Published in final edited form as:

Eur J Immunol. 2005 March ; 35(3): 786–795. doi:10.1002/eji.200425729.

T cell homeostasis requires G protein-coupled receptor-mediated access to trophic signals that promote growth and inhibit chemotaxis

Ryan M. Cinalli¹, Catherine E. Herman², Brian O. Lew², Heather L. Wieman², Craig B. Thompson¹, and Jeffrey C. Rathmell²

¹ Abramson Family Cancer Research Institute, Departments of Cancer Biology and Medicine, University of Pennsylvania, Philadelphia, USA

² Department of Pharmacology and Cancer Biology, Sarah W. Stedman Center for Nutrition and Metabolism, Duke University Medical Center, Durham, USA

Abstract

Signals that regulate T cell homeostasis are not fully understood. G protein-coupled receptors (GPCR), such as the chemokine receptors, may affect homeostasis by direct signaling or by guiding T cell migration to distinct location-restricted signals. Here, we show that blockade of G α i-associated GPCR signaling by treatment with pertussis toxin led to T cell atrophy and shortened life-span in T cell-replete hosts and prevented T cell homeostatic growth and proliferation in T cell-deficient hosts. *In vitro*, however, neither GPCR inhibition nor chemokine stimulation affected T cell atrophy, survival, or proliferation. These findings suggest that GPCR signals are not trophic stimuli, but instead may be required for migration to distinct trophic signals, such as IL-7 or self-peptide/MHC. Surprisingly, while chemokines did not affect atrophy, atrophic T cells displayed increased chemokine-induced chemotaxis that was prevented by IL-7 and submitogenic anti-CD3 antibody treatment. This increase in migration was associated with increased levels of GTP-bound Rac and the ability to remodel actin. These data suggest a novel mechanism of T cell homeostasis wherein GPCR may promote T cell migration to distinct location-restricted homeostatic trophic cues for T cell survival and growth. Homeostatic trophic signals, in turn, may suppress chemokine sensitivity and cytoskeletal remodeling, to inhibit further migration.

Keywords

G protein-coupled receptor; CXCR4; IL-7; Homeostasis; Rac

Introduction

Maintenance of lymphocyte homeostasis is crucial to allow rapid protective responses to foreign pathogens and simultaneously prevent diseases of the immune system, such as autoimmunity or immunodeficiency. One mechanism to maintain immune homeostasis is the requirement of cells to receive signals from their microenvironment to prevent activation of a default cell-intrinsic death pathway [1]. When deprived of these signals, cells undergo cellular atrophy prior to an ultimate commitment to apoptosis. Atrophy is characterized by decreased cell size and metabolism and inhibition of the cell cycle [2–4]. A second mechanism to maintain

immune homeostasis is the spontaneous proliferation of lymphocytes in lymphopenic animals [5]. This cytokine and self-antigen-driven cell expansion may play an important role to increase lymphocyte numbers when they are limiting. A third homeostatic process is regulation of T cell migration to allow even T cell distribution and immunosurveillance [6,7]. Lymphocyte chemotaxis is guided primarily by G α i-associated G protein-coupled receptor (GPCR) signaling mediated by chemokine receptors [7].

The γ_c cytokine IL-7 and interactions between the TCR and self-peptide/MHC maintain T cell viability, allow homeostatic proliferation *in vivo* and can maintain basal T cell size and metabolism *in vitro* [3,5,8,9]. The role of GPCR in these T cell homeostatic processes, however, is not known. These signals may directly affect T cell homeostasis through activation of similar signaling pathways as activated by γ_c cytokines and the TCR. In particular, the survival-promoting phosphatidylinositol 3-kinase/Akt [10], MAPK [11,12], NF- κ B [13], and Jak kinase [14] signaling pathways have been reported to become activated by chemokine receptor signaling. Each of these pathways may mediate a direct effect of GPCR on T cell homeostasis and account for observations of chemokine-mediated cell survival [12,15–17]. Alternatively, GPCR may affect T cell survival and homeostasis indirectly by chemokine-guided migration of cells to locations where they can gain access to distinct signals that provide trophic support, such as IL-7 or self-peptide/MHC.

Here, we show that G α i-associated GPCR and chemokines regulate T cell trophic state and homeostasis and suggest that this occurs indirectly by promoting migration of T cells to distinct trophic signals. T cells were treated with pertussis toxin (PTX) to ADP-ribosylate G α i proteins and irreversibly inhibit G α i-associated GPCR signaling [18]. This prevented lymphocytes from responding to chemokine cues *in vitro* as well as upon adoptive transfer *in vivo*. Treated T cells underwent atrophy and death-by-neglect in T cell-replete hosts and failed to undergo homeostatic growth and proliferation in lymphopenic hosts. *In vitro* blockade of GPCR or stimulation with chemokines, however, did not affect resting T cell size, survival, or proliferation. Surprisingly, in contrast to the inability of chemokines to affect the T cell trophic state, T cell neglect led to an increased sensitivity to chemokine-induced actin remodeling and migration. These data suggest that resting T cell homeostasis may be regulated by distinct GPCR-mediated directional cues and by location-restricted trophic cues that support T cell viability and growth but inhibit chemotaxis.

