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A Role for Stat5 in CD8\(^+\) T Cell Homeostasis

John Kelly, Rosanne Spolski, Kazunori Imada, Julie Bollenbacher, Stephen Lee, and Warren J. Leonard

Cytokine signals are known to contribute to CD8\(^+\) memory T cell homeostasis, but an exact understanding of the mechanism(s) has remained elusive. We have now investigated the role of Stat5 proteins in this process. Whereas Stat5\(a\) and Stat5\(b\) KO mice have decreased numbers of CD8\(^+\) T cells, Stat5\(a\)-transgenic mice have an increased number of these cells. Stat5\(b\)-transgenic mice exhibit increased Ag-induced cell death of CD4\(^+\) T cells and augmented proliferation and Bcl-2 expression in CD8\(^+\) T cells, providing a basis for this finding. Moreover, CD8\(^+\) memory T cells are substantially affected by Stat5 levels. These findings identify Stat5 proteins as critical signaling mediators used by cytokines to regulate CD8\(^+\) T cell homeostasis.


During the primary immune response, Ag-specific T cells proliferate to provide an effective response against invading pathogens (1, 2). At the end of this response, the majority of the effector cells undergo apoptosis, with a small minority persisting as Ag-specific memory cells (3, 4). The size of naive and activated T cell pools are regulated independently, assuring both diversity and ability to engage new Ags as well as a rapid response to previously encountered Ags (5). Unlike naive CD8\(^+\) T cells, memory CD8\(^+\) T cells do not require the presence of MHC class I for their maintenance (6). Accordingly, Ag-independent factors such as cytokines have been implicated as the critical determinants of CD8\(^+\) memory T cell survival and proliferation (7).

Cytokines signal via multiple pathways, including the Janus kinase/Stat pathway (8, 9). Seven STAT proteins have been identified to date: Stat1–4, Stat5\(a\), Stat5\(b\), and Stat6. Whereas Stat2, Stat4, and Stat6 have a narrow activation profile, Stat1, Stat3, Stat5\(a\), and Stat5\(b\) are activated by many cytokines. Stat5\(a\) and Stat5\(b\) are particularly distinctive in having unusually high (\(>90\%\)) amino acid identity and are located in a head-to-head orientation on human chromosome 17 and mouse chromosome 11 (9, 10). However, important functional differences between these two proteins may exist as evidenced by differences in phenotypes seen in gene-targeted mice. For example, Stat5\(a\) KO (Stat5\(a^{−/−}\)) mice exhibit defective prolactin signaling (11) and IL-2-induced expression of the IL-2R\(\alpha\) (12), while Stat5\(b^{−/−}\) mice have impaired growth responses (13) and exhibit more severe immunological abnormalities (14). Whether these findings result from intrinsic differences in protein structure and DNA binding and/or differences in tissue/cellular availability of these proteins is an area of ongoing investigation. Interestingly, both Stat5\(a^{−/−}\) and Stat5\(b^{−/−}\) mice have been reported to exhibit reduced splenic cellularity (12, 14) and Stat5b\(^{−/−}\) and Stat5a\(^{−/−}\)/Stat5b\(^{−/−}\) mice have reduced splenic CD8\(^+\) cells (14, 15), although no studies related to Stat5 and CD8\(^+\) memory T cell homeostasis have been reported. To investigate the role of Stat5 in T cell homeostasis and to determine the extent to which Stat5b could compensate for the absence of Stat5a, we reconstituted Stat5a\(^{−/−}\) mice with a transgene expressing Stat5b. Transgenic expression of Stat5b on a Stat5a\(^{−/−}\) background allowed us to achieve a dose response with regard to the effect of Stat5 levels on T cell homeostasis. We found that whereas Stat5a\(^{−/−}\) and Stat5b\(^{−/−}\) mice have decreased CD8\(^+\) memory cells, transgenic Stat5 expression markedly increases CD8\(^+\) memory T cell numbers, indicating a critical role for Stat5 proteins in regulating this population of cells.

