Published in final edited form as:

Exp Hematol. 2007 November ; 35(11): 1684–1694.

STAT5 requires the N-domain to maintain hematopoietic stem cell repopulating function and appropriate lymphoid-myeloid lineage output

Gegiang Li¹, Zhengqi Wang¹, Yi Zhang¹, Zizhen Kang¹, Eleonora Haviernikova¹, Yongzhi Cui², Lothar Hennighausen², Richard Moriggl³, Demin Wang^{4,5}, William Tse^{1,6,7}, and Kevin D. Bunting^{1,6,7}

¹ Department of Medicine, Division of Hematology/Oncology, Case Western Reserve University, School of Medicine, Cleveland, OH 44106, USA

² National Institute of Diabetes, Digestive, and Kidney Disease, NIH, Bethesda, MD 20892

³ Ludwig Boltzmann Institute for Cancer Research, A1090 Vienna, Austria

⁴ Blood Research Institute, BloodCenter of Wisconsin, Milwaukee, WI 53226, USA

⁵ Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, WI 53226, USA

⁶ Case Comprehensive Cancer Center, Cleveland, OH 44106, USA

⁷ Center for Stem Cell and Regenerative Medicine, Cleveland, OH 44106, USA

Abstract

Objective— STAT5 is a critical regulator of hematopoietic development and its impaired activation is associated with hematopoietic and immune cell defects. However, much of this information has been learned from knockout mice that still retain the potential for expression of STAT5 proteins that are N-terminally truncated due to alternative internal translation initiation codons. The goal of these studies was to use transplantation based assays to analyze the degree of STAT5ΔN activity in HSC and throughout lymphomyeloid development.

Methods— We have directly compared E14.5 fetal liver cells from mice with potential to express STAT5abΔN (STAT5ab^{ΔN/ΔN}) with mice completely lacking STAT5a and STAT5b (STAT5ab^{null/null}). We have also utilized retroviral complementation of STAT5ab^{null/null} fetal liver hematopoietic stem cells (HSC) to enforce expression of full-length STAT5a or STAT5a lacking the first 136 amino acids (STAT5aΔN).

Results— We report that STAT5 is required for HSC, lymphocyte, and erythrocyte development. We demonstrate that restored expression of STAT5a in STAT5ab^{null/null} HSC provides a strong selective advantage, correcting T/B lymphocyte and erythrocyte development. Interestingly, Gr-1⁺ blood cells were inversely correlated with B-lymphocytes and both were normalized by STAT5a expression. In contrast, transduction of STAT5aΔN only provided partial B-lymphocyte development.

To whom correspondence should be addressed: Kevin D. Bunting, Ph.D., Department of Medicine, Division of Hematology/Oncology, Case Western Reserve University, WRB 2-131, Cleveland, OH 44106-7284, Phone: (216) 368-5694, Fax: (216) 368-1166, Email: Kevin.Bunting@case.edu.

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Conclusions— These studies define the role of STAT5 in maintaining normal lymphoid vs. myeloid balance during hematopoiesis and highlight a major role for the N/domain in HSC function. The platform of retroviral complementation described here will be particularly useful for future studies to sub-define the N-domain regions that are critical for hematopoiesis.

Keywords

cytokine signaling; JAK/STAT; hematopoiesis; hematopoietic stem cell; retroviral transduction

INTRODUCTION

The common gamma chain (γ C) of the receptors for IL-2, 4, 7, 9, 15, and 21 is essential for lymphopoiesis and its loss of function mutation results in an early block of T and B lymphocyte development in mice [1]. The Janus kinase/Signal Transducer and Activator of Transcription pathway links γ C to downstream target gene expression through Src homology-2 (SH2) domain interactions. JAK3 tyrosine phosphorylation is essential for common gamma chain signaling and its deletion phenocopies that of γ C deficiency in mice [2]. Both γ C and JAK3 mutations are causative for X-linked severe combined immunodeficiency (X-SCID) in humans [3]. STATs are latent transcription factors that upon phosphorylation by JAKs, then form dimers, translocate to the nucleus, and regulate target gene expression. Tetramerization of STATs also occurs and the crystal structure of the STAT4 N-domain [4] supports cooperative DNA binding among dimers bound to two consecutive STAT binding sites. Tetramer formation by STAT5a [5,6,7] increases the stability of DNA binding, prolongs signaling, and enhances target gene expression. STAT5 tetramerization is essential for peripheral T cell proliferation and it promotes proto-oncogene induction in the context of a persistently activated STAT5a molecule [8].

