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A Closer Look at Homeostatic Proliferation of CD4+ T Cells: Costimulatory Requirements and Role in Memory Formation

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Ag-specific proliferation of CD4+ T cells is regulated, in part, by costimulatory signals through CD28. The proliferative response during primary activation is an important determinant of the ability of the T cell to respond to Ag re-encounter. Proliferation of mature CD4+ T cells during lymphopenia (homeostatic proliferation) requires interaction with endogenous peptide MHC. However, the role of costimulation during homeostatic proliferation is unclear, as is the ability of homeostatic proliferation to regulate secondary T cell responses. Using a TCR transgenic system and serial adoptive transfers we find that homeostatic proliferation of CD4+ T cells occurs for at least 5 wk after adoptive transfer into recombination-activating gene (RAG)-/- recipients. Two discrete populations of proliferating T cells can be resolved, one that is highly proliferative and dependent on CD28 signaling, and the other that contains cells undergoing low levels of CD28-independent proliferation. Importantly, naive CD4+ T cells that have undergone homeostatic proliferation acquire both phenotypic and functional characteristics of true memory cells. These studies indicate that functional memory T cells can be generated by encounters with endogenous Ags only. This mechanism of T cell regeneration is possibly active during lymphopenia due to viral infections, such as HIV, transplantation, or cancer therapy, and may explain selected autoimmune diseases. The Journal of Immunology, 2001, 167: 3699–3707.

Throughout adult life, the size of the peripheral lymphocyte pool is tightly regulated and remains relatively constant in the absence of disease. Recently, the mechanisms that regulate the pool have been the focus of intense investigation. This includes thymic output, peripheral T cell expansion, and death. Interestingly, the rate of export of T cells from the thymus, except under extreme conditions, is often not an important parameter regulating the size of the peripheral T cell pool (1). Indeed, regulation of the size of the peripheral T cell pool seems to occur outside the thymus and is the summation of T cell proliferation and/or survival and death. This process of maintaining the appropriate numbers of peripheral T lymphocytes is incompletely understood but seems to be a complex interaction between different components of the immune system. For example, the survival of peripheral CD4+ T lymphocytes is negatively affected by recent thymic emigrants as well as CD8+ T lymphocytes (1, 2). In addition, naive CD4+ T lymphocytes require expression of the selecting MHC class II molecules in the periphery for survival (3–5), as well as several transcription factors, cytokines, and survival proteins (6–9). Therefore, a picture evolves where the maintenance of naive peripheral T lymphocytes is both a complex and an active process requiring signaling through the TCR by a self-peptide-MHC complex.

Therefore, it is not surprising that the thymus will not only continue to export naive T cells during lymphopenia, but peripheral T cells will proliferate in the absence of exogenous stimulation (homeostatic proliferation) to fill the “empty” lymphoid space (10). This may occur physiologically following lymphoid ablation of normal animals (as part of tolerance-inducing protocols or cancer therapy). It is also observed in experimental systems where, upon transfer of CD4+ and CD8+ T lymphocytes into lymphopenic hosts, the T cells proliferate. Many of the same factors are necessary for homeostatic proliferation as for survival and maintenance of T lymphocytes when peripheral lymphocyte counts are normal. These include interaction with self-peptide-MHC complexes (11–13), as well as cytokines, particularly those that signal through the common cytokine receptor γ-chain (14, 15).

When naive peripheral CD4+ T lymphocytes encounter foreign peptides in a complex with self-MHC as well as a costimulatory signal, such as through CD28, a robust immune response is generated. This results in proliferation and differentiation into short-lived cytokine-secreting effector cells. However, some cells survive to become long-lived memory cells. The precise requirements for memory formation are unclear. Although somewhat controversial, most researchers agree that CD8+ T lymphocytes that have proliferated in a lymphopenic host in the absence of exogenous Ag have increased ability to produce cytokines and lyse targets compared with naive CD8+ lymphocytes (16, 17). Presently it is unclear whether the same holds true for CD4+ T lymphocytes. The requirement for costimulation during homeostatic proliferation of T lymphocytes is equally unclear. It has been shown that B7-deficient mice have decreased numbers of peripheral T cells, particularly CD4+ T cells, whereas the reverse is true for mice overexpressing B7 (18). One group (19) using CD28-knockout mice suggested that costimulation was not required for homeostatic proliferation of polyclonal populations of peripheral CD8+ T lymphocytes. In any event, it is unclear whether costimulation is required for homeostatic proliferation of Ag-specific peripheral CD4+ T cells.