Results

G α i-associated GPCR signals are required for resting T cells to enter into normal lymphoid environments, prevent atrophy and maintain cell life-span *in vivo*

GPCR regulate a variety of cellular processes and may affect T cell homeostasis through direct signaling mechanisms or indirectly by controlling T cell migration to distinct homeostatic cues. To test the role of GPCR in T cell homeostasis, GPCR that signal through G α i were blocked by treatment with PTX. PTX treatment prevented T cell chemotaxis *in vitro* to the chemokines CCL21 or CXCL12 in transwell cell migration assays (Fig. 1A). Similarly, analysis of adoptively transferred PTX-treated Thy1.1 T cells into Thy1.2 recipients demonstrated that PTX treatment prevented entry into the splenic white pulp or into lymph nodes at any point in these studies (Fig. 1B and data not shown). Cell size was analyzed as an indicator of the cellular trophic state because cell size incorporates multiple aspects of cell metabolism and is regulated by trophic factors [3,19]. Flow cytometric mean forward light scatter was measured to indicate cell size. Untreated adoptively transferred Thy1.1 T cells had similar forward light scatter and size as endogenous Thy1.2 T cells at each time point (data not shown). Transferred Thy1.1 CD4 and CD8 T cells that had been treated with PTX, however, had atrophied and were smaller than control Thy1.1 and endogenous Thy1.2 T cells (Fig. 1C and data not shown).

Ultimately, cellular neglect and atrophy can lead to apoptosis. To test if PTX-treated T cells had shortened life-spans *in vivo*, PTX-treated and untreated 5(6)-carboxyfluorescein succinimidylester (CFSE)⁺ T cells were adoptively transferred into syngeneic recipients. After one day, CFSE⁺ T cells were enumerated in recipient spleens to establish a baseline cell number in each recipient group. After transfer (1 or 2 weeks), spleens were analyzed and the number of transferred CFSE⁺ T cells was determined and expressed as the percentage remaining of that observed on day 1 (Fig. 1D). At no time were PTX-treated T cells found in lymph nodes. PTX-treated T cells underwent more rapid elimination than untreated T cells, with a recovery half-life of less than 1 week compared to a half-life of approximately 2 weeks. These data suggested that PTX treatment had inhibited a signal required to maintain T cell trophic state and viability [3,8].

T cells require GPCR signals to allow homeostatic cell growth and proliferation

To test if GPCR signals were also necessary for T cell growth *in vivo*, purified T cells were stained with CFSE, left untreated or treated with PTX and were adoptively transferred into *Rag1*^{-/-} recipients. Untreated T cells entered spleen and lymph nodes and underwent homeostatic proliferation in each tissue (Fig. 2A). PTX-treated T cells were unable to enter lymph nodes, but were readily observed in the spleen (Fig. 2A). Nevertheless, PTX treatment prevented homeostatic proliferation of both CD4 and CD8 T cells in the spleen. The requirement for GPCR signals to allow homeostatic proliferation was not due to direct inhibition of cell division by PTX. Similar to findings of others [20], PTX-treated T cells proliferated and produced the cytokines IL-2 and IFN- γ equivalently compared to control cells upon *in vitro* stimulation (Fig. 2B and data not shown).

It has become apparent that cell growth and division can be independently regulated [21]. Untreated and PTX-treated T cells that had not divided (based on CFSE staining) were analyzed in normal and lymphopenic adoptive transfer recipients to determine if GPCR signals are required for cell division-independent T cell growth. After transfer of CFSE-labeled untreated or PTX-treated T cells into T cell-replete (*Rag*^{+/+}) or T cell-deficient (*Rag*^{-/-}) hosts (1 week), spleens were analyzed to determine the size of cells that remained CFSE^{bright} (Fig. 2C). Untreated CFSE^{bright} T cells grew significantly in *Rag1*^{-/-} hosts compared to those injected into *Rag*^{+/+} hosts. In contrast, PTX-treated CFSE^{bright} T cells failed to grow or had atrophied *in vivo*, even in lymphopenic *Rag1*^{-/-} hosts. T cell growth, therefore, is homeostatically regulated independently of cell division and requires intact Gai-associated GPCR function.

Gai-associated GPCR and chemokines do not directly affect T cell atrophy or death-by-neglect

The *in vivo* atrophy and failure of PTX-treated T cells to undergo cell growth in lymphopenic hosts could have been caused either directly by inhibition of Gai-associated GPCR trophic and survival signals or indirectly by altering T cell migration to other signals that affect T cell growth and survival. To determine if GPCR blockade or chemokine stimulation could directly signal to affect T cell atrophy or growth, purified T cells were left untreated or were treated with PTX and analyzed *in vitro*. T cells were analyzed immediately after purification and treatment (Fresh) or were cultured for 1 or 2 days without stimulation (Neglect) or in the presence of various stimuli, including chemokines, IL-7, and submitogenic anti-CD3 antibody amounts. Results were similar after 1 or 2 days in culture, and results from day 2 are presented when effects were more pronounced (Fig. 3A, B and data not shown). Treatment of T cells with PTX had no effect on the viability or size of purified T cells in any condition. Culturing T cells with doses of the chemokines CCL21 and CXCL12 sufficient to promote robust chemotaxis (Fig. 1A) also had no effect on atrophy or survival in culture (Fig. 3A, B). In contrast, culturing T cells in the cytokine IL-7 potently inhibited both cellular atrophy and cell

death-by-neglect [8, 22, 23]. Atrophy and cell death were also attenuated in T cells cultured on plates that had been coated with submitogenic doses of anti-CD3 antibody.

