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*J Immunol* 1999; 162:766-773;  
http://www.jimmunol.org/content/162/2/766

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*The Journal of Immunology* is published twice each month by  
The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Functional Characteristics and Survival Requirements of Memory CD4\(^+\) T Lymphocytes In Vivo\(^1\)

Cheryl A. London, Victor L. Perez,\(^2\) and Abul K. Abbas\(^3\)

The phenotypic and functional characteristics of Ag-specific memory CD4\(^+\) lymphocytes are poorly defined. To examine the properties and cytokine responsiveness of these cells, we have developed an adoptive transfer system using in vitro-activated T cells expressing the DO.11 transgenic TCR specific for OVA\(_{323-339}\) I-A\(^\alpha\). In vitro-activated DO.11 CD4\(^+\) cells exhibit comparable survival patterns at 1, 6, and 10 wk after adoptive transfer, indicating that a stable population of memory cells has been generated. In the absence of Ag, previously activated T cells survive longer than their naive counterparts in vivo, rapidly revert to a partially naive phenotype, and maintain their effector cytokine profile. The DO.11 CD4\(^+\) memory cells are capable of proliferating in response to IL-2 and IL-4, while naive DO.11 CD4\(^+\) cells exhibit no such proliferative responses. *The Journal of Immunology*, 1999, 162: 766–773.

Upon exposure to Ag, specific CD4\(^+\) T cells are activated and differentiate into effector T cells, producing cytokines that ultimately determine the outcome of Ag challenge. A small fraction of these Ag-specific cells survive to become long-lived memory cells. Despite considerable interest in defining the stimuli for the development of memory cells, their characteristics and the factors that promote their survival remain poorly defined (1–6). One reason for this is that the phenotype of memory cells is not conclusively established and may not be fixed. For instance, early studies suggested that, in humans, naive T cells express the CD45RA isoform of the CD45 phosphatase, whereas memory cells express the CD45RO isoform (6–9). However, it is now clear that the expression of these CD45R isoforms is regulated by cell activation, and the phenotypes are interconvertible (10–16).

Naive T cells also express high levels of L-selectin (CD62L) and CD45RB (in mice) and low levels of CD44, whereas memory cells have the opposite phenotype (1, 2, 6, 7, 17). Again, however, it is not established if these markers are associated with differentiation into memory cells or if the changes are the result of activation.

While the phenotypic characterization of memory cells is controversial, virtually nothing is known concerning the requirements for the generation of memory cells during a primary immune response, or the factors that promote the survival of these cells once an immune response has been terminated. Some studies indicate that memory cells are long-lived in the absence of Ag exposure, whereas other studies indicate that memory populations are maintained in a noncycling state by continuous low-level stimulation by persistent or cross-reactive Ag (4, 5, 18–20). While it now appears that CD8\(^+\) memory cells do require the presence of MHC class I for their long-term survival (21), it is not clear if MHC class II is required for the persistence of memory CD4\(^+\) cells. Recent evidence also suggests that cytokines such as IFN-\(\alpha/\beta\) and IL-15 may play a role in maintaining CD8\(^+\), but not CD4\(^+\), memory cells (22–24). The cytokines, if any, that play a role in sustaining memory CD4\(^+\) cells have yet to be identified. The obvious importance of these problems is that lymphocyte memory is the basis of prophylactic immunization, and understanding the control and maintenance of memory may be valuable for enhancing protective immunity against microbial pathogens.

Detailed analysis of Ag-specific long-lived CD4\(^+\) cells is often hampered by their low numbers, and much of what is known about these cells is derived from limiting-dilution assays (4, 25–28). Moreover, in conventional systems it is difficult to quantitatively follow a cohort of Ag-specific T cells in vivo with and without exposure to Ag. The availability of Ag receptor transgenic mice has now made it possible to more critically examine the fate of an Ag-specific lymphocyte population in vivo. We have utilized CD4\(^+\) cells from the DO.11 TCR transgenic mouse as a source of T cells specific for a known Ag, and for which a clonotypic Ab is available. This experimental system allows us to define the phenotypic and functional characteristics of T cells recovered from mice by staining with the clonotypic Ab and studying responses to the cognate peptide. Our results demonstrate that a population of memory CD4\(^+\) T cells can be generated by in vitro Ag priming. In this paper we describe the phenotype and cytokine secretion profiles of these cells. Using a novel assay for cell survival and cycling, we show that memory, but not naive CD4\(^+\) cells, are capable of cycling in response to IL-2 and IL-4. Therefore, these cytokines may play a role in the maintenance of CD4\(^+\) memory cells 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 OVA\(_{323-339}\) in the context of the MHC class II molecule I-A\(^\alpha\), were obtained from Dr. D. Loh (Hoffmann-La Roche, Nutley, NJ). Mice deficient in the IgM heavy chain (\(\mu^{-/-}\), B cell deficient) on the BALB/c background were obtained from Dr. Steve Reiner (University of Chicago, Chicago, IL). 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.

