

Chapter 4: Lack of antigen non-specific competition when both antigens are presented by the same APC

Unlike B cells that recognise conformational epitopes, T cell responses are directed to only a small number of epitopes within a whole protein (Berkower et al. 1984; Hurwitz et al. 1984; Manca et al. 1984; Shimonkevitz et al. 1984; Solinger et al. 1979) or pathogen (Deng et al. 1997; Gotch et al. 1987; Townsend et al. 1986; Vijn and Pamer 1997).

Immunodominant hierarchies arise as dominant antigens elicit massive expansion of antigen specific cells while sub-dominant antigens induce significantly smaller responses (Yewdell and Bennink 1999). Factors that have been proposed to shape these immunodominant hierarchies include antigen processing, the level of antigen presentation and competition between cells within the responding repertoire of T cells. To date the only *in vivo* evidence of competition was between cells making simultaneous responses to different antigens derived from the same protein (Butz and Bevan 1998; Kedl et al. 2000), when both antigens were presented by the same DC (Kedl et al. 2002). In addition, Hayball (2004) demonstrated antigen non-specific competition when the competing response had an advantage in terms of early access to antigen. While antigenic competition is not a consistent feature in responses to multi-valent vaccines, increasing the number of antigenic epitopes has been shown in some cases to produce suppression of the response to particular epitopes (Fattom et al. 1999; Manoj et al. 2003; Raadsma et al. 1994). Together these data support the notion that the primary mechanism mediating competition between cells of different antigen specificity is access to APCs. Since peptides do not require processing prior to presentation on the surface of APCs (Colledge et al. 2001), the absence of antigen non-specific competition in the experiments described in the previous chapter may have been a result of T cell responses being focussed on different APCs. In the experiments described here, a recombinant protein containing the stimulatory epitopes of both HEL and MCC was used to ensure presentation of both epitopes on the same APCs. As described in chapter 2, HELMCC is a mutagenised form

of hen egg lysozyme (HEL) in which a hydrophilic loop at residues 83-93 was originally replaced with the stimulatory MCC epitope (residues 87-103). As a consequence, HELMCC contains the antigenic epitopes for both 3A9 and 5C.C7 TCR tg cells and can induce recruitment and proliferation of both types of TCR tg cells when protein is administered in CFA (Figure 2.10). The series of experiments in this chapter describe the comparison of T cell responses induced by immunisation with HELMCC compared to stimulation of T cells with a combination of HEL and MCC peptides.

4.1 The effect of antigen specific competition on proliferation of 5C.C7 cells

Utilising the model of *in vivo* competition established in the previous chapter, recipient mice were immunised with 3 doses of recombinant HELMCC protein (5 μ g, 0.5 μ g and 0.05 μ g) and received 1x10⁶ unlabelled 5C.C7 or 3A9 LN cells 24 hours later mice (Figure 4.1a). 1x10⁶ CFSE labelled Ly5.1⁻ 5C.C7 cells were transferred into all recipient mice a further 24 hours later, and draining LNs harvested after 3 or 10 days for analysis by flow cytometry.

At the a dose of 5 μ g or 0.5 μ g of HELMCC protein, transferring 5C.C7 cells 24 hours into an ongoing 5C.C7 response resulted in only a small reduction in the percentage of CFSE labelled 5C.C7 cells recruited into division. Thus at a dose of 5 μ g recruitment decreased from 92% to 82% (Figure 4.1c) and at 0.5 μ g recruitment from reduced from 80% to 73% (Figure 4.1g). Although there was only a small reduction in recruitment, at day 10 a 10-fold reduction in the total number of divided Ly5.1⁻ 5C.C7 cells was observed when cells were transferred 1 day into an ongoing 5C.C7 response (Figure 4.1e and i). At these two doses no change in recruitment or proliferation was seen when CFSE labelled 5C.C7 cells were transferred 1 day into an ongoing 3A9 response. There was also no difference in the total number of divided Ly5.1⁻ 5C.C7, and the increase in cell numbers between days 3 and 10 was comparable to the control group (Figure 4.1 e and i).

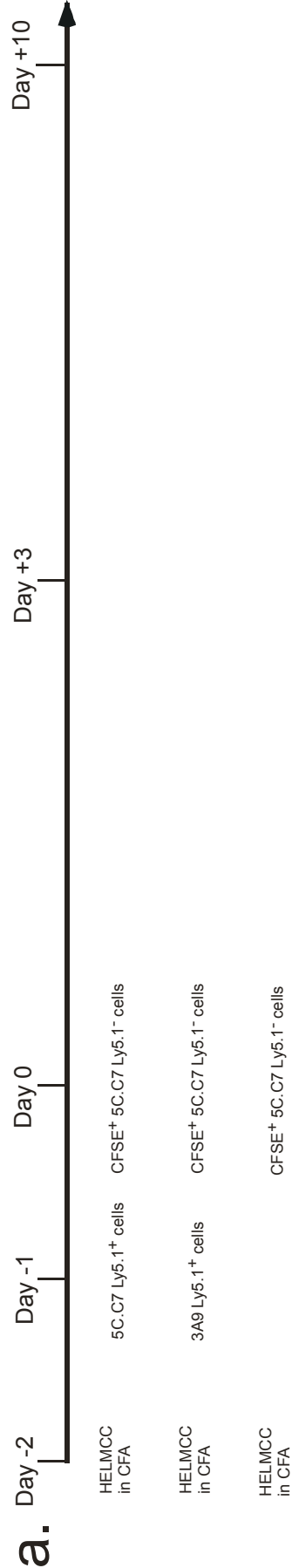


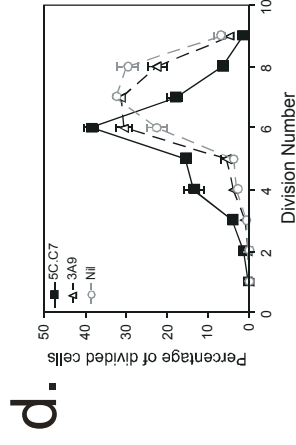
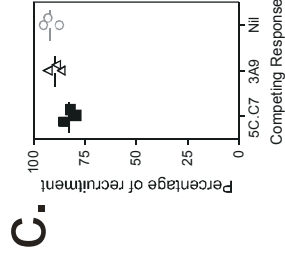
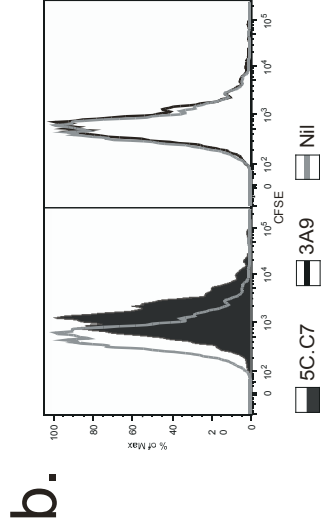
Figure 4.1: The effect of competition on the response of 5C.C7 cells stimulated with varying doses of recombinant protein HELMCC.

a. F1L recipient mice were immunized on day -2 with either 5 μ g, 0.5 μ g or 0.05 μ g of recombinant HELMCC protein. On day -1 unlabeled 5C.C7, 3A9 or no cells were transferred into recipient mice and 24 hours later mice received equal numbers of CFSE labelled 5C.C7 cells. On day 3 draining lymph nodes were harvested and analyzed by flow cytometry.

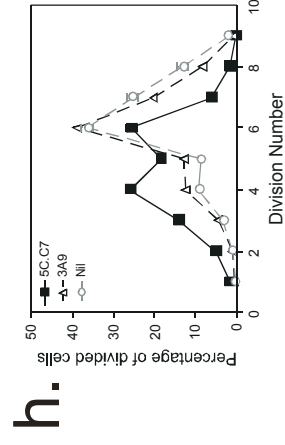
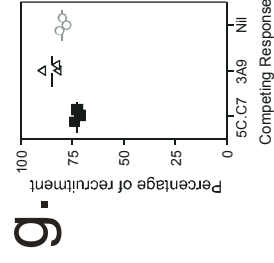
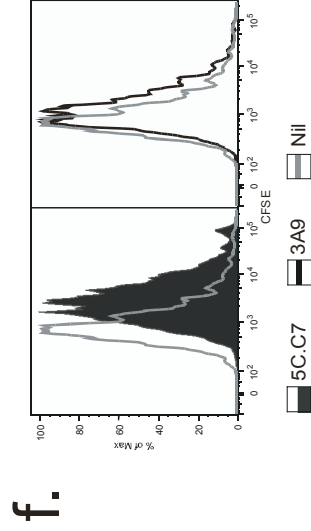
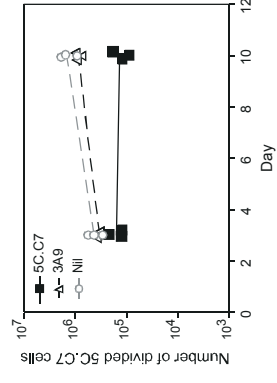
CFSE profiles of 5C.C7 T cells (**b, f, j**) transferred 1 day into a competing 5C.C7 (black filled), 3A9 (black open line) or no competing response (grey line). The percentage of 5C.C7 T cells recruited into division (**c, g, h**) and the percentage of divided 5C.C7 T cells per division (**d, h, i**) was calculated at the 3 different doses of HELMCC when cells were transferred 1 day into an ongoing 5C.C7, 3A9 or no competing response.

The total number of divided 5C.C7 cells (**e, i, m**) was calculated by multiplying the frequency of divided Ly5⁻, CD4⁺, KJ25⁺ cells per sample by the total organ count.

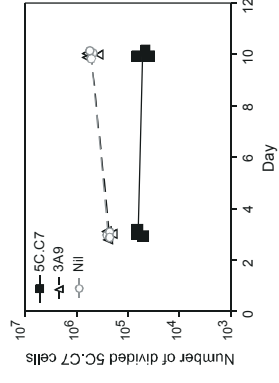
5 μ g HELMCC



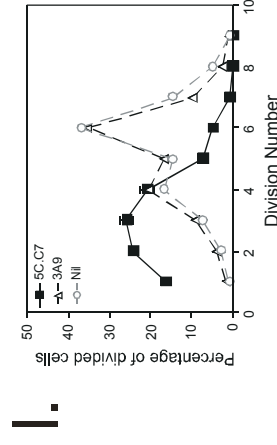
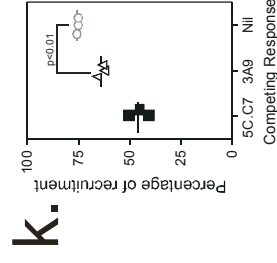
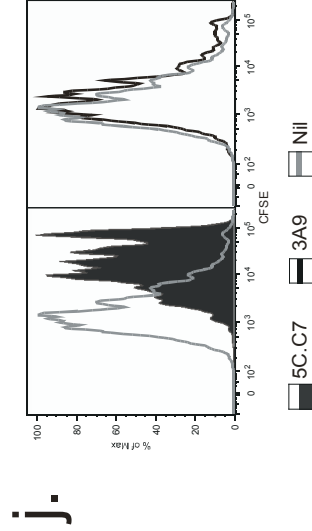
e.



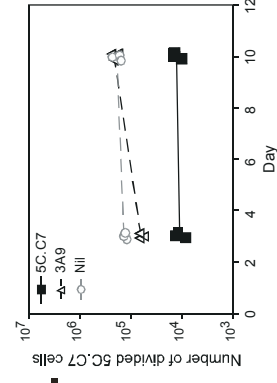
i.



0.05 μ g HELMCC



m.



At a dose of 0.05 μ g, transferring 5C.C7 cells 1 day into an ongoing 5C.C7 response reduced recruitment into division from 75% to 45% (Figure 4.1k) and divided cells progressed through fewer rounds of cell division by day 3 (Figure 4.1l). Consistent with this substantial reduction in recruitment, a 10-fold decrease in the total number of divided 5C.C7 cells was observed at days 3 and 10 (Figure 4.1m). In this experiment, recruitment into division was also reduced when 5C.C7 cells were transferred 1 day into an ongoing 3A9 response (from 75% to 64%), although proliferation of 5C.C7 cells by day 3 was not affected (Figure 4.1k). Despite a small decrease in the total number of divided 5C.C7 cells observed at day 3 (due to a small decline in recruitment), there was no difference in the cell number at day 10 when compared to the control group (Figure 4.1m).

In summary, a small degree of antigen non-specific competition was observed at the lowest dose of HELMCC and led to only a small reduction in recruitment and the total number of divided 5C.C7 cells at day 3. There were no long term effects of this initial reduction in recruitment, as by day 3 divided cells progressed through an equal number of division and there was an equal number of divided 5C.C7 cells in both groups by day 10.

4.2 The effect of antigen non-specific competition on the on the generation of memory cells

In the previous chapter, it was shown that the effect of antigen specific competition could be enhanced by transferring CFSE labelled cells at day 3 of an ongoing response (Figure 3.6). This experiment was repeated using a low dose of HELMCC rather than a mixture of HEL and MCC peptides (Figure 4.2.1).

The congenic marker Ly5.1 was used to distinguish CFSE labelled 5C.C7 or 3A9 cells from competing and host cells (Figure 4.2.1a). On day -3, Ly5.1⁺ recipient mice were immunised with 0.05 μ g HELMCC in CFA and received 2.5x10⁶ CD4⁺ Ly5.1⁺ 3A9 T cells. All recipient mice received 2.5x10⁶ CFSE labelled CD4⁺ Ly5.1⁻ 5C.C7 cells on day 0 and the draining LNs were harvested 3 days and 8 weeks later for analysis by flow cytometry.

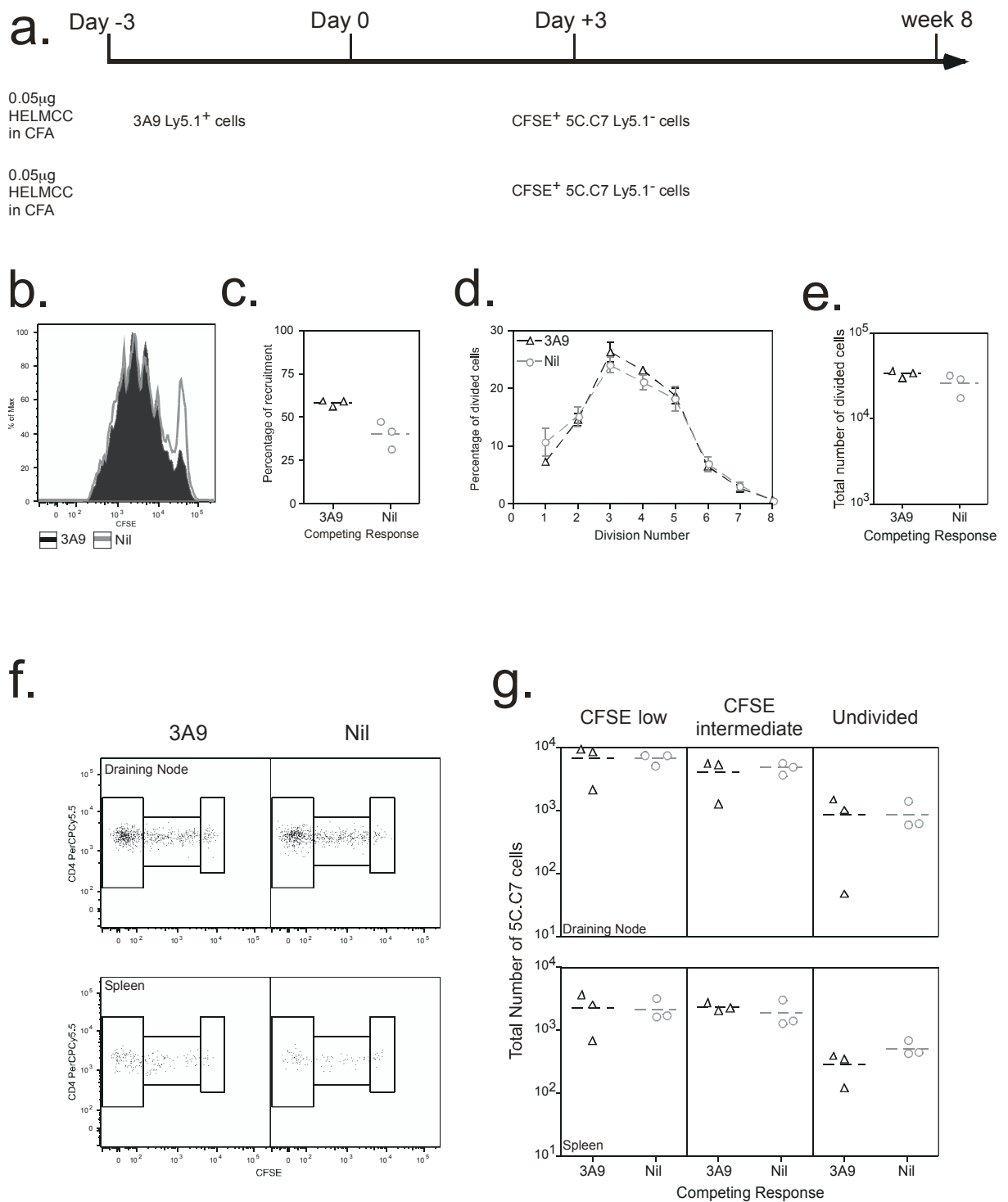


Figure 4.2.1: The effect of antigen non-specific competition on the response of 5C.C7 cells

a. On day -3, all recipient mice were immunized with 0.05 μ g HELMCC emulsified in CFA. At the same time one group received a total of 2.5×10^5 CD4⁺ unlabeled 3A9 competing cells with 2.5×10^5 CFSE labelled 5C.C7 cells transferred into all recipient mice on day 0. Draining lymph nodes and spleens were harvested at day 3 and week 8 for analysis by flow cytometry. CFSE profiles of 5C.C7 T cells (**b.**) transferred 3 days into a competing 3A9 (black open line) or no competing response (grey line). Graphs represent the percentage of 5C.C7 cells recruited into division (**c.**), the percentage of divided 5C.C7 cells per division (**d.**) (mean of three mice per group $\pm$ SEM) and the total number of divided 5C.C7 (**e.**) cells recovered from the draining node at day 3. (**f.**) 8 weeks after cell transfer the draining lymph nodes and spleens were harvested and the total number (**g.**) of fully divided (CFSE low) (left), CFSE intermediate (middle) and undivided (right) 5C.C7 cells calculated by multiplying the frequency of cells per sample by the total organ count.

Transferring 5C.C7 cells 3 days into an ongoing 3A9 response slightly enhanced recruitment into division (from 40% to 58%) (Figure 4.2.1c). However by day 3, divided 5C.C7 cells had progressed through an equal number of divisions in the two groups, and equal numbers of divided 5C.C7 cells were detected (Figure 4.2.1e). 8 weeks after cell transfer an equal number of 5C.C7 cells were recovered from the draining LNs and spleens of the two experimental groups (Figure 4.2.1g).

In this same experiment (Figure 4.2.2a), recipient Ly5.1⁻ mice were immunised with 0.05µg HELMCC in CFA and received 2.5×10^6 CD4⁺ Ly5.1⁻ 5C.C7 cells followed 3 days later with 2.5×10^6 CFSE labelled CD4⁺ Ly5.1⁺ 3A9 cells. Draining LNs were harvested 3 days and 8 weeks later for analysis by flow cytometry.

Transferring 3A9 cells 3 days into an ongoing 5C.C7 response reduced recruitment into division from 17% to 10% (Figure 4.2.2.c) ($p < 0.05$). While divided cells progressed through an equal number of divisions by day 3 (Figure 4.2.2d), there was a slight decrease in the total number of divided 3A9 cells seen at day 3 (Figure 4.2.2e) ($p < 0.01$). 8 weeks after transfer, no significant difference in the number of divided 3A9 cells between the two groups was observed (Figure 4.2.2g). A slightly higher number of undivided 3A9 cells was observed when 3A9 cells were transferred into an ongoing 5C.C7 response, but this was a reflection of the decreased percentage of cells recruited into division by day 3. In summary, while antigen non-specific competition slightly decreased the percentage of 3A9 cells recruited into division, it had no long-term effect on the total 3A9 response. Thus, despite the almost 2-fold decrease in the percentage of 3A9 cells recruited into division, there was no difference in the number of divided cells (Figure 4.2.3 g) or long-lived memory cells (Figure 4.2.3g).

4.3 Comparison of 5C.C7 and 3A9 cell responses to HELMCC and peptides.

Since antigen non-specific competition was seen at the lowest dose of HELMCC, it was important to establish whether this was due to competition between cells for access to

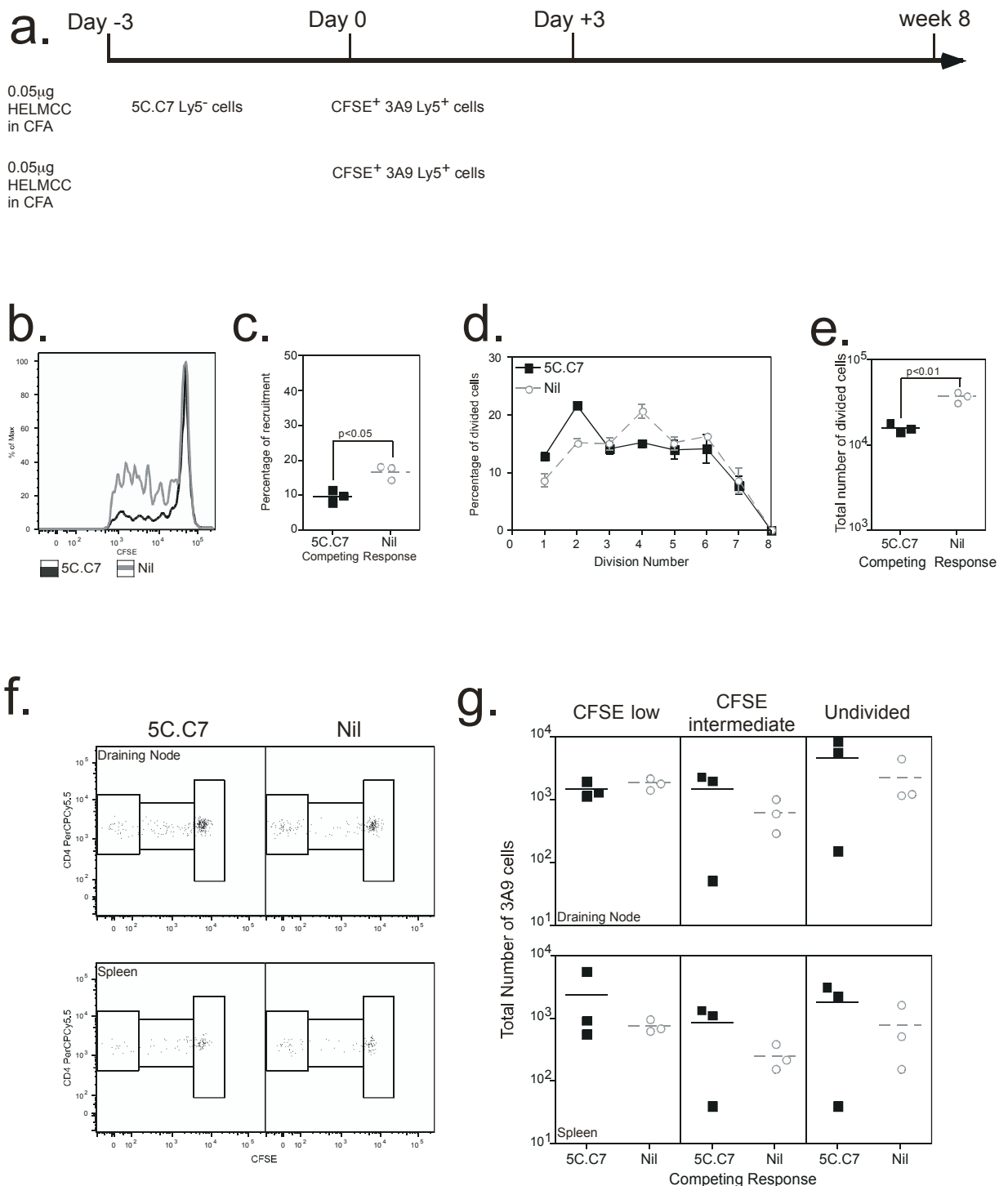


Figure 4.2.2: The effect of antigen non-specific competition on the response of 3A9 cells

a. On day -3, all recipient mice were immunized with 0.05 μ g HELMCC emulsified in CFA. At the same time one group received a total of 2.5×10^5 CD4⁺ unlabeled 5C.C7 competing cells with 2.5×10^5 CFSE labelled 3A9 cells transferred into all recipient mice on day 0. Draining lymph nodes and spleens were harvested at day 3 and week 8 for analysis by flow cytometry. CFSE profiles of 3A9 T cells (**b.**) transferred 3 days into a competing 5C.C7 (black filled line) or no competing response (grey line).

Graphs represent the percentage of 3A9 cells recruited into division (**c.**), the percentage of divided 3A9 cells per division (**d.**) (mean of three mice per group $\pm$ SEM) and the total number of divided 3A9 (**e.**) cells recovered from the draining node at day 3.

8 weeks after cell transfer the draining lymph nodes and spleens were harvested and the total number of fully divided (CFSE low) (left), CFSE intermediate (middle) and undivided (right) 3A9 cells calculated by multiplying the frequency of cells per sample by the total organ count.

APCs or due to use of a relatively smaller amount of antigen. To accurately compare antigen non-specific competition between T cells with linked (recombinant HELMCC) versus unlinked (peptide) antigen, the relative response of 5C.C7 and 3A9 cells to recombinant protein relative to peptide (Figure 4.3.1) was measured.

On day -1, 1×10^6 CD4⁺ CFSE labelled 5C.C7 or 3A9 cells were adoptively transferred into recipient mice. 24 hours later, recipient mice were immunised with graded doses of MCC peptide, HEL peptide or HELMCC protein emulsified in CFA. The percentage of 5C.C7 or 3A9 cells recruited into division at the different doses of antigen was calculated 3 days post transfer. The percentage of 5C.C7 cells recruited into division ranged from 20% at a dose of 0.001 μ g MCC peptide to 75% at a dose of 10 μ g MCC peptide (Figure 4.3.1). The percentage of 3A9 cells recruited into division ranged from 10% seen with a dose of 0.032 μ g HEL peptide to 30% with a dose of 32 μ g HEL (Figure 4.3.1). With a dose of 0.5 μ g HELMCC, 65% of 5C.C7 cells and 43% of 3A9 cells were recruited into division while at a dose of 0.05 μ g HELMCC, 55% of 5C.C7 cells and 23% of 3A9 cells were recruited into division (Figure 4.3.1).

The effect of graded antigen doses on the expression of activation markers CD25, CD44 and CD69 was also measured in this experiment. Expression of CD25 and CD44 on activated 5C.C7 cells remained relatively unchanged across all the doses of MCC peptide, except for a higher level of CD25 seen on 5C.C7 cells stimulated with 0.001 μ g of MCC (Figure 4.3.2). Stimulation of 5C.C7 cells with 0.05 μ g HELMCC induced a similar level of CD44 and CD25 expression. A similar pattern of CD44 and CD25 expression was also seen on 3A9 cells stimulated with a range of HEL peptide doses (Figure 4.3.3) or with 0.05 μ g HELMCC. Surface expression of CD69 on divided 5C.C7 cells appeared to inversely correlate with the dose of MCC peptide (Figure 4.3.2), with the highest level of CD69 expression seen at the lowest doses of peptide. This pattern of CD69 expression was also true for 3A9 cells (Figure 4.3.3).

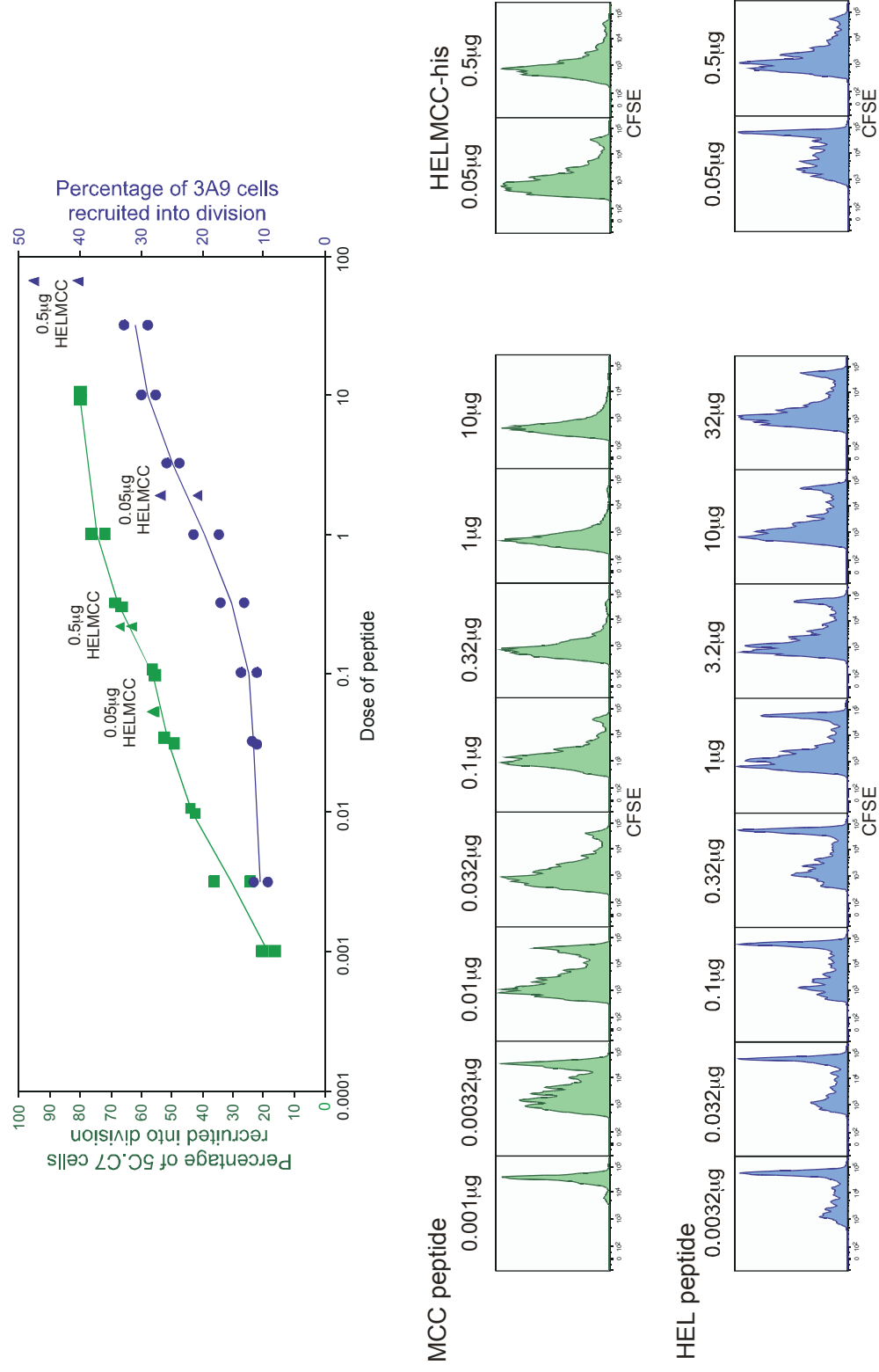


Figure 4.3.1: Response of 5C.C7 and 3A9 TCR tg cells to varying doses of peptide.

Recipient mice received 1×10^6 CD4^+ 5C.C7 or 1×10^6 CD4^+ 3A9 T cells followed by immunization with graded doses of MCC or HEL peptides 1 day later. Proliferation and recruitment of 5C.C7 cells (green) or 3A9 cells (blue) into division was calculated on day 3, lines represent the mean of 2 mice per dose of peptide. CFSE profiles show the proliferation of CD4^+ and CFSE $^+$ 5C.C7 or 3A9 cells at varying doses of either MCC or HEL peptides. The recruitment of 5C.C7 and 3A9 cells into division with 0.5 μg or 0.05 μg HELMCC-his was also calculated (triangles).

