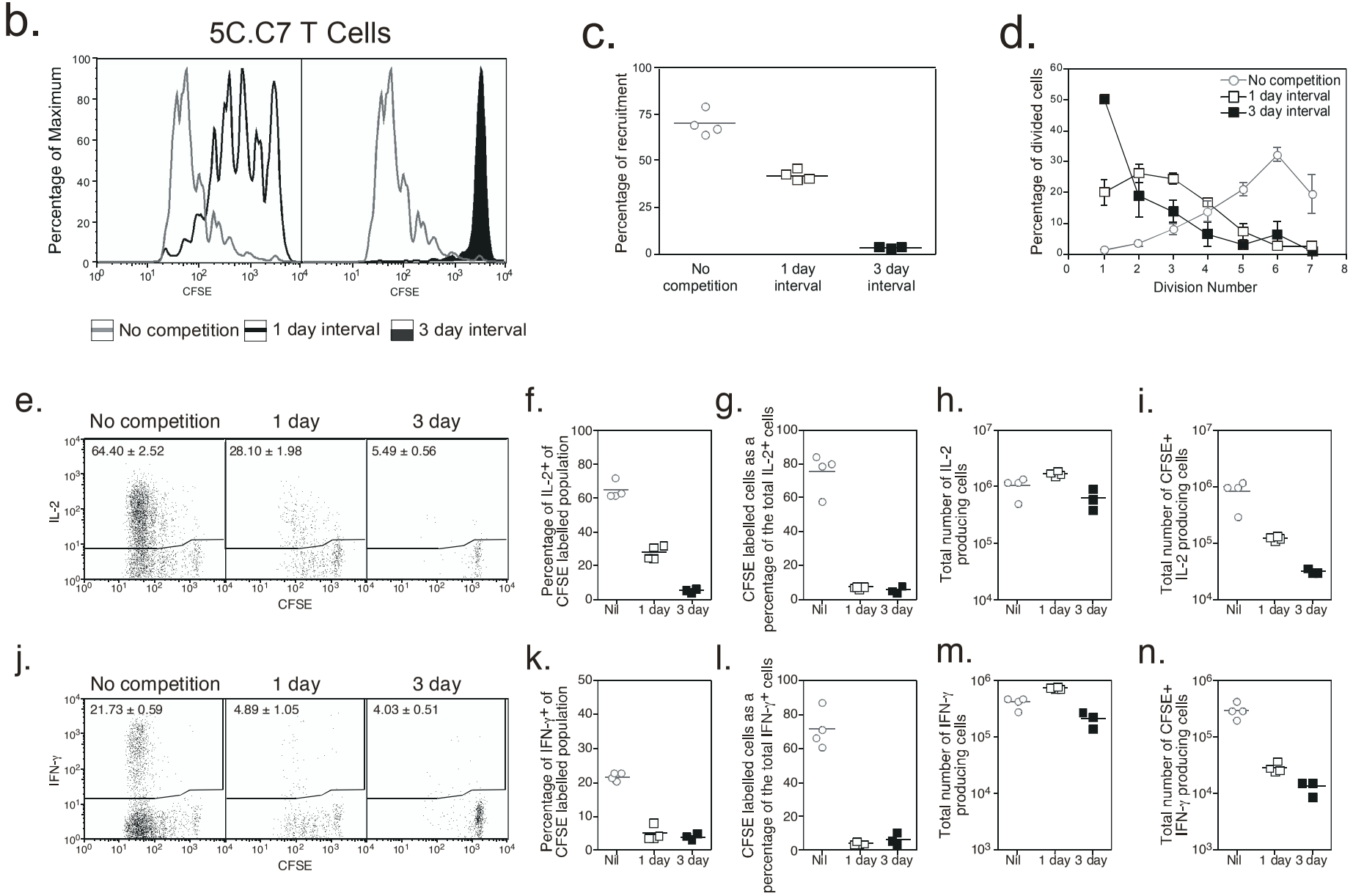


Figure 3.6: The effect of increasing time interval on high affinity competition
a. On day -3.5 recipient KU mice were immunised with 10μg MCC emulsified in CFA. 12 and 84 hours later competing 5C.C7 KU cells were adoptively transferred into host mice. On day 0, readout CFSE labelled 5C.C7 KU cells were transferred and 2.5 days later, draining nodes were harvested for analysis by flow cytometry. **b.** CFSE profiles of 5C.C7 cells transferred 1 day or 3 days after an initial 5C.C7 competing response. The percentage of 5C.C7 cells recruited into division (**c.**) and the percentage of divided 5C.C7 cells per cell division (**d.**) when transferred 1 day or 3 days after an initial 5C.C7 response.
(**e-n**) Production of IL-2 and IFN-γ, was measured by intracellular cytokine staining after 6 hours of ex vivo restimulation. (**e, j**) Representative dot plots of CFSE vs intracellular IL-2 or IFN-γ in CFSE-labelled TCR transgenic 5C.C7 cells, gated for live $CD4^+$ T cells. Numbers within dot plots represent the mean percentage +/- SEM of cells within the positive gate for each group. (**f, k**) Percentage of cytokine-producing cells, calculated from the profiles as in (**e, j**). (**g, l**) Percentage of total cytokine-producing cells derived from the CFSE-labelled cohort of $CD4^+$ TCR transgenic T cells, calculated by dividing the number of $CD4^+$ TCR transgenic CFSE-labelled cytokine-producing cells in each sample by the total number of $CD4^+$ cytokine-producing cells in the sample. (**h, m**) Total $CD4^+$ cytokine-producing cells in the draining lymph nodes of each mouse, calculated by multiplying the percentage of $CD4^+$ cytokine-producing cells in each sample by the number of cells harvested from the draining lymph nodes. (**i, n**) Total CFSE-labelled TCR transgenic $CD4^+$ cytokine-producing cells in the draining lymph nodes of each mouse, calculated by multiplying the percentage of CFSE-labelled TCR transgenic $CD4^+$ cytokine-producing cells in each sample by the number of cells harvested from the draining lymph nodes. For panels (**e-i**) and (**k-n**), each point represents an individual mouse and the group mean is indicated by the horizontal line.