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Received for publication August 6, 1998. Accepted for publication October 6, 1998.

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\(^1\) This work was supported by National Institutes of Health Grants AI37963 and AI25022 (to A.K.A.) and Grant K01RR-00121 (to C.A.L.).

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Adaptive transfers, immunization, and FACS analysis

Naive DO.11 T cells were prepared by harvesting lymph node and spleen cells pooled from DO.11 mice and running these over a nylon wool column to enrich for T cells before transfer. In vitro-activated cells were prepared by isolating CD4+ T cells from lymph node and spleen cells pooled from DO.11 mice using Dynabeads (Dynal, Oslo, Norway). The resultant CD4+ cells were cultured 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^{-2} M 2-ME, and 10% FBS (Sigma, St. Louis, MO). The cells were then harvested and ficollined (Lymphocyte Separation Medium, Oregon Teknika, Durham, NC) before transfer. The number of T cells expressing the DO.11 TCR was measured by staining with the clonotypic Ab, KJ1-26, and flow cytometry. Naive or in vitro-activated CD4+ T cells (15 × 10^6) were adoptively transferred into unirradiated BALB/c or μ−/− recipients by tail vein injection. Before transfer, the activated cells were either 75% KJ1-26+, and >95% CD4+. At various time points after transfer, recipients were either not immunized, or immunized with 100 μg of OVA peptide emulsified in IFA (Difco, Detroit, MI) by s.c. injection 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 3–4 days after immunization. For flow cytometry, the lymph node and spleen cell suspensions from each group were blocked with anti-CD16/CD32 (mouse Fc receptor), then stained with cytochrome c-labeled anti-CD4 mAb (PharMingen, San Diego, CA) and biotinylated KJ1-26 clonotypic Ab followed by streptavidin-FITC or streptavidin-phycytoerythrin. Samples were then stained with phycytoerythrin-labeled Abs to CD25, CD44, or L-selectin (PharMingen). Analyses were performed on a FACScan flow cytometer.

Fluorochrome labeling of cells and FACS analysis

To determine whether memory or naive cells would proliferate in response to cytokines in vitro, memory cells were labeled with 5-chloromethylfluorescein diacetate (CMFDA; 4 cell tracker green), and naive cells were labeled with BODIPY red (both from Molecular Probes, Eugene, OR). Cells were suspended at 10^6/ml in RPMI 1640 supplemented with 1 mM l-glutamine, penicillin, streptomycin, and 5 μg/ml of OVA peptide. Naive or in vitro-activated KJ1-26 cells were labeled with BODIPY red (both from Molecular Probes, Eugene, OR), then stained with cytochrome c-labeled anti-CD4 mAb (PharMingen, San Diego, CA) and biotinylated KJ1-26 clonotypic Ab followed by streptavidin-FITC or streptavidin-phycytoerythrin. Samples were then stained with phycytoerythrin-labeled Abs to CD25, CD44, and L-selectin (PharMingen). Analyses were performed on a FACScan flow cytometer.

In vitro proliferation and cytokine assays

To analyze proliferative responses of the adoptively transferred cells, 5 × 10^5 lymph node cells collected from transfer recipients before or after immunization were cultured in 0.2 ml of RPMI 1640 supplemented as described above in 96-well plates. Cells were restimulated with 0–1 μg/ml of OVA peptide. After 48 h, cultures were pulsed with 6 h with 1 μCi [3H]thymidine (New England Nuclear, Boston, MA), and the incorporation of radioactivity was measured in a Betaplate scintillation counter (Pharmacia LKB, Piscataway, NJ). To determine cytokine production, 4 × 10^4 lymph node cells collected from transfer recipients were cultured in 1 ml of medium in the presence of 0 or 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 (29).

Abbreviation used in this paper: CMFDA, 5-chloromethylfluorescein diacetate.

