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Functional Responses and Costimulator Dependence of Memory CD4+ T Cells

Cheryl A. London, Michael P. Lodge, and Abul K. Abbas

To examine the functional characteristics of memory CD4+ T cells, we used an adoptive transfer system to generate a stable population of Ag-specific memory cells in vivo and compared their responses to Ag with those of a similar population of Ag-specific naive cells. Memory cells localized to the spleen and lymph nodes of mice and exhibited extremely rapid recall responses to Ag in vivo, leaving the spleen within 3–5 days of Ag encounter. Unlike their naive counterparts, memory cells produced effector cytokines (IFN-γ, IL-4, IL-5) within 12–24 h of Ag exposure and did not require multiple cycles of cell division to do so. Memory cells proliferated at lower Ag concentrations than did naive cells, were less dependent on costimulation by B7 molecules, and independent of costimulation by CD40. Furthermore, effector cytokine production by memory cells also occurred in the absence of either B7 or CD40 costimulation. Lastly, memory cells were resistant to tolerance induction. Together, these findings suggest that the threshold for activation of memory CD4+ cells is lower than that of naive cells. This would permit memory cells to rapidly express their effector functions in vivo earlier in the course of a secondary immune response, when the levels of Ag and the availability of costimulation may be relatively low. The Journal of Immunology, 2000, 164: 265–272.

Protective immunity induced by vaccination or infection is largely mediated by memory cells generated by the Ag exposure. This realization has led to a large amount of work on the induction and properties of memory T and B lymphocytes (1–4). The observation that somatic hypermutation is characteristic of memory B cells has been useful for tracking these cells. However, no comparable marker exists for memory T cells, and it has been difficult to critically examine the development of T cell memory. Indeed, many questions about the requirements for T cell memory generation and maintenance and the functional phenotype and characteristics of memory cells remain unanswered.

One of the earliest features shown to distinguish memory and naive T cells was their migration pattern. Naive T cells preferentially migrate to peripheral lymph nodes, presumably due to high levels of L-selectin expression, whereas memory cells migrate to peripheral sites of inflammation (2, 4–6). Additionally, T cells exhibiting a “memory” phenotype can be identified both in the peripheral circulation and spleen (2, 7). While several studies have examined the homing of naive vs memory cells, these were all based on identification of populations by phenotypic markers (CD45RB and L-selectin) rather than Ag specificity (2, 6, 8, 9). Recent work suggests that such markers may be influenced by the state of activation of the cell, the manner in which the memory population was generated, and the presence of Ag, and thus may not be reliable for distinguishing naive, effector, and memory populations (10–18).

The functional capacities of memory cells are also not definitively established. It is generally accepted that memory responses are more rapid than primary responses, and this rapidity forms the basis for protective immunity. Memory CD8+ lymphocytes, induced by virus infection, can be readily induced to secrete cytokines and become active CTLs upon Ag re-encounter (3, 19–23). Unlike their naive counterparts, memory CD4+ cells produce effector cytokines such as IFN-γ and IL-4 upon Ag encounter, thus promoting vigorous phagocyte and B cell responses and elimination of the pathogen (2, 17, 24). The basis for this difference in response kinetics between naive and memory cells is unclear. By comparing responses of naive and memory T cells to Ags presented by different APCs, it has been proposed that memory cells are less costimulator dependent than naive cells, and that they may require lower doses of Ag for activation (2, 25, 26). Other characteristics of memory cells, such as their susceptibility to tolerance induction, are also unclear as different studies suggest that memory cells are more or less sensitive to anergy than naive cells (27–30).

We have previously demonstrated that adoptive transfer of in vitro-activated CD4+ T cells derived from the DO.11 TCR transgenic mouse into syngeneic BALB/c mice leads to the development of a stable population of OVA223–339-specific long-surviving cells that can be identified by staining with a clonotypic Ab (KJ1-26) (17). These long-lived KJ1-26+ cells possess characteristics believed to be typical of memory cells (17). Therefore, we used this adoptive transfer system to analyze the functional capacities of Ag-specific memory CD4+ T cells. Our results demonstrate that memory cells localize to both the spleen and peripheral lymph nodes of mice, and that memory cells present in the spleen are rapidly activated to migrate away from the spleen after subcutaneous Ag exposure. Furthermore, we show that memory cells produce effector cytokines in the absence of cell cycling, and proliferate at Ag concentrations significantly lower than those required by naive cells. Lastly, our data demonstrate that memory cells are less dependent on costimulation by B7 molecules than naive cells and, consequently, less sensitive to tolerance induction in vivo.
Materials and Methods

