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Effector T Cell Differentiation and Memory T Cell Maintenance Outside Secondary Lymphoid Organs

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Naive T cell circulation is restricted to secondary lymphoid organs. Effector and memory T cells, in contrast, acquire the ability to migrate to nonlymphoid tissues. In this study we examined whether nonlymphoid tissues contribute to the differentiation of effector T cells to memory cells and the long-term maintenance of memory T cells. We found that CD4, but not CD8, effector T cell differentiation to memory cells is impaired in adoptive hosts that lack secondary lymphoid organs. In contrast, established CD4 and CD8 memory T cells underwent basal homeostatic proliferation in the liver, lungs, and bone marrow, were maintained long-term, and functioned in the absence of secondary lymphoid organs. CD8 memory T cells found in nonlymphoid tissues expressed both central and effector memory phenotypes, whereas CD4 memory T cells displayed predominantly an effector memory phenotype. These findings indicate that secondary lymphoid organs are not necessary for the maintenance and function of memory T cell populations, whereas the optimal differentiation of CD4 effectors to memory T cells is dependent on these organs. The ability of memory T cells to persist and respond to foreign Ag independently of secondary lymphoid tissues supports the existence of nonlymphoid memory T cell pools that provide essential immune surveillance in the periphery. The Journal of Immunology, 2006, 176: 4051–4058.

Primary immune responses are initiated within secondary lymphoid organs, where naive CD4 and CD8 T cells encounter foreign Ags presented by professional APCs, are activated, and differentiate into effector lymphocytes (1). Effector T cells then migrate to nonlymphoid tissues, where they seek and eliminate foreign Ags (2–4). The majority of Ag-specific T cells that participate in the primary immune response undergo apoptosis (5). Only a small subset survives to become long-lived memory T cells (5–7). Unlike naive T cells, whose circulation is restricted to the blood and secondary lymphoid organs, memory T cells are found in both lymphoid and nonlymphoid sites and are readily activated by Ag in all tissues (3, 8–13). The widespread homing of memory T cells can be attributed to the presence of two memory cell types: the central memory population, which preferentially circulates through secondary lymphoid organs, and the effector memory cell population, which is found in nonlymphoid tissues and splenic red pulp (14–16). Homing of memory T cells to nonlymphoid tissues allows them to seek foreign Ags that persist outside secondary lymphoid organs (17).

The requirements for the generation and maintenance of memory T cells have been studied extensively (18). Current data suggest that the generation of CD8 memory T cells follows a linear differentiation pathway by which memory cells arise from effectors (19–21). Importantly, a single encounter between a naive CD8 T cell and its cognate Ag is sufficient to trigger this differentiation program (22). The generation of CD4 memory T cells from naive precursors, in contrast, is less clear. Both the linear differentiation of CD4 memory T cells from mature effectors (23), analogous to that described for CD8 T cells, as well as their divergent differentiation from early (uncommitted) activated T cells have been described (24–26). Once established, CD4 and CD8 memory T cell populations are maintained by similar mechanisms. Both populations persist long term because of the extended survival of individual cells and through basal homeostatic proliferation (15, 27). The latter process ensures that dying memory T cells are continuously replaced by new progeny with identical Ag specificity. Neither cognate Ag nor MHC molecules appear to be required for the long-term maintenance of either CD4 or CD8 memory T cells (28, 29). However, their maintenance is dependent on cytokines, specifically IL-7 for CD4 memory T cells and both IL-7 and IL-15 for CD8 memory T cells (30–33).

Where memory T cells are generated from effectors and maintained is less clear. Candidate sites include secondary lymphoid organs, primary lymphoid tissues (bone marrow), and nonlymphoid tissues (34–36). In this manuscript, we examined whether the generation and maintenance of CD4 and CD8 memory T cells can occur independently of secondary lymphoid organs. Although secondary lymphoid organs are essential for the generation of effector T cells from naive precursors, it is not known whether these tissues are also required for the differentiation of effectors into memory T cells or for the maintenance of established memory T cell populations. It is also unclear whether CD4 and CD8 effector or memory T cells share similar fates after they exit secondary lymphoid organs. Previous studies (37, 38) have shown that naive
CD4, but not CD8, T cells decline rapidly in the absence of secondary lymphoid organs, suggesting that survival requirements differ between the two populations. Whether these differences apply to effector or memory T cells is not known. To address these questions, we transferred polyclonal, alloreactive, effector, or memory T cells into congenic mice that lack all secondary lymphoid organs and studied their long-term maintenance, tissue distribution, phenotype, and function in the adoptive host. We report in this study that established CD4 and CD8 memory T cells are maintained and function independently of secondary lymphoid organs, but that the optimal differentiation of CD4 effectors into a stable CD4 memory T cell pool is dependent on these tissues.

