IL-7 Promotes the Transition of CD4 Effectors to Persistent Memory Cells

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Abstract

After transfer to adoptive hosts, in vitro–generated CD4 effectors can become long-lived memory cells, but the factors regulating this transition are unknown. We find that low doses of interleukin (IL) 7 enhance survival of effectors in vitro without driving their division. When in vitro–generated effectors are transferred to normal intact adoptive hosts, they survive and rapidly become small resting cells with a memory phenotype. CD4 effectors generated from wild-type versus IL-7 receptor$^{-/-}$ mice were transferred to adoptive hosts, including intact mice and those deficient in IL-7. In each case, the response to IL-7 was critical for good recovery of donor cells after 5–7 d. Recovery was also IL-7–dependent in Class II hosts where division was minimal. Blocking antibodies to IL-7 dramatically decreased short-term recovery of transferred effectors in vivo without affecting their division. These data indicate that IL-7 plays a critical role in promoting memory CD4 T cell generation by providing survival signals, which allow effectors to successfully become resting memory cells.

Key words: cytokines • CD4 T cells • immunity • effector cells • survival

Introduction

It is increasingly clear that the generation of CD4 memory cells from effectors is independent of antigen recognition, does not require recognition of self Class II, and is even independent of further division (1–3). In general, activated effector cells are highly susceptible to apoptosis, including both active activation-induced cell death (AICD) and passive mechanisms induced by withdrawal of growth/survival factor or nutrients referred to as programmed cell death (PCD) (4–6).

In vitro analyses have suggested a major role for Fas–FasL interactions in inducing AICD, but not PCD (7, 8), and of IL-2 and TGF$_\beta$ in blocking death (9, 10). In contrast, in vivo death of activated T cells and contraction of effector populations after a response is usually not mediated by Fas–FasL interactions, but has instead been found to rely on the presence of IFNγ (11–14) and to depend on mechanisms involving reactive oxygen and nitrogen (15). The decline of effectors after the expansion and effector generation phase of an immune response could either be initiated by AICD, as effectors reencounter antigen on infected cells, or by PCD, due to withdrawal of growth factors as the response subsides (4). PCD is often thought to occur because of a change in equilibrium between antiapoptotic factors, such as Bcl-2 and Bcl-xL, and in favor of proapoptotic

death family proteins such as Bim and Bax (4–6). Perhaps because it is difficult to study effector death in vivo, the role of these relative mechanisms and their possible regulation by other factors is still unknown.

No factors have been identified that overcome the susceptibility of effectors to apoptosis in vivo and could thus promote the transition from CD4 effector to memory cells (2). Previously, we have focused on the transition from activated effectors to those in a resting state (rested effectors) in vitro and on their subsequent in vivo conversion to persistent memory cells. Once in a resting state, CD4 T cells become long-lived memory cells after adoptive transfer with very high efficiency (2, 16), escaping the contraction phase that normally occurs. The memory cells are maintained indefinitely. The number of memory CD4 T cells generated is directly proportional to resting effector input. Thus, the efficiency of the escape of effectors from apoptosis and their successful transition to resting cells will determine how many memory cells are generated.

Cytokines that use the common γ chain (γc) of the IL-2 receptor are as follows: IL-2, IL-4, IL-7, IL-9, IL-15, and

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IL-21. These cytokines can maintain the survival of resting naive T cells (17–21). On the other hand, activated CD4 and CD8 T cells, such as effector cells, and resting CD8 T cells can directly proliferate in response to γc cytokines (22–28). IL-15 is strongly induced by activation of macrophages and dendritic cells in response to infection (28) and, therefore, we would predict that it serves primarily a non-homeostatic function. In contrast, IL-7 is produced constitutively by stromal cells (29) and would be more appropriate as a candidate for a survival factor that might promote memory generation (30). It has been suggested that IL-7 acts to induce Bcl-2 expression (21, 31–33) and counteract Bax-mediated death (34), which support the concept that it acts as a survival factor.

However, there is considerable controversy as to whether IL-7 has a physiologic role in supporting the homeostatic turnover of naive CD8 T cells (17, 24, 26) or memory CD4 T cells (24). Papers examining CD8 T cells from IL-7R$^{-/-}$ mice (18) or from cytokine-deficient hosts (24) support a role for both IL-7 and IL-15 in the homeostasis-driven division (HDD) and in the maintenance of memory CD8 T cells. However, these same papers argued against a role in CD4 memory cell HDD, but did not address survival or the efficiency of memory formation (24). However, one analysis in γc receptor–deficient mice (35) and one using blocking antibody (26) failed to find a role for IL-7. Thus, it is conceivable that the positive effects of IL-7 are dependent on lymphopenia and primarily due to enhancement of homeostatic division. Indeed, a recent work found that a combination of IL-7 and homeostatic TCR signaling combined to regulate memory CD4 T cell homeostasis (36).

