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Autologous Regulation of Naive T Cell Homeostasis Within the T Cell Compartment

Wolfgang Dummer, Bettina Ernst, Eric LeRoy, Dong-Sup Lee, and Charles D. Surh

Naive T cells undergo spontaneous slow proliferation on adoptive transfer into syngeneic T cell (T)-deficient hosts. Recent work has shown that such “homeostatic” T cell proliferation is driven by MHC molecules loaded with self-peptides rather than foreign peptides. Because naive T cells in normal T-sufficient hosts remain in interphase despite continuous contact with self-MHC/peptide ligands, T cells apparently inhibit homeostatic proliferation of neighboring T cells. To address this, we have investigated the requirements necessary for “bystander” T cells to inhibit homeostatic proliferation of other T cells. Three key findings are reported. First, homeostatic proliferation of T cells only occurs in specific microenvironments, namely the T cell compartment of the secondary lymphoid tissues. Second, direct entry into T cell compartments is also required for bystander inhibition of homeostatic proliferation. Third, bystander inhibition is mediated largely by naive rather than activated/memory T cells and does not require proliferation or TCR ligation. These findings suggest that homeostasis of naive T cells is unlikely to be regulated through competition for systemic soluble factors or for specific stimulatory self-MHC/peptide ligands. Rather, the data favor mechanisms that involve competition for local non-MHC stimulatory factors or direct cell-to-cell interactions between the T cells themselves within the T cell compartment. The Journal of Immunology, 2001, 166: 2460–2468.
invariant chain peptide (CLIP) (31–33). Thus, naïve CD4+ cells from wild-type C57BL/6 (B6) mice, which have been positively selected on MHC class II molecules loaded with diverse self-peptides, proliferated very poorly in H2-M− hosts, whereas naïve CD4+ cells from H2-M+ mice, positively selected to CLIP, proliferated efficiently in H2-M+ hosts (25, 27). Second, in TAPI mouse engineered to express Kb molecule loaded with specific peptides, homeostatic proliferation of naïve CD8+ OT-I TCR-transgenic T cells required that the T-depleted hosts express a low-affinity peptide that previously had been shown to induce positive selection of OT-I T cells in fetal thymic organ cultures (28).

Based on the above findings, we proposed that the weak TCR signals received by naïve T cells making contact with self-MHC/peptide ligands are translated differently depending on the total size of the T cell pool (27). Under normal T-sufficient conditions, the signals promote prolonged survival of T cells in a resting state, but under lymphopenic conditions the TCR signals induce naïve T cells to proliferate. However, why lymphopenia seems to augment TCR signals in residual T cells and causes these cells to enter cell cycle is still unclear. There are at least two broad possibilities. First, T cells may undergo homeostatic proliferation in response to increased availability of stimulatory factors. These factors could be either cell surface molecules, such as specific MHC/peptide ligands and costimulatory molecules on APC, or soluble factors such as cytokines that either work locally or systemically. Second, T cells could undergo proliferation because they have been liberated from constant inhibitory signals that dampen the overall accumulated signals received from continuous interaction with self-MHC/peptide ligands. Such suppressive cues could come either from direct interaction with other T cells or through a complex interaction involving a third-party cell, such as APC or stromal cells.

To examine this question, we have investigated whether there exists a spatial requirement for T cells to undergo homeostatic proliferation, and whether homeostatic proliferation can be controlled by the presence of large numbers of “bystander” T cells with different characteristics. Data from these experiments support a model in which homeostatic proliferation is regulated through short-range T-T interactions within specific regions of the lymphoid tissues, namely the T cell zones in spleen and lymph nodes (LN).

Materials and Methods

Mice

All mice were used at 6–12 wk of age. B6 (H2b), B6.PL (H2b, Thy-1.1+), and B6.Ly5.1+ mice were obtained from the breeding colony at The Scripps Research Institute (La Jolla, CA). H2-M2 and H2-M2.Thy1.1 in the B6 background (backcrossed six times to B6) were bred and maintained in our animal colony. B6.FasL− (glu) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.H-2K− (backcrossed 20 times to B6) and CTLA-4− (backcrossed six times to B6) mice were obtained from Dr. Terry Lauffer (University of Pennsylvania, Philadelphia, PA) and Dr. Craig Thompson (University of Pennsylvania), respectively. OT-II and AND TCR-transgenic mice were obtained from Dr. William Heath (Walter and Eliza Hall Institute, Melbourne, Australia) and Dr. J. Kaye (The Scripps Research Institute), respectively. CTLA-4− plus B6-mixed bone marrow (BM) chimeras were generated as recently described (34). Briefly, lethally irradiated (1000 cGy) B6.Ly5.1+ mice were injected with a mixture of CTLA-4− (Thy-1.2, Ly5.2+) and B6.PL (Thy-1.1, Ly5.2+) T-depleted BM cells. After 8–12 wk, CTLA-4− T cells and B6.PL T cells were purified from the host LN cells and used for inhibition experiments.