Recently, we have used the dye CFSE to label Ag-specific CD4+ T lymphocytes before adoptive transfer into syngeneic wild-type mice (20). This has enabled us to quantify proliferation of T lymphocytes during a response to foreign Ag in vivo, and better determine the role of CD28 costimulation in promoting proliferation and differentiation of responding T cells. The studies...
presented here use this approach to determine whether B7-mediated costimulation is required for homeostatic proliferation of Ag-specific CD4+ T cells, as well as to determine whether CD4+ T cells acquire phenotypic and functional changes consistent with memory cells as a result of homeostatic proliferation.

**Materials and Methods**

**Mice**

Recombination-deficient DO11.10 (Thy1.2+) mice (21) on BALB/c background (>20 generations) were either obtained as DO11.10/recombination-activating gene (RAG)−/− mice (W. Lee, State University of New York, Albany) or DO11.10 mice bred with SCID mice (The Jackson Laboratory, Bar Harbor, ME), and subsequently maintained as breeding colonies in our animal facility. PBL from all mice were screened by flow cytometry for the absence of B and nontransgenic T lymphocytes to ascertain that they were homozygotes for the SCID mutation or RAG deletion. RAG−/− mice were purchased from Taconic Farms (Germantown, NY). BALB/c (Thy1.1+) mice were bred in our animal facility. All mice were used at 6–12 wk of age.

**Peptide, Abs, and CFSE labeling**

CTLA4Ig (22) was provided by R. Peach (Bristol-Myers Squibb Pharmaceuticals, Princeton, NJ). The hybridoma producing anti-CD28 mAb (37,51) was provided by J. Allison (University of California, Berkeley, CA). Control hamster IgG and control human IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). OVA peptide (323–339) (OVAp) was synthesized by the Protein Chemistry Laboratory, University of Pennsylvania (Philadelphia PA). Fluorochrome-labeled mAbs against CD4, CD44, CD45RB, CD69, CD62 ligand (CD62L), IL-2, IFN-γ, CD16/CD32 (Fc block), and isotype controls were purchased from BD PharMingen (San Diego, CA). Fluorochrome-labeled anti-DO11.10 Ab, KJ1-26, was purchased from Caltag Laboratories (Burlingame, CA). CFSE labeling of lymphocytes was achieved as described (23), with some modifications (24).

**In vitro proliferation cultures**

Labeled DO11.10/RAG−/− lymphocytes were plated at 10–20 × 10^6 transgenic cells per well in a round-bottom 96-well plate in RPMI 1640 medium containing 10% FCS (HyClone Laboratories, Logan, UT), 2 mM l-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 5 µM 2-ME. They were stimulated with varying concentrations of OVAp for 4 days, with an excess of irradiated splenocytes as a source of APCs (APC/clonotypic T cell ratio >10) as well as anti-CD28 mAb and control human IgG or CTLA4Ig and control hamster IgG.

**Adoptive transfer and immunization**

A total of 1.5–15 × 10^6 CFSE-labeled transgenic cells (obtained from the lymph nodes and spleens of DO11.10/RAG−/− or DO11.10/SCID mice) were injected i.v. into RAG−/− mice in a total volume of 0.2 ml. One to four days later mice were either left unimmunized or immunized with 50 µg of OVAp mixed inIFA (OVApIFA) into two sites in the lower back, at the base of the tail, and three sites on the upper back (total of 300 µg of OVAp/mouse). In some cases the mice were treated at the time of adoptive transfer, before immunization, and twice per week thereafter with 200 µg i.p. of either CTLA4Ig or human IgG.

