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13. ABSTRACT (Maximum 200 Words)

We have previously shown using a mouse model of melanoma that homeostatic T-cell proliferation, a process occurring in response to lymphopenia and dependent on signaling by self-peptide/MHC and trophic cytokines, may promote effective anti-tumor autoimmunity if induced in conjunction with a tumor-cell challenge. We are currently investigating whether this principle can be applied to mouse models of advanced breast carcinoma, and whether the anti-tumor response can be enhanced using selected T-cell subpopulations, cytokines and tumor-vaccines. The results obtained during the first year of this project indicated that (a) lymphopenia and the associated homeostatic T-cell proliferation can be effectively induced using T cell-depleting anti-Thy1 antibodies; (b) lymphopenia-induced homeostatic T-cell proliferation inhibits subcutaneous tumor growth, lung metastasis and mortality in an ectopic model of breast carcinoma; (c) gamma/delta T cells, a lymphocyte subpopulation with significant anti-tumor activity, can also be iduced to undergo homeostatic proliferation, and this requires depletion of both alpha/beta and gamma/delta T cell compartments and availability of either IL-7 or IL-15; (d) the anti-tumor response is diminished in aged mice, and this correlates with inefficient homeostatic T-cell proliferation; (e) homeostatic T-cell proliferation in aged mice can be restored by provision of the trophic cytokine IL-7.

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Breast cancer, autoimmunity, T cell homeostasis, lymphopenia

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INTRODUCTION

Due to lack of efficient treatments, breast cancer is the second most common cause of cancer death among women. Current efforts in cancer immunotherapy focus on induction of autoimmune responses against tumor-associated antigens that are primarily encoded by normal unmutated genes. Breaking tolerance for self-antigens, however, remains a major challenge. Recent studies showed that, under lymphopenic conditions, peripheral T cells undergo "homeostatic proliferation" to re-establish appropriate cell numbers. Since homeostatic T-cell proliferation depends on recognition of self-peptide/MHC antigens and is accompanied by acquisition of effector functions, we hypothesized that induction of such process concurrently with a tumor cell challenge may be a way to preferentially expand and activate otherwise tolerant lymphocytes and, hence, elicit effective anti-tumor autoimmunity (reviewed in (1), see **appended manuscript # 1**). Our preliminary experiments with melanoma cell-challenged lymphopenic mice infused with small numbers of syngeneic polyclonal T cells indicate that this is indeed the case (2). Here, we wish to extend this novel observation and determine whether the principle of homeostatic T-cell proliferation can be used to inhibit progression of established breast tumors. Specific aims include (a) to apply the principle of homeostatic T-cell expansion to inhibit growth of established tumors in models of advanced breast cancer; (b) to enhance the efficacy of the response by manipulating the composition of the infused T cells; and (c) to potentiate the anti-tumor effect by using T cell survival and proliferation promoting cytokines, and/or by enhancing tumor-antigen presentation with efficient tumor vaccines. The results will define the role of homeostatic T-cell proliferation in tumor autoimmunity, and provide the basis for translation studies and for the design of new approaches to the treatment of breast cancer.

BODY

The results summarized below describe research accomplishments associated with tasks outlined in the approved Statement of Work.

Task 1.a. Define a protocol to induce lymphopenia using T cell-depleting antibodies for optimal homeostatic T-cell proliferation

An important question is whether homeostatic T cell expansion can be used therapeutically for the treatment of established tumors. In our initial studies, lymphopenia was induced before tumor challenge by sublethal irradiation (2). Because irradiation would affect tumor growth, assessment of the effect of homeostatic T cell proliferation in this setting would require induction of lymphopenia by other means than irradiation. Therefore, we evaluated the use of T cell-depleting antibodies as an alternative way of lymphopenia induction. C57BL/6 (B6) mice (15/group) were treated with either sublethal irradiation (600 rads, control) or T cell-depleting antibodies. As antibodies, we used anti-CD3 (145-2C11, 500 µg i.p.), anti-Thy1.2 (30-H12, 500 µg i.p.) or a combination of anti-CD4 (GK1.5, 200 µg i.p.) plus anti-CD8 (YTS169.4.2 200 µg i.p.), as described (3, 4). At days 1, 2, 3, 4 and 10 after treatment, 3 mice per group were sacrificed and cells in LNs and spleen enumerated. As shown in **Fig. 1**, both anti-CD3 and anti-Thy1.2 antibodies appeared efficient in inducing depletion of $CD4^+$ and $CD8^+$ T cell subsets, in both LNs and spleen. In contrast, depletion with anti-CD4 plus anti-CD8 was not as efficient (data not shown). Additionally, whereas anti-Thy1.2 did not cause obvious adverse effects on the mice, anti-CD3 treatment caused a toxic-like effect resembling that induced by bacterial superantigens. For both anti-CD3 and anti-Thy1.2, optimal depletion, comparable to that observed 1 day after sublethal irradiation, was achieved 4 days after injection. In a second experiment, B6 mice were similarly treated and transfused with 5×10^6 CFSE-labeled B6.PL ($Thy1.1^+$) LN cells. Transfusion was performed at day 1 after irradiation and at day 4 after antibody injection. At day 7 post-transfer, 3 mice/group were sacrificed, and LN and spleen cells enumerated and analyzed by FACS. As shown in **Fig. 2A**, homeostatic expansion of $CD4^+$ and $CD8^+$ T cells in mice that were T cell-depleted by anti-Thy1.2 and anti-CD3 was at least as efficient and that induced through sublethal irradiation. Recovery of donor cells in anti-Thy1.2-treated mice was also very efficient (**Fig. 2B**), whereas recovery in anti-CD3-treated mice was significantly reduced (<10%) compared to irradiated mice. Thus, anti-Thy1.2 antibodies efficiently induced T cell depletion and will be used for future experiments with mice bearing established tumors.

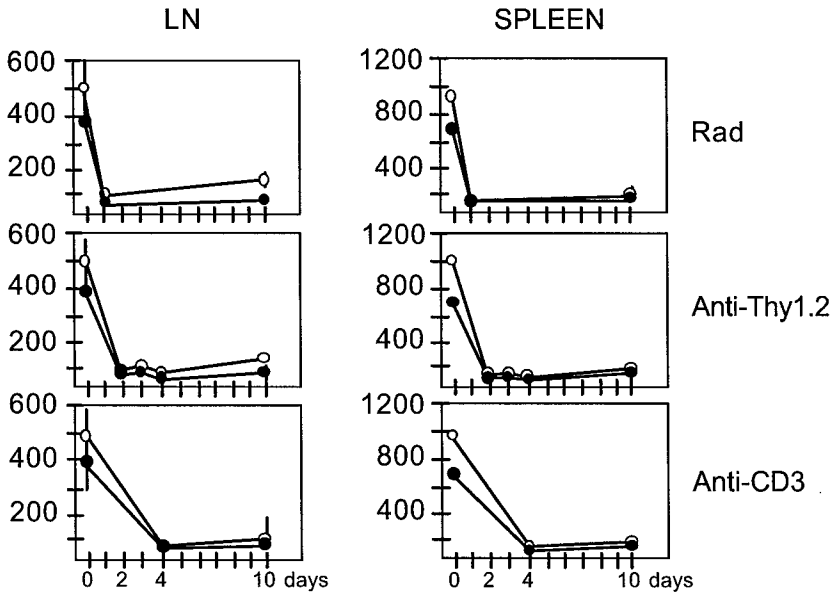

Figure 1. *Efficient T cell depletion is achieved with anti-Thy1.2 and anti-CD3 antibodies. B6 mice were treated (on day 0) with sublethal irradiation (600 rads), anti-Thy1.2 antibodies (500 µg i.p.) or anti-CD3 antibodies (500 µg i.p.). At the indicated days after treatment, mice were sacrificed, and LN and spleen CD4 (open circles) and CD8 (filled circles) T cells enumerated. Similar experiments were performed with mice treated with anti-CD4 and anti-CD8 antibodies (not depicted). Data represent T cell numbers × 10(-4) ± SD.*

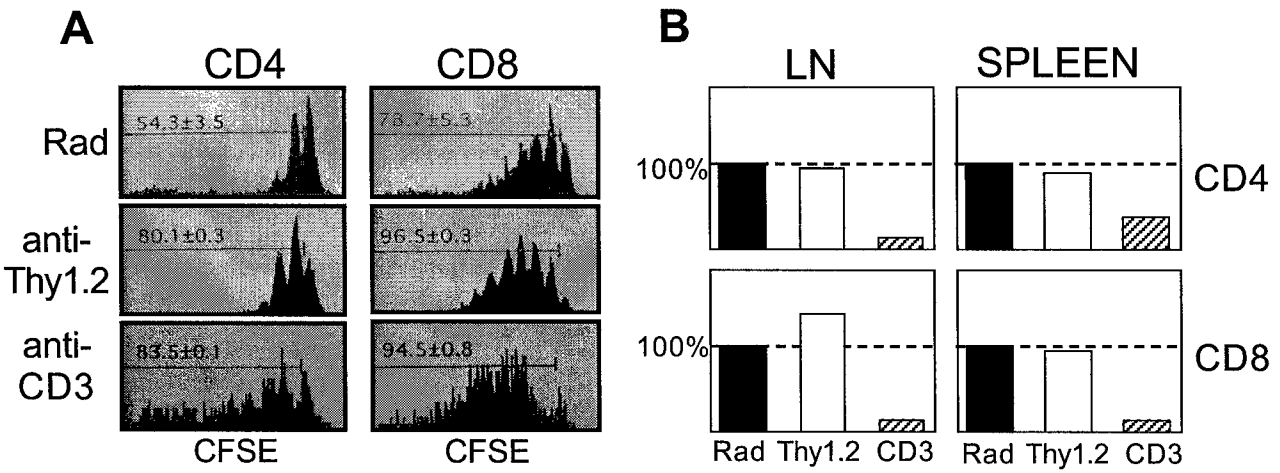

Figure 2. *Efficient homeostatic T cell proliferation in recipient mice rendered lymphopenic with anti-Thy1.2 antibodies. B6 mice were treated with sublethal irradiation (600 rads), anti-Thy1.2 antibodies (500 µg i.p.) or anti-CD3 antibodies (500 µg i.p.). At day 1 after irradiation, or at day 4 after antibody treatment, mice were transfused with 5 × 10(6) LN cells from Thy1.1(+) B6.PL mice. At day 7 after transfer, mice were sacrificed and LN and spleen cells enumerated and analyzed by FACS. (A) CFSE profiles on gated CD4(+) or CD8(+) T cells isolated from LN. Similar results were obtained by analyzing spleen cells. (B) Recovery of donor cells at day 7 post transfer expressed as a percentage (± SD) of cells recovered in sublethally irradiated control mice.*

Task 1.b. Evaluate the efficacy of homeostatic T-cell expansion on established tumors of increasing size using an ectopic model of breast cancer

The weakly immunogenic mammary carcinoma cell line 4T1, originally derived from BALB/c (B/c) mice by Dr. Fred Miller (5), was obtained and cultured as described (6). The growth kinetic of 4T1-induced tumors was initially established in unmanipulated female B/c mice (12/group). We found that subcutaneous (s.c.) injection of 10^5 4T1 cells was sufficient to induce tumors that reach 1,000 mm^3 in approximately 25 days. We next performed a series of experiments to define the efficacy of homeostatic T-cell proliferation in inhibiting tumor growth in this ectopic model of breast carcinoma. B/c mice (12/group) were rendered (or not)

lymphopenic, challenged s.c. with 10^5 4T1 cells, and then transfused with (a) no cells, (b) 5×10^6 LN cells from unmanipulated B/c mice, or (c) 50×10^6 LN cells from unmanipulated B/c mice. Mice were then followed for the presence of a palpable tumor, which was measured 2-3 times per week. At day 27 to 30 after tumor challenge, mice were sacrificed and examined histologically for the presence of tumor cells in various organs and by FACS for the frequency of CD4 and CD8 T cells and activated subsets defined on the basis of CD44, CD62L and CD25 expression. Tumor growth inhibition was observed in mice undergoing homeostatic expansion of transfused LN cells, as shown by reduced tumor volumes (**Fig. 3B and 3C**) and reduced tumor weight (not shown). In contrast, the anti-tumor effect was not observed in mice rendered lymphopenic but not transfused (**Fig. 3A**), i.e., in mice in which spontaneous T cell reconstitution by thymic exportation and peripheral homeostatic expansion occurs but is protracted. Importantly, lymphopenic mice receiving 50×10^6 LN cells also showed reductions in both spontaneous lung metastases and mortality compared to lymphopenic mice receiving 5×10^6 (or no) LN cells (not shown). Thus, homeostatic expansion of 50×10^6 LN seem to significantly affect tumor growth and metastases of 4T1 breast carcinoma cells. Current experiments are evaluating the therapeutic limits of this approach at later disease stages.