Materials and Methods

Mice

C57BL/6 (Thy1.2, H-2b; hereafter B6), B6-PL-Thy1a/Cy (Thy1.1, H-2d), C3H (H-2k), and BALB/c (H-2d) mice were purchased from The Jackson Laboratory. A lymphoplasia mice (Map3k1^−/−). Thy1.2, H-2b; hereafter alaly/aly) were purchased from CLEA Japan. All animals were bred and maintained under specific pathogen-free conditions according to Yale University institutional animal care and use committee guidelines.

Surgical procedures

Splenectomy was performed according to established techniques via a subcostal midaxillary incision (11). Completeness of splenectomy was verified postmortem. Partial thickness skin transplantation was performed using abdominal skin from donor BALB/c or C3H mice. s.c. layers of donor skin were removed by blunt scraping. A 1-cm² recipient graft bed was prepared by s.c. dissection, leaving an intact panniculus carnosus with uncompromised vasculature. Donor skin was cut to fit the prepared bed and was secured with sutures and bandages for 7 days. Grafts were monitored daily. Rejection was defined as >90% graft necrosis.

Generation, isolation, and adoptive transfer of effector and memory T cells

BALB/c spleens were harvested and crushed in PBS, followed by hypotonic lysis of RBCs to obtain splenocytes for immunization. B6-PL-Thy1a/Cy mice were immunized i.p. with 2 × 10⁵ BALB/c spleenocytes in PBS. Spleen and lymph node (LN)³ cells were harvested 7 or 15 days later to obtain effector T cells. To obtain memory T cells, B6-PL-Thy1a/Cy mice were immunized with BALB/c spleenocytes twice (3 wk apart), and spleen and LN cells were harvested >8 wk (range, 8–14 wk) later. T cells were enriched by negative selection via magnetic cell separation (Pan-T Cell Isolation Kit; Miltenyi Biotec). Purified T cells were labeled with Abs against CD4, CD8, and CD44 and were sorted for CD⁴⁺ CD44²⁺ and CD⁸⁺ CD44²⁺ populations (≥98% purity and all Thy1.1⁺) on a FACS-Vantage (BD Biosciences). Each donor mouse yielded ~1 × 10⁶ CD4 and 5 × 10⁶ CD8 effector or memory T cells. Sorted effector or memory CD4 or CD8 T cells (5 × 10⁵ to 1 × 10⁶) were injected i.v. into splenectomized homozygous alaly/aly (alaly/aly-spleen) or B6 hosts.

Cell harvest after transfer

Adoptive hosts were killed 1, 4, or ~11 wk (range, 8–14 wk) after cell transfer. Liver was perfused with 50 U/ml collagenase IV (Worthington Biochemical), excised, minced, passed through a cell strainer, and incubated with collagenase IV at 37°C for 40 min, and lymphocytes were separated on 25% Optiprep gradient (Accurate Chemical & Scientific). Lungs were perfused with PBS, excised, minced, digested in 50 U/ml collagenase IV at 37°C for 60 min, and strained through a 40-μm pore size filter to obtain lymphocytes. Femurs and tibiae were flushed with PBS to obtain bone marrow cells. Total body bone marrow cells were estimated by multiplying cells harvested from the femurs and tibiae by 5.4 (39).

Flow cytometry and intracellular cytokine staining

Fluorochrome-tagged CD4 (RM4-5), CD8α (53-67), CD44 (KM201), CD25 (7D4), CD62L (MEL-14), CD90.1 (OX-7), and IFN-γ (XMG1.2) were purchased from BD Pharmingen, eBioscience, or Southern Biotechnology Associates. To measure intracellular IFN-γ, lymphocytes were stimulated ex vivo with BALB/c splenocytes (1/1) for 5 h in the presence of brefeldin A. Cells were stained, washed for surface markers, fixed, permeabilized with 0.25% saponin, and incubated with anti-IFN-γ Ab for 1 h at room temperature. Flow acquisition was performed on LSRII analyzers (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

In vitro proliferation assay

Sorted T cells were labeled with CFSE (Molecular Probes) for 10 min at 37°C in PBS, quenched with 10% FCS in RPMI 1640, washed in PBS, and adoptively transferred to congenic splenectomized alaly/aly (alaly/aly-spleen) or B6 mice. Lymphocytes were harvested 3 wk later from lymphoid and nonlymphoid tissues, and the extent of cell proliferation was measured by CFSE dilution after gating on the Thy1.1⁺ transferred population.