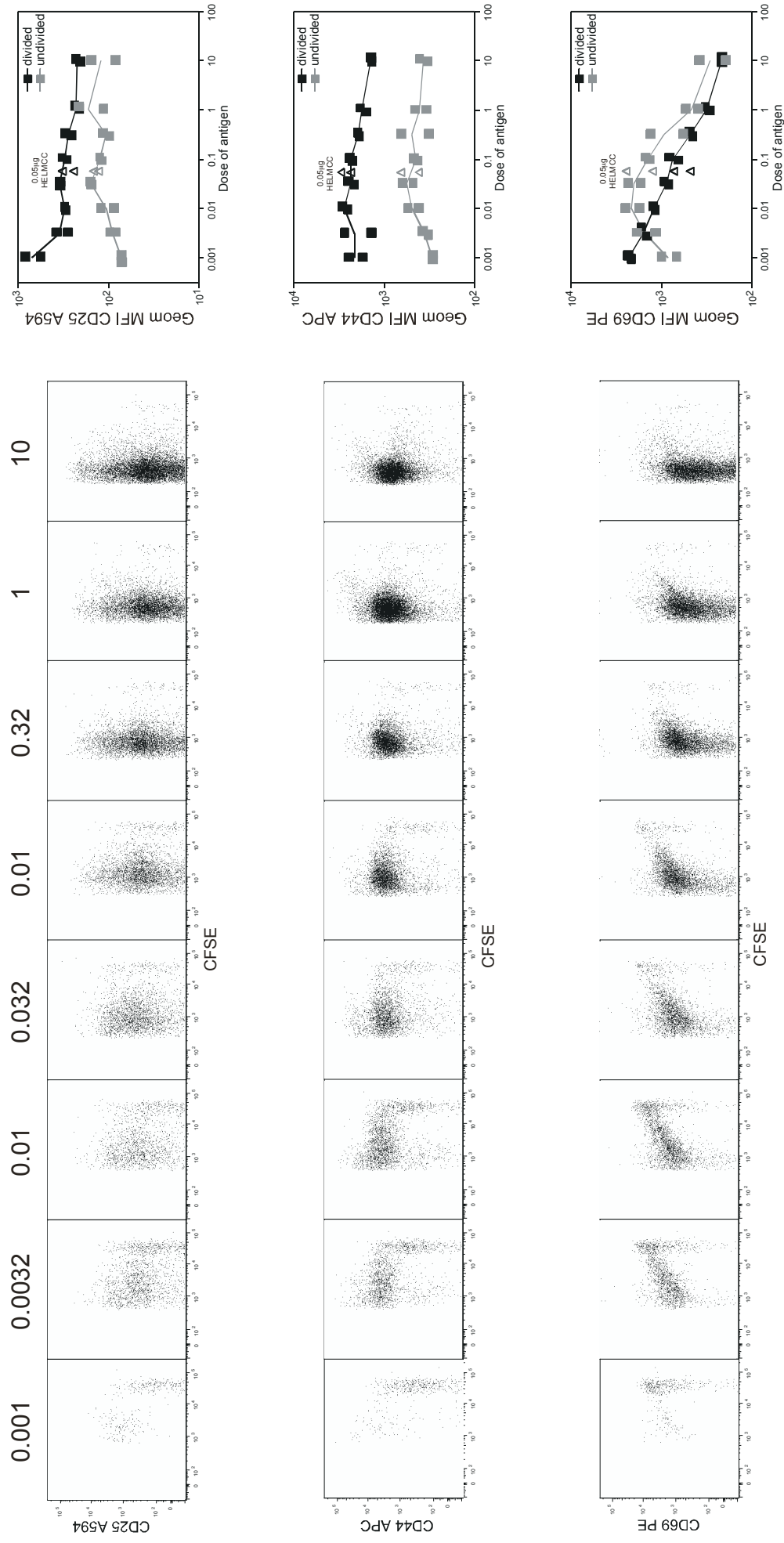


Figure 4.3.2: Expression of activation markers on 5C.7 cells at varying doses of MCC peptide

Recipient mice were immunised with graded doses of MCC peptide and 3 days later the expression of CD25 (top panel) CD44 (middle panel) and CD69 (bottom panel) measured by flow cytometry. Graphs represent the geometric mean fluorescence intensity (Geom MFI) of activation marker expression where the line represents the mean of 2 mice per group with individual mice shown as single point. Samples were gated on CFSE and CD4. Expression of each marker on 5C.7 cells stimulated with 0.05μg HELMCC is indicated by the open triangles.

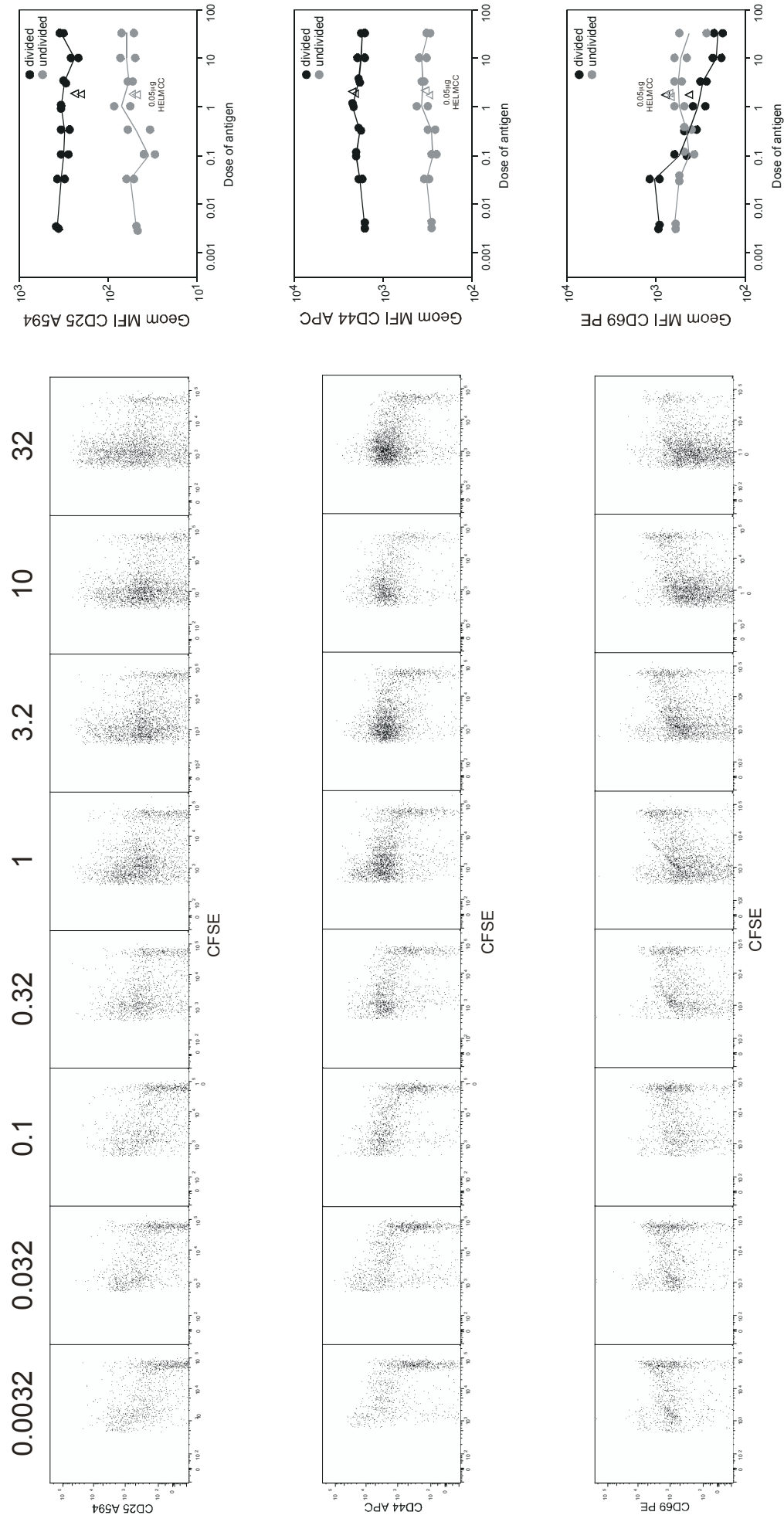


Figure 4.3.3: Expression of activation markers on 3A9 cells at varying doses of HEL peptide

Recipient mice were immunised with graded doses of HEL peptide and 3 days later the expression of CD25 (top panel) CD44 (middle panel) and CD69 (bottom panel) was measured by flow cytometry. Graphs represent the geometric mean fluorescence intensity (Geom MFI) of activation marker expression where the line represents the mean of 2 mice per group with each mouse shown as individual points. Samples were gated on CFSE and CD4. Expression of each marker on 3A9 cells stimulated with 0.05 µg HELMCC is indicated by the open triangles.

From the peptide dose response curves this placed the availability of the MCC epitope from 0.05 μ g HELMCC between that of 0.032 μ g and 0.1 μ g of MCC peptides, and the availability of the HEL epitope between that of 1 μ g and 3.2 μ g HEL peptides.

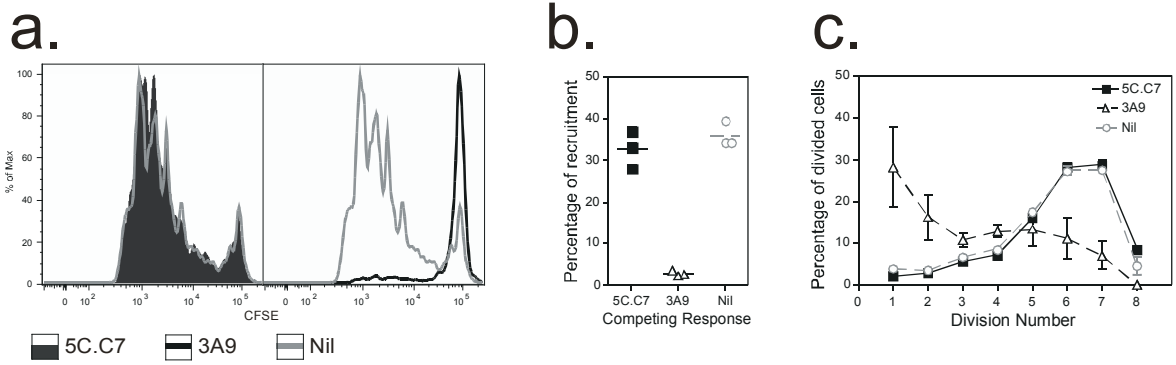
4.4 Competition between linked versus unlinked antigens

To test whether antigen non-specific competition could be seen when cells were stimulated with doses of HEL and MCC peptides equivalent to a very low dose of recombinant HELMCC protein, recipient mice were immunised with a total of 0.1 μ g MCC and 3.2 μ g HEL peptides in CFA, 0.032 μ g MCC and 1 μ g HEL peptides in CFA or 0.05 μ g HELMCC protein in CFA (Figure 4.4.1a). These doses were based on the experiment described above, and aimed to sandwich the HELMCC dose between 2 doses of each peptide. At this same timepoint, recipient mice received 1×10^6 CD4⁺ 5C.C7 cells, 1×10^6 CD4⁺ 3A9 cells or no cells. 3 days later all recipient mice received 0.5×10^6 CD4⁺ CFSE labelled 3A9 cells and draining LNs were harvested a further 2.5 days later for analysis by flow cytometry.

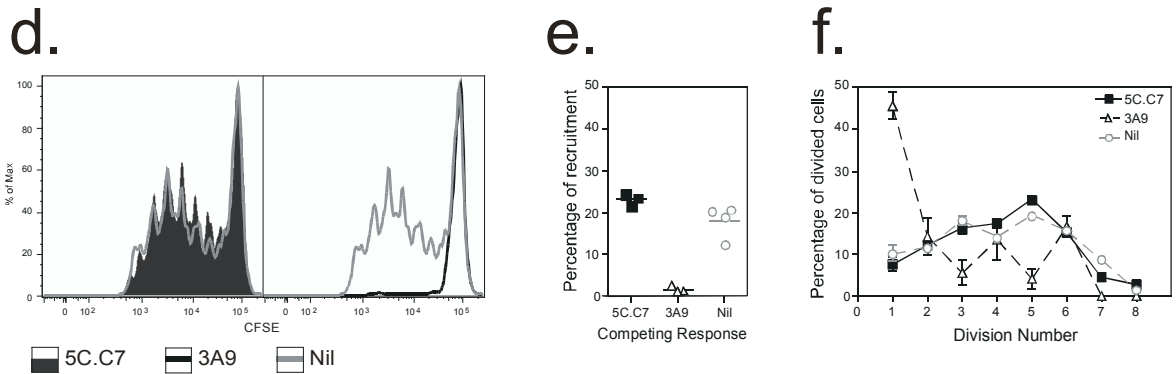
Consistent with the experiments described in the previous chapter, transferring 3A9 cells 3 days into an ongoing 3A9 response reduced recruitment into division and divided cells progressed through fewer rounds of division by day 3. This was true when cells were stimulated with either a mixture of MCC and HEL peptides (Figure 4.4.1 upper and lower panels), or recombinant HELMCC emulsified in CFA (Figure 4.4.1 middle panel). However in no case did transfer of 3A9 cells 3 days into an ongoing 5C.C7 response affect recruitment or proliferation by day 3 (Figure 4.4.1), in contrast to the results described in Figure 4.2.2.

This experiment was repeated with equal numbers of 5C.C7 and 3A9 cells (Figure 4.4.2a). Although the response of 3A9 cells to 0.05 μ g of HELMCC was slightly lower than had previously been seen, transferring 3A9 cells 3 days into an ongoing 5C.C7 response had no effect (Figure 4.4.2d, e and f). At both doses of MCC and HEL peptides (Figure

0.1 μ g MCC and 3.2 μ g HEL peptides



0.05 μ g HELMCC



0.032 μ g MCC and 1 μ g HEL peptides

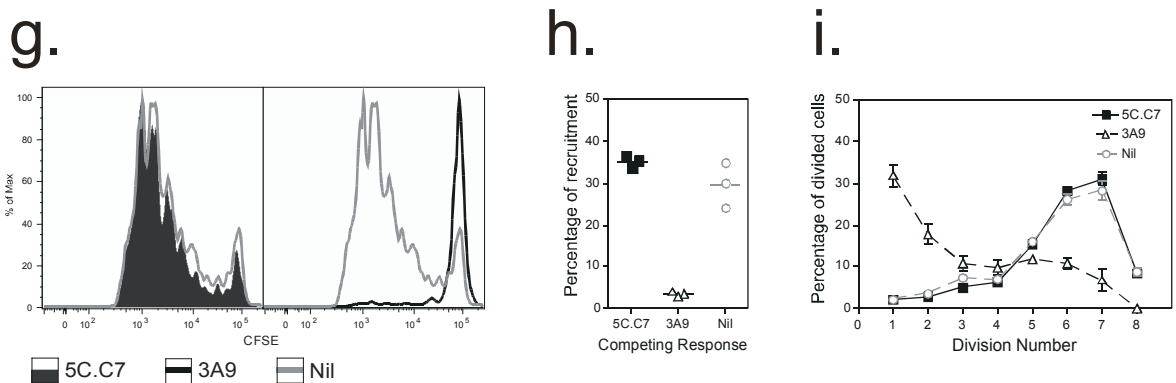
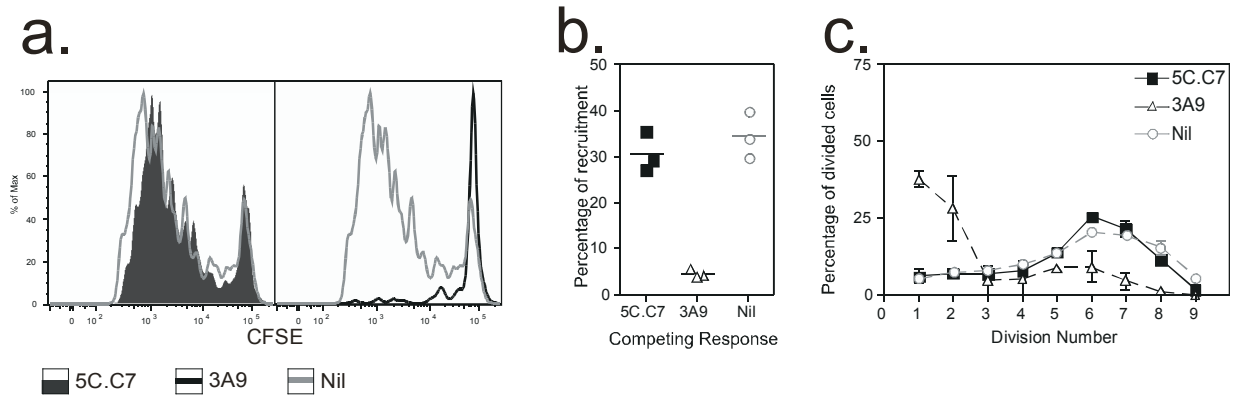


Figure 4.4.1: The effect of competition between linked versus unlinked antigens

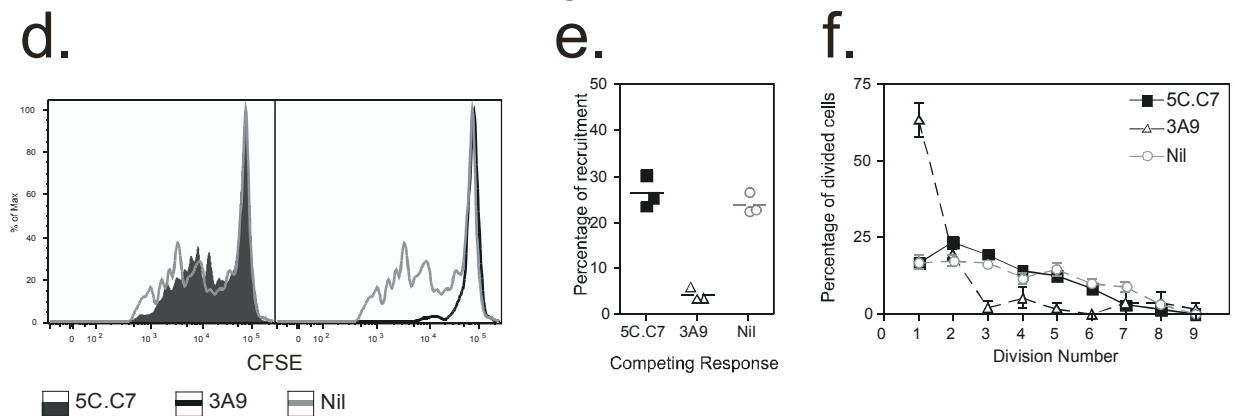
Recipient mice were immunized on day -3 with either 0.1 μ g MCC and 3.2 μ g HEL peptides, 0.05 μ g of recombinant HELMCC protein or 0.032 μ g MCC and 1 μ g HEL peptides and simultaneously received 1×10^6 CD4⁺ 5C.C7, 3A9 or no competing cells. On day 0 all recipient mice received 0.5×10^6 CFSE labelled 3A9 cells and 3 days later draining lymph nodes were harvested and analyzed by flow cytometry.

CFSE profiles of 5C.C7 T cells (**a**, **d**, **g**) transferred 1 day into a competing 5C.C7 (black filled), 3A9 (black open line) or no competing response (grey line). The percentage of 3A9 T cells recruited into division (**b**, **e**, **h**) and the percentage of divided 3A9 T cells per division was calculated (**c**, **f**, **i**) for each of the separate different immunization regimes.

0.1 μ g MCC and 3.2 μ g HEL peptides



0.05 μ g HELMCC



0.032 μ g MCC and 1 μ g HEL peptides

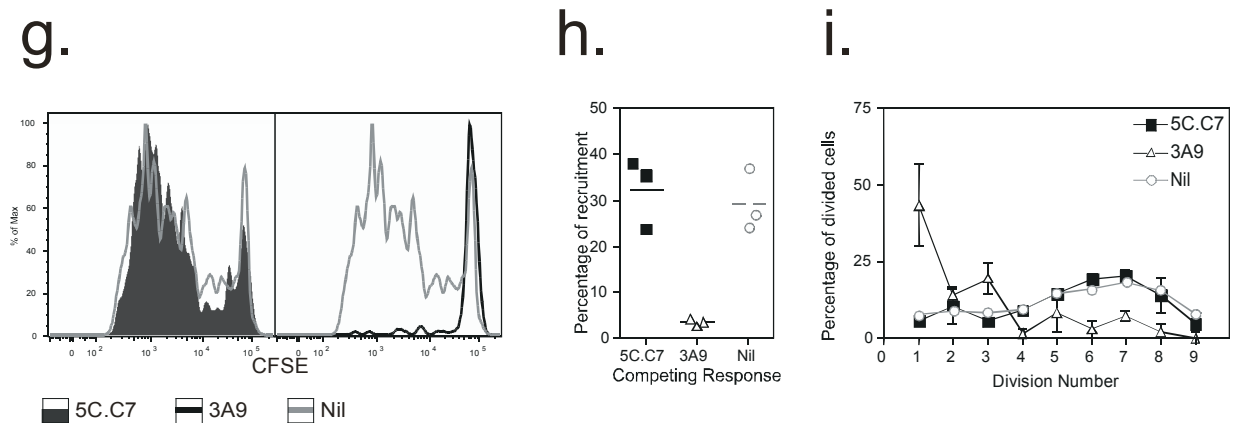


Figure 4.4.2: The effect of competition between linked versus unlinked antigens

Recipient mice were immunized on day -3 with either 0.1 μ g MCC and 3.2 μ g HEL peptides, 0.05 μ g of recombinant HELMCC protein or 0.032 μ g MCC and 1 μ g HEL peptides while simultaneously receiving 1×10^6 CD4⁺ 5C.C7, 3A9 or no competing cells. On day 0 all recipient mice received 1×10^6 CD4⁺ CFSE labelled 3A9 cells and 3 days later draining lymph nodes were harvested and analyzed by flow cytometry.

CFSE profiles of 5C.C7 T cells (**a, d, g**) transferred 1 day into a competing 5C.C7 (black filled), 3A9 (black open line) or no competing response (grey line). The percentage of 3A9 T cells recruited into division (**b, e, h**) and the percentage of divided 3A9 T cells per division was calculated (**c, f, i**) for each of the separate immunization regimes.

4.4.2 upper and lower panels), or with recombinant HELMCC (Figure 4.4.2 middle panel), reduced recruitment and proliferation was detected when CFSE labelled 3A9 cells were transferred 3 days into an ongoing 3A9 response.

Despite the use of a recombinant antigen driving presentation of both antigens on the same APC there was no evidence of antigen non-specific competition in the two experiments described in this section.

Discussion

The experiments described in this chapter endeavoured to investigate the effect of competition for access to APCs on the response of 5C.C7 and 3A9 TCR tg cells. To achieve this, a recombinant protein containing the epitopes recognised by 3A9 and 5C.C7 TCR tg cells was utilised. A small degree of competition between cells of different antigen specificity was detected with the use of a very small dose of recombinant HELMCC in two of the four experiments. However competition had only a minor effect on the early stages of the response without affecting the total number of divided cells or long-lived memory cells. In the first case, 10% fewer 5C.C7 cells were recruited into division (Figure 4.1k) although the total number of divided cells was equal to the control at day 10 (Figure 4.1m). However, in a second experiment recruitment of 5C.C7 cells was marginally enhanced (Figure 4.2.1c), while recruitment of 3A9 cells was reduced (Figure 4.2.2c). In this same experiment, the total number of memory cells 8 weeks post transfer was unaffected (Figure 4.2.2g). However, two further repeats of the 3A9 recruitment experiments did not confirm the existence of antigen non-specific competition by 5C.C7 cells (Figure 4.4.1 and Figure 4.4.2). These data demonstrate that antigen non-specific competition, if it occurs, has only a minor effect on the early response, and is not evident at 10 days or 8 weeks later.

While there is published evidence of competition between cells of different antigen specificity at early stages of a T cells response, day 3 for CD4⁺ T cells (Hayball et al.

2004) and day 5 for CD8⁺ T cells (Kedl et al. 2000; Kedl et al. 2002), an effect of competition on later timepoints of the response has not been documented (Probst et al. 2002). Unfortunately none of these studies investigated the effect of competition on both the early and late stages of the response, and therefore it is difficult to assess the effect of antigen non-specific competition on the overall T cell response. It is, however, clear that antigen specific competition has a far more potent effect on the response of CD4⁺ or CD8⁺ T cells. In experiments by Kedl (2000), antigen non-specific competition was not always evident and when it was, required presentation of both antigens by the same DCs and could be overcome by injection or additional peptide pulsed DCs. They interpreted this result as supporting a model in which ongoing T cell responses deprive other T cells of access to DCs, globally suppressing other simultaneous responses. However, only a tiny fraction of injected DCs actually reach the T cell zone of the draining LN, where they represent only 1-2% of total DCs (Smith and Fazekas de St. Groth 1999). Moreover, previous work from the lab has shown that the injection of pulsed DCs can distribute Class II-MHC peptide complexes to other endogenous DCs, possibly by means of exosomes (Smith and Fazekas de St. Groth 1999) (Roediger, Chklovskaya and Fazekas de St. Groth, unpublished). Thus, the result of Kedl et al. could simply have reflected an increase in the concentration of specific peptide-MHC complexes, without any significant change in access to the DCs presenting them. Importantly, antigen non-specific competition was only detected at the lowest dose of HELMCC, when overall T cell stimulation was low, and may have been an artefact related to small variations in the antigen dose at the steep point of the dose response curve.

The data suggest competition between the T cells responding to unrelated epitopes has a negligible influence on immunodominance hierarchies, as it is highly variable and affects only the early stages of the T cell response without altering the generation of memory cells. Immunodominance hierarchies are more likely to reflect antigen processing hierarchies, rather than T cell competition. This interpretation is supported by a series of papers demonstrating the domination of the secondary response to influenza virus by the response to NP₃₆₀₋₃₇₁/D^b (Crowe et al. 2003), which is primarily due to the faster kinetics of

antigen presentation (Probst et al. 2003). This effect could be overcome by reducing the level of NP presentation (Chen et al. 2004; La Gruta et al. 2006).

Alterations in the level of antigen presentation, due to an increased number of antigenic epitopes or through the use of different recombinant vectors, may explain the variability in the level of competition seen in different vaccine studies (Fattom et al. 1999; Grilli et al. 1997; Johansson 1999; Jones et al. 2002; Manoj et al. 2003; Morris et al. 2000; Raadsma et al. 1994; Sjolander et al. 1998). Therefore ensuring there is a sufficient level of antigen presentation, either through the use of individual vectors, selecting proteins that are presented by independent MHC molecules, or expressing each epitope under the control of different promoter, may circumvent the potential effect of competition for the efficiency of multi-valent vaccines.

Chapter 5: The effect of competition on the generation of effector and memory cells

Although the predicted possible number of different T cell receptor V(D)J and $\alpha\beta$ chain combinations is 10^{15} (Davis and Bjorkman 1988), elimination of non-functional and self-reactive clones by thymic selection is believed to lead to constriction of the peripheral repertoire by a factor of 10^8 (Ashwell et al. 1986; Casrouge et al. 2000). In response to antigen, there is further repertoire restriction, with conserved $V\alpha$ and $V\beta$ usage reported between different individuals (Chen et al. 1997; Deckhut et al. 1993; Rodewald et al. 1989) and CDR3 sequences between antigen specific clones (Casanova et al. 1992; Cose et al. 1995; Lehner et al. 1995).

Restriction of the T cell repertoire in response to antigen is paralleled by an increase in the overall strength of the response. Unlike B cell affinity maturation that occurs through 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, due to the selective expansion of high affinity clones (Busch and Pamer 1999; McHeyzer-Williams et al. 1999) or loss of lower affinity cells (Fasso et al. 2000; Malherbe et al. 2004; Savage et al. 1999).

In the previous chapters, only antigen specific competition between cells of relatively high affinity was consistently demonstrated, reducing recruitment into division, proliferation and cytokine production. When observed, antigen non-specific competition induced only minor reductions in recruitment without altering the burst size of the response or the generation of memory cells. The experiments described in this chapter were designed to investigate the effect of lowering the TCR affinity on the potency of antigen specific and antigen non-specific competition. In so doing the role of competition in T cell affinity maturation was examined. For this study a situation in which a clone of uniformly high affinity T cells competes against a polyclonal population containing a mixture of affinities was modelled.

This situation would arise during a normal response to a single epitope, and should mimic the process of competition that drives affinity maturation of the CD4⁺ T cell response.

The cells of mixed affinity were derived from TCR transgenic mice expressing only the β chain of the moth cytochrome C (MCC)-specific 5C.C7 receptor (Jorgensen et al. 1992). When paired with a repertoire of endogenously rearranged TCR α chains, the 5C.C7 β chain confers reactivity for MCC-IE^k on approximately 15% of CD4⁺ T cells, as evidenced by proliferation after exposure to MCC *in vivo* (Gutgemann et al. 1998). Because a range of different α chains are used by these T cells, the overall affinity is lower than that of CD4⁺ T cells derived from transgenic mice expressing both chains of the high affinity 5C.C7 TCR (Fazekas de St. Groth et al. 1992; Seder et al. 1992). To test the effect of relatively high affinity competition directed against a different antigen, we used T cells from transgenic mice expressing both chains of the 3A9 TCR specific for hen egg lysozyme (HEL) presented on I-A^k (Ho et al. 1994). To mimic competition during a natural response to a mixture of antigens, responses to both peptides were initiated simultaneously.

In all experiments, expression of the congenic marker Ly5.1 (CD45.1) was used to distinguish readout 5C.C7 β cells from competing and host cells. In general, Ly5.1⁻ recipient mice received 1×10^6 Ly5.1⁺ CFSE labelled 5C.C7 β cells together with an equal number of unlabelled Ly5.1⁻ 5C.C7 or Ly5.1⁻ 3A9 cells (Figure 5.0). 2 days later all recipient mice were immunised with 0.1 μ g MCC peptide emulsified together with 3.2 μ g HEL peptide in CFA. At subsequent time points post immunisation, organs were harvested and analysed by flow cytometry. The total number of divided or cytokine producing 5C.C7 β cells was calculated by multiplying the frequency of 5C.C7 β cells per sample (determined by flow cytometry) by the total organ count.

5.1: T cell competition at low precursor frequencies.

To test the potency of antigen specific and non-specific competition on the response of lower affinity CD4⁺ T cells, all recipient Ly5.1⁻ recipient mice received 1×10^6 LN cells from

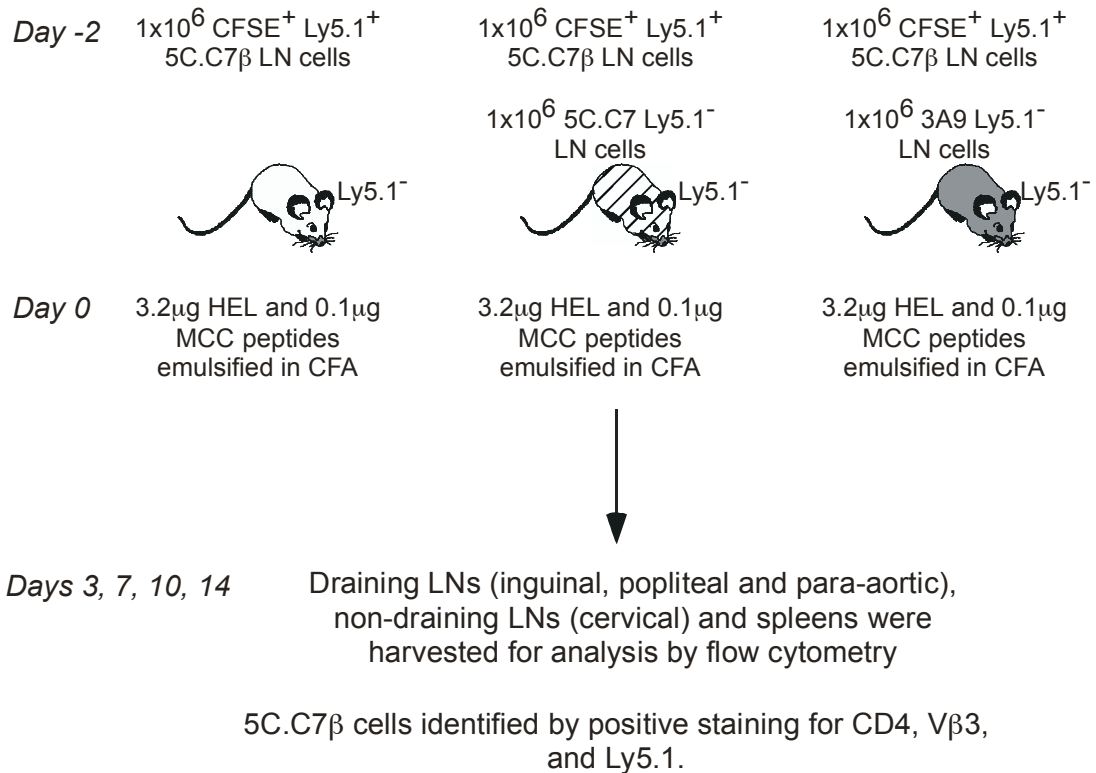


Figure 5.0: Experimental Protocol.