Abs and cell purification

The mAbs to H2-A2 (28-16-8s), heat-stable Ag (J11d; Thy-1.2; J1J), CD4 (GK1.5 and RL172), and CD8 (3.168) have been described previously (35). CD4+ cells were purified by killing with a cocktail of anti-CD8, anti-H2-A2, and anti-heat-stable Ag mAbs plus complement and then panning on plates coated with anti-CD4 mAbs (35).

Adoptive transfer of T cells

Small numbers (1–2×105/mouse) of whole LN cells or purified T cells were labeled with the intracellular fluorescent dye CFSE (Molecular Probes, Oregon) as described (36) and i.v. injected into host mice. For inhibition experiments, the CFSE-labeled “donor” cells were coinfected i.v. with large numbers (3–8×107 cells/mouse) of unlabeled whole LN cells or purified T cells. Host mice received a sublethal dose (600 cGy) of whole body irradiation 1 day before donor cell injection.

LN cells were treated with pertussis toxin (PTX) (List Biological Laboratories, Campbell, CA) as described (37). Briefly, cells were incubated in RPMI 1640 containing 2% FCS at 4×107 cells/ml with 100 ng/ml PTX at 37°C for 2 h and then washed three times with RPMI 1640 medium before use.

In vitro proliferation

Whole B6 cells treated with medium only or medium plus PTX were incubated at 2×107 cells/well with titrating doses of anti-CD3 mAb (2C11; BD Pharmingen, San Diego, CA) for 2 days. Some cells were incubated with irradiated (2000 cGy) T-depleted B6, B6.bm12, B6.bm1 or B10.BR spleen cells (7×105 cells/well) for 3–5 days. Cultures were pulsed with 1 μCi [3H]thymidine/well 8 h before harvest.

Histological analysis

Frozen spleen and LN sections were prepared and stained as described previously (38). Briefly, freshly cut 6-μm sections were dried and fixed with acetone and stained with mAbs. For immunofluorescence, sections were stained with biotinylated RA3-6B2 (anti-CD8), PE-conjugated streptavidin (Jackson ImmunoResearch), and Cy5-conjugated streptavidin (Jackson ImmunoResearch). As described previously (27), homeostasis-driven proliferation of OT-I TCR-transgenic T cells required that the T-depleted hosts express a low-affinity peptide that previously had been shown to induce positive selection of OT-I T cells in fetal thymic organ cultures (28).

FACS analysis

Host LN and spleen cell suspensions were stained for the donor cells by standard procedures as described (35). Briefly, donor T cells were detected by staining with either biotinylated OX-7 mAb (anti-Thy-1.1; BD Pharmingen), J1 (anti-Thy-1.2), or A20-1.7 (anti-Ly5.1). Cells then were double stained with PE-conjugated anti-CD4 (Life Technologies) mAbs and Cy5-conjugated streptavidin (Jackson ImmunoResearch). Ly5.1 congenic donor cells were stained further with CyChrome-conjugated anti-CD8 mAb (BD Pharmingen) to identify donor CD8+ cells. CFSE levels on donor cells are shown as histograms for gated donor CD4+ and CD8+ T cells (Thy-1.1 CD4+ or Ly5.2 CD8+). Dead cells were excluded by staining with propidium iodide. Background staining by donor-specific mAbs was negligible (no >0.01%) in cells from control irradiated mice that did not receive any donor cells (not shown).

Results

Spatial requirement for T cells to undergo homeostatic proliferation

As described previously (27), homeostasis-driven proliferation of naïve T cells can be readily observed by adoptively transferring small numbers of T cells into sublethally irradiated (600 cGy) syngeneic mice. Thus, in irradiated B6 (Thy-1.2) mice injected with 106 CFSE-labeled congenic B6.PL (Thy-1.1+) purified T cells, the proliferation of donor T cells, as indicated by the appearance of cells with progressive 2-fold reductions in CFSE intensity, begins within 2–3 days after injection and becomes prominent by 6–8 days after injection (Fig. 1). A population of donor
were injected i.v. into a group of B6 (Thy-1.2<sup>+</sup>; one other experiment showed similar results. The data are representative of two mice analyzed individually at each time point; one other experiment showed similar results.