Results

Maintenance and recall of CD4 and CD8 memory T cells in the absence of secondary lymphoid organs

To investigate the role of secondary lymphoid organs in the maintenance and recall of T cell memory, we immunized B6-PL-Thy1a/Cy mice with BALB/c spleenocytes and >8 wk later transferred sorted CD4 and/or CD8 CD44²⁺ T cells to congenic wild-type (wt) B6 mice or to congenic alaly/aly-spleen mice that lack LN, spleen, and Peyers’ patches (40). Phenotypic analysis of sorted CD⁴⁺ CD44²⁺ and CD⁸⁺ CD44²⁺ memory T cells before transfer confirmed that they were >98% CD44²⁺ and contained both CD62Llow and CD62Lhigh subpopulations (Fig. 1A), consistent with the presence of both central and effector memory subsets. One, 4, and ~11 wk (range, 8–14 wk) after transfer, alaly/aly-spleen hosts were killed, and tissues were analyzed for Thy1.1⁺ cells. As shown in Fig. 1B, the total number of CD4 and CD8 Thy1.1⁺ cells present per animal per 1 × 10⁶ cells transferred remained stable over time, indicating that transferred memory T cells are maintained in the absence of secondary lymphoid organs. Cotransferring CD4 and CD8 memory T cells did not significantly alter the yield of either CD4 or CD8 Thy1.1⁺ cells at any time point (data not shown). In addition, Thy1.1⁺ CD4 and CD8 memory T cells harvested from wt hosts 8 wk after transfer were similar in number to those harvested from alaly/aly-spleen mice (Fig. 1B), confirming that memory maintenance is independent of secondary lymphoid tissues.

Thy1.1⁺ CD44²⁺ T cells transferred from immunized B6 mice include not only Ag-induced memory T cells, but also naturally existing memory T cells of unknown specificities (41). To assess the contribution of naturally existing memory populations to the overall maintenance of Thy1.1⁺ CD44²⁺ cells transferred from immunized mice, we transferred CD4 or CD8 Thy1.1⁺ CD44²⁺ T cells from unimmunized mice to alaly/aly-spleen hosts and enumerated them ~12 wk later. As shown in Fig. 1B, naturally existing CD⁸⁺ CD44²⁺ T cells were maintained in the absence of secondary lymphoid organs, whereas naturally existing CD⁴⁺ CD44²⁺ were recovered at a significantly lower level than CD⁴⁺ CD44²⁺ cells transferred from immunized mice (Fig. 1B). To test whether Ag-induced CD8 memory T cells are also maintained in alaly/aly-spleen hosts, we measured the precursor frequency of alloreactive CD8 T cells in the Thy1.1⁺ CD44²⁺ CD8 T cell population before and after transfer. Harvested T cells were stimulated ex vivo with either allogeneic (BALB/c) or syngeneic (B6) splenocytes, and IFN-γ production was measured by flow analysis 5 h later. The precursor frequency of BALB/c-reactive CD8⁺ Thy1.1⁺ T cells was 2 ± 0.6% (n = 6) before transfer and 2.5 ± 0.9% (n = 2) >8 wk after transfer into alaly/aly-spleen hosts (Fig. 1C). In contrast, ~0.3% of naturally existing CD8⁺ CD44²⁺ T cells obtained from naive mice produced IFN-γ in response to stimulation with BALB/c splenocytes. These data show that both Ag-induced and