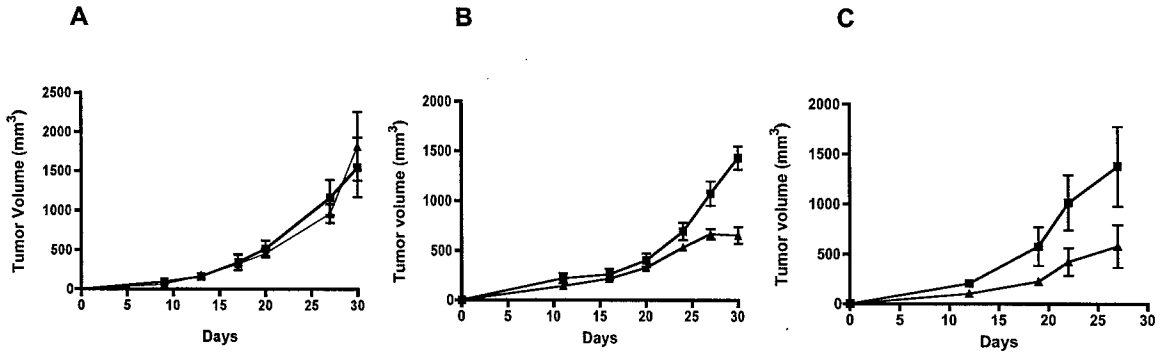

Figure 3. *Inhibition of breast carcinoma growth by homeostatic T-cell proliferation. BALB/c (B/c) mice were rendered lymphopenic, challenged subcutaneously with 10(5) 4T1 breast carcinoma cells (t = 0) and transfused intravenously with (A) no cells, (B) 5 × 10(6) LN cells, or (C) 50 × 10(6) LN cells (filled triangles). LN cells used for transfusion were obtained from LNs of unmanipulated syngeneic B/c mice. In each case, control mice (filled square) included tumor-challenged mice that were not lymphopenic and that received no transfusion. Mice were followed for tumor growth and, at the indicated time-points, tumors were measured using a caliper and volumes determined according to the formula $^1/_2 \times length \times width^2$. Data represent average tumor volume ± SE (n = 12/group).*

Task 2.b. Determine whether enrichment of TCRgamma/delta T cells in the infused cell population enhances the anti-tumor response in the ectopic model

T cells expressing γδ T cell receptor (TCR) constitute a significant fraction of lymphocytes in peripheral lymphoid organs and blood and dominate in mucosa and epithelia of various tissues. Considerable evidence indicates that γδ T cells exhibit significant anti-tumor activities (7-9). We hypothesized that the principle of homeostatic expansion could be used to selectively enrich and prime this T cell subpopulation, so as to more efficiently exploit the associated anti-tumor effects. Therefore, to explore this possibility, we initiated experiments to determine wether γδ T cells also undergo lymphopenia-induced proliferation and to characterize the homeostatic requirements of such process ((10), see **appended manuscript #2**).

γδ T cells were isolated by negative selection (to avoid activation) from LNs and spleen of unmanipulated B6 mice. These cells were then labelled with CFSE and injected into syngeneic recipients that were either unmanipulated or sublethally irradiated to induce lymphopenia. No proliferation was observed in non-lymphopenic recipients, suggesting competition with other lymphocyte populations. In contrast, homeostatic expansion of γδ T cells was detected in lymphopenic hosts. As previously observed with αβ T cells, γδ T cells also showed phenotypic changes during homeostatic proliferation, most notably upregulation of CD44 and downregulation of CD62L.

To identify the lymphocyte subsets that compete with γδ T cells during homeostatic proliferation, adoptive transfers were performed into mutant recipients that selectively lack specific cell populations. As expected, γδ cells proliferated in $RAG^{-/-}$ mice, lacking both αβ and γδ T cells. Surprisingly, however, no proliferation occurred in non-irradiated $TCR\delta^{-/-}$ mice lacking γδ T cells, suggesting competition with αβ T cells. To investigate the mechanisms underlying such competition, we evaluated the involvement of MHC/peptide ligands, IL-7 and IL-15, i.e., the main modulators of αβ T cell homeostasis. Consistent with the previous observation that γδ T cell populations are not decreased in MHC-deficient mice, we found normal homeostatic proliferation in $\beta_2M^{-/-}$ and $I\text{-}A\beta^{-/-}$ recipients. On the other hand, lack of homeostatic proliferation in double-deficient $IL\text{-}7^{-/-}IL\text{-}15^{-/-}$ mice, as opposed to normal expansion in single-deficient ($IL\text{-}7^{-/-}$ or $IL\text{-}15^{-/-}$) mice and in $IL\text{-}7^{-/-}IL\text{-}15^{-/-}$ mice treated with recombinant IL-7, suggested that γδ T cell homeostatic expansion requires either IL-7 (like most αβ T cells) or IL-15 (like memory $CD8^+$ αβ T cells).

If IL-7 and IL-15 were the only factors controlling γδ T cell homeostasis, γδ T cells would be expected to expand in non-irradiated $TCR\alpha^{-/-}$ recipients lacking αβ T cells. Availability of IL-7 and IL-15 in these mice was demonstrated by the fact that adoptively transferred αβ T cells extensively proliferated, at rates similar as in $RAG\text{-}1^{-/-}$ mice . Remarkably, however, no evidence of homeostatic expansion was observed for γδ T cells in non-irradiated $TCR\alpha^{-/-}$ mice. Because γδ T cells proliferated in $RAG\text{-}1^{-/-}$ hosts, these results indicate that, in addition to αβ T cells, γδ T cells also restrain acute homeostatic expansion of γδ T

cells. Thus, it appears that the size of the γδ T cell pool in lymphoid organs is defined by availability of cytokines commonly used by other lymphoid cells (i.e., IL-7 and IL-15) as well as by additional γδ T cell-specific ligands.

On the basis of these results, experiments currently ongoing are evaluating whether γδ T cells, expanded through lymphopenia-induced proliferation in the presence of either IL-7 or IL-15, exhibit anti-tumor responses in the 4T1 model of breast cancer, and whether this activity can be used to potentiate the anti-tumor effects of αβ T cells.

Task 3.a. Define whether trophic cytokines enhance T cell homeostatic survival and proliferation in young and aged mice

Because the incidence of breast cancer increases in women with advanced age, and aging is associated with defective immune functions and decreased production of trophic cytokines, we examined whether the anti-tumor effect of homeostatic T-cell proliferation is impaired in aged mice. Young (4 wks of age) and old (>80 wks of age) B6 mice were sublethally irradiated to induce lymphopenia, challenged s.c. with 5×10^5 B78D14 tumor cells and transfused i.v. with 5×10^6 LN cells from age-matched syngeneic donors. A control group included tumor-challenged non-lymphopenic young mice.

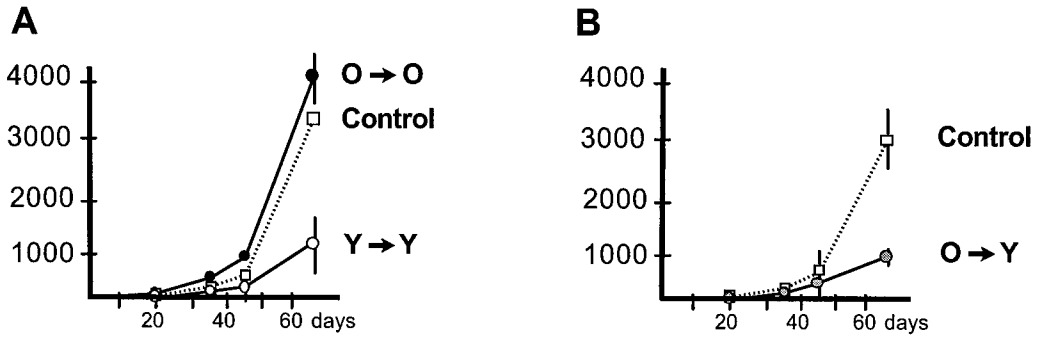

Figure 4. *The anti-tumor effect of homeostatic T-cell proliferation is impaired in aged mice. (A) Young (Y, 4 wks) and old (O, >80 wks) mice were sublethally irradiated, challenged s.c. with 5 × 10(5) B78D14 tumor cells (t = 0), and transfused i.v. with 5 × 10(6) total LN cells from age-matched donors. (B) Young (Y, 4 wks) mice were sublethally irradiated, tumor-challenged, and transfused with LN cells from old (O, >80 wks). In both experiments, control mice were non-lymphopenic and tumor-challenged. Data represent tumor volume in mm(3) ± SD.*

As shown in **Fig. 4A**, compared to young mice, tumor growth was not significantly inhibited in old mice. To determine whether the reduced anti-tumor response in aged mice was due to defects in the responding T cells or to age-related changes in the environment that supports such responses, additional groups were set in which young mice were rendered lymphopenic, tumor-challenged and transfused with LN cells from aged donors. The results indicated that impaired anti-tumor effects in the aged were not due to defective T cell responses, but rather to changes in the recipients, as T cells from old donors efficiently induced anti-tumor immunity when infused into lymphopenic young recipients (**Fig. 4B**).

Since aging is associated with considerable declines in the production of several cytokines, including the homeostasis-controlling IL-7 (11-14), we examined whether abnormal anti-tumor responses in aging correlate with reduced T cell homeostatic proliferation kinetics. CFSE-stained LN cells were transferred between allelically-different B6 and B6.PL mice in various age combinations (**Fig. 5A**). Indeed, the results indicated that aging is associated with impairment of homeostatic T cell proliferation. This abnormality appears not to be caused by a primary T cell defect but rather by changes in the microenvironment, since adoptively transferred T cells from aged donors displayed normal proliferation in lymphopenic young recipients.

To determine whether impaired homeostatic T cell proliferation in the aged can be corrected by supplementing cytokines, we treated old sublethally-irradiated recipients s.c. with 1μg recombinant mouse IL-7, either once (before transfusion) or 5 times (once before transfusion, then once a day for 4 days). Analysis at day 7 revealed that IL-7 given for 5 days significantly increased the frequency of proliferating T cells in aged recipients (virtually to levels registered in young recipients), whereas a single treatment had only marginal effects (**Fig. 5B**). We are currently examining whether the effect by IL-7 is long lasting (allowing full reconstitution of the recipients), whether other homeostasis affecting cytokines (like IL-15, IL-2 or combinations with IL-7) also exert similar effects and, importantly, and whether such treatments can improve anti-tumor responses following homeostatic T cell expansion.