T cells with no CFSE content, indicative of having undergone multiple rounds of cell division, is often visible after 6 days. This population appears to arise from expansion of donor memory T cells, as we have found previously that a prominent CFSE-negative population appears to arise from expansion of donor memory T cells with no CFSE content, indicative of having undergone multiple rounds of cell division.

Histological analysis of the lymphoid tissues revealed that the donor cells (as detected either by CFSE fluorescence or by Thy-1.1 expression) localized selectively within the T cell areas of the spleen and LN, i.e., the periaortiolar lymphocyte sheaths (pals) in the spleen and the paracortex in LN. This is illustrated in Fig. 2A, where a section of the spleen from a mouse 8 days after injection shows a cluster of CFSE<sup>+</sup> donor cells with variable CFSE intensity in the pals, indicating that the cells had undergone cell division.

The above finding suggests that T cells undergo homeostatic proliferation within the T cell areas. However, the possibility remains that the T cells had proliferated elsewhere before migrating into the T cell areas or received the stimulatory signals elsewhere only to complete cell division in the T cell areas. To determine whether entry into the T cell areas is essential for homeostatic proliferation, CFSE-labeled naive T cells were treated with PTX before injection into irradiated syngeneic normal B6 mice. Treatment with PTX is known to inactivate chemokine receptors and thus prevents lymphocytes from entering either LN or the white pulp in the spleen, although entry into the red pulp of the spleen is unimpaired (Fig. 2D). This effect of PTX is attributable to the inactivation of GTP-binding proteins (G proteins) by the ADP-ribosyltransferase of PTX, thereby neutralizing the G protein-coupled chemokine receptor 7 (CCR7) that recognizes two key chemokines for T cell trafficking: secondary lymphoid tissue chemokine, which attracts lymphocytes onto high endothelial venules for transmigration into the LN, and EBV-induced molecule I ligand chemokine, which in conjunction with secondary lymphoid tissue chemokine attracts T cells into the T cell zones in the LN and spleen (37). Strikingly, CFSE-labeled PTX-treated donor B6.Ly5.1 T cells obtained from the spleens of irradiated B6 mice 8 days after adoptive transfer remained uniformly CFSE<sup>high</sup>, whereas CFSE-labeled sham-treated B6.PL T cells that were injected into the same host underwent efficient proliferation (Fig. 3A). The inability of PTX-treated T cells to undergo homeostatic proliferation did not appear to be attributable to a general block in the cell cycle because these cells proliferated as efficiently as sham-treated control T cells in vitro in response to stimulation by anti-CD3 mAb or allogeneic spleen cells (Fig. 3B). It is important to emphasize that despite their lack of division, the PTX-treated T cells survived well after adoptive transfer; thus 10–20% of the injected cells were recovered on day 8, which was similar to the recovery of sham-treated cells in unirradiated hosts.

If entry into the T cell compartment is a prerequisite for T cells to undergo homeostatic proliferation, one would expect normal T cells not to proliferate in T cell-deficient mice that lack organized T cell compartments in the lymphoid tissues. To test this idea, mice that are deficient in expression of lymphotoxin (LT) α were used as hosts. LTα<sup>−/−</sup> is required for normal development of LNs and Peyer’s patches, and hence LTα<sup>−/−</sup> mice lack peripheral lymphoid tissues, except for the spleen, which is structurally disorganized and largely devoid of discrete T cell areas (39). After injection of CFSE-labeled B6.PL CD4<sup>+</sup> and CD8<sup>+</sup> cells into B6.LTα<sup>−/−</sup> mice, CFSE<sup>+</sup> donor T cells on day 8 were found in the spleen intermingled with the B cells in the disorganized white pulp (Fig. 2B). Notably, the Thy-1.1<sup>+</sup> donor T cells in B6.LTα<sup>−/−</sup> host spleen underwent only minimal cell division, whereas the donor T cells proliferated efficiently in irradiated normal B6 host spleen (Fig. 4).

