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CD4 T–cell memory can persist in the absence of class II

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THE ROYAL SOCIETY

CD4 T-cell memory can persist in the absence of class II

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To understand how memory CD4 T cells are generated we have re-examined the requirements for continuing antigen stimulation in the generation and persistence of this population. We find that specific antigen is only required for a short period during the activation of naive CD4 T cells and is not required for memory generation from activated CD4 T cells or for persistence of resting memory cells generated by transfer of activated CD4 to adoptive hosts. Moreover, transfer of activated CD4 T cells to class-II-deficient hosts, indicates that TcR–class II major histocompatibility interaction is also unnecessary for either the transition from activated CD4 T cell to resting memory cells or for persistence over an eight-week period. Thus the signals regulating generation and maintenance of memory are fundamentally different from those which regulate the expansion of effector CD4 T-cell populations which include antigen itself and the CD4 T-cell autocrine cytokines induced by antigen.

Keywords: memory; cytokines; T cell; apoptosis; life span

1. INTRODUCTION

We believe that the qualitative changes that occur in CD4 T cells, which are associated with their transition from so-called naive (non-antigen-experienced) T cells to memory T cells, are a critical component of protective immunity. We have identified several important functional properties of memory CD4 T cells that are consistent with their presumed ability to engage in faster, more efficient responses. These differences from naive cells include (i) rapid production of effector cytokines; (ii) induction by lower antigen dose and by cells such as B cells, which express low to moderate levels of co-stimulatory molecules; and (iii) resistance to rapid activation-induced cell death, a phenotype shared with naive but not effector CD4 T cells. These features would allow memory CD4 T cells to respond to low doses of antigen presented by specific memory B cells and would provide highly efficient help to such B cells immediately, inducing rapid production of specific antibody. Moreover, even before titres of previously encountered infectious agents became high, the responding memory CD4 T cells would have begun a rapid expansion phase so they could participate in other mechanisms involved in a successful immune response to an infectious agent.

2. RESULTS AND DISCUSSION

Our basic model to study the features of CD4 memory T cells and the factors regulating their generation and maintenance, uses a well-characterized population of naive CD4 T cells from a T-cell receptor for antigen (TcR) transgenic (Tg) mouse line AND, with TcR specific for a peptide of pigeon cytochrome C (PCCF) presented by the class II major histocompatibility (MHC) molecule I-E^k (Kaye *et al*. 1989). A homogeneous, naive antigen-specific CD4 T-cell population is purified from AND mice and stimulated *in vitro* with peptide antigen and highly co-stimulatory antigen-presenting cells (APC). The cytokine IL-2 is added to enhance response (Rogers *et al*. 1998) and the cells are polarized to become either Th1 (IL-2, IFNγ, LT) or Th2 (IL-4, IL-5, IL-10, IL-13) cytokine-producing cells by addition of IL-12 and anti-IL-4, or IL-4 and anti-IFNγ, respectively (Swain *et al*. 1996; Swain 1994). After four days of *in vitro* culture the activated cells, which include operationally defined effectors and 'pre-memory' cells (which may or may not overlap), are transferred to an adoptive host, where populations of Th1- or Th2-polarized memory cells develop (Swain 1994). We assess memory by determining the size and function of the donor CD4 T-cell population, re-isolated from the adoptive hosts at various times after transfer and analysed *ex vivo* by flow cytometry and re-stimulation with antigen. By varying the conditions and/or additions in the original *in vitro* culture we can determine what the activation requirements are for generating pre-memory cells, and by varying the adoptive host and *in vivo* conditions we can analyse what is necessary for pre-memory to memory transition and for persistence of the CD4 memory population. Moreover functional analysis of the well-defined memory population recovered can be used to identify what are the capabilities of a CD4 memory population. In most studies, we use Th2-polarized populations because in the mouse strains used, which are all on a C57BL/6 background (B6), there is no significant host production of Th2 cytokines such as IL-4 and IL-5, making production of these very useful markers of Tg CD4 memory cell activity.