b.
5C.C7 T Cells
Percentage of Maximum
CFSE
No competition
1 day interval
3 day interval
c.
Percentage of recruitment
No competition
1 day interval
3 day interval
d.
Percentage of divided cells
Division Number
No competition
1 day interval
3 day interval
e.
No competition
1 day
3 day
64.40 ± 2.52
28.10 ± 1.98
5.49 ± 0.56
IL-2
CFSE
f.
Percentage of IL-2+ of CFSE labelled population
Nil
1 day
3 day
g.
CFSE labelled cells as a percentage of the total IL-2+ cells
h.
Total number of IL-2 producing cells
i.
Total number of CFSE+ IL-2 producing cells
j.
No competition
1 day
3 day
21.73 ± 0.59
4.89 ± 1.05
4.03 ± 0.51
IFN-γ
CFSE
k.
Percentage of IFN-γ+ of CFSE labelled population
l.
CFSE labelled cells as a percentage of the total IFN-γ+ cells
m.
Total number of IFN-γ producing cells
n.
Total number of CFSE+ IFN-γ producing cells

Transferring CFSE labelled cells 3 days after immunisation did not significantly reduce the level of antigen presentation. 80% of CFSE labelled 5C.C7 cells were recruited into division and transferring CFSE labelled cells one day after an equal cohort of 5C.C7 cells reduced recruitment by 25% (Figure 3.6c). When the time between the initiation of a 5C.C7 response and the transfer of additional precursors was increased to 3 days, less than 5% of CFSE labelled 5C.C7 cells were recruited into division and 50% of divided cells had progressed through only 1 round of cell division in 2.5 days (Figure 3.6d).
In this experiment, the cytokine producing capacity of CFSE labelled 5C.C7 cells was measured by intracellular cytokine staining after 6 hours of *ex vivo* antigen specific restimulation. A 50% reduction in the frequency of IL-2 positive cells and a 15% reduction in IFN-γ positive cells was seen when CFSE labelled cells were transferred 1 day into an ongoing response (Figure 3.6e and f). Transferring 5C.C7 cells 3 days into the response decreased the frequency of IL-2 and IFN-γ-producing cells to less than 5% (Figure 3.6j and k). The contribution of CFSE labelled 5C.C7 cells to the total MCC specific response was determined by calculating the percentage of CFSE labelled cells within the total $CD4^+$ $IL\text{-}2^+$ or $IFN\text{-}\gamma^+$ population. The total number of effector cells was calculated by multiplying the frequency of cells per sample by the total organ count. Overall the contribution of CFSE labelled 5C.C7 cells to the total IL-2 (Figure 3.6g) or IFN-γ (Figure 3.6i) response was less than 5% when cells were transferred 1 or 3 days after the initiation of the response. However transferring additional precursors to an ongoing 5C.C7 response did not compromise the total MCC specific cytokine response. The size of the total MCC specific cytokine response (Figure 3.6h, m) was similar for all 3 groups, the slight decrease in the case of a 3 day interval being attributable to the kinetics of cytokine production in this model. The number of 5C.C7 cells secreting IFN-γ and IL-2 in the draining LN in response to MCC-CFA has previously been shown by other members of the lab to peak at day 4 after immunisation (Bugeja and Fazekas de St. Groth, unpublished data). Consistent with this, the highest absolute number of IL-2 or IFN-γ secreting cells was present after restimulation of cells from the second group (1 day interval), in which the majority of responder cells were injected 3.5 days before harvest,

rather than in the control group (Nil) (2.5 days before harvest) or third group (3 day interval) (5.5 days before harvest).

In summary, an ongoing 5C.C7 response inhibited the recruitment, proliferation and differentiation of additional 5C.C7 precursors without affecting the overall level of the response. Their contribution to the effector cell pool was inversely related to the size of the ongoing response. Thus $2.5x10^6$ 5C.C7 cells generated $1x10^6$ IL-2-producing and $3x10^5$ IFN-γ-producing cells when a polyclonal host response was initiated 3 days earlier (Nil group, Figure 3.6i, n), but only 3 $x10^4$ IL-2-producing and $1x10^4$ IFN-γ-producing cells when the response was initiated 3 days earlier by $2.5x10^6$ 5C.C7 cells (3-day interval, Figure 3.6i, n).

3.7 The effect of competition on early access to antigen presenting cells

In the previous series of experiments, increasing the time interval between competing cells was shown to enhance the degree of antigen specific competition. A number of papers have suggested that T cells compete primarily for access to APCs (Hayball et al. 2004; Kedl et al. 2000). A decreased ability of cells to access APCs, due to an increasing number of cells as the first cohort proliferated, may explain why the effect of antigen specific competition increases with time. To formally test this hypothesis, upregulation of surface CD69 expression (an early indicator of T cell activation) was measured 2 hours after transfer to investigate the ability of the second cohort to access APCs and receive early TCR stimulation (Figure 3.7).

According to the schema presented in Figure 3.7.1a, all recipient mice were immunised with a mixture containing 10μg MCC and 32μg HEL peptides emulsified together in CFA, and $2.5x10^6$ $CD4^+$ unlabelled 5C.C7 cells or 3A9 cells were transferred into recipient mice on day –4 or day -1. All recipient mice received $2.5x10^6$ $CD4^+$ $Ly5.1^-$ 3A9 cells on day 0 with CD69 expression analysed 2 hours later or proliferation a further 2.5 days later.

a.