Neglected T cells have enhanced sensitivity to chemotactic stimuli *in vitro* and *in vivo*

The inability of GPCR inhibition or stimulation by CCL21 and CXCL12 to directly affect T cell atrophy and survival *in vitro* suggested that T cell directional and trophic cues may be independent. To test this, purified T cells were analyzed immediately (Fresh) or after 2 days in culture in the absence of stimulation (Neglect) or in the presence of CXCL12 alone or in combination with IL-7 or on plates coated with submitogenic anti-CD3 antibody amounts (Fig. 4A, B). CXCL12 was unable to further enhance the survival and cell size effects of IL-7R or TCR stimulation. Comparable results were obtained using the chemokine CCL21 (data not shown).

Conversely, it was possible that changes in T cell trophic state could affect the ability of T cells to respond to chemotactic signals. T cells were purified and neglected *in vitro* for 1 day and compared to freshly isolated T cells for chemotactic responses to CXCL12 and CCL21. In contrast to their reduced metabolism and mitogenic ability [8], neglected T cells had increased chemotactic sensitivity to CXCL12 (Fig. 5A). This effect was not specific to CXCL12 and its receptor CXCR4, because neglected T cells also displayed enhanced migration to CCL21, which utilizes CCR7 as its receptor (Fig. 5B). The increase in T cell migration upon neglect *in vitro* began to develop after 1 h in culture, was half-maximal after 4 h and was largest after 20 h in culture (Fig. 5C).

Chemotaxis *in vitro* may not reflect *in vivo* T cell migration into lymphoid organs. Entry of fresh and neglected T cells into lymph nodes was, therefore, analyzed. Purified Thy1.1 T cells (Fresh) were mixed with neglected CFSE-labeled Thy1.2 T cells in a 1:1 ratio of live Thy1.1⁺ to CFSE⁺ cells. T cells were neglected for 1 day *in vitro*, because significant cellular atrophy occurs at this time point but a large fraction of cells remain viable. The mixed cell suspension was then adoptively transferred intravenously into Thy1.2 mice. The number of adoptively transferred CFSE⁺ (Neglected) and Thy1.1⁺ (Fresh) T cells was determined in lymph nodes of recipient mice at various times. Neglected T cells entered recipient lymph nodes faster and in greater numbers than freshly isolated T cells (Fig. 5D). This was particularly the case for CD8 T cells (data not shown).

Trophic signals inhibit chemotaxis

To determine if the increased chemotaxis of neglected T cells was due to deprivation of *in vivo* trophic stimuli, T cells were cultured in the presence of IL-7 and/or TCR stimuli, and chemotaxis was assayed (Fig. 6). Purified T cells were analyzed immediately after isolation or after 1 day in culture in the absence of stimulation or in the presence of IL-7, submitogenic anti-CD3 antibody amounts or IL-7 and submitogenic anti-CD3 antibody amounts together. While IL-7R and submitogenic TCR signals each were separately unable to prevent the increased chemotaxis that occurred when cells were isolated and placed in culture, the combination of IL-7R and TCR signaling, however, potentially inhibited the neglect-induced increase in chemokine GPCR-mediated cell migration.

Neglected T cells and chemokine receptor expression

The mechanism of the increased chemotactic response of neglected cells to CXCL12 and CCL21 may have been due to increased chemokine receptor expression or cell surface localization. Total levels of CXCR4, CCR7, and actin were found unchanged between freshly isolated and neglected T cells by Western blot of total cell lysates (Fig. 7A). T cells were also analyzed flow cytometrically for surface expression of CXCR4 and CCR7 (Fig. 7B). Although surface CXCR4 is present on freshly isolated T cells based on chemotactic responses to

CXCL12, it was not detectable by flow cytometry in freshly isolated T cells. A slight increase in CXCR4 staining was, however, observed in neglected T cells. In contrast, surface CCR7 levels appeared to decrease upon neglect.

Neglected T cells have increased GTP-Rac

Rho family small GTPases are key mediators of chemokine receptor signal transduction and actin cytoskeleton organization [24]. In particular, the small GTPase Rac has been implicated in lymphocyte motility [25]. Purified T cells were analyzed immediately after isolation or after 1 day of neglect for the presence of GTP-Rac (Fig. 8A). Neglected T cells had increased levels of GTP-Rac compared to freshly isolated T cells. After 30 s of stimulation with CXCL12, freshly isolated T cells displayed increased GTP-Rac, indicating that fresh cells could respond to the chemokine. Neglected T cells had undetectable changes in overall GTP-Rac levels at 30 s of stimulation with CXCL12.

Neglected T cells have dynamic actin cytoskeletons

Freshly isolated or neglected T cells were left untreated or were stimulated with CXCL12 for 1 min, fixed and stained with phalloidin to indicate polymerized actin. Despite similar total actin levels (Fig. 7A), neglected T cells had less polymerized actin than freshly isolated T cells prior to stimulation (Fig. 8B). Neglected T cells were, however, capable of rapid actin polymerization in response to CXCL12, which increased polymerized actin content above that of freshly isolated T cells. This rapid actin polymerization response resulted in increased actin polarization in neglected T cells. Actin polarization was observed by confocal microscopy of phalloidin-stained fresh and neglected T cells that were left unstimulated or were treated with CXCL12 for 5 min. Representative confocal images are shown in Fig. 8C. Images are individually contrasted to highlight the distribution of polymerized actin and do not indicate total polymerized actin levels. Cells were quantified as nonpolarized (even actin distribution around cells) or polarized (accumulation of polymerized actin on one side of the cell) (Fig. 8D). A low frequency of cells with a polarized actin cytoskeleton was present in both fresh and neglected T cell samples, but only neglected T cells responded to CXCL12 stimulation with an increased frequency of polarized cells.