Mice

BALB/c mice, 6–8 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). Transgenic mice expressing the DO.11.10 TCR (DO.11), specific for the chicken OVA peptide OVA232–239 in the context of the MHC class II molecule I-A^d, were obtained from Dr. D. Loh (Hoffmann-La Roche, Nutley, NJ) (31). Mice deficient in B7.1 and B7.2 on the BALB/c background were obtained from The Jackson Laboratory (33). These mice were bred in our pathogen- and viral Ab-free facility in accordance with the guidelines of the Committee on Animals of the Harvard Medical School, and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (Washington, DC). The mice were typed for the DO.11 TCR by staining peripheral blood cells with Abs against CD4 and Vβ8 (present in the transgenic TCR). In the DO.11 mouse, ~90% of the CD4^+ T cells express the transgene-derived Vβ8 gene compared with the wild-type syngeneic mouse (BALB/c), in which only 5% of the CD4^+ T cells are Vβ8^+.

Adoptive transfers and FACS analysis

All adoptive transfers were performed by injection of cells via the tail vein. Naive DO.11 T cells were prepared by harvesting peripheral lymph nodes from DO.11 mice and purifying CD4^+ cells using Dynabeads (Dynal, Oslo, Norway). In vitro-activated cells were prepared by culturing naive CD4^+ DO.11 T cells with mitomycin C-treated splenocytes from BALB/c mice (as APCs) and OVA peptide at 1 μg/ml in 24-well plates for 96 h in RPMI 1640 supplemented with 1 mM l-glutamine, penicillin, streptomycin, nonessential amino acids, sodium pyruvate, HEPES (all from Life Technologies, Grand Island, NY), 5 × 10^5 M 2-ME, and 10% FBS (Sigma, St. Louis, MO). The cells were then harvested andelicited (lymphocyte separation medium; Oregon Teknika, Durham, NC), before transfer. For adoptive transfer of naive cells into BALB/c recipients, lymph node and spleen cells were harvested from DO.11 mice. The number of T cells expressing the DO.11 TCR in the naive and activated DO.11 populations was measured by staining with the clonotypic Ab, KJ1–26, and flow cytometry. Next, 5–15 × 10^6 naive KJ1-26^+ CD4^+ T cells (in ~30–60 × 10^6 whole spleen/lymph node DO.11 mouse-derived cells) were transferred into BALB/c recipients, or, to generate memory cells, 20–25 × 10^6 in vitro-activated DO.11 T cells were transferred into BALB/c recipients. For the in vitro analyses of memory responses, the lymph nodes and spleens were collected from recipients of activated cells 6–7 mo after transfer and the CD4^+ cells were purified using Dynabeads. For flow cytometry, the cell suspensions were blocked with anti-CD16/CD32 (mouse Fc receptor), then stained with CyC-labeled anti-CD4 mAb (both from PharMingen, San Diego, CA) and biotinylated KJ1-26 clonotypic Ab followed by streptavidin-PE. Some samples were then stained with FITC-labeled Abs to CD25, CD44, CD45RB, CD40L, CD62L, IL-2Rα, B7.1, or B7.2 (PharMingen). Analyses were performed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA).