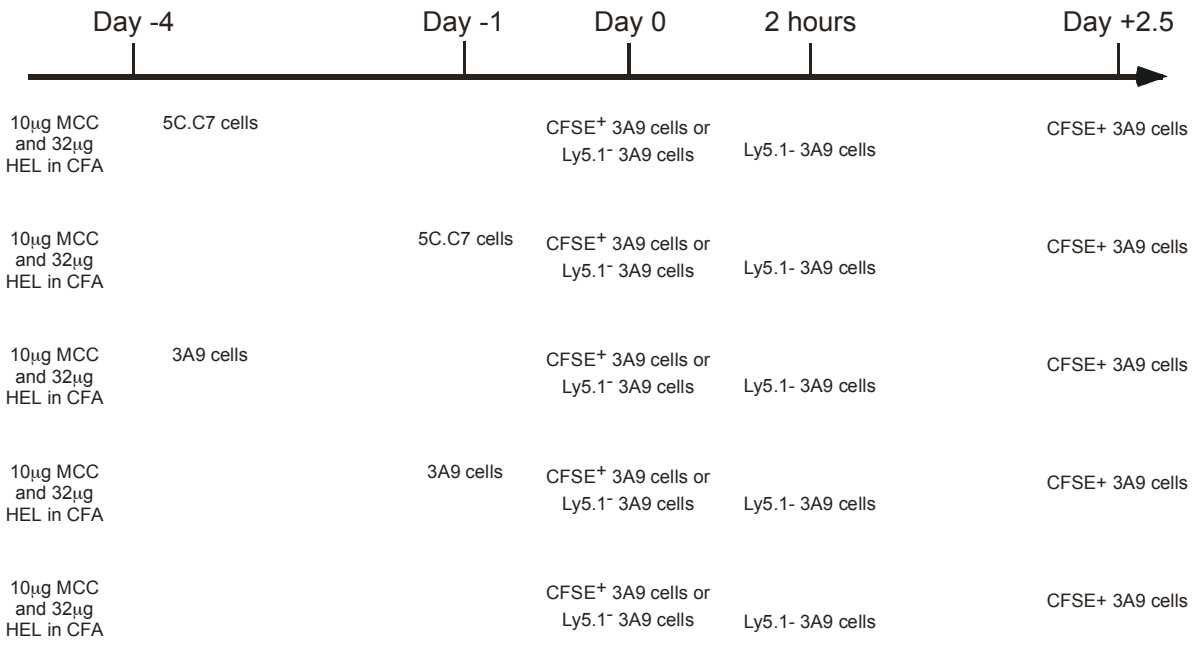
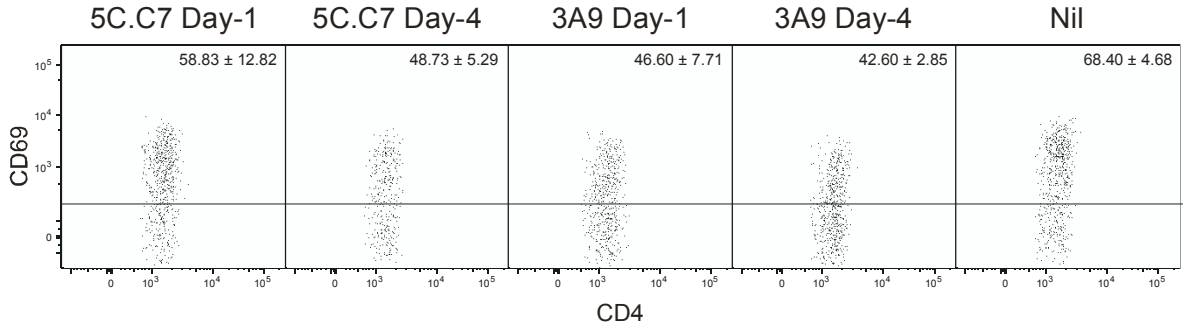


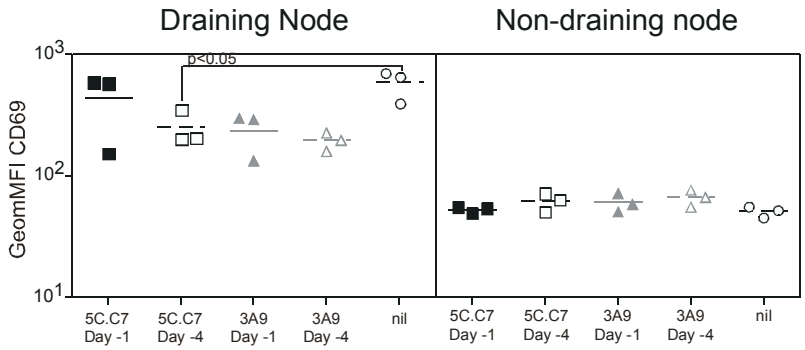
Figure 3.7.1: Expression of CD69 and division profiles of 3A9 T cells after transfer into varying competitive environments

a. On day -4 all recipient F1L mice were immunised with 10μg MCC and 32μg HEL emulsified together in CFA. On days -4 and -1, 5C.C7 F1L and 3A9 F1L cells were adoptively transferred into host mice. On day 0, readout CFSE labelled 3A9 BK or unlabeled Ly5.1$^-$ 3A9 BK cells were adoptively transferred into Ly5.1$^+$ recipient mice, 2 hours later mice that received CFSE unlabeled 3A9 cells were sacrificed to measure CD69 expression while the remainder of mice were sacrificed 2.5 days later to measure proliferation. Profiles (**b.**) illustrate the expression of CD69 on 3A9 T cells recovered from the draining node 2 hours after transfer into recipient mice. Numbers represent the mean percentage of CD69$^+$ cells of three mice per group +/- SEM. The geometric mean fluroscence intensity (GMFI) of CD69 expression on 3A9 T cells (**c.**) in the draining nodes (left panel) and the non-draining nodes (right panel) is displayed. Day 2.5 CFSE profiles of 3A9 T cells (**d.**) stimulated in the presence of a competing response (grey filled histogram) or in the absence of a competing response. From CFSE profiles the percentage of 3A9 cells recruited into division (**e.**) and the percentage of divided cells per division number (**f.**) was calculated.

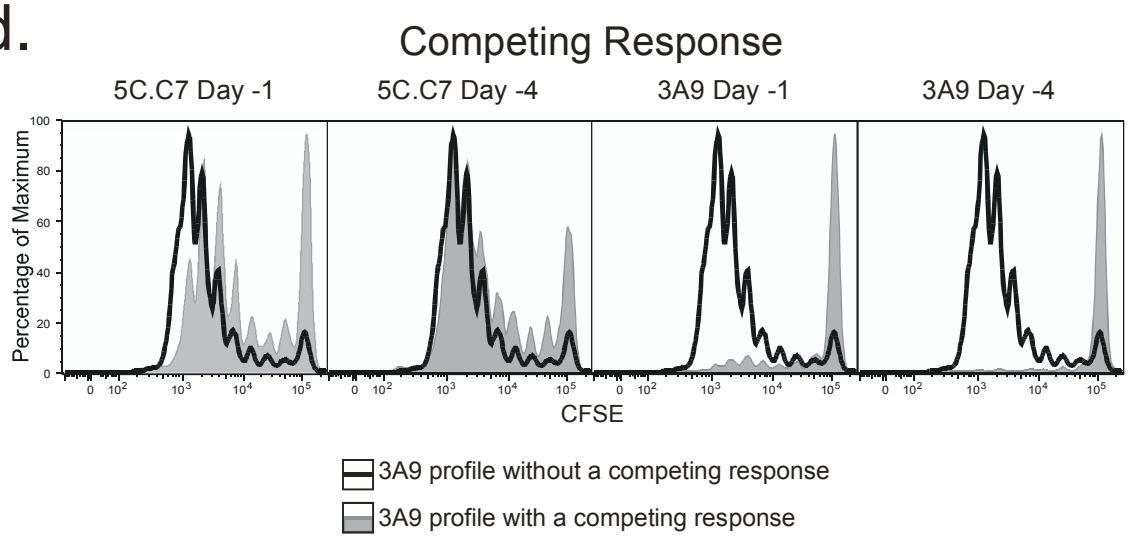
b.