Results

Previously activated Ag-specific T cells are longer lived in vivo than naive T cells

The experimental protocol we have used for generating long-lived T cells involves priming cells expressing a transgenic TCR in vitro, and transferring these primed cells into syngeneic mice. The advantages of such a system are that survival can be followed without overt Ag exposure or persistence, the Ag-specific cells can be identified with a clonotypic Ab and assayed for patterns of gene expression, and, in the future, selected mutations can be introduced into these cells.

To compare the survival of naive T cells vs activated T cells in vivo, we adoptively transferred 15 × 10^6 naive or in vitro-activated KJ1-26+ CD4+ T cells from DO.11 TCR transgenic donors into syngeneic BALB/c recipients. Transfer recipients were assayed for the numbers of DO.11 T cells in lymphoid tissues by staining and flow cytometry, without further exposure to Ag. At 1 wk after adoptive transfer, there are comparable numbers of naive and previously activated DO.11 T cells in the peripheral lymph nodes of adoptive transfer recipients (Fig. 1). In this representative experiment, naive KJ1-26+ cells represent 1.3% of the total lymph node population (3.9% of the CD4+ T cells, 3.1 × 10^6 KJ1-26+ cells per lymph node), while the in vitro-activated T cells are 1.8% of the lymph node population (5.2% of the CD4+ T cells, 3.8 × 10^6 KJ1-26+ cells per lymph node). However, at 10 wk after transfer, there are significantly fewer naive KJ1-26+ cells (0.5% of total, 1.6% of CD4+ cells, 0.8 × 10^6 KJ1-26+ cells) compared with the surviving previously activated KJ1-26+ cells (1.7% of total, 5.2% of CD4+ cells, 4.5 × 10^6 KJ1-26+ cells). Results of multiple experiments summarized in Table I confirm the prolonged survival of previously activated T cells. Therefore, the process of activation in vitro generates a population of cells that are stable for long periods in vivo.

As it is known that the KJ1-26+ CD4+ T cells in DO.11 mice may have rearranged an endogenous TCR α-chain leading to expression of a second TCR (30), it is possible that the long-term
survival of the in vitro-activated, transferred cells is due to interaction of the second TCR on the transgenic cells with endogenous Ags in the recipient BALB/c mice. To rule out this possibility, we performed identical experiments with DO.11 mice bred onto the SCID background. As these mice are unable to effectively rearrange both TCR and Ig genes, all the T cells should express only the transgenic TCR. As before, naive CD4+ cells were purified from the DO.11 SCID mice, activated in vitro, and adoptively transferred into BALB/c recipients. At both 1 and 10 wk after transfer, comparable numbers of SCID KJ1-26+ memory cells are found in the lymph nodes of transfer recipients as were seen with wild-type KJ1-26+ memory cells (Table I, Expts. 7 and 8). Therefore, expression of a second TCR on a proportion of the memory cells derived from wild-type DO.11 mice is not responsible for their long-term survival in the BALB/c recipient mice.

### Surface phenotypes and cytokine profiles of long-lived, previously activated T cells

In mice, naive T cells are known to express high levels of CD45RB and L-selectin and low levels of CD44 (1, 2, 6, 7, 17). Other markers, such as CD25 and CD69, correlate with the state and timing of T cell activation (7, 31). While the phenotype of naive cells is relatively well characterized, that of long-lived memory cells has remained controversial. To evaluate the phenotype of long-surviving CD4+ T cells, lymph node cells from DO.11 transfer recipients were stained for the expression of the clonotypic TCR and a panel of surface markers and examined by flow cytometry. Before adoptive transfer, the naive T cells were CD25low, L-selectinlow, and CD44low, while the activated cells were CD25high, L-selectinhig, and CD44high. Long-surviving T cells, at 6 or 10 wk after adoptive transfer, became CD25high but maintained high levels of CD44 (Fig. 2A). Comparison of the cells at 1 and 10 wk after transfer also shows that memory cells are CD44high (Fig. 2B). Interestingly, the memory KJ1-26+ cells present in lymph nodes gradually become L-selectinhig, and this is lost upon Ag challenge (Fig. 2B). Thus, as has been observed with CD8+ T cells (32), the expression of L-selectin varies with the state of activation of CD4+ cells, down-regulating upon Ag exposure and up-regulating upon entering a state of quiescence. Conversely, CD44 expression remains high on activated cells, even after they have resided in vivo for long periods of time (Fig. 2, A and B). Therefore, as with CD8+ memory cells, the most reliable phenotype for CD4+ memory cells appear to be CD25high and CD44high.