1×10^6 Ly5.1⁺ CFSE labelled 5C.C7 β lymph node cells were adoptively transferred into syngeneic Ly5.1⁻ host mice with 1×10^6 Ly5.1⁻ donor 5C.C7 or 3A9 lymph node cells. Two days later mice were subcutaneously immunised with a mixture of 3.2 μ g HEL peptide emulsified together with 0.1 μ g MCC peptide in Complete Freud's Adjuvant (CFA). Each mouse received a total volume of 200 μ l distributed between the hind foot-pads (50 μ l) and at the base of the tail (100 μ l). At subsequent timepoints during the response draining LNs, non-draining LNs and spleens were harvested and analysed by flow cytometry. 5C.C7 β cells were identified by positive staining for CD4, KJ25 (V β 3) and Ly5.1.

5C.C7 β TCR tg mice together with an equal number of Ly5.1⁻ 5C.C7 LN cells, 3A9 LN cells or no competing cells. 2 days later recipient mice were immunised with an emulsion containing a total of 0.1 μ g of MCC peptide together with 3.2 μ g of HEL peptide. At subsequent time points after immunisation, recipient mice were sacrificed and draining LNs and spleens were analysed by flow cytometry.

By day 3 of the response, 4% of CD4⁺ 5C.C7 β cells were recruited into division in all three experimental groups (Figure 5.1.1b), and divided 5C.C7 β cells had gone through an equal number of cell division (Figure 5.1.1c). In addition, the total number of divided 5C.C7 β cells in the draining LNs were unaffected by the presence of a competing response.

However after day 3, the expansion of 5C.C7 β cells in the draining LNs was significantly inhibited in the presence of a competing 5C.C7 response (5-fold reduction), whereas there was only a modest decrease in the presence of a 3A9 response (2-fold) (Figure 5.1.1d). Cell numbers in the spleen followed a similar pattern (Figure 5.1.1d lower panel).

To determine whether competition had affected the generation of effector cells, the cytokine-producing capacity of 5C.C7 β cells was measured by intracellular cytokine staining for IL-2 and IFN- γ after 6 hours of peptide restimulation *in vitro*. Unfortunately, with the transfer of 1×10^6 5C.C7 β cells, cell numbers at day 3 were too low to allow accurate measurement of cytokine-producing cells.

The frequency of IL-2 producing 5C.C7 β cells remained equivalent between all three groups over the course of the response (Figure 5.1.2b). At day 14, the presence of a high affinity 5C.C7 response led to a small (but not statistically significant) reduction in the percentage of IL-2 producing 5C.C7 β cells recovered from the draining LNs (Figure 5.1.2b). This was also true on days 10 and 14 in the spleen (Figure 5.1.2b). Using the data from Figure 5.1.1d, the total number of IL-2 secreting 5C.C7 β cells over the course of the response was calculated. Competition by high affinity 5C.C7 cells reduced the total number of IL-2 producing 5C.C7 β cells by around 5-fold in the draining LNs and a similar degree in the spleen (Figure 5.1.2c). In the presence of a high affinity HEL specific response, small decreases in the number of divided 5C.C7 β cells and percentage of IL-2

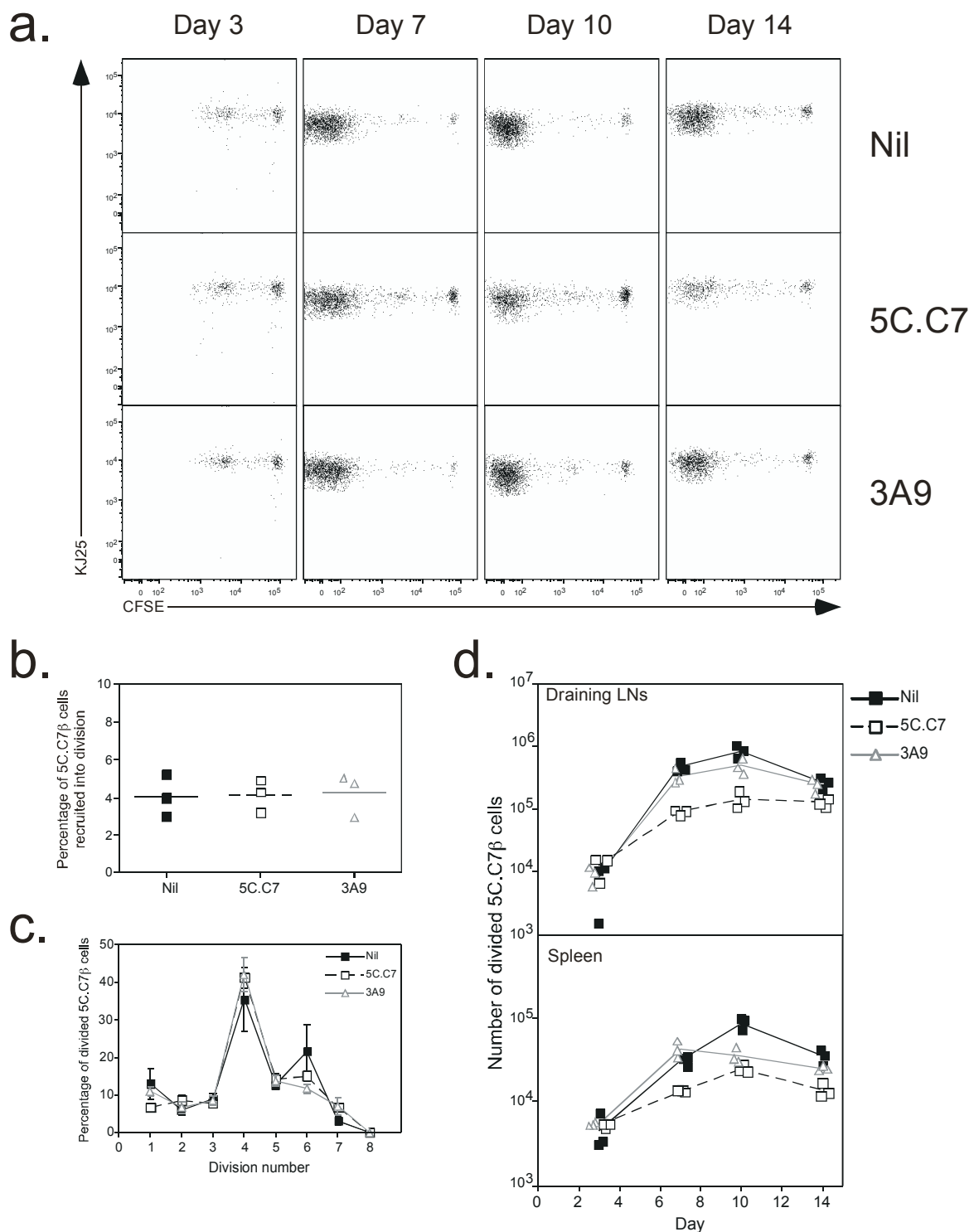


Figure 5.1.1: Division of lower affinity 5C.C7 β cells in the presence of a 5C.C7, 3A9 or no competing response

a. CFSE profiles of donor 5C.C7 β cells 3 days after adoptive transfer with equal numbers of 5C.C7 (middle panel) or 3A9 cells (bottom panel)

b. The percentage of 5C.C7 β cells recruited into division in the presence of a 5C.C7 (open squares), 3A9 (open grey triangles) or no competing response (filled black squares) calculated at day 3 of the response. Individual mice are represented as separate symbols and lines represents the mean of each group.

c. The percentage of divided 5C.C7 β cells per division number calculated at day 3. Each point represents the mean of three mice per group.

d. Kinetics of the response of 5C.C7 β cells when transferred with a equal cohort of 5C.C7, 3A9 or no competing cells. Pooled lymph nodes (upper panel) and spleens (lower panel) were removed at 3, 7, 10 and 14 days post-immunisation and the total number of divided donor 5C.C7 β cells was calculated by multiplying the percentage of 5C.C7 β cells per sample (determined by flow cytometry) by the total organ count.

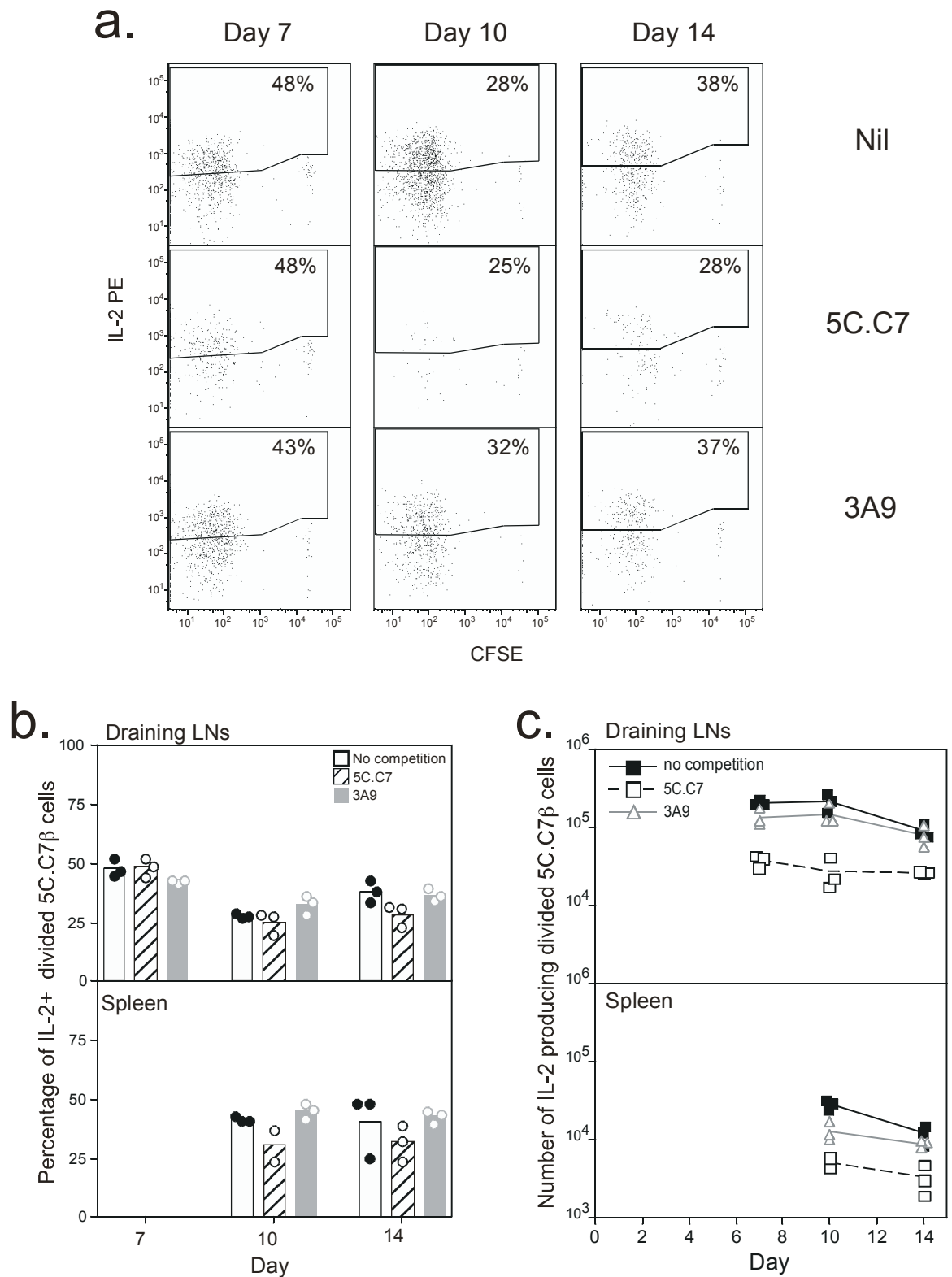


Figure 5.1.2: IL-2 production by 5C.C7 β cells

At 3, 7 and 10 days post immunisation, draining LNs and spleens were harvested and cells re-stimulated with 10 μ M MCC for 6 hours. Samples were gated for V β 3⁺, CD4⁺, Ly5⁻.

a. Dot plots show CD4 versus intracellular IL-2 of 5C.C7 β cells from the draining nodes stimulated in the absence of a competing response (top panel) or the presence of a competing 5C.C7 (middle panel) or 3A9 (bottom panel) response. The percentage of IL-2⁺ divided cells (**b.**) was measured by flow cytometry and the total number of IL-2 producing 5C.C7 β cells (**c.**) calculated by multiplying the percentage by the total organ count. Lines represent the mean of 3 mice per group with each individual mouse shown as a single point.

secreting cells, contributed to a decrease in the absolute number of IL-2 producing 5C.C7 β cells.

In the same experiment IFN- γ secretion was also measured by intracellular cytokine staining. On day 7, similar frequencies of IFN- γ -secreting 5C.C7 β cells were observed in all experimental groups (Figure 5.1.3b). By day 14, competition by high affinity 5C.C7 cells had resulted in a marked decrease in the percentage of IFN- γ -secreting 5C.C7 β cells (Figure 5.1.3b). Competition by 5C.C7 cells reduced the number of mixed affinity 5C.C7 β cells by approximately 10-fold on days 7, 10 and 14 of the response (Figure 5.1.3c). In the presence of a high affinity 3A9 response, small decreases in both the number of 5C.C7 β cells and the frequency of IFN- γ -secreting cells (Figure 5.1.3b), contributed to a decrease in the absolute number of IFN- γ -producing 5C.C7 β cells (Figure 5.1.3c).

Competition by higher affinity 5C.C7 or 3A9 cells did not alter recruitment into division nor division profiles at day 3 of the response. Competition did, however, decrease the generation of effector cells as measured by the total number of IFN- γ producing 5C.C7 β cells. The total number of effector cells in the presence of either a 5C.C7 or 3A9 response was a reflection of a reduced percentage of cytokine producing cells together with reduction in the total number of divided cells.

5.2: Competition at high precursor frequencies.

To measure the potency of competition on the early stages of the response, the number of transferred cells was increased from 1×10^6 to 1×10^7 and the dose of antigens increased accordingly. 4 days after the transfer of 1×10^7 CFSE labelled Ly5.1⁻ 5C.C7 β cells together with an equal number of Ly5.1⁺ 5C.C7 or Ly5.1⁻ 3A9 cells, all recipient mice received a total of 10 μ g of MCC peptide together with 32 μ g of HEL peptide emulsified together in CFA.

Consistent with the previous experiment, 5% of 5C.C7 β cells were recruited into cell division in all three groups (Figure 5.2.1a) and in the presence of a 3A9 or 5C.C7

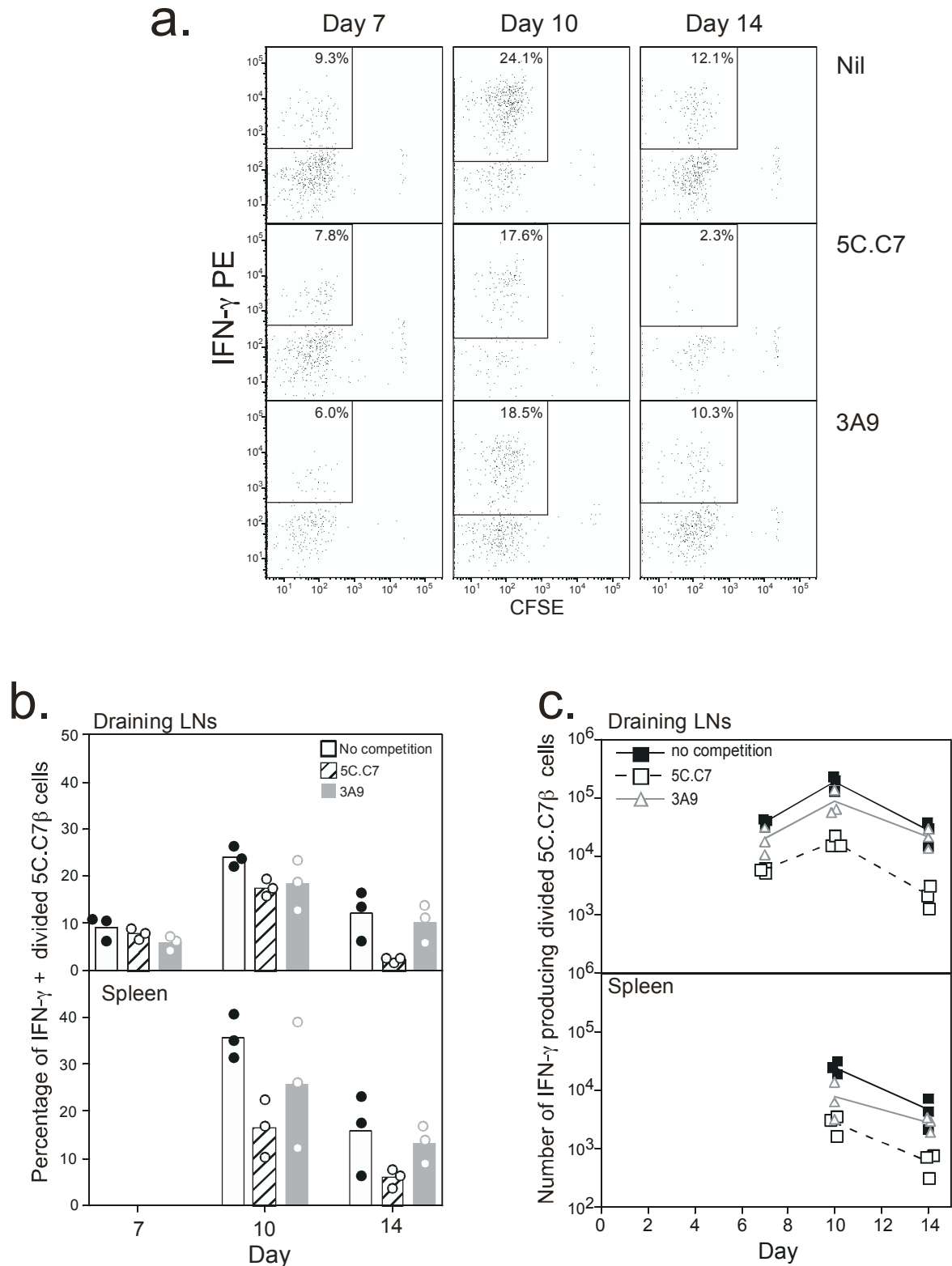


Figure 5.1.3: IFN- γ secretion by divided 5C.C7 β cells

At 3, 7 and 10 days post immunisation, draining LNs and spleens were harvested and cells re-stimulated *ex vivo* with 10 μ M MCC for 6 hours. Samples were gated for V β 3⁺, CD4⁺, Ly5⁺.

a. Dot plots show CD4 versus intracellular IFN- γ of 5C.C7 β cells stimulated in the absence of a competing response (top panel) or the presence of a competing 5C.C7 (middle panel) or 3A9 (bottom panel) response. The percentage of IFN- γ ⁺ divided cells (**b.**) was measured by flow cytometry and the total number of IFN- γ producing 5C.C7 β cells (**c.**) calculated by multiplying the percentage by the total organ count. Lines represent the mean of 3 mice per group with each individual mouse shown as a single point.

competing response, divided 5C.C7 β cells had progressed through one less round of cell division (Figure 5.2.1b). This did not alter the total number of divided 5C.C7 β cells in either the draining LNs or spleens at day 3 (Figure 5.2.1d). In the presence of a competing high affinity 5C.C7 response, a 10-fold and 5-fold reduction in the total number of 5C.C7 β cells in the draining LNs and spleen respectively was observed at day 7. Competition by 3A9 cells had no effect on the total number of divided 5C.C7 β cells (Figure 5.2.1d).

In a separate experiment, the effector function of 5C.C7 β cells was measured by intracellular cytokine staining for IL-2 and IFN- γ . With an increase in the number of cells transferred from 1×10^6 to 1×10^7 , cytokine production was detectable as early as day 3. In the presence of a competing 5C.C7 response, a reduced frequency of IL-2 $^+$ 5C.C7 β cells was observed at day 10 in the draining LNs, and day 10 and 14 in the spleen (Figure 5.2.2b). Consistent with the previous experiment, small decreases in the absolute number of 5C.C7 β cells together with reduced frequencies of IL-2 producing cells contributed to the overall reduction in the total number of IL-2 producing 5C.C7 β cells generated in the presence of a simultaneous 5C.C7 response (Figure 5.2.2b). Competition by 3A9 cells led to a reduction in the frequency of IL-2 $^+$ 5C.C7 β cells which was most apparent in the spleen (Figure 5.2.2b). Yet overall the total number of IL-2 producing 5C.C7 β cells was not affected by the presence of a competing 3A9 response (Figure 5.2.2c).

Although the frequency of IFN- γ $^+$ 5C.C7 β cells was initially enhanced at day 3 in the presence of a competing 3A9 or 5C.C7 response, there was no difference in the total number of effector cells between the three groups of mice at this early timepoint (Figure 5.2.3c). Consistent with previous data, competition by 5C.C7 cells reduced both the frequency (Figure 5.2.3b) and absolute number of IFN- γ producing 5C.C7 β cells (Figure 5.2.3c). A similar reduction was also seen in the presence of a 3A9 response, but was less marked than the effect of antigen specific competition.

In summary, increasing the number of transferred cells from 1×10^6 to 1×10^7 resulted in similar kinetics of competition and enabled the detection of cytokine production as early

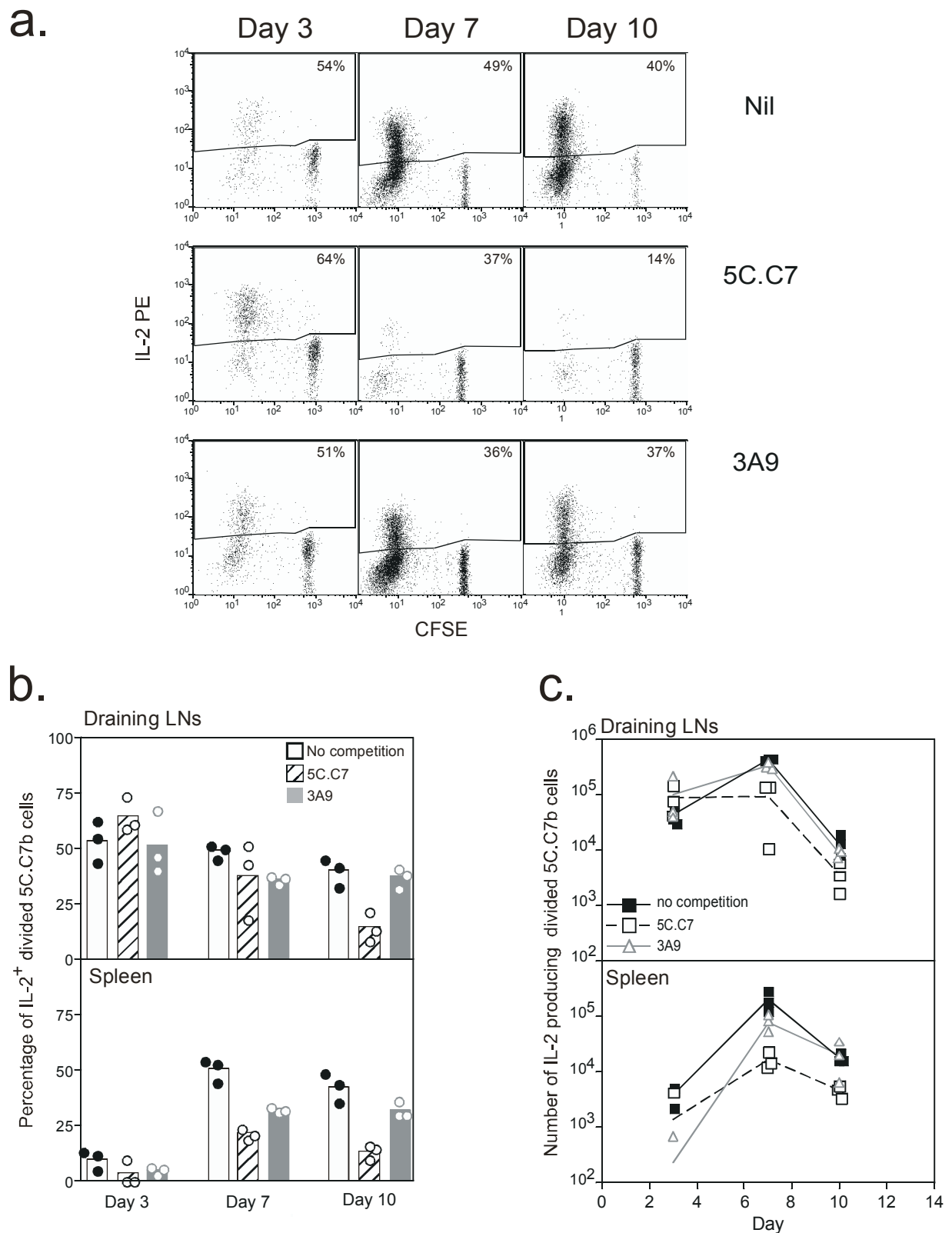


Figure 5.2.2: IL-2 secretion by divided 5C.C7 β cells

At 3, 7 and 10 days post immunisation, draining LNs and spleens were harvested and cells re-stimulated *ex vivo* with 10 μ M MCC for 6 hours. Samples were gated for V β 3⁺, CD4⁺, Ly5⁻.

a. Dot plots show CFSE versus intracellular IL-2 of 5C.C7 β cells stimulated in the absence of a competing response (top panel) or the presence of a competing 5C.C7 (middle panel) or 3A9 (bottom panel) response. The percentage of IL-2⁺ 5C.C7 β divided cells (**b.**) was determined by flow cytometry and the total number of IL-2 producing 5C.C7 β cells (**c.**) calculated by multiplying the percentage by the total organ count. Line represent the mean of 3 mice per group with each individual mouse shown as a single point.

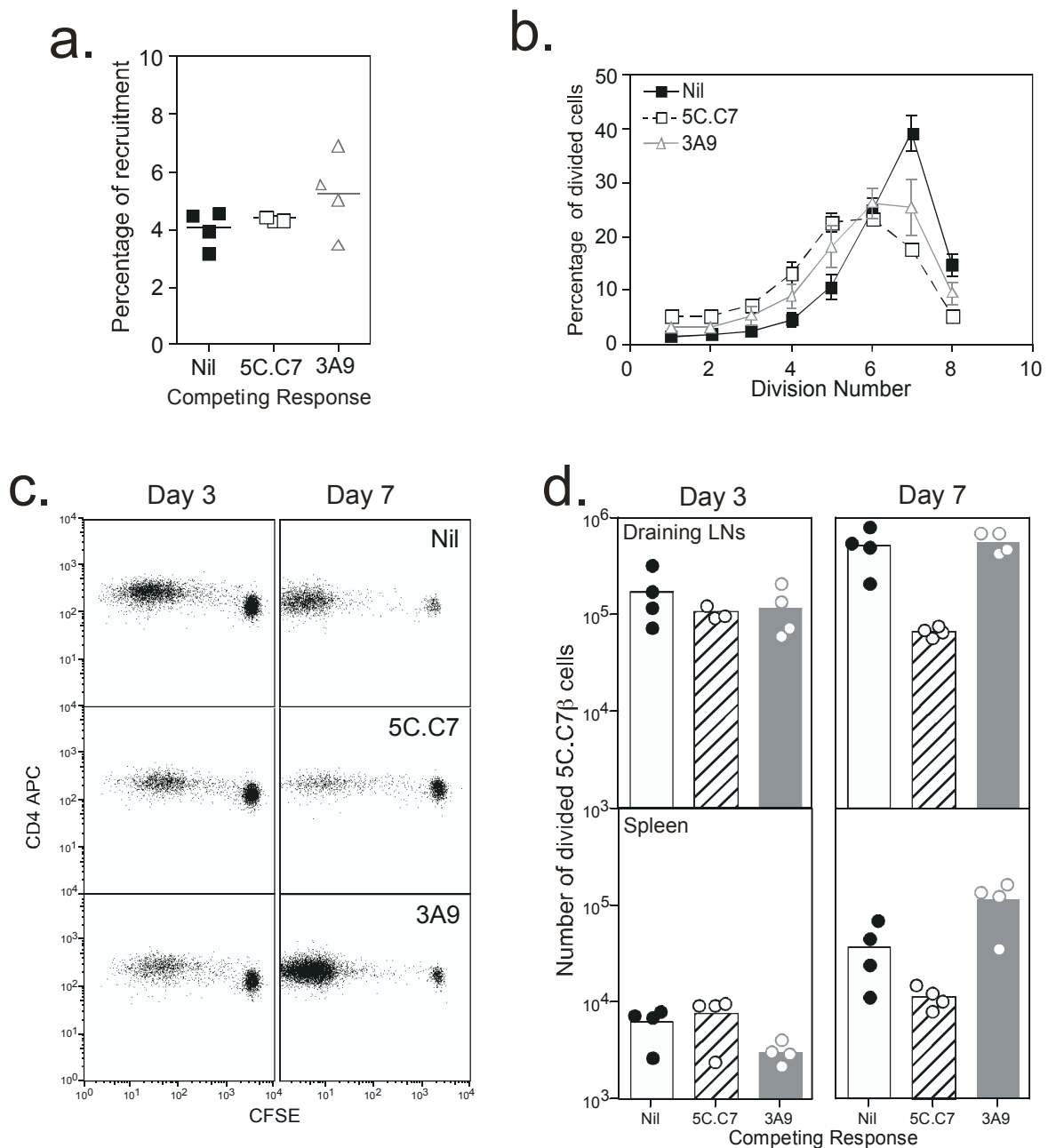


Figure 5.2.1: At high precursor frequencies competition is not detected at day3 post-immunization

1×10^7 CFSE labelled Ly5.1⁻ 5C.C7β lymph node cells were adoptively transferred with 1×10^7 Ly5.1⁺ 5C.C7 or 1×10^7 Ly5.1⁻ 3A9 lymph node cells into Ly5.1⁻ recipient mice. 4 days later mice were immunised with a total of 10μg MCC and 32μg HEL peptides emulsified together in CFA and organs harvested 3 and 7 days later for analysis by flow cytometry.

a. The percentage of 5C.C7β cells recruited into division was calculated at day 3 of the response.

b. The percentage of divided 5C.C7β cells per division when stimulated in the absence of a competing response (black filled) or in the presence of a competing 5C.C7 (black open) or 3A9 (grey filled) response.

c. CFSE profiles of 5C.C7β cells stimulated in the absence of a competing response (top panel) or in the presence of a competing 5C.C7 (middle panel) or 3A9 (bottom panel) response.

d. At day 3 (left panels) and day 7 (right panels) the number of divided 5C.C7β cells recovered from the draining LNs (top panel) or spleen (bottom panel) in the absence of a competing response (black filled) or in the presence of a competing 5C.C7 (black open) or 3A9 (grey open) response was calculated. Bars represent the mean of 4 mice per group with each individual mouse shown as a single point.