To resolve whether IL-7 effects were limited to supporting HDD, we have examined the role of IL-7 during the effector to memory transition under circumstances in which Class II–dependent HDD would be impossible or negligible. We asked whether IL-7 might provide critical survival signals to activated effectors, which could allow them to escape death and, thus, promote their transition to resting memory cells. We found that the addition of IL-7 at low doses in vitro dramatically promoted effector survival without division and allowed effector cells to become resting, consistent with such a potential role. Using both IL-7– and IL-7R–deficient mice and antibodies to IL-7, we found that IL-7 also plays a critical role in vivo by supporting the survival of effectors and their transition to resting cells, without necessarily impacting their division. These effects were seen both in intact mice, where HDD is limited, and in Class II KO, where TCR signaling and HDD are negligible, as well as after transfer of rested effectors, which divide very little.

Materials and Methods

Mice. We used naive CD4 T cells from AND mice transgenic (Tg) for a TCR specific for pigeon cytochrome *c* fragment (PCCF) 83-104 presented by I-E^k as described previously (2). This population is naive by both phenotypic and functional cri-

teria (1, 2). The AND mice have been backcrossed for over 10 generations to C57BL/6 (B6). For most experiments, the AND mice were crossed to B6.PL Thy1.1 mice resulting in AND.Thy1.1 mice. For some experiments, AND mice were crossed to B6.eGFP mice, produced by our colleague T. Randall (Trudeau Institute, Saranac Lake, NY; reference 37). All cells from these mice express high levels of green fluorescent protein (GFP). AND mice were also backcrossed to IL-7R$^{-/-}$ mice on the B6 background (B6.129S7–1L-7tmi; obtained from Jackson Laboratory), resulting in mice expressing the AND TCR and deficient in the IL-7R (AND.IL-7R$^{-/-}$). IL-7$^{-/-}$ mice on a B6 background (29) were obtained from L. Puddington (University of Connecticut School of Medicine, Farmington, CT), with permission from DNAX, and were used as recipients. All mice were bred in and obtained from Trudeau Institute's Animal Breeding Facility. Experimental procedures involving mice were approved by the Trudeau Institute Institutional Care and Use Committee.

Adult-thymectomized, bone marrow–reconstituted (ATXBM) mice were prepared by surgical thymectomy of B6 mice at 3–4 wk of age. Thymectomized mice were irradiated with 950 rad 1–3 wk after surgery and reconstituted with T cell–depleted syngeneic bone marrow. Donor cells were injected at 1–3 wk after bone marrow reconstitution.

Cytokines and mAbs. Recombinant murine cytokines IL-2 and IL-4 were obtained from culture supernatant of X63.Ag8-653 cells transfected with cDNA for the respective cytokines (38). Recombinant murine IL-12 was a gift from S. Wolf (Genetics Institute, Cambridge, MA). Recombinant murine IL-7 was purchased from PeproTech.

The hybridoma specific for IL-7, M25 (39) was sent to us by Immunex. Antibody was generated as supernatants and purified by protein G chromatography. Mouse IgG2b (Sigma-Aldrich) was used as isotype control for in vivo blocking.

Preparation of Th1 and Th2 Effector Cells In Vitro. CD4 effectors were generated by culturing purified naive CD4 cells obtained from 6–8-wk-old AND TCR Tg$^+$ mice as described previously (2, 8). In brief, 3×10^5 cells/ml naive CD4 cells were cultured with a fibroblast cell line expressing I-E^k and intracellular adhesion molecule–1 (DCEK-ICAM) as APCs (1.5×10^5 cells/ml) plus 5 μM KAERADLIAYLKQATAK (PCCF) peptides (New England Peptide Inc.). Th1 effectors were generated with 11 ng/ml IL-2, 2 ng/ml IL-12, and 10 μg/ml anti–IL-4 (clone 11B11). Th2 effectors were generated with IL-2, 15 ng/ml IL-4, and 10 μg/ml anti-IFNγ (clone XMG1.2). At the end of culture, live T cell recoveries were determined by direct cell counts of trypan blue–excluding cells. Effectors were periodically assessed for polarization by determination of their cytokine production.

Cytokine Detection. Culture supernatants were collected after 24 h from 5×10^5 cells/ml of effectors restimulated with 2.5×10^5 cells/ml Ag/APC or with 10 μg/ml of platebound anti-Vβ3. Supernatants were assayed for the presence of IL-2 in a bioassay with NK-3 cells and for IL-4, IL-5, and IFNγ by ELISA as described previously (1).

In Vitro Response of Effector Cells to IL-7. In vitro–generated Th1 and Th2 effector cells were harvested at day 4 and recultured at a concentration of 1–1.5 × 10^5/ml with 0–10 ng/ml murine rIL-7.

Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) Labeling, Cell Cycle Analysis, and Cell Recovery. To determine division of effectors in vivo, Th1 or Th2 effector cells were incubated at 3×10^7/ml with 2 μM CFSE in a 37°C water bath for 10 min and

washed thoroughly with media before adoptive transfer. Live cell recovery was determined at various times using trypan blue exclusion. Cell cycle analysis of T cell populations was analyzed after 3 d. For this analysis, 3×10^6/ml cells were washed twice in PBS supplemented with 0.1% glucose and fixed by ice-cold 70% ethanol at 4°C overnight. Cells were incubated in propidium iodide (PI) solution (50 μg/ml PI and 100 U/ml RNase A in PBS with 0.1% glucose) for 30 min at room temperature before FACS® analysis. Only single cells were analyzed.