5C.C7 Day-1
5C.C7 Day-4
3A9 Day-1
3A9 Day-4
Nil
58.83 ± 12.82
48.73 ± 5.29
46.60 ± 7.71
42.60 ± 2.85
68.40 ± 4.68
CD69
CD4

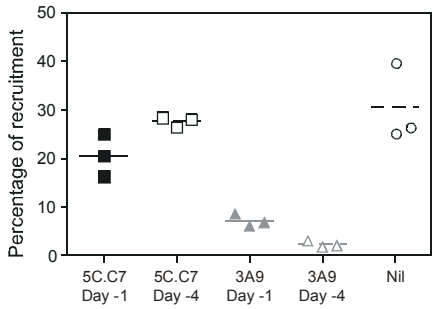
c.


Draining Node
Non-draining node
p<0.05
GeomMFI CD69
5C.C7 Day -1
5C.C7 Day -4
3A9 Day -1
3A9 Day -4
nil

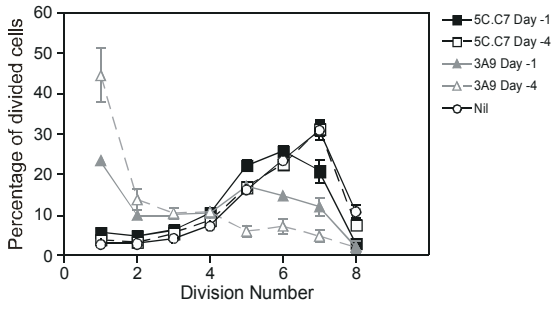
d.


Competing Response
5C.C7 Day -1
5C.C7 Day -4
3A9 Day -1
3A9 Day -4
Percentage of Maximum
CFSE
3A9 profile without a competing response
3A9 profile with a competing response

e.


Percentage of recruitment
5C.C7 Day -1
5C.C7 Day -4
3A9 Day -1
3A9 Day -4
Nil

f.


Percentage of divided cells
Division Number
5C.C7 Day -1
5C.C7 Day -4
3A9 Day -1
3A9 Day -4
Nil

In the absence of a competing response, 69% of 3A9 cells had upregulated CD69 2 hours after transfer (Figure 3.7.1b) and 30% of cells were recruited into division within the first 2.5 days of the response. 58% of 3A9 cells transferred 1 day into an ongoing 5C.C7 response had upregulated CD69 expression within the first 2 hours after transfer. There was a slight reduction in the percentage of 3A9 cells recruited into division but this was not statistically significant, and divided cells progressed through an equal number of cell divisions by day 2.5 (Figure 3.7.1f). 3A9 cells transferred 4 days into an ongoing 5C.C7 cell response had a slight reduction in the frequency of CD69 positive cells (Figure 3.7.1b) although not statistically significant and the overall level of surface CD69 expression ($p<0.05$) (Figure 3.7.1c) when compared to 3A9 cells transferred in the absence of a competing response. This reduction in CD69 expression 2 hours post transfer did not correlate with reduced proliferation of 3A9 cells since neither recruitment into division (Figure 3.7.1e) nor progression through division (Figure 3.7.1f) was altered by day 2.5.
In contrast, transferring 3A9 cells 1 or 4 days into an ongoing 3A9 response reduced the frequency of $CD69^+$ cells (Figure 3.7.1b) and the level of CD69 expression (Figure 3.7.1c) 2 hours post transfer. Consistent with previous data, transferring 3A9 cells into an ongoing response reduced recruitment into division (Figure 3.7.1e) and proliferation by day 2.5 (Figure 3.7.1f).
In a repeat of the previous experiment, but with a different cohort of readout cells, mice were immunised on day –3 with 32μg HEL and 10μg MCC emulsified in CFA (Figure 3.7.2). The different groups comprised of mice that had been injected with 3A9 cells on day –3 or 5C.C7 T cells transferred on days -1, -2 and -3, in addition to the control group in which no competing cells were transferred. All recipient mice received $2.5x10^6$ $CD4^+$ $Ly5.1^-$ 5C.C7 cells on day 0. Mice that received unlabelled 5C.C7 cells were sacrificed 2 hours after transfer to measure early upregulation of CD69 expression, while recipient mice that received CFSE labelled 5C.C7 cells were sacrificed on day 3 to measure proliferation.
In the control group, the percentage of cells that upregulated CD69 expression within the first 2 hours after transfer corresponded to the percentage recruited into division (Figure

3.7.2c, d). Thus 87% of 5C.C7 T cells upregulated CD69 and 88% entered cell division. However as the delay between the initiation of the high affinity response and the transfer of the CFSE-labelled cohort increased, it became apparent that not every cell expressing CD69 was recruited into division. With a 1 day delay between 5C.C7 cells, over 80% of cells upregulated CD69 but only 69% were recruited into division. The disparity was even greater when 5C.C7 cells were transferred 2 days into an ongoing 5C.C7 response. When the delay was 3 days, both CD69 expression and recruitment were reduced to less than 40%. CFSE labelled 5C.C7 cells transferred 1, 2 or 3 days into an ongoing 5C.C7 response progressed through fewer rounds of cell division by day 2.5 (Figure 3.7.2e) and maintained a higher level of CD69 expression when compared to the control group (Figure 3.7.2f).

In contrast to the effect of a 3 day delay between adoptive transfer of cohorts of 5C.C7 cells, initial transfer of a cohort of 3A9 cells had no effect on the subsequent response of 5C.C7 cells. Despite a minor but not statistically significant drop in the frequency of cells upregulating CD69 when 5C.C7 cells were transferred 3 days into an ongoing 3A9 response (from 87% to 81%) (Figure 3.7.2c), a similar percentage of 5C.C7 cells were recruited into division by day 5.5 (Figure 3.7.2d). Divided 5C.C7 cells progressed through an equal number of divisions (Figure 3.7.2e) and displayed similar expression of CD69 at day 2.5 (Figure 3.7.2f).

In the two experiments described in this section, the percentage of CD69 positive cells did not correlate with the percentage of cells recruited into division. Therefore reduced recruitment and proliferation of additional precursors (cells within the second cohort) could not solely be a result of reduced access to APCs.