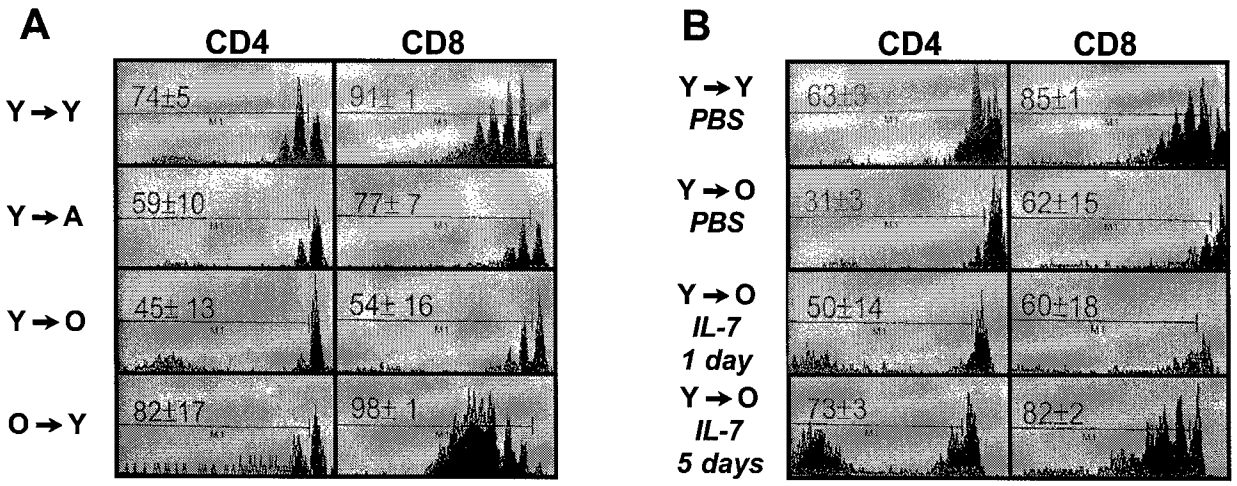

Figure 5. *Impaired homeostatic proliferation in aging and correction by IL-7. (A) CFSE-stained LN T cells (5 x 10^6) from young (Y, 4 wks) B6.PL mice were transferred into irradiated (600 rad) young (Y, 4 wks), adult (A, 48 wks) or old (O, 80 wks) B6 mice. Similarly, CFSE-stained LN T cells from old B6 mice (80 wks) were injected into young (4 wks) B6.PL mice (n = 3/group). CFSE profiles of donor (Thy1.2$^+$ or Thy1.1$^+$) CD4$^+$ and CD8$^+$ LN T cells were determined 7 days after transfer. Percentages of dividing cells are indicated. (B) CFSE-stained LN T cells (5 x 10^6) from young (Y, 4 wks) B6.PL mice were transferred into irradiated (600 rad) young (Y, 4 wks), or old (O, 108 wks) B6 mice treated s.c. with either PBS, IL-7 (1μg) for 1 day, or IL-7 for 5 days (n = 4/group). CFSE profiles were obtained at day 7 after transfer.*

KEY RESEARCH ACCOPLISHMENTS

- Lymphopenia and the associated homeostatic T-cell proliferation can be effectively induced using T cell-depleting anti-Thy1 antibodies
- Lymphopenia-induced homeostatic T-cell proliferation inhibits subcutaneous tumor growth, lung metastasis and mortality in an ectopic model of breast carcinoma
- γδ T cells, a lymphocyte subpopulation with significant anti-tumor activity, can also be iduced to undergo homeostatic proliferation, and this requires depletion of both alpha/beta and gamma/delta T cell compartments and availability of either IL-7 or IL-15
- The anti-tumor response is diminished in aged mice, and this correlates with inefficient homeostatic T-cell proliferation
- Homeostatic T-cell proliferation in aged mice can be restored by provision of the trophic cytokine IL-7.

REPORTABLE OUTCOMES

Baccala, R., R. Gonzalez-Quintial, W. Dummer, and A.N. Theofilopoulos. 2005. Tumor immunity via homeostatic T cell proliferation: mechanistic aspects and clinical perspectives. *Springer Semin Immunopathol.* PMID: 15666151

Baccala, R., D. Witherden, R. Gonzalez-Quintial, W. Dummer, C.D. Surh, W.L. Havran, and A.N. Theofilopoulos. 2005. Gamma delta T cell homeostasis is controlled by IL-7 and IL-15 together with subset-specific factors. *J Immunol* 174:4606-4612. PMID: 15814683

CONCLUSIONS

Considerable advances have been made in recent years in defining the nature of tumor antigens and devising innovative approaches for the immunotherapy of cancer. Nevertheless, clinical success has been limited primarily by the difficulty of overcoming T-cell tolerance for such antigens. Recent clarifications of the role of self-recognition in lymphopenia-induced homeostatic T-cell proliferation and the acquisition of a semi-activated state by the proliferating cells led us and others to suggest that T-cell priming through this process might overcome T-cell tolerance against tumor antigens, and data with various tumors exhibiting different immunologic and histologic characteristics strongly support this concept. We have shown that the principle of homeostatic T-cell proliferation can be applied to a mouse model of breast carcinoma, can be used to expand and activate subpopulations of lymphocytes with significant intrinsic anti-tumor activities, and can be improved in aged individuals through provision of exogenous IL-7. Future studies will provide a more detailed characterization of mechanistic issues such as the exact process by which tolerance is broken, direct or indirect means of tumor antigen presentation, efficacy of refined T-cell subsets, and the potentiating effects of homeostatic proliferation-promoting cytokines and specific vaccines. Nonetheless, the results thus far indicate that the process of tumor-specific vaccination and transfusion of naïve unprimed autologous T cells administered in conjunction with, or immediately after, conventional lymphopenia-inducing anti-cancer therapies may be a simple means to elicit effective tumor immunity.

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APPENDICES

Manuscript #1:
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Original Article

Tumor immunity via homeostatic T cell proliferation: mechanistic aspects and clinical perspectives

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Abstract Efforts to develop effective anti-tumor immunotherapies are hampered by the difficulty of overcoming tolerance against tumor antigens, which in most instances are normal gene products that are over-expressed, preferentially expressed or re-expressed in cancer cells. Considering that lymphopenia-induced homeostatic T cell proliferation is mediated by self-peptide/MHC recognition and that the expanded cells acquire some effector functions, we hypothesized that this process could be used to break tolerance against tumor antigens. Studies by us and others in several mouse models demonstrated that availability of tumor antigens during homeostatic T cell proliferation indeed leads to effective anti-tumor autoimmunity with specificity and memory. This effect appears to be mediated by reduction in the activation threshold of low-affinity tumor-specific T cells, leading to their preferential engagement and expansion. In its simplicity, this approach is likely to have application in humans, since it relies on conventional lymphopenia-inducing cancer therapies, infusion of autologous lymphocytes and, optimally, tumor-specific vaccination.

Keywords Lymphopenia - Melanoma - Cancer immunotherapy - Anti-tumor autoimmunity - Tumor antigens

Introduction

Immunotherapy holds significant promise for the treatment of cancer, particularly considering the efficacy with which a "properly" activated immune system attacks and destroys virally infected cells, allogeneic cells from transplanted tissues displaying minimal histocompatibility mismatches, and even normal cells during the acute phases of autoimmune diseases. The concept that the immune system may play a protective role against tumors was originally proposed by Burnet in his "cancer immunosurveillance" hypothesis [12]. Largely abandoned during the 1970s, this idea has found new support in recent years, particularly on the basis of studies with molecularly defined mouse models generated through gene targeting or transgenic approaches. As recently reviewed [18, 35], a large body of evidence demonstrates the effect of the immune system on developing tumors, including data showing that (a) more tumors arise in immunodeficient individuals, (b) tumors selected in immunodeficient mice tend to be rejected when transplanted into wild-type, immunocompetent mice, and (c) tumor escape variants often emerge, apparently to evade immune attack, including variants characterized by loss or down-regulation of MHC molecules and/or tumor antigens, defective death receptor signaling, lack of costimulation, production of immunosuppressive cytokines, and expression of molecules promoting T cell apoptosis. The characterization of tumor-associated antigens as self molecules primarily encoded by non-mutated genes, the remarkable progress in elucidating the molecular and cellular basis of immune recognition and regulation, and the discovery that self antigens play a critical

role in T cell repertoire selection during lymphopenia-induced homeostatic proliferation, have provided a rationale for the design of novel immunotherapeutic approaches to the treatment of cancer.

Cancer immunotherapy targeting tumor-associated self antigens

Considerable advances towards the development of effective cancer vaccines have been made following the identification of tumor-associated antigens. To a large extent, these antigens are tissue-specific or differentiation molecules encoded by normal genes and presented as MHC-bound peptides to $CD8^+$ cytotoxic and $CD4^+$ helper T cells [9, 53, 58, 63]. This has primarily been established with melanomas where, with rare exceptions, the associated antigens are either lineage-specific membrane proteins also expressed by normal melanocytes, or differentiation proteins expressed by melanoma cells and spermatozoa [32, 57]. Differentiation antigens are also involved in immunity to other tumors, including colon, breast, pancreatic and prostate carcinomas [24, 32].

Since tumor-associated antigens and the corresponding peptides are self antigens, immunotherapies are designed to elicit autoimmune responses. Induction of autoimmunity in otherwise normal individuals is, however, complicated by the organism's use of several mechanisms to confer tolerance to self constituents and avoid development of autoimmune diseases. Such tolerance mechanisms include negative selection in the thymus of strongly self-reactive T cell precursors, deletion or anergy of mature peripheral T cells mediated by incompetent antigen-presenting cells such as immature dendritic cells (DCs), and immunosuppression by regulatory/suppressor T cell subsets, most notably $CD4^+CD25^+$ T cells [24]. Several of these tolerance mechanisms have been invoked to explain the absence of or only weak immune responses to tumor-associated antigens. Nonetheless, even if central tolerance is properly applied, low-affinity self-reactive T cells do escape to the periphery. Moreover, because not all self antigens are present in the thymus, high-affinity self-reactive T cells might also be exported, but remain either quiescent due to peripheral tolerance mechanisms, or "ignorant" when the corresponding self antigens are sequestered behind anatomic barriers [9, 24, 32, 53, 57, 58, 63]. Therefore, current strategies for cancer immunotherapy attempt to target such low-affinity and/or ignorant self-reactive T cells for engagement, expansion and activation.

A number of approaches have been used to elicit T cell responses against tumor-associated self antigens, including vaccination with syngeneic or allogeneic tumor cells engineered to express various cytokines and costimulatory molecules [2, 15, 34, 49, 66, 71, 72], antigenic peptides modified to enhance MHC binding and TCR triggering, and delivered in conjunction with cytokines, chemokines and other immunomodulatory factors [1, 6, 21, 41, 61, 65, 84], and recombinant viral or cDNA-based vaccines [7, 32, 63]. DCs fused with tumor cells, pulsed with tumor lysates or synthetic peptide epitopes, or transfected with tumor-cell derived RNA or DNA have also been used [25, 30, 40, 54]. Additional therapeutic interventions were designed to target immunological checkpoints, including regulatory $CD4^+CD25^+$ T cells [22, 70] and T cell inhibitory receptors such as CTLA-4 [19, 59, 73]. Notwithstanding the encouraging results obtained with these approaches, effective immunotherapies that consistently induce objective clinical responses have not yet been developed for any type of malignancy [7, 8, 64], likely a reflection of the fact that breaking tolerance for self antigens remains a major challenge. As discussed below, such a process might be facilitated when T cell stimulation occurs under conditions of lymphopenia.

Role of self antigens in T cell homeostasis and lymphopenia-induced homeostatic proliferation

For many years it was thought that, subsequent to thymic selection and exportation to the periphery, mature T cells would ignore self-peptide/MHC ligands and remain dormant in the secondary lymphoid organs until engaged by activated antigen-presenting cells displaying foreign antigenic peptides. However, recent studies demonstrated that signals from self-peptide/MHC ligands and/or specific cytokines are required by peripheral T cells to survive under lymphocyte-sufficient conditions and homeostatically expand under lymphopenic conditions (Fig. 1). .