FACS® Analysis. Cells were resuspended in PBS, 1% BSA, and 0.1% NaN$_3$ at a concentration of 1–5 $\times$ 10^6/ml. 100 μl of this solution was incubated at 4°C for 30 min with monoclonal antibodies specific for the following antigens and coupled to the indicated fluorochromes: PE-CD44, PE-CD45, PE-CD25, PE-FAS, PE-CD62L, PE or FITC or PerCP-Thy1.1, and Cy-Chrome or allophycocyanin-CD4 (all from BD Biosciences). Relative fluorescence intensities were measured using the FACScan™ or FACScalibur™ and analyzed with CELLQuest™ Software or Flowjo (BD Biosciences).

For Bcl-2 staining, cells were surface stained, fixed with 4% paraformaldehyde for 20 min, and permeabilized in PBS containing 1% BSA and 0.1% saponin for 30 min, followed by staining with PE-conjugated hamster anti–mouse Bcl-2 monoclonal antibody (BD Biosciences) for 30 min at room temperature. Cells were washed once in permeabilization solution and once in PBS containing 1% BSA before analyses on the FACS® machine.

Adoptive Transfer of Effectors. Th2 effectors were generated in vitro (as aforementioned). They were harvested and washed, and 3–5 $\times$ 10^6 cells were injected i.v. into adoptive hosts as in described previously (2). For assessing the impact of IL-7R on survival of the transferred population, a mixture containing an equal number (5 $\times$ 10^6 total cells) of in vitro–generated AND. IL-7R$^{-/-}$ Th2 effector cells and AND.GFP effector cells was injected i.v. into Thy1.1$^+$ ATXBM hosts. The ratio of each set of the donor cells was analyzed by staining with monoclonal antibodies to Thy1.2-PE, CD4-CYC at various time points afterwards.

Results

We evaluated the role of IL-7 on the survival of in vitro–generated effectors in culture, and used a variety of approaches to search for similar roles in vivo.

IL-7 Promotes the Survival and Recovery of Effectors In Vitro. To determine the impact of IL-7 on effectors, we added from 0.1 to 10 ng/ml of IL-7 to in vitro–generated Th1 and Th2 effectors. Fig. 1 a shows that in the absence of added IL-7, Th1 and Th2 effector recovery decreases such that both effector populations have nearly disappeared by day 6 after reculture. The difference in the susceptibility of Th1 and Th2 effectors to PCD has been reported previously (8). As increased amounts of IL-7 were added to effectors, their recovery increased proportionately. At a concentration of 0.5 ng/ml, increased survival was readily apparent, and at higher doses, effectors expanded and persisted at higher levels. By adding IL-7 weekly, we were able to keep the major fraction of the population of effectors alive and functional for >4 wk (unpublished data). Because Th1 effectors initially declined at a greater rate in vitro, we concentrated subsequent in vivo studies on the impact of IL-7 mostly on Th2 effectors, where there are

the fewest dying cells in the transferred population. We were concerned that a substantial fraction of in vitro-generated Th1 effectors might be in the early stages of apoptosis (8), and that this might undermine our ability to discern the effect of in vivo survival factors such as IL-7. However many of the experiments have been done with Th1 effectors, and a similar pattern of results was observed (unpublished data).

Analysis of cell cycle status at day 3 of culture with IL-7 (Fig. 1 b), indicated that a low dose of IL-7 (0.5 ng/ml) induced only a small increase in progression through the cell cycle, but a dramatic decrease in the proportion of dead cells (Fig. 1 b). At 10-fold higher IL-7 doses (5.0 ng/ml), we found many cycling cells in G$_2$, S, and M phases of the cell cycle. Similar effects were seen at days 2 and 6 (unpublished data), and when Th1 effectors were cultured with IL-7 (unpublished data). Anti–IL-7 blocked the effects of addition of IL-7 (unpublished data). These data
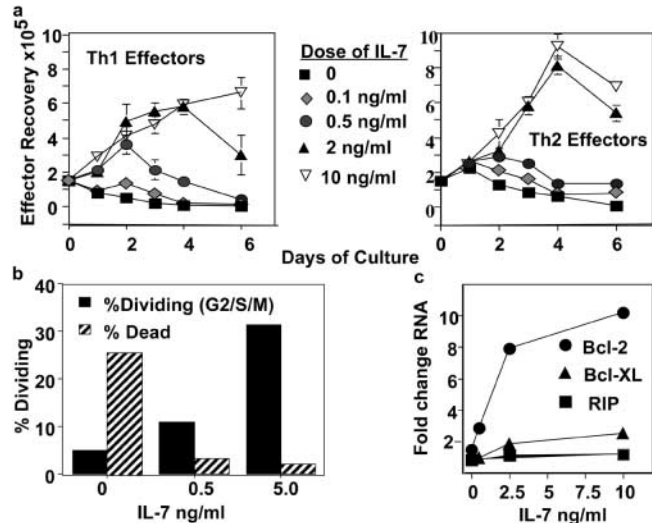