**Spatial requirement for bystander T cells to suppress homeostatic proliferation**

We have shown previously that proliferation of donor T cells in irradiated syngeneic hosts can be suppressed by coinjecting large numbers of unlabeled syngeneic T cells with CFSE-labeled donor cells. As shown here, the level of suppression is directly proportional to the dose of cojected “bystander” T cells: partial suppression can be achieved by coincubating as few as 0.5–1 × 10<sup>7</sup> bystander T cells (1–2 × 10<sup>7</sup> whole LN cells), whereas near-complete inhibition requires higher doses (Fig. 5). Because the data discussed above indicate that homeostatic proliferation occurs within the T cell areas of the secondary lymphoid tissues, it was of interest to determine whether bystander T cells must also gain entry into the T cell area to mediate suppression. To test this idea, bystander T cells were treated first with PTX to neutralize their ability to enter into the T cell areas and then were cojected with CFSE-labeled normal T cells into irradiated syngeneic mice. As shown in Fig. 6, coinjection of a large dose of B6 LN cells (8 × 10<sup>7</sup>) treated with PTX failed to suppress proliferation of CFSE-labeled B6.PL T cells in irradiated B6 hosts, whereas sham-treated control B6 LN cells efficiently inhibited proliferation. This finding applied not only to cells localizing in host LNs, where PTX-treated T cells failed to gain entry, but also to the cells in host spleens where PTX-treated T cells accumulated in large numbers only in the red pulp; data for the spleen are shown for Fig. 6.

**Bystander T cells need to be naive cells**

Because entry into the T cell areas of the lymphoid tissues applies to both naive and memory cells, the question arises whether both of these cell types have the capacity to inhibit homeostatic proliferation of naive T cells. It is difficult to prepare large numbers of activated or memory phenotype T cells from normal mice because of their low abundance; there are also problems with using in vitro-activated T cells because these cells survive poorly after injection in vivo. In view of these problems we used T cells from adult CTLA-4-deficient and Fas ligand (FasL)-deficient (B6.gld) mice as bystander...
cells; for these two strains, the vast majority of peripheral T cells have an activated/memory phenotype (40–44). The striking finding was that coinjection of large numbers of LN cells (7 3 10^7) from either CTLA-4− or FasL− mice failed to suppress proliferation of CFSE-labeled purified B6.PL T cells (red) with PE-conjugated anti-B220 mAb. Note that CFSE− donor cells (green) with variable intensity, indicative of varying numbers of cell divisions, are situated almost exclusively in perivascular lymphocyte sheath (pals); f denotes follicle. B, A Lto-deficient B6 mouse injected with 2 3 10^6 CFSE-labeled purified B6.PL CD4+ plus CD8+ cells stained for B cells with PE-conjugated anti-B220 mAb. Note that CFSE− donor cells are uniform in intensity and are scattered throughout the disorganized white pulps that contain no distinct B and T cell compartments. C, A B6 mouse injected with 2 3 10^6 B6.PL (Thy-1.1) LN cells stained for Thy-1.1 (red) and counterstained with hematoxylin. Note that donor T cells are localized in the pals with very few in the red pulp (rp) or in the follicles (f). D, A B6 mouse injected with 2 3 10^6 PTX-treated B6.PL LN cells stained for donor-type Thy-1.1 (red) and counterstained with hematoxylin. Note that PTX-treated donor T cells are excluded from entry into the white pulp including pals and reside mostly in red pulp (rp). E, B6.PL mouse injected with a mixture of 2.5 3 10^6 B6.Ly5.1 CD4+ cells and 5 3 10^6 B6.CTLA-4− CD4+ cells stained for Ly5.1 (red) and Thy-1.2 (blue) and B cells (gray). Note that both types of donor T cells are found scattered in pals around the central arteriole (ca); CTLA-4− cells are found in other compartments including the follicles (f). F, A serial section of the spleen stained as in E but without any primary mAbs. Only ca is visible as background staining. These data are representative of three or more mice analyzed individually.