(a) *Features of recovered CD4 memory T cells*

We have used the expression of both the transgenic TcRα and β chains (Swain 1994) and, more recently,

differences in Thy1 alleles to identify the donor-derived CD4 T-cell population recovered from adoptive hosts. In most experiments, we used adult thymectomized hosts, which had been lethally irradiated and then reconstituted with syngeneic bone marrow and which therefore could not generate their own CD4 T cells. The *in vitro* stimulated cells are transferred after washing and no additional antigen is introduced. We use staining with CD4, Vβ3 and Vβ11 to detect donor cells by flow cytometry and find an accumulation of donor cells in the spleen. Using additional staining and analysis the donor cells recovered from three weeks to one year are uniformly small resting cells, not expressing activation markers (CD69, IL-2R a), with high expression of CD44 (Swain *et al.* 1996; Swain 1994). The cells also show predominantly low expression of CD45RB and CD62L, a phenotype consistent with other functional definitions of memory in normal, antigen-primed mice (Swain *et al.* 1996).

The memory population persists with only a slow decrease in numbers and functional activity *ex vivo* for the duration of the study up to 45 weeks (Swain 1994).

The advantage of using this population, rather than populations derived from normal non-Tg mice, for analysing the properties of memory cells is that all the transferred cells have received a defined, optimized stimulation with antigen and co-stimulatory signals. This is in contrast to most non-Tg models or models where 'memory'-phenotype cells are derived from normal mice, for which the history of the population studied is unknown and in which the population is likely to contain effector cells, suboptimally stimulated cells and perhaps anergic cells as well as bona fide memory cells.

A major difference distinguishing memory from naive cells, is that the memory populations are able to secrete high titres of multiple cytokines which have pleiotropic activities, whereas naive CD4 T cells make only IL-2, which they consume as they respond (Swain *et al.* 1996). On a cell-to-cell basis, the memory Th1- and Th2-polarized populations secrete nearly as much of most cytokines on *ex vivo* re-stimulation as do highly activated Th1- and Th2-polarized effectors. In particular, we have examined IL-2, IFNγ, IL-4 and IL-5 at the protein level and in addition to those many cytokines at the RNA level. Thus memory cells can exert a series of functions shortly following stimulation, without several days of differentiation into effectors. When naive cells are stimulated, there is a notable lag period before they synthesize cytokine RNA and before they commence proliferation. We find, however, that memory cells are induced to synthesize RNA at a pace equivalent to effector cells, which is much faster than that for naive cells (figure 1). For instance the Th2-polarized memory cells synthesize IL-4 messenger RNA within an hour of activation with anti-Vβ3.

Another potentially critical difference between naive and memory CD4 T cells is the amount of co-stimulation and of antigen needed to induce response. Naive CD4 T cells require high antigen dose presented by APC expressing high levels of multiple co-stimulatory ligands (Dubey *et al.* 1995). In contrast, memory cells respond at high antigen dose or with anti-TcR reagents without co-stimulation. At lower doses they respond partially and they can respond to low antigen concentrations when co-stimulation is present. We find resting B cells are not