In addition, the degree of CD69 downregulation appeared to correlate with the time of transfer into an ongoing 5C.C7 response. Interestingly the level of CD69 expression at day 3 of the response appeared to correlate inversely to the dose of stimulating antigen (Figure 4.3.2). At the highest dose of sub-cutaneous MCC peptide, expression of CD69 at day 3 by divided 5C.C7 cells was lowest (Figure 4.3.2). On the other hand, cells stimulated with a low dose of MCC, even one which does not induce proliferation,

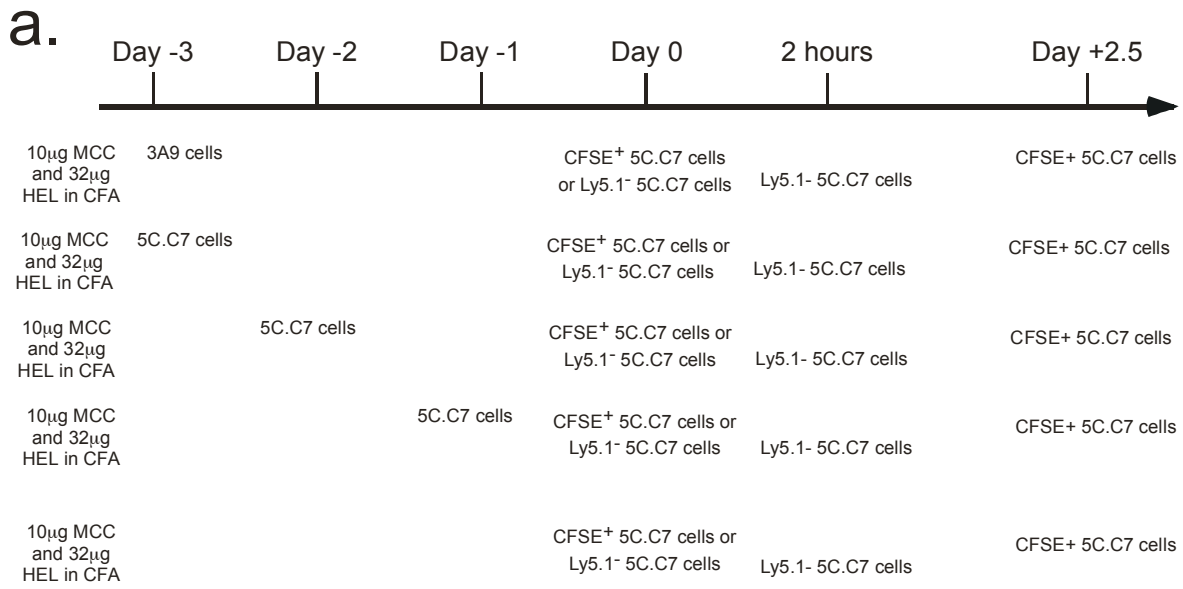
Figure 3.7.2: Ability of 5C.C7 cells to access APCs

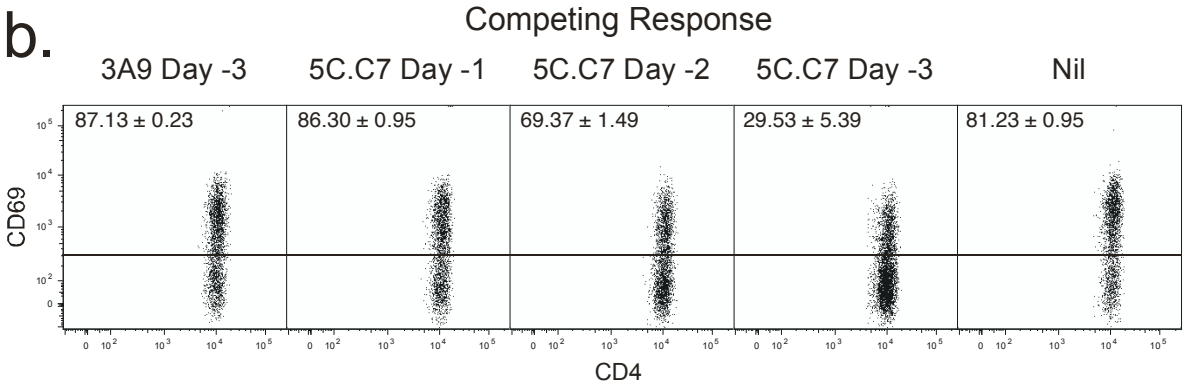
a. On day 3 all Ly5.1$^+$ recipient mice were immunised on day -3 with 10μg MCC and 32ug HEL peptides emulsified together in CFA. Three groups of recipient mice received unlabeled Ly5.1$^+$ 5C.C7 cells on days -3, -2 and -1 while another group of mice received unlabeled Ly5.1$^+$ 3A9 cells on day -3. On day 0, recipient mice received Ly5.1$^-$ 5C.C7 cells either unlabeled for analysis 2 hours later or CFSE labelled for analysis 3 days later. 5C.C7 cells were identified by positive staining of Vα11-bi av-Alexa 700 and CD4-APC while negative staining for Ly5.1-Pacific Blue.

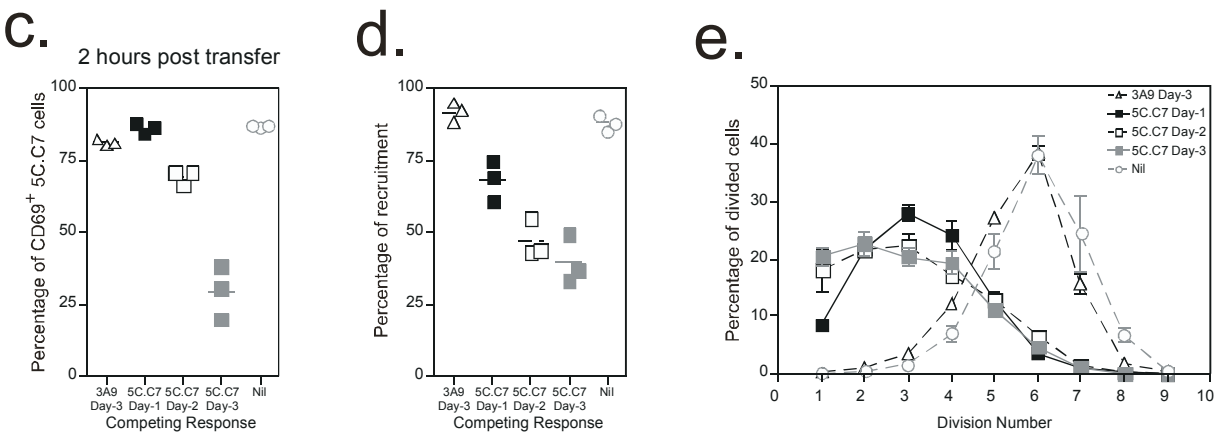
b. CD69 expression by Ly5.1$^-$ 5C.C7 cells 2 hours after adoptive transfer into recipient mice that had received a competing cohort of 3A9 cells on day -3, competing 5C.C7 cells on day -3. -2 or -1 or no competing cells. The mean percentage of CD69$^+$ 5C.C7 cells of three mice per group +/- SEM is shown.