FIGURE 2. Histological detection of T cells undergoing homeostatic proliferation in host spleens. Frozen spleen sections were stained as described in Materials and Methods. All host mice were irradiated (600 cGy) 1 day before i.v. injection of donor cells and spleens were harvested 8 days after injection of donor cells. A, A B6 mouse injected with 10^6 CFSE-labeled purified B6.PL T cells; section was stained for B cells (red) with PE-conjugated anti-B220 mAb. Note that CFSE− donor cells (green) with variable intensity, indicative of varying numbers of cell divisions, are situated almost exclusively in perivascular lymphocyte sheath (pals); f denotes follicle. B, A Lto-deficient B6 mouse injected with 2 3 10^6 CFSE-labeled purified B6.PL CD4+ plus CD8+ cells stained for B cells with PE-conjugated anti-B220 mAb. Note that CFSE− donor cells are uniform in intensity and are scattered throughout the disorganized white pulps that contain no distinct B and T cell compartments. C, A B6 mouse injected with 2 3 10^6 B6.PL (Thy-1.1) LN cells stained for Thy-1.1 (red) and counterstained with hematoxylin. Note that donor T cells are localized in the pals with very few in the red pulp (rp) or in the follicles (f). D, A B6 mouse injected with 2 3 10^6 PTX-treated B6.PL LN cells stained for donor-type Thy-1.1 (red) and counterstained with hematoxylin. Note that PTX-treated donor T cells are excluded from entry into the white pulp including pals and reside mostly in red pulp (rp). E, B6.PL mouse injected with a mixture of 2.5 3 10^6 B6.Ly5.1 CD4+ cells and 5 3 10^6 B6.CTLA-4− CD4+ cells stained for Ly5.1 (red) and Thy-1.2 (blue) and B cells (gray). Note that both types of donor T cells are found scattered in pals around the central arteriole (ca); CTLA-4− cells are found in other compartments including the follicles (f). F, A serial section of the spleen stained as in E but without any primary mAbs. Only ca is visible as background staining. These data are representative of three or more mice analyzed individually.
CD45RB, and CD25 expression (Ref. 34, and not shown). As shown in Fig. 7B, naive CTLA-4<sup>2</sup> and CTLA-4<sup>1</sup> bystander T cells from the BM chimeras were equally effective in inhibiting homeostatic proliferation of CFSE-labeled B6.Ly5.1<sup>1</sup> T cells in irradiated syngeneic B6 hosts. Based on these findings, it would appear that bystander inhibition of naive T cell homeostatic proliferation can be mediated only by naive cells and not by activated/memory cells.

**FIGURE 3.** PTX-treated T cells do not undergo homeostatic proliferation even though their ability to proliferate in response to stimulations in vitro is intact. A, Small doses (2 × 10⁶/mouse) of CFSE-labeled B6.PL LN cells previously treated for 2 h with either medium only or PTX (100 ng/ml) were i.v. injected into irradiated (600 cGy) syngeneic B6 hosts. Splenocytes from the hosts were harvested 8 days later and stained for Thy-1.1 and CD4; CFSE levels on gated donor T cells are shown. Data are representative of six mice per group analyzed individually in three separate experiments. B, Stimulation of PTX- or medium-treated B6.PL LN cells (4 × 10⁵/well) with titrating doses anti-CD3 mAb for 3 days (left) or with allogeneic spleen cells (7 × 10⁵/well) for 3–5 days (right). Cultures were pulsed with 1 μCi [³H]TdR/well 8 h before harvest. Stimulator spleen cells were depleted of T cells as described in Materials and Methods. One other experiment yielded similar results.

CD45RB, and CD25 expression (Ref. 34, and not shown). As shown in Fig. 7B, naive CTLA-4<sup>2</sup> and CTLA-4<sup>1</sup> bystander T cells from the BM chimeras were equally effective in inhibiting homeostatic proliferation of CFSE-labeled B6.Ly5.1<sup>1</sup> T cells in irradiated syngeneic B6 hosts. Based on these findings, it would appear that bystander inhibition of naive T cell homeostatic proliferation can be mediated only by naive cells and not by activated/memory cells.

**FIGURE 4.** Failure of normal T cells to undergo efficient homeostatic proliferation in LTα-deficient syngeneic hosts. Small doses (2 × 10⁶/mouse) of CFSE-labeled purified B6.PL CD4<sup>+</sup> and CD8<sup>+</sup> cells were i.v. injected into irradiated (600 cGy) B6 or B6.LTα<sup>−/−</sup> mice. Spleens from the hosts were harvested 8 days later and stained for Thy-1.1 and CD4; CFSE levels on gated donor T cells shown. Three other mice per group analyzed individually showed similar results in two separate experiments.

**FIGURE 5.** Dose-dependent inhibition of homeostatic T cell proliferation by co-injected bystander T cells. Small doses of (2 × 10⁶/mouse) CFSE-labeled B6.PL LN cells were i.v. injected into irradiated (600 cGy) B6 hosts either alone or supplemented with increasing numbers of B6 LN cells. Host LN cells were harvested 8 days later and stained for Thy-1.1 and CD4; CFSE levels on gated donor T cells are shown. The data are representative of three hosts per group analyzed individually in two separate experiments.