Materials and Methods

Generation of mice

The Stat5b-transgenic construct was under the control of the H-2K\(\beta\) promoter and H chain enhancer in pHSE (Fig. 1A). The HA1 epitope tag from hemagglutinin (HA)\(^2\) was inserted at the 5’ end of the Stat5b coding region to distinguish the transgenic product from endogenous Stat5b. Mice were generated by microinjection of C57BL/6 embryos at the National Institute of Allergy and Infectious Diseases transgenic facility. Presence of the transgene was initially detected by Southern blotting and subsequently by PCR. Protein expression was confirmed by Western blotting using an Ab to the HA1 epitope (12CA5 mAb; Boehringer Mannheim, Indianapolis, IN). In addition, Stat5b-specific Abs (16) were used to determine levels of total (endogenous and transgenic) Stat5b protein. Transgenic mice were crossed to Stat5a hypomorphic (\(+/−\)) mice. Stat5a\(^{−/−}\) females expressing the Stat5b transgene were then mated with Stat5a\(^{+/−}\) or Stat5a\(^{−/−}\) males to generate Stat5a\(^{−/−}\) mice that express the Stat5b transgene. Mice were analyzed at 8–12 wk of age. Transgenic mice were also crossed onto wild-type (WT, C57BL/6 or BALB/c) and common cytokine receptor \(\gamma\)-chain (\(\gamma\)) KO backgrounds. KO status was established by PCR. Because of the age-dependent expansion of CD4\(^+\) cells in \(\gamma\) KO mice, these mice and relevant controls were analyzed at 3–4 wk of age. To evaluate Ag-specific responses, mice were immunized i.p. with 1 mg of OVA (Pierce, Rockford, IL) mixed with 100 \(\mu\)g of monoclonal anti-CD40 (BD Pharmingen, San Diego, CA) (17). All experiments were performed under protocols approved by the National Institutes of Health Animal Use and Care Committee and followed the National Institutes of Health guidelines Using Animals in Intramural Research.

CD4\(^+\) and CD8\(^+\) cell separation

To determine Stat5b levels in thymic and splenocyte subpopulations, cells were separated using paramagnetic MicroBeads conjugated to monoclonal CD4 and CD8a anti-mouse Abs (MACS; Miltenyi Biotec, Auburn, CA). Positive and negative selection columns were used according to the manufacturer’s instructions. Purity (\(>90\%\)) was confirmed by flow cytometry.

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Levels of Stat5b were assessed in thymic subpopulations and in splenic CD4+ and CD8+ T cells with and without stimulation with either plate-bound anti-CD3ε (7.5 μg/ml) or a combination of soluble anti-CD3ε (2 μg/ml) and anti-CD28 (2 μg/ml).

**Western blotting**

Whole cell extracts (5–20 μg/sample) were fractionated on 8% polyacrylamide gels (Invitrogen/NOVEX, San Diego, CA) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). After blocking with 5% milk, the blots were incubated with rabbit anti-Stat5b or mouse anti-HA, washed, and incubated with a HRP-conjugated anti-rabbit or anti-mouse Ab (Nycomed; Amersham, Little Chalfont, Buckinghamshire, U.K.). Blots were developed with an ECL substrate (Pierce). For immunoprecipitation, whole cell extracts (200 μg/sample) were incubated with 2 μl of anti-HA Ab (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, followed by addition of protein A-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) for 90 min. After washing, Western blotting was performed as described above, on 15 μl of the immunoprecipitated samples, with anti-phospho-Stat5 (Tyr 694; Cell Signaling Technology, Beverly, MA).

** EMSAs and RNase protection assays**

Nuclear extracts were prepared and EMSAs were performed with a β casein probe as described previously (18). For supershifting assays, nuclear extracts were preincubated for 20 min with 2 μl of anti-Stat5b (Zymed, South San Francisco, CA). A multiprobe RNase protection assay system (RiboQuant; BD PharMingen) was used according to the manufacturer’s instructions. These tetramers are complexes of four MHC class I molecules associated with a specific peptide and bound to a fluorochrome (PE). They stain a specific set of TCRs on a subset of CD8+ T cells, allowing evaluation of Ag-specific responses (19, 20). For Bcl-2 staining, cells were fixed and permeabilized using Cytofix/Cytoperm solution, followed by FITC-conjugated Bcl-2 Ab staining (BD Pharmingen). 5-Bromo-2′-deoxyuridine (BrdU) incorporation was assessed following two injections of 800 μg of BrdU (Sigma-Aldrich, St. Louis, MO) at 16 and 10 h before sacrifice. Levels of BrdU were determined using a FITC-conjugated Ab (21). Apoptosis was assessed using the TACS Annexin V FITC apoptosis detection kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. For intracellular cytokine staining, cells were fixed and permeabilized using Cytofix/Cytoperm solution, followed by FITC-conjugated IFN-γ and PE-conjugated IL-4 Abs (BD Pharmingen).