**Flow cytometry**

Cells were washed in PBS containing 2% FCS and 0.02% azide at 4°C. Unlabeled anti-CD16/CD32 (anti-FcRγII/anti-FcRγII) were used to block FcR binding. Between 0.2 and 3.0 × 10^6 cells were stained with either 1) PE-conjugated anti-CD4 and CyChrome-conjugated anti-CD44 or 2) PE-conjugated Abs against CD45RB or L-selectin, and PerCP-conjugated anti-CD4. In both cases, cells were also stained with APC-conjugated Thy1.2 and/or FITC-conjugated KJ1–26 to identify the transgenic cells. Four-color flow cytometry was performed on a FACSCalibur (BD Immunocytometry Systems, San Jose, CA), and cells were acquired and analyzed using CellQuest and FlowJo software (BD Immunocytometry Systems). Between 20 × 10^3 and 1.5 × 10^6 events were collected.

**Intracellular cytokine staining**

Cells were stimulated in vitro with 2.5 µg/ml OVAp in the presence of 2 µg/ml anti-CD28 and 2 µM monensin (Boehringer Mannheim, Indianapolis, IN) (25). After staining for surface receptors, the cells were fixed in a final concentration of 1% formaldehyde at 4°C overnight. Subsequently they were washed in PBS, then in PBS containing 2% FCS, 0.02% azide, and 0.1% saponin (Sigma, St. Louis, MO). PE-conjugated anti-IL-2 or anti-IFN-γ mAb (BD PharMingen) mixed in 30 µl of PBS/saponin buffer was used to stain each sample for 60 min (30 min at 4°C followed by 30 min at room temperature).

**Quantitation of T cell-proliferative dynamics from CFSE profiles**

The quantitative analysis of proliferation using CFSE has been previously described (20, 24).

**Results**

**Homeostatic proliferation—kinetics**

To better understand the requirements for, and consequences of, homeostatic proliferation of CD4+ T cells, we adoptively transferred CFSE-labeled OVA-specific T lymphocytes into RAG−/− mice. As a source of naive Ag-specific CD4+ T cells, we used DO11.10/RAG−/− or DO11.10/SCID mice.

Three weeks after adoptive transfer we identified two populations of OVA-specific T lymphocytes based on CFSE brightness (Fig. 1). When 1.5 × 10^6 cells were adoptively transferred, some of the input cells divided minimally (0–2 rounds of division), and they are visualized as distinct peaks of proliferation, whereas others divided multiple times (>6–8 rounds of division, Fig. 1A). In the latter case, the CFSE stain became very diluted, and individual peaks of proliferation were no longer identified. The loss of discrete peaks in the multiply divided population precludes us from extrapolating the ratio of input cells that generated these two populations (20). However, the input cells that divided multiple times generated 75.5 ± 11.4% of the OVA-specific T lymphocytes present in the spleen (Fig. 1, A and G). The rest, or 24.3 ± 11.4%, were generated by input cells that proliferated minimally or not at all. It is unclear why two distinct homeostatic proliferative potential exist. As the OVA-specific T cells that we adoptively transferred were derived from TCR-transgenic mice on a RAG−/− background, this eliminates the possibility that the population generated by multiple rounds of division was the progeny of memory cells generated by exposure to an environmental Ag through stimulation of a second TCR (26). However, it is possible that the different proliferative behavior reflects distinct stimuli that the cells encountered in the recipient mouse, e.g., endogenous Ag presentation by professional vs nonprofessional APCs. Alternatively, the input cells may have an intrinsic variability in proliferative potential.

It was also apparent when comparing the proliferative profile of cells 3 and 5 wk after adoptive transfer that proliferation continued beyond 3 wk (compare Fig. 1, A and B, as well as C and D). However, despite this increase in proliferation, there was no difference in the total number of cells collected 3 and 5 wk after adoptive transfer (data not shown). This suggests that in the recipient RAG−/− mice homeostatic proliferation continues but is balanced by ongoing cell death.

