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Role of B cells in maintaining helper T-cell memory

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The cellular interactions involved in maintaining $CD4^+$ T-cell memory have hitherto not been identified. In this report, we have investigated the role played by B cells in this process. We show that that long-lasting helper T-cell memory depends on the presence of B cells, but that direct antigen presentation by B cells is not required. These findings provide new insights into the mechanisms which underlie helper T-cell memory. They also suggest that the efficacy of future vaccines will depend critically on the inclusion of B- as well as T-cell epitopes.

Keywords: B cells; $CD4^+$ T cells; antibody; antigen presentation; follicular dendritic cells; antigen dependence

1. INTRODUCTION

Immunological T-cell memory is an area of intense interest (for reviews, see Ahmed & Gray 1996; Sprent 1994), but a consensus has yet to emerge as to how it is generated and maintained. Rival theories state either that memory T cells are specialized, long-lived clones (Bruno *et al.* 1995), or that they comprise a population needing continual stimulation by antigen in order to persist (Gray & Matzinger 1991).

In the case of $CD8^+$ T-cell memory, recent reports suggest that persisting antigen may not be required (Mullbacher 1994; Lau *et al.* 1994; Hou *et. al.* 1994): instead, cross-reactivity with other antigens (Selin *et al.* 1994; Beverley 1990) and activation by cytokines (Tough *et al.* 1996; Zhang *et al.* 1998) have both been suggested as means by which $CD8^+$ memory T cells are maintained. In contrast, few studies have addressed the requirements for maintaining $CD4^+$ T-cell memory (Gray & Matzinger 1991). Bearing in mind the differences in the nature of antigens recognized by $CD8^+$ and $CD4^+$ T cells (intracellular versus extracellular), and in the cell types which can present them (almost all cells express major histocompatibility (MHC) class I, whereas only specialized antigen-presenting cells (APCs) express MHC class II), it seems likely that $CD8^+$ and $CD4^+$ T-cell memory could be maintained by different mechanisms.

It has long been suggested (Feldbush 1973) that intermittent stimulation by persisting antigen is necessary for the survival of both memory helper T cells (Gray & Matzinger 1991) and memory B cells (Gray & Skarvall 1988). The only known means by which non-replicating antigens can be retained *in vivo* for long periods is in the form of immune complexes on the surface of follicular dendritic cells (FDCs; Mandel *et al.* 1980). If these are required to maintain helper T-cell memory, it follows that this memory should depend on the production of antibodies by B cells. We tested this directly by examining the longevity of $CD4^+$ T-cell memory in mice which lack B cells.

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2. MATERIAL AND METHODS

(a) *Animals*

B6 μMT (Kitamura & Rajewsky 1992), B10 severe combined immunodeficient (SCID), C57BL/6 and B10.D2/n mice were six to ten weeks old at the start of each experiment. Mice were maintained on sterile food and bedding in filter-topped cages. Thymi were taken from C57BL/6 foetuses at day 14 of gestation and up to six were implanted under the kidney capsules of B10 SCID mice. Six to ten weeks later, reconstitution of T cells in peripheral blood leucocytes (PBL) was confirmed by fluorescence-activated cell sorter (FACS) analysis using fluorescein isothiocyanate (FITC)-labelled anti-Thy-1 (provided by H. von Boemer). B6 μMT mice were backcrossed with congenic B10.D2/n mice, and expression of $H\text{-}2K^b$ and $H\text{-}2D^d$ on PBLs was assessed by FACS analysis using biotinylated monoclonal antibodies AF6–88 and 19–191 (both provided by A. Livingstone), respectively, followed by PE-streptavidin. B220 expression was detected using FITC-labelled RA3–6B2 (Pharmingen, San Diego, CA, USA).

(b) *B-cell reconstitution and priming*

B10 SCID mice and B6 μMT mice were reconstituted intravenously with 10^7 B cells purified (Kapasi *et al.* 1993) from non-immune C57BL/6 mice. These mice were immunized intraperitoneally at the same time with 50 μg keyhole limpet haemocyanin (KLH) (Calbiochem, San Diego, CA, USA). KLH, possibly due to its large size, is one of the few antigens which is immunogenic in this form. To prevent possible attack by natural killer-cells, $H\text{-}2^{b/d}$ μMT mice were irradiated with 5 Gy one day before the injection of 10^7 B cells from C57BL/6 or

Bl0.D2/n mice. These mice were immunized one week later, to allow time for the regeneration of T cells. Two weeks after immunization, all mice were bled and serum immunoglobulin levels and anti-KLH IgG and IgM were measured as described (Gray *et al.* 1994). At the longest time-point analysed (12 weeks), donor B cells were still detectable in splenocytes by FACS analysis of B220 expression (as above).