**Proliferation assays**

Fresh splenocytes were cultured for 48 h in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, and antibiotics, with or without 2 nM IL-2, on 96-well flat-bottom plates (2 × 105 cells/well) with and without anti-CD3ε coating. Wells were pulsed with 1 μCi of [3H]thymidine (6.7 Ci/mmol; NEN, Boston, MA) for the last 9 h of culture. To evaluate the response to IL-15, transgenic mice (TG1) and WT mice were injected with either PBS or poly(I:C) (100 μg/mouse; Amersham Pharmacia Biotech), sacrificed 48 h later, and splenocytes were cultured (2 × 10^6 cells/well) in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, with antibiotics) with no cytokine, IL-2 (2 nM), or IL-15 (100 ng/ml) for 24 h and pulsed with 1 μCi of [3H]thymidine for the last 9 h of culture.

**Flow cytometric analysis**

Single-cell suspensions from spleen were stained and analyzed using a FACSort with CellQuest software (BD Biosciences, San Jose, CA). The following Abs, all from BD Pharmingen, were used: anti-CD4-PE, -PE, and -CyChrome; anti-CD8-PE and -allophycocyanin; anti-TCR-γ/δ-PE (H57-597 to TCRβ); anti-TCR-γ/δ-FITC; anti-IL-2-PE (CD25)-FITC and -PE; anti-CD44-CyChrome; anti-IL-2R-PE and -PE; anti-pan-NK cells (DX5)-FITC; and anti-CD3-allophycocyanin. In some experiments, dead cells were excluded by staining with propidium iodide (PI). Ag-specific CD8+ T cells were stained using the iTag MHC tetramer H-2Kb-OVA-(SIINFEKL)-SA-PE (Beckman Coulter, Fullerton, CA) according to the manufacturer’s instructions. In some experiments, dead cells were excluded by staining with propidium iodide (PI). Ag-specific CD8+ T cells were stained using the iTag MHC tetramer H-2Kb-OVA-(SIINFEKL)-SA-PE (Beckman Coulter, Fullerton, CA) according to the manufacturer’s instructions. These tetramers are complexes of four MHC class I molecules associated with a specific peptide and bound to a fluorochrome (PE). They stain a specific set of TCRs on a subset of CD8+ T cells, allowing evaluation of Ag-specific responses (19, 20). For Bcl-2 staining, cells were fixed and permeabilized using Cytofix/Cytoperm solution, followed by FITC-conjugated Bcl-2 Ab staining (BD Pharmingen). 5-Bromo-2′-deoxyuridine (BrdU) incorporation was assessed following two injections of 800 μg of BrdU (Sigma-Aldrich, St. Louis, MO) at 16 and 10 h before sacrifice. Levels of BrdU were determined using a FITC-conjugated Ab (21). Apoptosis was assessed using the TACS Annexin V FITC apoptosis detection kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. For intracellular cytokine staining, cells were fixed and permeabilized using Cytofix/Cytoperm solution, followed by FITC-conjugated IFN-γ and PE-conjugated IL-4 Abs (BD Pharmingen).

**FIGURE 1.** Transgenic expression of Stat5b. A, Schematic of the pHSF-Stat5b-transgenic vector. The XhoI site within the Stat5b coding region was mutated (without changing the amino acid sequence) to allow excision of the transgenic expression elements by XhoI. B, Anti-Stat5b Western blot using lysates from fresh splenocytes. Both TG1 (lane 1) and TG2 (lane 2) have more total Stat5b than WT (lanes 3–6). C, Anti-HA Western blotting of lysates from splenic and thymic T cell subpopulations of TG1 mice. WT subpopulations are included as controls. The HA-tagged Stat5b migrates close to a nonspecific band (NS). D, Stat5b levels are induced in WT splenic T cells following 48-h stimulation with anti-CD3/CD28, particularly in the CD8+ cells. Stat5a is similarly induced (data not shown). E, Immunoprecipitation with anti-HA followed by Western blotting with an anti-phospho-Stat5 Ab confirms IL-2-induced phosphorylation of Stat5a/5b (lanes 3 and 7) and the band was supershifted by anti-Stat5a (lanes 4 and 8) in both CD4+ and CD8+ cells.
IFN-γ production

Mice were immunized i.p. with 1 mg of OVA (Pierce) mixed with 50 μg of monochlonal anti-CD40 (BD PharMingen) (17). Six days later, fresh splenocytes (2 × 10^6 cells/well in RPMI 1640 containing 10% FBS, 2 mM l-glutamine, with antibiotics) were cultured for 48 h on 96-well plates with either a combination of soluble anti-CD3ε (2 μg/ml) and anti-CD28 (2 μg/ml) or 100 μg of soluble OVA. IFN-γ levels were measured using intracellular staining as outlined above.

