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Paul R. Rogers, Caroline Dubey and Susan L. Swain

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Qualitative Changes Accompany Memory T Cell Generation: Faster, More Effective Responses at Lower Doses of Antigen

Paul R. Rogers, 2 Caroline Dubey, 3 and Susan L. Swain 4

The generation of memory T cells is critically important for rapid clearance and neutralization of pathogens encountered previously by the immune system. We have studied the kinetics of response and Ag dose requirements for proliferation and cytokine secretion of CD4+ memory T cells to examine whether there are qualitative changes which might lead to improved immunity. TCR Tg CD4+ T cells were primed in vitro and transferred into T cell-deficient hosts. After 6 or more weeks, the persisting T cells were exclusively small resting cells with a memory phenotype: CD44hi CD62L +/− CD25−. Memory CD4 T cells showed a similar pattern of response as naive cells to peptide analogues with similar Ag dose requirements for IL-2 secretion. However, memory cells (derived from both Th2 and Th1 effectors) displayed faster kinetics of cytokine secretion, cell division, and proliferation, enhanced proliferation in response to low doses of Ag or peptide analogues, and production of IL-4, IL-5, and IFN-γ. These results suggest there is a much more efficient response of CD4 memory T cells to Ag re-exposure and that the expanded functional capacity of memory cells will promote a rapid development of effector functions, providing more rapid and effective immunity. The Journal of Immunology, 2000, 164: 2338–2346.

Memory CD4 T cells may contribute to systemic immunity for many reasons, including a high frequency of specific precursors, selection of cells with higher affinity for Ag, decreased requirements for stimulation, and secretion of a broad range of cytokines (reviewed in Refs. 1–5). In normal mice, it is difficult to visualize memory T cell responses because the frequency of Ag-specific T cells is very low (6–8) and because there is no stable cell surface marker that reliably distinguishes memory cells from naive and effector T cells (reviewed in Ref. 2). Moreover, in nontransgenic (Tg)2 mice primed with specific Ag, there is no way to ensure that the Ag injected in adjuvant is not persisting and that no activated or effector CD4 T cells specific for the Ag are present. The interpretation of studies using “memory phenotype” cells to study the properties of memory cells is hampered by the fact that the history of memory phenotype cells recovered from normal mice or humans is unknown, and it is likely that some of the cells identified by phenotype are primed under conditions of suboptimal stimulation and/or could include anergic or tolerant cells. In addition, homeostasis-driven division can also lead to memory phenotype cells (CD44 hi) which are not functionally memory cells (H. Hui and S. L. Swain, unpublished data). Moreover, in many cases, polyclonal stimuli such as anti-TCR reagents must be used to stimulate the memory cells, obscuring the roles of Ag dose and affinity in the response.

To address the activation requirements of memory T cells, we have developed an adoptive transfer system that allows recovery of a substantial number of resting, Ag-experienced, Ag-specific, TCR Tg T cells (9) with a known history of optimized stimulation with peptide Ag. The use of optimal doses of Ag and polarizing cytokines in vitro ensures that the memory cell population is derived from uniformly activated Ag-experienced cells. The host is Ag free and we can directly compare bona fide naive and resting memory CD4 T cells at the same frequency and with the same receptor so that qualitative differences between the two populations can be clearly delineated.

We have shown that when Th1- and Th2-polarized primary effector T cells are generated in vitro from naive CD4 T cells of pigeon cytochrome c (PCC)-specific TCR Tg mice (AND) and are transferred to adult thymectomized irradiated bone marrow reconstituted (ATXBM) recipients, the CD4+ T cells return to a resting state, persist for long periods of time (up to 1 year), and produce a cytokine profile similar to that of primary effectors (9). The persistence of this population, its resting memory phenotype, and the uniformly activated, Ag-experienced nature of the transferred population, as well as their enhanced functional capacity, identify these cells as canonical resting memory cells (2, 9).

Data from several earlier studies have argued that memory phenotype T cells, isolated based on cell surface markers, are less dependent on costimulation for activation compared with naive T cells (10–14), but the populations tested could have easily included cells that were activated or were responding to environmental Ags. Recent studies with TCR Tg CD8+ memory cells have presented evidence to suggest that these cells may be hypersensitive to Ag compared with naive cells (15). However, the requirements for Ag triggering and costimulatory interactions for induction of proliferation and cytokine secretion by isolated Ag-specific CD4+ memory T cells are largely unknown. Using a high dose of peptide, we previously showed that CD4+ memory T cells

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1 Paul R. Rogers, 2 Caroline Dubey, 3 and Susan L. Swain 4

2 Current address: La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA 92121.

3 Current address: Rhone-Poulenc Rorer, Centre de Recherche de Vitry-Alfortville, 13, quai Jules Guesde B.P. 14, 94403 Vitry sur Seine, France.