In vitro proliferation and cytokine assays

To analyze proliferative responses of the naive and memory cells, 5000 CD4^+ KJ1-26^+ naive cells (purified from DO.11 lymph nodes) or memory cells (purified from transfer recipients) were cocultured with 2.5 × 10^5 mitomycin C-treated BALB/c, B7/2/− or CD40−/− APCs in 0.2 ml of RPMI 1640 supplemented as described above in 96-well plates. Cells were stimulated with 0–1 μg/ml of OVA peptide. After 24, 48, 72, or 96 h, cultures were pulsed for 6 h with 1 μCi [3H]thymidine (New England Nuclear, Boston, MA), and incorporated radioactivity was measured in a Betaplate scintillation counter (LBK Pharmacia, Piscataway, NJ). To determine cytokine production, 5 × 10^5 KJ1-26^+ CD4^+ cells were cultured with 2.5 × 10^5 mitomycin C treated BALB/c APCs in 1 ml of medium in the presence of 0–1 μg/ml of OVA peptide. Supernatants were collected after 0, 24, 48, and 72 h, and levels of IL-2, IL-4, IL-5 and IFN-γ were assayed by ELISA as previously described (34). Proliferative responses of untreated, immunized or tolerized naive and memory cells were assessed as above, but by total incorporation of 1 μCi [3H]thymidine in each well in 96-well plate without additional APCs. To measure cytokine responses of untreated, immunized, or tolerized naive and memory cells, 4 × 10^4 total lymph node cells were cultured in 24-well plates without or with OVA peptide at 1 μg/ml, and cytokine levels in the supernatants were assayed as described above.

Fluorochrome labeling of cells and FACS analysis

To accurately assess cycling, naive cells from the lymph nodes of DO.11 mice or memory cells harvested from transfer recipients were labeled with chloromethylfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) (35). Cells were suspended at 10 × 10^6/ml in RPMI 1640 with no FCS, and the CFSE was added to a final concentration of 1 μM. The cells were incubated for 12 min at 37°C, at which time the labeling was terminated by the addition of cold RPMI 1640. CFSE is lipophilic and passively enters the cell where it is converted to the fluorescent 5-chloromethylfluorescein by esterase hydrolysis and can no longer diffuse out of the cell. With repeated cell division, the fluorochrome is gradually lost from the cell, thus serving as an indicator of cell cycle status. At 12, 36, 60, and 84 h after culture, the cells were collected, stained with biotinylated KJ-26 followed by streptavidin PE, and analyzed by flow cytometry.

Immunohistochemistry

A total of 15 × 10^6 naive or in vitro-activated KJ1-26^+ T cells were adoptively transferred into unirradiated BALB/c recipients by tail vein injection. At 10 wk after transfer, recipients were either not immunized or immunized with 150 μg of OVA peptide emulsified in IFA (Difco, Detroit, MI) by s.c. injection in four to six sites along the back. The peripheral lymph nodes (submandibular, axillary, brachial, inguinal, and popliteal for nonimmunized; axillary, brachial, and inguinal for immunized) and spleens were harvested on day 0, 1, 3, or 5 after immunization. The tissues were fixed in OCT (TissueTek; Miles, Elkhart, IN), flash frozen, cut into 6-μm thick sections, fixed in ice cold acetone for 10 min, and allowed to dry overnight. The sections were rehydrated in PBS with 0.1% Tween 20 for 10 min, then blocked with 5% BSA in PBS with anti-CD16/CD32 (mouse Fc receptor) added at 2.5 μg/ml for 60 min at room temperature. The sections were then incubated with FITC-labeled anti-B220 (PharMingen) at 5 μg/ml and biotinylated KJ1-26 at 2.5 μg/ml for 60 min at room temperature. The slides were washed with PBS for 15 min, and the alkaline phosphatase was developed first using the following mix: 5 mg naphth AS-MX phosphate in 250 μl N,N-dimethyl formamide was added to 40 ml 0.1 M Tris-HCl, pH 8.5; 10 mg fast blue base in 250 μl 2 N HCl and 250 μl 4% sodium nitrite was added to the Tris buffer, after which 10 mg of levamisole was added to inhibit endogenous alkaline phosphatase activity. The mixture was filtered, placed on the tissue sections, and the slides were incubated in the dark for 15–60 min. After the color had developed, the slides were washed in PBS for 15 min and the horseradish peroxidase was developed using the following mix: 4 mg of 3-aminophenylcarbazole dissolved in 250 μl of N,N-dimethyl formamide was added to 9.75 ml of 0.05 M sodium acetate buffer, pH 5.0. This was filtered, 5 μl of 30% H2O2 was added, and the buffer was placed on the tissue sections. All chemicals for color development were obtained from Sigma. Color developing was terminated by a final wash in PBS, after which the slides were mounted using Biomeza Crystal Mount (Biomeza, Foster City, CA).