To determine whether the long-lived KJ1-26+ cells maintain the pattern of cytokine production that was present before adoptive transfer, lymph node cells were harvested from adoptive transfer recipients of naive or activated cells at 1 and 10 wk after transfer and restimulated in vitro with OVA323–339 peptide. As the frequency of T cells specific for OVA 323–339 in normal BALB/c mice is undetectable, all cytokine production in this assay is attributable to the remaining KJ1-26+ T cells (data not shown). As shown in Fig. 3, at both 1 and 10 wk after transfer, the in vitro-activated T cells produce a mix of cytokines, including IL-2, IFN-γ, IL-4, and IL-5, identical to those produced after in vitro activation and before adoptive transfer (data not shown). In contrast, the naive KJ1-26+ T cells produce IL-2 and IFN-γ at 1 wk after transfer and low

### Table I. Survival of in vitro-activated Ag-specific CD4+ T cells after adoptive transfer

<table>
<thead>
<tr>
<th>KJ1-26+ Cells Per Lymph Node</th>
<th>1 wk post AT</th>
<th>6 wk post AT</th>
<th>10 wk post AT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td>3.8 × 10^4</td>
<td>3.0 × 10^4</td>
<td>3.6 × 10^4</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>4.4 × 10^4</td>
<td>3.5 × 10^4</td>
<td>5.0 × 10^4</td>
</tr>
<tr>
<td>Expt. 3</td>
<td>ND</td>
<td>5.8 × 10^4</td>
<td>3.4 × 10^4</td>
</tr>
<tr>
<td>Expt. 4</td>
<td>ND</td>
<td>4.4 × 10^4</td>
<td>6.8 × 10^4</td>
</tr>
<tr>
<td>Expt. 5</td>
<td>ND</td>
<td>5.2 × 10^4</td>
<td>4.5 × 10^4</td>
</tr>
<tr>
<td>Expt. 6</td>
<td>ND</td>
<td>ND</td>
<td>4.4 × 10^4</td>
</tr>
<tr>
<td>Expt. 7</td>
<td>6.1 × 10^4</td>
<td>ND</td>
<td>3.5 × 10^4</td>
</tr>
<tr>
<td>Expt. 8</td>
<td>ND</td>
<td>ND</td>
<td>3.0 × 10^4</td>
</tr>
<tr>
<td>Mean</td>
<td>4.77 × 10^4</td>
<td>4.38 × 10^4</td>
<td>4.28 × 10^4</td>
</tr>
<tr>
<td>SD</td>
<td>1.19 × 10^4</td>
<td>1.16 × 10^4</td>
<td>1.22 × 10^4</td>
</tr>
</tbody>
</table>

*In vitro-activated wild-type (Expts. 1–6) or SCID (Expts. 7–8, in italic) DO.11 CD4+ cells (15 × 10^6) were adoptively transferred into BALB/c mice. At 1, 6, and 10 wk after transfer, the mice were sacrificed, and the peripheral lymph node cells were harvested. Cells were stained with KJ1-26 and anti-CD4 and analyzed by flow cytometry. The total numbers of KJ1-26+ cells in the harvested lymph nodes are indicated. AT, adoptive transfer; ND, not done.

**FIGURE 2.** In vitro-activated DO.11 lymphocytes rapidly convert to a partially naive phenotype after adoptive transfer. A, Naive KJ1-26+ cells (derived from the lymph nodes of DO.11 mice), memory KJ1-26+ cells (derived from mice adoptively transferred with in vitro-activated cells 6 wk before collection), and activated cells (naive KJ1-26+ cells activated in vitro for 96 h with OVA peptide and spleen cells) were assayed for the expression of CD25 and CD44. B, Naive or in vitro-activated DO.11 CD4+ T cells (15 × 10^6) were adoptively transferred into BALB/c mice. One week after transfer, the mice in each group were sacrificed, and lymph node cells were collected and analyzed for percent of KJ1-26+ cells and expression of L-selectin and CD44 on the gated KJ1-26+ population. At 10 wk after transfer, mice were either untreated or immunized by s.c. injection of OVA peptide in IFA. The draining lymph node cells were collected from the untreated and immunized mice 3 days later and analyzed for percent KJ1-26+ cells and expression of L-selectin and CD44. A representative example of two experiments (naive adoptive transfers) and six experiments (activated adoptive transfers) is shown. As L-selectin expression is usually bimodal, the percent of KJ1-26+ cells that were L-selectinhig in each experiment is also shown, indicated by the number above the L-selectin bar graph. AT, adoptive transfer; imm, immunized.
levels at 10 wk after transfer; no IL-4 or IL-5 was detected. Therefore, the effector phenotype of the in vitro-activated T cells is stable, even after prolonged residence in vivo.