4 Address correspondence and reprint requests to Dr. Susan L. Swain at her current address: Trudeau Institute, P.O. Box 59, 100 Algonquin Avenue, Saranac Lake, NY 12983. E-mail address: sswain@northnet.org

5 Abbreviations used in this paper: Tg, transgenic; PCC, pigeon cytochrome c; AND, PCC-specific TCR Tg mouse; ATXBM, adult thymectomized irradiated bone marrow reconstituted; PCCF, PCC (fragment 88-104); CFSE, 5- and 6-carboxyfluorescein diacetate succinimidyl ester.

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12983. E-mail address: sswain@northnet.org

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could proliferate in response to a variety of APC and that stimulation with a highly cross-linking, high-affinity reagent, anti-CD3, could induce proliferation in the absence of APC (16). Similarly, resting memory cells were less dependent on costimulatory molecule interactions for cytokine secretion (3) when anti-CD3 was used as a source of a TCR signal. In contrast, recently generated effector T cells can proliferate and secrete many cytokines in the apparent absence of costimulation (17), although B7-1 (CD80) and ICAM-1 (CD54) costimulation are still required for optimal IL-2 secretion (16–19).

Although it is well known that systemic Ab responses in primed animals are faster than primary responses, this could be due to increased frequency of Ag-specific T and B cells rather than a qualitative change in the kinetics of response. The kinetics of memory CD4 T cell response to Ag has not been carefully examined. A rapid response of memory CD4 T cells that produce high quantities of an array of effector cytokines could contribute to the speed of immune responses by immediately inducing B cell and macrophage function and thus promoting a much more rapid development of Ab and effector cells, leading to an increase in protection against pathogens.

In this study, we have measured the kinetics of T cell response and determined how the activation of CD4+ memory cells is influenced by Ag dose and by TCR affinity. We have directly compared resting memory cells to resting naive cells and effector cells. We find that memory CD4 T cells can be qualitatively distinguished from naive cells (which express the same TCR) by their faster production of cytokines, which leads to enhanced proliferation and cell division, as well as by the ability to respond to APC presenting lower densities of Ag. These results have important implications for understanding how the generation of memory CD4 T cells contribute to secondary responses and confer immune protection following natural or therapeutic immunization.

Materials and Methods

Mice

H-2<sup>b</sup>and H-2<sup>κ</sup>Vaa11/Vβ3 AND TCR Tg mice (Tg) were bred in the animal facilities at the University of California, San Diego or at the Trudeau Institute and were used at 2–6 mo of age. Tg males (H-2<sup>b</sup>), on a H-2b/k offspring. Tg H-2<sup>b</sup>/k mice were bred repeatedly (21, 23) using 3.155 (anti-CD8), RL172.4 (anti-CD4), HO13.4 (anti-Thy1.2), and FD56K (anti-Thyl.2). For B cell preparations, 33D1 and M1/70 Abs were used in addition to the above Abs and were cross-linked with MAR18.5. The dense fraction of cells (from the 80/63 interface) was collected on a Percoll gradient and then adhered to plastic at 37°C for 1 h. Resulting cells were small and ≥95% B220<sup>+</sup>. APC populations were treated with mitomycin C (50–100 μg/ml; Sigma) for 45 min at 37°C before use.

Analysis of in vitro responses

Cells were cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 200 μg/ml penicillin, 200 μl/ml streptomycin, 4 mM glutamine, 5 × 10<sup>−5</sup> M 2-mercaptoethanol, 10 mM HEPES, and 7.5% FCS (HyClone, Logan, UT). An equal number of CD4+Vβ3+ T cells (naive, effector, or memory) (1–3 × 10<sup>5</sup>/ml), determined by FACS analysis before plating, were stimulated in 0.2-ml volumes with mitomycin C-treated APC (0.3–1 × 10<sup>6</sup>/ml fibroblast APC/ml) and various doses of PFCF (0.001–100 μM). In some experiments, APC were pulsed with peptide for 2 h before culture with T cells. Proliferation was measured at 48, 72, or 96 h of culture by incorporation of [3H]thymidine (1 μCi/well; ICN Pharmaceuticals, Irvine, CA) during the last 12–18 h. To follow cell division, T cells were labeled with 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE, C-1157; Molecular Probes, Eugene, OR) and analyzed after 1–3 days by flow cytometry. Briefly, cells were labeled with 0.05 μM CFSE for 15 min at 37°C and then washed with cold PBS before use (24). T cells were counterstained with PE-labeled Vβ3 (PharMingen, San Diego, CA) before flow cytometry analysis. CFSE histograms were gated on Vβ3-positive cells. Studies using CFSE-labeled cells in vivo have shown an 80–90% loss of fluorescence after 1 day followed by stabilization and virtually no loss of fluorescence at later times (up to 8 wk) (25).