Abbreviations used in this paper: LN, lymph node; 2C, CD8 TCR-tg lymphocytes reactive to L4 of BALB/c Ig; transgenic; wt, wild type.
Maintenance and function of established CD4 and CD8 memory T cells in the absence of secondary lymphoid organs. Alloreactive memory T cells were generated in B6.PL-Thy1a/Cy mice by immunization with BALB/c splenocytes. LN and spleen cells were harvested ≥8 wk later, and CD4⁺ CD44<sup>high</sup> and CD8⁺ CD44<sup>high</sup> T cells were sorted before transfer into aly/aly-spleen or wt hosts (Thy1.2). Naive CD4⁺ CD44<sup>low</sup> and CD8⁺ CD44<sup>low</sup> T cells and natural CD4⁺ CD44<sup>high</sup> and CD8⁺ CD44<sup>high</sup> memory T cells were sorted from unimmunized B6.PL-Thy1a/Cy mice. A, Phenotype of sorted CD4 and CD8 populations before transfer. B, Enumeration of Thy1.1⁺ cells present in the adoptive hosts at 1, 4, and −11 wk (range, 8–14 wk) after transferring 5 × 10<sup>5</sup> to 2 × 10<sup>6</sup> Ag-induced CD4 or CD8 memory T cells or natural memory T cells. Cells were harvested from the liver, lungs, bone marrow, and blood at the indicated time points. Harvested cells were counted using a hemacytometer to estimate the number of harvested cells. The percentage of Thy1.1⁺ cells within the harvested cell population was determined by flow analysis. The absolute number of harvested Thy1.1⁺ cells from each tissue was estimated by multiplying the percentage of Thy1.1⁺ cells present by the number of cells harvested from that tissue. The Thy1.1⁺ cells from each tissue were added to estimate the absolute number of Thy1.1⁺ cells harvested from that recipient. Values are the mean ± SD of three to five mice per time point. C, Ag-specific memory CD8 T cells are maintained in the absence of secondary lymphoid organs. The precursor frequency of BALB/c-reactive Thy1.1⁺ memory T cells was quantitated before and after adoptive transfer by stimulating sorted CD8⁺ CD44<sup>high</sup> T cells ex vivo with either syngeneic (B6, Thy1.2) or allogeneic (BALB/c, Thy1.2) splenocytes. IFN-γ production by Thy1.1⁺ cells was determined by intracellular cytokine staining. Representative plots of two to six experiments are shown. The percentages shown are those of IFN-γ-producing Thy1.1⁺ cells compared with the total Thy1.1⁺ population. D, Tissue distribution of transferred cells. Cells were harvested from the liver, lungs, bone marrow, and blood at the indicated time points. The number of Thy1.1⁺ cells harvested from each tissue was estimated as described in B. Values are the means of three to five mice per time point. E, Phenotype of memory T cells harvested from the liver of aly/aly-spleen mice or liver and spleen of wt mice −11 wk after transfer. CD4, CD45, and CD62L expression on both transferred Thy1.1⁺ and host (Thy1.1⁺) T cells after gating on either the CD4 or CD8 population is shown. Representative plots of three to five experiments are shown. The percentages shown are of CD44<sup>high</sup>, CD25<sup>high</sup>, and CD62L<sup>high</sup> Thy1.1⁺ cells compared with the total Thy1.1⁺ population. F, Memory T cell function measured by the ability of adoptively transferred hosts to reject skin allografts. At least 8 wk after receiving CD4 or CD8 memory T cells, aly/aly-spleen hosts were transplanted with BALB/c skin grafts. Control groups consisted of mice that received 1) CD4<sup>high</sup>CD4 and CD8 T cells, 2) CD4<sup>low</sup>CD4 and CD8 T cells sorted from unimmunized mice (natural memory), or 3) memory T cells generated in B6 mice immunized with third-party alloantigen (C3H splenocytes). None of the control mice rejected their grafts up to 100 days after transplantation. Sorted cells (5 × 10<sup>5</sup> to 1 × 10<sup>6</sup>) were transferred per mouse (three or four mice per group). Naturally existing CD8 memory T cells are maintained in the absence of secondary lymphoid organs. In contrast, Ag-induced CD4 memory T cells are significantly better maintained in the absence of secondary lymphoid organs than their naturally existing counterparts.

The tissue distribution of CD4 and CD8 Thy1.1⁺ cells in the adoptive hosts is shown in Fig. 1D. Transferred CD8 memory T cells were found predominantly in the bone marrow, liver, lungs, and, to a lesser extent, blood. The distribution of CD4 memory T cells was similar to that of CD8, except that a significant proportion of CD4 memory T cells localized to the bone marrow at 1 wk after transfer, but redistributed to nonlymphoid tissues (liver and lungs) at later time points. Neither CD4 nor CD8 memory T cells were detected in measurable numbers in the thymus, salivary glands, kidneys, peritoneal cavity, or gut.

To confirm that CD4 and CD8 Thy1.1⁺ cells present in adoptive hosts −11 wk after transfer are memory T cells, the phenotype and function of harvested cells were studied. As shown in Fig. 1E, both CD4 and CD8 Thy1.1⁺ cells harvested from the liver were exclusively CD44<sup>high</sup> and CD25<sup>low</sup>, consistent with the memory phenotype. CD4 memory T cells (Thy1.1⁺CD4⁺CD44<sup>high</sup>) that persisted in aly/aly-spleen mice were predominantly CD62L<sup>low</sup> (96 ± 3%), whereas the CD8 memory population (Thy1.1⁺CD4⁺CD44<sup>high</sup>) contained both CD62L<sup>low</sup> (55 ± 5%) and CD62L<sup>high</sup> (45 ± 5%) cells. In contrast, both CD62L<sup>low</sup> and CD62L<sup>high</sup> CD4 (77 ± 5% CD62L<sup>low</sup> and 23 ± 4% CD62L<sup>high</sup>) and CD8 (33 ±
7% CD62L<sup>low</sup> and 67 ± 5% CD62L<sup>high</sup>) memory T cells were maintained in wt B6 hosts (Fig. 1E). The relative proportions of CD62L<sup>low</sup> and CD62L<sup>high</sup> memory T cells found in the spleen and liver of wt B6 hosts (Fig. 1E) were commensurate with those observed in the pretransfer populations (Fig. 1A). These findings provide evidence that the maintenance of CD4 central memory T cells is dependent on secondary lymphoid organs, whereas both CD8 central and effector memory T cells persist in nonlymphoid tissues. Phenotypic analysis of Thy1.1<sup>+</sup> T cells that homed to the lungs or bone marrow yielded similar results (data not shown).

There was no evidence of significant sequestration of CD4<sup>+</sup>CD44<sup>high</sup>CD62L<sup>high</sup> (central memory) cells in the bone marrow (data not shown).

