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Constitutive Activation of STAT5 Supersedes the Requirement for Cytokine and TCR Engagement of CD4+ T Cells in Steady-State Homeostasis

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The transcription factor STAT5 is one of several signaling mediators activated via common γ-chain cytokine receptors. As such, it plays an important role in lymphocyte survival and proliferation during normal homeostasis as well as under lymphopenic conditions. Transgenic mice expressing a constitutively activated form of STAT5b have been shown previously to contain increased numbers of peripheral CD4+CD25− T cells. To define the mechanism(s) for this occurrence, we have used adoptive transfer studies to examine the effects of STAT5 activity on steady-state CD4+ T cell homeostasis. We observed that constitutive STAT5 signaling induced 4- to 7-fold increased levels of basal steady-state proliferation, which was accompanied by a comparable increase in T cell recovery. Most strikingly, steady-state CD4 T cell proliferation occurred independently of both MHC class II and IL-15. These observations demonstrate that the STAT5-driven pathway is important to lymphocyte homeostasis and can supersede the need for both TCR engagement and cytokine stimulation. This suggests that the need for TCR stimulation to induce common γ-chain cytokine receptor expression, and thus STAT5 activation, is a key factor in maintaining normal CD4+ T cell homeostasis. The Journal of Immunology, 2006, 177: 2216–2223.

Basal and lymphopenia-induced (homeostatic) proliferation are important parameters that act cooperatively to control peripheral lymphocyte populations and maintain steady-state lymphocyte numbers. Lymphocyte homeostasis may be perturbed by factors such as reduced thymic output and infection (1–7). Under these conditions of lymphopenia, homeostatic pressure promotes clonal proliferation until an equilibrium is re-established (8, 9). The signals that drive homeostatic proliferation include increased availability of both Ag/MHC complexes and growth factors in the form of the common γ-chain cytokines (including IL-2, IL-7, IL-15) and the chemokine CCL21 (10–22). Different T cell subsets require distinct signals for homeostatic expansion. Whereas naive CD8+ T cells require both MHC class I and IL-7, naive CD4+ T cells require MHC class II (MHC II) along with both IL-7 and CCL21 (or in the case of CD4+CD25+ regulatory T cells, MHC II and IL-2 (23–25)). Memory CD4+ and CD8+ T cells have been shown to undergo lymphopenia-induced expansion independently of MHC molecules, and although a requirement for IL-7/IL-15 has been demonstrated in CD8+ memory T cells, a comparable requirement for memory CD4+ T cells has not been identified to date.

The role of specific intracellular signaling molecules triggered by both the TCR and cytokine receptors in homeostatic proliferation has begun to be characterized (26–29). For example, the TCR signaling associated molecule p56lck was shown recently to be necessary for homeostatic proliferation (29, 30). Studies using an inducible lck transgenic model have confirmed that in the absence of lck and fyn TCR signaling failed to elicit efficient homeostatic proliferation (29). Among the downstream pathways activated by γc cytokine receptors is the JAK/STAT signaling cascade, which induces activation of the transcription factor STAT5 (16, 31). This ultimately results in the translation of genes important in proliferation and survival such as cyclins D2, D3 and E, bcl-2, and bcl-xL (32–34).

Mouse models either deficient in, overexpressing, or expressing constitutively active STAT5 have highlighted its importance in lymphocyte homeostasis (26, 35–42). For example, in mice expressing a constitutively activated form of STAT5b as a transgene targeted to the T cell lineage (STAT5b-CA mice), CD8 T cells display an activated/memory phenotype and undergo lymphopenia-induced proliferation even in the absence of both IL-7 and IL-15 (26). These mice also have markedly elevated numbers of CD4+CD25+ regulatory T cells, and an almost 2-fold increase in numbers of naive CD4+ T cells in secondary lymphoid organs (26). Intriguingly, nonregulatory T cells from STAT5b-CA mice exhibited enhanced proliferation in response to anti-CD3 stimulation. Given the role of basal self-Ag/MHC interactions with the TCR in maintaining T cell homeostasis, these data collectively suggested that there might be alterations in survival and/or expansion of mature peripheral CD4+ T cells under nonlymphopenic conditions.