**Do bystander cells compete for stimulatory MHC/peptide ligands or factors?**

The requirement for naive T cells to enter the T cell zones to undergo homeostatic proliferation and to suppress homeostatic proliferation of other T cells suggests that certain components of the T cell zones, e.g., dendritic and/or stromal cells, may regulate T cell homeostasis. Contact with MHC molecules on dendritic cells (DC) is likely because for CD4<sup>+</sup> cells, exclusive expression of MHC class II molecules on DC prevents the disappearance of CD4<sup>+</sup> cells transferred to MHC class II<sup>−</sup> hosts (11). Considering that homeostatic proliferation is driven by specific self-MHC/peptide ligands and requires T-depletion of the host, it is possible that the major driving force behind such a proliferation is the increased availability of specific self-MHC/peptide ligands on DC because of a lack of competition from bystander T cells. If this idea is correct, bystander T cells with below-normal affinity for self-MHC/peptide ligands would be unable to compete at the DC level.

**FIGURE 6.** Coinjecting PTX-treated bystander T cells fails to suppress homeostatic proliferation of normal T cells. Small doses (2 × 10⁶/mouse) of CFSE-labeled B6.PL LN cells were i.v. injected into irradiated (600 cGy) B6 mice either alone or supplemented with B6 LN cells previously treated in vitro for 2 h with medium only or medium containing PTX (100 ng/ml). LN cells from the hosts were harvested 8 days later and stained for Thy-1.1 and CD4; CFSE levels on gated donor T cells are shown. The data are representative of six hosts per group analyzed individually in three separate experiments.
and thus would fail to suppress homeostatic proliferation of T cells. To test this possibility, we took advantage of the previous finding that wild-type B6 CD4+ cells fail to undergo efficient homeostatic proliferation in T-depleted H2-M+ hosts, which express normal levels of syngeneic MHC class II molecules (H2-Ak) loaded predominantly with a single species of self-peptides, CLIP (25, 27). By contrast, CD4+ cells from H2-M+ mice do undergo efficient homeostatic proliferation in T-depleted H2-M+ hosts. Collectively, these data suggest that because of differences in the ligands controlling positive selection, most wild-type B6 CD4+ cells display significantly lower affinity toward CLIP+H2-Ak complex than H2-M+CD4+ cells. Based on these findings, we tested whether homeostatic proliferation of H2-M+CD4+ cells in T-depleted H2-M+ hosts can be inhibited by coinjecting large numbers (5 × 10^7) of purified wild-type B6 CD4+ cells. Interestingly, these cells mediated strong inhibition of H2-M+CD4+ cells (Fig. 8A), implying that the inhibitory function of bystander cells is relatively independent of the TCR affinity of these cells for self-MHC/peptide ligands.

In addition to blocking proliferation of H2-M+CD4+ cells, bystander B6 CD4+ cells also suppressed proliferation of CFSE-labeled donor CD8+ cells that were coinjected as controls. The inhibition of CD8+ cells by bystander CD4+ cells confirms our previous report that CD4+ and CD8+ subsets can largely cross-regulate each other in terms of homeostatic proliferation (27). However, the past experiments were performed in normal B6 hosts where the bystander T cells can interact sufficiently with appropriate self-MHC molecules (class I for CD8+ cells and class II for CD4+ cells). In accounting for the capacity of wild-type B6 CD4+ cells to inhibit proliferation of H2-M+CD4+ cells in H2-M+ hosts, it is possible that even very low TCR affinity for self-MHC/peptide ligand (CLIP+H2-Ak) is sufficient to signal the bystander cells to mediate their inhibitory function. To investigate this question, we tested whether B6 CD4+ cells are able to block homeostatic proliferation of CD8+ cells in MHC class I+II+ hosts, i.e., in hosts lacking the TCR ligands for CD4+cells. As shown in Fig. 8B, this was indeed the case. Thus, B6 CD4+ cells suppressed homeostatic proliferation of CD8+ cells with equal efficiency in either MHC class I+II+ hosts. The bystander T cells thus have the capacity to mediate inhibition without receiving signals through their TCR.

Even without the involvement of self-MHC/peptide ligands, bystander T cells could mediate inhibition by sequestering other stimulatory factors essential for cell proliferation. This raises the question whether T cells must undergo homeostatic proliferation themselves to serve as efficient bystander cells. Considering that bystander T cells need to be injected in large doses (>3 × 10^7 cells/host), which partially restores the overall size of the T cell pool, it is unlikely that these T cells themselves undergo a significant level of homeostatic proliferation. Nevertheless, a small fraction of bystander T cells might undergo proliferation, even in hosts deficient in expression of H2-M or MHC molecules, and this could be sufficient to inhibit homeostatic proliferation. Hence, we tested whether TCR-transgenic T cells (AND and OT-II), which are almost completely unable to proliferate in irradiated hosts (Ref. 27 and our unpublished observation), can serve as bystander T cells. This was found to be the case, as coinjection of either AND or OT-II cells significantly suppressed proliferation of both normal B6 CD4+ and CD8+ cells in irradiated syngeneic hosts (Fig. 8C).