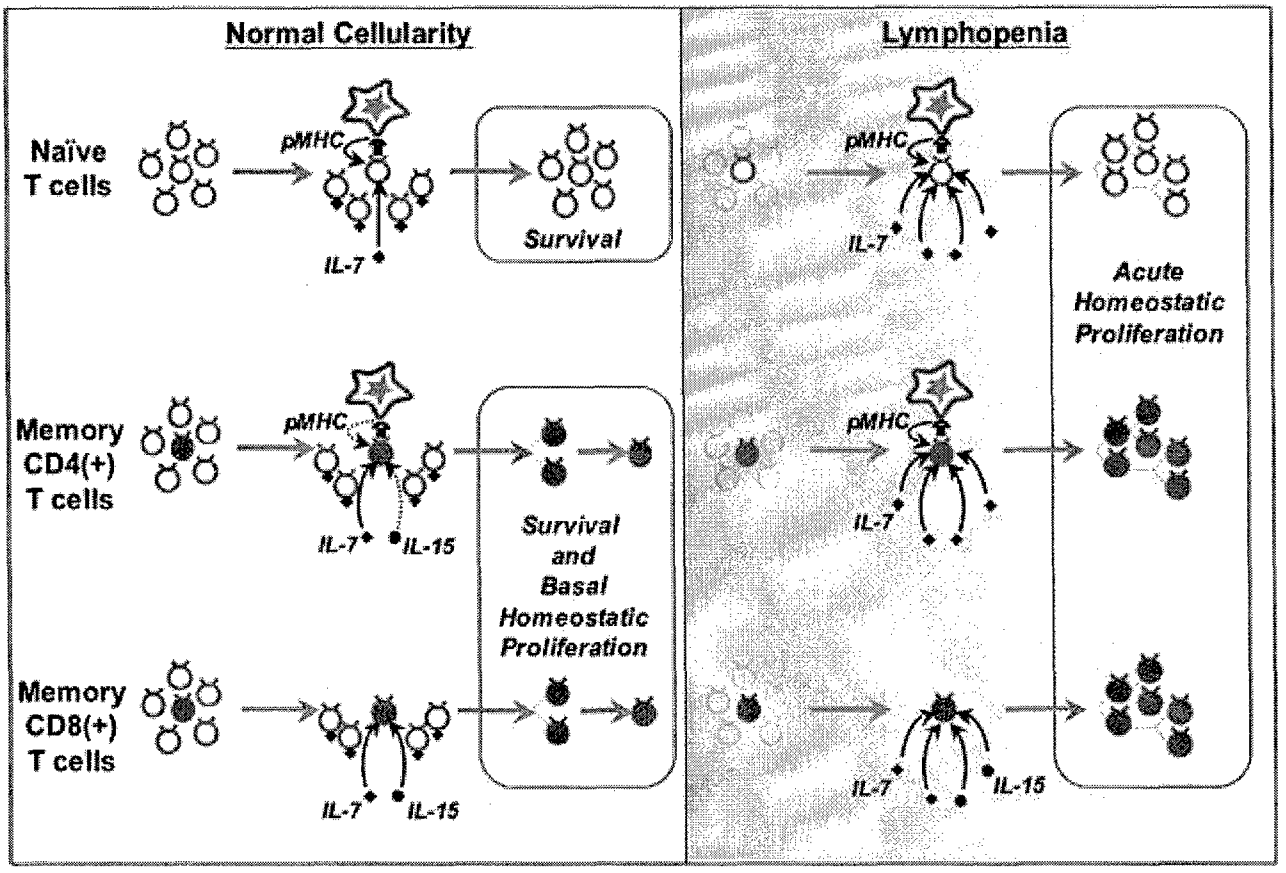

Fig. 1 T cell homeostasis under lymphocyte-sufficient and lymphopenic environments. In a lymphocyte-sufficient state (*left panel*), survival of naïve T cells requires recognition of self-peptide-MHC (*pMHC*) and IL-7 for $CD8^+$ cells, and IL-7 for $CD4^+$ cells. Similarly, IL-7 and, partially, IL-15 and pMHC are required for survival and basal homeostatic proliferation of memory $CD4^+$ T cells, whereas IL-15 or IL-7 are needed for survival and basal homeostatic proliferation of memory $CD8^+$ T cells. Under conditions of lymphopenia (*right panel*), an excess of trophic cytokines (and perhaps reduced competition for pMHC) promotes acute homeostatic proliferation of the remnant (or infused) T cells, with pMHC and IL-7 required for proliferation of naïve T cells, pMHC or IL-7 required for proliferation of memory $CD4^+$ T cells, and IL-15 or IL-7 required for proliferation of memory $CD8^+$ T cells

In a lymphocyte-sufficient state, survival of naïve $CD4^+$ T cells requires IL-7, whereas survival of $CD8^+$ T cells depends on IL-7 and TCR interaction with self-peptide/MHC ligands [11, 29, 39, 62, 67, 69, 75, 77, 81, 82, 83]. In contrast, survival of memory T cells is MHC independent, but requires IL-7 for $CD4^+$ cells, and IL-7 or IL-15 for $CD8^+$ cells [42, 46, 52, 60, 69, 74]. Furthermore, the continuous stem cell-like self renewal of memory T cells, termed "basal homeostatic proliferation", which assures the long-term maintenance of the memory T cell pool, is dependent on IL-7 (and, to a lesser extent, IL-15 and MHC) for memory $CD4^+$ T cells, and on IL-15 (and, to a lesser extent, IL-7) for memory $CD8^+$ T cells [5, 42, 43, 46, 68, 85].

Additional studies showed that, under lymphopenic conditions, the remaining naïve and memory T cells proliferate to reconstitute a nearly normal lymphocyte pool, a process termed "acute homeostatic proliferation". This proliferation requires self-peptide/MHC recognition and IL-7 for naïve $CD4^+$ and $CD8^+$ T cells [20, 26, 36, 81], whereas proliferation of memory $CD4^+$ T cells requires either self-peptide/MHC or IL-7, and proliferation of memory $CD8^+$ T cells needs either IL-7 or IL-15 [28, 37, 68, 76].

T cell proliferation in lymphopenic, but not lymphocyte-sufficient, states is likely due to the excess of the required cytokines and, possibly, self-peptide/MHC ligands. As the lymphocyte pool size is restored, increased competition among cells reduces availability of these factors, leading to progressive inhibition of proliferation. Importantly, not all T cells appear to undergo lymphopenia-induced homeostatic expansion with the same efficiency, and experiments with TCR-transgenic T cells suggest that this could be due to a preferential engagement of cells exhibiting higher affinity for self [23, 47, 50, 80]. This observation suggests that provision of large amounts of defined antigens through vaccination or other means is likely to result in a preferential engagement and expansion of T cell clonotypes exhibiting increased affinity for such antigens.

An additional relevant observation is that the expanded T cells acquire a significant number of activation/memory phenotype markers, as well as effector functions [13, 14, 23, 27, 51, 56]. Thus, TCR-transgenic $CD8^+$ T cells that had undergone homeostatic proliferation were shown to kill target cells in vivo in a peptide- and TCR-dependent manner, and to produce IFN-γ after in vitro stimulation with anti-CD3 [13, 56]. Similarly, after (but not before) homeostatic proliferation, polyclonal $CD8^+$ T cells rapidly secreted IFN-γ in response to in vitro anti-CD3 stimulation and killed concanavalin A-coated syngeneic targets [13]. It seems, therefore, that lymphopenia not only promotes homeostatic proliferation, but also reduces the activation threshold of specific T cells, which will then be more easily recruited into specific immune responses, including those directed against self antigens when these are available in sufficient amounts.

Homeostatic T cell proliferation and pathogenic autoimmunity

Considering that lymphopenia-induced homeostatic proliferation favors expansion of T cells with increased affinity for self-peptide/MHC ligands and generates a population of semi-activated T cells with significant effector functions, we have hypothesized that chronic recurrence of this process and availability of specific self-antigens in predisposed individuals might contribute to the initiation and/or progression of certain autoimmune diseases [4, 79]. There is indeed considerable evidence suggesting that lymphopenia might be associated with autoimmunity. In humans, lymphopenia has been detected in patients with type-1 diabetes, rheumatoid arthritis, Sjögren's syndrome and lupus, and autoimmune manifestations are frequently observed in lymphopenic patients with AIDS. In rodent models, lymphopenia-associated autoimmunity was observed after neonatal thymectomy, discontinuation of cyclosporine treatment, total lymphoid irradiation, or infection with a mouse T lymphotropic virus [79].

To obtain direct experimental evidence that, in specific conditions and genetic backgrounds, homeostatic T cell proliferation may lead to autoimmune manifestations, studies were performed in lupus-predisposed BXSB mice [45]. Due to an undefined Y-chromosome-associated autoimmunity accelerator gene termed *Yaa*, male BXSB mice develop a T cell-mediated early lupus-like autoimmunity, whereas females (Yaa^-) are relatively free of disease. Yet, like Yaa^+ T cells, Yaa^- T cells from female mice induced accelerated disease after adoptive transfer and homeostatic expansion in lymphopenic TCR α-deficient BXSB males. Additional support for the role of homeostatic T cell proliferation in autoimmunity has been provided by more recent studies in non-obese diabetic (NOD) mice [38]. Unlike non-diabetic controls, NOD mice spontaneously exhibited a considerable level of lymphopenia, with modestly decreased numbers of $CD4^+$ T cells and B cells, and supported homeostatic expansion of adoptively transferred TCR-transgenic T cells. Lymphopenia and the compensating homeostatic T cell proliferation in NOD mice were attributed to defective survival of activated T cells caused by increased production of, and response to, IL-21, a cytokine that supports T cell proliferation but not survival. Importantly, lymphopenia was corrected, homeostatic proliferation inhibited, and diabetes prevented when the number of T cells in NOD mice was increased by infusion of large numbers of $CD4^+$ T cells from isogeneic donors.

The combined findings clearly indicate that autoimmunity might ensue in predisposed backgrounds as a consequence of homeostatic T cell proliferation. This outcome might be the result of either increased capacity to activate T cells (BXSB male environment), or defective central tolerance together with repeated cycles of T cell proliferation and apoptosis (NOD environment). As we have recently reviewed [4], other contributing factors, such as frequency of lymphopenic episodes, shape of the T cell repertoire, enrichment of high-affinity clones and availability of specific self-antigens, will determine whether pathogenic autoimmunity will develop and the type of the consequent clinical entity.

Homeostatic T cell proliferation and beneficial autoimmunity in cancer

Based on the observation that under lymphopenic conditions self-peptides can induce homeostatic proliferation and activation of T cells and even promote autoimmune manifestations, we tested whether induction of homeostatic T cell proliferation in the presence of tumor-derived antigens could lead to an efficient anti-tumor autoimmune response that, in this case, would obviously be beneficial to the host [17]. C57BL/6 (B6) mice were sublethally irradiated, transfused 24 h later with either 5×10^6 or 5×10^7 syngeneic lymph node (LN) cells, and subsequently challenged subcutaneously (s.c.) with 5×10^5 B16-derived B78D14 melanoma cells. Transfusion of the smaller LN cell dose led to a reduction in tumor size compared to non-transfused, sublethally irradiated mice. Tumor growth inhibition was even more evident in mice transfused with 5×10^7 LN cells, with tumors reduced to almost undetectable levels in ~50% of the animals at 30 days post-challenge.

The specificity of the anti-tumor response elicited through homeostatic T cell proliferation was evaluated by assessing in vitro cytotoxicity and IFN-γ production in T cells isolated from B78D14 tumor-bearing mice [17]. A strong cytotoxic response was elicited against B78D14 cells,

whereas lysis of control MC-38 colon carcinoma cells was marginal, even at high effector-to-target cell ratios. Similarly, high IFN-γ production was detected after in vitro restimulation with B78D14 tumor cells, but not with control MC-38 cells. The specificity of the effect was further defined by studying memory responses. When rechallenged with the same melanoma cells and with control MC-38 cells 80 and 200 days after the first challenge, mice completely rejected the melanoma cells, but not the colon tumor cells. Additional signs of anti-melanoma autoimmunity included vitiligo, primarily in the area above the tumor cell injection site, as seen in other types of melanoma immunotherapy and generally considered to reflect therapeutic efficacy. No other macroscopic evidence of autoimmunity was noted in experimental animals.

The observed anti-tumor response was not mediated by a nonspecific effect of irradiation, since B6.RAG$^{-/-}$ mice (lacking T and B cells) showed identical tumor growth whether irradiated or not prior to tumor inoculation, whereas significant inhibition was observed upon T cell transfusion in non-irradiated B6.RAG$^{-/-}$ mice [17]. T cell transfusion without homeostatic expansion was also ineffective, since tumor growth was unimpeded in non-irradiated lymphocyte-sufficient mice transfused with 5×10^6 or 5×10^7 syngeneic LN cells. Instead, the process required homeostatic expansion of polyclonal T cells, which likely include tumor-specific T cells. In fact, compared to mice treated with irradiation alone where homeostatic proliferation of the remnant T cells occurs, but is protracted, tumor growth was not further impaired following transfusion of TCR transgenic T cells with unrelated specificity despite efficient homeostatic expansion. Additional experiments indicated that homeostatic expansion of highly purified (>99%) CD8$^+$ T cells efficiently inhibited melanoma growth, suggesting a limited participation of helper CD4$^+$ T cells.

We also showed that the anti-tumor response required T cell proliferation in the regional LNs, the likely site where tumor antigen-presenting cells are encountered [17]. Thus, the effect was impaired in recipient mice lacking LNs due to deletion of the lymphotoxin-α gene (LT-$\alpha^{-/-}$) despite efficient proliferation of the transfused B6 T cells in the spleen. More importantly, transfusion of T cells lacking β7 integrin and L-selectin, which do not home into LNs but home and proliferate efficiently in the spleen, did not enhance the anti-tumor response compared to irradiation.

These results provide the theoretical explanation for earlier observations in mice that lymphopenia induced by sublethal irradiation [31] or treatment with cyclophosphamide [55] might be beneficial for the treatment of tumors (reviewed in [48, 78]). More recently, a stronger anti-tumor effect was observed when mice were vaccinated with modified autologous tumor cells immediately after myeloablative therapy and bone marrow reconstitution [10]. Similarly, using a mouse breast tumor model, it was shown that immunization with DCs pulsed with whole tumor cell lysates in the early phase of bone marrow reconstitution led to efficient anti-tumor responses [3]. Finally, it was shown that tumor-specific T cells can preferentially expand in lymphopenic RAG1$^{-/-}$ mice following a melanoma vaccine [33]. The possibility that tumor vaccination in humans could be improved when combined with bone marrow cell transplantation has also been discussed [44]. Interestingly, a recent study reported high response rates in patients with metastatic melanoma after non-myeloablative lymphodepletion and transfusion with highly selected anti-tumor T cells [16]. Unlike studies described above, however, this latter approach required the in vitro expansion and antigen-based selection of specific tumor-infiltrating lymphocytes, an expensive and time-consuming procedure that cannot be easily implemented in a large number of patients. We propose that selection of tumor-specific T cells may occur spontaneously in vivo during homeostatic expansion, provided that sufficient tumor-antigens are presented in the draining LN under conditions of lymphopenia, wherein enhanced availability of cytokines and peptide/MHC ligands might result in decreased T cell activation thresholds (Fig. 2).