To address these questions, we have used STAT5b-CA mice to examine the effects of STAT5 activation on steady-state homeostasis of naive CD4+ T cells. We observed that naive CD4+ T cells...
from STAT5b-CA transgenic mice exhibited enhanced in vivo steady-state levels of proliferation. Moreover, the "spontaneous" basal proliferation of these naïve CD4+ T cells was independent of MHC II molecules and IL-15. These observations provide a mechanistic basis for the enhanced naïve CD4+ T cell numbers observed in the periphery of STAT5b-CA mice. They further demonstrate that STAT5-dependent signaling pathways can bypass the need for TCR triggering during peripheral homeostasis of CD4+ T cells, and suggest that the need for TCR stimulation for cytokine receptor expression, and thus STAT5 activation, is a key factor in maintaining normal CD4+ T cell homeostasis.

**Materials and Methods**

**Mice**

STAT5b-CA transgenic mice on a C57BL/6 background have been previously described (26). IL-15 knockout mice (IL-15−/−) on a C57BL/6 background were obtained from Taconic Farms upon securing a material transfer agreement from Amgen. Both C57BL/6 congenic Thy 1.1+ and CD45.1+ mice were obtained from either The Jackson Laboratory or Taconic Farms. C57BL/6 CD45.1+ MHC II-deficient mice were purchased from Taconic Farms. All mice used in experiments were between 6 and 12 wk of age and housed and bred under specific pathogen-free conditions at the University of Pennsylvania (Philadelphia, PA). Experimental protocols were conducted in compliance with the University of Pennsylvania Institutional Animal Care and Use Committee.

**Flow cytometry**

Flow cytometric analysis was done using the following labeled Abs: FITC, PE, allophycocyanin, or allophycocyanin-Cy7-anti-CD4 (GK1.5), PerCP-anti-CD69 (H1.2F3), allophycocyanin-anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD25 (PC61), anti-CD45RB (16A), anti-CD8 (53–6.7), biotin-anti-IL-7R (B12–1), streptavidin-conjugated PE (all purchased from BD Pharmingen) and biotin-anti-IL-15Rα (Chimerigen), and allophycocyanin-anti-mouse/rat Foxp3 (eBioscience). Stained cells were analyzed on a LSR II flow cytometer (BD Biosciences). Data analysis and display was done using FlowJo software (Tree Star).

**Adoptive transfers**

Naïve (CD4+CD45RBhighCD25−) T cells obtained from the spleen and lymph nodes of donor STAT5b-CA and littermate Thy-1.2+ and CD45.2+ C57 BL/6 mice were sorted using a FACSVantage (BD Biosciences) to a purity of ≥97%. Cells were labeled with 1 µM CFSE (Molecular Probes), and 1–2 × 10^6 cells were transferred into recipient C57BL6 Thy-1.1+ or CD45.1+ unirradiated congenic mice via the tail vein in 200 µl of sterile PBS. Mice were sacrificed on either day 8 or 14 and lymphocytes harvested from spleen and lymph nodes for flow cytometric analysis. Donor STAT5b-CA cells were detected using either allophycocyanin-conjugated anti-Thy-1.2 (clone 53–2.1), or PerCP-Cy5.5-conjugated anti-CD45.2 (clone 104) Abs (BD Biosciences).

**Western blotting**

Cell lysates were prepared using a lysis buffer composed of 50 mM Tris-HCl (pH 6.8), 0.2% 2-ME, 20% glycerol, 4% SDS, and 0.001% bromophenol blue (Sigma-Aldrich) at a concentration of 1 × 10^6 cells/30 µl of lysis buffer. The supernatant was boiled for 5 min and loaded at 1 × 10^6 cell equivalents/well on a 10% SDS-PAGE gel (Bio-Rad Laboratories) for fractionation. Proteins were transferred onto ECL nitrocellulose membranes (Amersham Biosciences) for analysis. Blocking reagent (Boehringer Mannheim) was first used to block membranes for 1 h at room temperature or overnight at 4°C. Membranes were subsequently probed with Abs of interest at specified dilutions, washed, and incubated with either HRP-conjugated anti-rabbit or anti-mouse Abs at a 1/1000 to 1/2000 dilution for 1 h at room temperature. Protein detection was done using ECL chemiluminescence kit (Roche Diagnostics) according to the manufacturer’s protocol and using and X-OMAT Film (Kodak) or Hyperfilm ECL (Amersham Biosciences). Blots were reprobed after stripping with Restore Western blot stripping buffer (Pierce).