Discussion

Here, we show that homeostasis of the resting T cell repertoire requires Gai-associated GPCR in addition to trophic stimuli such as IL-7 and TCR. *In vitro* and *in vivo* results suggest that GPCR, such as the chemokine receptors for CXCL12 and CLC21, may provide critical directional signals for T cells to gain access to trophic signals. Although GPCR signals neither played a direct role in regulation of the resting T cell trophic state nor modified the effects of known trophic signals, trophic signals inhibited T cell responses to chemokine stimulation of GPCR. While the precise mechanism of this inhibition is uncertain, changes in basal GTP-Rac levels and alterations in the actin cytoskeleton may play prominent roles. These data suggest a model of T cell homeostasis in which the maintenance of T cell trophic state, growth, and proliferation are dependent on chemokine GPCR-mediated T cell migration to distinct location-restricted trophic signals. T cells that fail to migrate to proper trophic signals are, however, not committed to death by atrophy, but instead gain increased chemotactic ability and potential to locate trophic signals at other locations.

PTX-treated T cells accumulate in the splenic red pulp, in close proximity to normal T cell areas in the white pulp. Nevertheless, PTX treatment leads to T cell atrophy, suggesting that relevant GPCR-independent trophic factors are location restricted and are not readily diffusible. *In vitro*, IL-7R and TCR signals may play important trophic support roles *in vivo*. IL-7 is required for *in vivo* T cell survival [5] and has been shown to be produced by dendritic

cells present in T cell zones [26,27]. Although IL-7 is a soluble protein, it can bind extracellular matrix components [28,29], which may restrict IL-7 diffusion *in vivo*. TCR signals are initiated by peptide/MHC complexes on the surface of antigen-presenting cells, such as dendritic cells. MHC class II expression is restricted to professional antigen-presenting cells, and it has been shown that CD4 T cells require self-peptide/MHC class II expression on dendritic cells to maintain their viability [30]. CD8 T cells, in contrast, require the ubiquitously expressed MHC class I to maintain their viability [5]. Nevertheless, CD8 T cells underwent cellular atrophy *in vivo* similar to CD4 T cells when treated with PTX. It is possible that CD8 T cells may require TCR stimulation in conjunction with an additional physically restricted signal.

T cell homeostatic growth and proliferation were also inhibited *in vivo* when GPCR signals were blocked with PTX. T cell proliferation and cytokine production *in vitro*, however, were not affected (Fig. 2 and data not shown). Failed homeostatic proliferation of CD4 and CD8 PTX-treated T cells was also observed by Dummer et al. [20]. Here, we expand on these findings to show that T cells undergo homeostatic growth independently of cell division. T cells require GPCR signals to undergo both processes. The inability of PTX inhibition of GPCR or treatment with CXCL12 or CCL21 to act cooperatively with IL-7R- or TCR-induced signals *in vitro* to prevent atrophy and increased T cell growth *in vitro* suggests that GPCR may act indirectly to promote homeostatic growth by providing directional cues towards trophic support signals.

A surprising result of these studies was the finding that neglected T cells have enhanced chemokine sensitivity and chemotaxis. This increase in chemokine sensitivity occurred as a consequence of decreased stimulation of trophic signal receptors, because it could be blocked by culture of T cells with the trophic stimuli IL-7 and submitogenic anti-CD3 antibody amounts. Similarly, it has been shown by *in vivo* two-photon confocal microscopy and by *in vitro* analyses of T cell chemotaxis that T cell migration is inhibited when specific antigen or mitogenic stimuli are encountered [31–34]. Together, these studies show that access to both mitogenic stimuli and homeostatic trophic signals, such as combined IL-7 and non-mitogenic TCR stimulation, inhibit chemotaxis.

The mechanism of increased chemokine sensitivity of neglected T cells is uncertain. T cell chemotaxis can be altered by receptors, such as Robo [35] or the sphingosine 1-phosphate receptor [36], and requires the generation of phospholipids that may be altered by changes in expression of lipid kinases or phosphatases [10]. It is unclear, however, what regulatory role these proteins may play to allow increased migration of neglected T cells. Changes in actin polymerization and GTP-Rac levels may also affect the motility of neglected T cells. T cell actin polarization is Rac dependent [25]. Rac in freshly isolated T cells is predominantly in empty or GDP-bound inactive forms. While chemokine signaling in freshly isolated T cells led to GTP loading of Rac, this was insufficient to increase filamentous actin formation or to induce T cell polarization. In contrast, neglected T cells had a significant portion of their actin in an unpolymerized form, with GTP-bound Rac present. Upon chemokine stimulation, neglected T cells rapidly polymerized actin, leading to T cell actin polarization. GTP-Rac levels were not observed to further increase in neglected T cells after chemokine stimulation. It is possible that a transient increase in GTP-Rac levels occurred, but the appropriate time point was not observed. Alternatively, GTP-Rac levels may be near maximal in neglected T cells prior to stimulation. GTP-Rac must, however, interact with effector proteins to mediate cytoskeletal rearrangements, and these interactions may be altered by neglect. In this case, increased basal GTP-Rac levels may allow increased interactions with Rac effector proteins to allow rapid actin remodeling and polarization in neglected T cells.