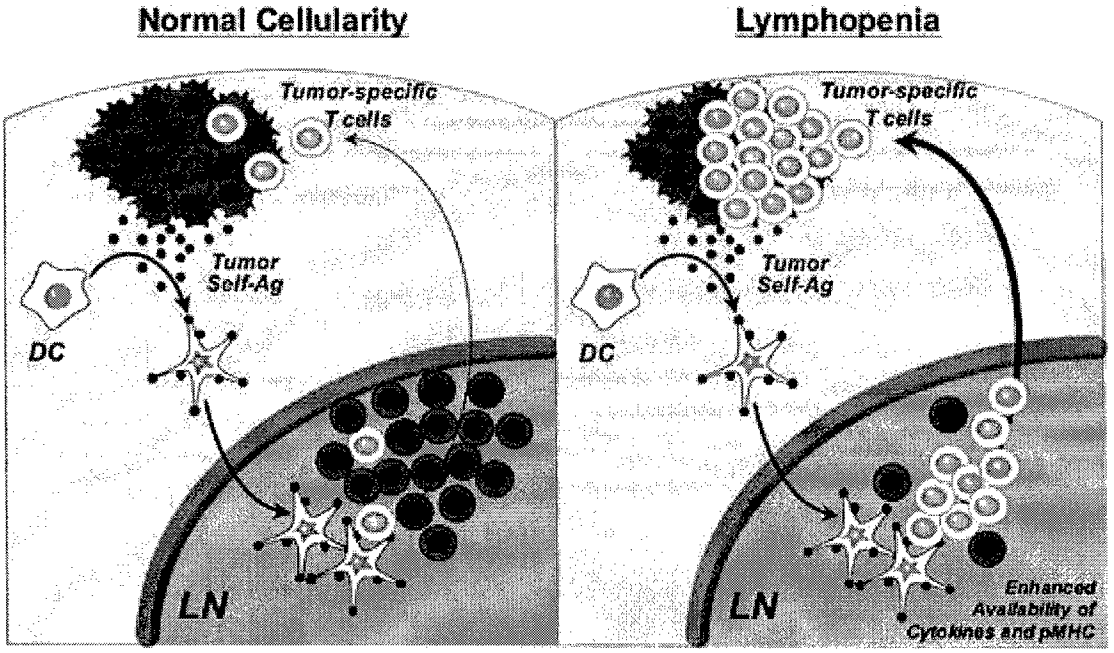

Fig. 2 Postulated mechanism by which homeostatic T cell proliferation promotes anti-tumor autoimmunity. In a lymphocyte-sufficient state (*left panel*), the low-affinity T cells that recognize self tumor-antigens are inefficiently engaged and only a few migrate into the tumor. In contrast, under conditions of lymphopenia (*right panel*), wherein self-peptide/MHC-based polyclonal expansion of T cells occurs, enhanced availability of cytokines and peptide/MHC (*pMHC*) ligands presented by tumor antigen-displaying DCs in the draining LNs decreases the activation threshold of tumor-specific T cells, leading to their preferential engagement and expansion (*LN* lymph node)

Conclusions

Considerable advances have been made in recent years in defining the nature of tumor antigens and devising innovative approaches for the immunotherapy of cancer. Nevertheless, clinical success has been limited primarily by the difficulty of overcoming T cell tolerance for such antigens. Recent clarifications of the role of self recognition in lymphopenia-induced homeostatic T cell proliferation and the acquisition of a semi-activated state by the proliferating cells led us and others to suggest that T cell priming through this process might overcome T cell tolerance against tumor antigens, and data in various tumor models strongly support this concept. Future studies will determine whether this approach can be applied to tumors with different immunological and histological characteristics, including primary, metastatic and established solid tumors, and will provide a more detailed characterization of mechanistic issues such as the exact process by which tolerance is broken, direct or indirect means of tumor antigen presentation, efficacy of refined T cell subsets, and the potentiating effects of homeostatic proliferation-promoting cytokines. Nonetheless, the results thus far indicate that the process of tumor-specific vaccination and transfusion of naïve unprimed autologous T cells administered in conjunction with, or immediately after, conventional lymphopenia-inducing anti-cancer therapies may be a simple means to elicit effective tumor immunity.

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γδ T Cell Homeostasis Is Controlled by IL-7 and IL-15 Together with Subset-Specific Factors[1]

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Among T cell subsets, γδ T cells uniquely display an Ag receptor-based tissue distribution, but what defines their preferential homing and homeostasis is unknown. To address this question, we studied the resources that control γδ T cell homeostasis in secondary lymphoid organs. We found that γδ and αβ T cells are controlled by partially overlapping resources, because acute homeostatic proliferation of γδ T cells was inhibited by an intact αβ T cell compartment, and both populations were dependent on IL-7 and IL-15. Significantly, to undergo acute homeostatic proliferation, γδ T cells also required their own depletion. Thus, γδ T cell homeostasis is maintained by trophic cytokines commonly used by other types of lymphoid cells, as well as by additional, as yet unidentified, γδ-specific factors. *The Journal of Immunology,* **2005, 174: 4606–4612.**

T cells expressing γδ TCR constitute a significant fraction of lymphocytes in secondary lymphoid organs and blood, and predominate in mucosa and epithelia of various tissues (reviewed in Refs. 1–4). In support of the role played by these cells in both innate and adaptive immunity, mice lacking γδ T cells show a variety of abnormalities, including enhanced susceptibility to bacterial and viral infections, higher incidence of tumor development, defective wound healing, airway hyperresponsiveness, and increased inflammatory reactions (reviewed in Refs. 3 and 5). γδ T cells with pro- or anti-inflammatory functions have also been implicated in various experimental and spontaneous models of autoimmunity (reviewed in Ref. 6).

A unique feature of γδ T cells is the correlation between TCR structure and tissue localization. In mice, for example, Vγ3 is primarily expressed by T cells found in the skin, Vγ4 is mostly expressed in the female reproductive tract and the tongue, Vγ1 and Vγ2 predominate in lymph nodes (LNs),[5] spleen, and blood, whereas Vγ5 defines T cells in the intestinal epithelium (1). Intriguingly, the various γδ T cell subsets are not produced simultaneously, but rather in sequential waves during defined periods of fetal, neonatal, or adult life, a phenomenon apparently regulated by specific programs of preferential Vγ gene rearrangements. The fact that γδ T cell subsets produced early in life persist in the adult and can even become the dominant lymphocyte populations in the corresponding tissues implies the existence of specific homeostatic mechanisms that define the size of these cell subsets by controlling preferential homing, survival, and/or expansion. However, the factors that govern the homeostasis of γδ T cell populations have thus far not been explored.

Lymphocyte homeostasis is a fundamental process by which the overall T and B cell pool sizes are maintained at remarkably constant levels despite the changes throughout life, including age-associated declines in lymphogenesis, expansions and contractions of Ag-engaged cell populations, conversion of selected clonotypes into long-lived memory cells, and loss of cellular functions through anergy, exhaustion, or senescence. Several studies, primarily focusing on αβ T cells, have recently provided molecular and cellular explanations of lymphocyte homeostasis regulation (reviewed in Refs. 7–11). Under lymphocyte-sufficient conditions, survival (without proliferation) of naive $CD4^+$ and $CD8^+$ T cells depends on at least two independent signals, i.e., TCR interaction with self-peptide/MHC ligands and availability of IL-7. In contrast, survival of memory $CD4^+$ and $CD8^+$ T cells is MHC independent, but requires IL-7. Furthermore, the continuous stem cell-like self-renewal of memory T cells, termed "basal homeostatic proliferation", that assures the long-term maintenance of the memory T cell pool is dependent on IL-7 (and, to a lesser extent, on IL-15 and MHC recognition) for memory $CD4^+$ T cells and on IL-15 (and, to a lesser extent, on IL-7) for memory $CD8^+$ T cells. Additional studies showed that, under conditions of lymphopenia, αβ T cells proliferate to reconstitute a nearly normal lymphocyte pool, a process termed "acute homeostatic proliferation". This proliferation requires self-peptide/MHC recognition and IL-7 for naive $CD4^+$ and $CD8^+$ T cells, whereas proliferation of memory $CD4^+$ T cells requires either MHC or IL-7 and proliferation of memory $CD8^+$ T cells needs either IL-7 or IL-15. Similar approaches have been used to define the homeostatic requirements for NK cells, NKT cells, and B cells. For homeostatic expansion in lymphopenic mice, NK cells require IL-15 (12), NKT cells require IL-15 (and less IL-7) but not the Ag-presenting CD1d molecule (13), and B cells require Btk-mediated signals but not IL-7 (14).

In the present study, we sought to define the homeostatic requirements of γδ T cells. We show that, like all other major lymphocyte types, γδ T cells from secondary lymphoid organs undergo homeostatic expansion after adoptive transfer into lymphopenic recipients. We also demonstrate that homeostatic γδ T cell proliferation requires depletion of both γδ and αβ T cell

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[5] Abbreviations used in this paper: LN, lymph node; β_2M, β_2-microglobulin.

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populations, signaling from either IL-7 or IL-15, as well as additional γδ T cell-specific factors.

Materials and Methods

Mice

C57BL/6 (B6, Thy1.2$^+$ CD45.2$^+$), B6.PL (Thy1.1$^+$), and B6.Ly5a (CD45.1$^+$) congenic mice were obtained from the breeding facility of The Scripps Research Institute. B6.RAG-1$^{-/-}$, B6.TCRδ$^{-/-}$, B6.TCRα$^{-/-}$, and B6.β_2M$^{-/-}$ mice were purchased from The Jackson Laboratory. B6.I-Aβ$^{-/-}$, B6.IL-7$^{-/-}$, B6.IL-15$^{-/-}$, and double-deficient B6.IL-7$^{-/-}$IL-15$^{-/-}$ mice have been previously described (15). All mice were maintained under specific pathogen-free conditions and all experimental protocols were approved by the Institutional Animal Care Committee.

Donor cells

Peripheral γδ T cells were enriched by negative selection using panning- and magnetic bead-based procedures. Briefly, single cell suspensions were prepared from spleen and inguinal, axillary, brachial, and cervical LN of B6.PL or B6.Ly5a mice. Splenocytes were purified by density gradient centrifugation on Lympholyte-M (Cedarlane Laboratories) and pooled with LN cells. The resulting cell suspensions were first incubated (1 h at room temperature) in flasks coated with anti-mouse IgG and anti-mouse IgM Abs (Caltag Laboratories). Unbound cells were then incubated (45 min, rotating at 4°C) with rat Abs (BD Pharmingen) specific for mouse CD4 (RM4-5), CD8 (53-6.7), CD45R (RA3-6B2), and MHC class II (M5/114.15.2). After washing with DMEM-2% FCS, cells were incubated (45 min, rotating at 4°C) with magnetic beads coated with anti-rat Ig (BioMag; Qiagen). Bead-coated cells were removed using a magnet (Advanced Magnetics), and the unbound cells were washed in DMEM-2% FCS and purified by Lympholyte-M gradient centrifugation. As assessed by flow cytometry, γδ T cells were typically enriched to 40–50%.

Adoptive cell transfers

Donor cells consisting of either enriched γδ T cells or total LN cells were stained with CFSE (Molecular Probes). Briefly, cells were washed in PBS-0.1% BSA, resuspended to 10^7 cells/ml in prewarmed (37°C) PBS-0.1% BSA containing 10 μM CFSE, incubated for 10 min at 37°C, and washed twice with cold DMEM-20% FCS and DMEM. Aliquots of 1.5–3 × 10^6 cells were injected i.v. into either unmanipulated or sublethally irradiated mice (exposed to 600 rad whole body irradiation 1 day before). At the indicated time points, mice were sacrificed, and LN and spleen cells were harvested and analyzed by flow cytometry. In some experiments, recipient mice were injected with recombinant mouse IL-7 (R&D Systems; 1 μg per day s.c.) for five days, starting before cell transfusion.