Figure 1. IL-7 promotes the survival and long term recovery of Th1 and Th2 effectors in vitro. (a) Effect of IL-7 on effector recovery in vitro. Th1 and Th2 effectors were generated from purified naive CD4 T cells from AND TCR Tg mice as in previous papers (references 2, 8). 10^5/ml Th1 (left) and Th2 (right) effector cells were stimulated with 0, 0.1, 0.5, 2, and 10 ng/ml of IL-7 for 1, 2, 3, 4, and 6 d. Live cell recoveries were determined by counting live, trypan blue–excluding cells at the times indicated. Results are the mean count from triplicate cultures with standard deviations. This experiment is representative of three separate experiments. (b) The effect of IL-7 on effector division and viability in vitro. Cell cycle analysis. Th1 and Th2 effector cells were generated as described in a. After 4 d, the effector cell populations were collected and stimulated with or without high (5 ng/ml) or low doses (0.5 ng/ml) of IL-7 for 3 d. Recovered cells were stained with PI after ethanol fixation. We determined the fractions of cells in G0 or G1 (not depicted), in S, G2, or M (% dividing), and cells that were dead (% dead). Only single cells were analyzed. Results are representative of five separate experiments. (c) IL-7 up-regulates Bcl-2 gene expression. Th2 effector cells were stimulated without or with high (5 ng/ml) or low (0.5 ng/ml) doses of IL-7 for 16 h. RNA was isolated and expression of mRNA for a panel of death genes was analyzed by RNase protection assay using the mouse apoptosis-related template probe set (BD Biosciences). Results were similar in Th1 effectors and in a repeat experiment. The fold change relative to the L32 housekeeping gene for Bcl-2, Bcl-xL, and RIP at different doses of IL-7 is shown.

suggest that at low doses, in the range of 0.5 ng/ml, IL-7 can act on effectors to prevent death without driving substantial division.

We also investigated whether exposure of effectors to IL-7 would result in an up-regulation of expression of anti-apoptotic molecules. We cultured Th1 and Th2 effectors with various concentrations of IL-7 for 16 h, isolated RNA and determined levels of expression of a panel of death-related genes by RNase protection assay (Fig. 1 c). Bcl-2 RNA was dramatically increased in Th2 effectors, and in Th1 effectors (unpublished data). The biggest increase in expression occurred from the 0 to the 2.5 ng/ml dose, which gave an eightfold increase in intensity. Bcl-xL was increased only modestly (Fig. 2 b). Messages for proapoptotic proteins, RIP (Fig. 1 c), Bcl-w, Bfl-1, and FAP were not increased or increased only slightly by IL-7 (not depicted). Bcl-2 protein was also increased as determined by staining with specific antibodies at days 2–4, 6, and 8 for both Th1 and Th2 effectors (unpublished data).

Effectors Rapidly Transition to Resting Cells and Up-regulate IL-7 In Vivo. Our hypothesis of possible IL-7 action would suggest that effectors transferred to adoptive hosts would encounter IL-7 at low levels, which would support
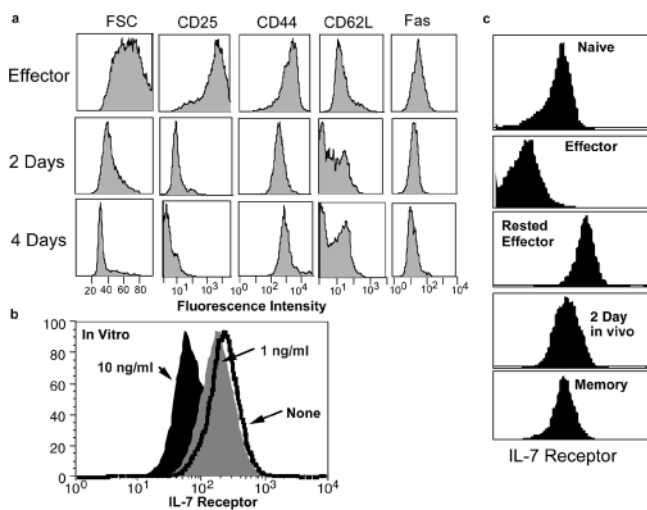

Figure 2. Effectors become rested effector/memory cells after transfer. (a) Phenotypic shifts after transfer to adoptive hosts. Th2 effectors generated from AND.Thy1.1 donors were analyzed for FSC and expression of CD25, CD44, CD62L, and Fas by staining and FACS® analysis (effector row). Effectors were transferred to intact B6 mice. After 2 and 4 d, hosts were analyzed. Cells from the spleen and lymph node were recovered and stained, and the coexpression of CD4+ Thy1.1+ (donor cells) and the markers was determined. (b) Effect of IL-7 on IL-7Rα expression (in vitro). In vitro–generated Th2 effectors were incubated with no cytokine (none), and low (1 ng/ml) or high (10 ng/ml) doses of IL-7 for 2 d. The expression of IL-7R was determined by staining and FACS® analysis. (c) Expression of IL-7Rα on CD4 T cell subsets. The expression of IL-7R was determined on fresh naive CD4 T cells (naive) and on in vitro–generated Th2 effectors (effector), and those same cells rested for 2 d in vitro (rested effector). Th2 effectors were also transferred to intact B6 mice and evaluated after 2 d (2 d in vivo). For memory cells, Th2 effectors were transferred to Class II KO hosts, and the resulting memory cells evaluated after 8 wk (memory). IL-7R expression was determined on each of these populations.

survival but allow them to become resting within a few days. To test this possibility, we followed the size and cell surface phenotype of donor effectors after transfer to normal intact hosts (Fig. 2 a). The large effectors (Fig. 2 a, effector and FSC) became much smaller by day 2 and were very small by day 4. CD25 (IL-2Rα, an activation marker) was down-regulated by day 2, as was Fas expression, consistent with a transition to a resting, more death-resistant phenotype. The marker CD44, associated with a history of response to antigen and with recruitment of effectors, remained relatively high, and CD62L remained low, both consistent with the expected memory CD4 T cell phenotypes. These results support the concept that effectors that survive rapidly become resting cells in situ and have already become "memory-like" within 4 d. Because we predict this is the critical transition, further studies concentrate on the effects of IL-7 over the first 4–7 d after transfer.