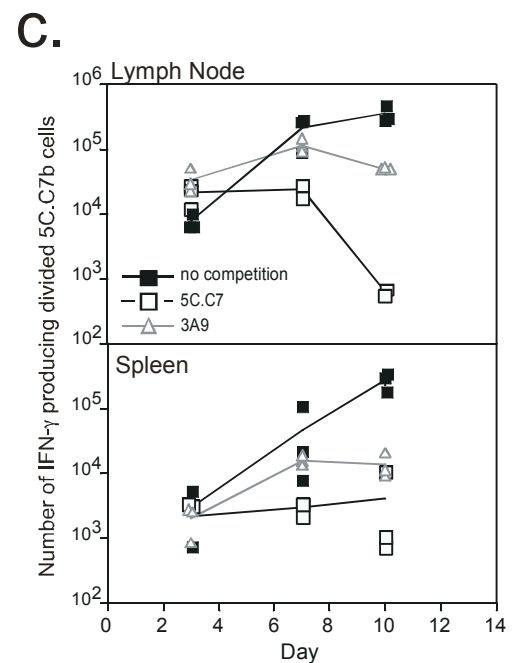
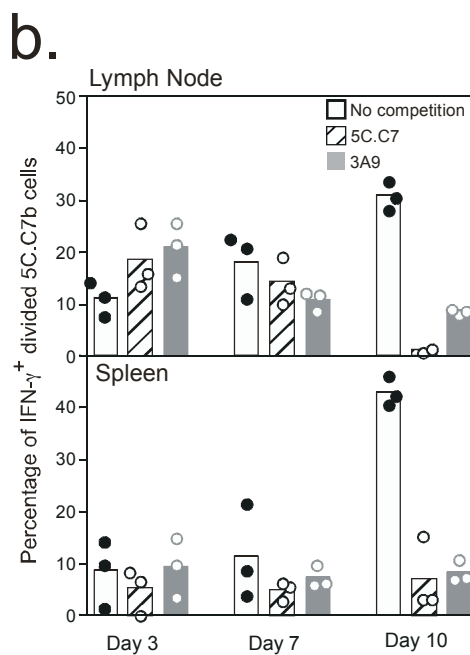
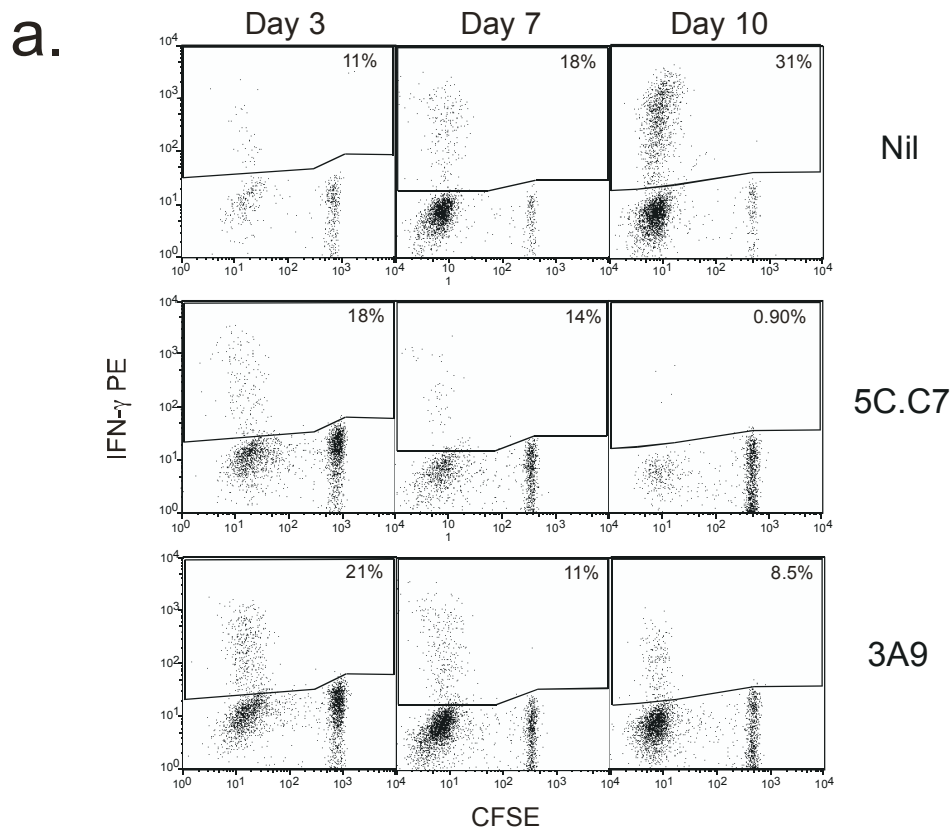


Figure 5.2.3: IFN- γ secretion by divided 5C.C7 β cells

At 3, 7 and 10 days post immunization, draining lymph nodes and spleens were harvested and cells re-stimulated *ex vivo* with 10 μ M MCC for 6 hours. Samples were gated for V β 3 $^{+}$, CD4 $^{+}$, Ly5 $^{-}$.

a. Dot plots show CFSE versus IFN- γ intracellular of 5C.C7 β cells stimulated in the absence of a competing response (top panel) or the presence of a competing 5C.C7 (middle panel) or 3A9 (bottom panel) response. The percentage of IFN- γ $^{+}$ 5C.C7 β divided cells (**b.**) was determined by flow cytometry and the total number of IFN- γ producing 5C.C7 β cells (**c.**) calculated by multiplying the percentage by the total organ count. Lines represent the mean of 3 mice per group with each individual mouse shown as a single point.

as day 3 of the response. At this timepoint, both antigen specific and antigen non-specific competition reduced proliferation and IFN- γ production although the percentage of cells recruited into division was unaltered.

5.3: The effect of competition as measured by the expression of activation markers

The experiments described above suggested that competition manifested relatively late in the response. To test whether changes in the expression of activation markers or interleukin receptors preceded changes in cell number and cytokine production, the cell surface characteristics of 5C.C7 β cells were examined at day 3 of the response. 1×10^7 CFSE labelled Ly5.1 $^-$ 5C.C7 β cells were adoptively transferred into Ly5.1 $^+$ recipient mice together with an equal number of competing Ly5.1 $^+$ 5C.C7 or Ly5.1 $^+$ 3A9 cells, 6 hours prior to immunisation with 10 μ g MCC and 32 μ g HEL peptides in CFA. 3 days later draining LNs were analysed for the expression of a panel of activation markers. CD5 and CD69 expression was also investigated in this study with no difference seen between the three groups.

In response to antigen, 5C.C7 β cells recruited into division upregulated the level of surface CD4 (Figure 5.3.1a) and CD44 expression (Figure 5.3.1c) as indicated by higher level of expression seen on divided versus undivided 5C.C7 β cells. An increase in expression of costimulatory ligand Ox-40 (CD134) (Figure 5.3.1e) was also observed in response to antigen. In the presence of a competing 5C.C7 response there was a reduced level of CD4, CD44 and CD134 expression. The presence of a competing 3A9 response did not, however, alter the expression of any of these activation markers.

In the same experiment the expression of a number of cytokine receptors was also examined. Antigen activated and divided 5C.C7 β cells upregulated the surface expression of IL-2R α chain (CD25) (GMFI of 195 on divided cells compared to GMFI of 25 on

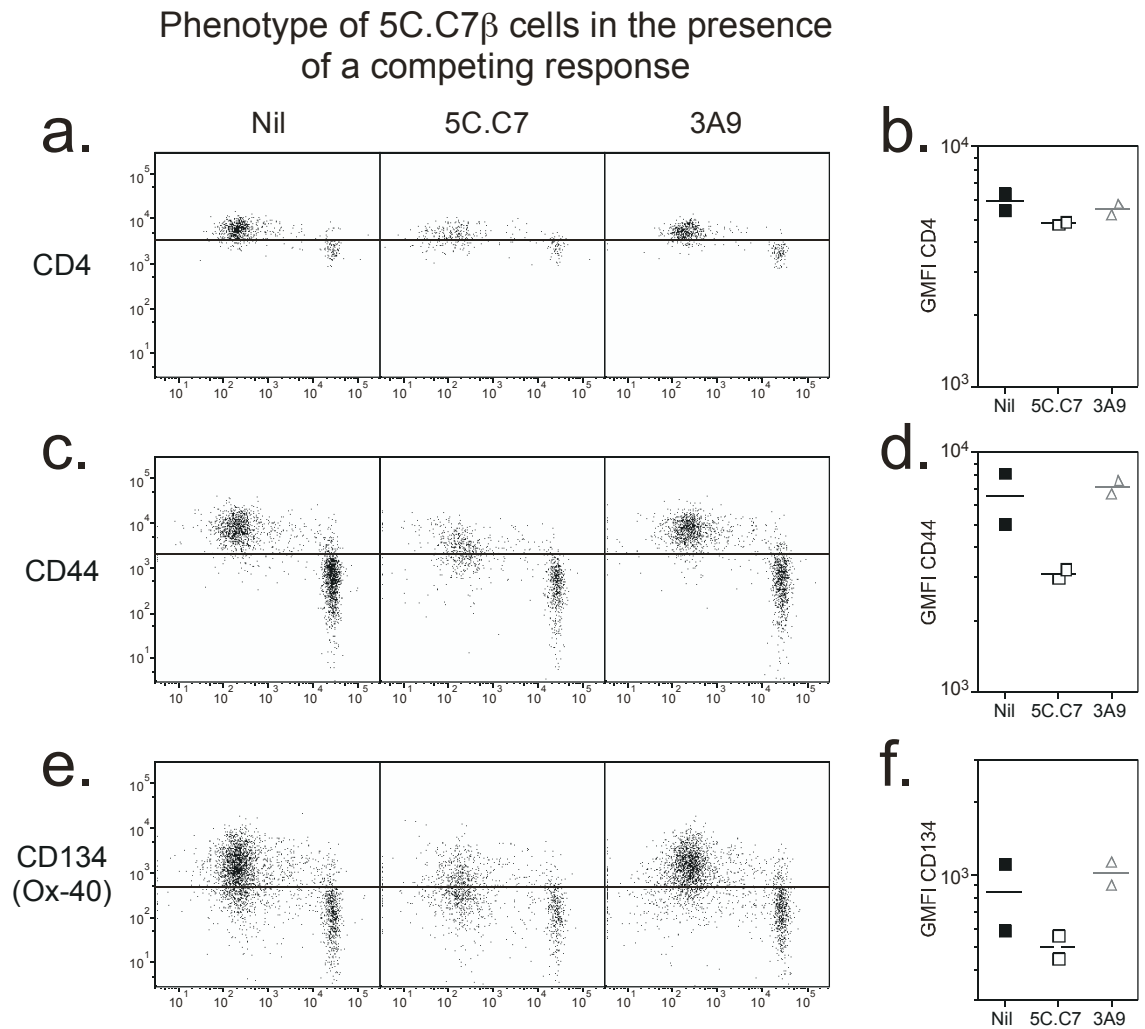


Figure 5.3.1: Expression of activation markers CD4, CD44 and CD134 (Ox-40).

1×10^7 donor CFSE⁺ Ly5.1⁻ 5C.C7 β cells and 1×10^7 competing Ly5.1⁺ 5C.C7 or Ly5.1⁺ 3A9 cells were adoptively transferred into Ly5.1⁺ host mice 6 hours prior to immunisation with 10 μ g MCC and 32 μ g HEL peptides emulsified together in CFA. 3 days later, the expression of CD4, CD44 and CD134 on 5C.C7 β cells recovered from the draining LNs was analyzed by flow cytometry. 5C.C7 β cells were identified by positive staining for CD4, V β 3 and negative for Ly5.1. Dot plots demonstrate the expression of CD4 (**a.**), CD44 (**c.**) or CD134 (**e.**) versus CFSE of 5C.C7 β cells in the absence of a competing response or in the presence of a competing 5C.C7 (middle panel) or 3A9 (right panel). Graphs represent the GMFI of divided 5C.C7 β cells for CD4 (**b.**), CD44 (**d.**) and CD134 (**f.**).

undivided cells), IL-2R β chain (CD122) (GMFI 380 on divided cells compared to 117 on undivided cells) and IL-R common γ chain (CD132) (GMFI 340 on divided cells compared to on 114 undivided cells) (Figure 5.3.2). Activated 5C.C7 β cells downregulated expression of IL-7R (CD127) by day 3 (Figure 5.3.2c). In the presence of a competing 5C.C7 response, divided 5C.C7 β cells expressed lower levels of all chains of the IL-2R with no significant difference in the level IL-7R downregulation. Competition by 3A9 cells did not alter the level of expression of any of the cytokine receptors investigated. Taken together these data demonstrate that competition by 5C.C7 cells led to an overall reduction in the stimulation of 5C.C7 β cells as early as day 3 of the response, as indicated by reduced upregulation CD4, CD44, reduced expression of co-stimulatory receptor Ox-40 (CD134) and reduced expression of all three chains of the IL-2R (CD25, CD122 and CD132).

5.4: The effect of blocking apoptosis pathways on antigen specific competition

In an *in vitro* model, Gett and colleagues showed that reduced anti-CD3-mediated stimulation increased susceptibility to apoptosis via decreased expression of the anti-apoptotic molecule Bcl-x_L (Gett et al. 2003). This suggested that increased apoptosis might account for the reduced number of mixed affinity T cells in the presence of a high affinity response to the same peptide-MHC complex, particularly as reduced expression of activation markers on day 3 of the response suggested an early reduction in TCR-dependent stimulation. To test this model, 5C.C7 β transgenic mice were crossed with 2 strains of transgenic mice in which apoptosis of T cells is inhibited: the Bcl-2 strain overexpressing Bcl-2 (Strasser et al. 1991), an anti-apoptotic molecule of the BH3-only family, and the CrmA strain expressing cowpox virus-encoded protein CrmA (Smith et al. 1996), shown to prevent Fas-mediated apoptosis *in vitro*. In both of the experiments

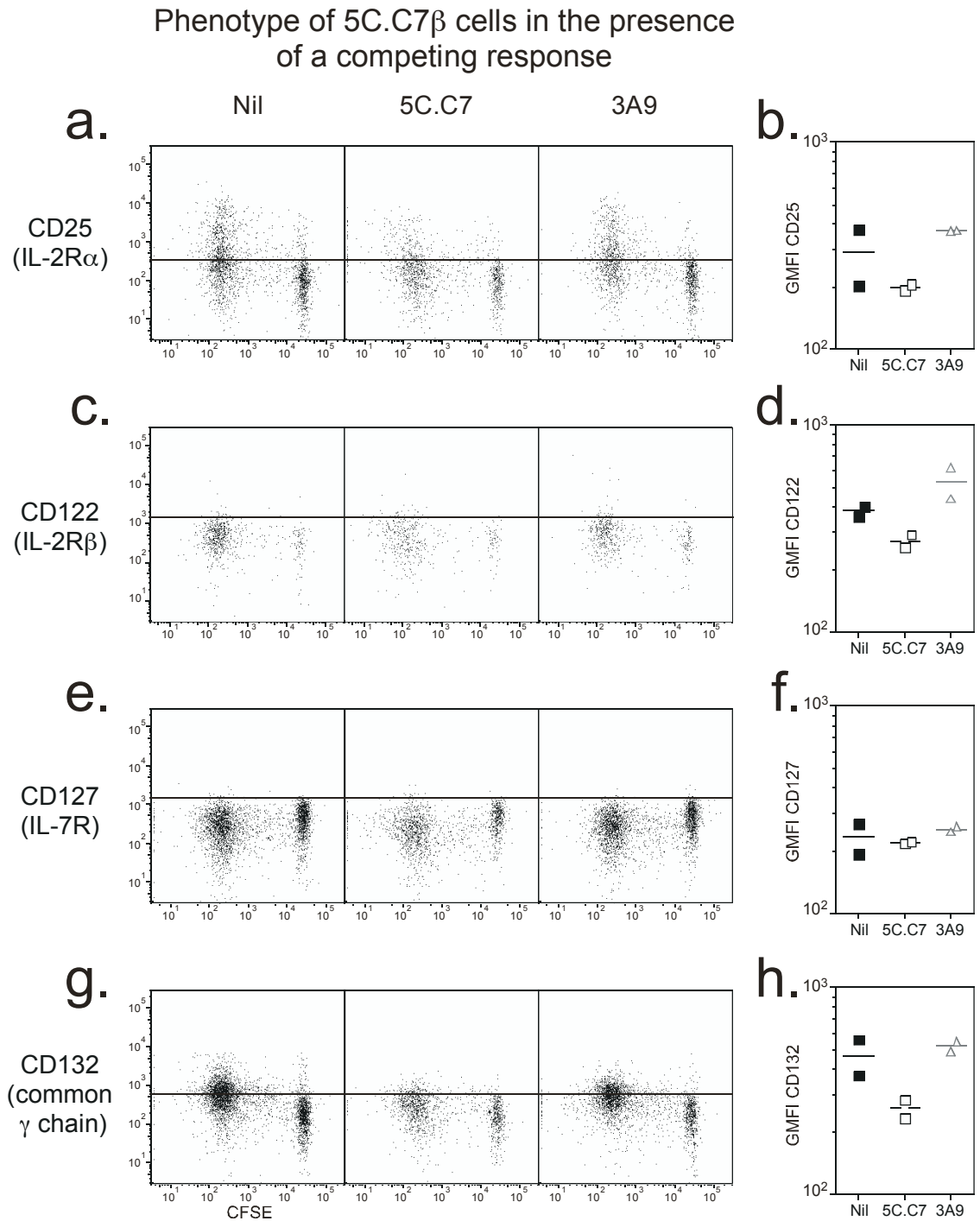


Figure 5.3.2: Cytokine Receptors IL-2R α (CD25), IL-2R β (CD122), IL-7 (CD127) and the IL-2, IL-4, IL-7, IL-9 and IL-15 common γ chain (CD132).

1x10⁷ donor CFSE⁺ Ly5.1⁺ 5C.C7 β cells and 1x10⁷ competing Ly5.1⁺ 5C.C7 or Ly5.1⁺ 3A9 cells were adoptively transferred into Ly5.1⁺ host mice 6 hours prior to immunisation with 10 μ g MCC and 32 μ g HEL peptides emulsified together in CFA. 3 days later, the expression of CD25, CD122, CD127 and CD132 on 5C.C7 β cells recovered from the draining LNs was analyzed by flow cytometry. 5C.C7 β cells were identified by positive staining for CD4, V β 3 and negative for Ly5.1.

Dot plots demonstrate the expression of CD25 (**a.**), CD122 (**c.**), CD127 (**e.**) or CD132 (**g.**) versus CFSE of 5C.C7 β cells in the absence of a competing response or in the presence of a competing 5C.C7 (middle panel) or 3A9 (right panel). Graphs represent the GMFI of divided 5C.C7 β cells for CD25 (**b.**), CD122 (**d.**), CD127 (**f.**) and CD132 (**h.**).

described below, the percentage of CD4⁺ cells within each fraction of donor LN cells was determined by flow cytometry and the number of cells transferred adjusted accordingly to ensure equivalent number of CD4⁺ cells were transferred.

Recipient mice received either 1×10^7 CD4⁺ 5C.C7 β or 5C.C7 β Bcl-2⁺ LN cells with or without 1×10^7 CD4⁺ 5C.C7 cells. 2 days later, recipient mice were immunised with 10 μ g of MCC peptide in CFA, and a further 10 days later, draining LNs and spleens harvested and the total number of either 5C.C7 β or 5C.C7 β Bcl-2⁺ cells determined by flow cytometry. In the presence of a competing 5C.C7 response, a 10-fold reduction in both the number of 5C.C7 β and 5C.C7 β Bcl-2⁺ divided cells was observed in the draining LNs (Figure 5.4.1a upper panel) with only a 5-fold reduction seen in the spleen (Figure 5.4.1a lower panel). There was no difference in the total number of undivided 5C.C7 β or 5C.C7 β Bcl-2⁺ cell (Figure 5.4.1b), indicating equal numbers of CD4⁺ were originally transferred and recruitment into division was equivalent between all four groups.

In a separate experiment 2.5×10^6 CD4⁺ 5C.C7 β or 5C.C7 β CrmA⁺ cells were adoptively transferred into recipient mice with or without an equal number of competing 5C.C7 cells. 2 days later recipient mice were immunised with 10 μ g MCC in CFA, and draining LNs and spleens were harvested on day 10. In the presence of a competing 5C.C7 response, a 5-fold reduction in the total number of divided 5C.C7 β cells was observed in both the draining LNs and spleens at day 10 of the response (Figure 5.4.2a). Expression of CrmA did not change the kinetics of competition as a 5-fold decrease in the number of divided 5C.C7 β CrmA⁺ cells was also seen in the presence of a competing 5C.C7 response (Figure 5.4.2a).

In summary, both of these experiments demonstrated that the reduction in the total number of divided 5C.C7 β cells seen in the presence of a competing 5C.C7 response was not due to apoptosis of 5C.C7 β cells via either the mitochondrial or Fas-FasL induced cell death pathways.

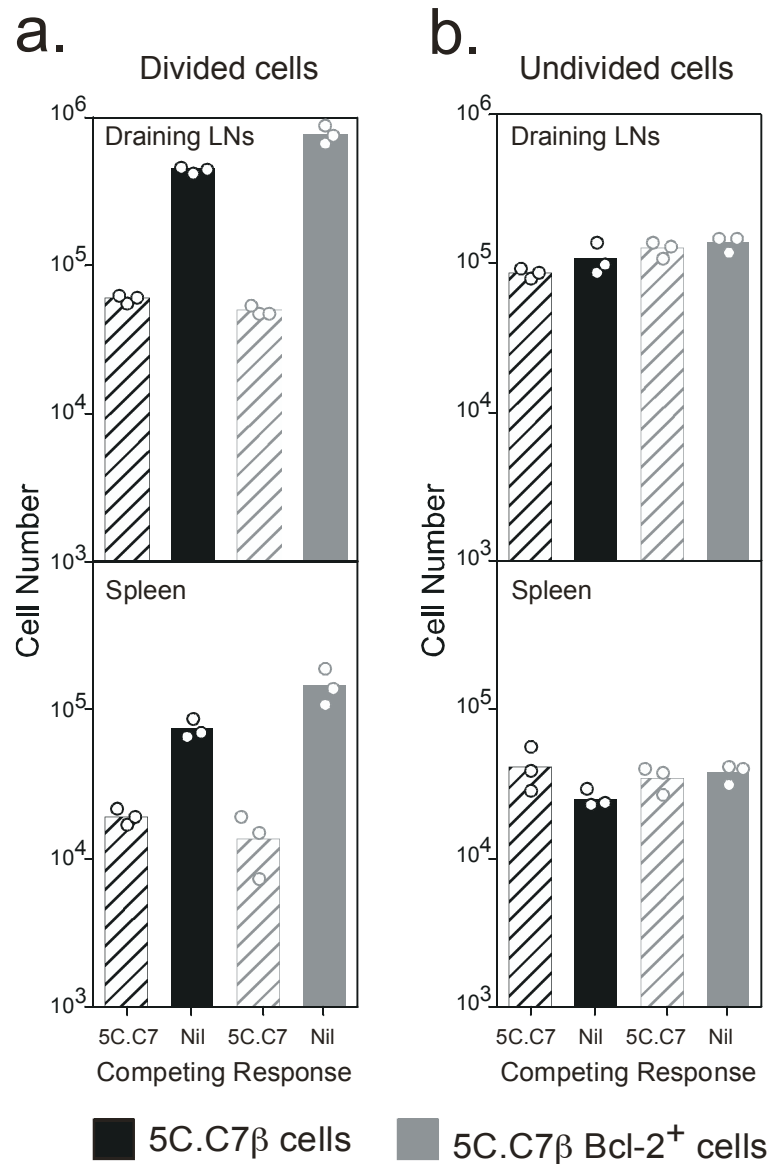


Figure 5.4.1: 5C.C7β and 5C.C7β.Bcl-2⁺ cell numbers at day 10 of the response

1 × 10⁷ CD4⁺ CFSE labelled 5C.C7β or 5C.C7β.Bcl-2⁺ LN cells were adoptively transferred with or without equal numbers of competing 5C.C7 Ly5.1⁺ cells into Ly5.1⁺ recipient mice. 2 days later mice were immunised with 10 μg of MCC peptide emulsified in CFA and 10 days later draining LNs (top panels) and spleens (lower panels) were harvested for analysis by flow cytometry. Graphs represent the total number of divided (**a.**) and undivided (**b.**) 5C.C7β (black) and 5C.C7β.Bcl-2⁺ (grey) cells when cells were stimulated with (striped) or without (filled) a competing 5C.C7 response. Bars represent the mean of 3 mice per group with each individual mouse shown as a single point.

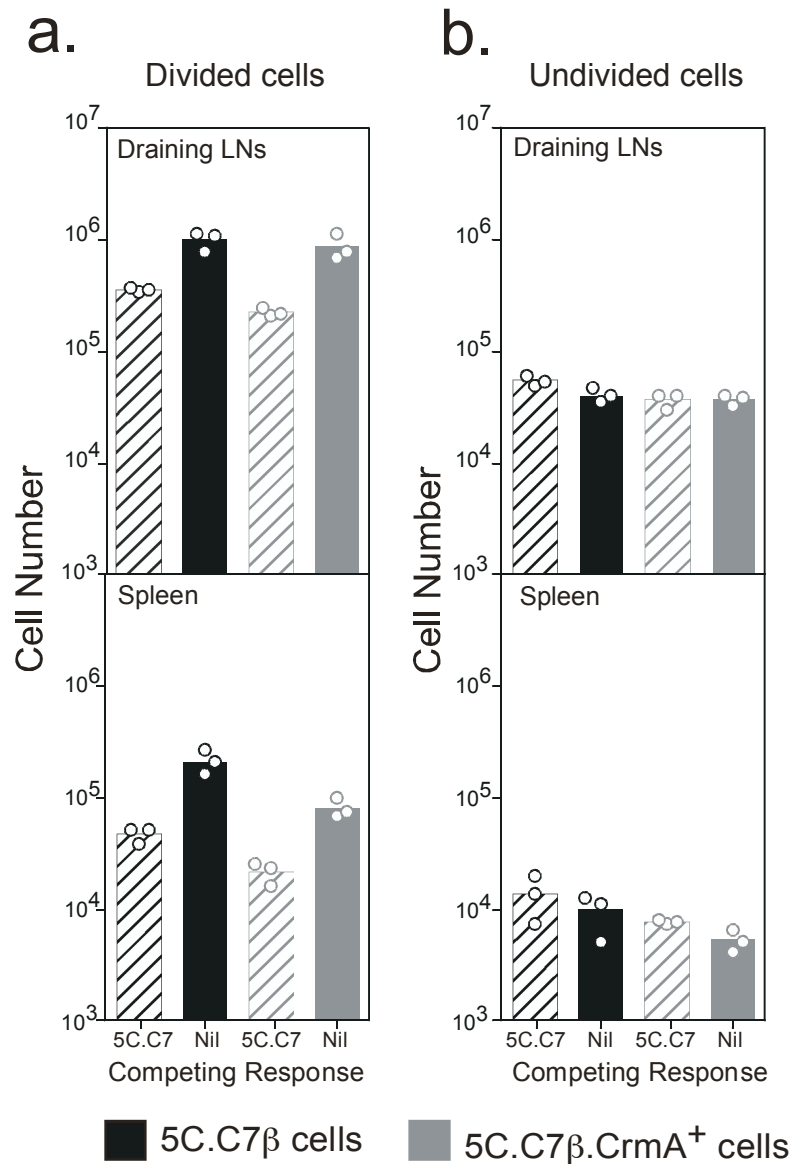


Figure 5.4.2: 5C.C7β and 5C.C7βCrmA⁺ cell numbers at day 10 of the response

2.5×10^6 CD4⁺ 5C.C7β or 5C.C7βCrmA⁺ lymph node cells were adoptively transferred with or without equal numbers of competing 5C.C7 cells. 2 days later mice were immunised with 10μg of MCC peptide emulsified in CFA 10 days later draining LNs (top panels) and spleens (lower panels) were harvested for analysis by flow cytometry. Graphs represent the total number of divided (**a.**) and undivided (**b.**) 5C.C7β (black) and 5C.C7βCrmA⁺ (grey) cells present when cells were stimulated with (striped) or without (filled) a competing 5C.C7 response. Bars represent the mean of 3 mice per group with each individual mouse shown as a single point.

5.5: The effect of competition on the proliferative capacity of

5C.C7 β cells

Since the previous experiment indicated that the effect of high affinity T cell competition was not mediated via an increase in apoptosis, BrdU uptake was used to test whether a reduction in cell division was involved. BrdU is a brominated analogue of thymidine that is selectively incorporated into a cell's DNA during the S phase of cell cycling. Incorporation of BrdU can be subsequently measured by intracellular staining with a monoclonal antibody against BrdU. While it does not provide an indication of the speed of cell division, it indicates the percentage of cells within a population that have entered the S phase of division during the pulse period, and thereby can be used to determine the number of cells dividing. In the same experiment as described in section 5.3, 1×10^7 CFSE labelled 5C.C7 β cells were adoptively transferred into recipient mice with an equal number of competing 5C.C7 or 3A9 cells and 6 hours later recipient mice were immunised with 10 μ g MCC and 32 μ g HEL in CFA. On day 3 of the response, recipient mice received 2mg of BrdU (via intraperitoneal injection) or an equal volume of PBS and 6 hours later mice were sacrificed for analysis of BrdU incorporation by flow cytometry.

In the control group, approximately 70% of 5C.C7 β cells had incorporated BrdU in the 6 hours pulsing period (Figure 5.5b). A similar percentage of BrdU uptake was observed when 5C.C7 β cells were stimulated in the presence of a competing 3A9 response (Figure 5.5b), while competition by 5C.C7 cells reduced BrdU incorporation by 15%. In the non-draining LNs there was no difference in the percentage of BrdU incorporation (Figure 5.5d).

Together with the apoptosis data, these experiments demonstrated that the reduction in the number of divided 5C.C7 β cells observed in the presence of a competing high affinity 5C.C7 response was due to a reduction in cell division rather than an increase in apoptosis.

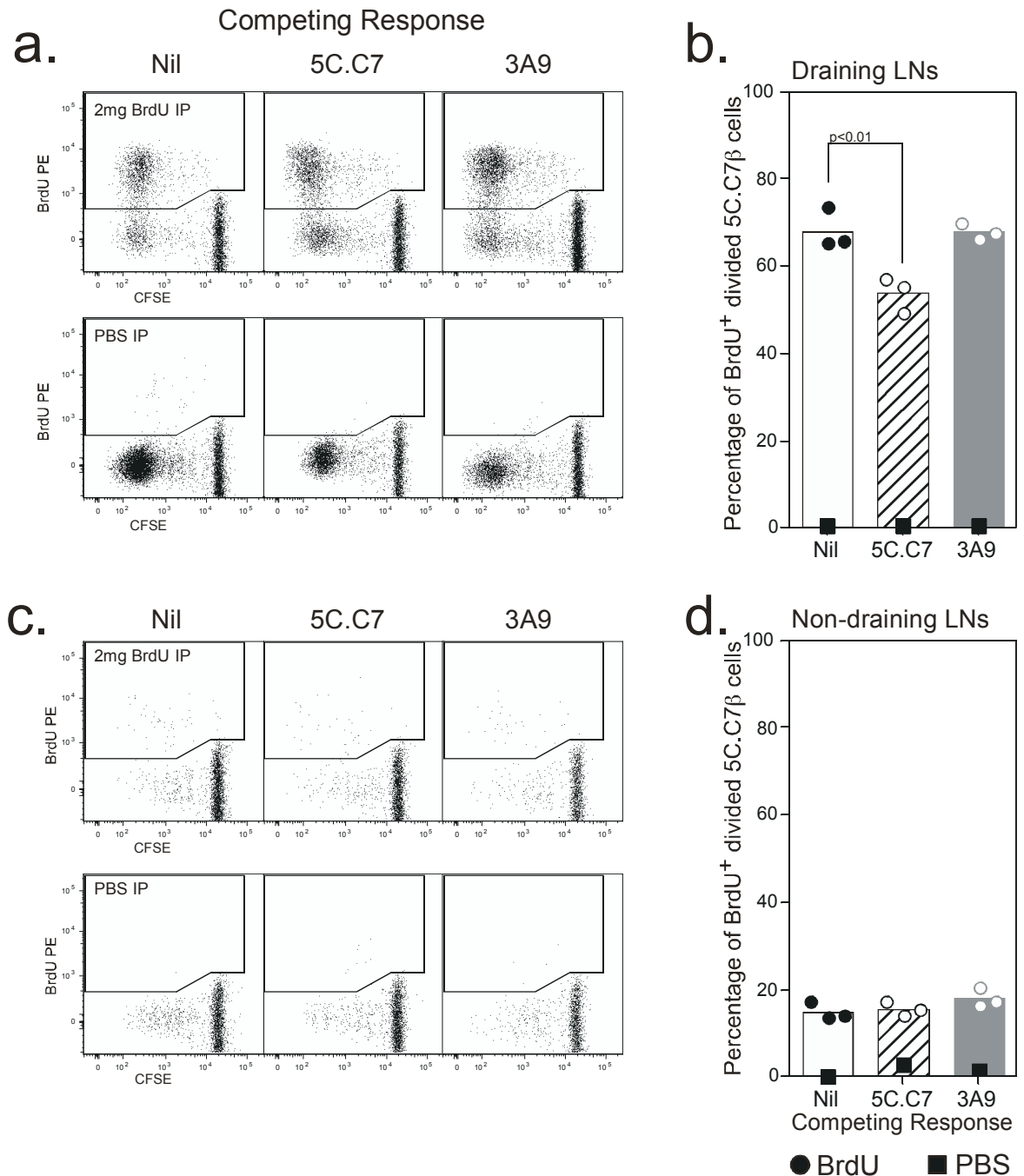


Figure 5.5: BrdU incorporation by 5C.C7 β cells at day 3 of the response.