**Homeostatic proliferation—requirements**

Two parameters that are known to regulate Ag-driven T cell proliferation in vivo are the availability of costimulation through molecules such as CD28, and the relative vacancy of the lymphoid compartment (13, 19, 27–37). To understand how these factors regulate homeostatic proliferation, we altered the number of input cells that were adoptively transferred and/or blocked B7-mediated costimulation.
The proliferative capacity of the input cells was clearly affected by the number of cells adoptively transferred into the RAG−/− mice. When 10 times more cells (15 × 10^6) were adoptively transferred, the input cells again proliferated either minimally or multiple times and generated two discrete populations of cells. However, the relative size of these two populations was very different compared with when fewer cells were adoptively transferred (Fig. 1, A, E, and G). Thus, only 30.0 ± 1.5% of the population was generated by input cells that had proliferated multiple times compared with 75.5 ± 11.4% when 10 times fewer cells were adoptively transferred (p = 0.05). CD28 costimulation signals also regulated the size of the highly proliferative pool. When 1.5 × 10^6 input cells were adoptively transferred and mice were treated with CTLA4Ig, only 9.0 ± 6.0% of the OVA-specific T lymphocyte population was generated by cells that had proliferated maximally (p < 0.05) (Fig. 1F). The effect of CTLA4Ig was less evident when 15 × 10^6 cells were adoptively transferred, although the difference seen in the spleen did reach statistical difference (p < 0.05) (Fig. 1G). Taken together, these data suggest that two known checkpoints of Ag-specific proliferation, costimulation and pool size, also regulate homeostatic proliferation.

Surface phenotype of Ag-specific CD4+ T lymphocytes after homeostatic proliferation and after priming by the specific Ag

When naive T lymphocytes encounter their specific Ag they increase in size and up-regulate “early activation markers,” such as CD69. Subsequently, they proliferate and differentiate into effector and memory cells. To determine whether the same holds true for naive cells that have undergone homeostatic proliferation we examined expression of early activation markers as well as expression of classical markers of memory.

OVA-specific T lymphocytes were adoptively transferred into syngeneic RAG−/− mice. One to two days after adoptive transfer, the mice were either left untreated (adoptively transferred–untreated) or immunized with OVAp mixed in IFA s.c. (adoptively transferred–immunized) (Fig. 2). The OVA-specific T cells exposed to the OVAp became activated as noted by their increase in size and up-regulation of CD69 as early as 24 h after immunization. In contrast, the OVA-specific T lymphocytes that were adoptively transferred and not exposed to OVAp remained small and did not up-regulate CD69 at 48 h after adoptive transfer. Thus, consistent with previous reports (16, 17, 38, 39), we find that...
OVA-specific T lymphocytes adoptively transferred into lymphopenic hosts do not show hallmark signs of early activation after adoptive transfer. However, at later time points (4–5 wk), the OVA-specific T cells that had undergone maximal levels of homeostatic proliferation were enlarged, consistent with blastogenesis (data not shown).

To determine the expression of the classical markers of memory, we compared the expression of CD45RB and CD62L (L-selectin) on OVA-specific T lymphocytes that were adaptively transferred into syngeneic RAG−/− mice that were left untreated (homeostatic prolif.-naive) or immunized with OVAp/IFA (effector/memory), as well as freshly isolated OVA-specific T lymphocytes (fresh naïve) (Fig. 3). Interestingly, OVA-specific T lymphocytes exposed to OVAp/IFA in vivo changed their surface phenotype within 4 wk after adoptive transfer from naïve (CD45RBhigh and CD62Lhigh) to that of memory/effector cells (CD45RBlow and CD62Llow) (n = 7, Fig. 3). Interestingly, the surface phenotype of OVA-specific T lymphocytes that had undergone homeostatic proliferation also converted to that of memory cells, but this conversion required a minimum of 6 wk (n = 3). It should be noted that although the entire population of OVA-specific T cells undergoing homeostatic proliferation down-regulated CD45, only a proportion down-regulated CD62L. This also holds true for the memory/effector population, although in this instance a larger proportion down-regulated CD62L. Partial down-regulation of CD62L may in part be due to the triphasic expression of CD62L (40). In addition, down-regulation of both CD45 and CD62L is associated with cell cycle number, as both were most markedly reduced in the cells that had divided multiple times (Fig. 3B). These data suggest that specific foreign Ag is not required for conversion from a naïve to a memory surface phenotype. However, the rate of conversion is slower than that of T lymphocytes primed with the specific peptide, possibly as a result of much slower progression through the cell cycle (our unpublished observations and Ref. 39).

**Functional analyses of Ag-specific CD4⁺ T lymphocytes undergoing homeostatic proliferation**

Memory markers are expressed on both memory and effector T lymphocytes and do not inform us of the ability of expressing T cells to produce cytokines or proliferate. Having found that OVA-specific T cells that had undergone homeostatic proliferation acquired phenotypic characteristics of memory cells, we next wished to determine their functional characteristics as well. To study this, we adoptively transferred OVA-specific T lymphocytes into RAG−/− mice as described above, and either left the mice untreated (homeostatic prolif.-naive) or immunized the mice with OVAp/IFA s.c. to generate the memory/effector population. The function of these cells was then compared with that of freshly isolated OVA-specific T lymphocytes (fresh naïve).