Tolerance induction

A total of 20–25 × 10^6 in vitro-activated DO.11 T cells or 5 × 10^6 naive cells were prepared as described above and adoptively transferred into BALB/c. At 6 wk or 9 mo after transfer of activated cells, or 1–2 days after transfer of naive cells, recipients were either not immunized, immunized with 150 μg of OVA peptide emulsified in IFA by s.c. injection along the back, or tolerized by tail vein injection of 300 μg of OVA peptide in PBS (this dose of OVA peptide has been previously shown to induce tolerance in the peripheral lymph node cells of adoptively transferred naive DO.11 T cells (34, 36, 37)). The peripheral lymph nodes (submandibular, axillary, brachial, inguinal, and popliteal for untreated and tolerized; axillary, brachial, and inguinal for immunized) were harvested 3 days after immunization. In vitro proliferation and cytokine assays were performed as described above.

Abbreviation used in this paper: CFSE, chloromethylfluorescein diacetate succinimidyl ester.
Results
Memory cells proliferate at lower Ag concentrations than naive cells, and do not require multiple cycles of cell division to produce effector cytokines

To provide a source of Ag-specific memory CD4\(^+\) T cells, DO.11 T cells were activated in vitro with peptide and syngeneic APCs and adoptively transferred into syngeneic mice (BALB/c). This results in the development of a long-lived population of quiescent cells that survive in the absence of overt Ag exposure and can be detected as long as 9 mo after transfer, without additional Ag exposure (Fig. 1A and Ref. 17). These cells express high levels of CD44 and low levels of CD25 (Fig. 1B), while naive KJ1-26\(^+\) CD4\(^+\) cells purified from the peripheral lymph nodes of DO.11 mice express low levels of CD44 and CD25 (Fig. 1B). Additionally, we found that the memory KJ1-26\(^+\) cells express identical levels of CD45RB, IL-2R\(\beta\), and CD40L as those expressed by their naive counterparts (Fig. 1B). L-selectin (CD62L) expression is biphasic on the KJ1-26\(^+\) memory cells, with the majority of the population having up-regulated expression to levels similar to those on naive cells (Fig. 1B and Ref. 17). Interestingly, memory cells express slightly higher levels of B7-1 and B7-2 than naive cells. In summary, as with CD8\(^+\) cells express slightly higher levels of B7-1 and B7-2 than naive cells. In summary, as with CD8\(^+\) cells, CD4\(^+\) memory cells was noted at 0.1 \(\mu\)g/ml of OVA. These experiments indicate that memory KJ1-26\(^+\) cells are capable of proliferating more rapidly and at a log lower concentration of OVA than their naive counterparts.

Supernatants derived from these cultures were analyzed for secreted cytokines to correlate cycling with effector responses. Both naive and memory cells were secreting IL-2 within 12 h of Ag exposure at both 0.1 and 1 \(\mu\)g/ml of OVA, and proliferation of naive cells was noted at 0.1 \(\mu\)g/ml of OVA. These experiments indicate that memory KJ1-26\(^+\) cells are capable of proliferating more rapidly and at a log lower concentration of OVA than their naive counterparts.

Superantigens derived from these cultures were analyzed for secreted cytokines to correlate cycling with effector responses. Both naive and memory cells were secreting IL-2 within 12 h of Ag challenge (Fig. 3). Interestingly, the memory cells also began producing IL-4 at 12 h of Ag exposure at both 0.1 and 1 \(\mu\)g/ml of OVA.

FIGURE 1. Purification and phenotype of naive and memory DO.11 lymphocytes. A, Naive DO.11 CD4\(^+\) cells were activated in vitro as described in Materials and Methods and transferred into BALB/c recipients. At 6 mo after transfer, the CD4\(^+\) cells from transfer recipients (referred to as memory cells) were purified from lymph node and spleen cells. Naive cells were cultured with the peripheral lymph nodes of intact DO.11 mice. Both populations were analyzed for percent of CD4\(^+\) KJ1-26\(^+\) cells by flow cytometry. B, The naive and memory populations purified above were stained with Abs to CD25, CD40L, CD44, CD45RB, CD62L, IL-2R\(\beta\), B7.1, and B7.2.