1×10^7 donor CFSE labelled 5C.C7 β cells were transferred with an equal number of competing 5C.C7 or 3A9 cells 6 hours prior to immunisation with $10 \mu\text{g}$ MCC and $32 \mu\text{g}$ HEL together in CFA. On day 3 of the response, mice received an intraperitoneal injection of 2mg BrdU or an equal volume of PBS. 6 hours later draining LNs and non-draining LNs were harvested to measure the percentage of BrdU incorporation by flow cytometry. (a, c) Plots show one representative mouse per group. (b, d) Graphs represent the percentage of BrdU $^+$ divided 5C.C7 β cells when pulsed with either BrdU (circles) or PBS (squares). Bars represent the mean of 3 mice per group with individual mice represented as single points.

5.6: The effect of competition on the generation of memory cells.

If T cell affinity maturation occurred through a competition dependent mechanism, then one would expect that the presence of high affinity 5C.C7 cells would reduce the generation of memory cells derived from the mixed affinity 5C.C7 β cells. Therefore the number and cytokine-producing capacity of memory 5C.C7 β cells 8 weeks after primary stimulation in the presence of a competing high affinity 5C.C7 or 3A9 response was measured.

1×10^6 CFSE labelled Ly5.1 $^-$ 5C.C7 β cells were adoptively transferred with an equal number of Ly5 $^+$ 3A9 or Ly5 $^+$ 5C.C7 cells into Ly5.1 $^+$ recipient mice. 3 days later, recipient mice were immunised with 0.1 μ g MCC and 3.2 μ g HEL peptides in CFA. 8 weeks after immunisation, the total number of memory 5C.C7 β cells (CD44 ^{hi}) residing in draining LNs and spleens were calculated by flow cytometry. LNs and spleens cells were also restimulated *ex vivo* to determine the cytokine producing capacity of these long-lived memory cells.

In the absence of a competing response, greater than 10^4 CFSE ^{lo} CD44 ^{hi} 5C.C7 β cells were recovered from the draining LNs of mice (Figure 5.6.1b). The presence of a competing 3A9 response did not alter the generation 5C.C7 β memory cells, as indicated by equal numbers of CD44 ^{hi} 5C.C7 β cells recovered from both the draining LNs and spleens. The presence of a competing MCC-specific response however, led to a 2-fold and 4-fold reduction in the number of divided 5C.C7 β memory cells recovered from the draining LNs and spleen respectively (Figure 5.6.1).

The frequency of IFN- γ -producing cells within the divided CD44 ^{hi} population decreased from 7% to less than 1% in the face of 5C.C7 competition (Figure 5.6.1), leading to a 10-fold reduction in the number of 5C.C7 β memory cells capable of producing IFN- γ at week 8 (Figure 5.6.1f, g, h). Competition by 3A9 cells had no effect on the frequency or number of IFN- γ -producing cells (Figure 5.6f, g, h). The frequency of IL-2-producing cells was more variable than that of IFN- γ -producing cells (Figure 5.6.1c, d, e), so that the 2-fold

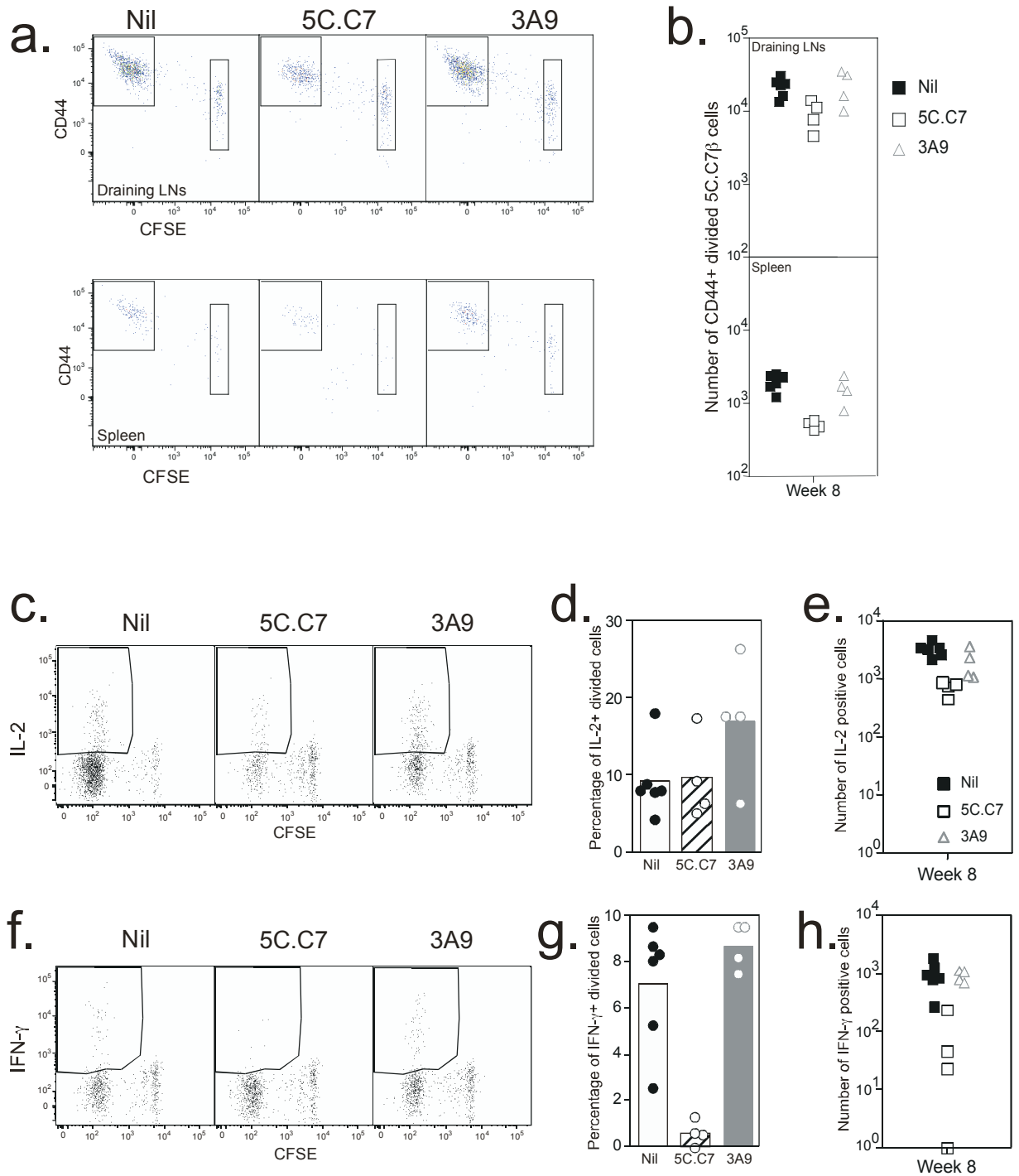


Figure 5.6.1: 5C.C7β memory cells 8 weeks post-immunization

Recipient mice received 1×10^6 CFSE labelled 5C.C7β cells with an equal number of 5C.C7 or 3A9 cells. Mice were immunised with 0.1μg MCC and 3.2μg HEL peptides and 8 weeks later draining LNs and spleen harvested for analysis by flow cytometry. **a.** CD44 expression of 5C.C7β cells isolated from the draining LNs (top panel) and spleens (bottom panels) when cells were stimulated in the presence of a 5C.C7 (middle panel), 3A9 (right panel) or no competing response (right panel). **b.** Graphs represent the total number of CD44^{hi} divided 5C.C7β cells isolated from the draining LNs (top panel) and spleen (bottom panel) 8 weeks after immunisation. The effector capacity of 5C.C7β memory cells was determined by measuring the percentage of intracellular IL-2 (**c, d**) and IFN-γ (**f, g**) positive 5C.C7β cells after 16 hours of *ex vivo* stimulation with 10μM MCC. The total number of IL-2 (**e**) and IFN-γ (**h**) producing 5C.C7β memory cells was calculated by multiplying the percentage of positive cells per sample by the total organ count.

decrease in the total number of IL-2-producing cells (Figure 5.6.1e) was due primarily to the reduction in the total number of 5C.C7 β cells in the face of 5C.C7 competition. Once again, competition by 3A9 cells had no effect on the frequency or number of IL-2-producing cells. These data demonstrate that the generation of divided cells with a memory phenotype and the capacity to produce effector cytokines was affected by competition for peptide-MHC, but not by competition mediated by cells that recognised a different antigen.

It was important to establish whether the reduction in the total number of 5C.C7 β cells capable of producing IFN- γ was a result of reduced differentiation of 5C.C7 β cells into either a CD62L^{lo} effector memory (T_{EM}) or CD62L^{hi} central memory (T_{CM}) phenotype (Sallusto et al. 1999). Therefore the total number and cytokine producing capacity of 5C.C7 β CD62L^{lo} and CD62L^{hi} was measured 9 weeks after immunisation. The presence of a competing high affinity 5C.C7 response reduced the frequency of T_{EM} 5C.C7 β cells from 81% to 51% with a 5-fold reduction in the total number of CD62L^{lo} cells (Figure 5.6.2b). An increase in the percentage of CD62L^{hi} was not due to preferential differentiation of 5C.C7 β cells into a central memory phenotype as an equal number of CD62L^{hi} divided 5C.C7 β cells was observed between all three groups (Figure 5.6.2c). As would be predicted from earlier data, the presence of a high affinity 3A9 response did not alter the frequency or total number of either sub-type of memory 5C.C7 β cells.

To confirm that the CD62L^{lo} phenotype corresponded to the T_{EM} population, IFN- γ and IL-2 production was assessed. In all three groups, cytokine production was restricted to the CD62L^{lo} T_{EM} subset of 5C.C7 β cells (Figure 5.6.2e and f). Consistent with earlier experiments, competition by high affinity 5C.C7 cells reduced the frequency of IL-2 and IFN- γ producing 5C.C7 β cells, resulting in a 10-fold reduction in the total number of CD62L^{lo} IL-2 (Figure 5.6.2f) and IFN- γ producing 5C.C7 β cells (Figure 5.6.2i). Once again, competition by high affinity 3A9 cells did not alter the frequency or absolute number of CD62L^{lo} IFN- γ or IL-2 producing 5C.C7 β cells.

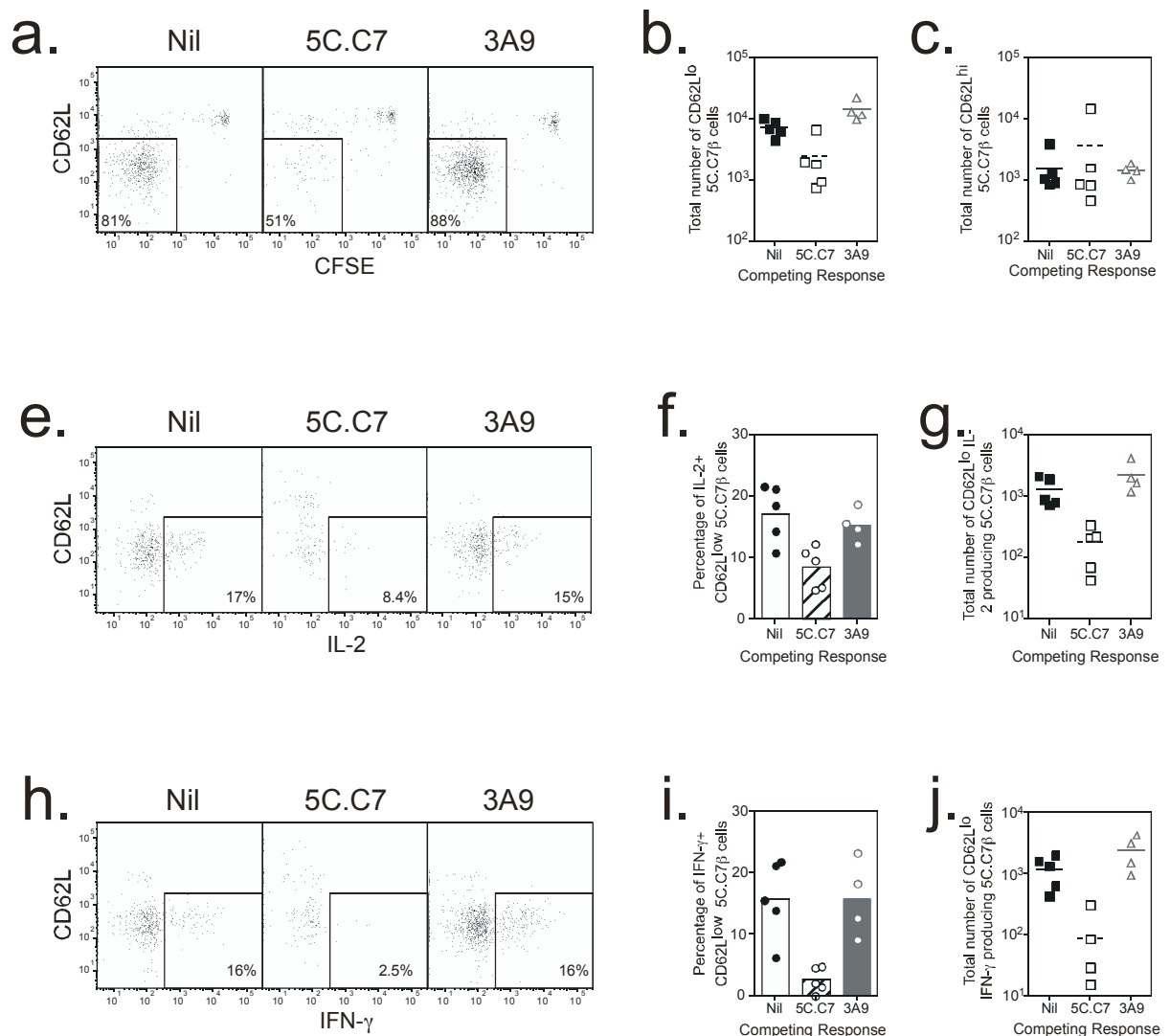


Figure 5.6.2: The effect of high affinity competition on the generation of central or effector memory cells

1×10^6 Ly5.1⁻ 5C.C7 β cells were adoptively transferred with an equal number of Ly5.1⁺ 5C.C7 or 3A9 cells 1 day prior to immunisation with $0.1 \mu\text{g}$ MCC and $3.2 \mu\text{g}$ HEL peptides in CFA. 9 weeks after immunisation draining LNs were harvested and analysed by flow cytometry.

a. Dot plots demonstrate the expression of CD62L versus CFSE of 5C.C7 β cells (gated as Ly5.1⁻ and CD4⁺), numbers represent the mean percentage of CD62L^{lo} divided 5C.C7 β cells. The total number of CD62L^{lo} 5C.C7 β cells (**b.**) and CD62L^{hi} 5C.C7 β cells (**c.**) was calculated by multiplying the frequency by the total organ count.

The production of IFN- γ and IL-2 was measured by intracellular cytokine staining after 16 hours of ex vivo restimulation with $10 \mu\text{M}$ MCC peptide. Plots represent CD62L expression (y-axis) versus IL-2 (**e.**) or IFN- γ (**h.**), numbers represent the mean percentage of IL-2⁺ or IFN- γ ⁺ divided CD62L^{lo} 5C.C7 β cells. Graphs represent the percentage of IL-2⁺ (**f.**) or IFN- γ ⁺ (**i.**) divided CD62L^{low} 5C.C7 β cells with the total number of IL-2 (**g.**) or IFN- γ (**j.**) producing divided CD62L^{lo} 5C.C7 β cells calculated as previously described.

In summary, competition by high affinity 5C.C7 cells reduced the generation of effector memory 5C.C7 β cells as determined by expression of CD62L. In addition to a reduction in the number of CD62L^{lo} T_{EM}, these cells maintained a reduced capacity for IFN- γ secretion and therefore the reduced frequency of IFN- γ was not solely due to a reduced differentiation into a T_{EM} phenotype. Competition by high affinity 5C.C7 cells did not, however, enhance differentiation of 5C.C7 β cells into a central memory phenotype, as an equal number of T_{CM} were present in all three groups.

5.7: Enhancing the effect of antigen non-specific competition

In the previous series of experiments, antigen non-specific competition had a minor impact on the number of effector cells in the primary response, with no effect on the number or function of memory cells.

To determine whether antigen non-specific competition could be enhanced by presentation of both HEL and MCC epitopes by the same APCs, cells were stimulated with recombinant HELMCC. Recipient mice received 1×10^6 CFSE labelled Ly5.1⁻ 5C.C7 β cells, together with an equal number of 5C.C7 or 3A9 cells. 2 days later recipient mice were immunised with 0.05 μ g of HELMCC, and draining LNs were harvested a further 10 days later for analysis by flow cytometry. Consistent with the previous data, competition by 5C.C7 cells reduced the total number of divided 5C.C7 β cells by 10-fold (Figure 5.7.1b), but surprisingly competition by 3A9 cells had no effect on cell numbers. Competition by 5C.C7 cells reduced the frequency and absolute number of both IL-2 and IFN- γ producing 5C.C7 β cells (Figure 5.7.1c-h), while competition by 3A9 cells had no effect.

In a separate experiment, the effect of antigen non-specific competition on the generation of memory 5C.C7 β cells was assessed when both epitopes were presented on the same APCs. As predicted from earlier data, competition by 5C.C7 cells reduced both the frequency (Figure 5.7.2a) and absolute number (Figure 5.7.2b) of CD62L^{lo} effector

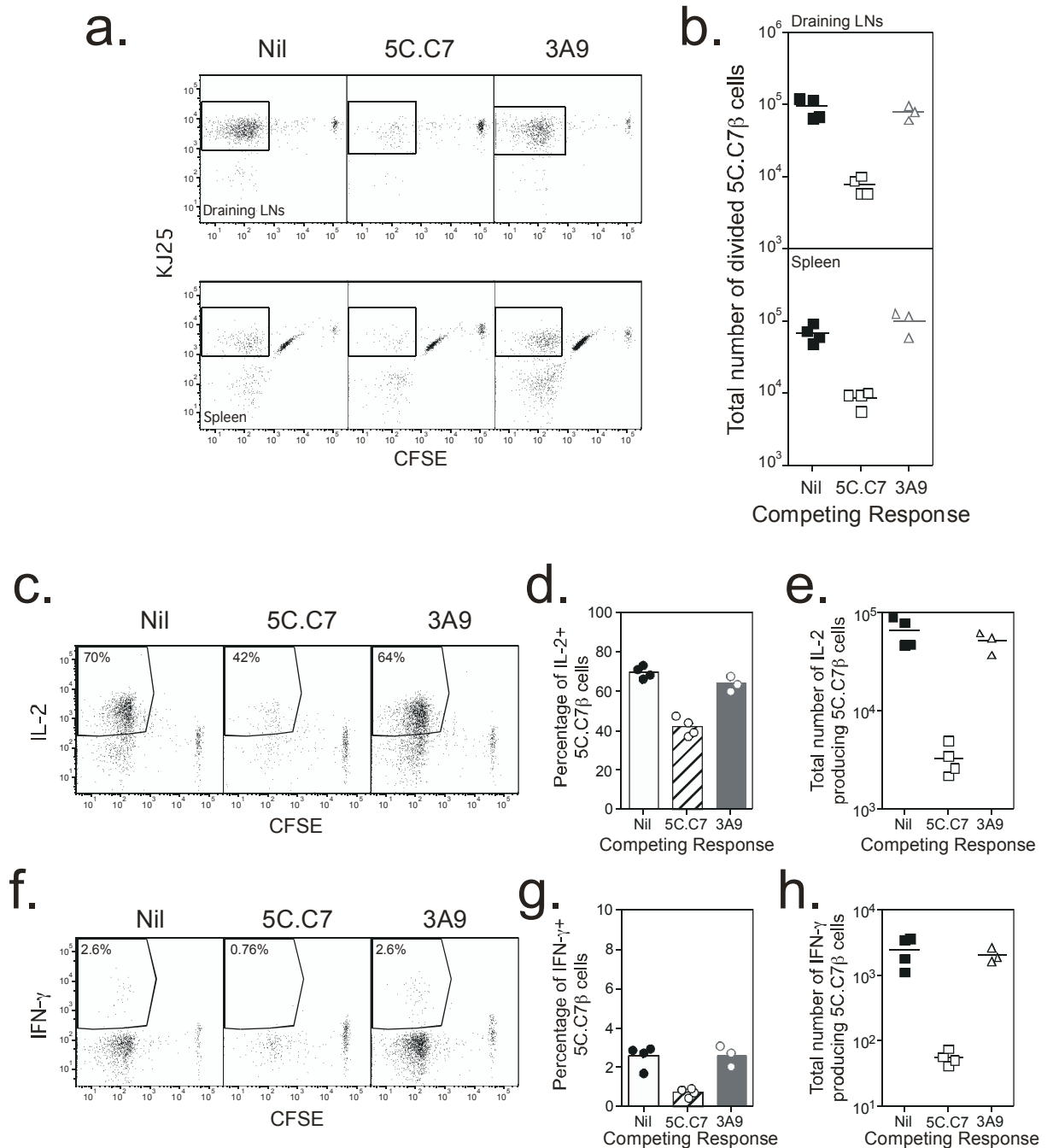


Figure 5.7.1: The effect of antigen non-specific competition when both antigens presented on the same APCs

1x10⁶ CFSE labelled 5C.C7β LN cells were adoptively transferred with an equal number of 5C.C7 or 3A9 LN cells. 2 days later recipient mice were immunised with 0.05μg HELMCC-his in CFA with draining lymph nodes and spleen analysed 10 days later.

a. Dot plots demonstrate CFSE versus KJ25 expression gated on CD4⁺, Ly5⁻ of draining LNs and spleen samples harvested at day 10.

b. The total number of 5C.C7β cells was calculated by multiplying the frequency of CD4⁺, Ly5⁺, KJ25⁺, CFSE^{neg} cells per sample by the total organ count.

IL-2 and IFN-γ secretion were measured by intracellular cytokine staining. Number represent the mean percentage of IL-2 (**c.** and **d.**) or IFN-γ+ (**f.** and **g.**) divided 5C.C7β cells. The total number of IL-2 (**e.**) and IFN-γ (**h.**) secreting 5C.C7β cells was calculated as previously described.

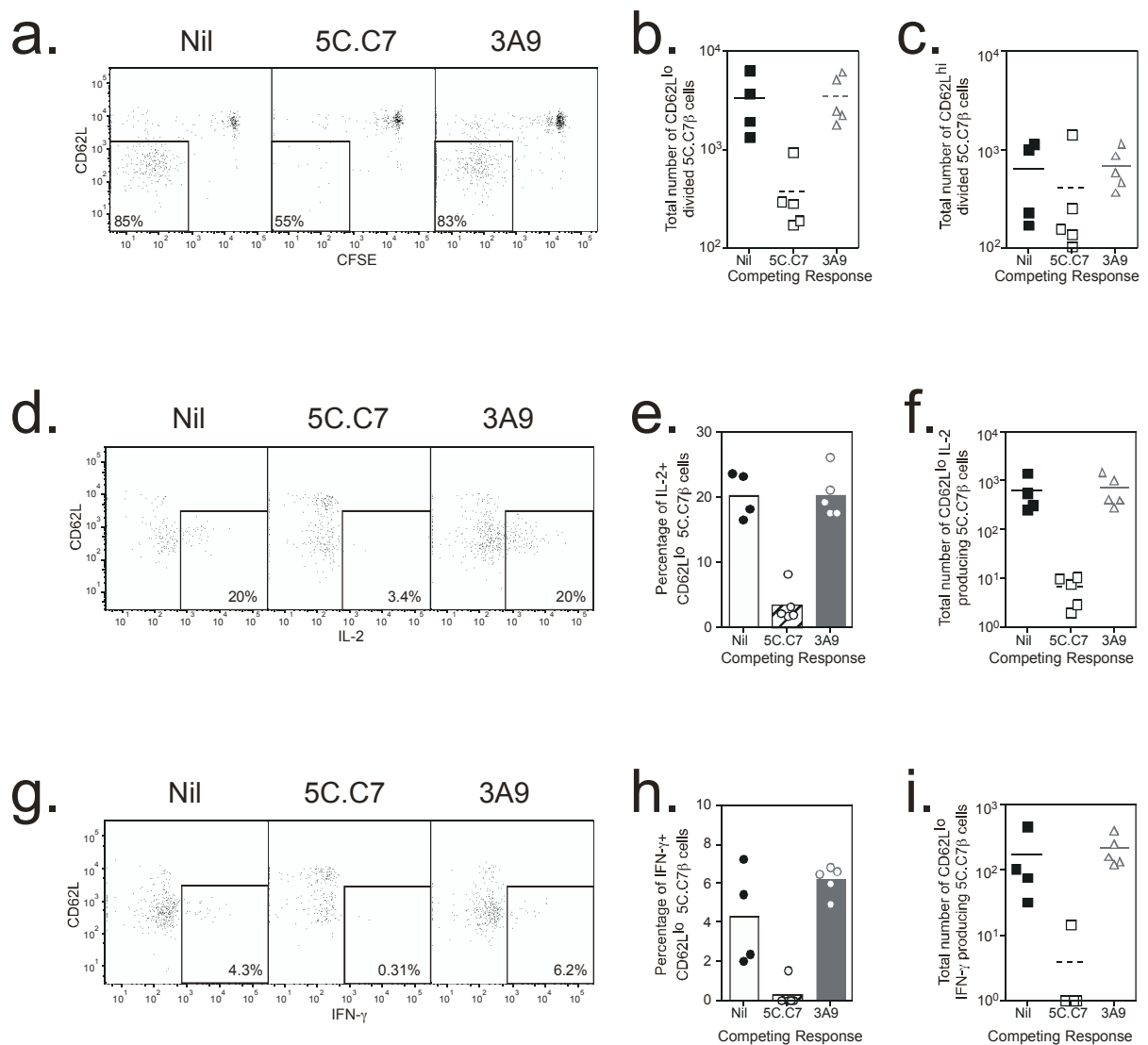


Figure 5.7.2: The effect antigen non-specific competition on the generation of central or effector memory cells when both antigens are presented on the same APCs

1x10⁶ Ly5.1⁻ 5C.C7β cells were adoptively transferred with an equal number of Ly5.1⁺ 5C.C7 or 3A9 cells 1 day prior to immunisation with 0.005μg HELMCC-his in CFA. 9 weeks after immunisation draining LNs were harvested and analysed by flow cytometry.

a. Dot plots demonstrate the expression of CD62L versus CFSE of 5C.C7β cells (gated as Ly5.1⁻ and CD4⁺), numbers represent the mean percentage of CD62L^{lo} divided 5C.C7β cells. The total number of CD62L^{lo} 5C.C7β cells (**b.**) and CD62L^{hi} 5C.C7β cells (**c.**) was calculated by multiplying the frequency by the total organ count.

The production of IFN-γ and IL-2 was measured by intracellular cytokine staining after 16 hours of ex vivo restimulation with 10μM MCC peptide. Plots represent CD62L expression (y-axis) versus IL-2 (**e.**) or IFN-γ (**h.**), numbers represent the mean percentage of IL-2⁺ or IFN-γ⁺ divided CD62L^{lo} 5C.C7β cells. Graphs represent the percentage of IL-2⁺ (**e.**) or IFN-γ⁺ (**h.**) divided CD62L^{lo} 5C.C7β cells with the total number of IL-2 (**f.**) or IFN-γ (**i.**) producing divided CD62L^{lo} 5C.C7β cells calculated as previously described.

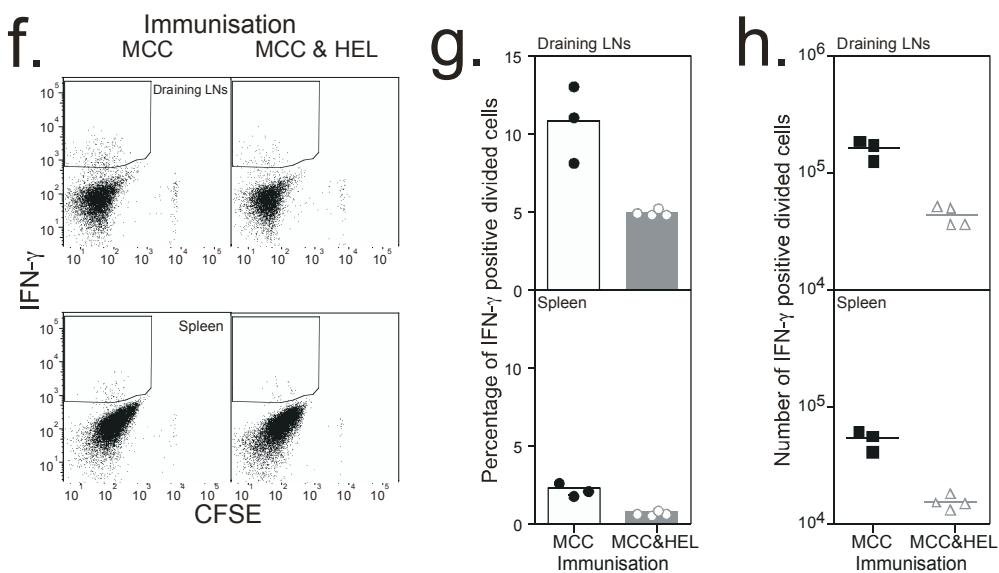
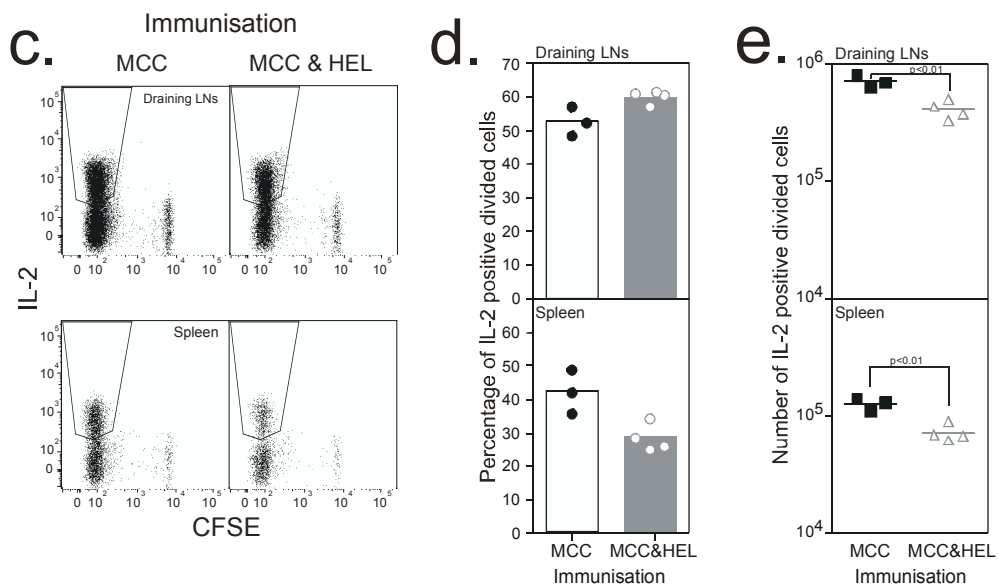
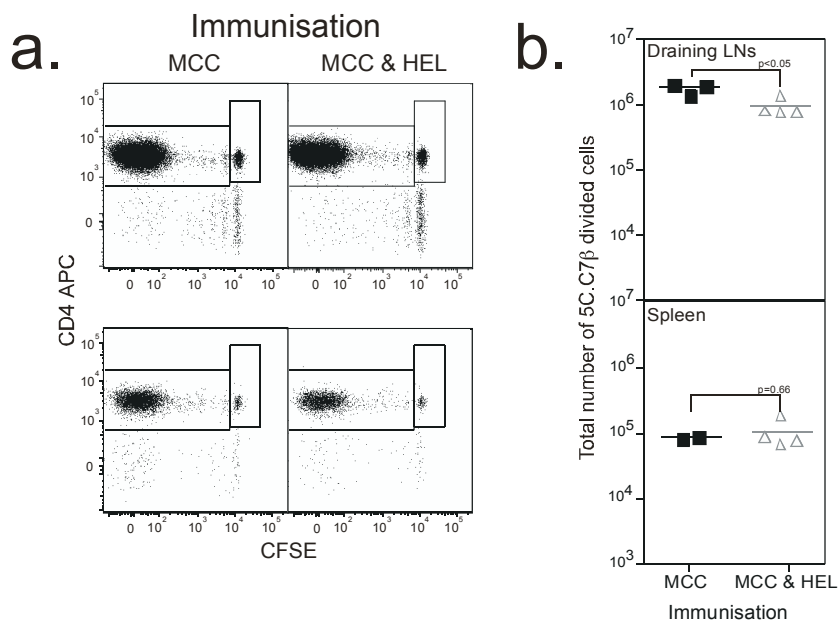
memory cells 10 weeks after immunisation, while the total number of central memory cells was not affected (Figure 5.7.2c). In contrast, competition by 3A9 cells had no effect on either the percentage or total number of either memory T cell subset.