**FIGURE 1.** Phenotype of CD4 T cells from STAT5b-CA mice. A, Single-cell suspension from spleen and lymph node of STAT5b-CA and WT control mice were stained for CD4, CD45RB, and CD25, or CD4, CD45RB, CD25, and Foxp3 and analyzed by flow cytometry. B, In addition to Abs used in A, cells were additionally stained with Abs to CD62L, CD44, and CD69. Histograms were generated by gating on CD4+ naïve T cells (shaded, STAT5b-CA; open, WT). Data are representative of three experiments.
In vitro survival and proliferation

Cells were either labeled with 1 μM CFSE or cultured unlabelled in RPMI complete medium comprising RPMI 1640 (Invitrogen Life Technologies), with 10% heat-inactivated FCS (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1.5 μM 2-ME (Sigma-Aldrich), at 37°C in 5% CO₂. Cells were cultured at a concentration of 1–2 × 10^6 cells/ml in 96-well U-bottom plates (Costar) in the presence or absence of specified amount of murine rIL-2, rIL-7, or rIL-15 (R&D Systems) for 96 h. Cells were harvested, washed, and either counterstained with vital dye 7-amino-actinomycin D (7-AAD; BD Biosciences) to assess viability or analyzed by flow cytometry to assess proliferation.

BrdU labeling

Mice were injected i.p. with 1 mg/day BrdU in PBS for 4 days (to assess the 1-wk time point) and for 7 days (to assess the 2-wk time point) before sacrifice. Cells were harvested from lymph nodes and spleens and stained with Abs Thy-1.2 or CD45.2 to identify donor cells. Data expressed are the percentage of live nucleated donor cells in host lymph nodes or spleens and are representative of three experiments.
with anti-Thy1.2. BrdU labeling was assessed using aliphycocyanin BrdU Flow kits (BD Biosciences) according to the manufacturer's instructions.

Results

Constitutive activation of STAT5 leads to basal proliferation of naive CD4+ T cells

To investigate the role of STAT5 in the steady-state homeostasis of naive T cells, we studied CD4+CD45RBhigh cells from STAT5b-CA mice. Consistent with our previous report (26), STAT5b-CA mice had elevated proportions of CD4+CD25Foxp3+ regulatory cells within the CD4+ T cell compartment (Fig. 1A). Nonregulatory naive CD4+CD45RBhigh cells expressed similar levels of CD44, CD62L, and CD69, as did cells from wild-type control (WT) mice (Fig. 1B).

To examine whether constitutive activation of STAT5 affected steady-state homeostasis in the CD4 compartment, we adoptively transferred FACS-sorted (purity, $\geq$97%), CFSE-labeled naive CD4+ T cells (CD4+CD25CD45RBhigh) from STAT5b-CA and WT mice into congenic hosts. By day 8 after transfer, naive CD4+ T cells from STAT5b-CA mice had begun to proliferate, whereas those of the WT mice had not (Fig. 2A, 1 wk). By day 14, we detected extensive division of STAT5b-CA cells (Fig. 2A, 2 wk). At this time point, $>70$% of the naive CD4+ T cells from STAT5b-CA mice had divided compared with only 6–20% of cells from littermate controls (Fig. 2A, 2 wk). Table I displays detailed proliferation parameters observed at day 14 and demonstrates that CD4+ T cells from STAT5b-CA mice experienced a 4- to 7-fold greater proliferation frequency compared with CD4+ T cells from WT. These results demonstrate that constitutive activation of STAT5 can elicit extensive proliferation of CD4+ T cells in a steady-state environment.

STAT5 is known to induce the expression of prosurvival molecules such as bcl-xL (43–46) (Fig. 2D), suggesting that it might also modulate the in vivo survival of STAT5b-CA naive CD4+ T cells. Therefore, for the adoptive transfer experiments described above, we also quantified cell recovery. We observed that STAT5b-CA naive CD4+ T cells displayed a statistically significant 2- to 3-fold increase in recovery compared with littermate control CD4+ T cells (Fig. 2B). In vivo proliferation was further confirmed by BrdU labeling studies, which demonstrated a 2- to 5-fold higher percentage of BrdU-positive STAT5b-CA naive T cells compared with WT littermate CD4+ naive T cells (Fig. 2C). The increase in bcl-xL expression in STAT5b-CA CD4+ T cells (Fig. 2D) raised the possibility that enhanced survival, as well as proliferation, contributed to the increase in cell recovery of this population following adoptive transfer. Notably, however, this increase in cell recovery of STAT5b-CA CD4+ T cells does not exceed their degree of enhanced proliferation (Table I). Thus, it is likely that, despite the increased levels of bcl-xL expression observed in STAT5b-CA CD4+ T cells, augmented survival plays little if any role in their enhanced numbers following adoptive transfer.