By 36 h of culture, a small amount of proliferation (as assessed by loss of CFSE content) was noted in the memory KJ1-26\(^+\) cells cultured with 1 \(\mu\)g/ml of OVA peptide; no cycling was noted in the naive population (Fig. 2). At 60 h of culture, significant cycling of the memory cells cultured with 1 \(\mu\)g/ml of OVA was evident; at lower Ag concentrations (0.1 \(\mu\)g/ml), the memory cells showed much more cycling than the naive cells. By 84 h, proliferation of memory cells was noted at 0.01 \(\mu\)g/ml of OVA, and proliferation

Materials and Methods

Purification and phenotype of naive and memory DO.11 lymphocytes. By diluting the naive cells with purified BALB/c CD4\(^+\) cells, naive KJ1-26\(^+\) cells were collected and analyzed by flow cytometry to compare the responses of Ag-specific naive and memory cells to Ag challenge. The naive and memory populations purified above were stained with Abs to CD25, CD40L, CD44, CD45RB, CD62L, IL-2R\(\beta\), B7.1, and B7.2.

Results
Memory cells proliferate at lower Ag concentrations than naive cells, and do not require multiple cycles of cell division to produce effector cytokines

To provide a source of Ag-specific memory CD4\(^+\) T cells, DO.11 T cells were activated in vitro with peptide and syngeneic APCs and adoptively transferred into syngeneic mice (BALB/c). This results in the development of a long-lived population of quiescent cells that survive in the absence of overt Ag exposure and can be detected as long as 9 mo after transfer, without additional Ag exposure (Fig. 1A and Ref. 17). These cells express high levels of CD44 and low levels of CD25 (Fig. 1B), while naive KJ1-26\(^+\) CD4\(^+\) cells purified from the peripheral lymph nodes of DO.11 mice express low levels of CD44 and CD25 (Fig. 1B). Additionally, we found that the memory KJ1-26\(^+\) cells express identical levels of CD45RB, IL-2R\(\beta\), and CD40L as those expressed by their naive counterparts (Fig. 1B). L-selectin (CD62L) expression is biphasic on the KJ1-26\(^+\) memory cells, with the majority of the population having up-regulated expression to levels similar to those on naive cells (Fig. 1B and Ref. 17). Interestingly, memory cells express slightly higher levels of B7-1 and B7-2 than naive cells. In summary, as with CD8\(^+\) cells express slightly higher levels of B7-1 and B7-2 than naive cells. In summary, as with CD8\(^+\) cells, CD4\(^+\) memory cells was noted at 0.1 \(\mu\)g/ml of OVA. These experiments indicate that memory KJ1-26\(^+\) cells are capable of proliferating more rapidly and at a log lower concentration of OVA than their naive counterparts.

Superantigens derived from these cultures were analyzed for secreted cytokines to correlate cycling with effector responses. Both naive and memory cells were secreting IL-2 within 12 h of Ag challenge (Fig. 3). Interestingly, the memory cells also began producing IL-4 at 12 h of Ag exposure at both 0.1 and 1 \(\mu\)g/ml of OVA.
stimulator-deficient APCs. We chose to use APCs deficient in B7.1/(as described above) and analyzed their proliferative responses to co-
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costimulators in the activation of memory cells has not been ex-
tory cells are less reliant than naive cells on costimulation for ac-
dendritic cells or B cells as APCs, it has been postulated that mem-
naive population with BALB/c CD4
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1

FIGURE 2. Memory cells cycle more rapidly and at lower Ag concen-
trations than naive cells. The CD4+ naive and memory cells, purified as in
Fig. 1, were corrected for the percentage of KJ1-26+ cells by diluting the
naive population with BALB/c CD4+ cells as described in Materials and
Methods. In each experiment, the KJ1-26+ memory cells were 5–7% of the
total CD4+ population. To assess cycling status, the naive and memory
populations were labeled with CFSE and 1 × 10^6 CD4+ cells (5 × 10^4
KJ1-26+ cells) of each were cocultured with 2.5 × 10^5 mitomycin C-
treated BALB/c APCs and no peptide or OVA peptide at 0.01, 0.1, or 1
µg/ml. At 12, 36, 60, or 84 h, the cells were collected and analyzed for
CFSE expression in the CD4+ KJ1-26+ population by flow cytometry. A
representative example of two experiments is shown; each time point was
performed in duplicate for each experiment.