The findings here suggest a possible model in which T cells utilize both directional and location-restricted trophic cues to maintain homeostasis. The interplay between T cell trophic

signals, such as IL-7 and self-peptide/MHC, and chemokine-mediated directional signals may play an important role in survival and distribution of T cells and will be important for future study.

Materials and methods

Mice

Wild-type non-transgenic C57BL/6J (Thy1.2, *RagI*^{+/+}), and congenic C57BL/6J-*IgH*^a-*Thy1*^a-*GpiI*^a (Thy1.1) and C57BL/6J-*RagI*^{-/-} mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were analyzed between 8 and 16 weeks of age.

T cell purification and culture

T cells were purified by negative selection as described [3] (StemCell Technologies, Vancouver, BC, Canada). Some T cells were treated cultured in 100 ng/ml PTX (List Biological Laboratories, Campbell, CA) for 2 h at 37°C in RPMI with 10% FCS, then washed three times. T cells were plated at 10⁶ cells/ml without additional stimulation, in CCL21 (R&D Systems, Minneapolis, MN), CXCL12 (R&D Systems), IL-7 (BD-PharMingen, San Diego, CA), or on tissue culture plates pre-coated with 0.5 µg/ml anti-CD3 antibody (clone 145–2C11; BD-PharMingen) at the indicated doses. T cell viability and size were measured as described [8]. Some T cells were labeled with CFSE (Molecular Probes, Eugene, OR), as described [3]. *In vitro* T cell proliferation was measured on plates coated with 5 µg/ml anti-CD3 (clone 145–2C11; BD-PharMingen) and anti-CD28 antibody (clone 37.51; BD-PharMingen) for 3 days. Cell surface CCR7 and CXCR4 staining were measured flow cytometrically by staining with goat anti-CCR7 polyclonal IgG (Abcam, Cambridge, MA) or goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA), followed by Donkey anti-goat IgG-FITC (Jackson ImmunoResearch, West Grove, PA) or anti-CXCR4-PE antibody (clone 2B11/CXCR4; BD-PharMingen) with a rat IgG2b-PE isotype control (clone A95–1; BD-PharMingen).

Chemotaxis assays

Chemotaxis was assayed in transwell plates with 5 µm pore size (Costar; Fisher, Atlanta, GA) with 600 µl medium in the lower chambers and 5×10⁵ live T cells in 100 µl added to the upper chambers for 2 h at 37°C. Cells in the lower chambers were quantified with a Coulter Z₂ (Beckman-Coulter).

Adoptive transfers

For analysis of T cell atrophy *in vivo*, Thy1.2 recipients were injected with 2×10⁶ donor Thy1.1 T cells via tail vein. Spleen and lymph node cell suspensions from triplicate recipient mice were analyzed on days 1, 3, and 7. Cell suspensions were stained with anti-Thy1.1-PE (clone OX-7; BD-PharMingen), anti-CD4-CyChrome (clone RM4–5; BD-PharMingen), anti-CD8-allophycocyanin (clone 53–6.7; BD-PharMingen) antibodies. To measure T cell survival *in vivo*, CFSE-labeled T cells were left untreated or were treated with PTX, and 2.5×10⁶ T cells were transferred via tail vein. Recipient lymph node suspensions were analyzed flow cytometrically to enumerate CFSE⁺ T cells. To study homeostatic T cell growth and proliferation, 2.5×10⁶ CFSE-labeled T cells were left untreated or were treated with PTX and transferred into congenic *RagI*^{+/+} or *RagI*^{-/-} recipients via tail vein. Lymph node and spleen cell suspensions were analyzed flow cytometrically to observe CFSE dilution and mean forward angle light scatter after 7 days. To observe chemotaxis *in vivo*, Thy1.2 T cells were purified, stained with CFSE and neglected *in vitro* for 1 day. Thy1.1 T cells were purified and mixed with neglected CFSE-stained Thy1.2 cells at a 1:1 ratio of live cells and injected via tail vein into Thy1.2 recipients. After transfer (1, 3, 6 h), recipient spleens and lymph nodes were

harvested and analyzed to determine the number of lymphocytes that were CFSE⁺ and Thy1.1⁺.

Fluorescence microscopy

Frozen spleen sections were stained with anti-Thy1.1-PE (clone OX-7; BD-PharMingen) and anti-Thy1.2-FITC (clone 53-2.1; BD-PharMingen) antibodies. To image F-actin, T cells were fixed with 1% paraformaldehyde in PBS, permeabilized with 0.1% saponin in PBS and stained with Alexa Fluor 488 phalloidin (Molecular Probes). Ten microscopic fields of 10–35 cells were imaged for each condition.

Western blots and determination of GTP-Rac levels

Purified live T cells were lysed as described [8]; 40 µg protein was loaded on a 10–20% SDS-PAGE Criterion gel (Bio-Rad), and samples were analyzed by Western blot with rabbit anti-CXCR4 (Abcam), goat anti-CCR7 (Abcam), and rabbit anti-actin (Santa Cruz Biotechnology) antibodies.