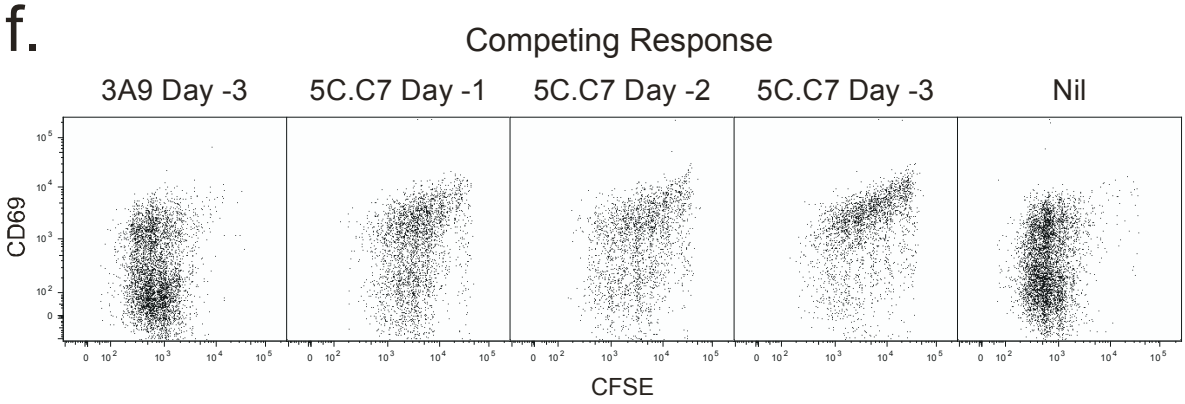
Graphs represent (**c.**) the percentage of CD69$^+$ Ly5.1$^-$ 5C.C7 2 hours after adoptive transfer (**d.**) the percentage of 5C.C7 cells recruited into division and (**e.**) the percentage of divided 5C.C7 cells per division calculated at day 3 of the response.

(**f.**) Dot plots represent the level of surface CD69 expression versus CFSE of Ly5.1$^-$ 5C.C7 cells recovered from the draining lymph nodes on day 3.


a.
Day -3
Day -2
Day -1
Day 0
2 hours
Day +2.5
10μg MCC and 32μg HEL in CFA
3A9 cells
CFSE+ 5C.C7 cells or Ly5.1- 5C.C7 cells
Ly5.1- 5C.C7 cells
CFSE+ 5C.C7 cells
10μg MCC and 32μg HEL in CFA
5C.C7 cells
CFSE+ 5C.C7 cells or Ly5.1- 5C.C7 cells
Ly5.1- 5C.C7 cells
CFSE+ 5C.C7 cells
10μg MCC and 32μg HEL in CFA
5C.C7 cells
CFSE+ 5C.C7 cells or Ly5.1- 5C.C7 cells
Ly5.1- 5C.C7 cells
CFSE+ 5C.C7 cells
10μg MCC and 32μg HEL in CFA
5C.C7 cells
CFSE+ 5C.C7 cells or Ly5.1- 5C.C7 cells
Ly5.1- 5C.C7 cells
CFSE+ 5C.C7 cells
10μg MCC and 32μg HEL in CFA
CFSE+ 5C.C7 cells or Ly5.1- 5C.C7 cells
Ly5.1- 5C.C7 cells
CFSE+ 5C.C7 cells


b.
Competing Response
3A9 Day -3
5C.C7 Day -1
5C.C7 Day -2
5C.C7 Day -3
Nil
87.13 ± 0.23
86.30 ± 0.95
69.37 ± 1.49
29.53 ± 5.39
81.23 ± 0.95
CD69
CD4


c.
2 hours post transfer
Percentage of CD69+ 5C.C7 cells
3A9 Day-3
5C.C7 Day-1
5C.C7 Day-2
5C.C7 Day-3
Nil
Competing Response
d.
Percentage of recruitment
Competing Response
e.
Percentage of divided cells
Division Number
3A9 Day-3
5C.C7 Day-1
5C.C7 Day-2
5C.C7 Day-3
Nil


f.
Competing Response
3A9 Day -3
5C.C7 Day -1
5C.C7 Day -2
5C.C7 Day -3
Nil
CD69
CFSE

maintained a high level of CD69 on day 3 of the response. Hence the level of CD69 expression at day 3 may indicate the level of T cell stimulation over the course of an ongoing response.

3.8 The effect of additional antigen on recruitment and proliferation

A number of papers have suggested that antigen presentation by DCs is relatively short lived (Hermans et al. 2000; Ingulli et al. 1997; Norbury et al. 2002). CTL mediated killing of antigen pulsed DCs has been proposed as the primary mechanism controlling the size of the CD8 response as this leads to a reduction in the total level of antigen presentation (Hafalla et al. 2002; Wong and Pamer 2003). To determine whether the reduced recruitment of additional cells into an ongoing response was related to the level of antigen presentation, additional antigen was administered at the time of adoptive transfer.
4 days before the transfer of CFSE labelled 5C.C7 cells, recipient mice received a total of 10μg of MCC peptide in CFA shared between the two hind-footpads (Figure 3.8a). At this timepoint two groups of recipient mice also received $2.5x10^6$ $CD4^+$ unlabelled 5C.C7 cells. 4 hours prior to the transfer of CFSE labelled 5C.C7 cells, mice received a second sub-cutaneous injection at the base of the tail containing 10μg MCC in CFA or PBS alone in CFA. 3 days later mice were sacrificed and the para-aortic and inguinal LNs pooled together for analysis by flow cytometry. Consistent with previous experiments, transferring 5C.C7 cells 4 days into an ongoing response reduced recruitment from 90% to 40% (Figure 3.8c) and 60% of divided cells had only progressed through 1 to 3 rounds of division by day 3 (Figure 3.8d). Additional antigen administered at the time of cell transfer restored recruitment of CFSE labelled 5C.C7 cells to 90%, comparable to the level of recruitment seen in the control group (Figure 3.8c). Despite restoration of recruitment, proliferation of CFSE labelled 5C.C7 cells transferred into an ongoing response was reduced compared to the control group (Figure 3.8). These data suggest that restricted