OVA. A small amount of IFN-γ was noted at 12 h when memory
cells were cultured with 1 µg/ml of OVA; this increased dramatically
by 36 h at both high and low peptide concentrations. Pro-
duction of IL-5 by memory cells appeared later and correlated with

cell cycling, as IL-5 was not detected until 60 h of culture. No
IFN-γ, IL-4, or IL-5 was found in supernatants from the naive cell
cultures. These results indicate that memory cells produce effector
cytokines before they begin to cycle, and thus can initiate an effector
response without the need for multiple rounds of clonal expansion.

Memory cells are less dependent on costimulation than naive
cells

Based on experiments using CD45RBlowCD4+ T cells cultured on
dendritic cells or B cells as APCs, it has been postulated that mem-
ory cells are less reliant than naive cells on costimulation for ac-
tivation, presumably because their threshold for activation is lower
than that of naive cells (25, 26). However, the role of defined

stimulators in the activation of memory cells has not been ex-
aminined. To directly test this, we generated populations of CD4+ cells
containing equal numbers of naive and memory KJ1-26+ cells
(as described above) and analyzed their proliferative responses to co-
stimulator-deficient APCs. We chose to use APCs deficient in B7.1/
cells is much lower than that of naive cells, as they are capable of proliferating in the presence of extremely small numbers of APCs.

To determine the role of costimulators in the production of effector cytokines, naive and memory CD4⁺ cells containing identical numbers of KJ1-26⁺ cells were cocultured with wild-type, B7.1/B7.2⁻/⁻, or CD40⁻/⁻ APCs, and supernatants were collected over 4 days. The absence of B7 or CD40 did not impair any cytokine production in memory cells, while naive cells exhibited deficits in IL-2 production (Fig. 5). These results support the notion that the effector function of memory cells is not dependent on costimulation through CD28:B7 or CD40:CD40L interactions.

**Memory cells exhibit rapid recall responses to Ag in vivo**

The tissue localization of memory cells in vivo has not been clearly defined. While flow cytometric analysis of tissues containing Ag-specific memory cells is useful for quantitation, it provides no information about the anatomic compartmentalization of the population. To examine the location of memory cells in vivo, we used immunohistochemical analysis of lymphoid tissues to identify KJ1-26⁺ cells in mice 6 to 10 wk after adoptive transfer of in vitro-activated DO.11 T cells. For comparison, tissues from mice that had received naive KJ1-26⁺ cells were similarly analyzed. To follow the responses of the naive or memory cells to Ag, transfer recipients were immunized with OVA peptide in IFA by s.c. injection and lymphoid tissues were collected at day 0, 1, and 5 after Ag exposure.

At 10 wk after transfer, the in vitro-primed DO.11 lymphocytes (memory cells) were detectable in the T cell zones of the lymph nodes and spleen (Fig. 6) as well as circulating in the blood (data not shown). Upon Ag challenge, there was a rapid expansion of the T cells in the regional lymph nodes, beginning 24 h after immunization and increasing significantly by 5 days postchallenge. Strikingly, in the spleen, the memory cells began expanding 24 h after immunization, but then decreased in number between days 3 and 5 postchallenge. This is most likely due to rapid migration from the spleen to the site of Ag exposure and probably to the lymph nodes draining the site of Ag challenge. At the same time (10 wk postransfer), naive DO.11 cells were virtually undetectable by immunohistochemistry. However, they are present, because they too could be expanded by Ag challenge (Fig. 6). Interestingly, at the time when memory cells had left the spleen (day 5), the naive cells (now activated) begin expanding in the spleens of recipient mice. These results indicate that the more rapid activation of memory cells seen in vitro is mirrored in their responses to Ag challenge in vivo. The rapid migration of memory cells from the spleen may be due to the fact that these cells express adhesion molecules that permit immediate entry into the vasculature upon activation.

**Memory cells are resistant to tolerance induction**

The question of whether or not memory cells can be tolerized is largely unresolved. The system of adoptively transferring TCR
transgenic T cells is well-suited to studies of tolerance induction in vivo. Previous work from several laboratories has demonstrated that the administration of aqueous OVA peptide by i.v. injection to adoptive transfer recipients of naive DO.11 T cells leads to functional unresponsiveness in these T cells (34, 36, 37, 40, 41). This is manifested by an abortive expansion of KJ1-26+ cells in lymph nodes, and failure of these cells to enter B cell follicles. Functionally, the tolerized cells respond poorly to Ag restimulation in vitro and fail to produce either IL-2 or IFN-γ (34, 36, 37, 40, 41).