FACS analysis

mAbs to mouse Thy1.1, CD45.1, γδTCR, CD44, CD25, CD69, CD62L, IFN-γ, TCRVγ2, CD127, CD27, CD122, and streptavidin, either biotinylated or conjugated to PE, PerCP or APC, were all purchased from BD Pharmingen. For surface staining, cells were sequentially incubated with various combinations of Abs or streptavidin, washed, and analyzed on a four-color FACSCalibur (BD Biosciences). Calibration and color compensation were performed using fluorescent beads and single color controls per standard procedures (BD Biosciences). For intracellular staining of IFN-γ, cells were incubated with brefeldin A to inhibit intracellular protein transport and subsequently stained with appropriate Abs to surface molecules, fixed in formaldehyde, resuspended in saponin buffer containing 1 μg of anti-IFN-γ Ab, and analyzed on a FACSCalibur (BD Biosciences).

Immunohistochemistry

LNs and spleen from 6- to 8-wk-old B6 mice were frozen in Tissue-Tek compound (Sakura Finetek). Sections (6 μm) were acetone-fixed, air-dried, rehydrated in wash buffer (PBS supplemented with 0.7% FCS, 0.07% sodium-azide and 0.1% Tween 20) and stained for 30 min with FITC- or PE-conjugated Abs to γδTCR, αβTCR, or CD45R. After 20 min washing, sections were fixed in PBS-1% paraformaldehyde, mounted (Dako mounting medium; DakoCytomation), and examined with a Zeiss axiovert microscope and Spot 32 software.

Results

γδ T cells undergo homeostatic expansion in sublethally irradiated mice

To study γδ T cell homeostasis, peripheral γδ T cells were isolated from LN and spleen of unmanipulated B6.PL (Thy1.1$^+$) or B6.Ly5a (CD45.1$^+$) mice, stained with CFSE, and transferred into either nonirradiated or sublethally irradiated B6 (Thy1.2$^+$, CD45.2$^+$) recipients. Because cell sorting using γδ TCR-specific Abs tends to activate target cells, enrichment of γδ T cells was accomplished by negative selection. Seven days after transfer into nonirradiated recipients, no evidence of cell division was observed, with all donor γδ T cells uniformly retaining high levels of CFSE (Fig. 1*a*). In contrast, γδ T cell homeostatic proliferation was detected in hosts rendered T cell-deficient by sublethal irradiation, as indicated by multiple peaks of decreasing CFSE intensity (Fig. 1, *a* and *b*). Thus, ~60% of donor γδ T cells recovered 7 days after transfer had undergone one to four cell divisions, with no significant differences between LN and spleen. After 14 days, the frequency of γδ T cells that had divided increased to ~86%, with >20% of the donor cells exhibiting undetectable CFSE levels, which typically indicates at least seven cell divisions.

Comparisons with αβ T cell subsets at day 7 posttransfer indicated a similar frequency of proliferating cells between γδ T cells

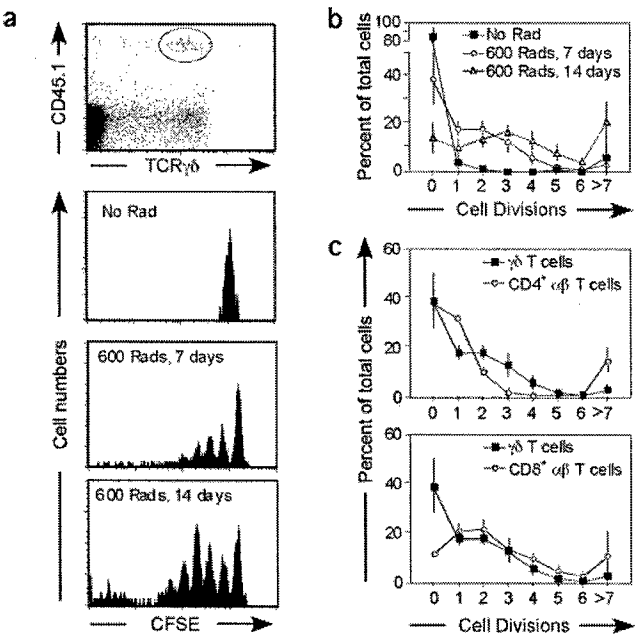

FIGURE 1. Peripheral γδ T cells undergo homeostatic expansion in lymphopenic recipients. Donor γδ T cells isolated from LN and spleen of B6.Ly5a (CD45.1$^+$) or B6.PL (Thy1.1$^+$) mice, were labeled with CFSE and adoptively transferred into either unmanipulated or sublethally irradiated (600 rad), allelically different (CD45.2$^+$, Thy1.2$^+$) B6 recipients. Control mice were injected with LN cells containing CD4$^+$ and CD8$^+$ αβ T cells. At day 7 or 14 posttransfer, LN and spleen cells were stained for either donor (CD45.1 or Thy1.1) γδ T cells or donor CD4$^+$ and CD8$^+$ αβ T cells and analyzed by FACS. *a*, Analysis of donor γδ T cells present in the LNs of recipient mice, 7 or 14 days after infusion. Shown are a representative dot plot of live-gated cells from nonirradiated hosts (*top panel*), and CFSE profiles of gated donor γδ T cells after 7 days in a nonirradiated recipient (*second panel*), or after 7 days (*third panel*) or 14 days (*forth panel*) in irradiated recipients. Similar data were obtained by analyzing donor γδ T cells present in the spleen of recipient mice. *b*, Percentage of donor γδ T cells in the various cell divisions as determined by CFSE profile analysis. Data represent average ± SD. ■, Nonirradiated hosts, 7 days posttransfer (n = 5); ○, irradiated hosts, 7 days posttransfer (n = 16); △, irradiated hosts, 14 days posttransfer (n = 7). *c*, Comparison of homeostatic expansions of γδ vs αβ T cells. Data are percentages (average ± SD) of donor cells in the various cell divisions as determined by CFSE profile analysis 7 days after transfer into irradiated hosts. ■, γδ T cells (n = 16); ○, CD4$^+$ (n = 4) or CD8$^+$ (n = 5) αβ T cells.

and CD4$^+$ αβ T cells, although γδ T cells had more cells in divisions 2, 3, and 4 (Fig. 1*c*, *top panel*). Moreover, although more CD8$^+$ T cells had proliferated (88%), the overall CFSE profiles of cells in divisions 1 through >7 were similar to γδ T cells, suggesting comparable proliferation kinetics (Fig. 1*c*, *bottom panel*).

During homeostatic proliferation, conventional αβ T cells acquire a memory-like phenotype characterized by increased expression of CD44, but not CD69 and CD25, and loss of CD62L (9). Similar phenotypic changes were observed with γδ T cells (Fig. 2). Before transfer, most γδ T cells expressed low levels of CD44, CD25, and CD69, and approximately one-third exhibited high levels of CD62L (Fig. 2*a*). At day 14 posttransfer, CD44 expression was also low for γδ T cells that had not divided (CFSE^{high}), but progressively increased during the first three cell divisions, remaining high thereafter (Fig. 2*b*, *top panels*). Expression of CD25 and CD69 remained low after transfer, without significant changes between undivided and divided cells. However, as previously reported for purified CD4$^+$CD25$^-$ αβ T cells (16), a slight increase in the expression of CD25 was observed in the transferred γδ T cells. Expression of CD62L was high in γδ T cells that had divided one to five times, but was drastically reduced in cells that had undergone more divisions, with kinetics similar to αβ T cells. In contrast, no changes were observed in the frequency of IFN-γ-producing cells, as determined by intracellular staining before and after homeostatic expansion (data not shown).

Additional changes were revealed by expression analysis of TCR Vγ2, one of the two dominant Vγ in lymphoid organs (1). Before transfer, Vγ2$^+$ T cells represented 27.4 ± 1.7% of LN γδ T cells (n = 5). However, 14 days after transfer into irradiated hosts, Vγ2$^+$ T cells represented 39.5 ± 3.5% of nondivided (CFSE^{high}) γδ T cells and 50.2 ± 5.0% of cells that had divided up to five times, but only 13.5 ± 2.1% of γδ T cells in division 6, and 10.5 ± 3.5% of cells that divided more than seven times (n = 2). These results suggest that Vγ2$^+$ T cells exhibit slower proliferation rates compared with Vγ2$^-$ (presumably Vγ1$^+$) γδ T cells.

γδ T cells compete with αβ T cells during homeostatic proliferation

The above experiments indicated that peripheral γδ T cells undergo homeostatic expansion in lymphopenic recipients, but not in lymphocyte-sufficient hosts, suggesting competition with other cells for space, ligands, or trophic cytokines. To characterize the cell types competing with γδ T cells for homeostasis-controlling factors, γδ T cells were adoptively transferred into mutant recipients that selectively lacked specific lymphocyte subsets. γδ T cells proliferated in nonirradiated syngeneic RAG-1$^{-/-}$ mice lacking both γδ and αβ T cells (Fig. 3, *upper panels*). Because RAG-1$^{-/-}$ mice retain NK cells, these results suggest that, similar to αβ T cells, but unlike NK cells (12), γδ T cells are not significantly inhibited by NK cells during homeostatic proliferation. In addition, although more γδ T cells proliferated in RAG-1$^{-/-}$ than in irradiated B6 mice (~77 vs 60%), CFSE profiles indicated similar cell division kinetics in these recipients. This contrasted with αβ T cells, which showed a dramatic increase in proliferation rates in RAG-1$^{-/-}$ compared with irradiated B6 hosts (Fig. 3, *right upper panel*).

We next examined whether selective depletion of γδ T cells in TCRδ$^{-/-}$ mice is sufficient to initiate homeostatic proliferation of γδ T cells. As expected, control CD4$^+$ and CD8$^+$ αβ T cells did not proliferate in nonirradiated TCRδ$^{-/-}$ recipients, most likely due to inhibition by host αβ T cells. Surprisingly, γδ T cells also failed to proliferate when transfused in these recipients (Fig. 3, *lower panels*). In contrast, both γδ and αβ T cells proliferated in TCRδ$^{-/-}$ mice rendered broadly lymphopenic by sublethal irradiation (data not shown). Thus, αβ T cells are sufficient to completely inhibit homeostatic proliferation of γδ T cells in TCRδ$^{-/-}$ hosts.

Homeostatic proliferation of γδ T cells is MHC independent

The experiments with TCRδ$^{-/-}$ recipients suggested that γδ and αβ T cell populations are controlled by overlapping homeostatic mechanisms. Homeostasis of αβ T cells depends on TCR interactions with MHC/peptide-ligands and/or availability of cytokines

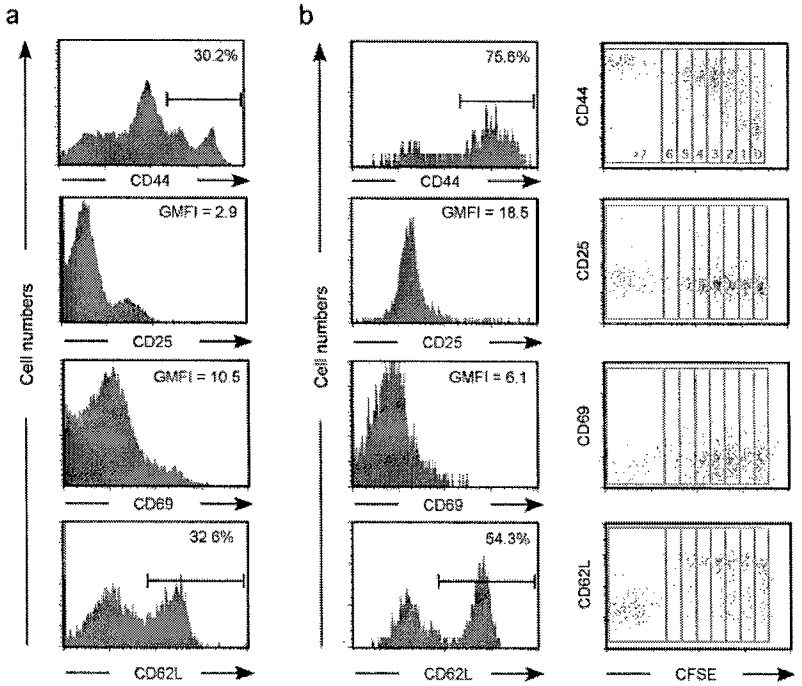

FIGURE 2. γδ T cells acquire a memory-like phenotype during homeostatic expansion. *a*, Expression of CD44, CD25, CD69, and CD62L by γδ T cells freshly isolated from LNs of unmanipulated (nonlymphopenic) B6.PL mice. Numbers represent the percentage of cells expressing high levels of CD44 and CD62L, or the geometric mean of fluorescence intensity (GMFI) of CD25 and CD69 expression (n = 6). *b*, Changes in activation-marker expression during γδ T cell homeostatic proliferation in lymphopenic irradiated mice. Peripheral γδ T cells from B6.PL mice were labeled with CFSE and transfused into irradiated B6 recipients. After 14 days, donor γδ T cells present in the LNs of recipient mice were analyzed by FACS. Expression profiles of CD44, CD25, CD69, and CD62L on gated donor γδ T cells are shown as histograms (*left column*) or as dot plots as a function of CFSE intensity and cell division (*right column*).