Cytokines and cytokine assays

Recombinant cytokines IL-2, IL-4, IFN-γ, and IL-5 were obtained from culture supernatants of X63.Ag8–653 cells transfected with murine cDNA for the respective cytokines (26). Recombinant murine IL-12 was kindly provided by Dr. Stanley Wolf (Genetics Institute, Cambridge, MA). The following anti-cytokine Abs were purified from ascites or were prepared by Amicon (W. R. Grace & Co., Beverly, MA) concentration of hybridoma supernatants: 11B11 (anti-IL-4), XMG1.2 and R46A2 (anti-IFN-γ), and TRFK4 and TRFK5 (anti-IL-5). IL-4, IFN-γ, and IL-5 were detected by ELISA using 11B11, R46A2, and TRFK5, respectively, as coating Abs, and biotinylated-anti-mouse IL-4 (BVD4) (PharMingen), biotinylated-XMG1.2, and biotinylated-TRFK4, respectively, as second step reagents. The data were quantitated from standard curves using recombinant cytokines and were expressed in ng/ml. One nanogram of IFN-γ equals 0.9 U of protein. Supernatants were collected at 24–28 h unless noted otherwise. IL-2 was detected by bioassay as described previously (6) by measuring proliferation of the NK cell line in the presence of 11B11 (anti-IL-4). IL-2 was quantitated from a standard
### Results

To evaluate the changes that occur when naive T cells become memory cells, we have used an adoptive transfer model to generate TCR Tg+ memory cells from in vitro-generated effectors. In each experiment, we have compared recovered memory populations to freshly isolated naive CD4 T cells from the AND Tg mice and in some cases compared the responses to Th1 and Th2 effectors generated 4 days previously. The different CD4 T cell populations were restimulated under identical conditions to allow a direct comparison. In our adoptive transfer model, memory CD4 T cells expressing a Vβ3/Vα11 Tg TCR (specific for PPC peptide PCCF) can be reisolated from the recipients of in vitro-generated Th1 or Th2 effectors. The recovered cells have a memory phenotype and retain their Th1/Th2 polarization (9). Naive cells are originally stimulated only with peptide Ag, and no Ag is introduced into the adoptive hosts. Moreover, there is good evidence that no environmental Ags stimulate the Tg T cells (21, 22). These facts and the fact that the recovered Tg+ cells are uniformly small, resting cells which do not express activation Ags, but do express markers of Ag experience such as CD44 (3, 9), identify them as resting memory cells. We will refer to the T cells recovered from adoptive hosts after 6 or more weeks as CD4 memory cells. Expression of CD4 and Vβ3 on the T cell subsets used in this study is shown in Fig. 1 (top panel). Approximately 80% of the recovered memory CD4+ cells retained expression of the Vβ3 and Vα11 TCR chains as shown in Fig. 1 (bottom panels) with TCR expression on memory cells similar or only slightly lower compared with those of naive T cells (bottom panels). In four other experiments, expression of the Tg TCR chains was equivalent or slightly lower on memory cells vs naive cells (data not shown). In each of the experiments described, the cell density has been readjusted before restimulation so that the responses measured are from an equal number of CD4+Vβ3+ cells from each group.

The non-Tg (Vβ3- ) CD4+ T cells in the memory population apparently come from the few original contaminating Tg+ effector cells. The outgrowth of non-Tg TCR CD4 T cells that express endogenous TCR chains has been seen by other investigators that have analyzed TCR Tg mice and seems to represent an eventual selection for the few cells that express endogenous TCR chains (29) that may have responded to environmental Ags. We have found that the non-Tg cells expressing endogenous TCRs do not react with PCCF. There is no in vitro response to PCCF detected in non-Tg mice and no response of the few transgene-negative CD4 cells from AND mice to PCCF (22). Since all of the stimulations reported herein are done with specific peptide (the native peptide, PCCF or PCCF analogues), the cells bearing non-Tg TCRs should not make a significant contribution or affect the interpretation of the results. In support of this hypothesis, we have transferred RAG-deficient AND T cells into ATXBM hosts and have seen no differences (vs RAG-positive cells) in cytokine profiles of recovered cells (30). Therefore, the lack of endogenous TCR expression had no impact on the generation or persistence of memory cells. Further evidence that the non-transgene-bearing memory cells (CD4+Vβ3- ) do not respond to specific Ag is found in the homogeneity of memory effector cells generated by stimulating the recovered memory cells in 4-day culture with APC and Ag. The recovered memory effector cells analyzed after restimulation in vitro in five experiments were >95% CD4+Vβ3+ and <1% (CD4+Vβ3-) and were similar to primary effectors in phenotype and cytokine production (our unpublished data).