(c) *Limiting dilution analysis*

Frequencies of KLH-specific IL-2 producing T cells were measured in reconstituted SCID and H-2^b μMT mice as described (Van Essen *et al.* 1995). All LDAs had χ^2-test probabilities of >0.05, indicating that the data was well fitted by a Poisson distribution of responder cells. To measure H-2^b- and H-2^d-restricted T-cell frequencies in reconstituted H-$2^{b/d}$ μMT mice, $CD4^+$ T cells were highly purified from responder splenocytes by depletion of MHC class II^+, $CD8^+$, $CD11c^+$ and IgM^+ cells using biotinylated monoclonal antibodies M5/114 (ATCC), 53.6.72 (ATCC) and N418 (32), and goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, UK), respectively, followed by two rounds of MACS separation (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of sorted cells was routinely $>99.5\%$, and these cells did not proliferate in response to either KLH or immobilized anti-CD_3 (145.2C11) unless exogenous APCs were added. Otherwise, limiting dilution analysis was as above. It should be noted that T cells restricted by hybrid H-2^b/H-2^d MHC molecules (e.g. I-E^b) will not be detected in these analyses.

(d) *Analysis of MHC restriction of antigen-specific T cells*

2×10^6 purified $CD4^+$ T cells, from an H-$2^{b/d}$ μMT mouse given H-2^d (C57BL/6) B cells, were cultured with an equal number of irradiated (30 Gy) H-2^d (Bl0.D2/n) APCs plus 100 μg ml^{-1} KLH and 100 U ml^{-1} IL-2. Two weeks later, cells were washed and re-cultured at 100 cells per well with 2×10^5 APCs (either H-2^b or H-2^d) and 100 U ml^{-1} IL-2, with and without KLH. After a further two weeks, wells were scored microscopically for cell growth. Numbers of positive wells per number of total wells: H-2^b APC + KLH, 0/96; H-2^b APC alone, 0/96; H-2^d APC + KLH, 16/96; H-2^d APC alone, 0/96 ($p<10^{-4}$, Fisher's exact test).

3. RESULTS AND DISCUSSION

SCID mice have neither T nor B cells, but T cells can be regenerated in these mice by implantation of foetal thymi from wild-type mice. We immunized mice treated in this way with soluble KLH, and injected half of them at the same time with syngeneic B cells. Soluble antigen was used in order to minimize the possibility of forming any artificial deposits *in vivo*—thus, retention of antigen should occur solely by the trapping of immune complexes on FDCs. Two weeks after immunization, all the mice which had received B cells had produced serum anti-KLH IgM and IgG antibodies, indicating successful T- and B-cell priming (not shown). Ten weeks after immunization, we measured the frequencies of KLH-specific helper T cells in each mouse. At this stage, the primary immune response has long since finished, and the continued presence of elevated numbers of antigen-specific cells is indicative of immunological memory. We found that those mice which had received B cells had significantly higher numbers of KLH-specific helper T cells than those which had not (figure 1). This demonstrates that $CD4^+$ T-cell memory is impaired in the absence of B cells.

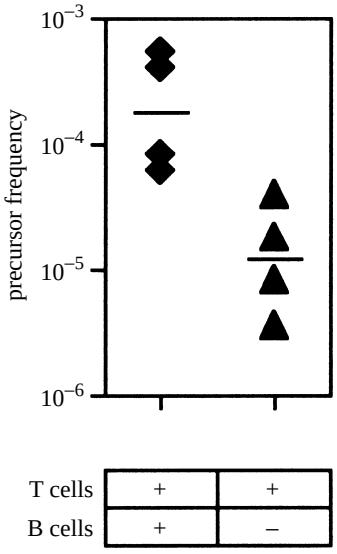

Figure 1. $CD4^+$ T-cell memory is impaired in T-cell-reconstituted SCID mice without B cells. Frequencies of KLH-specific helper T cells were assayed by limiting dilution analysis of IL-2 producing cells, ten weeks after immunization with 50 μg soluble KLH. SCID mice were grafted with thymi from syngeneic C57BL/6 mice, and half received 10^7 C57BL/6 B cells intravenously at the same time as immunization (diamonds). The remainder did not receive any B cells (triangles). Each point represents the frequency in one mouse. Geometric mean frequencies (lines): SCID + T cells and B cells, 1.8×10^{-4}, SCID + T cells only, 1.2×10^{-5} ($p=0.012$, two-tailed, unpaired Student's *t*-test). Unimmunized mice typically exhibit frequencies of less than 2×10^{-6} (not shown).