Despite the major role of γ C and JAK3 in activation of STAT5, previous studies using the mice generated at St. Jude, lacking the first methionine and upstream regulatory regions, lacked the same phenotypes in lymphocyte development as γ C^{-/-} or JAK3^{-/-} mice [9]. However, since N-terminally deleted STAT5 has been reported in lymphoid and erythroid cells from these STAT5-deficient mice, we now refer to them as STAT5ab^{ΔN/ΔN} [10,11]. Hematopoietic phenotypes of mice with targeted deletion of STAT5 but still permitting expression of an N-domain deleted STAT5 were severe and included defective T cell proliferation, severe reduction of NK cells [12,13], impaired myeloid cell survival [14], and impaired survival and proliferation of mast cells [15].

Transplantation of major histocompatibility complex (MHC) matched donors in patients suffering from various hematologic disorders is possible, but often engraftment problems occur. For successful repopulation after transplantation, HSCs must transit through the peripheral blood, extravasate through the vessel walls, and find their correct microenvironment in the BM niche and then respond to growth factors and produce progeny of all hematopoietic lineages. STAT5 is important in this complex functional setting. In bone marrow (BM) transplant experiments, STAT5ab^{ΔN/ΔN} BM HSCs were capable of engraftment, although this was associated with significant host T cell chimerism [16,17] that prevented development of autoimmunity and these chimeras were healthy and lived long-term. At the HSC level, STAT5ab^{ΔN/ΔN} mice had normal HSC phenotype and BM and fetal liver (FL) HSCs from these mice could engraft into irradiated hosts [17] but were deficient in self-renewal capacity during serial transplantation [18]. In competitive repopulation, these grafts were greatly inferior to wild-type BM indicating defects in HSC multilineage differentiation [17,19]. Defective cytokine responsiveness of STAT5ab^{ΔN/ΔN} HSCs also led to reduced *in vitro* growth response to IL-3 and SCF and reduced *in vivo* sensitivity to the S-phase active anticancer drug, 5-fluorouracil [20].

Recent deletion of the entire STAT5ab locus has permitted for the first time analyses to uncover STAT5 functions without compensation from STAT5ΔN [21]. This new STAT5-deficient mouse model referred to as STAT5ab^{null/null} showed markedly increased fetal anemia [21] and are growth retarded and die perinatally. These mice also had more severe defects in lymphocyte development and were unable to produce $\gamma\delta$ or CD8⁺ T cells and had much lower CD4⁺ T cells than STAT5ab^{ΔN/ΔN} mice [22,23]. In addition, STAT5ab^{null/null} cells were refractory to transformation via BCR-ABL and Tel-PDGFβ fusion proteins [23,24].

To date there has not been a comparative study of the two STAT5 knockout mouse lines to understand the contribution of the STAT5 N-domain to hematopoiesis. Since prior reports highlighted potential function for STAT5ΔN, we have investigated this further through head-to-head comparisons and retroviral complementation studies. We report that the N-domain of STAT5 is critical for the majority of activity in HSC and is critical for maintaining the normal balance between lymphoid and myeloid lineage development.

MATERIALS AND METHODS

Animals

Control mice were used as previously described [16]. The STAT5ab^{null/null} mice on the C57BL/6 background [21] and the STAT5ab^{ΔN/ΔN} mice [9] on the C57BL/6 [17] and HW80 [16] background have been described. Mice were housed in a specific pathogen-free environment. All studies were approved by the Case Institutional Animal Care and Use Committee.

Retroviral vector transduction and analysis

FL cells were cultured for 2 days in IMDM medium/15% fetal bovine serum with recombinant murine IL-3 (20 ng/ml), recombinant human IL-6 (50 ng/ml), and recombinant SCF (50 ng/ml) and concentrated retrovirus. Retrovirus-containing supernatants were collected from GP+E86 producer cell lines and were concentrated 20-fold by precipitating with polyethylene glycol 8000 (PEG 8000) overnight. For *in vivo* complementation studies, cells were transduced with two rounds of concentrated IRES-GFP (IRGFP) expressing virus *ex vivo* for two days only and then transplanted into lethally-irradiated recipient mice. Some FL cells were maintained in culture for an additional two days followed by flow cytometry to determine the percentage of GFP⁺ cells. Expression of STAT5 was documented in some experiments following mast cell culture from transduced FL cells and Western blotting using an antibody to the C-terminus (C-17; Santa Cruz) as described [15].