### Phenotype of T cells recovered from ATXBM recipients

Previously, we have shown that T cells with a memory phenotype can be recovered from adoptively transferred ATXBM mice and that the cell surface phenotype was stable over the 3–8 wk analyzed. As shown in Fig. 2, the CD4+Vβ3+ T cells recovered from ATXBM recipients were small (low forward light scatter (FSC)) and expressed intermediate levels of CD44 (100% IL-2Rα (CD25)). Recovered memory cells also down-regulated expression of CD45RB; however, this marker was not reliable in distinguishing memory from naive cells in this system. As seen previously in a similar adoptive transfer model (31), a subset of recovered T cells (approximately half) retained L-selectin (CD62L) expression and may represent T cells that homed to lymph nodes. Before transfer, effector T cells were nearly all L-selectin + (see Fig. 2). Compared with naive cells, memory cells expressed higher levels of CD44 and higher levels (2–3-fold) of both LFA-1 and ICAM-1 (Refs.32–34, and P. R. Rogers and S. L. Swain, unpublished data), but lower levels of CD45RB and L-selectin compared with naive T cells. Memory cells could also be distinguished from effectors by cell size and expression of IL-2Rα.

When memory cells are activated in vitro with APC and Ag to become memory effector cells, they up-regulate expression of CD45RB (100% IL-2Rα (CD25)), IL-2Rα (100% IL-2Rα), and L-selectin (65%) and retain high levels of CD44 (data not shown). In addition to phenotype, memory cells differ from naive cells by producing higher levels of cytokines other than IL-2 (3, 9).
Cytokine secretion from representative T cell populations stimulated with specific Ag (PCCF) and B7-1 ICAM-1 APC is shown in Table I. Naive T cells secreted predominantly IL-2, whereas memory T cells secreted IL-4, IL-5, and IFN-γ, in addition to IL-2. However, in contrast to their Th1 and Th2 effector counterparts, memory T cells made 5–30 times less IL-4, IL-5, and IFN-γ. Table I also shows that memory T cells retained their original Th1 and Th2 polarization as shown previously in this model (9). Polarization was even retained after 4 days of activation in vitro (data not shown). We did not detect any significant differences in the levels of cytokines secreted from memory T cells recovered at 7 wk vs 21 wk after transfer (data not shown).

**Memory T cells are more responsive than naive cells at low doses of Ag**

Several studies have suggested that memory CD8 T cells are more responsive to lower dose Ag than naive cells (13–15) using proliferation as a readout. To confirm these observations in the CD4 system, we directly compared naive and memory CD4 T cells for their response to doses of native peptide (PCCF). Fig. 3 shows the proliferative response of memory and naive T cells to B7-1 ICAM-1 APC presenting various doses of peptide (PCCF) over a 2–4-day time course. Fig. 3a shows the response of naive and memory T cells to both high-dose (2 μM) and low-dose (0.002 μM) Ag at 48, 72, and 96 h. Both subsets showed a dose-dependent increase in DNA synthesis with high-dose Ag over the 48–96-h time period. However, at low Ag dose (0.002 μM), memory T cells responded significantly better than naive T cells at 48 and 72 h, with responses ending in both populations at 96 h. Both subsets showed a decrease in DNA synthesis at 96 h, which may be due to cytokine depletion and cessation of proliferation. When analyzed over a wide range of doses, as shown in Fig. 3b, both subsets responded in a dose-dependent manner to increasing doses of Ag at 48 (left) and 72 (right) h but memory T cells responded better than naive T cells at low doses of Ag (0.0002–0.02 μM). Thus, the memory population is clearly able to respond better and with more rapid kinetics when Ag is limiting.

The more rapid and larger proliferative response of memory cells could be due to more cells entering the responding pool or the ability of memory cells to cycle sooner compared with naive cells. To address these possibilities, T cells were labeled with CFSE, a dye which covalently couples with intracellular proteins and partitions equally between daughter cells after cell division. Thus, we could follow cell division over time by flow cytometry. In Fig. 4, CFSE-labeled T cells were cultured with B7-1 ICAM-1 APC and 1 μM PCCF peptide for up to 3 days. At each time point (days 1–3), cells were counterstained with Vβ3-PE and the number of cell divisions was determined by flow cytometry. Fig. 4 shows histograms of CFSE-labeled (Vβ3-gated) cells on days 0–3. After 1 day, few or no naive (Fig. 4, top) and memory (Fig. 4, bottom) cells divided, although both populations showed diminished CFSE staining due to decay of fluorescence (see Materials and Methods) (25). Importantly, after 2 days, naive cells underwent up to two divisions whereas memory cells underwent up to three divisions. For naive T cells, on day 2, 35% of cells divided twice, 32% divided once, and 23% of T cells showed no division (overlapping with the profile at day 1). In contrast to naive T cells, a large fraction of memory cells have undergone three divisions. For memory T cells, 30% of cells divided three times, 31% twice, 15% once, and ~17% showed no division. After 3 days, nearly all T cells had divided at least once, but memory cells still showed more cell divisions compared with naive T cells. Therefore, under these Ag dose conditions, approximately equal numbers of naive and memory cells have entered the dividing pool; however, memory cells appeared to be going through more cell divisions or were dividing sooner vs naive T cells. Under conditions of limiting Ag dose or costimulation, it remains possible that more memory cells
are initially recruited into the dividing pool of cells in addition to showing more or sooner cell divisions.