Cytokine exposure often leads to changes in cytokine receptor expression, which in turn dictate responsiveness of the target cells to the cytokine. We had noted previously an up-regulation of IL-7Rα mRNA when we "rested" effectors (unpublished data). We first evaluated the effects of IL-7 exposure in vitro on the expression of IL-7Rα (Fig. 2 b). In vitro–generated Th2 effectors, which initially express little IL-7R (Fig. 2 c), were cultured for 2 d with no cytokine or either a high or low dose of IL-7. Effectors cultured without cytokine or with a low dose of IL-7 expressed moderately high IL-7R levels after 2 d (Fig. 2, b and c). In contrast, a high dose of IL-7 (10 ng/ml) led to little induction of IL-7 receptor.

We further analyzed whether the populations of cells that might require IL-7 for survival would have high IL-7Rα and especially if the transition to a resting population in vivo would result in the up-regulation of IL-7R after the effector transfer. Naive cells, in vitro–generated effector cells (effectors), in vitro–rested effectors, 2-d in vivo–rested effectors, and recovered long-lived memory cells were compared for IL-7Rα expression by staining with anti–IL-7Rα. Effectors expressed little IL-7R, but all the other populations expressed IL-7Rα at moderately high levels, supporting the concept that cells in a resting state all have the capacity to respond to IL-7 and that ambient levels in vivo are not sufficiently high as to down-regulate IL-7R.

Effectors Deficient in IL-7R Expression Survive Poorly after Transfer to Adoptive Hosts. To analyze the requirement for IL-7, we first compared effectors that expressed IL-7R to those that did not. Conventional IL-7R−/− mice have very few peripheral lymphocytes (31–33, 39), however, when IL-7R−/− mice are crossed with the AND TCR Tg mice, a dominant population of CD4+Tg+ cells is found in the periphery. Approximately 40–50% of peripheral blood cells are CD4+Tg+ (unpublished data), and we recover ~35–40 × 10⁶ CD4+, Vβ3+ cells per mouse. Similar rescue of naive CD4 T cells has also been seen in another TCR Tg model (18). We found that the naive AND.IL-7R−/− CD4 T cells had a comparable naive phenotype as similar nondefective naive cells. They also re-
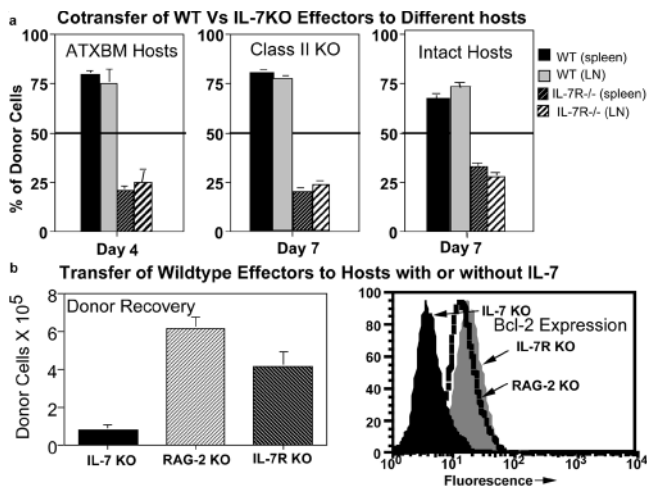
Figure 3. Effector cells that lack IL-7 response or are transferred to IL-7–deficient hosts survive poorly. (a) Transfer of effectors deficient in IL-7R expression. In vitro–generated AND IL-7R$^{-/-}$ Th2 effector cells (Thy1.2) and WT AND Th2 effector cells (GFP$^+$, Thy1.2) were mixed in equal proportions and injected into ATXBM hosts (Thy1.1) (left). middle, Class II KO hosts. right, intact hosts. After 4–7 d, spleen and lymph node cells were recovered and the percentage of the donor cells that were WT (GFP$^+$, Thy1.2) versus IL-7R$^{-/-}$ (GFP$^-$, Thy1.2) is shown. Data are the means of three mice per group, with standard deviation. Similar effects were seen in two replicate experiments. (b) Effectors divide less and survive less well in IL-7–deficient hosts (left). WT in vitro–generated Th2 effectors were CFSE-labeled and transferred to groups of hosts, including IL-7$^{-/-}$, IL-7R$^{-/-}$, and Rag-2 KO mice. The number of donor cells recovered were determined by gating on CD4-CYC and Thy1.1-PE. Donor cell recovery at day 5 from the experiment described in panel a is shown (left). Similar reduction in the recovery of donor cells in IL-7$^{-/-}$ compared with IL-7R$^{-/-}$ was seen in the other experiments and at days 2 and 7 and at 6 mo. Results are representative of three separate experiments. Bcl-2 expression in different hosts (right). In the same experimental design, Th2 effectors (Thy1.1) were transferred into IL-7$^{-/-}$, IL-7R$^{-/-}$, and Rag-2$^{-/-}$ hosts. Cells were recovered from the pooled spleen and lymph node at day 4 after transfer. Donor cell Bcl-2 expression was determined by intracellular staining with Ab to Bcl-2. Isotype control Ab staining (not depicted) was below 10 mean index of fluorescence in cells recovered from each host.