As seen in the previous experiment, both IL-2 (Figure 5.7.2d) and IFN- γ (Figure 5.7.2g) secretion was confined to the CD62L^{lo} population of memory cells. As expected, competition by 5C.C7 reduced both the frequencies and absolute numbers of IL-2 and IFN- γ producing T_{EM} 5C.C7 β cells, while competition by 3A9 cells did not affect either the frequency or total number of IL-2 or IFN- γ producing cells. Despite predicting antigen non-specific would be enhanced by presentation of both antigens on the same APCs, an effect of competition by 3A9 cells was not observed at either the peak of the primary response or 10 weeks after immunisation.

To determine whether an effect of antigen specific competition would be enhanced if the number of cells responding to a different antigen were increased, 1×10^6 CFSE labelled 5C.C7 β cells were transferred into intact 3A9 $\alpha\beta$ TCR transgenic mice so that the ratio of host HEL-specific to transferred 5C.C7 β cells would be greater than 100:1. Mice were immunised with either 0.1 μ g MCC peptide in CFA or a mixture of 0.1 μ g MCC peptide and 1mg HEL protein in CFA. Despite the 100-fold increase in the number of competing HEL-specific cells, the number of divided 5C.C7 β cells recovered from the draining LNs at day 10 (Figure 5.7.3) was decreased to a similar extent as in the experiment illustrated in Figure 5.1.1 and Figure 5.2.1 in which the starting cell ratio was 1:1, with no difference in the total number of 5C.C7 β cells observed in the spleen. The drop in the frequency of IFN- γ -producing cells was slightly more marked at 100:1 (Figure 5.7.3) than at 1:1 (Figure 5.7.3), leading to a 5-fold decrease in the total number of IFN- γ -producing cells at day 10 (Figure 5.7.3). However, when draining LNs were harvested 8 weeks after immunisation, a slightly higher number of 5C.C7 β cells was recovered from mice immunised with MCC and HEL (Figure 5.7.4). At this late timepoint, the frequency and total number of IFN- γ -producing 5C.C7 β cells (Figure 5.7.4) were unaffected by the competing response of HEL-specific cells. Thus, competition between CD4⁺ T cells of different antigenic

Figure 5.7.3: Response of 5C.C7 β cells with or without an endogenous 3A9 response.

1×10^6 CFSE labelled Ly5.1⁻ 5C.C7 β cells were adoptively transferred into 3A9 TCR tg host mice. Mice were immunised with either 0.1 μ g MCC peptide in CFA or a mixture containing 0.1 μ g MCC and 1mg of whole HEL protein. **a.** Draining LNs and spleens were harvested on day 10 of the response and analysed by flow cytometry. The total number of divided cells (**b.**) was determined by multiplying the percentage of 5C.C7 β cells per sample (determined by flow cytometry) by the total organ count. Cells from the draining LNs and spleen were restimulated *ex vivo* to assess the production of intracellular IL-2 (**c.**) and IFN- γ (**f.**), graphs show one representative mouse per group gated on Ly5.1⁻ CD4⁺ and V β 3⁺. The percentage of IL-2⁺ (**d.**) and IFN- γ ⁺ (**g.**) divided 5C.C7 β cells in the draining nodes and the spleen was determined by flow cytometry and the total number of IL-2 producing (**e.**) or IFN- γ producing (**h.**) was determined by multiplying the frequency by the total organ count.



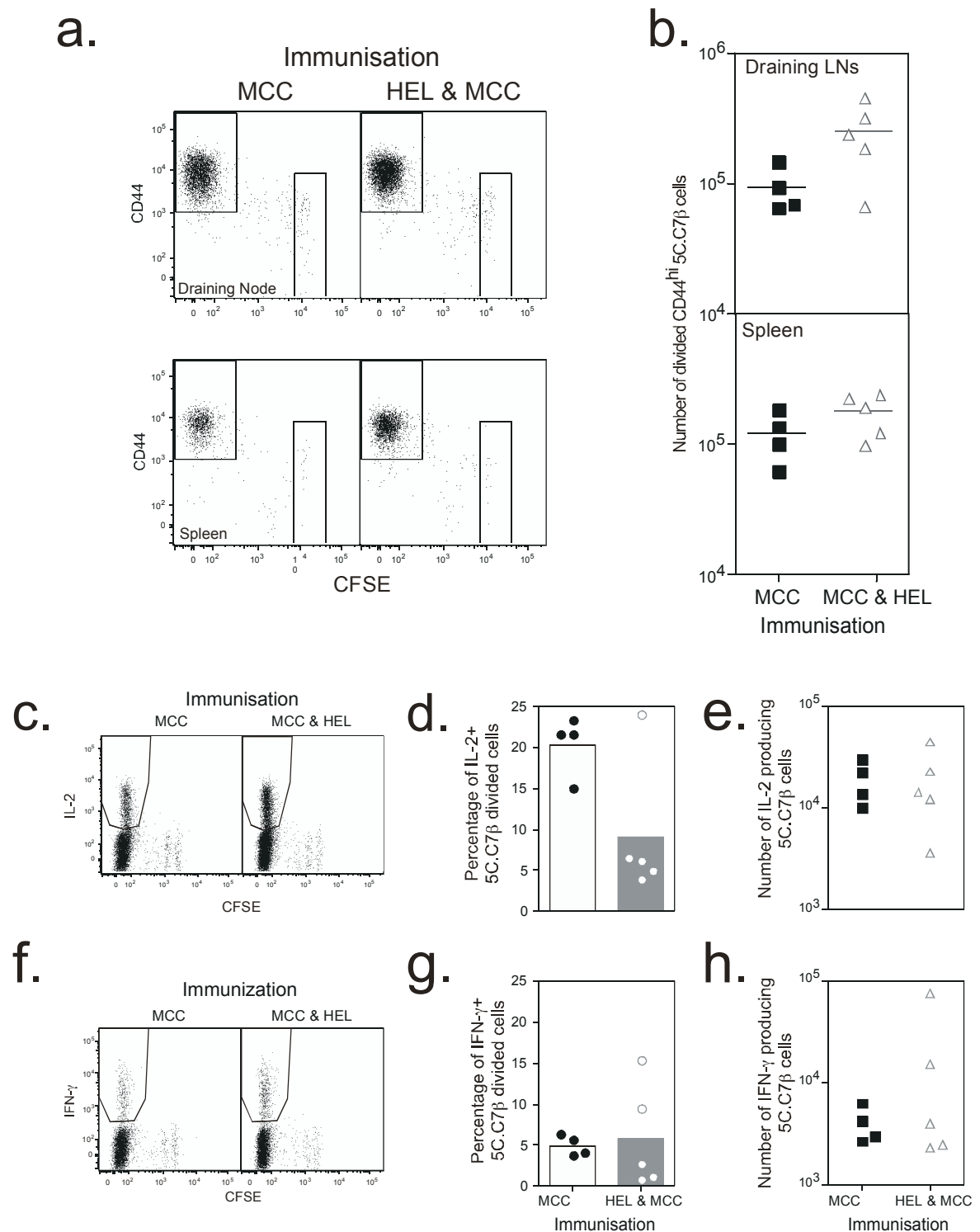


Figure 5.7.4: Generation of memory 5C.C7β cells in the presence or absence of an endogenous 3A9 response.

1x10⁶ CFSE labelled Ly5.1⁻ 5C.C7β cells were adoptively transferred into 3A9 TCR tg host mice. Mice were immunised with either 0.1μg MCC peptide in CFA or a mixture containing 0.1μg MCC and 1mg of whole HEL protein. **a.** Draining LNs and spleens were harvested 8 weeks post-immunization and analyzed by flow cytometry. The total number of CD44^{hi} divided cells (**b.**) was determined by multiplying the percentage of 5C.C7β cells per sample (determined by flow cytometry) by the total organ count.

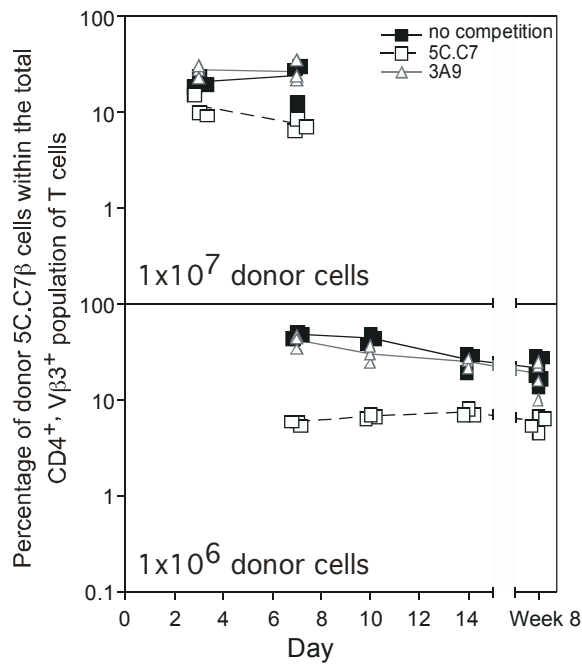
Cells from the draining LNs were restimulated *ex vivo* to assess the production of intracellular IL-2 (**c.**) and IFN-γ (**f.**). The percentage of IL-2⁺ (**d.**) and IFN-γ⁺ (**g.**) 5C.C7β cells was determined by flow cytometry and the total number of IL-2 producing (**e.**) or IFN-γ producing (**h.**) was determined by multiplying the frequency by the total organ count.

specificities can decrease the burst size to a limited extent, and reduce the percentage of effector cells within the dividing population, but no long lasting effect on the generation of memory cells can be detected. Therefore the major source of T cell competition during the course of a primary immune response is from cells of the same specificity, and it is this antigen-specific competition that drives affinity maturation by inhibiting the proliferation of lower affinity cells and the consequent generation of effector and memory populations.

5.8: The contribution of 5C.C7 β cells to the total MCC specific response

A recent publication from Malherbe et al (2004) suggested that competition for antigen is independent of affinity above an initial affinity threshold. If all MCC-reactive cells above a certain affinity threshold competed equally for antigen throughout the course of the response, then 5C.C7 β cells would represent a constant fraction of the response over time, both in absolute number of cells and number of cytokine-producing cells. To test whether this was the case, the data from the experiments described in section 5.1. 5.2 and 5.6 were reanalysed to determine the contribution of 5C.C7 β cells to the total number MCC-specific IFN- γ -producing cells. The data from the experiments involving transfer of 1×10^7 and 1×10^6 5C.C7 β cells both showed a gradual reduction in the contribution of 5C.C7 β cells to the total number of MCC specific cells (Figure 5.8a) and total number of IFN- γ producing MCC specific cells (Figure 5.8b) in both the control group and in the presence of a 3A9 response. However, in the presence of a high affinity MCC specific response (5C.C7), the contribution of 5C.C7 β cells to the total number of MCC specific cells remained stable from day 7 onwards. In contrast, the contribution of 5C.C7 β cells to the IFN- γ response declined over the course of the primary response and during the generation of memory cells (Figure 5.8b). These data support a model in which competition for peptide-MHC operates continuously throughout the primary response and

a.



b.

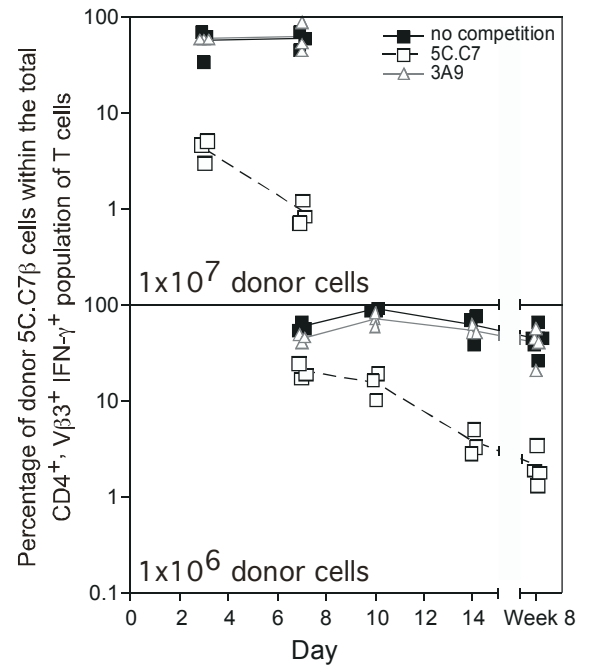


Figure 5.8: Contribution of donor 5C.C7β cells to the total anti-MCC response.

To determine the contribution of 5C.C7β cells to the total MCC response, each sample was gated for CD4⁺, KJ25⁺ or CD4⁺, KJ25⁺ and IFN-γ⁺ cells before the frequency of donor 5C.C7β cells within this population was determined. 1x10⁷ (top panel) or 1x10⁶ (bottom panel) donor 5C.C7β cells were adoptively transferred simultaneously with an equal number of 3A9 (triangles) or 5C.C7 (open squares) cells.

the generation of memory cells, leading to a gradual loss of cytokine producing capacity without substantially altering the number of MCC specific cells.

Discussion

In this chapter, the impact of antigen specific and non-specific competition on the generation of effector and memory cells was investigated. In so doing, an *in vivo* model of competition was established whereby the response of a mixed affinity population of MCC specific cells was measured when a competing cohort of high affinity MCC or HEL specific cells were present. Consistent with earlier work (Chapters 3,4), antigen specific competition was shown to have a more profound effect on the response of T cells than competition by cells of different antigen specificity (Chapter 3 and 4) (Kedl et al. 2002). Recruitment of 5C.C7 β cells into division was unaltered by the presence of a high affinity response, but by day 3, mixed affinity 5C.C7 β cells expressed lower levels of CD4, CD44, Ox-40 and all three chains of the IL-2R (α , β and γ), suggesting an overall reduction in TCR stimulation within the first 3 days of the response. Mixed affinity 5C.C7 β cells also displayed a reduced ability to proliferate, as measured by BrdU incorporation, which resulted in a reduction in the absolute number of 5C.C7 β cells from day 7 onwards. The most profound effect of antigen specific competition was on the generation of effector and memory cells. By day 14, less than 3% of 5C.C7 β cells were shown to secrete IFN- γ in the presence of a high affinity 5C.C7 response (Figure 5.1.3). By week 8 this was further reduced to less than 1% (Figure 5.6.1). At this timepoint, there was also a reduction in the total number of CD62L^{lo} effector memory cells (Figure 5.6.2). However a reduced absolute number of IFN- γ secreting cells was not solely due to a reduction in the number of effector memory cells, as a reduced frequency of IFN- γ producing cells was also observed within this population of cells. A reduction in the number of effector memory T cells is consistent with two recent reports in which reducing the level of TCR stimulation either by increasing the number of precursors (Marzo et al. 2005) or expression of antigen

in an attenuated vector (van Faassen et al. 2005) induced differentiation of CD8 cells primarily into central memory phenotype. However, both of these studies investigated the ratio of T_{EM} to T_{CM} without any measurement of the total number of each subset. In this study the increase in the frequency of T_{CM} cells was due to a reduction in the total number of T_{EM} and not preferential differentiation into a T_{CM} phenotype. As these cells have the capacity to secrete cytokines with longer periods of antigen restimulation (Sallusto et al. 1999), it would suggest that long-term memory is maintained despite the reduction in effector memory cells. Overall, the generation of a reduced number of effector memory cells in the presence of a high affinity response supports a role for competition in affinity maturation of the T cell response.

Affinity maturation of T cell responses has generally been studied by surveying the repertoire of TCR usage at various times throughout the response. The occurrence of affinity maturation has been inferred either by narrowing of the repertoire (Callan et al. 1998; Fasso et al. 2000; Maryanski et al. 1996) or increase in the usage of TCR sequences known to be of very high affinity (McHeyzer-Williams et al. 1999; McHeyzer-Williams and Davis 1995; Zheng et al. 1996). More recently, binding and dissociation rates of peptide-MHC-tetramers have provided a measurement of the affinity of populations of T cells (Busch and Pamer 1999; Malherbe et al. 2004; Savage et al. 1999). These studies have led to differing conclusions regarding the timing of affinity maturation. Some indicate that repertoire narrowing occurs early in the primary response (Callan et al. 2000; Fasso et al. 2000; Maryanski et al. 1996; McHeyzer-Williams et al. 1999), while others saw a further contraction during selection of memory cells in the secondary response (Busch and Pamer 1999; Busch et al. 1998; McHeyzer-Williams et al. 1996). In a recent report, Malherbe (2004) suggested that cells above a certain threshold were selected early in the response and then proceeded to divide with no further competition or affinity maturation.

In the experiments described in this chapter, analysis of the contribution of 5C.C7 β cells to simply the total number of MCC specific cells (Figure 5.8a) would suggest that selection had occurred by day 7 without any further selective events, in line with previously

published reports (Callan et al. 1998; Fasso et al. 2000; Malherbe et al. 2004; Maryanski et al. 1996; McHeyzer-Williams et al. 1996). However, analysis of effector function by IFN- γ production demonstrated a declining contribution of 5C.C7 β cells to the total IFN- γ response with time, supporting a model of continuous selection (Busch and Pamer 1999; Busch et al. 1998; McHeyzer-Williams et al. 1996). Moreover, the larger the number of cells competing for antigen, the earlier competition becomes evident. Thus when 3×10^6 T cells of high versus mixed affinity are responding to antigen, a decrease in proliferation, expression of activation markers, and cytokine production by cells of mixed affinity is evident at day 3, whereas the number of cells recruited into cell division is unaffected. In contrast, when only 3×10^5 T cells of high versus mixed affinity are competing, proliferation on day 3 is unaffected, whereas cell number and cytokine production is reduced on day 7. Finally when antigen specific competition is pushed to the extreme, for example in the presence of a 45-fold excess of responding cells, then an effect can be measured as early as day 3 (Smith et al. 2000). Together these data demonstrates that affinity maturation is driven by competition between responding T cells for continued peptide-MHC stimulation. Affinity maturation is not mediated by active loss of lower affinity cells by apoptosis, but due to an overall reduction in the level of TCR stimulation in the presence of high affinity cells, leading to a gradual loss of cells of lower affinity throughout the response. Consequently, this induces an increase in the overall strength of the response to a particular antigen.

In contrast to the potent effect of high affinity T cells responding to the same peptide-MHC complex, a simultaneous response to an unrelated epitope had only a limited effect on the generation of effector cells during the primary response, consistent with earlier reports (Kedl et al. 2000; Kedl et al. 2002). Importantly antigen non-specific competition displayed no detectable effect on the generation of memory cells, in terms of numbers, cytokine production or memory cell sub-types. This would also suggest that while Ag-MHC independent signals may enhance the size of the primary response these signals play only a minor role in the generation of memory cells. In the absence of either Ox-40 (Chen et al. 1999; Kopf et al. 1999; Linton et al. 2003; Murata et al. 2000; Pippig et al. 1999) or

4-1BB (DeBenedette et al. 1999; Kwon et al. 2002; Tan et al. 1999; Tan et al. 2000), a reduction in the overall size of the primary response has been observed. Reduced secondary or memory responses have also been observed in the complete absence of Ox-40 and 4-1BB (Bertram et al. 2002; Gaspal et al. 2005; Gramaglia et al. 2000; Hendriks et al. 2005). Unfortunately, there is currently no data on the effect of reduced costimulatory signals on the generation of memory cells. There has, however, been one report in which enhanced antigen-independent expansion of effector cells was shown not to increase the generation of memory cells, suggesting antigen specific signalling is the critical signal driving the generation of memory cells (Busch et al. 2000).

Importantly, antigen non-specific competition displayed no detectable effect on the generation of memory cells, in terms of numbers, cytokine production or memory cell subtypes. Thus competition between CD4⁺ T cells of different specificities should not prove to be a significant obstacle in the design of multi-epitope vaccines. Despite this finding, there is a considerable literature relating to competitive effects during immunisation with multiple antigens (Fattom et al. 1999; Manoj et al. 2003; Raadsma et al. 1994). These effects appear to be derived from two sources: interference between antigens for access to antigen-processing machinery or binding to particular MHC alleles, and interference between lymphocytes responding to closely related sources.

Competition for antigen processing is well established as the major contributing factor to the generation of immunodominance hierarchies (Basler et al. 2004; Busch and Pamer 1998; Chen et al. 2001; Deng et al. 1997; Levitsky et al. 1996; Niedermann et al. 1995). In fact, changing the overall level of presentation of the 2 dominant epitopes can reverse the hierarchy seen in response to Influenza A (Chen et al. 2004; La Gruta et al. 2006). There have been only two reports of antigen non-specific competition occurring in vivo when antigens were not derived from the same protein or presented on the same MHC allele. The first paper (Kedl et al. 2002) demonstrated a minimal reduction in the frequency of endogenous cells (from approximately 0.60% to 0.17%) in the presence of a high number of antigen specific cells. This analysis was performed at day 5 of the response with no indication of the long-term effect of this competition. In the second paper (Hayball et al.

2004), transferring HNT cells 18 hours into an ongoing OT-II response increased the frequency of undivided cells, while transferring OT-II cells into an ongoing HNT response was shown to have more of an effect than antigen specific competition, questioning the reproducibility of this data. Given the minimal effect of antigen non-specific competition, it would suggest that immunodominance hierarchies are predominantly mediated by antigen processing events and not interference between responding T cells.

In summary, the driving force behind the generation of effector and memory cells is signalling via the TCR. While signals independent of MHC, for example costimulatory or survival signals, may enhance the size of the primary response, antigen non-specific competition will not effect the generation of memory cells. Importantly higher affinity cells dominate the response by maintaining their access to peptide-MHC while limiting that of lower affinity cells, which leads to an increase in the overall affinity of the response.

Chapter 6: DC marker expression over the course of an antigen specific response

Competition for peptide-MHC and costimulatory signals was shown in the previous chapters to reduce the level of T cell stimulation, proliferation and generation of effector and memory cells. This effect could not solely be explained by a reduced ability of either late-arriving or lower affinity cells to access APCs, rather suggested a change in the level of peptide-MHC was involved in limiting the size of the CD4 response. CD8⁺ T cells are believed to regulate the size of an ongoing response by developing the capacity to destroy antigen presenting DCs (Hafalla et al. 2003; Wong and Pamer 2003; Yang et al. 2006). However it is unlikely that CD4⁺ T cells operate in a similar manner in the two models described in this thesis due to the minimal effect of antigen non-specific competition. A number of recent *in vitro* studies have demonstrated T cell induced down-regulation of specific peptide-MHC complexes either due to internalisation or removal of complexes from the surface of the DCs (Huang et al. 1999; Kedl et al. 2002; Patel et al. 1999; Wetzel et al. 2005). For CD8⁺ T cells there is some evidence that this phenomenon occurs *in vivo* (Kedl et al. 2002). As such it could potentially provide an antigen specific means of controlling the size of an ongoing response without affecting the immune system's ability to mount a simultaneous response to other antigens. If T cells can induce the down-regulation of MHC via the DC, then activated T cells might also be able to effect the level of costimulatory marker expression. As a direct test of whether molecules other than peptide-MHC could be involved in the down-regulation of the immune response, the expression of a number of costimulatory molecules on the surface of DCs were investigated over the course of an adjuvant-enhanced response.

In a preliminary experiment, 1×10^7 5C.C7 cells were adoptively transferred into non-tg recipient mice prior to immunisation with 10 μ g of MCC peptide or PBS alone in CFA. In this experiment, no difference in the level of costimulatory marker expression was observed between the two groups of mice, at any of the timepoints examined (data not

shown). It was hypothesised that the small number of responder cells may have been insufficient to induce detectable changes. Therefore in a subsequent experiment, 5C.C7 TCR tg mice or non-tg mice were immunized with 10 μ g of MCC peptide or PBS alone in CFA, and the expression of a number of markers analysed. Mice were immunised 7, 4, and 1 day prior to sacrifice with analysis of draining LNs by flow cytometry. This minimised the differences in the mean fluorescence intensity (MFI) that can occur between different assays due to staining or machine variability.

6.1: MHC Class II expression

DCs were identified by negative staining for B220 and PI, and positive expression of CD11c (Figure 6.1). Within this population there was further segregation of DCs expressing high (^{hi}) levels of MHC Class II, and a population expressing intermediate (^{int}) levels (gated in Figure 6.1a). More than 80% of all CD11c⁺ cells were MHC Class II^{hi} on day 0. However as the response progressed, the percentage of MHC Class II^{hi} cells declined, and by day 7 less than 50% of CD11c⁺ cells were MHC Class II^{hi} (Figure 6.1a). This decline in the frequency of MHC Class II^{hi} DCs was seen in all four groups of mice, with the largest reduction seen in 5C.C7 TCR tg mice immunised with MCC peptide in CFA (Figure 6.1a top panel). Over the course of the response, there was also a decrease in the level of MHC Class II expression within each of the gated DC populations. The lowest level of MHC Class II was once again seen in 5C.C7 TCR tg mice immunised with MCC in CFA (Figure 6.1b and c). In summary, over the course of a adjuvant-enhanced response, there was a decrease in the level of surface MHC. Not only did the frequency of CD11c⁺ MHC Class II^{hi} cells decline, but the level of MHC Class II expression on both MHC Class II^{hi} and MHC Class II^{int} DCs also declined.

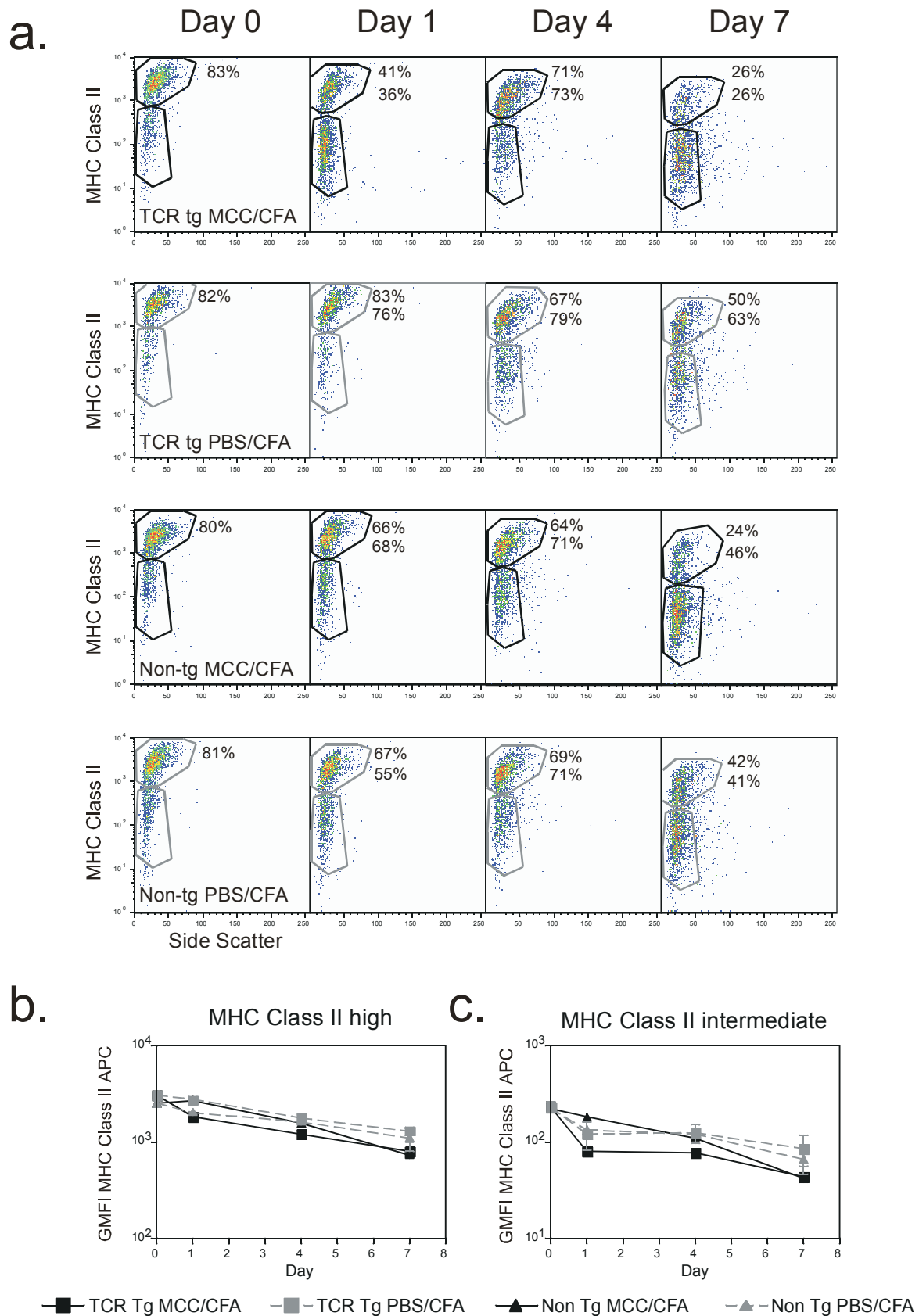


Figure 6.1: Expression of MHC Class II over the course of a adjuvant enhanced response.

On days -7, -4 and -1 5C.C7 TCR tg or non-tg recipient mice were immunized with either 10 μ g MCC peptide in CFA or PBS alone emulsified in CFA. On day 0 all mice were sacrificed and draining lymph nodes analyzed by flow cytometry. DCs were identified by positive expression of CD11c but negative staining for B220 and PI. **a.** Dot plots show the expression of MHC Class II versus side scatter with the percentage of MHC Class II^{hi} DCs for the 2 mice per group included. Graphs show the mean fluorescence of MHC Class II on the population of (**b.**) MHC Class II^{hi} and (**c.**) MHC Class II^{int}. Symbols represent the mean of 2 mice per group +/-SEM.

6.2: B7 Superfamily members

In the experiment described in section 6.1, the expression of three members of the B7 superfamily was investigated. As discussed in chapter 1, CD80 and CD86 both bind to CD28 and CTLA-4, while B7RP-1 (ICOS-L) binds only to its specific ligand ICOS. The level of ligand was calculated for both subsets MHC Class II^{hi} and MHC Class II^{int} of CD11c⁺B220⁻ cells.