**Proliferation of OVA-specific T lymphocytes**

In contrast to effector cells, which undergo activation-induced cell death upon Ag encounter (41–44), the proliferative response of memory cells to specific foreign Ags is faster and quantitatively greater than that of naive cells (45–47). Memory cells also proliferate in response to a lower dose of Ag and are less dependent on costimulatory signals compared with naive cells (47). To determine whether cells that had undergone homeostatic proliferation had a similar proliferative potential as memory cells, we labeled OVA-specific T lymphocytes that had previously undergone homeostatic proliferation or been primed in vivo with specific peptide (memory/effector) or freshly isolated OVA-specific T lymphocytes (fresh naïve) with CFSE and stimulated them in vitro at varying doses of OVAp for 4 days (Fig. 4). An excess of irradiated splenocytes served as a source of APCs, and cultures were stimulated either in the presence of anti-CD28 to provide maximal costimulation, or CTLA4Ig to block costimulation.

In the absence of costimulation, freshly isolated naive OVA-specific T lymphocytes proliferated minimally at low doses of peptide (Fig. 4A). In fact, quantifying proliferation based on CFSE brightness showed that only 36% of the input OVA-specific T lymphocytes initiated proliferation. However, when maximal costimulation was provided, this response increased to 78%. In contrast, the memory/effector population proliferated efficiently, independent of costimulatory signals. In the absence of costimulation, 69% of the input OVA-specific memory/effector T lymphocytes initiated proliferation. When maximal costimulation was provided there was a minor increment to 76%. Therefore, the proliferative potential of the effector/memory population was clearly greater than that of freshly isolated naive OVA-specific T lymphocytes. Proliferation of OVA-specific T lymphocytes that had previously undergone homeostatic proliferation was similar to that of the memory/effector population. In the absence of costimulation, 66%
of the input OVA-specific T lymphocytes initiated proliferation. When maximal costimulation was provided, this response increased to 82%. Taken together, these data suggest that the proliferative response of Ag-specific CD4\(^{+}\) T lymphocytes that have undergone homeostatic proliferation is similar to that of memory/effecter CD4\(^{+}\) T lymphocytes.

We also examined proliferative responses as a function of Ag concentration under conditions of CD28 blockade. This showed that in the absence of costimulation, freshly isolated OVA-specific T lymphocytes required 10-fold more peptide to obtain the same amount of proliferation compared with the memory/effecter cell population (Fig. 4B). This is consistent with published data comparing proliferation of memory and naive T lymphocytes (45–47). The proliferative response of OVA-specific T lymphocytes that had previously undergone homeostatic proliferation was again comparable to the memory/effecter cell population. In the absence of CD28-mediated costimulation they required a 10-fold lower Ag concentration to achieve a similar proliferative response as freshly isolated naive CD4\(^{+}\) T cells. Taken together, these data show that CD4\(^{+}\) T lymphocytes that have previously undergone homeostatic proliferation show a similar proliferative response as our memory/effecter cell population.

**Cytokine production by OVA-specific T lymphocytes**

To determine whether the OVA-specific T lymphocytes that had undergone homeostatic proliferation had comparable ability to make cytokines as memory cells, we restimulated these cell
populations for a short period (4–16 h) in vitro with OVAp and anti-CD28 in the presence of irradiated splenocytes.

When naive, OVA-specific T lymphocytes freshly isolated from the spleen were stimulated in vitro, only 3.4 ± 5.8 and 0.43 ± 0.2% made IL-2 or IFN-γ, respectively (Fig. 5 and Table I). This is consistent with published data indicating that during a brief activation period only a few naive T cells produce cytokines (48, 49). In contrast, 33.8 ± 5.9 and 20.5 ± 13.1% of effector/memory cells isolated from the spleen made these cytokines (p < 0.05). The percentage of OVA-specific T lymphocytes that had undergone homeostatic proliferation and made these cytokines was intermediate between the freshly isolated naive and memory/effector population. Thus, 18.1 ± 12.6 and 9.1 ± 6.5%, respectively, made IL-2 and IFN-γ. Therefore, the data suggested that Ag-specific CD4+ T lymphocytes that had undergone homeostatic proliferation acquired the ability to make the cytokines IL-2 and IFN-γ. However, they were not quite as likely to make cytokines as the memory/effector T cells.