sponded in a comparable fashion by expanding 7–12-fold under Th2-polarizing conditions to PCCFs (peptide antigen) and APCs (unpublished data).

The recovered effector population at day 4 was comparable in phenotype (CD44^{hi}, CD62L^{lo}, and CD45RB^{hi}) to those from WT mice, and they made equivalent levels of Th2 cytokines (unpublished data). Thus, we could generate effectors that were genetically unable to respond to IL-7 and directly compare them to those that could respond.

Th2 effectors generated from WT and IL-7R$^{-/-}$ AND TCR Tg mice were transferred to adoptive hosts, and their survival was compared (Fig. 3 a). To better understand the mechanisms of any IL-7 effects we might see, we compared intact hosts, ATXBM hosts (which lack T cells and are, thus, somewhat lymphopenic and may undergo enhanced HDD), and Class II KO hosts in which the effectors cannot recognize major histocompatibility molecules (2) and, therefore, do not undergo TCR–Class II interaction–dependent HDD.

To directly compare the populations, we cotransferred WT and IL-7R–deficient Th2 effector populations to the same host. To visualize the donor populations in the adoptive host, we used AND.GFP mice, which we derived by crossing the AND Tg mice to B6.eGFP mice developed by T. Randall (38). The recovery in the host of the WT (GFP$^+$) donor cells was compared with that of the IL-7R–deficient donor cells (GFP$^-$). To ensure that all mice received the same ratio of WT to IL-7R$^{-/-}$ donor cells, the effector cells were premixed at a 1:1 ratio, and aliquots were injected into groups of intact, ATXBM, or Class II KO hosts. Recipient mice were killed between 4 and 7 d, and the recovery of both types of donor cells in spleen and lymph node was determined. The two donor Thy1.2$^+$/CD4$^+$ populations were easily distinguished and quantified in each case, and the percent WT versus total donor cells is shown. Although WT and IL-7R KO populations were initially equal (determined by staining on day 1 after transfer; unpublished data), WT cells increased to 70–80% of the donor cells in each group of recipients and in both sites, whereas the IL-7 KO cells decreased proportionally to 20–30% of the total. Similar results were seen in three replicate experiments. The superior survival of WT versus IL-7R KO donor cells was obvious as early as day 2 (unpublished data). Importantly, the relative loss of those effectors unable to respond to IL-7 was seen not only in ATXBM hosts, but at equivalent levels in intact hosts and Class II$^{-/-}$ hosts, despite the expectation that HDD would occur only in the ATXBM host, especially over this short time frame.

Effectors Survive Poorly in IL-7–deficient Hosts. To gain additional confirmation of the role of IL-7 without using donor cells that might have been somehow influenced by their lack of IL-7, we transferred WT Th2 effectors into IL-7$^{-/-}$ hosts (highly lymphopenic, no IL-7), comparably lymphopenic IL-7R$^{-/-}$ hosts that express IL-7, and IL-7–sufficient, highly lymphopenic RAG-2$^{-/-}$ hosts. We reasoned that the IL-7R$^{-/-}$ and RAG-2$^{-/-}$ hosts would be the most appropriate control for the IL-7$^{-/-}$ hosts because they share the same lymphocyte deficiencies.

We analyzed donor cell recovery at day 5 after effector transfer. The donor cell recovery at this early time point was approximately sixfold greater in IL-7R$^{-/-}$ mice than in IL-7$^{-/-}$ mice (Fig. 3 b). A similar pattern of recovery in these same set of different hosts was seen at days 2 and 7, at later times, and in several repeat experiments.

To further evaluate the hypothesis that a major effect of IL-7 is to enhance survival and prevent PCD, we examined the expression of Bcl-2 in vivo by staining cells recovered from the IL-7$^{-/-}$, IL-7R$^{-/-}$, and RAG-2$^{-/-}$ hosts (Fig. 3 b, right). Results from representative mice from each host out of 3–4 mice per group are shown. Levels of Bcl-2 were higher in cells recovered from IL-7–sufficient hosts than in cells recovered from IL-7–deficient ones, supporting an in vivo role of IL-7 in inducing Bcl-2 on effectors in vivo.