**B cells are not required for the survival of CD4\(^+\) memory cells**

Recent work suggests that CD8\(^+\) memory cells require the presence of MHC class I molecules for survival, as memory cells do not persist in TAP-1 deficient mice (21). It has been postulated that CD4\(^+\) memory cells may be maintained by persistent Ag that is presumably trapped in the form of Ag-Ab complexes, permitting TCR on memory cells to intermittently encounter their cognate MHC class II-peptide complexes (4). It is therefore possible that B cells are responsible for this intermittent representation, as follicular dendritic cell-derived Ag/Ab complexes are thought to serve as a source for positive selection of B cells emerging from germinal centers. To determine whether B cells and/or immune-complexed Ag were required for the survival of memory cells, equal numbers of in vitro-activated KJ1-26\(^+\) cells were adoptively transferred into BALB/c recipients and at 6 or 10 wk after transfer, CD4\(^+\) cells were purified from the lymph nodes and spleens of recipient mice. For comparison, naive KJ1-26\(^+\) cells were obtained by purification of CD4\(^+\) cells from DO.11 SCID mice, or from peripheral lymph nodes (only) of wild-type DO.11 mice. The percentages of naive and memory KJ1-26\(^+\) cells in the respective purified CD4\(^+\) cells were determined by FACS analysis (data not shown). As expected, the memory KJ1-26\(^+\) cells in the respective purified CD4\(^+\) cells were determined by FACS analysis (data not shown). Therefore, B cells and immune-complexed Ag are not required for the maintenance of CD4\(^+\) memory cells in vivo.

**CD4\(^+\) memory cells proliferate vigorously in response to IL-2 and IL-4**

In the next series of experiments, we wished to identify cytokines that induced the survival or proliferation of CD4\(^+\) memory cells. Studies with CD8\(^+\) memory cells have demonstrated that they express high levels of the IL-2R\(\beta\)-chain and proliferate in response to IL-15 in the absence of Ag (24). In vitro-activated KJ1-26\(^+\) cells were adoptively transferred into BALB/c recipients and at 6 or 10 wk after transfer, CD4\(^+\) cells were purified from the lymph nodes and spleens of recipient mice. For comparison, naive KJ1-26\(^+\) cells were obtained by purification of CD4\(^+\) cells from DO.11 SCID mice, or from peripheral lymph nodes (only) of wild-type DO.11 mice. The percentages of naive and memory KJ1-26\(^+\) cells in the respective purified CD4\(^+\) cells were determined by FACS analysis (data not shown). As expected, the memory KJ1-26\(^+\) population expressed high levels of CD44, while the corresponding naive population expressed the expected low levels of CD44, and both populations expressed similar low levels of CD25 when compared with activated cells (Fig. 2A).

We chose to use CD4\(^+\) cells purified from intact DO.11 or DO.11 SCID mice as a source of naive cells rather than those present in an adoptive transfer recipient (i.e., naive cells into

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**FIGURE 3.** In vitro-activated DO.11 CD4\(^+\) lymphocytes maintain their effector cytokine profile after adoptive transfer. BALB/c mice were given 15 × 10\(^6\) naive or in vitro-activated KJ1-26\(^+\) T cells. At 1 wk (upper graphs) or 10 wk (lower graphs) after transfer, the recipients were sacrificed and lymph node cells were harvested and restimulated in vitro with 1 \(\mu\)g/ml of OVA\(_{323-339}\) peptide. Supernatants were collected on days 0, 1, 2, 3, and analyzed for the presence of IL-2, IFN-\(\gamma\), IL-4, and IL-5 by ELISA. Proliferative responses of the memory and naive cells are shown at both 1 and 10 wk, corrected for KJ1-26\(^+\) cell input. The error bars represent SDs calculated from duplicate wells. A representative example of two experiments (naive adoptive transfers) and six experiments (activated adoptive transfers) is shown.