Because we had found a dichotomy in the mitotic potential of DO11.10 T cells undergoing homeostatic proliferation (Fig. 1), we next wished to determine whether these two populations differed in their functional capacities. As we (20) and others (50, 51) have recently shown a link between cell division history and the ability of cells to produce cytokines such as IFN-γ and IL-4, but not IL-2, we chose cytokine production as a functional readout. For that purpose we adoptively transferred CFSE-labeled OVA-specific T cells into RAG−/− mice. Four weeks later we sacrificed the mice and briefly restimulated the T cells in vitro before intracellular staining for IL-2 and IFN-γ. As seen in Fig. 6, there was a strong relationship between cell division and the ability of the T cells to produce IFN-γ. Only the OVA-specific T cells that divided more than four to five times were able to produce IFN-γ. This was in sharp contrast to the capacity for IL-2 production, which was not associated with cell cycle production.

Interestingly, even cells that did not undergo homeostatic proliferation in the immunodeficient animals (i.e., failed to divide), were efficient IL-2 producers (Fig. 6, C and E), unlike freshly isolated naive OVA-specific T cells (Fig. 5, lower left panel), which do not produce IL-2 under the conditions studied. This suggests that a maturational/differentiative process has occurred uncoupled from cell division.

As noted above, although cells that had undergone homeostatic proliferation developed functional capabilities similar to cells that had been stimulated by Ag, on a cell-by-cell basis they had a lower frequency of IL-2 and IFN-γ production than Ag-activated memory/effector cells. One possibility is that this difference was due to the presence of effector cells in the latter population, which among CD4+ T lymphocytes, are the most efficient cytokine producers (52, 53). This might occur if the OVAp was not cleared adequately from the immunized mouse during this time, as it could continuously stimulate proliferation of OVA-specific T lymphocytes and differentiation into effector cells. To test that, we adoptively transferred 10 × 10⁶ CFSE-labeled OVA-specific T lymphocytes into RAG−/− mice that 4 wk earlier had received unlabeled OVA-specific T lymphocytes and were either immunized with OVA/IFA s.c. or left untreated (as control). The CFSE-labeled OVA-specific T lymphocytes that were adoptively transferred into the previously immunized RAG−/− mice did not proliferate, whereas the OVA-specific T lymphocytes that were adoptively transferred into the previously immunized RAG−/− mice increased in size and proliferated (data not shown). These proliferating T cells became 10% of the CFSE-labeled population. This clearly suggests that residual OVAp is present 4 wk after immunization of the RAG−/− mice, in quantities large enough to stimulate blast formation and proliferation of a proportion of OVA-specific T lymphocytes, and thus suggests the presence of effector cells in the memory/effector population.

To determine whether OVA-specific T lymphocytes that had undergone homeostatic proliferation produced cytokine comparable to “true” memory cells, it was important to eliminate the effector cells from our primed memory/effector population. To do so, we adoptively transferred OVA-specific T lymphocytes from the RAG−/− mice (previously adoptively transferred with OVA-specific T lymphocytes and either immunized with OVAp/IFA or left unimmunized) into wild-type Thy 1.1+ BALB/c mice (Fig. 7). This allows the OVA-specific T cells to rest in a physiologic environment, away from the specific peptide. By labeling the cells before adoptive transfer with CFSE, we confirmed that the cells were resting (as measured by lack of division) 4 and 7 days after adoptive transfer into BALB/c mice (data not shown). Due to down-regulation of the TCR, after in vitro culture we used Thy 1.1+ BALB/c mice to better identify our cell population, which is Thy 1.2+. IL-2 production by OVA-specific T lymphocytes that had previously undergone homeostatic proliferation was now equal to the IL-2 production of the memory cell population (12.4 ± 7.7% and 15.9 ± 5.6% in the lymph nodes and 26.68 ± 18.63% and 30.15 ± 21.57% in the spleen, respectively). This was significantly higher than that of freshly isolated OVA-specific T lymphocytes adoptively transferred into BALB/c mice (1.4 ± 0.8%) (Fig. 7). Collectively, these data show that Ag-specific CD4+ T lymphocytes that have undergone homeostatic proliferation are equally efficient cytokine producers as resting memory CD4+ T lymphocytes.