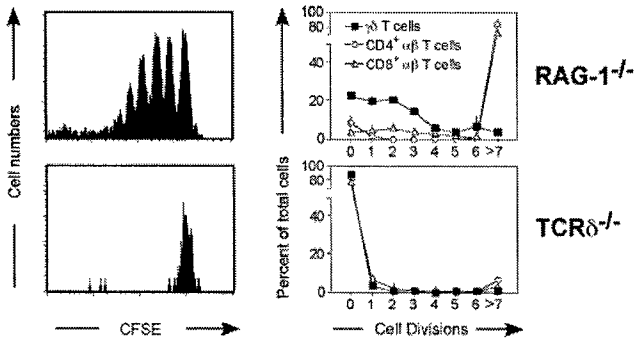

FIGURE 3. γδ T cell homeostatic expansion requires depletion of αβ T cells. γδ T cells from LNs and spleen of B6.Ly5a mice were labeled with CFSE and transfused into nonirradiated RAG-1$^{-/-}$ (*top row*, $n = 3$) or TCRδ$^{-/-}$ (*bottom row*, $n = 3$) congenic mice. Control mice were injected with LN cells containing CD4$^+$ and CD8$^+$ αβ T cells ($n = 3$–5/group). After 7 days, donor γδ T cells present in LNs and spleen of recipient mice were analyzed by FACS. Data are shown as profiles of CFSE intensity on gated donor γδ T cells (*left column*) or as percentage (average ± SD) of cells in various cell divisions as determined by CFSE profile analysis (*right column*).

(9). Although MHC-deficient mice exhibit normal γδ T cell numbers (17), γδ T cell subsets specific for either MHC class II or nonclassical MHC molecules have been identified (18–21). To determine whether MHC molecules play any role in γδ T cell homeostatic proliferation, adoptive transfer experiments were performed using β_2-microglobulin$^{-/-}$ ($\beta_2M^{-/-}$) and I-Aβ$^{-/-}$ mice (Fig. 4). As expected, control CD4$^+$ αβ T cells proliferated normally in irradiated $\beta_2M^{-/-}$ mice, but less efficiently in I-Aβ$^{-/-}$ mice, while the converse was observed for CD8$^+$ αβ T cells (Fig. 4 and data not shown). In contrast, γδ T cells showed virtually identical homeostatic proliferation kinetics in $\beta_2M^{-/-}$, I-Aβ$^{-/-}$, and wild-type B6 mice. These results clearly indicate that homeostatic proliferation of most peripheral γδ T cells is not dependent on MHC recognition.

γδ T cells require either IL-7 or IL-15 for homeostatic proliferation

We next examined the possibility that inhibition of γδ T cell homeostatic expansion by αβ T cells could be explained by competition for IL-7 and IL-15, the main controllers of αβ T cell homeostasis. Both IL-7 and IL-15 are known to play significant roles in γδ T cell development and/or tissue localization (22–28), but their effect on the overall γδ T cell homeostasis has not been studied. Like conventional αβ T cells, most γδ T cells in LN and spleen expressed significant levels of the IL-7Rα chain CD127 (Fig. 5*a*). In addition, a small proportion of γδ T cells expressed higher levels of CD127 and CD44 in both LNs (3–23%, average 9.0 ± 7.1%, $n = 6$) and spleen (3–16%, average 6.9 ± 5.7%, $n = 6$). A similar cell population was almost undetectable among αβ T cells of unmanipulated B6 mice (0.7 ± 0.2% in LNs and 0.9 ± 0.2% in spleen, $n = 6$), and may define an activated subset of Ag-experienced γδ T cells (29). In support of this idea, CD127^{high}CD44^{high} γδ T cells expressed low levels of CD62L and CD27 (Fig. 5*a*). Most γδ T cells also expressed the IL-15Rβ chain (CD122) at levels similar to those exhibited by most αβ T cells (Fig. 5*b*). In addition, a subset of both γδ T cells (12.9 ± 6.4% in LNs and 19.9 ± 6.7% in spleen, $n = 6$) and αβ T cells (4.8 ± 1.4% in LNs and 8.1 ± 1.8% in spleen, $n = 6$) expressed high levels of CD122, intermediate levels of CD44, and high levels of CD62L (Fig. 5*b* and data not shown). Previous studies with αβ T cells indicated that this subset corresponds to memory-phenotype CD8$^+$ T cells (29, 30).

To directly establish the role of IL-7 and IL-15 in γδ T cell homeostatic proliferation, adoptive transfer studies were performed using recipients lacking one or both these cytokines. γδ T cells proliferated in sublethally irradiated IL-7$^{-/-}$ recipients at least as efficiently as in irradiated B6 recipients (Fig. 5*c*). In fact, a small population of CFSE-negative γδ T cells was detected in the LNs of IL-7$^{-/-}$ recipients, and this could reflect faster proliferation rates of IL-7-independent cells in these severely lymphopenic mice. Likewise, γδ T cells showed normal homeostatic proliferation upon adoptive transfer into irradiated IL-15$^{-/-}$ mice. In contrast, homeostatic proliferation of γδ T cells was barely detectable in irradiated IL-7$^{-/-}$IL-15$^{-/-}$ hosts (Fig. 5*c*).

In view of the fundamental roles played by IL-7 and IL-15 in the development of several cell types, including αβ T cells, B cells, NK cells, and NKT cells, and considering the interdependence of lymphocyte cellularity and lymphoid tissue development, it is conceivable that lack of homeostatic expansion in the absence of these cytokines is not due to a direct effect on γδ T cells, but rather to a developmental defect resulting in the absence of specific cell populations required for this function. To exclude this possibility, we tested whether γδ T cell homeostatic proliferation could be corrected in IL-7$^{-/-}$IL-15$^{-/-}$ hosts through short-term provision of exogenous IL-7 (Fig. 5*c*). Indeed, homeostatic expansion of γδ

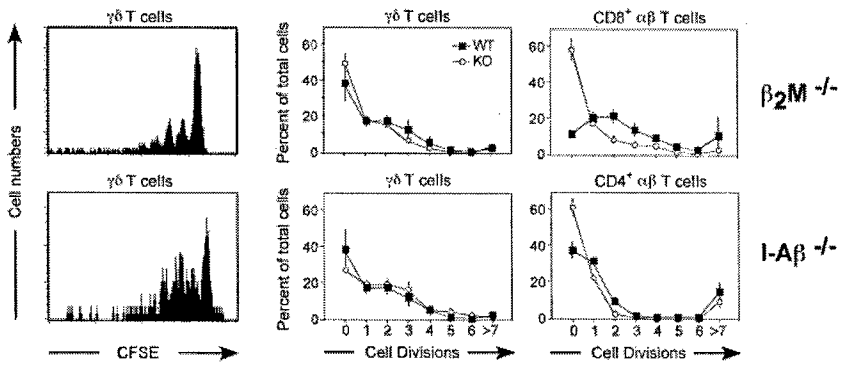

FIGURE 4. γδ T cell homeostatic expansion is MHC independent. γδ T cells from LNs and spleen of B6.Ly5a or B6.PL mice were labeled with CFSE and transfused into sublethally irradiated (600 rad) congenic $\beta_2M^{-/-}$ (*top row*, $n = 4$), I-Aβ$^{-/-}$ (*bottom row*, $n = 3$) or wild-type recipient mice. Control mice were injected with LN cells containing CD4$^+$ and CD8$^+$ αβ T cells ($n = 3$/group). After 7 days, donor γδ T cells present in LNs and spleen of recipient mice were analyzed by FACS. Data represent profiles of CFSE intensity on gated donor γδ T cells (*left column*) or percentages (average ± SD) of cells in various cell divisions as determined by CFSE profile analysis (*middle* and *right columns*). Comparisons are made between percentage of cells dividing in wild-type B6 recipients (wild type (WT), ■) vs $\beta_2M^{-/-}$ or I-Aβ$^{-/-}$ (knockout (KO), ○) recipients.

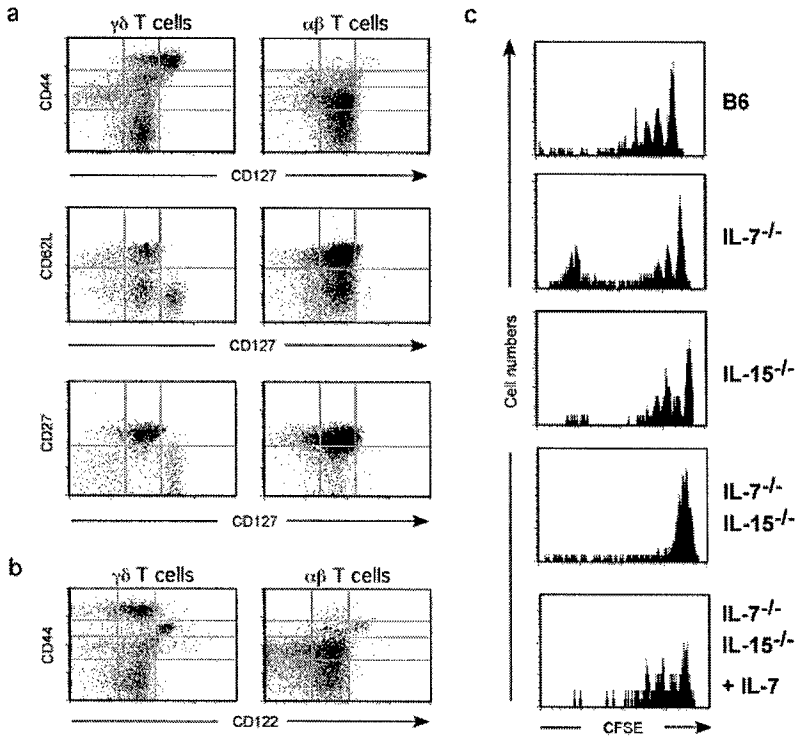

FIGURE 5. γδ T cell homeostatic expansion requires either IL-7 or IL-15. *a*, Expression of IL-7Rα (CD127) vs CD44, CD62L or CD27 on gated γδ T cells (*left column*) or αβ T cells (*right column*) freshly isolated from LNs of unmanipulated (nonlymphopenic) B6.Ly5a mice. Data are representative of two independent experiments (n = 3 per experiment). Similar profiles were obtained with spleen cells. *b*, Expression of IL-15Rβ (CD122) vs CD44. *c*, γδ T cell homeostatic proliferation is inhibited in mice lacking both IL-7 and IL-15. γδ T cells from LN and spleen of B6.Ly5a mice were labeled with CFSE and transfused into either IL-$7^{-/-}$, IL-$15^{-/-}$, or IL-$7^{-/-}$IL-$15^{-/-}$ mice, or into IL-$7^{-/-}$IL-$15^{-/-}$ mice injected with IL-7 (1 μg before transfusion, then 1 μg/day for 5 days). All mice were sublethally irradiated (600 rad) one day before transfusion. At day 7, LN and spleen cells were analyzed by FACS. Representative CFSE profiles of gated donor γδ T cells in the LNs are shown.

T cells was restored if the double-deficient recipients were treated with rIL-7 (1 μg/day, starting on the day of transfusion). Thus, to undergo lymphopenia-induced homeostatic expansion, γδ T cells require either IL-7, as most αβ T cells, or IL-15, as memory $CD8^+$ αβ T cells.

Requirement of γδ T cell-specific factors for homeostatic expansion

If IL-7 and IL-15 were the only factors controlling γδ T cell homeostasis, γδ T cells would be expected to expand in nonirradiated TCR$\alpha^{-/-}$ recipients lacking αβ T cells. Availability of IL-7 and IL-15 in these mice was demonstrated by the fact that adoptively transferred αβ T cells extensively proliferated, at rates similar as in RAG-$1^{-/-}$ mice (Fig. 6). Remarkably, however, no evidence of homeostatic expansion was observed for γδ T cells in nonirradiated TCR$\alpha^{-/-}$ mice (Fig. 6). Because γδ T cells proliferated in RAG-$1^{-/-}$ hosts (Fig. 3), these results indicate that, in addition to αβ T cells, γδ T cells also restrain acute homeostatic expansion of γδ T cells. One possibility for the γδ T cell-mediated inhibition of homeostatic proliferation is that these cells occupy specific niches that need to be accessible during this process. However, as previously reported in humans (31), immunohistochemical analysis of mouse spleen and LNs showed that γδ T cells do not segregate into defined zones (data not shown). Thus, it appears that the size of the γδ T cell pool in lymphoid organs is defined by availability of cytokines commonly used by other lymphoid cells (i.e., IL-7 and IL-15) as well as by additional γδ T cell-specific factors.