Following immunisation with MCC peptide or PBS alone in CFA, CD80 expression increased on both MHC Class II^{hi} and MHC Class II^{int} DCs, then declined again (Figure 6.2.1). On MHC Class II^{hi} DCs, the highest level of CD80 expression was detected at day 4, except in TCR tg mice immunized with PBS in CFA where the level peaked at day 1 (there was, however, only one mouse in this group). Expression of CD80 by MHC Class II^{int} DCs peaked at day 1 in TCR tg mice, and day 4 in non-tg mice. Interestingly, immunisation with MCC in CFA led to a second increase in the level of CD80 between days 4 and 7 (Figure 6.2.1b).

The expression of surface CD86 remained relatively unchanged over the course of the response, with comparable levels seen between all four groups of mice (Figure 6.2.2). In general, there was a slight increase above baseline levels on MHC Class II^{hi} DCs at days 1 and 4 (Figure 6.2.2b), and days 1,4 and 7 on MHC Class II^{int} DCs (Figure 6.2.2c). In contrast to CD80, which was expressed at similar level by the two subsets of DCs, CD11c⁺ MHC Class II^{hi} cells displayed 10x more CD86 than MHC Class II^{int} cells (Figure 6.2.2b).

The pattern of B7RP-1 expression was similar to that of CD80 expression (Figure 6.2.3). B7RP-1 expression by MHC Class II^{hi} cells increased from day 0 to day 1 and then declined over the remainder of the response. At day 0, B7RP-1 expression on MHC Class II^{hi} DCs was higher on cells derived from 5C.C7 TCR tg mice (Figure 6.2.3b). MHC Class II^{int} DC expression of B7RP-1 increased between days 0 and 1 and then declined in the population of MHC Class II^{int} cells (Figure 6.2.3c). In mice immunised with MCC in CFA, a second rise in B7RP-1 expression was observed between days 4 and 7.

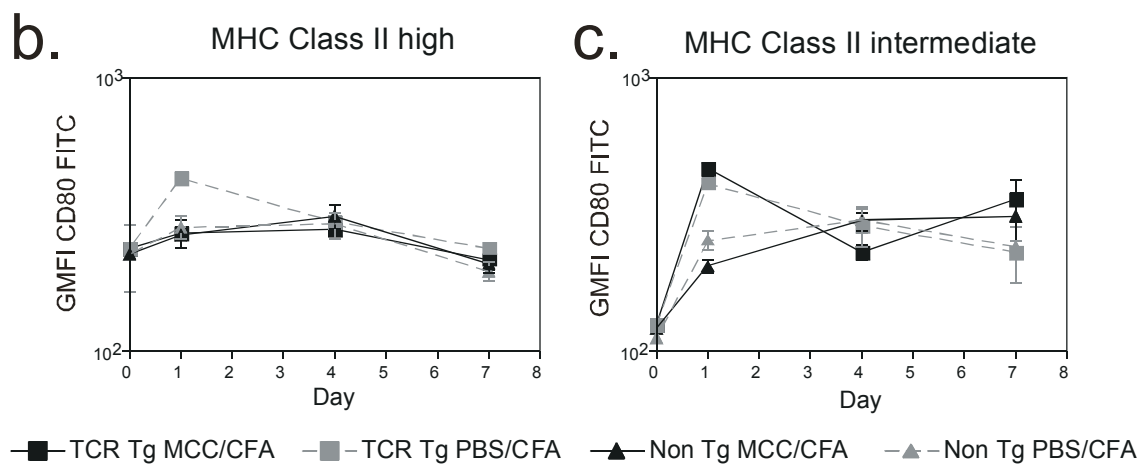
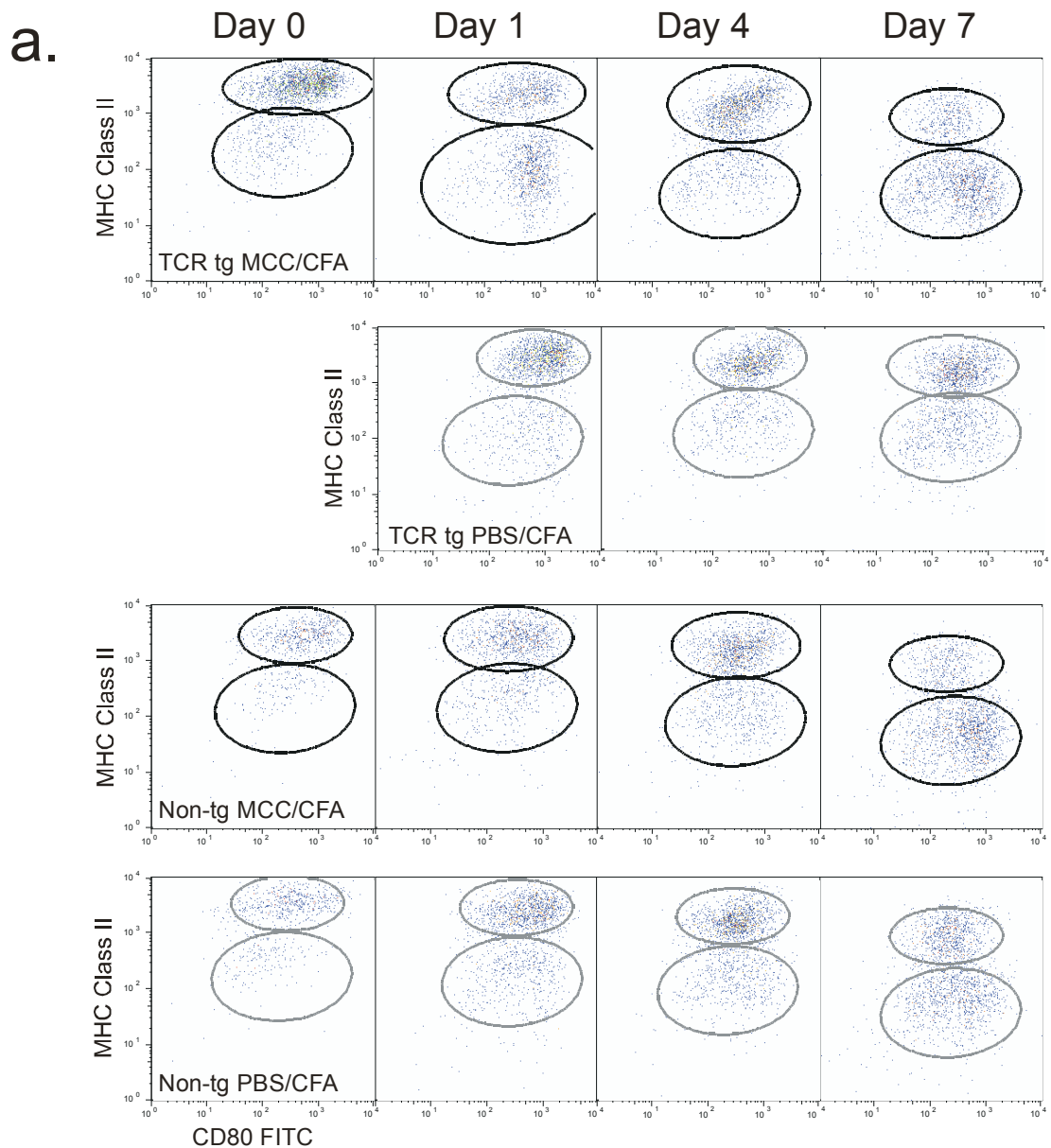


Figure 6.2.1: CD80 expression

In the same experiment as described in Figure 6.1.1, draining lymph nodes were gated on B220 and PI⁻ and CD11c⁺. (a.) Dot plots show the expression of CD80 on the x axis versus MHC Class II. Graphs show the mean fluorescence of CD80 on the population of (b.) MHC Class II^{hi} and (c.) MHC Class II^{int}. Symbols represent the mean of 2 mice per group +/- SEM.

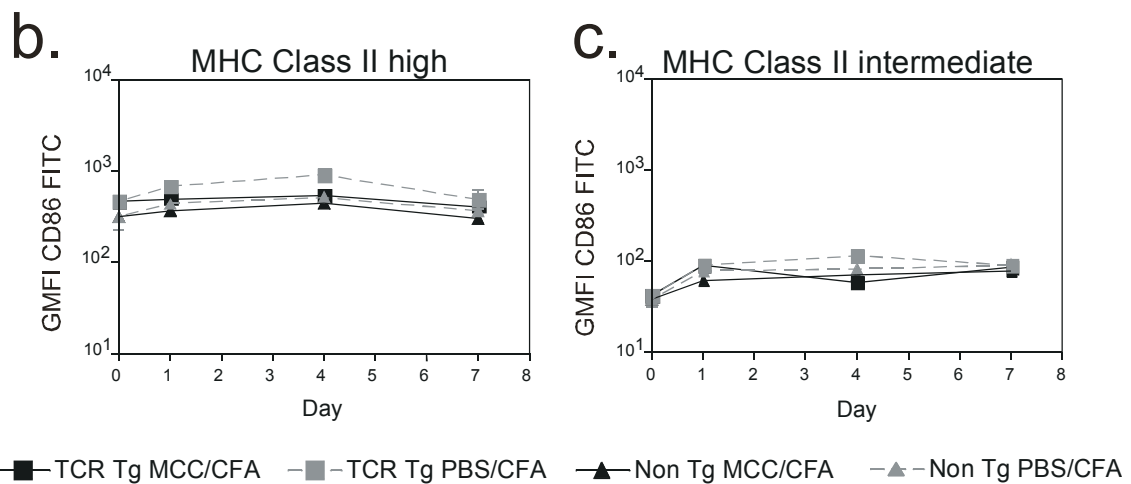
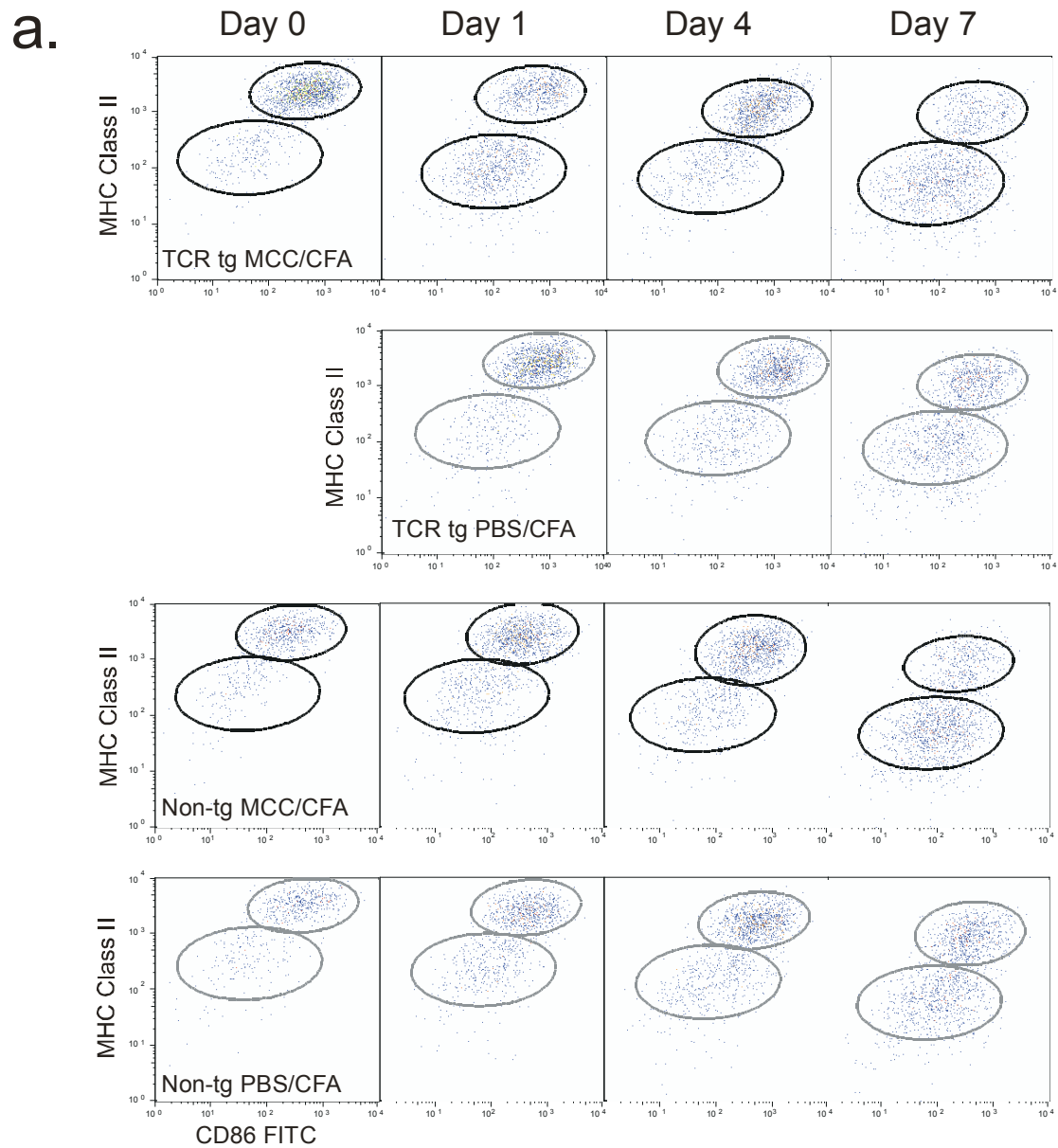


Figure 6.2.2: CD86 expression.

In the same experiment as described in Figure 6.1.1, draining lymph nodes were gated on B220 and PI⁻ and CD11c⁺. (a.) Dot plots show the expression of CD86 on the x axis versus MHC Class II. Graphs show the mean fluorescence of CD86 on the population of (b.) MHC Class II^{hi} and (c.) MHC Class II^{int}. Symbols represent the mean of 2 mice per group +/- SEM.

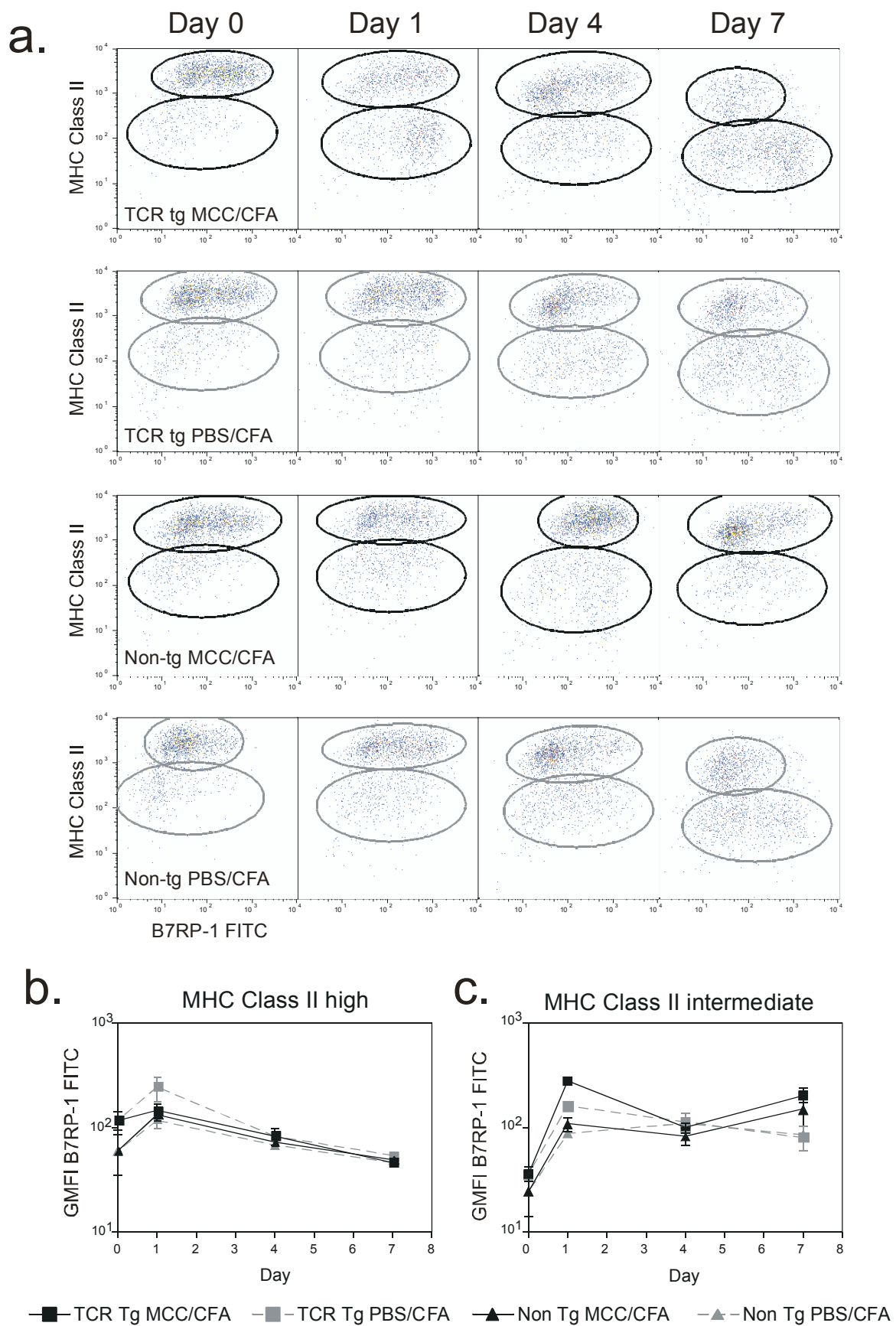


Figure 6.2.3: B7RP-1 expression

In the same experiment as described in Figure 6.1, draining lymph nodes were gated on B220 and PI^- and $CD11c^+$. (a.) Dot plots show the expression of B7RP-1 on the x axis versus MHC Class II. Graphs show the mean fluorescence of B7RP-1 on the population of (b.) MHC Class II^{hi} and (c.) MHC Class II^{int}. Symbols represent the mean of 2 mice per group $\pm$ SEM.

In general the changes in B7 superfamily expression in this experiment was small, and much of the variability could have been due to small changes in sample populations and staining. There was a small increase in the level of expression in the early stages of the response followed by a decline back towards baseline levels. The most apparent difference was in the population of MHC Class II^{int} cells, where in mice immunised with MCC in CFA, a second increase in both CD80 and B7RP-1 expression was detected between days 4 and 7.

6.3: TNFR Superfamily members

In the experiment described in sections 6.1 and 6.2, the expression of four members of the TNFR superfamily was also examined; CD70 (the ligand for CD27) (Figure 6.3.1), CD153 (the ligand for CD30) (Figure 6.3.2), Ox-40L (the ligand for Ox-40) (Figure 6.3.3) and 4-1BBL (the ligand for 4-1BB) (Figure 6.3.4). DCs were once again identified by gating on CD11c⁺ B220⁻ cells, and the DC populations were sub-divided based on the level of surface MHC Class II expression.

The expression of the 4 molecules generally paralleled that of CD80 and ICOSL, with peak expression at day 1, followed by a decline, except on CD11c⁺B220⁻ MHC Class II^{int} cells where a second increase between days 4 and 7 was consistently observed in mice immunised with MCC in CFA.

6.4: Expression of I-E^k versus I-A^b

In the previous experiment, MHC Class II expression on DCs was shown to decline after immunisation with either MCC peptide or PBS in CFA. It was not clear whether this decrease was T cell dependent, as the antibody against MHC Class II (M5-114 directly conjugated to APC) binds to multiple MHC Class II alleles. Therefore the experiment was

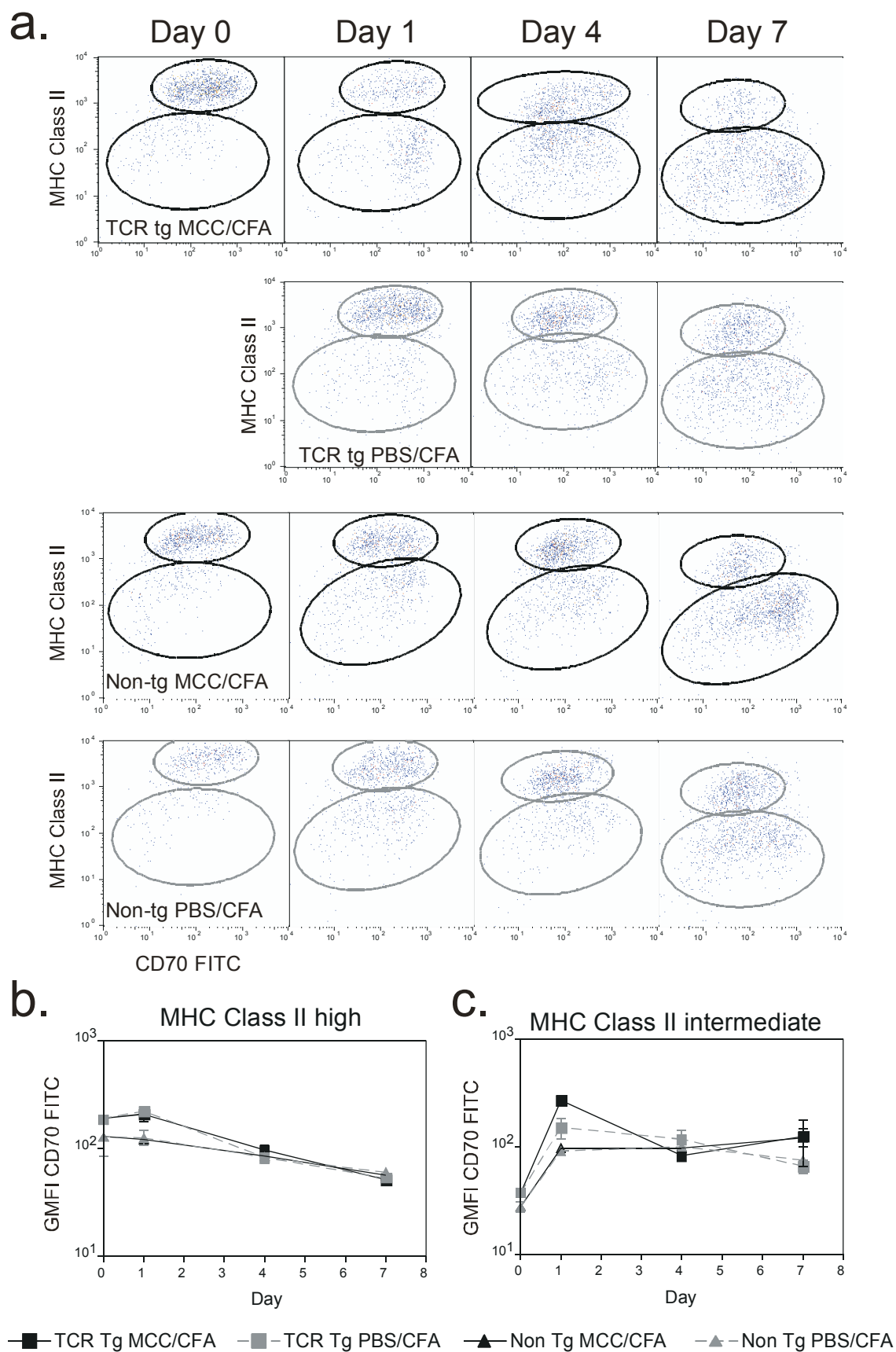


Figure 6.3.1: CD70 expression.

In the same experiment as described in Figure 6.1, draining lymph nodes were gated on B220 and PI⁻ and CD11c⁺. (a.) Dot plots show the expression of CD70 on the x axis versus MHC Class II. Graphs show the mean fluorescence of CD70 on the population of (b.) MHC Class II^{hi} and (c.) MHC Class II^{int}. Symbols represent the mean of 2 mice per group +/- SEM.

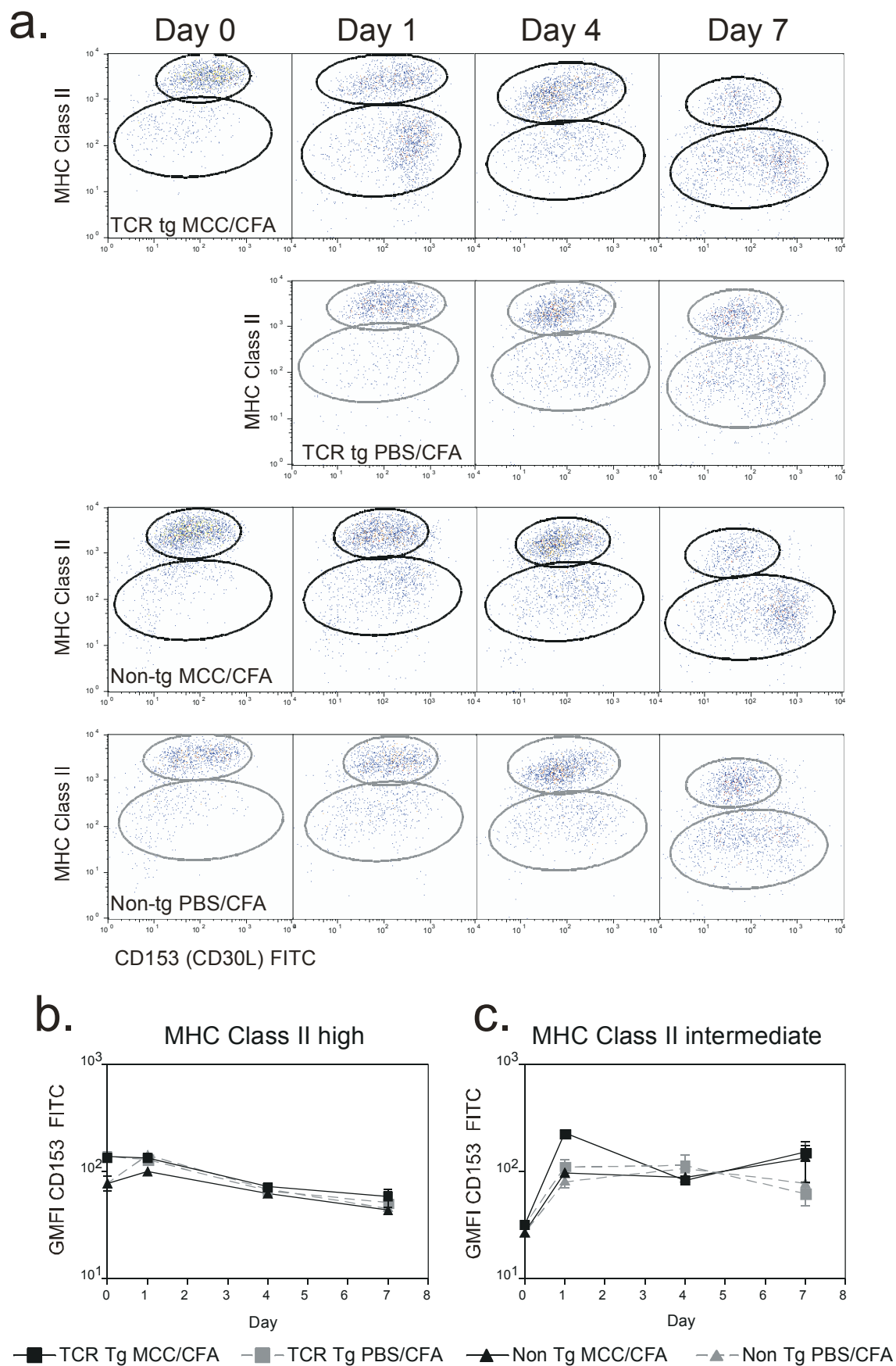


Figure 6.3.2: CD153 expression.

In the same experiment as described in Figure 6.1.1, draining lymph nodes were gated on B220 and PI⁻ and CD11c⁺. (a.) Dot plots show the expression of CD153 on the x axis versus MHC Class II. Graphs show the mean fluorescence of CD153 on the population of (b.) MHC Class II^{hi} and (c.) MHC Class II^{int}. Symbols represent the mean of 2 mice per group +/- SEM.

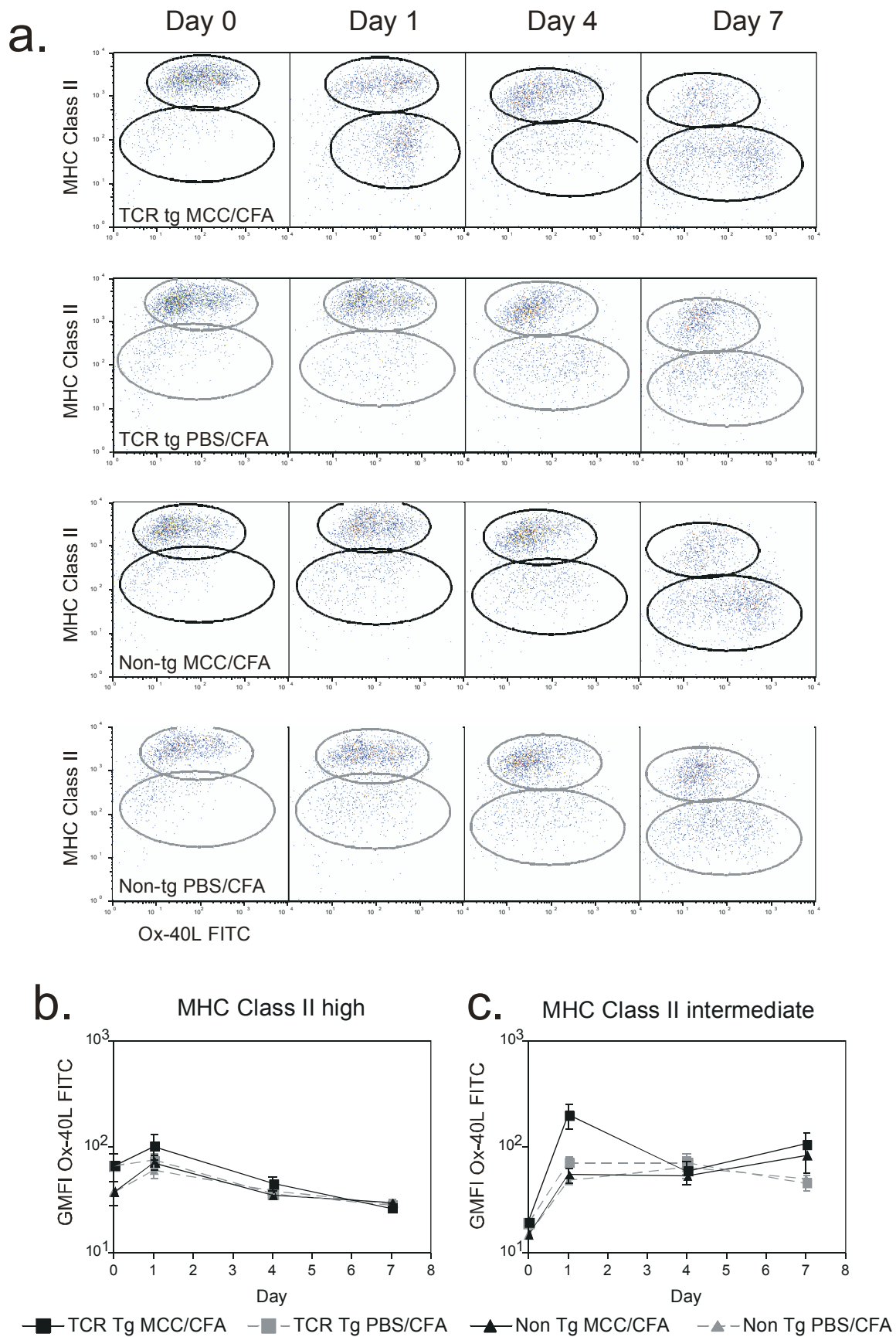


Figure 6.3.3: Ox-40L expression

In the same experiment as described in Figure 6.1.1, draining lymph nodes were gated on B220 and PI^- and $CD11c^+$. (a.) Dot plots show the expression of Ox-40L on the x axis versus MHC Class II. Graphs show the mean fluorescence of Ox-40L on the population of (b.) MHC Class II^{hi} and (c.) MHC Class II^{int}. Symbols represent the mean of 2 mice per group $\pm$ SEM.