Results

Generation of Stat5b-transgenic mice

To investigate the effect of augmented Stat5 expression within the immune system, we generated Stat5b-transgenic mice using pHSE (Fig. 1A), a transgenic vector which contains the H2-Kb promoter and IgM enhancer. This plasmid preferentially directs expression in T cells, B cells, and NK cells (22, 23). Two founder lines (TG1, TG2) expressing the Stat5b transgene in both thymus and spleen were evaluated for splenic Stat5b protein expression using an anti-Stat5b-specific antiserum. In both founders, levels of total Stat5b protein were higher than those of endogenous Stat5b in WT mice (Fig. 1B, lanes 1 and 2 vs 3–6). In splenocyte subpopulations, transgenic Stat5b expression was comparable in CD4+ and CD8+ T cells (Fig. 1C, lanes 8 vs 10). Stat5b was also expressed in thymic subpopulations (Fig. 1C, lanes 3, 4, and 6, and data not shown). Interestingly, levels of endogenous Stat5a and Stat5b protein were increased in WT mice in response to TCR activation with anti-CD3 (Fig. 1D and data not shown), consistent with a report of increased Stat5b mRNA in response to TCR stimulation (24). Such up-regulation may prime cells for optimum responses to cytokines following TCR engagement. To demonstrate that the transgene is activated in a manner similar to endogenous Stat5, we examined both phosphorylation and DNA binding in response to IL-2 stimulation. Immunoprecipitation with anti-HA Ab followed by Western blotting with an anti-phospho-Stat5 Ab confirmed phosphorylation of the transgene in response to IL-2 (Fig. 1E, lane 4). The absence of such a band in the WT splenocytes confirmed the specificity of the HA immunoprecipitation (Fig. 1E, lane 2). Similarly, EMSAs with a contaminated GAS motif from the β casein gene probe confirmed Stat5 DNA binding activity in response to IL-2 in transgenic mice at levels higher than those observed in WT mice (Fig. 1F, lanes 3, 7, and 11 vs 14). DNA binding activity was seen in both CD4+ and CD8+ T cells from transgenic mice lacking endogenous Stat5a and Stat5b (Fig. 1F, lanes 3 and 7), and supershift experiments with anti-Stat5b demonstrated that these complexes contained Stat5b (lanes 4 and 8).

Levels of Stat5 affect total cellularity and the CD4+:CD8+ ratio in the spleen

Thymocyte numbers were similar in WT, Stat5a−/− mice, and Stat5b-transgenic mice. Flow cytometric analysis of thymocytes from each of these mice revealed normal populations of CD4+ and CD8+ T cells (data not shown and see below). In contrast, in the periphery, both Stat5a−/− and Stat5b−/− mice tended to have slightly decreased splenocyte numbers (Fig. 2A), as previously reported (12, 14). Expression of the Stat5b transgene on either Stat5a−/− or WT backgrounds increased splenocyte numbers to higher than those of WT levels (Fig. 2A). Similarly, whereas CD8+ splenocytes were modestly reduced in both Stat5a−/− and Stat5b−/− mice (means of 6 ± 3 × 10^3 and 4 ± 2 × 10^3, respectively, vs 8 ± 3 ± 10^6 in WT mice, p < 0.01), Stat5a−/− mice expressing the transgenes had increased numbers of CD8+ T cells (means of 22 ± 7 ± 10^6 and 43 ± 18 ± 10^6, respectively, for TG2 and TG1, p < 0.0001 compared with WT). Moreover, whereas the CD4+ :CD8+ ratio was increased in Stat5a−/− and Stat5b−/− mice (average ratios, 2.7 ± 1 and 3.0 ± 1, respectively) as compared with WT (average ratio, 2.0 ± 0.4, p < 0.0001), Stat5a−/− mice.
expressing the Stat5b transgene had a decreased CD4⁺:CD8⁺ ratio (average values for TG2 and TG1, 1.2 ± 0.4 and 0.8 ± 0.4, respectively, p < 0.0001 compared with wild type; Fig. 2, B and C). Similar reductions in CD4⁺:CD8⁺ ratios were seen by expression of a Stat5a transgene either on WT (Fig. 2B) or Stat5b⁻/⁻ backgrounds (data not shown). CD4⁺ T cells were not reduced; therefore, the altered ratio was due to an increase in CD8⁺ T cells.