**Memory T cells proliferate better than naive T cells in response to peptide analogues**

To further explore memory cell response requirements, we examined whether the ability of T cells to proliferate varies as the TCR affinity for the peptide/MHC complex changes. We used peptide analogues which have altered affinity for the TCR (25, 32). Native peptide (PCCF) or PCCF analogues with higher (QASA) and lower (K99A) abilities to stimulate T cells were added to Tg T cell subsets cultured with a fixed number of APC (B7-1 ICAM-1). The peptide analogues have similar affinity for MHC (I-Ek) but have either a substitution in a TCR contact residue (K to A at residue 99) or an insertion of four amino acids (QASA) between residues 99 and 100 (27, 28, 35). The K99A analogue triggers cells less well and presumably binds the AND TCR with lower affinity, whereas the QASA analogue triggers more efficiently and presumably binds with higher affinity. The response of naive and memory cells to doses of PCCF and peptide analogues presented by the costimulatory fibroblast (B7-1 ICAM-1) is shown in Fig. 5 (top panels) and by relatively costimulation-poor small B cells in the bottom panels. In the top panel, memory cells responded at lower doses of peptide and with higher maximum response compared with naive T cells with each peptide tested, but response to the “low affinity peptide” (K99A) was only seen at the very highest dose. As expected, maximal responses to the high affinity peptide (QASA) occurred at low dose, to the native PCCF at a slightly higher dose, and to the weak affinity peptide only at the very highest dose. Thus, memory cells show the same hierarchy in response to peptide analogues or peptide affinity but show a greater proliferative response vs naive cells. Memory cells are very sensitive to affinity, but are triggered either at low doses of high affinity (QASA) or high doses of low-affinity peptide (K99A).

Th2 memory cells also showed enhanced responses compared with naive cells when small B cells were the APC, as shown in Fig. 5 (bottom panels). In this experiment, the APC were prepulsed with peptide to ensure exclusive presentation by the purified small B cells. Whereas naive T cells responded poorly to peptide (even the high-affinity analogue QASA), memory cells responded very well. These data are in agreement with and extend our previous studies (16) which showed that memory cells could respond to resting B cells presenting a single high dose of peptide or to CD3 cross-linking in the absence of costimulation. In addition to Th2 memory cells (Figs. 3 and 5), Th1 memory cells also showed enhanced responses with low doses of peptide. As seen in Fig. 6, Th1 memory cells incorporated more thymidine vs naive T cells in response to both B7-1 ICAM-1 fibroblast (Fig. 6, left) and B cell APC (Fig. 6, right). In addition to increased responses at low doses of peptide (0.01–1 µM), maximal thymidine incorporation was seen at a 10-fold lower dose of peptide in memory cells (0.1 µM vs 1 µM for fibroblast APC and 1 µM vs 10 µM for B cell APC).
Ag dose requirements for cytokine production of memory cells

Although naive and memory cells secrete cytokines in response to high doses of Ag, we wanted to determine whether memory cells were more like naive cells or effectors in response to varying doses of Ag presented on highly costimulatory APC. We assessed the effect of peptide dose in cultures of CD4 subsets stimulated with highly costimulatory APC (B7-1+ ICAM-1+). In Fig. 7, results are presented normalized for maximum cytokine production achieved by each population. Since the quantities of IFN-γ (from Th1 subsets) and IL-4 (from Th2 subsets) produced by naive CD4 T cells are negligible, these cytokines are not shown for naive cells. For the same reasons, since the production of Th1 cytokines by Th2 cells and vice versa was negligible they are not shown. All CD4 T cell subsets showed an Ag dose-dependent increase in cytokine secretion. Naive CD4 cells required 1 μM PCCF for optimum IL-2 production, whereas effectors secreted near optimum levels at 0.1 μM peptide. Memory cells were more like naive cells than effectors in the dose of Ag required for half maximal IL-2 secretion. Memory and naive cells required ~0.06 μM peptide for half maximal IL-2 secretion, and memory cell secretion of IL-4 occurred at a similar level of Ag (Fig. 7, top, and bottom, respectively). Thus, memory and naive CD4 T cells seem to have similar Ag dose requirements for cytokine accumulation. The dose of Ag required for half maximal IFN-γ secretion was somewhat higher (Fig. 7, middle). In contrast, effector cells only required ~0.01 μM peptide for half maximal IL-2 production. In addition, effectors required ~5–15-fold less Ag than memory cells for half maximal secretion.