Discussion

In this study, we evaluated mechanisms potentially involved in the homeostatic control of γδ T cell populations. We found that spleen and LN γδ T cells survive, but do not proliferate, after transfusion into lymphocyte-sufficient recipients. Similarly, no γδ T cell expansion was observed after transfer into mice lacking either γδ or αβ T cells. In contrast, reductions in the cellularity of both γδ and αβ T cell compartments promoted γδ T cell homeostatic proliferation, but only in mice expressing either IL-7 or IL-15. Thus, in secondary lymphoid organs, γδ T cell homeostasis is controlled, in part, by the size of both the αβ T cell pool, which defines availability of specific cytokines, and the γδ T cell pool, which defines availability of additional γδ T cell ligands. Together with previous reports documenting lymphopenia-induced homeostatic expansion of αβ T cells (9), NKT cells (13), NK cells (12), and B cells (14), the present findings provide further support to the notion that the lymphocyte repertoire is not static, but subject to continuous dynamic changes, partially determined by the symbiotic relationship and interdependence among the various cell types with regard to overlapping resources available in limited supply.

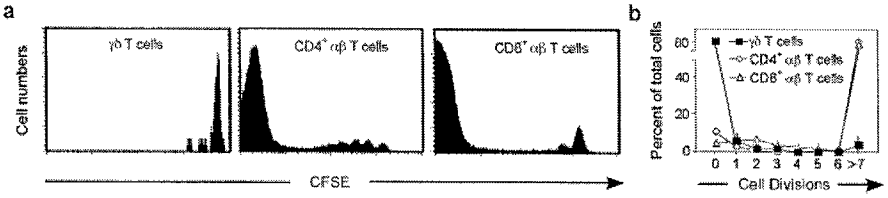

FIGURE 6. γδ T cell homeostatic expansion requires depletion of γδ T cells. γδ T cells from LNs and spleen of B6.Ly5a mice were labeled with CFSE and transfused into nonirradiated TCR$\alpha^{-/-}$ (n = 4) congenic mice. Control mice were injected with LN cells containing $CD4^+$ and $CD8^+$ αβ T cells (n = 3–5/group). After 7 days, donor T cells present in LNs and spleen of recipient mice were analyzed by FACS. Data are shown as profiles of CFSE intensity on gated donor γδ T cells, $CD4^+$ αβ T cells, or $CD8^+$ αβ T cells (*a*) or as percentage (average ± SD) of cells in various cell divisions (*b*) as determined by CFSE profile analysis.

The physiological significance of lymphopenia-induced homeostatic proliferation remains a matter of speculation. Clearly, it occurs in several clinical conditions, such as in lymphodepleted cancer patients during reconstitution with bone marrow or peripheral T cells. However, it is also possible that episodes of lymphopenia targeting subpopulations of cells in defined tissues are common during normal life, as a result of exposure to infectious agents, cytotoxic compounds, radiation or apoptosis-inducing signals. Considering that, during homeostatic expansion, T cell clonotypes with increased affinity for highly represented ligands have a selective advantage (32–34) and acquire a preactivated phenotype (9), it is conceivable that homeostatic proliferation is a mechanism that evolved, in part, to allow a rapid resetting of the lymphocyte repertoire for faster and more efficient immune responses. In support of this possibility, we (35) and others (36, 37) have shown that homeostatic expansion concurrent with immunization generates T cell populations enriched for $CD8^+$ effectors with enhanced antitumor activities. Here, we report that $\gamma\delta$ T cells acquire several activation markers during homeostatic expansion, but whether this principle can be used to more efficiently exploit the anti-tumor potential of these cells remains to be investigated. An additional situation in which homeostatic proliferation may be part of normal physiology is during ontogeny, when the first waves of thymus-derived T cells begin populating secondary lymphoid organs. Indeed, a recent study using bone marrow and thymic-graft models showed that early thymic emigrants expressing $\alpha\beta$ TCR proliferate in neonatal mice in a way regulated by the interaction with self-peptide/MHC and by the size of the peripheral T cell pool (38). Our study suggests that $\gamma\delta$ T cells, known to be among the first to be produced early in life, are also likely to proliferate in the T cell-devoid periphery of neonates, and identifies IL-7 and IL-15 as possible modulators of such expansion.

Unlike NK cells (12), $\gamma\delta$ T cells strongly proliferated in nonirradiated RAG-$1^{-/-}$ recipients, indicating no (or limited) inhibition by NK cells. In contrast, lack of homeostatic expansion in TCR$\delta^{-/-}$ mice suggested that $\gamma\delta$ T cells must compete with $\alpha\beta$ T cells for homeostasis controlling factors. To investigate the mechanisms underlying such competition, we evaluated the involvement of MHC/peptide ligands, IL-7 and IL-15, i.e., the main modulators of $\alpha\beta$ T cell homeostasis. Consistent with the previous observation that $\gamma\delta$ T cell populations are not decreased in MHC-deficient mice (17), we found normal homeostatic proliferation in $\beta_2M^{-/-}$ and I-A$\beta^{-/-}$ recipients. Although we cannot exclude the possibility that $\gamma\delta$ T cell subpopulations reactive, for example, with T10/T22 or class II MHC (18–21), were impaired in these transfers, the results indicated that homeostatic proliferation of most peripheral $\gamma\delta$ T cells is MHC-independent and, hence, that inhibition by $\alpha\beta$ T cells cannot be explained by competition for TCR-ligands. In contrast, lack of homeostatic proliferation in double-deficient IL-$7^{-/-}$IL-$15^{-/-}$ mice, as opposed to normal expansion in single-deficient (IL-$7^{-/-}$ or IL-$15^{-/-}$) mice and in IL-$7^{-/-}$ IL-$15^{-/-}$ mice treated with rIL-7, suggested that $\gamma\delta$ T cell homeostatic expansion requires either IL-7 (like most $\alpha\beta$ T cells) or IL-15 (like memory $CD8^+$ $\alpha\beta$ T cells). Other examples of homeostatic proliferation inhibition among functionally different lymphocyte subsets, such as NK, NKT, and memory $CD8^+$ $\alpha\beta$ T cells have also been interpreted as reflecting overlapping cytokine requirements (13). Thus, whereas inhibition of $\gamma\delta$ T cell homeostatic expansion by $\alpha\beta$ T cells is consistent with their consumption of IL-7 and IL-15, lack of inhibition by the IL-15-dependent NK cells is likely due to the small number of NK cells, to the fact that $\gamma\delta$ T cells can use IL-7 when IL-15 is not available, or to different localization of $\gamma\delta$ and NK cells.

It is of interest that, whereas $\alpha\beta$ T cells inhibited $\gamma\delta$ T cell homeostatic expansion in TCR$\delta^{-/-}$ mice, the opposite (i.e., inhibition of $\alpha\beta$ T cell expansion by $\gamma\delta$ T cells) was not observed in TCR$\alpha^{-/-}$ mice. One possibility could be that $\gamma\delta$ T cells developing in TCR$\alpha^{-/-}$ mice are defective, as reported for $\gamma\delta$ T cells of TCR$\beta^{-/-}$ mice (39). However, unlike their counterpart in TCR$\beta^{-/-}$ mice, $\gamma\delta$ T cells of TCR$\alpha^{-/-}$ mice exhibited an apparently normal gene expression profile (39), and inhibited homeostatic expansion of adoptively transferred $\gamma\delta$ T cells (this study). A more likely explanation for this unidirectional inhibition is that the larger $\alpha\beta$ T cell population may efficiently deplete IL-7 and IL-15, whereas the smaller $\gamma\delta$ T cell pool leaves sufficient levels of these factors to allow proliferation of $\alpha\beta$ T cells.

A significant finding in this study was that the control of the $\gamma\delta$ T cell pool size cannot be solely explained on the basis of competition with $\alpha\beta$ T cells for survival- and proliferation-promoting factors. Indeed, lack of $\alpha\beta$ T cells in TCR$\alpha^{-/-}$ mice was not associated with a significant expansion of endogenous $\gamma\delta$ T cells (data not shown). Moreover, unlike $\alpha\beta$ T cells, transfused $\gamma\delta$ T cells did not expand in TCR$\alpha^{-/-}$ hosts despite availability of IL-7 and IL-15. Thus, additional elements acting in conjunction with IL-7 and IL-15 seem to define $\gamma\delta$ T cell niches, including other cytokines, chemokines, adhesion molecules, or ligands for the $\gamma\delta$ TCR.

A major evidence suggesting that $\gamma\delta$ TCR ligands may be involved in $\gamma\delta$ T cell homeostasis is the restricted TCR usage in defined tissues. Although the mechanisms responsible for this restriction remain unknown, the involvement of specific Ags seems plausible. For example, it was shown that in the skin of mice lacking the prototypic V$\gamma3^+$ TCR, part of the substitute $\gamma\delta$ T cells seem to express a TCR similar in structure (and possibly Ag specificity) to the prototypic TCR, as suggested by shared reactivity with a clonotypic Ab (40). Additional evidence includes changes in Vγ/Vδ usage during fetal and neonatal life (41, 42), the abnormal expansion of $\gamma\delta$ T cells in mice homozygous for a mutated form of the TCR signaling-adaptor linker for activation of T cells (43), as well as cellular turnover differences between TCR-transgenic and polyclonal $\gamma\delta$ T cells (44). The present results indicating that $\gamma\delta$ T cells inhibit each other during homeostatic expansion and suggesting that the expressed TCR Vγ affects homeostatic proliferation kinetics may provide further support for a role of the TCR in $\gamma\delta$ T cell homeostasis.

IL-7 and IL-15 were previously recognized as playing essential roles in the biology of $\gamma\delta$ T cells. IL-7 signaling was shown to be absolutely required for the initiation of TCRγ gene rearrangements (24). Consistent with this observation, $\gamma\delta$ T cells were absent in IL-$7^{-/-}$ and IL-7R$\alpha^{-/-}$ mice (22, 23), but could be restored through introduction of a rearranged $\gamma\delta$ TCR transgene (25, 45). Likewise, $\gamma\delta$ T cell generation was rescued in IL-$7^{-/-}$ mice upon grafting of an IL-7-expressing thymus (46). Additional BrdU labeling studies with $\gamma\delta$ TCR-transgenic IL-$7^{-/-}$ mice suggested that IL-7, although not required for $\gamma\delta$ T cell survival, may increase the life span of proliferating cells, whereas other cytokines, such as IL-15, may be important for driving this proliferation (25), a prediction consistent with our present results. In regard to IL-15, studies indicated reductions in $\gamma\delta$ T cell subsets in IL-$15^{-/-}$, IL-15R$\alpha^{-/-}$, and IL-15R$\beta^{-/-}$ mice (26, 47, 48). It was also shown that lack of V$\gamma3^+$ dendritic epidermal T cells in IL-$15^{-/-}$ mice could not be corrected by adoptive transfer of wild-type thymocytes (27), and that a V$\gamma3^+$ transgenic TCR could restore this cell subset in IL-7R$\alpha^{-/-}$ mice, but not in IL-15R$\beta^{-/-}$ mice (45), further suggesting that IL-15 signals are redundant for $\gamma\delta$ T cell maturation, but required for localization in the skin. More recent studies using mixed bone marrow-chimera indicated that development of intraepithelial $\gamma\delta$ T cells requires IL-15Rβ expression by bone

marrow-derived cells, and IL-15 and IL-15Rα expression by parenchymal cells, consistent with a model in which IL-15 is *trans*-presented by IL-15Rα-expressing cells to IL-15Rβ/γ_c-expressing cells (28). The present findings extend this information revealing additional functions for IL-7 and IL-15 in γδ T cell biology.

Considerable evidence indicates that, like other cells of the innate immune system, γδ T cells are part of the first line of defense to infection and play central regulatory roles in the maintenance of tissue integrity. γδ T cells also share several characteristics with memory αβ T cells, particularly within the CD8 subset. As shown by phenotypic characterization, γδ T cells appear constitutively activated and gene profile analysis indicated high expression of several cytolytic effector molecules (reviewed in Ref. 3). The present results on the homeostasis requirements provide additional evidence for the resemblance of γδ T cells with cells of both the innate and adaptive immune systems.

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