Memory cells are recognized by surface phenotypic markers that indicate prior activation via their TCR. Whereas Stat5a⁻/⁻ and Stat5b⁻/⁻ mice had fewer CD44highIL-2Rβhigh T cells than did WT mice, introduction of either a Stat5a or Stat5a transgene on either a Stat5a⁻/⁻ (Fig. 2D) or Stat5b⁻/⁻ (data not shown) background markedly increased this memory cell population. As anticipated, the CD8⁺ T cell expansion also occurred when the Stat5b transgene was expressed on a WT background (Fig. 2, A and B). The Stat5b (TG1) transgene conferred an increase in splenic cellularity in mice as young as 12 days old, with evidence of a decreased CD4⁺:CD8⁺ ratio (Fig. 2E, d vs c) and increased CD8⁺ memory T cells (f vs e). As remains true as the mice age, no change in thymic CD4⁺:CD8⁺ ratio was observed (Fig. 2E, b vs a).

**Stat5b overexpression increases proliferation and survival of CD8⁺ cells**

To examine the mechanisms by which Stat5 influences T cell homeostasis, we first evaluated anti-CD3-induced proliferation of fresh Stat5a⁻/⁻ splenocytes reconstituted with Stat5b (Fig. 3A). As previously reported, anti-CD3-induced proliferation is diminished in Stat5a⁻/⁻ splenocytes stimulated with anti-CD3 (25), and we now show that introduction of a Stat5b transgene corrected this defect (Fig. 3A). Previous studies of splenocytes from Stat5a⁻/⁻ mice demonstrated that both CD4⁺ and CD8⁺ T cells have decreased IL-2Rα induction following anti-CD3 stimulation, resulting in diminished high-affinity IL-2 receptors and therefore in diminished responsiveness to low concentrations of IL-2 (14, 25). Although Stat5a⁻/⁻ CD4⁺ and CD8⁺ T cells had lower levels of IL-2Rα than WT cells, expression of the Stat5b transgene increased anti-CD3-induced IL-2Rα expression to levels that were either similar to (TG2) or higher than (TG1) WT levels (data not shown). Consistent with the effect of the transgene on the Stat5a⁻/⁻ background, the presence of the Stat5b transgene on a WT background also enhanced the proliferation of fresh splenocytes in response to anti-CD3 (Fig. 3B). Because poly(I:C) stimulates proliferation of CD8⁺ cells in vivo (7) through the induction of cytokines, including IL-15, we measured in vitro proliferation in response to IL-2 or IL-15 in splenocytes of mice that were treated with poly(I:C). Consistent with increased CD44highIL-2Rβhigh memory T cell numbers in Stat5b-transgenic mice, the Stat5b transgenic splenocytes exhibited greater proliferation in response to both IL-2 and IL-15 than did WT splenocytes, and this proliferation was greatly enhanced in Stat5b-transgenic mice injected with poly(I:C) (Fig. 3C). To evaluate proliferation in vivo, we measured BrdU incorporation (Fig. 3D). Although there was no difference in basal BrdU incorporation between WT and transgenic splenocytes (Fig. 3D, histogram c vs a) following poly(I:C) injection, there was a marked increase in BrdU incorporation in the Stat5b-transgenic CD8⁺ splenocytes (Fig. 3D, histogram d vs b). However, similar BrdU incorporation was seen in the CD44highIL-2Rβhigh (which are comprised mainly of CD8⁺ cells) populations of both Stat5b-transgenic and WT mice stimulated with poly(I:C) (Fig. 3D, histograms f and h). Thus, the increased BrdU incorporation in Stat5b-transgenic CD8⁺ cells following poly(I:C) appears to reflect the increased IL-2RβhighCD44high population seen with Stat5b overexpression.