Antibodies to IL-7 Block Recovery of Transferred Effectors. Earlier blocking analyses assessing IL-7 effects primarily measured the division of the T cells rather than their sur-
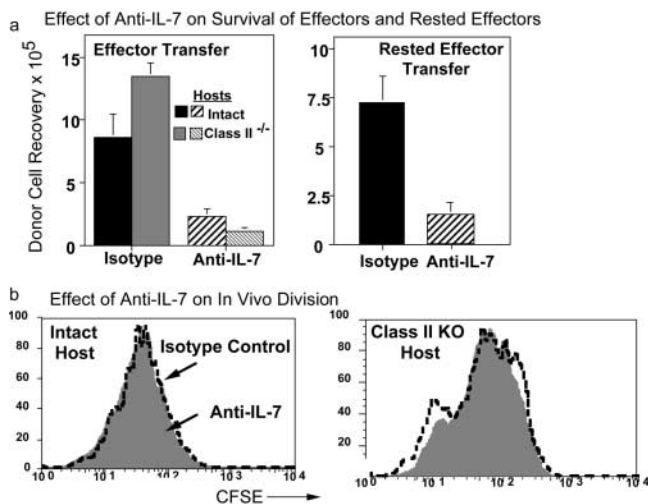
Figure 4. Antibodies to IL-7 block recovery of transferred effectors without affecting division. In vitro–generated Th2 effector cells (Thy1.1) were adoptively transferred into Thy1.2 hosts including: intact B6 and Class II$^{-/-}$ (Class II KO). (a) Blocking with anti–IL-7 in different hosts. Experimental groups were injected i.p. daily with anti–IL-7 (0.5 mg/mouse/d) from day −1, control groups were treated the same way, but with isotype-matched antibody. Results represent the mean donor cell recovery from individual animals (three to five mice per group), with standard deviations. Similar results were seen in a duplicate experiment and in other hosts, including RAG$^{-/-}$ and ATXBM hosts (not depicted). (b) CFSE-labeled Th2 effector cells (Thy1.1) were adoptively transferred into intact and Class II$^{-/-}$ (Class II KO) hosts. Experimental groups were injected i.p. with anti–IL-7 (0.5 mg/mouse/d) from day −1, control groups were treated the same way, but with isotype-matched antibody. After 3 d, CFSE staining of donor Thy1.1$^+$ cells was assessed. Similar results were seen in a repeat experiment. No difference in CFSE profile between anti–IL-7–treated and isotype-treated hosts was seen after 5 d (not depicted).

vival, and they failed to identify a role for IL-7 in the homeostatic division of naive CD8 T cells (26) or memory CD4 cells (24). This may suggest that the role of IL-7 we see is mostly due to improved survival rather than division.

To further establish the role for IL-7 in survival of effectors so they can become memory and address whether it can occur in the absence of effector division, we used an additional model in which we used anti–IL-7 antibodies to block IL-7 response in vivo (39). This model has the advantage that it did not depend on genetic depletion of IL-7 or its receptor. We transferred effectors or rested effectors, which divide very little after transfer (2), into intact hosts and to Class II$^{-/-}$ hosts (Fig. 4 a). Blocking antibodies to IL-7 or isotype-matched control antibodies were injected daily, starting 1 d before transfer.

Anti–IL-7 dramatically decreased the recovery of transferred effectors in both intact hosts and in Class II$^{-/-}$ hosts (Fig. 4 a, left), suggesting that IL-7 regulates survival independent of Class II recognition by the effector. To further confirm the independence of the survival effects on IL-7 from those that might induce division, we transferred in vitro–rested effectors to intact mice (Fig. 4 a, right). We have established previously that such effectors divide little after transfer to adoptive hosts (2). Anti–IL-7 blocking was also very effective in reducing donor cell recovery in this instance.

To confirm the independence of the IL-7 effect on donor cell division, we examined in situ division in the blocking experiments (Fig. 4 b). Effectors were labeled with CFSE before transfer, and the levels of CFSE were determined in the donor cells recovered from adoptive hosts at days 3 and 5 (unpublished data). The degree of division in the blocked and unblocked populations in intact (Fig. 4 b, left) and Class II$^{-/-}$ hosts (Fig. 4 b, right) was equivalent, indicating that the difference in recovery due to addition of anti–IL-7 was not due to different rates of division.

Discussion

The factors regulating the survival of effectors in vivo and their transition to resting memory cells, as well as long-term survival of those memory cells, are largely unknown. The results presented here indicate that one critical component, which promotes effector CD4 survival through their transition to memory cells, is IL-7. A role for IL-7 in enhancing recovery of effectors was seen in vitro, where IL-7 at low doses induced persistence of otherwise short-lived effectors, and this was accompanied by inhibition of death and up-regulation of Bcl-2. A similar role was supported in vivo for the following reasons: (a) recovery of IL-7R–deficient effectors was much reduced compared with their WT counterparts in adoptive hosts, including both intact and Class II–deficient recipients; (b) WT effectors survived in smaller numbers in IL-7$^{-/-}$ hosts versus comparably lymphopenic IL-7R$^{-/-}$ or RAG-2$^{-/-}$ hosts, and they expressed lower levels of Bcl-2 in the IL-7–deficient hosts; and (c) WT effector recovery was strikingly reduced in hosts treated with anti–IL-7 antibodies. In the blocking experiments, dramatic blocking was also noted in Class II$^{-/-}$ hosts, whereas there was no difference in situ division, strongly suggesting that the role of IL-7 is not dependent on MHC Class-II driven homeostatic division. Moreover, recovery of donor cells after transfer of rested effector cells, which barely divide, was also blocked by anti–IL-7 treatment. Together, these results provide strong evidence that IL-7 normally acts in situ to enhance the transition of activated effector to resting memory cells by enhancing survival during this transition. Moreover, the results suggest that this mechanism is independent of TCR-generated homeostatic signals.