To generate a population of memory cells for the tolerance experiments, 20–25 × 10^6 in vitro-activated DO.11 T cells were transferred into BALB/c recipients. These cells were allowed to remain quiescent in the recipient mice for 6 wk or 9 mo. Three days before tolerance induction, an additional group of BALB/c mice was transferred with 5 × 10^6 naive KJ1-26+ cells. The mice were either left untreated or given OVA peptide in IFA by s.c. injection (immunized) or OVA peptide in PBS by tail vein injection (tolerized). After 3 days, the peripheral lymph nodes from the mice were collected and analyzed for the remaining percent of KJ1-26+ cells. Untreated recipients of naive and memory cells had similar numbers of KJ1-26+ cells in the lymph nodes (Fig. 7A).

Upon encounter with Ag in adjuvant, the naive and memory KJ1-26+ cells expanded equivalently. The functional responses of the untreated, immunized, and tolerized populations were assayed by restimulation with OVA peptide ex vivo. After exposure to tolerogenic Ag, the naive DO.11 T cells failed to proliferate or produce cytokines upon Ag challenge (Fig. 7B). In striking contrast, the memory T cells showed no significant

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**FIGURE 6.** Immunohistochemical analysis of in vivo responses of naive and memory CD4⁺ T cells. Recipients of naive DO.11 cells (Naive) or in vitro-primed DO.11 T cells (Memory) 10 wk after transfer were left untreated or challenged with OVA peptide in IFA by s.c. injection. Lymph nodes and spleens were harvested from the untreated mice (left) or 1 day (middle) or 5 days (right) after Ag challenge. Frozen sections were stained with KJ1-26 (blue, alkaline phosphatase) and anti-B220 (brown, peroxidase). Note that memory DO.11 T cells are readily detected in lymph nodes and spleens without Ag challenge, expand rapidly within 1 day, and appear to leave the spleen within 5 days. Naive DO.11 cells are virtually undetectable in untreated recipients, and they expand much more slowly upon challenge.
additional BALB/c mice were transferred with 5 × 10^3 of cytokine).

OVA peptide in IFA or tolerized by i.v. injection of 300 μg/ml for 48 h and pulsing plates for 6 h with [3H]thymidine. Proliferation was corrected for KJ1-26 cells in the lymph node population analyzed. B. Proliferative responses of untreated, immunized, and tolerized KJ1-26 cells were assessed by culturing 5 × 10^5 lymph node cells with OVA peptide at 0–1 μg/ml for 48 h and pulsing plates for 6 h with [3H]thymidine. Proliferation is corrected for KJ1-26 cell input. Cytokine secretion was assayed in supernatants of the same cultures collected at days 0, 1, 2, and 3 and analyzed for the presence of IL-2 and IFN-γ by ELISA (expressed as U/ml of cytokine).

**FIGURE 7.** Memory cells are less susceptible to tolerance induction than naive cells. BALB/c mice were adoptively transferred with 20–25 × 10^6 in vitro-activated DO.11 T cells (memory mice). After 6 wk or 9 mo, additional BALB/c mice were transferred with 5 × 10^6 naive DO.11 lymphocytes (naive mice). Mice were left untreated, immunized with 150 μg of OVA peptide in IFA or tolerized by i.v. injection of 300 μg of OVA peptide in PBS (according to previously published protocols for tolerance induction in vivo) (34, 37). The peripheral lymph nodes were collected from the naive and memory mice 3 days after treatment and analyzed. A. The lymph node cells from untreated, immunized, and tolerized naive and memory mice were stained for KJ1-26 expression and analyzed by flow cytometry. The number in the FACS plot indicates the percent of memory T cells expressing a memory phenotype entered the cell cycle upon Ag exposure (Fig. 6).