To determine whether decreased CD8⁺ T cell death might be contributing to the increased CD8⁺ cell number and diminished CD4⁺:CD8⁺ ratio seen in Stat5b-transgenic mice, freshly isolated splenocytes from Stat5b-transgenic mice (TG1) on the WT background were preactivated with anti-CD3 for 48 h, washed, and then cultured at 1 x 10⁶/ml (Fig. 4A, day 0). The number of viable cells after subsequent culture of 1–7 days, as assessed by trypan
blue exclusion, was higher in the transgenic mice than in the WT mice (Fig. 4A). Correspondingly, levels of Bcl-2, which promotes T cell survival (3), were increased in freshly isolated transgenic CD8$^+$ T cells (Fig. 4B, histogram f) and reduced in Stat5a$^{-/-}$ mice (histogram e), as compared with WT mice (histogram d). Levels of Bcl-2 were also elevated on transgenic CD4$^+$ cells but to a lesser degree (Fig. 4B, histogram c). Bcl-2 is expressed at high levels in memory cells (26), raising the possibility that the increase in Bcl-2 expression in Stat5b-transgenic CD8$^+$ T cells was related to an increase in memory T cell numbers. It is possible that the augmented IL-2R$^b_{\text{high}}$ cells in Stat5b-transgenic mice allowed greater IL-15-dependent CD8$^+$ memory T cell development/expansion, favoring the increased survival of these cells. Gating on the CD4$^{b_{\text{high}}}$$^{b_{\text{high}}}$$^{b_{\text{high}}}$ cells revealed increased Bcl-2 mean fluorescent intensity in this memory cell population in the Stat5b-transgenic mice (histogram i, black overlay), and conversely the nontransgenic Stat5a$^{-/-}$ memory cells had decreased Bcl-2 expression (histogram h, black overlay). In addition, gating on the brighter peak of the biphase Bcl-2 CD8$^+$ gated cells (histogram f) revealed that this peak is comprised entirely of memory cells (data not shown).

Twenty-four hours after anti-CD3 stimulation, there was already significant expansion of the transgenic splenocytes that continued to increase until day 3 (Fig. 4A). We therefore examined the relative viability of these cells 24 h after anti-CD3 stimulation and found increased annexin V$^+$PI$^+$ cells in the Stat5b-transgenic CD8$^+$ T cell population (Fig. 4C, histogram c vs a). In contrast, the percent annexin V$^+$PI$^+$CD8$^+$ T cells was very low in WT, Stat5a$^{-/-}$, and Stat5b-transgenic mice (Fig. 4C, histograms d, e, and f). Subsequent stimulation with IL-2 (Fig. 4D) or IL-15 (data not shown) followed by a secondary 6-h stimulation with anti-CD3 resulted in an even more profound increase in annexin V$^+$PI$^+$CD8$^+$ T cells in the Stat5b-transgenic mice (Fig. 4D, histogram c), whereas the CD8$^+$ T cells exhibited minimal numbers of this population (histogram f).

**Stat5 can induce CD8$^+$ T cell expansion in γ$c$ KO mice, albeit at an attenuated level**

Given the observed effects of IL-2 and IL-15 on CD8$^+$ T cell expansion in vitro, we next investigated the contribution of γ$c$-dependent cytokines in driving the CD8$^+$ memory T cell expansion in vivo by crossing the TG1 Stat5b transgene onto the γ$c$ KO background. γ$c$ KO mice are known to exhibit defective lymphoid development with decreased CD8$^+$ cells (27). Interestingly, the presence of the Stat5b transgene increased the splenic cellularity on both WT and γ$c$ KO backgrounds (Fig. 5A). Although the...
FIGURE 5. Overexpression of Stat5b does not alter the CD4\(^+\):CD8\(^+\) ratio in γ\(_c\) KO mice. A. The γ\(_c\) KO mice had reduced splenic cellularity compared with WT mice at 4 wk of age. The presence of the transgene increased the cellularity on both WT and γ\(_c\) KO backgrounds. B. The Stat5b transgene on the γ\(_c\) KO background did not give rise to the altered CD4\(^+\):CD8\(^+\) ratio seen on the WT background. However, when total splenic cellularity is taken into account, there is an effect on both total lymphocyte and memory cell numbers. C. Fresh splenocytes were cultured in plates that were neither coated or coated with anti-CD3 as described in Materials and Methods, and thymidine incorporation was determined. Proliferation in response to 2 nM IL-2 served as a control for γ\(_c\) function. The splenocytes from Stat5b-transgenic mice on WT and γ\(_c\) KO backgrounds proliferated to a greater degree in response to anti-CD3 than did the γ\(_c\) KO mice lacking the transgene. As expected, these mice did not respond to IL-2. Representative data are shown from one of three similar experiments, with two mice in each group. The p values are shown above TG1/WT bar graphs for comparisons between WT and TG1/WT. Similarly, the p value above TG1/γ\(_c\) KO represents the comparison between γ\(_c\) and TG1/γ\(_c\) KO.