GTP-Rac levels were measured as described [37] in equivalent numbers of live freshly purified or neglected T cells that were left unstimulated or were stimulated for 30 s with 10 ng/ml CXCL12. Equivalent quantities of protein from each cell lysate were used to pull down active GTP-bound Rac with Sepharose 4B-glutathione beads bound with GST-PBD (generously provided by Keith Burrige; University of North Carolina, Chapel Hill, NC). Proteins were analyzed by Western blot with mouse-anti-Rac antibody (BD-Transduction Laboratories).

Actin polymerization

To measure actin polymerization, T cells were fixed without treatment with chemokine or after 1 h in the presence of 10 ng/ml CXCL12 at 37°C. Cells were then permeabilized and stained with Alexa Fluor 488 phalloidin (Molecular Probes).

Acknowledgements

The authors wish to thank Peter S. Hammerman and Dr. Kenneth Frauwirth. We also thank Dr. Keith Burrige and Lisa Sharek (University of North Carolina, Chapel Hill, NC) for generously providing reagents for analysis of Rac activity. We thank Drs. Patrick Casey, Tannishtha Reya, Motonari Kondo, and Carlijn Voermans for critical reading of the manuscript. We also thank Peter Rathmell for technical assistance and image analysis. This work was supported by the National Cancer Institute. J.C.R. was supported by the Irvington Institute for Immunological Research, a Howard Temin Career Development Award from the National Cancer Institute, a Sidney Kimmel Scholar Award, and a V Foundation Scholar Award.

Abbreviations

GPCR	G protein-coupled receptor
PTX	Pertussis toxin

References

1. Raff MC. Social controls on cell survival and cell death. *Nature* 1992;356:397–400. [PubMed: 1557121]
2. Kan O, Baldwin SA, Whetton AD. Apoptosis is regulated by the rate of glucose transport in an interleukin 3 dependent cell line. *J Exp Med* 1994;180:917–923. [PubMed: 8064240]

3. Rathmell JC, Vander Heiden MG, Harris MH, Frauwirth KA, Thompson CB. In the absence of extrinsic signals, nutrient utilization by lymphocytes is insufficient to maintain either cell size or viability. *Mol Cell* 2000;6:683–692. [PubMed: 11030347]
4. Rathmell JC, Fox CJ, Plas DR, Hammerman P, Cinalli RM, Thompson CB. Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival. *Mol Cell Biol* 2003;23:7315–7328. [PubMed: 14517300]
5. Jameson SC. Maintaining the norm: T cell homeostasis. *Nat Rev Immunol* 2002;2:547–556. [PubMed: 12154374]
6. Cyster JG. Chemokines and cell migration in secondary lymphoid organs. *Science* 1999;286:2098–2102. [PubMed: 10617422]
7. Moser B, Loetscher P. Lymphocyte traffic control by chemokines. *Nat Immunol* 2001;2:123–128. [PubMed: 11175804]
8. Rathmell JC, Farkash EA, Gao W, Thompson CB. IL-7 enhances the survival and maintains the size of naive T cells. *J Immunol* 2001;167:6869–6876. [PubMed: 11739504]
9. Khaled AR, Durum SK. Death and Baxes: mechanisms of lymphotropic cytokines. *Immunol Rev* 2003;193:48–57. [PubMed: 12752670]
10. Ward SG. Do phosphoinositide 3-kinases direct lymphocyte navigation? *Trends Immunol* 2004;25:67–74. [PubMed: 15102365]
11. Tilton B, Ho L, Oberlin E, Loetscher P, Baleux F, Clark-Lewis I, Thelen M. Signal transduction by CXC chemokine receptor 4: Stromal cell-derived factor 1 stimulates prolonged protein kinase B and extracellular signal-regulated kinase 2 activation in T lymphocytes. *J Exp Med* 2000;192:313–324. [PubMed: 10934220]
12. Suzuki Y, Rahman M, Mitsuya H. Diverse transcriptional response of CD4(+) T cells to stromal cell-derived factor (SDF)-1: cell survival promotion and priming effects of SDF-1 on CD4(+) T cells. *J Immunol* 2001;167:3064–3073. [PubMed: 11544290]
13. Sanchez-Sanchez N, Riolo-Blanco L, De La Rosa G, Puig-Kroger A, Garcia-Bordas J, Martin D, Longo N, Cuadrado A, Cabanas C, Corbi AL, et al. Chemokine receptor CCR7 induces intracellular signaling that inhibits apoptosis of mature dendritic cells. *Blood* 2004;104:619–625. [PubMed: 15059845]
14. Soriano SF, Serrano A, Hernanz-Falcon P, Martin de Ana A, Monterrubio M, Martinez C, Rodriguez-Frade JM, Mellado M. Chemokines integrate JAK/STAT and G-protein pathways during chemotaxis and calcium flux responses. *Eur J Immunol* 2003;33:1328–1333. [PubMed: 12731058]
15. Vlahakis SR, Villasis-Keever A, Gomez T, Vanegas M, Vlahakis N, Paya CV. G protein-coupled chemokine receptors induce both survival and apoptotic signaling pathways. *J Immunol* 2002;169:5546–5554. [PubMed: 12421931]
16. Louahed J, Struyf S, Demoulin JB, Parmentier M, Van Snick J, Van Damme J, Renauld JC. CCR8-dependent activation of RAS/MAPK pathway mediates anti-apoptotic activity of I-309/CCL1 and vMIP-I. *Eur J Immunol* 2003;33:494–501. [PubMed: 12645948]
17. Spinetti G, Bernardini G, Camarda G, Mangoni A, Santoni A, Capogrossi MC, Napolitano M. The chemokine receptor CCR8 mediates rescue from dexamethasone-induced apoptosis via an ERK-dependent pathway. *J Leukoc Biol* 2003;73:201–207. [PubMed: 12525579]
18. Cyster JG, Goodnow CC. Pertussis toxin inhibits migration of B and T lymphocytes into splenic white pulp cords. *J Exp Med* 1995;182:581–586. [PubMed: 7629515]
19. Vander Heiden MG, Plas DR, Rathmell JC, Fox CJ, Harris MH, Thompson CB. Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol Cell Biol* 2001;21:5899–5912. [PubMed: 11486029]
20. Dummer W, Ernst B, LeRoy E, Lee D, Surh C. Autologous regulation of naive T cell homeostasis within the T cell compartment. *J Immunol* 2001;166:2460–2468. [PubMed: 11160306]
21. Conlon IJ, Dunn GA, Mudge AW, Raff MC. Extracellular control of cell size. *Nat Cell Biol* 2001;3:918–921. [PubMed: 11584274]
22. Boise LH, Minn AJ, June CH, Lindsten T, Thompson CB. Growth factors can enhance lymphocyte survival without committing the cell to undergo cell division. *Proc Natl Acad Sci USA* 1995;92:5491–5495. [PubMed: 777536]