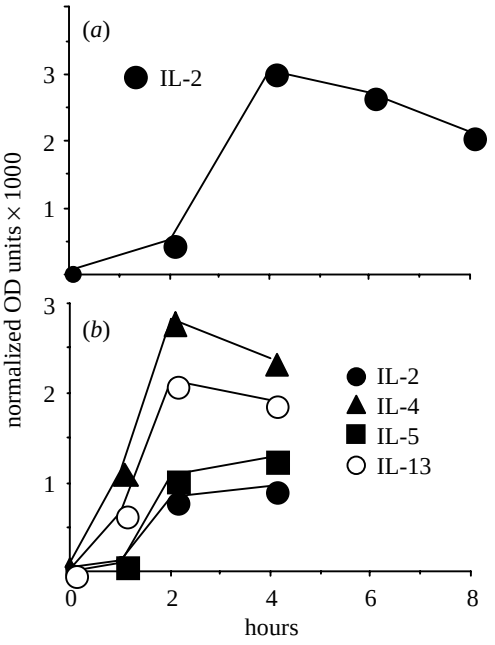

Figure 1. Kinetics of RNA synthesis. (*a*) Naive and (*b*) memory cells were isolated and re-stimulated with anti-Vβ3 (effector and memory cells) or anti-Vβ3 plus anti-CD28 (naive cells). RNA was isolated initially (0) and at 1, 2, 4 and 8 h after stimulation and cytokine RNA determined by RNAse protection (RNA-P) assays. Bands from RNAse protection assay were quantitated by phospho-imaging and normalized for loading with L32. The normalized data are shown in the figure. Data for IL-2, IL-4, IL-5 and IL-13 are shown. No IL-4, IL-5 or IL-13 RNA was detected in naive cells. Similar experiments with Th1 effectors and memory showed similar acceleration compared with naive CD4 T cells (N. Lepak and S. L. Swain, unpublished results).

able to act as APC for naive cells but are good APCs for memory cells (figure 2).

These data together (see table 1 for a summary) support our hypothesis that in a response where memory CD4 T cells are available, efficient CD4 T-cell–B-cell interactions, which drive both CD4 T-cell cytokine production and expansion, and B-cell expansion and differentiation into antibody-secreting cells, can occur almost immediately on introduction of antigen, without the several day delay which occurs in the primary response (Garside *et al.* 1998).

Another critical difference among CD4 T-cell subsets is there susceptibility to activation-induced cell death (AICD) and spontaneous cell death (SCD). As naive CD4 T cells differentiate into effectors, they acquire susceptibility to rapid AICD and a slightly slower SCD, mediated by a Fas–FasL mechanism (Lenardo 1991; Zhang *et al.* 1995). Interestingly fully polarized Th2 effectors are not nearly as susceptible (figure 3) (Zhang *et al.* 1997). Importantly, after transfer to hosts and transition to resting memory this susceptibility is lost. Thus when re-stimulated, memory cells will expand rather than undergo apoptosis.

(b) *No requirement for antigen in the generation and persistence of memory CD4 T cells*

In our model, naive cells are stimulated *in vitro* with peptide antigen and an APC population treated with

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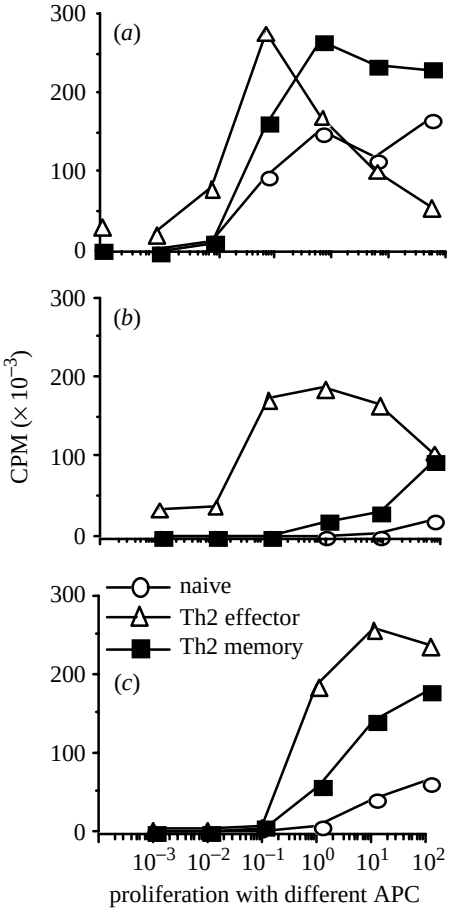

Figure 2. Memory cells respond to B cells as APC. Naive, Th2 effector and Th2 memory cells were stimulated with three types of APC presenting PCCF peptide at different doses. They were (*a*) DCEK-ICAM (expressing both B7.1 and ICAM-1), (*b*) DCEK-RG (expressing neither, with no known co-stimulatory ligands) and (*c*) purified small B cells which express low levels of both (P. Rogers, C. Dubey, X. Zhang, G. Huston and S. L. Swain, unpublished results).