In addition to their memory phenotype, CD4 and CD8 Thy1.1<sup>+</sup> T cells that were maintained long term in <i>aly/aly-spleen</i> hosts exhibited memory function; that is, they were capable of mounting an in vivo, Ag-specific, recall response in the absence of secondary lymphoid organs (11). As shown in Fig. 1F, <i>aly/aly-spleen</i> hosts that received either CD4 or CD8 memory T cells ~11 wk previously rejected BALB/c skin allografts, indicating the presence of functional, allospecific, memory T cells. Control <i>aly/aly-spleen</i> hosts that received either CD44<sup>low</sup> (naive) or CD44<sup>high</sup> (natural memory) T cells from unimmunized B6 mice did not reject BALB/c skin allografts. Likewise, rejection was not observed in control <i>aly/aly-spleen</i> mice that received CD44<sup>high</sup> T cells from B6 mice immunized with third-party alloantigens (C3H splenocytes). The rejection data, therefore, demonstrate that functional, Ag-specific, memory T cells are maintained in the absence of secondary lymphoid organs.

**CD4 and CD8 memory T cells undergo basal homeostatic proliferation in the absence of secondary lymphoid organs**

Basal homeostatic proliferation is an essential mechanism by which memory T cell populations are maintained (15). To confirm that CD4 and CD8 memory T cells are maintained independently of secondary lymphoid organs, we transferred CFSE-labeled, Thy1.1<sup>+</sup>, naive T cells (CD44<sup>low</sup> cells sorted from unimmunized mice) or memory T cells (CD44<sup>high</sup> cells sorted from immunized mice) to wt or <i>aly/aly-spleen</i> B6 hosts and analyzed their proliferation 21 days later. As shown in Fig. 2A, proliferation of CD4 and CD8 memory T cells was observed in the livers of both wt and <i>aly/aly-spleen</i> mice in the absence of antigenic stimulation. Similar proliferation was observed in the bone marrow of <i>aly/aly-spleen</i> hosts (Fig. 2A). In contrast, naive T cells did not proliferate in either host during the same period of time (Fig. 2A). Basal homeostatic proliferation of CD4 and CD8 memory T cells was also observed in the lungs of wt and <i>aly/aly-spleen</i> mice (data not shown). These data demonstrate that CD4 and CD8 memory T cell populations undergo basal homeostatic proliferation in nonlymphoid tissues and bone marrow, independently of secondary lymphoid organs. Interestingly, naturally existing CD8, but not CD4, memory T cells (CD44<sup>high</sup> cells sorted from unimmunized mice) underwent homeostatic proliferation in the absence of secondary lymphoid organs (Fig. 2B). This finding is consistent with the observation that naturally existing CD4 memory T cells are poorly maintained in <i>aly/aly-spleen</i> hosts (Fig. 1B).

As presented in Fig. 2, we observed that a subpopulation of transferred CD4 memory T cells divides to negative CFSE in the absence of known stimulation with cognate Ag. This phenomenon has been noted by others (33). It is not clear whether the observed proliferation is an intrinsic property of the CD4 memory T cells or is a response to cross-reactive environmental or self-Ags. To ensure that the observed proliferation was not due to the cotransfer of cognate Ag, we investigated whether immunized B6 mice (which serve as the source of memory T cells) clear BALB/c alloantigens before harvesting memory populations. CFSE-labeled naive (CD44<sup>low</sup>; CD8 TCR-transgenic (TCR-tg) lymphocytes reactive to L<sub>d</sub> of BALB/c (2C)) cells were transferred into B6 mice either on the day of immunization with BALB/c splenocytes or 6 days later. As shown in Fig. 3, 2C cells transferred on the day of immunization proliferated in the spleen and LN, but did not divide when transferred 6 days after immunization. This suggests that the proliferation of memory T cells in the adoptive host was not due to cotransfered BALB/c alloantigens. Therefore, our data indicate that CD4 and CD8 memory T cell populations undergo basal homeostatic proliferation independently of secondary lymphoid tissues and alloantigen.