Mouse peripheral blood hematology

Peripheral blood was obtained following puncture of the retroorbital venous sinus using a microcapillary tube. The microcapillary tubes were spun in a microcentrifuge (Stat-Spin Inc.) and hematocrits were read manually. For red and white blood cell counts, cells were diluted in isotonic saline solution and analyzed using a Coulter counter (Beckman Coulter Inc.).

Hematopoietic colony-forming assays

Embryonic day 14.5 fetal liver cells were plated at 2×10^5 cells per 3 ml in methylcellulose medium (Stem Cell Technologies, EPO-containing M3334, Vancouver, Canada) with added recombinant murine interleukin (IL)-3 (20 ng/ml), IL-6 (50 ng/ml), and stem cell factor (50 ng/ml). Seven days later the colonies were counted using an inverted microscope. Colonies were scored for CFU-GM, CFU-GEMM, and BFU-E according to the manufacturer's instructions.

Hematopoietic stem cell transplantation

FL competitive and non-competitive transplants were performed as previously described [17]. For head-to-head competitive repopulation between any two of the STAT5ab^{+/+}, STAT5ab^{ΔN/ΔN}, and STAT5ab^{null/null} mice, FL cells from both HW80 and C57BL/6 background mice were mixed thoroughly at a 1:1 donor equivalent ratio and then injected via the lateral tail-vein into lethally irradiated (1100 rads) recipient mice. Beginning at 8 weeks post-transplant, mice were bled from the retroorbital venous plexus. Hemoglobin patterns were analyzed from packed peripheral red blood cells by electrophoresis on cellulose acetate gels. For competitive limiting dilution assays, E14.5 FL cells were transplanted at a range of cell doses mixed with a radioprotective dose of 2×10^5 CD45.1 adult BM cells.

Analyses of hematopoietic engraftment

To analyze donor engraftment (chimerism), the BM and peripheral blood of the recipient mice were stained with CD45.2 antibody and subjected to flow cytometry analysis. CD45 is the Ly5 locus and a polymorphism on this gene makes it convenient to track donor cells by flow cytometry in all hematopoietic lineages by co-staining with lineage specific antibodies. Donor chimerism is the percentage of hematopoietic cells of that lineage that were donor derived (CD45.2). The absolute number of engrafted donor cells was calculated as the %CD45.2 positive cells multiplied by the total white blood cell count per microliter of blood. For limiting dilution experiments, the frequency of HSC was calculated using Poisson statistics using L-Calcul software (Stem Cell Technologies) as described [25]. Repopulating unit (RU) calculations were calculated as described [26].

Antibody/fluorescent dye staining and flow cytometry

For multi-lineage analysis, peripheral blood cells were stained with a cocktail of antibodies as described previously [16]. For competitive engraftment analyses peripheral blood cells of recipient mice were stained with FITC-conjugated CD45.2. Cells were then analyzed on a fluorescence activated cell sorter (FACS) LSR I flow cytometer (BD Biosciences) using CellQuest software.

Southern blot

Genomic DNA was prepared, digested, and analyzed as described previously [17].

RESULTS

Endogenous STAT5ΔN compensates for loss of function in lymphocyte and erythrocyte development following FL transplantation

As described in *Methods*, we utilized CD45.2 positive E14.5 FL cells from either wild-type, STAT5ab^{ΔN/ΔN} or STAT5ab^{null/null} fetuses and transplanted into lethally irradiated CD45.1 recipient mice. The recipient mice (10 mice per group total, 2 separate transplant experiments) were bled at 12 weeks post transplantation and the peripheral blood hematology was analyzed. Fig. 1A shows the results of one representative experiment (n=5 recipients). The hematocrit of the STAT5ab^{ΔN/ΔN} transplanted mice was $43 \pm 1\%$ compared to $48 \pm 0.4\%$ for wild-type mice, while the hematocrit of STAT5ab^{null/null} mice was reduced to $32 \pm 1.4\%$. The absolute red blood cell count was similar to the hematocrit, with STAT5ab^{ΔN/ΔN} transplanted mice being mildly reduced ($2.7 \pm 0.2 \times 10^6/\mu\text{l}$), while STAT5ab^{null/null} was $1.7 \pm 0.1 \times 10^6/\mu\text{l}$, which was half of wild-type ($3.3 \pm 0.4 \times 10^6/\mu\text{l}$). Both STAT5ab^{ΔN/ΔN} ($5 \pm 0.6 \times 10^3/\mu\text{l}$) and STAT5ab^{null/null} ($3 \pm 0.7 \times 10^3/\mu\text{l}$) mice showed a significant reduction in absolute white blood cell count compared with wild-type mice ($12 \pm 2 \times 10^3/\mu\text{l}$).