Table 1. *Distinctions between naive and memory*

	cytokines	speed of response	antigen dose/ co-stimulation	rapid ACID
naive	IL-2	slow	high–high	no
memory	Th0,Th1, Th2	fast	low–low	no

mitomycin C. Few traces of the APC population are detectable in these cultures after one to two days (Zhang *et al*. 1995), suggesting that no further antigen stimulation is occurring. Recently we have analysed both how long peptide presentation continues in primary culture and what duration of peptide presentation is optimum for effector generation and memory development following transfer. We find antigen is presented for less than 48 h and that 48 h is sufficient for optimum effector development (H. Hu and S. L. Swain, unpublished results). Moreover in preliminary results, 36–48 h of exposure of naive CD4 T cells to antigen is also sufficient for memory generation (D. J. Gibbs and S. L. Swain, preliminary results). We find no evidence for antigens which

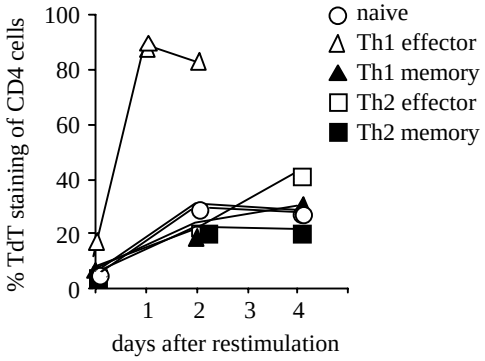

Figure 3. Only effectors die by rapid AICD. Kinetics of death of different subsets was determined. CD4 subsets were isolated and stimulated with PCCF and DCEK-ICAM. The percentage of apoptotic TdT+ cells after different days is shown. Memory cells only died slowly, even if they were Th1 polarized (X. Zhang, C. Dubey, P. Rogers, G. Huston and S. L. Swain, unpublished results).

cross-react with the TcR in our AND Tg and indeed few if any Tg$^+$ memory cells develop in the Tg animals even with age (Linton *et al*. 1996). Thus we consider the chance of antigen stimulation in adoptive hosts to be minimal.

To further support the lack of requirement for antigen, except during the initial *in vitro* stimulation, we derived AND Tg on a RAG-2 deficient background and used naive cells from such hosts compared to normal AND mice for the generation of pre-memory cells. The naive cells from RAG-2-deficient mice have no possibility of expressing endogenous, non-Tg TcR receptors, which could potentially cross-react on environmental antigens. When these pre-memory cells were transferred to adoptive hosts, both developed into donor Tg$^+$ CD4 memory-cell populations of the same size and with comparable abilities to produce cytokines after antigen stimulation of re-isolated cells *ex vivo*. Thus we conclude that the only antigen stimulation needed for the transition from a naive to memory CD4 T cell is the initial stimulation which is required to initiate their activation and expansion.

(c) *No requirement for host MHC class II expression for the generation and persistence of memory CD4 T cells*

Several recent publications have suggested that the survival of naive CD4 (Rooke *et al*. 1997) and CD8 (Tanchot *et al*. 1997) T cells is dependent on their interaction with class II or class I MHC, respectively. The authors envision a kind of interaction with self-peptides, which occurred during positive selection in the thymus. The studies have also presented evidence supporting a need for continued CD8:class I (Tanchot *et al*. 1997; Markiewicz *et al*. 1998) for survival of CD8 memory cells and hence maintenance of the memory populations. These studies can be difficult to interpret, because when the class-I-positive CD8 T cells are transferred into class-I-deficient hosts, a host versus graft reaction is possible. It is also possible that in the absence of class I, NK cells develop which are reactive against class I. The question of continuing requirement for MHC is more straightforward to study on transfer of CD4 T cells into class II KO, because in the mouse such donor cells are class II negative.