**Differentiation of CD4 and CD8 effector T cells to memory in the absence of secondary lymphoid organs**

To investigate whether secondary lymphoid organs are required for the generation of memory T cells from effectors, we transferred Thy1.1<sup>+</sup> effector CD4 and/or CD8 T cell populations to congenic <i>aly/aly-spleen</i> mice. The effector T cell populations used in these experiments were harvested from B6 mice 7 days after immunization with BALB/c splenocytes. Phenotypic analysis of sorted CD4 and CD8 CD44<sup>high</sup> T cells before transfer showed that they were >98% CD44<sup>high</sup> and either CD62L<sup>low</sup> or CD62L<sup>high</sup> (Fig. 3). To study basal homeostatic proliferation, 1 × 10<sup>6</sup> memory T cells were generated and sorted as described in Fig. 1. Cells were labeled with CFSE and transferred to either <i>aly/aly-spleen</i> or wt hosts. Control <i>aly/aly-spleen</i> and wt mice received an equal number of sorted naive (CD44<sup>low</sup>) T cells. Twenty-one days later, spleen and liver (wt host) or liver and bone marrow (<i>aly/aly-spleen</i> host) cells were harvested, and CFSE dilution was analyzed after gating on Thy1.1<sup>+</sup> cells. Similar results were obtained when lung cells were analyzed (two to four mice per group). B, Basal homeostatic proliferation of natural memory T cells was studied in <i>aly/aly-spleen</i> mice as described in A, except that CD44<sup>high</sup> T cells were harvested from unimmunized mice (n = 3).
CD25low, consistent with the memory phenotype. Analogous to H11003 and H11004 factor T cells and not naturally existing memory T cells. One, 4, referred to aly/aly-spleen mice were predominantly CD62L low (93%, CD4 memory T cells recovered from what was shown in Fig. 1C, a long-lived population of CD8 Thy1.1+ cells was detected ~11 wk after transfer, whereas CD4 Thy1.1+ T cells declined in number after the fourth week. Cotransferring CD4 and CD8 effector T cells did not significantly increase the yield of either CD4 or CD8 Thy1.1+ cells (data not shown). Similarly, transferring up to ~4 x 10^6 effector CD4 T cells did not increase the yield of CD4 T cells ~11 wk later (data not shown). In contrast, CD4 Thy1.1+ effector T cells were recovered from wt hosts 12 wk after transfer (Fig. 4C), suggesting that the differentiation of CD4 effectors into long-lived memory is dependent on secondary lymphoid organs.

The tissue distribution of Thy1.1+ T cells after the transfer of either CD4 or CD8 effectors into aly/aly-spleen hosts is shown in Fig. 4D. CD8 Thy1.1+ T cells were found predominantly in the liver, lungs, and bone marrow and, to a lesser extent, in the blood. This distribution is similar to that observed after the transfer of Thy1.1+ memory T cells (Fig. 1C). Conversely, the number of CD4 Thy1.1+ T cells present in nonlymphoid tissues declined significantly by ~11 wk (~100 cells/organ). Again, neither CD4 nor CD8 transferred T cells were detected in measurable numbers in the thymus, salivary glands, kidneys, peritoneal cavity, or gut. To confirm that Thy1.1+ T cells present in the adoptive hosts ~11 wk after transfer had indeed differentiated to memory, the phenotype and function of harvested cells were studied. The phenotype of Thy1.1+ T cells isolated from the liver is shown in Fig. 4E. Both CD4 and CD8 Thy1.1+ T cells were exclusively CD44high and CD25low, consistent with the memory phenotype. Analogous to what was shown in Fig. 1E, CD4 memory T cells recovered from aly/aly-spleen mice were predominantly CD62Llow (93% ± 4%), whereas the CD8 memory population contained both CD62Llow (70% ± 3%) and CD62Lhigh (30% ± 5%) cells (Fig. 4E). CD4 memory T cells recovered from wt hosts, in contrast, contained both CD62Lhigh and CD62Llow populations in the liver (15% ± 3% CD62Lhigh and 85% ± 2% CD62Llow) and spleen (27% ± 5% CD62Lhigh and 73% ± 5% CD62Llow). These proportions are commensurate with those observed after the transfer of established CD4 memory T cells into wt hosts (Fig. 1E). Phenotypic analysis of Thy1.1+ T cells that homed to the lungs or bone marrow yielded similar results (data not shown).

CD8 Thy1.1+ memory-phenotype T cells generated in aly/aly-spleen hosts from effectors also exhibited memory function. aly/aly-spleen hosts that received either CD8 or CD8 plus CD4 effector T cells ~11 wk earlier rejected BALB/c skin allografts (Fig. 4F), indicating the presence of functional allospecific memory T cells. In contrast, rejection in the group that received CD4 effectors did not occur consistently, even when the number of transferred cells was doubled (Fig. 4F). This finding is consistent with the observed decline in the number of CD4 effector T cells transferred to aly/aly-spleen hosts (Fig. 4C) and suggests that the differentiation of CD4 effectors into long-lived, functional, memory T cells in the absence of secondary lymphoid organs is not as efficient as that of CD8 effectors. Control aly/aly-spleen hosts that received either CD44low (naive) or CD44high (natural memory) T cells from unimmunized B6 mice ~11 wk previously did not reject BALB/c skin allografts. Likewise, rejection was not observed in aly/aly-spleen mice that received CD44high T cells from B6 mice immunized with third-party alloantigens (C3H splenocytes).