23. Vella A, Teague TK, Ihle J, Kappler J, Marrack P. Interleukin 4 (IL-4) or IL-7 prevents the death of resting T cells: Stat6 is probably not required for the effect of IL-4. *J Exp Med* 1997;186:325–330. [PubMed: 9221762]
24. Burridge K, Wennerberg K. Rho and Rac take center stage. *Cell* 2004;116:167–179. [PubMed: 14744429]
25. Reif K, Cyster J. The CDM protein DOCK2 in lymphocyte migration. *Trends Cell Biol* 2002;12:368–373. [PubMed: 12191913]
26. de Saint-Vis B, Fugier-Vivier I, Massacrier C, Gaillard C, Vanbervliet B, Ait-Yahia S, Banchereau J, Liu YJ, Lebecque S, Caux C. The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. *J Immunol* 1998;160:1666–1676. [PubMed: 9469423]
27. Sorg RV, McLellan AD, Hock BD, Fearnley DB, Hart DN. Human dendritic cells express functional interleukin-7. *Immunobiology* 1998;198:514–526. [PubMed: 9561370]
28. Ariel A, Hershkovich R, Cahalon L, Williams DE, Akiyama SK, Yamada KM, Chen C, Alon R, Lapidot T, Lider O. Induction of T cell adhesion to extracellular matrix or endothelial cell ligands by soluble or matrix-bound interleukin-7. *Eur J Immunol* 1997;27:2562–2570. [PubMed: 9368611]
29. Banwell CM, Partington KM, Jenkinson EJ, Anderson G. Studies on the role of IL-7 presentation by mesenchymal fibroblasts during early thymocyte development. *Eur J Immunol* 2000;30:2125–2129. [PubMed: 10940902]
30. Brouck T. Survival of mature CD4 T lymphocytes is dependent on major histocompatibility complex class II-expressing dendritic cells. *J Exp Med* 1997;186:1222–1232.
31. Peacock JW, Jirik FR. TCR activation inhibits chemotaxis toward stromal cell-derived factor-1: evidence for reciprocal regulation between CXCR4 and the TCR. *J Immunol* 1999;162:215–223. [PubMed: 9886389]
32. Miller MJ, Wei SH, Parker I, Cahalan MD. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 2002;296:1869–1873. [PubMed: 12016203]
33. Bousso P, Robey E. Dynamics of CD8⁺ T cell priming by dendritic cells in intact lymph nodes. *Nat Immunol* 2003;4:579–585. [PubMed: 12730692]
34. Stoll S, Delon J, Brotz TM, Germain RN. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science* 2002;296:1873–1876. [PubMed: 12052961]
35. Wu JY, Feng L, Park HT, Havlioglu N, Wen L, Tang H, Bacon KB, Jiang Z, Zhang X, Rao Y. The neuronal repellent Slit inhibits leukocyte chemotaxis induced by chemotactic factors. *Nature* 2001;410:948–952. [PubMed: 11309622]
36. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, Proia RL, Cyster JG. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* 2004;427:355–360. [PubMed: 14737169]
37. Wennerberg K, Ellerbroek SM, Liu RY, Karnoub AE, Burridge K, Der CJ. RhoG signals in parallel with Rac1 and Cdc42. *J Biol Chem* 2002;277:47810–1147817. [PubMed: 12376551]

Fig. 1.