FIGURE 5. Th2 memory cells show enhanced responses to peptide analogues. T cells (3 × 10^5/ml) were cultured with B7-1+ ICAM-1+ APC (1 × 10^5/ml) and soluble doses of peptides (top panels). In the bottom panels, T cells (1 × 10^5/ml) were cultured with small B cells (2.5 × 10^5/ml) that were prepulsed for 2 h with doses of peptide (PCCF or QASA). Triplicate wells were pulsed with [3H]thymidine for 12 h and proliferation was measured at 63 h. Data are representative of two experiments.

FIGURE 6. Th1 memory cells show enhanced proliferative response to low doses of peptide. T cells (2 × 10^5/ml) were cultured with half as many B7-1+ ICAM-1+ fibroblast APC or four times as many B cells and soluble doses of peptide (PCCF). Wells were pulsed with [3H]thymidine for 12 h and proliferation was measured at 48 h. Data are representative of three experiments.

FIGURE 7. Ag dose requirements for CD4 subset cytokine secretion. T cells (2 × 10^5/ml) were stimulated with B7-1+ ICAM-1+ APC (1 × 10^5/ml) and various doses of PCCF. Supernatants from triplicate wells, collected at 24 h, were pooled and assayed for IL-2, IL-4, and IFN-γ. IL-2 and IFN-γ were assayed from naive, Th1 effector, and Th1 memory cells. IL-4 was assayed from naive, Th2 effector, and Th2 memory cells. Data are representative of three experiments.
of IL-4 and IFN-γ. Therefore, at low Ag doses (10⁻³–10⁻² μM), only effector cells can secrete detectable levels of cytokines.

Kinetics of memory cell cytokine production is more rapid than that of naive cells

To determine whether resting memory cells can actually respond faster than naive cells, and thus behave more like effector cells, we compared the kinetics of cytokine secretion and accumulation in cultures of memory CD4 T cells to that of naive and effector cells, each activated with highly costimulatory APC presenting a high dose of Ag (10 μM). Representative results from one of several experiments, this one using Th2-polarized effectors and memory cells, are shown in Fig. 8. Naive T cells secreted IL-2 that was only barely detectable after 4 h with maximal accumulation of IL-2 in supernatants at 24–48 h. No IL-4 was detected in culture supernatants, even up to 60 h (Fig. 8 and data not shown). Th2 memory cells secreted similar amounts of IL-2 (as shown in Table I), but supernatant accumulations reached peak levels more rapidly compared with naive T cells, with significant amounts of IL-2 already detected by 4 h and peak amounts found at 12 h. In addition to IL-2, Th2 memory cells secreted substantial amounts of IL-4 (and IL-5; data not shown) which were initially detectable by 2 h. IL-4 accumulation was maximal at 24 h whereas IL-5 accumulation (data not shown) was maximal at 48 h. Compared with memory cells, effector T cells made much higher amounts of IL-2, IL-4 (Fig. 8), and IL-5 (Table I) but with similar or only slightly faster kinetics (Fig. 8). Memory cells were also similar to effector cells in the kinetics of cytokine mRNA induction with maximal transcripts seen at 1–2 h after stimulation (X. Zhang et al., manuscript in preparation). Therefore, it appears that memory T cells produced cytokines at a rapid pace more like effector than naive cells. IL-2 and IL-4 are known to be autocrine growth factors for T cells, and the early production of cytokines by memory cells may lead to faster or enhanced proliferation of memory vs naive T cells.

Discussion

The studies presented here support the concept that memory cells, even those which have reverted to a totally resting state and have not encountered Ag for months, are qualitatively distinct in important ways from resting naive cells which express the same TCR. The differences we find have important implications for when and under what circumstances CD4 T cells can be activated when exposed to Ag. One important difference is found in the ability of memory cells to synthesize and secrete a variety of polarized cytokines with very rapid kinetics, while naive CD4 T cells exhibit a lag period before production of only IL-2 (Table I, Fig. 8, and our unpublished data). In addition, memory cells are able to proliferate in response to APC at lower Ag dose and to peptides, which results in lower affinity interactions. Taken together, these properties suggest that a key component of the enhanced protection against re-infection, which accompanies sppecific immunization, is the ability of memory CD4 T cells to “jump start” the secondary immune response by quickly providing an array of highly effective cytokines to B cells and other APC, such as macrophages, before these cells are themselves highly activated (i.e., without the requirement for APC with high levels of costimulation) and before available Ag has risen to high levels. This is in marked contrast to the behavior of naive CD4 T cells in the primary response, which require recognition of high densities of Ag on professional APC such as dendritic cells (36, 37), and which are found to interact with B cells only after several days. These changes can be expected to be of particular importance in memory responses to infectious agents, because the response potentially could be triggered early in infection and protective responses mounted before the infectious agent replicates extensively.