There are two alternative explanations for the above result: B cells could be needed (i) for the maintenance of helper T-cell memory, or (ii) for the initial clonal expansion of antigen-specific T cells. We investigated the latter possibility by measuring the frequencies of antigen-specific T cells at the peak of the primary response, two weeks after immunization. The experimental protocol was essentially as above, except that μMT mice were used in place of SCID mice. μMT mice have a disrupted immunoglobulin $C\mu$ gene, resulting in the complete absence of mature B cells in the periphery (Kitamura & Rajewsky 1992). Again, mice were immunized with soluble KLH, and half were injected with syngeneic B cells. Two weeks later, only those mice which had received B cells had produced serum anti-KLH antibodies (not shown). We measured the frequencies of KLH-specific helper T cells after two weeks, to assess their initial clonal expansion, and again after 12 weeks, to test for T-cell memory. As shown in figure 2*a*, two weeks after immunization there was no difference in the level of T-cell priming between mice which had, and which did not have, B cells. This is in line with another recent report on T-cell priming in B-cell-deficient mice (Epstein *et al.* 1995). After 12 weeks, as with the SCID mice, the number of antigen-specific T cells has decayed more rapidly in μMT mice which have not been given B cells (figure 2*b*). These data confirm that while B cells are not needed for the initial expansion of antigen-specific T cells,

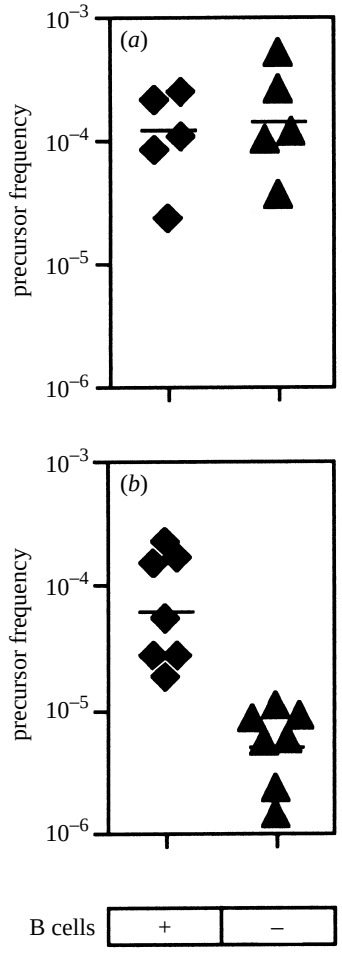

Figure 2. $CD4^+$ T-cell memory, but not priming, is impaired in μMT mice without B cells. Frequencies of KLH-specific helper T cells in μMT mice, (*a*) two weeks, and (*b*) 12 weeks after immunization with KLH. Mice were immunized and given C57BL/6 B cells as in figure 1. Geometric mean frequencies: (*a*) μMTs + B cells (diamonds) 1.4×10^{-4}, μMTs without B cells (triangles), 1.3×10^{-4} ($p = 0.81$); (*b*) μMTs + B cells, 6.3×10^{-5}, μMTs without B cells, 5.1×10^{-6} ($p = 2.6 \times 10^{-4}$).

they are vital for the maintenance of helper T-cell memory.

Topham *et al.* (1996) have previously studied $CD4^+$ T-cell memory to influenza virus infection in μMT and wild-type mice. In contrast to the results presented above, they concluded that B cells were not required for long-lasting helper T-cell memory. However, the use of live virus as an immunogen makes it difficult to exclude the possibility of incomplete viral clearance, thereby bypassing any need for antigen localization on FDCs. This is particularly relevant in immunocompromised animals, and indeed several reports suggest that B cells are important for the elimination of influenza viruses (Iwasaki & Nozima 1977; Kris *et al.* 1988; Palladino *et al.* 1995). Although the authors found it to be effectively cleared from the site of infection (lungs), this virus is able to infect all cell types, as its receptor, sialic acid, is ubiquitously expressed.