### Discussion

Recently it has become increasingly clear that both CD8+ and CD4+ T lymphocytes will proliferate in a lymphopenic host in the absence of the specific exogenous Ag (27, 30). It has been found that this requires interaction with endogenous peptide MHC complex (11, 12). In this study we have identified two CD4+ T cell populations based on their proliferative history during lymphopenia. One population was generated by input cells that underwent numerous cell divisions, whereas the other was generated by cells that proliferated minimally. Both costimulation and pool size, known checkpoints of Ag-specific proliferation, regulated this proliferative response. Importantly, we also found that the CD4+ T cells generated during lymphopenia acquired proliferative and cytokine-producing functions in response to antigenic stimuli that were comparable to that of true memory T lymphocytes.

Several groups have studied homeostatic proliferation both in CD8+ and CD4+ T lymphocytes. Most of the studies on CD8+ T cells indicate that they change their surface phenotype to that of
memory cells (11, 17, 54). Some studies report no functional alteration of CD8$^+$ T cells during homeostatic proliferation compared with naive cells (54), whereas others disagree (16, 17, 19). The discrepancy in the reported studies is probably due to differences in the experimental system. Thus, it was recently shown that adoptive transfer of transgenic CD8$^+$ T lymphocytes into irradiated wild-type mice allowed cells to proliferate and reach the "optimum" number of peripheral cells (17). During this proliferation, the CD8$^+$ T cells changed their surface phenotype to that of memory cells and acquired increased CTL activity and the ability to make IFN-γ. When the peripheral pool was "full", the T cells reverted back to quiescence, converted back to a naive surface phenotype, and were less efficient in lysing targets and making IFN-γ. However, when the same CD8$^+$ T cells were adoptively transferred into RAG$^-/-$ mice. Four weeks later, the cells were restimulated in vitro with OVAp and anti-CD28 as described (A, C, and D) or with medium alone (B). Density plots are gated on CD4$^+$ and Thy 1.2$^+$ cells. Graph E outlines the percentage ratio of CD4$^+$ T cells that make cytokines IL-2 and IFN-γ as a function of division. Representative results from two experiments are shown.

FIGURE 6. Cytokine production as a function of cell cycle progression. OVA-specific T cells were labeled with CFSE and adoptively transferred into RAG$^-/-$ mice. Four weeks later, the cells were restimulated in vitro with OVAp and anti-CD28 as described (A, C, and D) or with medium alone (B). Density plots are gated on CD4$^+$ and Thy 1.2$^+$ cells. Graph E outlines the percentage ratio of CD4$^+$ T cells that make cytokines IL-2 and IFN-γ as a function of division. Representative results from two experiments are shown.

FIGURE 7. IL-2 production by OVA-specific T cells. OVA-specific memory/effector cells, generated as in Fig. 5, were adoptively transferred into BALB/c mice for 4–7 days to remove them from OVAp to create a resting memory population. Cells that had undergone homeostatic proliferation, as well as "fresh-naive" cells were similarly adoptively transferred as controls. Four to seven days later, all three populations were recovered and stimulated briefly in vitro with OVAp and anti-CD28 before cytokine staining. Contour plots show live clonotypic (KJ1-26$^+$) CD4$^+$ T cells collected from the spleen. Similar results were obtained from lymph nodes. Representative results of four separate experiments are shown.
transferred into RAG−/− mice, the behavior of the cells was quite different. They never reached the same “desired” peripheral number of cells and never ceased proliferating. Accordingly, the T cells never reverted back to a naive phenotype and never lost the ability to lyse targets or make IFN-γ. Others have also reported that the amount of proliferation of transgenic CD8+ T cells is dependent on the number of cells adoptively transferred as well as the time (19). Therefore, the discrepancy in the published studies can be explained by differences in factors such as the length of time since adoptive transfer, numbers of cells that were adoptively transferred, as well as the type of recipient mouse used.