**Differentiation of effector CD4 T cells to memory is enhanced by extended access to secondary lymphoid organs**

To explore why effector CD4 T cells do not efficiently differentiate to memory in the absence of secondary lymphoid organs, we asked whether extended access of activated T cells to secondary lymphoid organs is required for the optimal generation of CD4 memory. To test this possibility, we harvested CD44high CD4 effector T cells either 7 or 15 days after immunizing mice and transferred them to aly/aly-spleen hosts. Thy1.1+ T cells present in the adoptive hosts were enumerated ~12 wk (range, 8–16 wk) after transfer. As shown in Fig. 5A, CD4 effector T cells transferred to aly/aly-spleen mice 15 days after immunization generated 40-fold more memory T cells than those transferred on day 7, comparable to that observed if the cells were transferred into wt hosts. Similarly, the transfer of day 7 CD4 effector T cells into wt B6 mice significantly increased the number of memory CD4 T cells recovered (Figs. 4C and 5A). These findings suggest that secondary lymphoid organs facilitate the optimal differentiation of CD4 effector T cells to memory. In contrast to CD4 effector T cells, day 7 and 15 CD8 effectors differentiated into memory T cells regardless of whether they were transferred to a wt host or a host that lacks secondary lymphoid organs (Figs. 4C and 5A). Because cell division may influence memory commitment and the number of memory T cells generated (18), we postulated that impaired differentiation of CD4 effector T cells to memory in aly/aly-spleen hosts is due to their reduced proliferation in the absence of secondary lymphoid organs. To test this hypothesis, we harvested CD4+CD44high and CD8+CD44high T cells from B6.PL-Thy1a/Cy mice 7 days after immunization, labeled them with CFSE, and transferred them into either B6 or aly/aly-spleen hosts. Although CD8 T cells continued to divide in either adoptive host, transferred CD4 T cells underwent additional proliferation only if secondary lymphoid organs were present (Fig. 5B). These data suggest that the optimal generation of CD4 memory is dependent on the continued proliferation of Ag-stimulated CD4 T cells within secondary lymphoid organs.
are of CD44high, CD25high, and CD62Lhigh Thy1.1 liver of aly/aly-spleen. Values are the mean of three to six mice per time point. from each tissue is estimated as described in Fig. 1 mice. Also shown is the recovery of CD4 and CD8 effector T cells aly/aly-spleen or B6 (syngeneic) splenocytes, spleen cells were harvested, and CFSE dilution was analyzed after gating on Thy1.1. Mice in the experimental groups received 5F11003 of adoptively transferred hosts to reject skin allografts. Experiments were conducted as described in Fig. 1.

Discussion
The goal of this study was to investigate the role of secondary lymphoid organs in the maintenance of memory T cells and the differentiation of effector T cells to memory. To do so, we transferred either memory or effector T cells into congenic hosts that lack secondary lymphoid organs and analyzed their number, phenotype, and function >8 wk later. We found that established CD4 and CD8 memory T cells are maintained long term and function (reject allogeneic skin grafts) in the absence of secondary lymphoid organs. Although CD8 effectors differentiated to memory cells independently of secondary lymphoid organs, the optimal differentiation of CD4 effectors into a stable memory pool was dependent on these tissues.

We used in this study an in vivo polyclonal system to investigate memory T cells, because of its similarities to physiological immune responses. Immune responses, such as those responsible for allograft rejection, target a diverse array of antigenic epitopes and are therefore polyclonal (42). Moreover, CD4 and CD8 responses to the same Ag can be compared concurrently in a polyclonal system. Monoclonal TCR-tg cells, in contrast, may not accurately reflect physiological immune responses and do not allow for unbiased comparisons between CD4 and CD8 T cells. A potential drawback of the polyclonal system, however, is the presence of both Ag-specific and naturally existing memory populations among CD44high T cells (41). In the present study, we were able to distinguish between these populations by measuring both their ex vivo cytokine production and their in vivo function. We found that CD44high T cells from alloimmunized mice contain allospecific T cells, which produce IFN-γ in response to Ag and mediate allograft rejection in the absence of secondary lymphoid organs. In contrast, naturally existing CD44high T cells from unimmunized mice contained many fewer allospecific T cells and did not mediate allograft rejection in a host that lacks secondary lymphoid organs. We also observed that the majority of the polyclonal CD44high pool had divided shortly after immunization, implying that sorting for CD44high T cells before adoptive transfer significantly enriches for Ag-induced effector T cells. Therefore, polyclonal experimental systems can be quite useful for studying Ag-specific memory T cells, especially in the setting of alloimmunity.