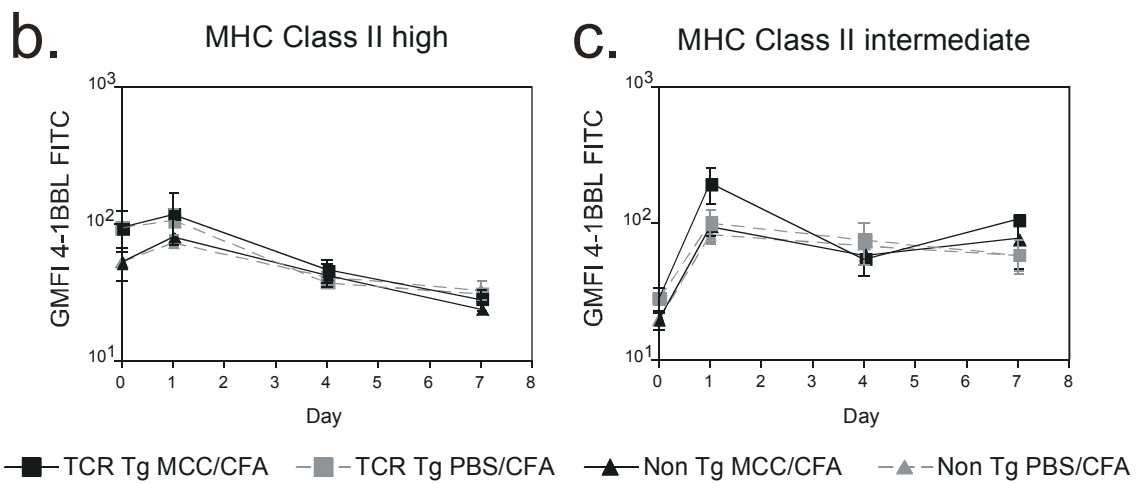
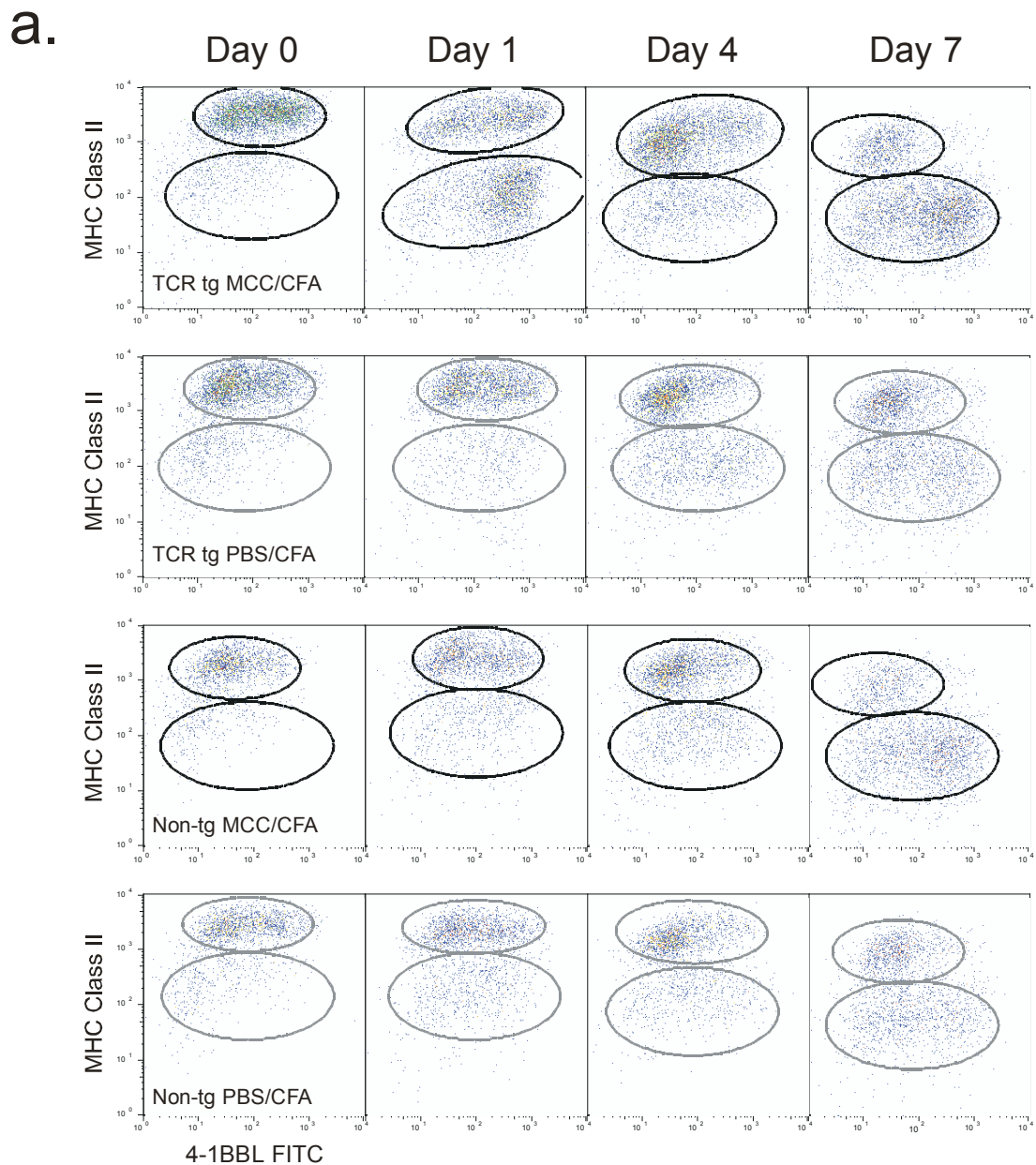


Figure 6.3.4: 4-1BBL expression

In the same experiment as described in Figure 6.1.1, draining lymph nodes were gated on B220 and PI^- and $CD11c^+$. (a.) Dot plots show the expression of 4-1BBL on the x axis versus MHC Class II. Graphs show the mean fluorescence of 4-1BBL on the population of (b.) MHC Class II^{hi} and (c.) MHC Class II^{int}. Symbols represent the mean of 2 mice per group +/- SEM.

repeated, and the expression of I-E^k and I-A^b tested independently. In this experiment only 5C.C7 TCR tg mice were examined as TCR tg and non-tg mice had displayed similar patterns of expression in the previous experiment. 5C.C7 TCR tg mice were immunised with either 10µg of MCC peptide emulsified in CFA or PBS alone in CFA 7, 4 and 1 days prior to sacrifice and analysis of draining LNs by flow cytometry. In this experiment, staining against CD80, B7RP-1, CD70 and Ox-40L was also performed, and while the staining against CD70, B7RP-1 and Ox-40L was not successful, CD80 expression displayed the same pattern as seen in the previous experiment (data not shown). DCs were gated for CD11c⁺ and MHC Class II⁺ (M5-114), B220⁻ and PI⁻.

Initially the level of both I-E^k and I-A^b expressed on the surface of DCs increased in mice immunised with MCC-CFA, compared with PBS-CFA. MHC Class II expression declined subsequently and by day 7 was below the baseline levels seen at day 0. However the expression of the allele presenting specific peptide to the majority of T cells (I-E^k) was higher than that of a control allele (I-A^b), indicating no decrease in the expression of the allele being recognised by T cells.

In summary after immunisation of 5C.C7 TCR tg mice with either MCC peptide or PBS alone in CFA, surface expression of MHC Class II on the total population of DCs was shown to decline. Surface I-E^k, I-A^b and total MHC Class II all declined in parallel.

Discussion

In this chapter the expression of a number of surface markers over the course of an adjuvant-enhanced T cell response were investigated. For all the markers analysed, changes in expression were independent of an ongoing T cell response, since the pattern of expression was similar to the control even in the absence of peptide (PBS/CFA) or antigen specific T cells (non-tg recipient). The adjuvant CFA is a mixture of heat killed *Mycobacterium tuberculosis* dissolved in mineral oil (Freund and McDermott 1942) and was originally shown to enhance immunoglobulin production due to its strong immune

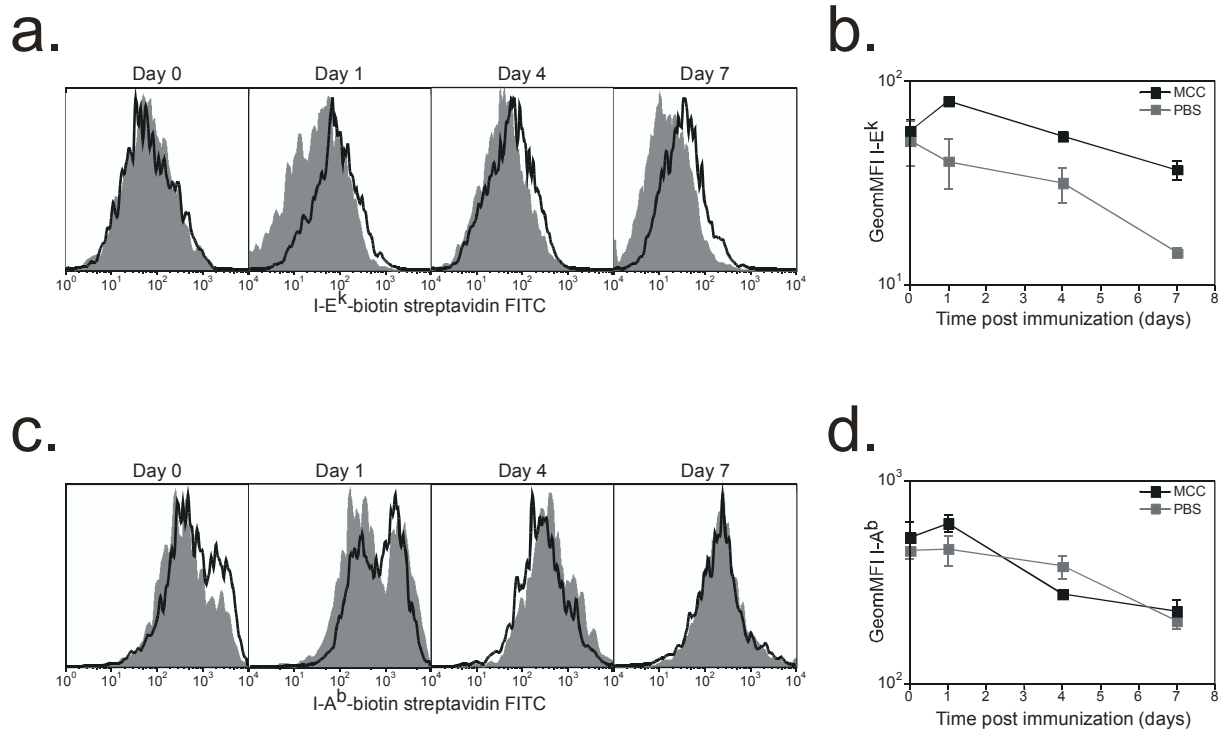


Figure 6.4. Expression of different MHC Class II alleles over the course of a response

Intact 5C.C7 TCR tg mice were immunized with 10 μ g MCC peptide in CFA or PBS alone in CFA 1, 4 and 7 days prior to sacrifice. Draining lymph nodes were harvested and the level of surface I-E^k and I-A^b measured by flow cytometry. Overlays represent the level of surface I-E^k or I-A^b expression on CD11c⁺, MHC Class II⁺ (M5-114⁺), B220⁻ cells from mice treated with MCC peptide in CFA (black) or PBS alone in CFA (grey). Graphs represent the geometric mean fluorescence intensity $\pm$ SEM (2 mice per group).

stimulatory capacity. This mixture contains a number different stimuli for PRR and TLR recognition systems of the innate immune system. It is therefore not surprising that immunisation with CFA alone induced upregulation of a number of costimulatory molecules. In fact the expression of all costimulatory molecules investigated has been shown to increase when DCs are treated with LPS or other inflammatory signals (Bullock and Yagita 2005; Futagawa et al. 2002; Goldie-Cregan et al. 2002; Hendriks et al. 2005; Hertz et al. 2001; Hochweller and Anderton 2005; Lee et al. 2003; Michelsen et al. 2001; Zhou et al. 2005). In one particular study, DCs were shown to require an inflammatory signal to upregulate the surface expression of CD80 and CD86 (Hochweller and Anderton 2005), whereas administration of PBS or peptide alone was not sufficient to increase expression and the effect of LPS alone was not measured. One of the primary roles of the immune system is to enhance the level of immune activation during infections. Therefore it is not surprising that a T cell independent enhancement of costimulatory molecules occurs when mice are injected with CFA.

For all the markers studied, an initial increase in surface expression was followed by a steady decline. A peak in the expression of most markers was observed at day 1. In mice immunised with MCC in CFA, an increase in the frequency of MHC Class II^{int} DCs and a second increase in the expression of a number of markers was observed between days 4 and 7. One potential explanation for this change could be due to an influx of new DCs to the draining LN, due to DC turnover during the primary response. In the earliest work characterising DCs it was shown that these cells have a a half-life of 5 days in the spleen (Steinman et al. 1974). More recent studies have estimated that the half-life of different DC subsets ranges from 1.5 to 2.9 days in the spleen (Kamath et al. 2000), while turnover time is slightly longer in cutaneous LNs (Kamath et al. 2002). Importantly both of these studies demonstrated enhanced turnover of a number of different DC subsets upon treatment with LPS (Kamath et al. 2002; Kamath et al. 2000). Interestingly, upon activation of plasmacytoid DCs (pDCs) with microbial signals, they can assume a more archetypal DC phenotype (O'Keeffe et al. 2002), so that the increase in the frequency of DCs expressing intermediate levels of MHC Class II could also be due to differentiation of

pDCs. The fact that there is a high degree of DC turnover under inflammatory conditions highlights the difficulty in measuring changes in the level of DC marker expression at the level of the total population.

In contrast to T cells, DCs do not readily home to the original site after purification and reinjection. Indeed some sub-types of DCs are unable to migrate into LNs from afferent lymphatics (Smith and Fazekas de St. Groth 1999). While DCs that are capable of migration into the LN do not migrate to the same extent as Langerhans or dermal DCs and represent only 1-2% of total DCs (Smith and Fazekas de St. Groth 1999) (Roediger, Chklovskaya and Fazekas unpublished). Therefore even if labelled DCs migrate back into the LN there is no guarantee that these will be the only DCs involved in the response. Secondly, membrane transfer between migrating DCs and resident DCs occurs upon injection of labelled DCs (Bedford et al. 1999; Knight et al. 1998) and under normal conditions (Roediger and Fazekas de St Groth unpublished data). Given that peptide-MHC transfer occurs between DCs, it would be difficult to distinguish between changes in the level of expression dependent on the T cell response or due to membrane transfer between DCs. While histology provides some idea about *in situ* localization of DCs and responding T cells, it does not provide the quantitative means to measure subtle changes in the expression of costimulatory molecules or MHC Class II. Finally, while an antibody against MCC/I-E^k has been used for in a number of *in vitro* systems (Colledge et al. 2001; Reay et al. 2000), this antibody has a high degree of cross-reactivity and is not sensitive enough to measure MCC-IE^k on freshly isolated LN samples (data not shown). Herein lies the current limitation in this system to determine whether the effect of competition is mediated by changes in peptide-MHC expression at the DC level.

One of the most interesting results seen in this series of experiments was the decline in surface MHC Class II expression after immunisation with CFA. At first glance this decline in MHC Class II fits nicely with the data in Chapter 3, where competition by a preactivated T cell response was shown to limit the recruitment of additional cells into an ongoing response. Since this inhibition could be overcome, to some extent, by injection of more antigen, the level of antigen presentation appeared to be mediating this suppressive

effect. However the decline in MHC Class II expression seen after injection of CFA was independent of an ongoing T cell response (Figure 6.4). Not only was there a reduction in the level of surface MHC Class II (Figure 6.1), but a simultaneous decline in expression of at least two MHC Class II alleles, I-E^k and I-A^b was also observed (Figure 6.4).

To date there has been only a single report of a specific decline in the peptide-MHC expression by DCs as a result of an ongoing T cell response (Kedl et al. 2002). In this experiment, GFP tagged DCs were pulsed with OVA peptide, injected into recipient mice and then the level of K^b-OVA expression on reisolated GFP-DCs was measured with a monoclonal antibody against K^b-OVA. The decline in K^b-OVA was shown to be highly antigen specific, and did not result in an overall decline in either K^b or total MHC Class I expression. As CD4 stimulation can be achieved with as few as 200 to 400 peptide-MHC complexes (Reay et al. 2000), it is not surprising that a specific reduction in a peptide-MHC complex does not result in an overall change in MHC expression. Therefore it is possible that an underlying change in the specific expression of MHC/peptide complexes could still be occurring in this system. Unfortunately, it is below the level of detection with the only available antibody against MHC/peptide or I-E^k.

In summary, it appears that the driving force behind the upregulation in the expression of costimulatory molecules is stimulation of DCs by microbial products binding to PRR and TLRs. While an overall decline in MHC Class II expression over the course of an adjuvant-enhanced response has consistently been observed in response to CFA injection, currently we do not have the means to track a relative change in the level of MHC/peptide expression.

Chapter 7: General Conclusions

Competition between lymphocytes is fundamental to the immune response ensuring that the most effective response is mounted to each challenge. For B cells, a competitive mechanism to select higher affinity clones is as necessary for the process of affinity maturation as somatic hypermutation of immunoglobulin genes (Berek et al. 1991; Jacob et al. 1991). For T cells, competition is first seen during ontogeny in the thymus, where T cells compete for access to MHC during positive selection (Berg et al. 1989). Competition between lymphocytes of different antigenic specificities has been postulated for many years, principally as a result of early studies in which the antibody titers in response to vaccination were lower if additional antigens were included in to the vaccine (reviewed in (Liacopoulos and Ben-Efraim 1975; Taussig 1973)). However, the mechanism of such competition, which could involve complex interactions between antigen and APCs, T cells and B cells, remains undefined. The experiments described in this thesis provide a detailed characterisation of competition between CD4⁺ T cells *in vivo*. By investigating multiple stages of the response, these experiments have provided insights into a number of general aspects of T cell biology.

Two types of competition

T cells may compete for space, antigen-MHC complexes, costimulatory molecules and/or APC derived factors. While competition for peptide-MHC, that is antigen specific competition, is a well-established aspect of both CD4⁺ and CD8⁺ T cell responses, the existence of antigen non-specific competition has been more difficult to determine.

Antigen Specific Competition

In these studies, competition between cells recognising the same antigen-MHC complex was shown to have a profound effect throughout the CD4⁺ T cell response (Table 7.1.1). Transferring high affinity CD4⁺ cells 24 hours or more into an ongoing response was shown to reduce recruitment of additional cells into the response, and divided cells progressed through fewer rounds of cell division. When an intermediate response was competing with a simultaneous high affinity response, both the frequency and total number of cytokine producing cells derived from lower affinity cells was reduced although the overall antigen specific response was not compromised. These findings are consistent with two recent reports in which competition led to a reduction in level of T cell stimulation and correspondingly decreased IFN- γ production (Foulds and Shen 2006; Obst et al. 2005). Interestingly the most profound effect of antigen specific competition was on the generation of memory cells. At first glance the data indicated that the presence of a high affinity response induced preferential differentiation of mixed affinity 5C.C7 β cells into a CD62L^{hi} T_{CM} phenotype, as has been previously reported to occur under conditions of reduced T cell stimulation (Marzo et al. 2005; van Faassen et al. 2005). However, after calculating the total number of T_{EM} and T_{CM} cells it became apparent that the change in the ratios of memory cell subsets was in fact due to a reduced number of T_{EM} cells. In addition to the reduction in the number of T_{EM} cells, the ability of these cells to secrete IFN- γ upon *ex vivo* restimulation was also reduced. Interestingly a stable population of T_{CM} cells were generated even in the face of a simultaneous high affinity response. This suggests that long-term memory is maintained even under conditions of reduced T cell stimulation (Bouneaud et al. 2005).

These experiments also demonstrated affinity maturation of the CD4 response through a competition-dependent mechanism operating continuously throughout the response. Affinity maturation was not due to active deletion of lower affinity cells, but rather involved reduced proliferation of 5C.C7 β cells. It is important to note that selection occurred predominantly within the effector population of CD4⁺ cells. This helps to explain the

Table 7.1.1: Summary of Antigen Specific Competition

Readout Response	Competing Response	Immunisation	Timing	Time between cohorts	Effect	Chapter
5C.C7 cells	5C.C7 cells	Peptide or protein	Day 3	24 hours	Reduced recruitment into division Reduced proliferation Reduced IFN- γ production	3 and 4
3A9 cells	3A9 cells	Peptide or protein	Day 3	24 hours	Decreased recruitment into division and proliferation by day 3	3 and 4
5C.C7 β cells	5C.C7 cells	Peptide	Days 7 onwards	No time interval	Decrease in expression of activation markers Reduced IFN- γ production Reduced proliferation (BrdU) Decreased total number of effector cells Decreased number of effector memory cells	5

Table 7.1.2: Summary of Antigen Non-specific Competition

Readout Response	Competing Response	Immunisation	Timing	Time between cohorts	Effect	Chapter
5C.C7 cells	3A9 cells	Recombinant protein	Day 3	24 hours	Minor decrease in recruitment into division with no difference in cell numbers by day 10	4
3A9 cells	5C.C7 cells	Peptide	2 hours	4 days	Decreased percentage of CD69 ⁺ cells without any effect on recruitment or proliferation	3
3A9 cells		Recombinant protein	Day 3	3 days	Reduced recruitment into division but no effect on the total number of memory cells	4
5C.C7 β cells	3A9 cells	Peptide	Day 10	No time interval	Decreased total number of effector cells No effect on the generation of memory cells	5

discrepancy between previously published reports as to the timing of affinity maturation. Some indicate that repertoire narrowing occurs early in the primary response (Callan et al. 2000; Fasso et al. 2000; Malherbe et al. 2004; Maryanski et al. 1996; McHeyzer-Williams et al. 1999), while others saw a further contraction during the selection of memory cells in to the secondary response (Busch and Pamer 1999; Busch et al. 1998; McHeyzer-Williams and Davis 1995). Importantly most of these studies did not measure affinity maturation based on effector function, and therefore provided an estimate based solely on numbers, which in the experiments reported here did not change from day 7 onwards (Figure 5.8).

Competition between cells recognising the same antigen-MHC complex reduced the size of the response per cell without affecting the overall size of the response. Previous experiments by Adrian Smith observed a similar restriction to the overall size of the response (Smith 2002; Smith et al. 2000). When increasing numbers of adoptively transferred 5C.C7 cells (10^4 , 10^5 , 10^6 , 10^7) were stimulated with a set dose of MCC ($10\mu\text{g}$), the starting 1000-fold difference in precursor frequency was reduced to only 12-fold by day 7, and by day 42 there was only a 2-fold difference in antigen specific cell numbers between those that received the highest and lowest number of cells. At high precursors frequencies resulting from transfer of 10^7 cells, interclonal competition markedly limited the expansion of 5C.C7 cells, while the absence of competition at low precursor frequencies enabled the unrestricted expansion of 5C.C7 cells. Another group has recently shown a similar effect of interclonal competition (Catron et al. 2006). By transferring graded numbers of antigen specific cells or increasing the dose of antigen, the proliferation of each antigen-specific cell was enhanced. Together with the experiments described in this thesis, these data demonstrate an important role of competition for peptide-MHC in controlling the size of the antigen specific response. The profound effect of antigen specific competition at multiple stages of the response highlights the reliance of CD4^+ T cells on continued TCR stimulation, providing the immune system with a means to regulate the size of the response according to the availability of antigen. The experiments described in this thesis, together with a number of

recent reports (Hafalla et al. 2002; Hermans et al. 2000; Norbury et al. 2002) suggest that an ongoing response regulates the level of antigen presentation, thereby maintaining control over the size of the response. It has been suggested that CD8⁺ T cells can regulate the size of the response by developing the capacity to destroy antigen presenting DCs (Hafalla et al. 2003; Hafalla et al. 2002; Hermans et al. 2000; Wong and Pamer 2003; Yang et al. 2006). However a number of recent *in vitro* reports suggest that CD4⁺ T cells may control the level of antigen by actively removing peptide-MHC complexes from the surface of APCs (Huang et al. 1999; Hudrisier et al. 2001; Hwang et al. 2000; Patel et al. 1999; Tsang et al. 2003; Wetzel et al. 2005). Unfortunately the available mAb against MCC/IE^k complexes could not reliably detect *in vivo* expression of this antigen-MHC complex (data not shown). However two publications have reported a decline in specific antigen-MHC complexes over the course of an *in vivo* response (Catron et al. 2006; Kedl et al. 2002). Although difficult to study *in vivo*, antigen/MHC capture provides an elegant means by which the immune system can control the size of an ongoing response without affecting the response to other antigens.

In summary, antigen specific competition is a consistent and important feature of both CD4⁺ and CD8⁺ T cell responses. Competition provides a means of controlling the burst size of precursors, while its effects on affinity distribution increase the overall strength of the response. Importantly, selection occurs primarily in the pool of cytokine secreting T cells, ensuring that a stable population of memory cells is maintained even in the face of competition.

Antigen Non-specific Competition

In contrast to the profound effect of competition between cells recognising the same antigen-MHC complex, antigen non-specific competition affected only the burst size of the primary response, but not the generation of long-lived memory cells (Table 7.1.2). In the

first series of experiments, antigen non-specific competition induced only a minor change in recruitment even when both antigens were presented on the same APCs. In the second series of experiments, competition by a high affinity 3A9 response led to only a minor reduction in the size of the effector 5C.C7 β response. Even with a 100-fold excess in the number of competing cells, the generation of memory cells was not affected by a simultaneous high affinity response to an independent antigen (Figure 5.7.4).

Importantly, all stages of the CD4⁺ response were investigated in this study. In contrast, many other studies investigated only one timepoint or parameter (Creusot et al. 2003; Hayball et al. 2004; Kedl et al. 2000; Kedl et al. 2002; Probst et al. 2002) (Table 7.2.1 and 7.2.2). In this study, antigen non-specific competition was not observed between cells of relatively high affinity, consistent with the results of Probst (2002). However, with a relatively low affinity response, competition by 3A9 cells was shown to have a detectable effect on the total number of 5C.C7 β cells at day 10 of the response (2-fold reduction) similar to the level of reduction observed in other studies (Kedl et al. 2000; Kedl et al. 2002). However the frequency of cytokine producing cells showed no effect of antigen non-specific competition, while enhancement of the frequency of cytokine production was observed in the presence of a 100-fold excess of competing 3A9 cells (Figure 5.7.4), similar to a previous report (Creusot et al. 2003). This illustrates the importance of calculating responder cell numbers as well as frequency. A second complication in interpreting previous studies is the fact that the competing cohorts of T cells recognise epitopes derived from the same protein or antigens presented by the same MHC allele. In the work of Kedl (2000), both peptides were presented by the same MHC Class I allele, therefore underlying competition for presentation was also involved. Taken together these results presented here support the conclusion that the primary source of competition seen between viral proteins or coadministered proteins is in fact a result of antigen processing events and not competition between responding T cells. Since antigen non-specific competition induced only a minimal effect on burst size, it would also suggest that signals independent of MHC serve primarily to enhance the size of the effector pool (Busch et al. 2000). Importantly the generation of memory cells is not reliant of the level of

Table 7.2.1: Summary of *in vivo* CD4 competition papers

Paper	Readout Response	Competition	Competing Response	Cell Number	Antigen	Time	Effect
Smith 2000	Transferred 5C.C7 or MBP cells	Ag specific	Endogenous TCR tg population of 5C.C7 or MBP cells	5-10x10 ⁶	200ng MCC and 55µg MBP peptides in CFA	Day 3	Reduced proliferation and total number of dividing cells
Creusot 2003	Endogenous OVA or PCC response or transferred OVA cells	Enhancement of Ag non-specific response	Transferred OVA or PCC specific cells	10 ⁴ , 10 ⁵ or 10 ⁶ cells	Gold/DNA construct encoding OVA, PCC or OVA and PCC	Day 5	Increased in number of IFN-γ, IL-2 and IL-4 positive cells (measured by ELISPOT)
Hayball 2004	Transferred HNT or OT-II cells	Ag specific and Ag non-specific	HNT or OT-II cells transferred 20 hours before readout cohort	20x10 ⁷	3µg HA and 10µg OVA peptides in Incomplete Freund's Adjuvant	Day 3	Reduced proliferation and increase in percentage of undivided cells
Foulds 2006	CFSE labelled OT-II or D011.10 cells	Ag specific	Unlabelled cohort of OT-II or D011.10	2x10 ⁵	rLM-OVA	Day 3 and 8	Proliferation and percentage of IFN-γ producing cells

Table 7.2.2: Summary of *in vivo* CD8 competition papers

Paper	Readout Response	Competition	Competing Response	Cell Number	Antigen	Time	Effect
Butz 1998	Endogenous gp276 response	Ag non-specific	Transferred P14 cells (gp33 specific)	1x10 ⁶	LCMV infection	Day 8	Decreased Cr release on ex vivo stimulation with gp276
Kedl 2000	Endogenous K ^b /SIINFEKL tetramer positive cells	Ag specific	Transferred OT-I cells	0.2-1x10 ⁶	VV-OVA (vaccina virus expressing OVA)	Day 9	Decreased percentage of tetramer positive cells within the total CD8 response
	Endogenous K ^b /KVVRDKL response	Ag non-specific	Transferred OT-I cells	5x10 ⁵	VV-OVA	Day 9	Decreased percentage of tetramer positive cells within the total CD8 response
Probst 2002	Endogenous K ^b /SIYRYGYL and K ^b /SIINFEKL	Ag non-specific	Transferred OT-I cells	1-2x10 ⁶	1.5x10 ⁶ DCs pulsed with 0.5µg of SIINFEKL SIYRYGYL or both	Day 5	Decreased percentage of tetramer positive cells within the total CD8 response
	Endogenous D ^b gp33-41, K ^b gp34-41 or D ^b gp276-286	Ag specific with no evidence of non-specific	Transferred 318 (gp33-41 specific cells)	10 ⁴ , 10 ⁵ or 10 ⁶ cells	LCMV infection	Day 12	Decreased percentage of tetramer positive cells
Kedl 2002	Endogenous OVA or gp33 response	Ag specific and non-specific	Transferred OT-I or P14 cells	2x10 ⁶	10 ⁵ DCs expressing gp1-60 LCMV or VV expressing gp protein	Day 7	
	Endogenous Kb-SIY or Kb-OVA cells	Ag specific and non-specific	Transferred OT-I cells	2.5x10 ⁵ , 1x10 ⁶ and 2x10 ⁶	5x10 ⁵ DCs pulsed with 10-100µg/ml OVA and GP33 peptides	Day 9	Reduced percentage of tetramer+ cells (K ^b -OVA or D ^b -GP33)
Wong 2003	Memory p60 specific cells (WP11.12)	Ag specific	Endogenous p60 ₄₄₉₋₄₅₇ or LLO ₉₁₋₉₉ specific cells	3x10 ⁶	DCs pulsed with OVA and SIY	Day 5	Reduced percentage of K ^b -SIY and K ^b -OVA cells within the total CD8 ⁺ T cells
Hafalla 2003	malTg T cells	Ag specific	Cohort of malTg cells transferred 2 days earlier	2x10 ⁶	2000 <i>L. monocytogenes</i> per animal	Day 4	Reduced proliferation, specific lysis
Marzo 2005	OT-I Tg cells	Ag specific	OT-I cells	1x10 ⁷ , 1x10 ⁴ , 5x10 ²	<i>P.yoei</i> malaria parasites	Day 3	Reduced proliferation
					Infected with VSV-OVA and rested for 198 days, rechallenged with LM-OVA	Day 40 (challenge)	Increased frequency of CD62L ^{hi} central memory cells

costimulatory marker expression or survival factors, so that the presence of multiple ongoing responses does not prejudice the memory response.

In summary, by investigating multiple stages of the CD4 response, namely recruitment, proliferation, generation of cytokine producing cells and generation of memory cells, conclusions about the overall effect of competition on the total CD4 response can be drawn. These data have shown that competition for the same antigen-MHC complex operates continuously throughout the response to control the overall size of the response and increase its overall affinity, ensuring that the most efficient response is generated. In contrast, competition between cells of different antigen specificity has only a minimal effect on the primary response, and none at all on the generation of memory cells. Therefore the immune system retains its capacity to respond fully to secondary infections or antigens expressed only late in the infection. Since the size of the response is regulated by the continued availability of antigens, the future design of multi-epitope vaccines will need to ensure high level, long-term antigen presentation in order to generate effective T_{EM} responses.

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