Somewhat to our surprise, we identified two populations of Ag-specific T lymphocytes that were generated during homeostatic proliferation. There are several factors that might explain why this has not been a consistent finding in published papers. Clearly, the ability to identify these two populations is dependent on the ability to differentiate the input cells from the cells of the recipient by a specific marker, because the population generated by highly proliferative cells becomes CFSE negative. Also, the appearance of this population is dependent on the number of cells that are adoptively transferred, as well as on time. Others studying polyclonal unsorted CD4+ T lymphocytes have identified two populations (39). Interestingly, in these studies the population generated by multiple divisions (more than six to eight times) expressed a surface phenotype consistent with that of memory cells. In contrast, the population generated by slowly dividing cells expressed a surface phenotype consistent with naive cells. Thus it was proposed that the population of cells that divided maximally was generated by homeostatic proliferation of memory cells within the polyclonal CD4+ T cell population. Our data would argue that the highly proliferative cells are not derived from pre-existing memory cells because we used DO11.10/RAG−/− or DO11.10/SCID cells. These cells are unable to express a second TCR on their surface, which in the presence of an unknown foreign Ag can lead to memory T cell formation (26). Consistently, all the DO11.10 cells express a naive phenotype. Rather, our data would suggest that either a subset of the donor CD4+ T lymphocytes are already inherently different or, alternatively, that the cells are exposed to different signals in the lymphopenic mouse. This would include competition for signals delivered by professional APCs as has been found to occur in vivo (55, 56). Although not mutually exclusive, we do favor the latter explanation because the highly proliferative donor cells were dependent upon B7 signaling for their proliferation, whereas the other population was not. It is controversial whether or not CD4+ T cells that divide in lymphopenic hosts acquire phenotypic and functional changes comparable to that of memory cells. Most studies find that naive CD4+ T lymphocytes do not convert to a surface memory phenotype during homeostatic proliferation (14, 39, 57); however, some have reported partial up-regulation of CD44 (11). Our data would suggest that CD4+ T cells might need a long period of time (>4 wk) to acquire the phenotypic changes of memory cells. This is in contrast to CD8+ T lymphocytes, which convert to a surface phenotype much earlier, possibly because of a brisker proliferative response during lymphopenia (11). Our data also suggest that CD4+ T cells that have undergone homeostatic proliferation acquire function comparable to memory T cells. First, their ability to proliferate in response to low doses of Ag and relative independence of costimulation is similar to the memory population. Second, their ability to make the cytokines IL-2 and IFN-γ in a short restimulation assay is greater than that of naive CD4+ T cells, and their IL-2 production is similar to that of memory cells. Finally, functional memory-like cells are more likely to be found following homeostatic proliferation in the population of cells that have divided multiple times and whose generation requires CD28 signals. This conclusion is based on the finding that the multiply divided cells have the greatest down-regulation of CD45RB (Fig. 3B), and more importantly, are the only population of cells that produced IFN-γ (Fig. 6A).

The question remains whether or how proliferation and differentiation are mechanistically linked. Signals from both the TCR and from cytokine receptors are critical in determining the cytokine profile of naive T cells. Multiple investigators have shown a correlation between cell cycle progression and cytokine production (20, 50, 58). This may be explained in part by the remodeling of chromatin during cell cycle progression (50, 51). Despite the chromatin changes that take place during proliferation, some have found that cell cycle progression is not required for cytokine production of naive T cells (59, 60). The controversy may, in part, be explained by different nonphysiologic situations used by the investigators to induce cytokine production in the absence of division. There are also multiple known factors, such as transcription factors and their repressors that might induce cytokine production independent of proliferation (61–68). Taken together, cell division seems to be an important parameter determining differentiation of naive T cells into effector cells, by remodeling DNA. It remains to be determined whether these epigenetic modifications of DNA are stably expressed in resting memory cells and whether similar changes occur in T lymphocytes that have undergone homeostatic proliferation.

Collectively therefore, the data suggest that the specific foreign Ag is not required for formation of memory CD4+ T cells. Rather, we find that endogenous peptides and necessary costimulatory molecules during lymphopenia are sufficient to stimulate formation of memory cells. It remains to be determined whether their ability to survive and compete for niches is similar to that of memory cells generated after encounter with the specific exogenous Ag.

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