IL-7 has been found previously to play an important role in the generation of naive T and B cells (31–33) and is implicated in the survival of resting naive CD4 and CD8 T cells (17–21). Other γc binding factors can replace IL-7 as survival factors for naive T cells in vitro (17), but they do not replace its role in lymphocyte generation (20, 40). The studies here suggest that IL-7 may be largely responsible for the survival and transition of effectors to memory cells in vivo because the recovery of transferred cells when IL-7 action was absent was only 10–30% of the levels achieved under conditions of normal ambient IL-7. On the other hand, interaction with TCR played a minor role, if any, over this time frame because levels recovered in Class II–

deficient mice were in fact slightly greater than in intact mice (Fig. 4 a). In contrast, homeostatic division of memory T cells seems to be more dependent on TCR–mediated signals (36, 41).

There are some published data that suggest a role of IL-7 in CD8 T cell memory maintenance. Memory cells of the CD8 lineage are increased in IL-7 Tg mice (30) and CD8 memory generation from IL-7R$^{-/-}$ naive CD8 T cells is impaired (18). A recent paper indicated joint roles for homeostatic TCR triggering and IL-7 in CD4 memory T cell persistence (36). Recent studies from Bradley's laboratory also indicate that CD4 memory maintenance is enhanced by IL-7 (42). None of the previous works supporting a role for IL-7 pinpoint when in the process of generation and maintenance IL-7 acts, or whether it acts as a survival factor or a factor supporting homeostatic division. Therefore, the role of IL-7 in the transition of CD4 effector to memory cells and in survival independent of HDD has not been directly addressed. This may help to explain the studies in γc receptor–deficient mice that argue against a role for cytokines binding this receptor (35) in memory T cell persistence. In that case, HDD, rather than survival, may be the factor most influencing the memory cell recovery.

Here, we included studies in intact mice where lymphopenia is absent and in Class II$^{-/-}$ mice where TCR–self MHC interaction does not occur, and found that IL-7 clearly has a predominant role in supporting efficient effector to memory transition. Importantly, we show that IL-7 can exert this effect independent of division, both by using hosts where division is very limited (intact and Class II deficient; Figs. 3 a and 4) and by transferring rested effectors that do not divide appreciably (Fig. 4 a; reference 2). We also directly demonstrated that there was no difference in in situ division when IL-7 blocking has dramatically reduced recovery (Fig. 4 b). The induction of Bcl-2, as well as the preceding considerations, points strongly to a critical physiologic role of IL-7 mediated by enhancement of survival of effector cells.

Our experiments focus primarily on the generation of CD4 memory that occurs with the transition of CD4 effectors to long-lived resting cells and their short-term survival. In the effector-to-memory transition, the cell survival effects of IL-7 are likely to be of particular relevance because a large fraction of effectors disappear as a result of apoptosis, most likely due to PCD. The early loss of effectors that do not express IL-7R between days 1 and 4 after transfer corresponds to the time in vitro in which populations of effectors cease turning over and contract dramatically (41). The fact that the impact of IL-7 is noted within a few days after transfer implies that IL-7 plays a particularly critical role during this transition from death-sensitive activated effectors, to resting memory-like cells that are more resistant to death.

Our experiments do not rule out the possibility that there may be additional reasons for the conflicting results noted in the preceding paragraphs. Whether IL-7 acts only

as a survival factor or acts in addition as a growth factor is likely to depend on the activation status of the cell and the related expression of IL-7 receptors, the presence of TCR signaling (36, 41), and, most importantly, on the available levels of IL-7. Our in vitro studies suggest that low doses of IL-7 are able to mediate effector survival without inducing division and without causing the down-regulation of IL-7R. We show that higher doses of IL-7 induce proliferation (Fig. 1) and cause down-regulation of IL-7R (Fig. 2 b). Conditions in vivo in intact mice apparently mimic the effects of the low doses because transferred effectors do not proliferate in the short term response to ambient IL-7 (Fig. 4), and because the levels of IL-7R they expressed were moderately high and comparable to those expressed by naive and memory cells directly ex vivo and by effectors rested in vitro in media for 2 d (Fig. 2 b). Thus, we suggest that in intact mice, ambient levels of IL-7 are usually too low to support division. In contrast, we predict that IL-7 could support some level of division when it is overexpressed. We predict that in lymphopenic models, where other lymphocytes do not compete for and consume IL-7, the ambient concentration of IL-7, which is produced constitutively by stromal cells, could be increased and might be sufficient to drive division. This division would not be physiological in normal circumstances where lymphopenia was not present. Neither the relative or absolute level of ambient IL-7 has been determined in the different situations.

In summary, we suggest that our experiments strongly support the conclusion that IL-7 plays a critical role in the survival of activated CD4 effectors so they can become resting, long-lived memory cells, and that it is likely that IL-7 promotes the generation of resting memory cells that can persist for many months or years.

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