**FIGURE 4.** B cells and Ab are not required for the survival of KJ1-26\(^+\) memory cells. In vitro-activated KJ1-26\(^+\) cells (15 × 10\(^6\)) were adoptively transferred into BALB/c mice or \(\mu^-\) mice. Mice were analyzed at 6 and 10 wk after transfer and found to have comparable numbers of memory KJ1-26\(^+\) cells remaining in the lymph nodes of recipients (Fig. 4). The memory cells remaining in the B cell-deficient mice were functionally competent, proliferating in response to Ag stimulation in vitro, and rapidly producing the effector cytokines IFN-\(\gamma\) and IL-4 (data not shown). Therefore, B cells and immune-complexed Ag are not required for the maintenance of CD4\(^+\) memory cells in vivo.
BALB/C mice) primarily due to the fact that the percent of naive KJ1-26\(^+\) cells is much higher in the CD4\(^+\) cell population from intact DO.11 mice. Moreover, SCID DO.11 mice have reduced numbers of CD4\(^+\) cells (despite the presence of the TCR transgene), precluding adoptive transfer into BALB/c mice unless extremely large numbers of donor mice are used. We and others have performed many experiments in which naive DO.11 T cells are adoptively transferred into BALB/c recipients and CD4\(^+\) cells were purified from the lymph nodes and spleens of these mice 6 wk after transfer to provide a source of memory KJ1-26\(^+\) cells. Naive KJ1-26\(^+\) cells were obtained by purifying CD4\(^+\) cells from the peripheral lymph nodes of 4-wk-old DO.11 mice. A. The naive and memory CD4\(^+\) cells were labeled with BODIPY red and tracker FITC, respectively. The labeled naive and memory cells were combined so that equal numbers of naive KJ1-26\(^+\) and memory KJ1-26\(^+\) cells were present. B. CD4\(^+\) cells (1.8 \times 10^6; containing equal numbers of BODIPY red-labeled naive and tracker FITC-labeled memory KJ1-26\(^+\)) cells were placed in 24-well plates. To these wells, no cytokine, IL-2 (20 U/ml), IL-4 (100 U/ml), or IL-15 (1 ng/ml) was added. Cells were collected at 4 and 8 days after culture and assayed by flow cytometry for percent of BODIPY red and tracker FITC expressing KJ1-26\(^+\) cells. Histograms are shown gating on KJ1-26\(^+\) or KJ1-26\(^-\) cells containing either BODIPY red (naive) or tracker FITC (memory). Data are from one representative experiment of four.

FIGURE 5. KJ1-26\(^+\) CD4\(^+\) memory cells proliferate in response to IL-2 and IL-4. In vitro-activated KJ1-26\(^+\) cells were adoptively transferred into BALB/c recipients and CD4\(^+\) cells were purified from the lymph nodes and spleens of these mice 6 wk after transfer to provide a source of memory KJ1-26\(^+\) cells. Naive KJ1-26\(^+\) cells were obtained by purifying CD4\(^+\) cells from the peripheral lymph nodes of 4-wk-old DO.11 mice. A. The naive and memory CD4\(^+\) cells were labeled with BODIPY red and tracker FITC, respectively. The labeled naive and memory cells were combined so that equal numbers of naive KJ1-26\(^+\) and memory KJ1-26\(^+\) cells were present. B. CD4\(^+\) cells (1.8 \times 10^6; containing equal numbers of BODIPY red-labeled naive and tracker FITC-labeled memory KJ1-26\(^+\)) cells were placed in 24-well plates. To these wells, no cytokine, IL-2 (20 U/ml), IL-4 (100 U/ml), or IL-15 (1 ng/ml) was added. Cells were collected at 4 and 8 days after culture and assayed by flow cytometry for percent of BODIPY red and tracker FITC expressing KJ1-26\(^+\) cells. Histograms are shown gating on KJ1-26\(^+\) or KJ1-26\(^-\) cells containing either BODIPY red (naive) or tracker FITC (memory). Data are from one representative experiment of four.
purified from the lymph nodes and spleens of adoptive transfer recipients to provide a source of memory cells. In this population, only 10–20% of the cells are KJ1-26+.

Conversely, naive KJ1-26+ cells were obtained by purifying CD4+ cells from the peripheral lymph nodes of intact DO.11 mice, and the resultant population is ~50% KJ1-26+. Therefore, fewer BODIPY-labeled cells are added to the cell suspension to obtain a final mix of equal numbers of naive (BODIPY red) and memory (tracker FITC) KJ1-26+ cells. Additionally, the presence of the nontransgenic (KJ1-26-) cells in the cultures serves as a critical internal control, allowing us to separately quantitate the cytokine responsiveness of an Ag-specific population of memory cells, an Ag-specific population of naive cells, and a population of cells with unknown Ag specificity. Cells were collected at 4 and 8 days after culture and analyzed for the percent of remaining memory or naive KJ1-26+ cells, as well as for the extent of cell cycling by measuring loss of tracker FITC or BODIPY red fluorescence.