We have previously shown that naive CD4 T cells from TCR Tg mice can be activated in vitro, transferred to recipient mice (ATXBM), and recovered up to 1 year later. Recovered T cells in this and earlier studies display a memory phenotype (9), proliferate, and secrete cytokines in response to specific peptide presented by APC. We and others have shown that memory cells (recovered from adoptively transferred hosts) are resting cells in the G₀ state of the cell cycle. The definition of these cells as memory T cells is based on several major criteria which include Ag-experience (assured by the protocol for generating the cells), phenotype (shown in Fig. 2), and function. When naive Tg cells are cultured in vitro with Ag and costimulatory APC, no resting cells are found after 4 days and the recovered cells are uniformly transgene positive (Fig. 1, effector) and have all undergone multiple rounds of division (D. Jelley-Gibbs et al., unpublished data). Thus, the Tg cells derived from this effector population after transfer to adoptive hosts are by definition Ag experienced. Moreover, we know that the cells have all been exposed to optimal levels of Ag in the presence of high levels of costimulation. This is critical because memory phenotype cells isolated from normal mice, not purposely stimulated with Ag, that have been used in many previous studies have an unknown history.

Memory T cells, as defined operationally by their ability to give strong response to previously injected Ag, have been previously characterized as small resting cells that are CD4⁺CD8⁻ L-selectinlow CD45RBlow (or CD45RO⁺ in human) (33) and high for expression of many integrin and adhesion molecules (34, 38). LFA-1 and Ly6C, in addition to CD44 and CD25, have been useful in discriminating memory cells in a CD8 adoptive transfer model (34). In our adoptive transfer model, the CD4 cells recovered after 7–21 wk were small resting IL-2Rα⁻ CD4⁺CD45RBlowLFA-1high (Ref. 9, Fig. 2, and our unpublished data). The phenotype of T cells recovered from our model is very similar to that of cells that have been activated in vivo in response to Ag (24, 39, 40). Using an adoptive transfer model with normal mouse recipients, Gudmundsdottir et al. (24) showed that administration of Ag induced Tg T cell to up-regulate CD44 and down-regulate both CD62L and CD45RB. Moreover, recent studies show that memory cells which develop in intact, ATXBM, and class II-deficient recipients are similar in phenotype and function (help and cytokine secretion) (2, 9, 30). These cells are not distinguishable from cells generated in vivo in response to Ag plus adjuvant (6). Most important, the recovered cells displayed memory function in that they responded to specific restimulation with Ag by proliferating, by secreting effector cytokines similar to memory cells generated in vivo in response to Ag or pathogens in non-Tg models (6, 41), and by providing effective

FIGURE 8. Kinetics of CD4 subset cytokine secretion. Naive, Th2 effector, and Th2 memory T cells (5 × 10³/ml) were stimulated with half as many B7-1⁺ ICAM-1⁺ APC and 10 μM PCCF peptide. Supernatant was collected at the indicated times and assayed for IL-2 and IL-4. Data are representative of three experiments.
help to B cells in situ (9). Thus, we are confident that the cell population we analyzed in these studies corresponds to long-lived resting memory that is traditionally generated in non-Tg models by stimulation with Ag presented in adjuvant. One potential drawback of these earlier experiments was the possibility that Ag persisted and that activated Ag-specific cells were also part of the population. That is not a concern in the current studies because the adoptive hosts have never received Ag and the original naive CD4 T cells were primed only with peptide.

Our studies focus on how the properties of memory cells can determine their behavior and give us insights into how the secondary response is regulated. The kinetics of cytokine production and Ag-specific proliferation have not previously been described for purified populations of Ag-specific CD4 memory cells, although memory cells generated in vivo have been shown to produce similar quantities of IL-2 as naive cells (6). Our results demonstrate that resting memory cells respond to Ag with faster kinetics of cytokine RNA accumulation (X. Zhang et al., manuscript in preparation) and protein secretion than naive T cells. The kinetics of proliferation (Fig. 3) and cytokine accumulation (Fig. 8) from memory cells was faster than that of naive cells and was more similar to that of effector cells. This rapid response is also seen in vivo, when Ag is given to recipient mice reconstituted with Tg T cells (X. Zhang et al., manuscript in preparation). Memory Th2 cells produced significant amounts of IL-2 and IL-4 (and IFN-γ from Th1 memory cells; data not shown) by 4 h, whereas naive cells required 12 h to accumulate equivalent amounts of IL-2. This rapid cytokine production is seen despite the fact that the phenotypic profile of memory cells indicated that they are homogeneously small resting cells expressing negligible levels of activation makers such as CD25 and despite the fact that much lower levels of cytokines are produced by memory cells compared with effectors (Table I). The rapid pace of cytokine production will allow memory cells to provide regulatory functions within a few hours of re-encountering Ag when they may be able to directly target the cytokines to the APC, so that the low levels of cytokine produced may be sufficient or even optimum for biological activity. In support of this data, recent results from Rocha and colleagues (42) suggest that memory CD8 cells secrete IFN-γ more rapidly than naive cells. A possible explanation for the rapid production of cytokines is suggested by studies which showed that differentiation of T cells is accompanied by demethylation of the cytokine promoter regions (43, 44). Heritable changes like demethylation, if they occur in cytokine promoters in general, could be responsible for the induction of rapid cytokine synthesis without a lag period such as we observe in memory CD4 T cells.