While FDCs can bind and retain immune complexes on their surface for long periods of time, they are unable to endocytose and process them for presentation to T cells (Gray *et al.* 1991). For this, an intermediary APC is needed. Previous studies have shown that B cells are able to acquire antigen by the endocytosis of bead-like, immune-complex-coated bodies (iccosomes), derived from the dendrites of FDCs (Szakal *et al.* 1988), and that they can present this antigen to $CD4^+$ T cells *in vitro* (Gray *et al.* 1991; Kosco *et al.* 1988). We therefore investigated the requirement for antigen presentation by B cells to maintain helper T-cell memory. Since mice which lack B cells not only cannot form immune complexes, but also cannot support the development of FDCs (MacLennan & Gray 1986; Kapasi *et al.* 1993), we generated chimaeric mice which contained B cells, but in which half of the T cells could not recognize the antigen presented by them. We backcrossed μMT mice ($H\text{-}2^b$) onto the $H\text{-}2^d$ genetic background, and generated F_1 mice expressing both *b* and *d* alleles at the H-2 locus ($H\text{-}2^{b/d}$ μMT mice). These mice contain T cells which recognize antigen presented on either $H\text{-}2^b$ or $H\text{-}2^d$ MHC molecules. We then reconstituted these mice with $H\text{-}2^b$ B cells: the resulting chimaeric animals generated antibody responses when immunized (not shown), but only $H\text{-}2^b$-restricted T cells can recognize antigen presented by B cells. We reconstituted a second group of mice in the reverse fashion, using $H\text{-}2^d$ B cells. All mice were then immunized as before, with soluble KLH.

Two weeks and 12 weeks later, we performed limiting dilution analyses of the $CD4^+$ T cell from these mice, using either $H\text{-}2^b$- or $H\text{-}2^d$-expressing APCs. In this way, we were able to independently determine the frequencies of $H\text{-}2^b$- and $H\text{-}2^d$-restricted KLH-specific T cells. Figure 3*a*,*b* illustrates that two weeks after immunization, there was no difference in the level of T-cell priming between these two populations. This corroborates our earlier finding that clonal expansion proceeds normally in the absence of B cells. Twelve weeks after immunization, we found, to our surprise, that antigen-specific T cells which could not recognize antigen presented by B cells were still present at elevated levels, which were comparable to the levels of antigen-specific T cells that could do so (figure 3*c*,*d*). Thus, it seems that while B cells are required for the survival of $CD4^+$ memory T-cells (figures 1 and 2), they are not needed as APCs (figure 3). It remained possible that the same T cell could recognize KLH-derived peptides presented on both $H\text{-}2^b$ and $H\text{-}2^d$ MHC molecules. This is unlikely, in view of the differences in structure and in peptide-binding preferences (Brusic *et al.* 1997) between $I\text{-}A^b$ and $I\text{-}A^d$ molecules. Nevertheless, we verified that this did not occur by stimulating bulk cultures of $CD4^+$ T cells from an $H\text{-}2^{b/d}$ μMT mouse given $H\text{-}2^d$ B cells, using $H\text{-}2^d$-expressing APCs. After two weeks, we purified and re-stimulated these T cells under limiting dilution conditions using either $H\text{-}2^d$- or $H\text{-}2^b$-expressing APCs. Whereas clones of T cells were readily stimulated by APCs expressing $H\text{-}2^d$, none were stimulated by APCs expressing $H\text{-}2^b$. This confirms that $H\text{-}2^d$-restricted T cells in this mouse could not recognize antigen presented on $H\text{-}2^b$ MHC molecules, thereby strengthening the notion that $CD4^+$ T-cell memory does not depend on antigen presentation by B cells.

How, then, does the presence of B cells affect the persistence of helper T-cell memory? The data outlined here support a model in which antibody production by B cells is needed for generation of immune complexes,