**Discussion**

The studies presented in this paper were undertaken to characterize the functional responses of Ag-specific memory CD4+ lymphocytes in vivo and in vitro. The experimental model we have used employs an adoptive transfer system in which DO.11 T cells are activated in vitro and transferred into syngeneic BALB/c recipients. The process of in vitro activation permits a population of the cells to survive for long periods in vivo without overt exposure to cognate Ag. These long-surviving cells are CD25^hi^CD44^low_, localize to the spleens and lymph nodes of transfer recipients, and produce effector cytokines upon Ag challenge (17). Thus, these long-surviving cells have the phenotypic and functional characteristics of memory cells.

Our data indicate that memory cells enter the cell cycle upon Ag stimulation much more rapidly than do naive cells and produce effector cytokines such as IFN-γ and IL-4 before cycling (Figs. 2 and 3). This suggests that memory T cells require little or no cycling to produce effector cytokines; this is in direct contrast to naive cells, which require at least four cell divisions to produce IL-4 (42). As effector cytokine production by naive cells has been demonstrated to be dependent on changes in the chromatin, including alterations in methylation status and accessibility for transcription factors (42), our results suggest that the chromatin changes induced during T cell activation may be maintained during memory. Thus, as activated cells progress into quiescence and memory, the status of the effector cytokine genes may not be significantly altered (e.g., methylated), allowing rapid cytokine gene expression upon reactivation. This would permit rapid effector function upon Ag challenge, without a need for clonal expansion.

It has been suggested that memory cells require less costimulation than do naive cells for activation upon Ag encounter. This conclusion is based on comparing different APCs for their ability to activate effector and/or memory cells (2, 25, 26). Thus, the role of costimulation has been inferred from the expected properties of the APCs. We have examined this question directly, using APCs from knockout mice in which the two major costimulatory pathways for T cells are deleted. Our data indicate that memory cells are less dependent on B7 costimulation than their naive counterparts (Fig. 4). Furthermore, the activation of memory cells does not require the presence of CD40 on APCs (Fig. 4). Our results also demonstrate that memory cells are activated at lower peptide concentrations than naive cells (Figs. 2 and 4). These characteristics of memory cells would permit the induction of a memory response in the presence of limiting Ag and/or inflammation, as may be the case very early after pathogen encounter. The rapid subsequent effector response would serve to eliminate the pathogen.

Immunohistochemical analysis of memory T cells shows that contrary to previous reports, memory cells do localize to the lymph nodes (Fig. 6). This is likely because memory T cells re-express L-selectin when they reach a stage of quiescence (Fig. 1B and Ref. 17). Indeed, recent experiments on memory cells in rats demonstrated that CD4^+^ T cells expressing a memory phenotype entered lymph nodes across the high endothelial venules as well as via afferent lymphatics (43, 44). It is also clear that memory cells are capable of mobilizing rapidly in response to distant Ag, as the KJ1-26^+^ memory cells present in the spleens of transfer recipients were rapidly activated and left the spleen within 3–5 days of s.c. Ag exposure (Fig. 6).
Finally, our data demonstrate that memory cells are resistant to tolerance induction (Fig. 7). Anergy induction in naive cells in vivo is believed to occur when these cells encounter Ag under conditions of limited costimulation (i.e., low levels of B7 and absence of inflammation) (34, 37). In this situation, B7-CTLA-4 interactions would predominate over B7:CD28 interactions, leading to inhibition of IL-2 production and cell cycling (45, 46). As memory cells are less dependent on B7 costimulation, the differential in B7 expression normally important in controlling naive T cell responses would be less consequential for memory cells. The reason for a decreased dependence on B7 costimulation for the activation of memory cells is not known. It is possible that memory cells differ from naive T cells in TCR-induced signaling pathways, resulting in a lower activation threshold. The rapid production of IFN-γ or IL-4 by memory cells without cycling would also serve to limit the capacity for anergy induction in this population.

In conclusion, the studies presented in this paper provide direct evidence that memory cells are capable of inducing rapid effector responses in the presence of limiting Ag and costimulation. Furthermore, unlike the case with naive cells, production of effector cytokines by memory cells does not require multiple rounds of cell division. These properties of memory cells ensure an expeditious and effective response to Ag challenge. The relative costimulator independence and tolerance resistance of memory T cells also raises the possibility that strategies for tolerance induction in naive cells, such as costimulator blockade in transplantation, may not be as effective for established immune responses.

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References