**Discussion**

It is currently unknown how severe T cell deficiency induces naive T cells to undergo homeostatic proliferation in response to self-MHC/peptide ligands. As mentioned in the Introduction, such proliferation may reflect either increased availability of stimulatory factors or absence of suppressive signals that normally prevent cell division. For the former, an increase in stimulatory factors could reflect either 1) enhanced availability of soluble factors (either systemic or short-range), or 2) more frequent contact with APC as the result of decreased “congestion” around APC, thus leading to increased contact with self-MHC/peptide complexes or with other ligands on APC.
The possibility that homeostatic proliferation is driven by a surplus of systemic soluble factor(s) was tested using PTX-treated T cells, with the rationale that these cells will still be able to respond to systemic factors even though they no longer migrate into the LNs or into the T cell compartments of the spleen. Strikingly, PTX-treated T cells failed to undergo proliferation in lymphopenic hosts despite their entry into the non-T cell compartments of the spleen, namely the red pulp areas that contain numerous MHC+ cells. Because PTX-treated T cells survived well in vivo and were unimpaired in their ability to undergo proliferation when stimulated through the TCR in vitro, the failure of PTX-treated T cells to undergo homeostatic proliferation in lymphopenic hosts presumably reflected that these cells are unable to enter the T cell compartments. The requirement for entry into the T cell compartments also applied for the bystander cells that mediate inhibition of homeostatic proliferation. Thus, in contrast to normal T cells, coinjection of large numbers of PTX-treated bystander naive T cells failed to block homeostatic proliferation of untreated normal naive T cells in lymphopenic hosts. This was apparent despite the finding that injected PTX-treated T cells engrailed efficiently and persistently in large numbers in the blood, BM, and the splenic red pulp of the hosts. Considering the fact that G proteins are coupled with a wide spectrum of receptors, it should be mentioned that we cannot completely rule out the possibility that the effect observed with PTX treatments is due to neutralization of receptors directly involved in homeostatic proliferation. Despite this caveat, the above findings collectively argue against the idea that homeostatic proliferation is induced by increased availability of some soluble factors that work systemically in a hormone-like fashion throughout the body.

The finding that naive T cells must enter specific T cell compartments to undergo homeostatic proliferation also suggests that the DC resident in this site are the primary APC that present self-MHC/peptide ligands to drive this response. The idea that DC are the crucial APC for homeostatic proliferation is in accord with the findings that long-term survival of naive CD4+ cells can be maintained by exclusive expression of MHC class II molecules only on DC (11). On this point, it is notable that homeostatic proliferation failed to occur efficiently in LTα− mice. Although this finding could be a reflection of various defects in these mice, the finding is of interest because the disorganized white pulp of LTα− mice shows a marked reduction in DC (45). Collectively, these data suggest that homeostatic proliferation in T cell compartment is largely under the control of DC. Whether other cell types, e.g., stromal cells, are involved is still unclear. Macrophages and B cells are probably not necessary because macrophages are rare in T cell compartments and homeostatic proliferation can occur in B cell-deficient mice, e.g., in Rag−/− mice.

Because homeostatic proliferation is directed to specific self-MHC/peptide ligands, the onset of proliferation in T-deficient hosts may be initiated as the result of decreased “congestion” around the DC. We assessed this possibility by determining whether bystander inhibition of homeostatic proliferation could be