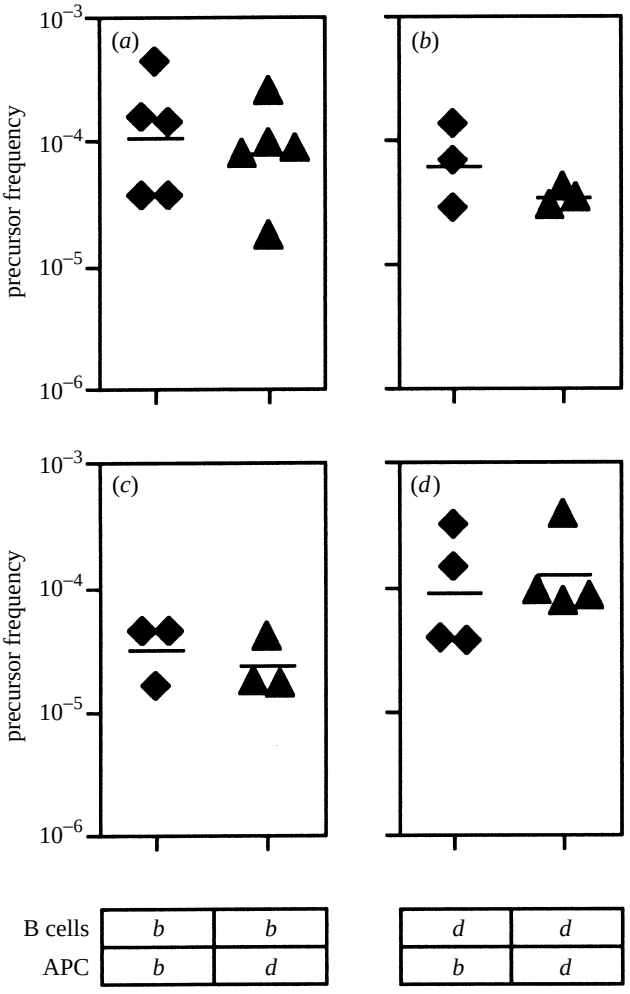

Figure 3. $CD4^+$ T-cell memory does not require antigen presentation by B cells. Frequencies of H-2^b- and H-2^d-restricted KLH-specific helper T cells in H-$2^{b/d}$ μMT mice at (*a*,*b*) two weeks and (*c*,*d*) 12 weeks after immunization with KLH. H-$2^{b/d}$ μMT mice were lightly irradiated and reconstituted with 10^7 B cells from congenic H-2^b (C57BL/6 (*a*,*c*)), or H-2^d (B10.D2/n (*b*,*d*)) donors. One week later they were immunized as in figure 1. Geometric mean frequencies: (*a*) H-2^b-restricted (diamonds), 1.1×10^{-4}, H-2^d-restricted (triangles), 7.9×10^{-5} ($p = 0.41$, two-tailed, paired Student's *t*-test); (*b*) H-2^b-restricted, 6.2×10^{-5}, H-2^d-restricted, 3.4×10^{-5} ($p = 0.40$); (*c*) H-2^b-restricted, 3.2×10^{-5}, H-2^d-restricted, 2.3×10^{-5} ($p = 0.53$); (*d*) H-2^b-restricted, 9.1×10^{-5}, H-2^d-restricted, 1.2×10^{-4} ($p = 0.66$).

and thus for long-term retention of antigen. This antigen is periodically presented to memory $CD4^+$ T cells, providing a stimulus for their survival, by APCs which are not B cells. This theory demands that there are cell types other than B cells which can acquire antigen from FDCs and present it to T cells. One promising candidate for this is the recently described germinal centre dendritic cell (Grouard *et al.* 1996). These cells express complement and F_c receptors and can bind immune complexes, but unlike interdigitating dendritic cells they have minimal ability to take up soluble antigens. *In vivo* they are found in germinal centres, in close proximity both to T cells and to FDCs. Experiments are underway to investigate whether these cells are involved in the maintenance of T-cell memory. The results do not rule out the possibility that B cells provide a trophic signal (cytokine?) that enhances $CD4^+$ memory T-cell survival in a manner similar to the influence of type I interferons and IL-15 on $CD8^+$ memory cells (Tough *et al.* 1996; Zhang *et al.* 1998). However, recent results (D. van Essen, A. Cutler and D. Gray, unpublished data) have shown that Clq-deficient mice (Cutler *et al.* 1998), which do not localize antigens to FDC, also have impaired maintenance of $CD4^+$ T-cell memory.

In conclusion, we have shown that $CD4^+$ T-cell memory decays in the absence of B cells. This is markedly different from the published reports on $CD8^+$ T-cell memory (Asano & Ahmed 1996; Di Rosa & Matzinger 1996), implying that $CD4^+$ and $CD8^+$ memory T cells are not maintained by the same mechanism. Moreover, we have demonstrated that the role of B cells in prolonging helper T-cell memory is not as APCs. This poses an important new question: What cell type(s) are required to present antigen to $CD4^+$ memory T cells?

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