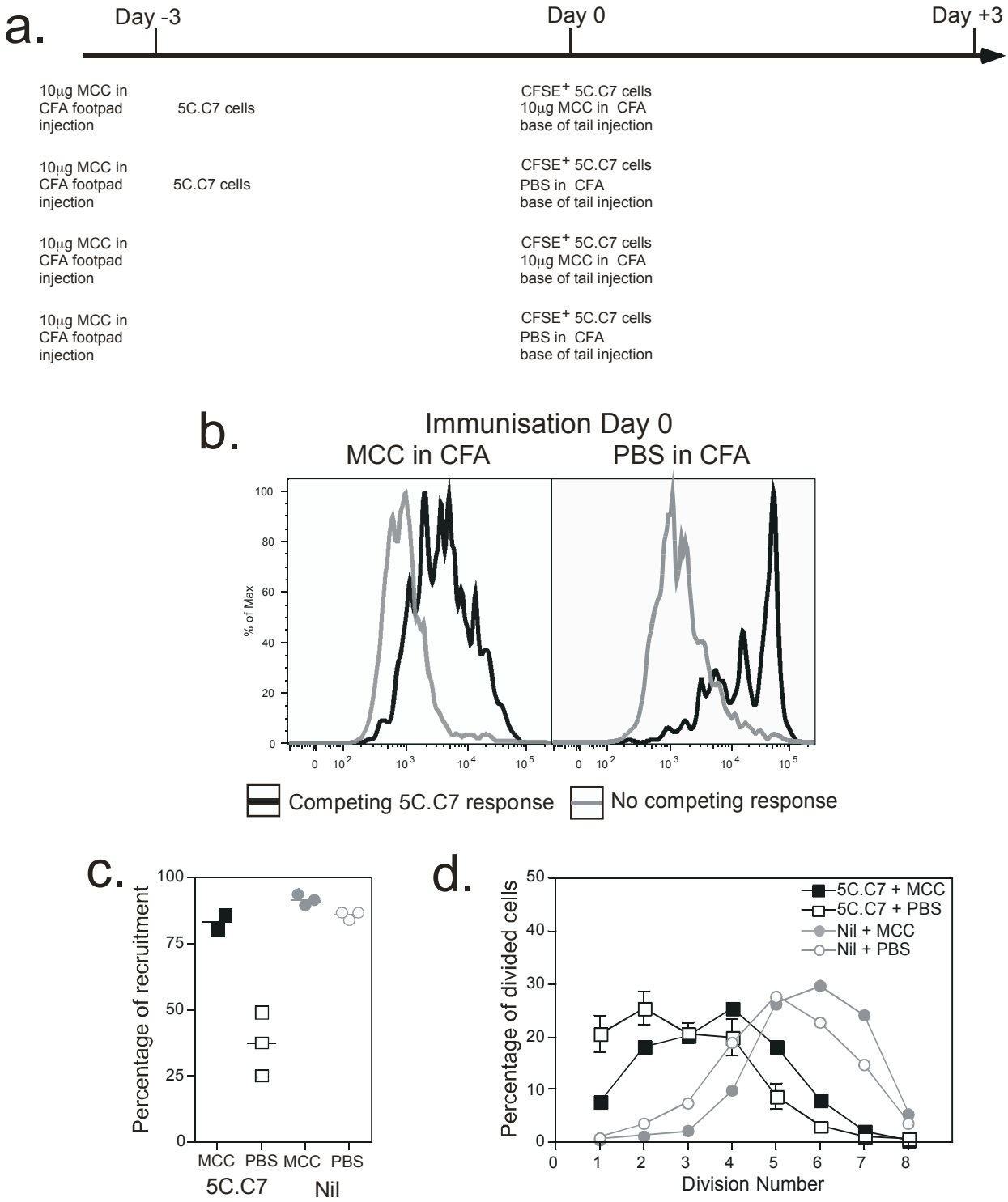


Figure 3.8: Overcoming competition by injecting more antigen
a. All recipient mice were immunised on day -3 with a total of 10μg MCC peptide emulsified in CFA shared between the two hind-footpads. $1x10^6$ competing 5C.C7 were adoptively transferred into two groups of recipient mice a few hours later. On day 0 all recipient mice received either 10μg MCC in CFA or PBS in CFA via a base of tail injection. Readout CFSE labelled 5C.C7 cells were transferred a few hours later and recipient mice approximately 2.5 days later recipient mice were sacrificed for the analysis by flow cytometry.
b. CFSE profiles of 5C.C7 T cells (grey line) transferred 3 days after a competing 5C.C7 (black line) response with a second immunization of MCC in CFA (left panel) or PBS in CFA (right panel).
c. Percentage of 5C.C7 cells recruited into division in the presence or absence of a competing 5C.C7 response stimulated 3 days earlier with a second injection of MCC in CFA or PBS in CFA.
d. The percentage of divided 5C.C7 cells in each division in the presence of a competing 5C.C7 with a second dose of MCC (black filled square), 5C.C7 with PBS (black open square), no competing response but with a second dose of MCC (grey filled circles) or no competing response with PBS (grey open circle).

access to antigen was at least partially responsible for the failure to recruit additional 5C.C7 cells into the ongoing response. We would predict that continued administration of MCC peptide would restore the proliferation of 5C.C7 cells transferred at late timepoints of the MCC response to that seen in the control group.

Discussion

The experiments described in this chapter were designed to investigate the relative contributions of TCR and costimulatory signals to the early stages of a $CD4^+$ T cell response. While there was no evidence that competition for signals independent of MHC could affect the early stages of a CD4 response, the data showed a critical role for continued stimulation of CD4 cells via the TCR for recruitment into division, proliferation and differentiation into effector cells.

In this study, a number of different manoeuvres were used to increase the sensitivity of the experimental model to possible competitive effects between cells directed to different MHC Class II restricted epitopes. Antigen doses were reduced so that cells would not be exposed to optimal concentrations and the delay between the initiation of responses was increased to 4 days so that the competing response was at its peak when the second response was initiated. Under none of these conditions could antigen-non-specific competition be detected. It must be noted that the different TCR tg $CD4^+$ T cells used in these studies were all of relatively high affinity for antigen and as a consequence would be predicted to have a low level requirement for costimulatory signals. Although MBP cells are considered low affinity because a higher dose of MBP peptide is required to stimulate a similar level of proliferation to MCC cells stimulated with low dose of MCC peptide (Figure 3.0), this is probably related to the poor binding efficiency of $MBP_{Ac1\text{-}11}$ to MHC Class II allele $I\text{-}A^u$ (Fairchild et al. 1993; Scott et al. 1994). This may also explain why no effect of antigen specific competition was seen between MBP cells (Figure 3.2).