By 4 days of culture, the memory KJ1-26+ cells were beginning to proliferate in response to both IL-2 (at all concentrations used; data shown for 20 U/ml) and IL-4 (at both 100 and 500 U/ml; data shown for 100 U/ml), but no proliferation was noted in the presence of IL-15 (at all concentrations used; data shown for 1 ng/ml) (Fig. 5B). In contrast, cycling was not observed in the naive population of KJ1-26+ cells, or in the population of BODIPY red or tracker FITC-labeled KJ1-26+ cells, in the presence of any cytokine. At 8 days of culture, the majority of the memory cells had undergone cell division in the presence of IL-2 and IL-4 (Figs. 5B and 6A). Furthermore, some proliferation was noted in the presence of IL-15, especially at higher concentrations (50 ng/ml, data not shown), although this was significantly less than that seen with the other cytokines. The numbers of remaining naive cells were reduced by day 8 of culture, most likely due to passive cell death. A very small amount of proliferation was noted in the tracker FITC-labeled KJ1-26+ population at day 8 when cultured in the presence of IL-2 and IL-4. However, these cells were purified from both the lymph nodes and spleens (as opposed to only lymph nodes for the naive cells) of adoptive transfer recipients. In this population, there are a small number of CD4+ KJ1-26+ memory (or activated) cells present within this population that respond to the added cytokine. We have also performed similar experiments in which the naive and memory cells were both labeled with tracker FITC and cultured in separate wells with the various cytokines. As in the studies described above in which the populations were cocultured, when cultured separately, only the KJ1-26+ memory cells were found to proliferate in both IL-2 and IL-4 (data not shown). We and others have utilized fluorochrome cytoplasmic labeling agents to follow the proliferation of naive cells in response to Ag both in vivo and in vitro (36–38). It is clear that these agents do not interfere with the activation and subsequent cycling of naive cells. Therefore, the lack of proliferation we observed in the naive KJ1-26+ population is not due to an effect of the BODIPY red labeling procedure.

Proliferative responses of nonfluorochrome-labeled naive, memory, and activated KJ1-26+ cells to cytokines were also evaluated at day 4 by [3H]thymidine incorporation. As expected, the memory cells proliferated well in response to IL-2 and IL-4, with lower levels of proliferation seen in the presence of IL-15 (Fig. 6B). Interestingly, the in vitro-activated cells proliferated much more vigorously in response to IL-2 than IL-4, exhibiting no response to IL-15. These results confirmed the data presented in Fig. 5, demonstrating that, unlike naive cells, memory cells retain the ability to respond to IL-2 and IL-4, and to a lesser degree IL-15.

Discussion

The studies presented in this paper were undertaken to characterize the phenotypic and functional characteristics of long-lived, Ag-specific CD4+ T cells. The experimental model we have used involves activation of TCR transgenic T cells in vitro, followed by adoptive transfer of these cells into syngeneic, nontransgenic recipients. The process of in vitro activation induces a change in the T cells that allows a population of the cells to survive for prolonged periods in vivo without additional overt exposure to cognate Ag (Fig. 1 and Table I). This system enables us to define the
phenotypic characteristics of long-surviving T cells and evaluate the signals required for their survival and functional responses to Ag challenge.

We have chosen to use the in vitro activation and adoptive transfer system for several reasons. Our experience, and that of many other investigators, is that intact TCR transgenic mice cannot be primed normally due to apoptosis of activated cells secondary to the high frequency of responding cells and the subsequent replacement of cells with new thymic emigrants (naive cells). The only other option is to transfer naive DO.11 T cells into one set of recipients, prime these, recover the in vivo-activated KJ1-26+ T cells, and transfer these into a second set of recipients. We have actually tried such experiments, but it is logistically impossible to do these routinely, especially for many of the in vitro cytokine sensitivity experiments. Furthermore, the issue of Ag persistence in the immunized mice may lead to carry-over of Ag into the in vitro restimulation experiments. Note that key to our studies is the ability to study T cell survival in the absence of Ag exposure, and this can only be accomplished using the in vitro activation and adoptive transfer system we have employed. The long-lived cells generated by in vitro activation and adoptive transfer possess several characteristics believed to be typical of memory cells: 1) by 10 wk after transfer, a stable population of DO.11 T cells is generated after in vitro activation, whereas similar numbers of adoptively transferred naive DO.11 T cells are markedly reduced (Fig. 1); 2) the surviving DO.11 T cells express markers typical of memory T cells (CD25lowCD44high, Fig. 2); and 3) the surviving DO.11 T cells exhibit functional responses believed to be characteristic of memory cells including rapid effector cytokine production upon restimulation (Fig. 3) and localization to the spleen with rapid migration out of the spleen upon Ag exposure (data not shown). Indeed, other investigators have successfully used the method of in vitro activation and adoptive transfer (39). In conclusion, the approach we have chosen is the only practical way of doing these experiments in a quantitative, reproducible manner.