FIGURE 4. Differentiation of CD4 and CD8 effector T cells to memory in the absence of secondary lymphoid organs. Effector T cells were generated in B6.PL-Thy1a/Cy mice by immunization with BALB/c splenocytes. LN and spleen cells were harvested 7 days after immunization, and CD4+ CD44high and CD8+ CD44high T cells were sorted before transfer into aly/aly-spleen or wt congenic hosts (Thy1.2). A, Phenotypes of sorted CD4 and CD8 populations before transfer. B, The majority of the CD44high population consists of divided T cells 7 days after alloimmunization. CFSE-labeled naive (CD44low) CD4 and CD8 Thy1.1+ T cells (10 × 106) were adoptively transferred to congenic wt (Thy1.2) hosts. Seven days after immunization with BALB/c (allogenic) or B6 (syngeneic) splenocytes, spleen cells were harvested, and CFSE dilution was analyzed after gating on Thy1.1+ cells. C, Enumeration of Thy1.1+ T cells present in the adoptive hosts at 1, 4, and −11 wk (range, 8–14 wk) after transferring 5 × 106 to 1 × 107 CD4 or CD8 effector T cells into aly/aly-spleen mice. Also shown is the recovery of CD4 and CD8 effector T cells −12 wk after transfer to wt hosts. The total number of Thy1.1+ T cells harvested from each recipient is estimated as described in Fig. 1B. Values are the mean ± SD of three to six mice per time point. D, Tissue distribution of transferred cells. Cells were harvested from the liver, lungs, bone marrow, and blood at the indicated time points. The number of Thy1.1+ T cells harvested from each tissue is estimated as described in Fig. 1B. Values are the mean of three to six mice per time point. E, Phenotype of T cells harvested from the liver of aly/aly-spleen mice or from the liver and spleen of wt mice −11 wk after transfer. CD44, CD25, and CD62L expression on both transferred Thy1.1+ and host (Thy1.1+) T cells after gating on either the CD4 or CD8 population is shown (representative of three to six experiments). The percentages shown are of CD44high, CD25high, and CD62Lhigh Thy1.1+ cells compared with the total Thy1.1+ population. F, Memory T cell function measured by the ability of adoptively transferred hosts to reject skin allografts. Experiments were conducted as described in Fig. 1F. Mice in the experimental groups received 5 × 106 sorted T cells unless indicated otherwise (three to six mice per group). Controls were the same as in Fig. 1F.
lymphoid organs, CD4 and CD8 CD44high T cells were sorted 7 days after proliferation of recently activated CD4 T cells is dependent on secondary are the mean/H11006/H11001 analyzed after gating on Thy1.1 ala/aly-spleen host) cells were harvested, and CFSE dilution was ther immunizing B6.PL-Thy1a/Cy mice, CFSE labeled, and transferred to ei-

FIGURE 5. Role of secondary lymphoid organs in the differentiation of CD4 effector T cells to memory. A, To investigate whether extended access of effector T cells to secondary lymphoid organs is required for the optimal generation of CD4 memory, we sorted $0.5 \times 10^6$ to $1 \times 10^6$ CD4$^+$CD44high or CD8$^+$CD44high $^+$ T cells 7 or 15 days after immunizing Thy1.1 mice and transferred them into either ala/aly-spleen or wt hosts. Eight to 14 wk later, recovered Thy1.1 T cells were enumerated. Values are the mean $\pm$ SD (three mice per group). B, To investigate whether the proliferation of recently activated CD4 T cells is dependent on secondary lymphoid organs, CD4 and CD8 CD44high T cells were sorted 7 days after immunizing B6.PL-Thy1a/Cy mice, CFSE labeled, and transferred to ei-

Our observation that both CD4 and CD8 memory T cells home to nonlymphoid tissues is consistent with published data. In addition to homing to secondary lymphoid organs, established CD8 memory T cells circulate through the bone marrow and nonlymphoid tissues, such as the liver and lungs (8, 13, 34–36, 43). Unrestricted access of CD8 memory T cells to the liver and lungs has been demonstrated in paraibiotic mice in the absence of antigenic rechallenge (13), suggesting that trafficking to nonlymphoid organs is a physiological attribute of memory T cells. Similarly, Reinhardt et al. (9) found CD4 memory T cells in lymphoid and nonlymphoid tissues of mice months after primary antigenic chal-

The data presented in this manuscript also indicate that CD4 and CD8 central (CD62L$^+$) and effector (CD62L$^-$) memory T cell subsets have differential maintenance requirements. After the transfer of memory T cell populations containing both CD62L$^+$ and CD62L$^-$ phenotypes to hosts that lack secondary lymphoid organs, we detected primarily CD62L$^+$ and very few CD62L$^-$ CD4 memory T cells in the liver, lungs, and bone marrow. This finding raises the possibility that CD4 central memory T cells either die or differentiate to the effector memory phenotype in the absence of secondary lymphoid organs. In contrast, both central and effector CD8 memory T cells were maintained in hosts that lack secondary lymphoid organs, indicating that either CD8 memory T cell subset can migrate to and survive in nonlymphoid organs.

In summary, we have shown that both CD4 and CD8 memory T cells are maintained and function independently of secondary lymphoid organs. These findings emphasize the resilience of established memory T cells and underscore their important role in immune surveillance of nonlymphoid organs. In contrast, CD4, but not CD8, effector differentiation to memory T cells is impaired in hosts that lack secondary lymphoid organs. The latter finding suggests that CD4 T cells require extended nurturing within secondary lymphoid organs before generating a stable memory pool.

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Disclosures
The authors have no financial conflict of interest.

References
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