Stat5b transgene on the γ\(_c\) KO background (Fig. 5Bd) did not reverse the CD4\(^+\):CD8\(^+\) ratio as it does on the WT background (Fig. 5Bb), it nevertheless partially increased both total lymphocyte and memory cell numbers (histogram h vs g). Moreover, although fresh splenocytes from γ\(_c\) KO expressing the Stat5b transgene proliferated less than those of WT Stat5b-transgenic mice, they exhibited more vigorous proliferation in response to anti-CD3 (p < 0.01) than did splenocytes from γ\(_c\) KO mice lacking the transgene (Fig. 5C). Although the absolute changes in cellularity and proliferation in the transgenic γ\(_c\) KO mice were less than those in the transgenic WT mice, the fold increase resulting from the Stat5b transgene expression on the γ\(_c\) KO background was greater than in WT mice for both cellularity (6.7 vs 3.8-fold) and anti-CD3 induced proliferation (2.0 vs 1.5-fold). These results support a role for γ\(_c\)-dependent cytokines in CD8\(^+\) T cell homeostasis, but suggest that Stat5b can also mediate γ\(_c\)-independent effects as well.

Enhanced RANTES and IFN-γ expression in CD8\(^+\) T cells

To help characterize the nature of the expanded CD8\(^+\) population, we performed ribonuclease protection assays using multiprobe sets. Although IL-2, IL-4, IL-6, IL-10, IL-15, and IFN-γ levels were not consistently up-regulated in freshly isolated Stat5b-transgenic splenocytes, the expression of the chemokine RANTES was elevated in Stat5b CD8\(^+\) but not CD4\(^+\) T cells (Fig. 6a, lane 4 vs 5 and 6). This is consistent with the known production of this chemokine by CD8\(^+\) T cells (28). CD8\(^+\) Ag-specific T cells were evaluated using a fluorochrome-conjugated OVA MHC class I tetramer (20) 6 days after immunization with 1 mg of OVA mixed with 50 μg of monoclonal anti-CD40. The Stat5b-transgenic mice developed more Ag-specific CD8\(^+\) T cells than did WT mice (Fig. 6b, b vs a). Since memory cells secrete IFN-γ in higher amounts than naive cells (29), we measured IFN-γ levels, in response to 24 h of stimulation with either 100 μg of OVA or anti-CD3, in splenocytes from unimmunized control mice and mice that were previously immunized with OVA/anti-CD40. Following stimulation with either OVA (Fig. 6c, b vs a) or anti-CD3/CD28 (b vs e), flow cytometric analysis of intracellular staining revealed higher IFN-γ levels in Stat5b-transgenic CD8\(^+\) T cells than in WT CD8\(^+\) T cells. OVA-specific CTL activity was also higher in immunized Stat5b-transgenic mice compared with WT controls (data not shown). Thus, the Stat5b-transgenic CD8\(^+\) cells display functional characteristics of memory cells, and Stat5b-transgenic mice develop enhanced immune responses.

Discussion

The survival and proliferation of memory CD8\(^+\) T cells appears in large part to be controlled by γ\(_c\)-dependent cytokines, including IL-2 (30), IL-7 (31–33), and IL-15 (32–35), each of which activates multiple signaling pathways, including Stat5a and Stat5b. Strikingly, whereas Stat5a\(^{-/-}\) and Stat5b\(^{-/-}\) mice have decreased numbers of CD8\(^+\) splenocytes, transgenic expression of Stat5 increased the number of CD8\(^+\) memory T cells. Although CD8\(^+\) T cell expansion was substantially decreased in Stat5b-transgenic mice on the γ\(_c\) KO background as compared with the WT background, some expansion occurred, suggesting that γ\(_c\)-independent cytokines also can affect CD8\(^+\) T cell homeostasis in a Stat5-dependent fashion.