T cells require Gai-associated GPCR signals to enter lymphoid T cell zones and maintain their trophic state. (A) Purified T cells were left untreated or were treated with PTX, and chemotaxis was measured *in vitro* to media alone or to 100 ng/ml chemokine, in transwell assays. (B, C) Purified Thy1.1 T cells were left untreated or were treated with PTX *in vitro*, and cells were injected into Thy1.2 recipients. After transfer (3 days), spleens were analyzed by immunofluorescence at 100 $\times$ magnification to detect transferred Thy1.1 T cells (in green) and endogenous Thy1.2 T cells (in red). Bars represent 100 μ m (B). Splenocytes were analyzed flow cytometrically to determine the mean forward scatter of transferred CD4 and CD8 T cells (C). (D) Purified T cells were labeled with CFSE, left untreated or treated with PTX and transferred into recipient mice to determine the number of transferred T cells in each spleen over time. Triplicate means and standard deviations are shown.

Fig. 2.

Inhibition of GPCR signals prevents homeostatic growth and proliferation. Purified T cells were CFSE-labeled and left untreated or treated with PTX. (A) T cells were adoptively transferred into *Rag1*^{-/-} recipients. After 7 days, spleens and lymph nodes were analyzed flow cytometrically to measure homeostatic cell proliferation. Percentages are shown for T cells that did not divide and are representative of triplicate mice. (B) T cells were cultured *in vitro* on uncoated (No Stim) or anti-CD3 and anti-CD28 antibody-coated plates (CD3 + CD28). The percentages of T cells that had undergone cell division after 2 days *in vitro* are shown. (C) Purified T cells were CFSE-labeled, left untreated or treated with PTX and adoptively transferred into *Rag*^{+/+} or *Rag*^{-/-} recipients. The mean forward scatter of T cells that remained CFSE^{bright} was measured. Means and standard deviations of each group are shown (*n*=3 for PTX-treated T cells transferred into *Rag*^{+/+} recipients; *n*=4 for all others).

Fig. 3.

Death-by-neglect and atrophy of resting T cells are not affected by GPCR signals. Purified T cells were left untreated or were treated with PTX and analyzed flow cytometrically immediately (Fresh) or after *in vitro* culture in the absence of stimulation (Neglect) or in the presence of 100 ng/ml CCL21 or CXCL12, 1 ng/ml IL-7, or on plates coated with 0.5 µg/ml anti-CD3 antibody, either alone or in combination with 1 ng/ml IL-7. (A) T cell viability was determined by propidium iodide exclusion. (B) T cell size was determined by mean forward scatter. Triplicate means and standard deviations are shown.

Fig. 4. Chemokines do not cooperate with IL-7R or TCR-induced signals to promote T cell survival or inhibit atrophy. Purified T cells were analyzed immediately (Fresh) or cultured for 2 days prior to analysis in the absence of stimulation (Neglect) or in the presence of 100 ng/ml CXCL12, on plates coated with 0.5 μ g/ml anti-CD3 antibody, or 1 ng/ml IL-7 alone or in combinations. (A) T cell viability was determined by propidium iodide exclusion. (B) T cell size was determined by mean forward scatter. Means and standard deviations are shown from triplicate samples.

Fig. 5.

Atrophic T cells have increased chemotactic responses *in vitro* and *in vivo*. (A, B) *In vitro* chemotaxis of freshly isolated or neglected T cells to CXCL12 (A) and CCL21 (B). (C) Chemotaxis in trans-well cultures to media (No Chemokine) or with 10 ng/ml CXCL12 after various times in culture. (D) Purified Thy1.2 T cells were labeled with CFSE and cultured overnight (Neglect) and the following day mixed in equal live cell numbers with freshly purified Thy1.1 T cells (Fresh). The cell suspension was transferred into Thy1.2 recipients, and lymph nodes from recipient mice were analyzed to quantitate transferred T cells. Means and standard deviations of triplicate samples or recipient mice are shown.

Fig. 6.

Increased chemotaxis occurs in response to deprivation of trophic signals or growth factors. (A) Purified T cells were analyzed immediately (Fresh) after isolation or were cultured for 1 day in the absence of stimulation (Neglect), in 1 ng/ml IL-7, or on plates coated with 0.5 μ g/ml anti-CD3 antibody, either alone or with 1 ng/ml IL-7. T cell chemotaxis to 10 ng/ml CXCL12 was determined in transwell assays.

Fig. 7. Chemokine receptor expression during neglect. (A) Purified T cells were lysed immediately (Fresh) or were cultured for 1 day prior to lysis (Neglect). Equivalent numbers of live cells were analyzed by Western blot for CXCR4, CCR7, and actin. (B) Surface CXCR4 and CCR7 expression was measured flow cytometrically on freshly isolated or neglected live T cells. Isotype controls are shown in grey, and chemokine receptors are shown in black.

Fig. 8.

Neglected T cells have increased GTP-Rac and enhanced actin polymerization and polarization responses to chemokine. (A) Fresh or neglected T cells were left untreated (None) or were stimulated for 30 s with CXCL12 (10 ng/ml) and analyzed to show total cellular Rac and GTP-bound Rac levels by Western blot. (B–D) Fresh or neglected T cells were left untreated (None) or were stimulated with CXCL12 (10 ng/ml), then stained with phalloidin and analyzed flow cytometrically 1 min after stimulation (B) or by confocal microscopy 5 min after stimulation (C). The frequency of cells with polarized actin was then quantified (D). Means and standard deviations are shown (* indicates $p < 0.005$).