One of the most dramatic and likely most important differences that distinguishes naive from memory CD4 T cells is the ability of the latter to secrete IL-4, IL-5, and IFN-γ upon restimulation (Table I, Figs. 7 and 8, and Ref. 3). Our in vitro results are in agreement with in vivo studies which show that upon initial restimulation, resting CD4 memory T cells from normal animals make IL-2 in titers roughly equivalent to naive CD4 cells and also produce detectable quantities of IL-4 and IFN-γ (2, 45, 46). As shown in Table I, memory T cells can produce multiple cytokines upon restimulation. These “effector” cytokines can directly mediate effector function such as B cell help (47). Ig secretion, macrophage activation, and Th1/Th2 polarization. Thus, the potential impact of the interaction of a memory CD4 T cell with an APC is great because the APC will be rapidly exposed to a range of cytokines that can drive its activation or differentiation.

In our studies, the cytokine production of memory cells increased with Ag dose, and memory and naive cells had equivalent requirements for peptide concentration to induce each cytokine as did naive cells for IL-2 induction (Fig. 7). These requirements were higher than those of the corresponding effector population, which produced detectable and maximal cytokines at a lower dose (generally at least a 10-fold lower dose). Thus, in this respect, memory CD4 T cells seem to retain the Ag dose restriction of naive cells for TCR triggering for cytokine production.

Although the kinetics of memory cell and naive cell proliferation with high doses of Ag presented by a costimulatory APC is similar, memory CD4 T cells can be triggered to begin proliferation at lower doses of Ag than naive CD4 T cells (Fig. 3), and their responses at the low doses are greater than those of naive CD4 T cells. The difference between naïve and memory CD4 T cells with respect to Ag dose is also seen when peptide analogues are used to stimulate the two subsets (Fig. 5). For both the analogue giving higher affinity (QASA) and that giving lower affinity (K99A) interaction, memory cells gave detectable responses at lower doses compared with naive CD4 T cells and at low doses they incorporate more radiolabel, suggesting a higher rate of proliferation. Our results are in agreement with previous studies which suggested that memory or primed CD8 cells proliferate in vitro to lower peptide doses (15) and display more rapid effector function (48) compared with naive T cells.

How much of these differences in response to low doses of peptide are due to cytokine availability and how much to possible differences in efficiency of TCR triggering is unknown. This enhanced proliferative capacity is not likely due only to the amount of autocrine IL-2 secreted (which is quite similar, see Table I), or increased levels of transgene (TCR) expression (Fig. 1), but may be influenced by the more rapid kinetics of cytokine secretion (Fig. 8) and lower costimulatory requirements. It is also possible that in memory cells there is altered tyrosine phosphorylation and/or different coupling of the TCR to downstream signaling cascades (49). Our results are in agreement with studies both in vivo (13, 50) and in vitro (15) which show that CD8 memory cells give a more vigorous and sustained response than naive cells. However, in contrast to one report (15), the increased response of memory cells we observe does not require addition of exogenous IL-2. The ability of weak agonist peptide such as K99A or low doses of PCCF (or QASA) to activate memory but not naive cells also suggests that memory cells are triggered more easily, or are able to form more stable cell-cell contact, or are able to sustain signaling long enough to activate downstream messengers. This lower threshold of activation may allow memory cells to divide occasionally in the presence of low levels of cognate or perhaps cross-reacting Ag. Such potential responses to cross-reacting Ag may be critical in inducing fast responses to viruses which often vary their major Ags to evade the immune system.

The ability of memory T cells to respond to Ag with faster kinetics often has been attributed to their increased precursor frequency in vivo (5, 7, 51, 52) and to selection for higher affinity (53). However, we suggest that the more rapid kinetics of cytokine secretion and proliferation is intrinsic to the memory phenotype and that coupled with the ability to secrete increased amounts of effector cytokines, the ability to respond to B cells, and the ability to respond to lower doses of Ag enable memory CD4 T cells to respond sooner and with greater effectiveness. This in turn would drive a faster response of B cells and other targets of T cell help. We thus postulate that these qualitative differences change the characteristics of the overall response such that infectious organisms are dispatched rapidly, accomplishing host protection.

References

QUALITATIVE CHANGES IN MEMORY CD4 T CELLS

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