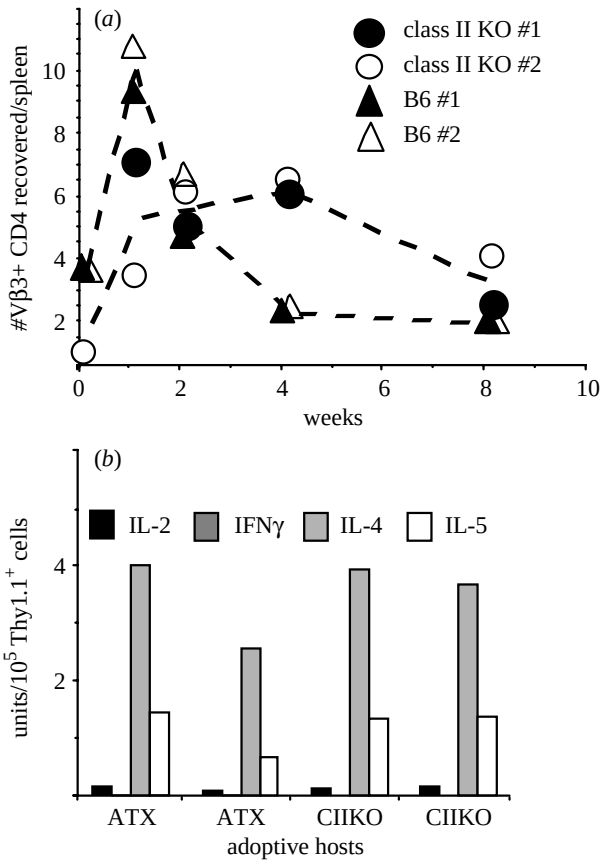

Figure 4. The transition from pre-memory to memory is not dependent on class II. Comparison of spleen recovery $\times 10^5$ (L) and function (R) after transfer to ATXBM and class II KO hosts. (*a*) Recovery of Tg^+ CD4 T cells; (*b*) cytokine profile after three weeks. Individual recipients are shown. Lines represent means (L only) (H. Hui, G. Huston and S. L. Swain, unpublished results).

To evaluate the requirement for continuing interaction of CD4 memory cells with class II, we transferred *in-vitro*-generated Th2 pre-memory cells to class-II-deficient B6 mice and to our usual recipient ATXBM mice. Neither of these has host class-II-restricted CD4 T cells, and the ATXBM but not the class II KO expresses class II. In both cases, equivalent donor memory CD4 T-cell populations developed and persisted for eight weeks (figure 4). When the donor cells were re-isolated at six weeks and stimulated *in vitro*, both made equivalent levels of Th2 cytokines (figure 4). Similar studies with Th1-polarized donor cells gave similar results. We also showed that no positive selection of CD4 T cells occurred when Tg bone marrow was transferred to irradiated class-II-deficient mice, confirming that no alternate TcR–MHC interaction within the range required for mediating positive selection was present.

Thus we conclude that CD4 T-cell memory generation from pre-memory cells and maintenance of the resultant memory populations is not dependent on any recurrent interaction of the TcR with class II or other MHC restriction element. We also showed that after the first week following transfer, there was only very slow turnover of the donor CD4 T-cell population indicating that the cells in the memory population were long-lived resting cells.

It should be pointed out that these studies do not in any way exclude the possibility that occasional stimulation with antigen might expand the memory population or give it a competitive advantage relative to other memory cells. Experiments to test these later possibilities are in progress.

3. CONCLUSIONS

Our studies support the hypothesis that initial stimulation with antigen/APC for as little as two days is sufficient to drive the generation of pre-memory cells, which can develop into long-lived resting memory cells after transfer to adoptive hosts. The generation and persistence of the resting memory CD4 T-cell population is also independent of further MHC class II interaction. The resting memory population is qualitatively different from naive cells (and from activated effectors) in that it responds rapidly by making high titres of multiple cytokines (like effector cells), requires less antigen and co-stimulation (with requirements intermediate between effector and naive cells), but is like naive cells in its resistance to cell death. Thus memory cells can be considered a separate stage of differentiation which is specialized to participate in fast, effective secondary immune responses.

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