The absence of antigen non-specific competition in experiments described in this chapter appears to contradict previous *in vitro* studies performed during my honours year and *in vitro* and *in vivo* studies by Lake and colleagues (Hayball et al. 2004; Lake et al. 1999). In our *in vitro* system, preactivated CD4 were capable of reducing the recruitment of additional cells into the response regardless of each cells specificity or affinity for antigen. However this effect was due to the consumption of soluble factors (unpublished data), a phenomenon unlikely to occur *in vivo*. One possible explanation for the differing *in vivo* results may be due the different level of stimulation due to administration of peptide antigens in Complete Freund's Adjuvant (CFA) (in this study) versus Incomplete Freund's Adjuvant (IFA) (Hayball et al. 2004) .

In the experiments described in this chapter, peptide antigens were always administered in CFA, a suspension of heat killed *Mycobacterium tuberculosis* in mineral oil (Freund and McDermott 1942). BCG is an intracellular bacteria that contains a large number of pathogenic structures that induce activation of the innate immune system via pattern recognition receptors (PRR) or Toll-like receptors (TLRs). Immunising mice with PBS in CFA induces the upregulation of a number of costimulatory markers (see chapter 7 for more detail) and therefore administration of peptide antigens in a highly stimulatory emulsion may have masked any effect of competition for costimulatory molecules in these experiments.

Although in this system no effect of competition for costimulatory signals was seen, the data clearly demonstrate the importance of TCR stimulation for both the initiation and maintenance of a CD4 response. Recruitment of additional precursors into an ongoing response was progressively inhibited with time. Not only were fewer cells recruited into the response but these cells underwent fewer rounds of division and fewer cells differentiated into cytokine producing cells (Figure 3.6). As a consequence these cells made only a minor contribution (less than 5%) to the total MCC specific response (Figure 3.6). Reduced recruitment of additional precursors into an ongoing response was not due to spacial constraints mediated by an increase in the total number of activated cells, as an ongoing response to an independent antigen was unaffected. It was also not due to an

inability of naïve cells to access APCs as has been suggested by a number of other studies (Hayball et al. 2004; Kedl et al. 2000). Recruitment was however a reflection of the level of antigen presentation and could be increased with administration of additional antigen at the time of T cell transfer. With a small number of precursors, such as are present with an endogenous repertoire, exogenous naïve high affinity T cells can be recruited into a response 4 days after immunisation (Figure 3.8). This is consistent with previous data in which CD4 cells transferred up to 3 weeks after influenza infection had a high level of recruitment into division, although slightly reduced compared to the control group (Jelley-Gibbs et al. 2005). However when the response was initiated with a relatively large number of high affinity precursors, recruitment of further cells into the response was significantly inhibited just 24 hours later (Figure 3.10) and is consistent with data demonstrating reduced recruitment and IFN-γ production of $CD4^+$ T cells in the face of a large number of competing precursors (Foulds and Shen 2006; Smith et al. 2000). Interestingly both activated $CD4^+$ (Lee et al. 2002) and $CD8^+$ (Kaech and Ahmed 2001; van Stipdonk et al. 2001; Wong and Pamer 2001) T cells can continue to divide *in vitro* after removal of antigen. Memory cell differentiation is also believed to be independent of antigen-MHC (Murali-Krishna et al. 1999; Swain et al. 1999). However the experiments described here suggest that optimal proliferation and differentiation *in vivo* requires ongoing access to antigen-MHC (Obst et al. 2005). After acute activation leading to surface expression of CD69, not all activated cells were recruited into cell division within the next 2.5 days (Figures 3.7.1 and 3.7.2). Moreover, ongoing cell division and differentiation to cytokine secretion was even more profoundly affected by T cell competition than initial recruitment. For example, 40% of precursors were recruited 1 day into an ongoing response in the experiment described in Figure 3.6, compared with 70% in the control group (Figure 3.6). However the mean number of divisions per recruited cell was only 2.5, compared with >6 in the control (Figure 3.6d). The number of IL-2-secreting cells was reduced from $1x10^6$ to $1x10^5$ (Figure 3.6i) and IFN-γ-secreting cells dropped from $3x10^5$ to $2x10^4$ (Figure 3.6n). While recruitment of additional precursors into division was fully restored by the administration of additional antigen at the time of cell transfer

(Figure 3.8), subsequent proliferation was still decreased compared to the control (Figure 3.8). Taken together, these results suggest that the level of available antigen not only controls initial T cell activation, but subsequent proliferation and differentiation into effector cells.

Previous studies in which antigen was administered by means of injecting peptide-pulsed or virus infected DCs have suggested that antigen presentation in the context of MHC class I is relatively short-lived (Hafalla et al. 2002; Hermans et al. 2000; Norbury et al. 2002). The ability of $CD8^+$ T cells to kill DCs presenting specific antigen is believed to contribute to the rapid loss of peptide-bearing DCs (Hafalla et al. 2003; Wong and Pamer 2003; Yang et al. 2006). In some studies, $CD4^+$ T cells have also been shown to decrease the survival of DCs presenting specific antigen (Ingulli et al. 1997) whereas in others, $CD4^+$ T cell recognition increased DC survival (De Smedt et al. 1998). Both $CD4^+$ and $CD8^+$ T cells have been shown to remove specific peptide-MHC complexes from the surface of APCs (Huang et al. 1999; Patel et al. 1999; Wetzel et al. 2005). Indeed, Kedl (2002) suggested that this process was responsible for antigen-specific competition between $CD8^+$ T cells *in vivo*. Whether this is responsible for changes in antigen presentation *in vivo* has yet to be determined.

Finally these data demonstrate that competition does not solely occur at one single stage of the response and is a constant parameter of the early response. $CD4^+$ T cells compete for initial access to peptide-MHC signals, which can affect early TCR signalling, while continued competition during the first 24 hours alters the percentage of cells recruited into division. These data suggest that competition between divided cells for continued access to peptide-MHC can affect the extent of T cell proliferation and differentiation (Obst et al. 2005). It is a continued requirement for peptide-MHC stimulation that enables the immune system to control the size of an antigen specific response. In conclusion these data illustrate the mechanism whereby the immune system responds efficiently to late phase antigens or secondary infections, while exerting tight control over the size and kinetics of each individual antigen specific response.