FIGURE 8. Naive T cells with limited affinity for self-MHC/peptide ligands can serve as inhibitory bystander T cells. A. Coinjection of purified B6 CD4+ cells inhibits homeostatic proliferation of H2-M− CD4+ cells in irradiated H2-M− hosts. Small doses (2 × 10⁵/mouse) of CFSE-labeled H2-M+ Thy-1.1 LN cells were injected into irradiated H2-M− hosts either alone or together with purified B6 CD4+ T cells, which are unable to undergo efficient proliferation in irradiated H2-M− mice (25, 27). LN cells from hosts were harvested 8 days later and stained for Thy-1.1 and CD4; CFSE levels on gated donor H2-M− T cells are shown. The data are representative of six hosts per group analyzed individually in three separate experiments. B. Coinjection of purified B6 CD4+ cells inhibits homeostatic proliferation of B6 CD8+ cells in irradiated syngeneic MHC class I−II− hosts. B6 or B6.H2-A− hosts were injected with small numbers (2 × 10⁶/mouse) of CFSE-labeled purified B6.PL CD4+ and CD8+ T cells alone or together with large numbers of unlabelled purified B6 CD4+ T cells. Host LN cells were harvested 8 days later and stained for Thy-1.1 and CD4; CFSE levels on gated donor T cells are shown. Note that CD8+ T cells cannot undergo efficient homeostatic proliferation in irradiated B6.H2-A− hosts. The data are representative of six hosts per group analyzed individually in three separate experiments. C. Coinjection of TCR-transgenic T cells that cannot undergo homeostatic proliferation suppresses homeostatic proliferation of normal T cells. Irradiated (600 cGy) B6 mice were injected with small doses (2 × 10⁶/mouse) of CFSE-labeled B6.PL donor cells either alone or together with large numbers of T cells from B6, OT-II, or AND mice; both lines of transgenic T cells do not undergo homeostatic proliferation in irradiated syngeneic B6 hosts (Ref. 27 and our unpublished observation). Host LN cells were harvested 8 days later and stained for Thy-1.1 and CD4; CFSE levels on gated donor B6.PL T cells are shown. The data are representative of four hosts per group analyzed individually in two separate experiments.
mediated by CTLA-4 and FasL- T cells; these cells were able to efficiently migrate into the T cell compartments and therefore were presumably able to create congestion around DC. Significantly, however, coinjection of large doses of activated/memory phenotype CTLA-4 and FasL- bystander T cells did not inhibit homeostatic proliferation of naive T cells; by contrast, naive phenotype CTLA-4 bystander T cells did efficiently inhibit homeostatic proliferation. Despite the concern that activate/memory phenotype CTLA-4 and FasL- T cells may not represent real memory T cells in terms of their homeostasis requirements, these data nevertheless strengthen the view that homeostasis of naive and memory T cells is independently regulated (46). In support of this idea, we have recently observed that homeostatic proliferation of normal B6 memory phenotype T cells cannot be suppressed by coinjecting large numbers of only naive phenotype bystander T cells (unpublished observation). For CTLA-4 T cells, it is of particular interest that naive phenotype CTLA-4 T cells shared the capacity of normal naive T cells to inhibit bystander proliferation of naive T cells. This finding is thus against the idea that homeostatic proliferation of naive T cells is under the control of CTLA-4 molecule (44). Whether CTLA-4 influences homeostatic proliferation of memory phenotype cells has yet to be studied.

The notion that bystander inhibition of homeostatic proliferation reflects competition for MHC/peptide ligands on APC predicts that bystander inhibition would be reduced if the T cells used as bystanders had limited affinity for the ligands inducing homeostatic proliferation. Three findings are against this idea. First, homeostatic proliferation of H2-M- CD4 T cells to CLIP/H2-A in H2-M- hosts was efficiently blocked by normal B6 CD4+ cells, i.e., by cells that presumably have very limited affinity for CLIP/H2-A. Second, the capacity of normal B6 CD4+ cells to inhibit bystander proliferation of CD8+ cells applied in MHC I+II hosts, i.e., hosts where the bystander CD4+ cells failed to see MHC II molecules. Third, T cells with only very low affinity for self-MHC/peptide ligands, i.e., particular transgenic T cells that exhibit little or no capacity to undergo homeostatic proliferation in T-deficient hosts, were nevertheless able to mediate efficient bystander inhibition of normal T cells. These findings, especially the data with MHC I+II hosts, imply that bystander inhibition does not depend on TCR ligation.

Although the above data argue that bystander inhibition does not reflect competition for MHC/peptide ligands on APC, there may still be competition for other ligands, e.g., cell-associated or soluble factors released from APC in the T cell zones. For soluble factors, it is possible that cytokines known to promote survival of naive T cells in vitro, such as IL-4, IL-6, and IL-7 (47–49), are involved in regulating naive T cell homeostasis. This possibility is currently under investigation.

An alternative idea is that bystander inhibition is mediated as the result of direct T-T interactions in the T cell zone. Based on the data presented here, such inhibition would be MHC independent and requires T-T interactions at the level of naive T cells. This notion hinges on the assumption that interactions between complementary molecules on T cells during T-T contact inhibit proliferation. Currently, there is little if any direct data that favors or refutes this possibility.

Note added in proof. While this manuscript was in press, Schluns et al. reported that efficient homeostatic proliferation fails to occur in syngenic IL-7-deficient hosts, thus strongly implicating that homeostasis of naive T cells is regulated mainly by IL-7 (50). Accordingly, homeostatic proliferation is presumably induced by increased availability of IL-7 in T-depleted hosts and blocked by the removal of the excess IL-7 by the bystander cells.

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