Effects of STAT5 activation on IL-7R and IL-15R expression

The γc cytokines IL-7 and IL-15 are important in survival and homeostatic proliferation of both naive and memory T cells (16, 19, 20, 31, 47–51). Given the enhanced spontaneous proliferation of STAT5b-CA naive CD4 T cells in vivo, and the known ability of STAT5 to enhance IL-2Rα chain expression (52), we next asked whether these cells expressed elevated levels of IL-7Rα or IL-15Rα chains. Flow-cytometric analysis revealed no changes in IL-7Rα expression (Fig. 3A). However, a pronounced difference was observed for the IL-15Rα chain, where naive CD4+ T cells from STAT5b-CA mice expressed ~4-fold higher IL-15Rα levels compared with littermate controls (Fig. 3A).

Having observed increased expression of IL-15Rα on STAT5b-CA naive CD4+ T cells, we next evaluated the response of these cells to stimulation with IL-15. IL-15 served as a control cytokine, because expression of its receptor was not altered by STAT5 activation. To this end, we cultured sorted CFSE-labeled cells with optimal concentrations of recombinant cytokines and evaluated proliferation after 96 h (Fig. 3B). IL-7 had only a minimal effect on the proliferation of naive CD4+ T cells from both STAT5b-CA mice and littermate controls. However, IL-15 induced modest but reproducible proliferation in naive CD4+ T cell from STAT5b-CA mice (Fig. 3B). Together, these results indicate that IL-15Rα expression is preferentially elevated on STAT5b-CA CD4+ T cells and may contribute to their proliferation.

IL-15- and MHC II-independent proliferation of STAT5b-CA CD4+ T cells in vivo

Our previous studies on STAT5b-CA mice demonstrated enhanced homeostatic proliferation of CD8+ T cells in the presence of IL-7 and IL-15 signaling, but also revealed the ability of these cells to undergo homeostatic expansion even in the absence of these cytokines (26). These results suggested that naive CD4+ T cells might be able to expand in vivo under steady-state conditions even in the absence of IL-15. To test this hypothesis, we adoptively transferred FACS-purified, CFSE-labeled naive CD4+ T cells into IL-15-deficient mice. After 14 days, mice were sacrificed and proliferation was assessed via CFSE dilution. As seen in Fig. 4 naive CD4+ T cells from STAT5b-CA mice proliferated readily even in the absence of IL-15, whereas no proliferation was observed in littermate control naive CD4+ T cells. Importantly, the degree of proliferation seen was comparable to that observed in IL-15-sufficient hosts (compare Figs. 2 and 4). Thus, despite increased expression of IL-15Rα and increased responsiveness to IL-15, the enhanced turnover of STAT5b-CA naive CD4+ T cells in lymphopenic mice is not dependent upon IL-15.

To examine whether MHC II molecules are required for the observed increase in basal proliferation of STAT5b-CA CD4+ T cells in a nonlymphopenic environment, FACS-sorted, CFSE-labeled CD4+ T cell subsets were transferred into congenic MHC

Table I. Frequency of undivided and divided naive CD4+ T cells 14 days after adoptive transfer

<table>
<thead>
<tr>
<th>Site</th>
<th>Naive STAT5b-CA CD4+ T Cells</th>
<th>Naive WT Control CD4+ T Cells</th>
<th>Naive STAT5b-CA CD4+ T Cells</th>
<th>Naive WT Control CD4+ T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency of undivided (%)</td>
<td>Lymph node</td>
<td>25.8 ± 2.45</td>
<td>82.4 ± 12.4</td>
<td>22.1 ± 5.78</td>
</tr>
<tr>
<td>Frequency of divided (%)</td>
<td>Lymph node</td>
<td>72.0 ± 2.04</td>
<td>10.7 ± 3.54</td>
<td>70.6 ± 11.8</td>
</tr>
</tbody>
</table>

*Sorted CFSE-labeled CD4+ naive T cells (CD4+CD45RBhigh/CD25-): from C57BL/6 STAT5b-CA and WT control donors (either Thy1.2+ or CD45.2+) were adoptively transferred into nonlymphopenic congenic C57BL/6 (either Thy1.1+ or CD45.1+) hosts. On day 14, cells were stained with Abs to Thy1.2 or CD45.2 and analyzed by flow cytometry. Gates were drawn on the CFSE histogram to define undivided and divided cells. Data are derived from four experiments.
II-deficient hosts (Fig. 5). We found that 75–85% of naive CD4⁺ T cells from STAT5b-CA mice had undergone division by day 14, whereas essentially no division was seen in littermate control CD4⁺ T cells. This lack of division of littersmate control naive CD4⁺ T cells is similar to that which is observed in the MHC II-sufficient mice (Figs. 2 and 5). Interestingly, STAT5b-CA naive CD4⁺ T cells proliferated equally well in an MHC II-null background as they did in a MHC II-sufficient environment (compare Figs. 2 and 5). This result suggests that the constitutive activation of STAT5 supports a pathway that may bypass the need for MHC II in the steady-state proliferation of naive CD4⁺ T cells.