Of the γ\(_c\)-dependent cytokines implicated as influencing CD8\(^+\) T cell homeostasis, IL-2, IL-7, and IL-15 can potently activate Stat5 (8), and IL-4, while primarily activating Stat6, also can activate Stat5 (36). Several lines of evidence suggest an important role for IL-15 in regulating CD8\(^+\) T cell homeostasis. First, mice lacking either IL-15 (37) or IL-15Rα (35, 38) have reduced CD8\(^+\) memory T cells. Second, these cells are increased in IL-15-transgenic mice (39, 40) or following in vivo administration of IL-15 (34, 41), which correlates with (42) reduced levels of Bcl-2 in Stat5a\(^{-/-}\) splenocytes and increased levels of Bcl-2 in Stat5b-transgenic mice. In contrast to IL-15, IL-2 may have dual effects related to CD8\(^+\) memory T cell homeostasis (30). First, IL-2 has been reported to inhibit CD8\(^+\) memory T cell expansion in vivo (41), possibly indirectly through up-regulation of CD4\(^+\)/CD25\(^+\) regulatory T cells (43). Second, as a T cell growth factor, IL-2 likely has a direct effect on CD8\(^+\) memory T cell growth following primary activation by Ag (30). The higher expression of IL-2Rβ on CD8\(^+\) than on CD4\(^+\) T cells (34) may contribute to the preferential increase in CD8\(^+\) T cell numbers and altered CD4\(^+\)/CD8\(^+\) ratio seen in Stat5b-transgenic mice. IL-2Rα also plays an important role in peripheral T cell homeostasis (44) and Stat5 plays a key
role in regulating IL-2Rα expression (12). Despite major differences in the phenotypes of Stat5a−/− and Stat5b−/− mice (11–14), we found that transgenic Stat5b could complement for the absence of Stat5α in normalization of IL-2-induced IL-2Rα expression and proliferation, indicating at least partial redundant actions for Stat5a and Stat5b. The ability of both Stat5α and Stat5b to bind to the IL-2 response elements in the gene encoding IL-2Rα explains why both of these Stat5 proteins can affect IL-2Rα expression and thus influence proliferation (18).

Although γc-dependent cytokine signaling likely accounts for most, if not all, of the effect of Stat5 on T cell homeostasis, our study reveals that γc-independent cytokines that activate Stat5 could also contribute. Stat5 expression is important for responsiveness of splenocytes to anti-CD3, based on the profoundly diminished responses in Stat5a−/−/Stat5b−/− mice (15, 18) and the augmented responses in Stat5b-transgenic mice we have observed. Interestingly, Stat5b augments anti-CD3-induced proliferation even on a γc KO background, consistent with a role for TCR-mediated Stat5-dependent signaling (45), even though TCR-mediated activation of primary murine (46) or human (47) cells does not induce tyrosine phosphorylation of Stat5. Thus, Stat5 plays a role, direct or indirect, in TCR-mediated signaling.

The increased splenocyte numbers we observed in Stat5b-transgenic mice following anti-CD3 stimulation could result from either increased proliferation or decreased cell death. In fact, these mice exhibited augmented proliferation of splenocytes and increased activation-induced cell death of CD4+ T cells. In contrast, Stat5b-transgenic CD8+ T cells do not exhibit increased apoptosis, consistent with the fact that high-dose Ag-induced death of CD8+ cells likely involves a TNF-dependent mechanism rather than the Stat5-dependent IL-2-induced Fas-mediated death observed in CD4+ T cells (48). Moreover, signaling through the IL-2R in CD8+ T cells after Ag stimulation causes proliferation and expansion rather than cell death (49). Interestingly, CD4+ T cells are not decreased in number in transgenic mice despite the increased activation-induced cell death, suggesting increased IL-2- and/or IL-15-dependent proliferation. Proliferation of both CD4+ and CD8+ T cells but preferential apoptosis of the CD4+ T cells likely explains the altered CD4+ :CD8+ ratio seen in Stat5b-transgenic mice. Another important difference seen in the CD8+ vs CD4+ T cells is the high expression of RANTES mRNA in the CD8+ but not CD4+ Stat5b-transgenic CD8 T cells. Chemokines are chemotactic cytokines involved in leukocyte migration (50). RANTES is produced by CD8+ T cells (28) and can act as an Ag-independent activator of T cells in vitro (51). The increase in RANTES is consistent with its preferential chemotraction of memory T cells (52). Like RANTES, IFN-γ is associated with memory cell function (29). The ability of Stat5b-transgenic splenocytes to produce higher IFN-γ levels is consistent with increased functional memory cells in Stat5-transgenic mice.

In conclusion, the decreased CD8+ memory T cells in Stat5a−/− and Stat5b−/− mice and increased CD8+ memory T cells in Stat5b-transgenic mice demonstrate that Stat5 proteins are critical mediators of cytokine-dependent CD8+ T cell homeostasis, and we show that this occurs via effects on both cell proliferation and survival. Furthermore, our results indicate that defective Stat5-dependent signaling at least partially accounts for the preferential loss of CD8+ cells in γc and Janus kinase 3-deficient mice.

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