Phenotypic analysis of the long-surviving T cells shows that by 10 wk after adoptive transfer, they express high levels of L-selectin, similar to naive T cells, and high levels of CD44, which distinguishes them from naive cells. Upon Ag stimulation the cells rapidly down-regulate L-selectin (Fig. 2). Similar findings have been reported in models of CD8+ T cell memory, in which virus-specific CD8+ T cells gradually return to a CD45RBhigh, L-selectinlow state within 3 mo after virus challenge and recovery (26, 32). Our results support the notion that L-selectin is a surface marker that reflects the state of activation of T cells, rather than a naive or memory phenotype. As previously reported (39), the long-surviving DO.11 T cells also retain the cytokine profiles that were induced in vitro priming (Fig. 3). We have not addressed the question of whether these cells are irreversibly committed to one effector program, or are capable of differentiating into either Th1 or Th2 subset upon subsequent Ag challenge.

It has been recently demonstrated that B cells and Ag-Ab complexes are not essential for the maintenance of CD8+ CTL memory (40, 41). Our data indicate that B cells are not required for the survival of the KJ1-26+ memory cells. Therefore, if MHC class II is necessary for intermittent stimulation of memory cells, it must be provided by other professional APCs, such as macrophages and dendritic cells. However, persistent Ag is unlikely to play a role in memory cell maintenance for several reasons. It is known that class II MHC/peptide complexes on APCs are rapidly recycled/internalized unless the APCs are activated (mature) in an inflammatory environment, after which time the complexes may remain on the cell surface for over 100 h (42). This process serves to allow APCs to efficiently present foreign Ags to T cells during an immune response. In the noninflammatory environment, any remaining persisting Ag in class II MHC complexes would likely be rapidly recycled, thus limiting the chances of memory cells to encounter the relevant APC. Furthermore, Ag remaining after challenge would be expected to persist complexed to Ab. As the μ−/− mice do not have circulating Ab, Ag-Ab complexes cannot form. Therefore, the survival of the KJ1-26+ memory T cells cannot be due to either Ag presentation by B cells or Ag in the form of immune complexes. Since the TCR transgenic T cells we have examined are restricted by the I-Aα allele, we cannot study their survival in mice lacking class II MHC molecules (I-Aα’s deficient) (43) in which the cells would be incapable of recognizing any Ag.

The possibility that memory cells are maintained by exposure to cytokines is an interesting one. Recent work has demonstrated that IL-15 can induce the proliferation of CD8+ memory cells in vitro, and administration of IFN-α/β to mice can induce their proliferation in vivo (22–24). Our results show that IL-15 has little effect on memory CD4+ cells except at high (50 ng/ml) concentrations. However, both IL-2 and IL-4 are able to induce the survival and proliferation of memory cells, to a much greater extent than that of naive cells. It is interesting that memory cells express almost the same level of CD25 as do naive cells (Fig. 2B), yet only the memory cells respond to IL-2. This raises the possibility that cytokine responsiveness is determined by factors in addition to the levels of receptor expression.

Finally, whereas in vitro-activated T cells show prolonged survival in vivo, the population of adoptively transferred naive T cells that homes to the lymph nodes of adoptive transfer recipients gradually decreases over time. Therefore, the process of in vitro activation must induce a genetic program capable of supporting long-term survival in the absence of proliferation or overt Ag exposure. These results also indicate that previously activated T cells possess survival signals that are not present in naive cells. We do not know the nature of the change induced in the T cells as a result of in vitro priming which results in their enhanced survival. By isolating T cells expressing the specific Ag receptor at various times after adoptive transfer, it should be possible to define the genetic and biochemical characteristics of long-surviving T cells and the features that distinguish them from naive cells. Such studies are currently in progress.

Acknowledgments
We thank Drs. Alex McAdam and Luk van Parijs for helpful discussions and critical review of the manuscript.

References