Discussion

Minimal proliferation of naive T cells occurs at the steady state while these cells undergo spontaneous proliferation in a lymphopenic environment to restore and maintain T cell homeostasis (11, 13, 17, 53–59). Lymphocyte homeostasis at the steady state or during lymphopenia is controlled by the TCR complex, and cytokine and chemokine receptors (11, 13, 17, 22, 60). TCR signaling results in down-regulation of suppressor of cytokine signal-1, thereby allowing γc cytokines (such as IL-7 and IL-5 which are produced constitutively but at low levels) to activate STAT5 and promote homeostasis (61). In this study, we investigated how a specific signal delivered downstream of γc cytokines is important in regulating naive CD4⁺ T cell homeostasis. We found that the constitutive activation of the STAT5 transcription factor increased steady-state proliferation of naive CD4⁺ T cells, which could occur independent of MHC II and IL-15.

The STAT5 transcription factor is associated with γc cytokines such as IL-2, IL-7, and IL-15 (40, 62). The γc cytokine receptor complex is composed of two to three subunits, which include common β (for IL-2 and IL-15) and γ-chains with a private α-chain for each individual cytokine (63–67). Signal initiation following receptor ligation is controlled by the JAK/STAT molecular cascade. Dimerization of both the β- and γ-chains occurs in response to cytokine engagement and provides docking sites for JAK1 and JAK3 tyrosine kinases, respectively. The phosphorylation of specific tyrosine residues Tyr³³⁸, Tyr⁹⁰³, or Tyr⁵¹⁰ on the β-chain allows it to in turn serve as a docking port for downstream signaling molecules, one of which is the transcription factor STAT5 (68). The activation and dimerization of STAT5 facilitates its nuclear translocation resulting in the transcription of genes such as cyclins D2, D3, E, cdk 6, bcl-2, and bcl-xL, which are important in lymphocyte proliferation and survival enhancement (32–34, 40, 68).

The importance of STAT5 in lymphocyte homeostasis has been well established by both overexpression and knockout studies. STAT5-deficient mice display dramatic decreases in splenic cellularity and defective IL-2 signaling (38–40). They also have significant reduction in common lymphoid progenitors, pro-B cells, pro-T cells, CD8⁺ T cells, and an absence of CD4⁺CD25⁺ regulatory T cells (35–38, 40–42, 69, 70). Conversely, overexpression of WT STAT5b may lead to a dramatic increase in CD8⁺ T cell numbers coupled with heightened proliferation and survival (37). Recently, we have observed expansion in both pro- and pre-B cells, dramatic increases in both CD8 T cells and CD4⁺CD25⁺ regulatory T cells, and more subtle changes in naive CD4⁺ T cell numbers (26, 70). Collectively, these models demonstrate the importance of STAT5 in lymphocyte homeostasis.
We observed that STAT5b-CA naive CD4+ T cells displayed a 4-fold greater expression of IL-15Rα chain on their surface when compared with WT T cells. Although the regulation of the IL-15Rα receptor by STAT5 is not well studied, that of the IL-2Rα is very well characterized (39, 71, 72). It has been demonstrated that IL-2 regulates the transcription of the IL-2Rα, which is critical for high-affinity IL-2 binding (71, 72). This occurs via an IL-2-responsive enhancer site that contains two STAT5 binding domains (71). The binding of STAT5 to these domains following IL-2Rα ligation by IL-2 leads to further IL-2Rα up-regulation (52, 71, 72). Interestingly, it was demonstrated that IL-15 could also elicit the expression of the IL-2Rα albeit in an in vitro system (73). IL-15 is often characterized as a T cell growth factor and was identified as Bcl-6, have been shown to be involved in STAT5-induced proliferation under conditions of lymphopenia and Ag activation (82–84). Bcl-6 was demonstrated to be able to act as an amplifier for the generation and proliferation of CD8+ T cells and contains a STAT5 binding site in its promoter region (82–84). In conclusion, this study demonstrated that constitutive activation of STAT5 is sufficient to enable steady-state proliferation of naive CD4+ T cells, even in the absence of MHC II molecules or IL-15. The ability of STAT5 signaling to bypass the need for TCR or IL-15 signaling in the induction of T cell proliferation highlights the importance of TCR-mediated regulation of γc cytokine receptor expression.

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Disclosures
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