The relative engraftment in multiple lineages in wild-type, STAT5ab^{ΔN/ΔN} and STAT5ab^{null/null} FL transplanted mice was analyzed by FACS at 12 weeks post transplantation and the results are shown in Fig. 1B adjusted to absolute counts by hematology. Reconstitution with the donor graft was roughly the same in cells expressing Gr-1 among all three groups of FL-transplanted mice (Wild-type 1236 ± 407/μl; STAT5ab^{ΔN/ΔN} 881 ± 212/μl; STAT5ab^{null/null} 907 ± 307/μl). However, the absolute number of Ter119⁺, CD4⁺ and B220⁺ was much lower in the STAT5ab^{null/null} FL-transplanted mice (1013 ± 379/μl; 25 ± 5/μl; 118 ± 28/μl) relative to wild-type (6785 ± 1121/μl; 1294 ± 273/μl; 6703 ± 1441/μl). In contrast, in STAT5ab^{ΔN/ΔN} FL-transplanted mice, the absolute number of cells of Ter119⁺, CD4⁺ and B220⁺ were intermediately reduced (3122 ± 488/μl; 206 ± 40/μl; 2450 ± 521/μl). Overall, the STAT5ab^{ΔN/ΔN} FL-transplanted mice showed only mild reduction in both B and erythroid cells, indicating that STAT5ΔN could compensate maximally for loss of STAT5 in these lineages. However, STAT5ΔN was less capable of compensating for loss of T cell repopulation. STAT5ab^{null/null} primary recipient BM engrafted into secondary recipients contributing primarily to Gr-1⁺ cells (Fig. 2). Our previous studies have demonstrated that STAT5ab^{ΔN/ΔN} mice can be engrafted through serial transplant into tertiary recipients [18].

We next compared FL cells from STAT5ab^{null/null} mice with those from STAT5ab^{ΔN/ΔN} mice by direct head-to-head competitive repopulation. STAT5ab^{ΔN/ΔN} or littermate wild-type FL (HW80 background; Hb_d) were competed at a 1:1 ratio with C57BL/6 STAT5ab^{null/null} FL (Hb_s) as previously described [16]. The relative levels of donor engraftment were analyzed 12 weeks after transplantation by hemoglobin gel electrophoresis (10 mice total per group, 2 independent experiments). As seen in Fig. 3A, control C57BL/6 wild-type (WT) versus HW80 WT competitions resulted in 35 ± 3% Hb_s and Hb_d engraftment (n = 5 recipients) (Fig. 3A, lane 14–18). In head-to-head competition between STAT5ab^{ΔN/ΔN} and STAT5ab^{null/null}, the contribution of the engraftment of STAT5ab^{ΔN/ΔN} was always 100% (Fig. 3A, lane 9–13), indicating that STAT5ab^{null/null} FL cells were more deficient in hematopoietic repopulation of the erythroid lineage.

We also performed colony-forming assays using 6 wild-type and 5 STAT5ab^{null/null} FL plated in IL-3, IL-6, SCF, and EPO. Similar to our prior studies of STAT5ab^{ΔN/ΔN} [16], there was not a significant reduction in CFU-GM frequency for STAT5ab^{null/null} FL (99 ± 63/2 × 10⁵ cells) relative to wild-type FL cells (90 ± 15/2 × 10⁵ cells) with P=0.74. Although mild reductions in CFU-GEMM (wild-type 33 ± 18 vs. null/null 18 ± 15/2 × 10⁵ cells) and BFU-E (wild-type 33 ± 18 vs. null/null 15 ± 15/2 × 10⁵ cells) frequency were observed these did not reach statistical significance (P=0.17 and P=0.11 respectively).

The STAT5 N-domain is responsible for most STAT5 function in HSC maintenance and repopulating activity

Since the erythroid defects were more severe in STAT5ab^{null/null} mice, we also examined non-erythroid repopulation of BM and spleen from mice that received head-to-head competitive transplant of STAT5ab^{ΔN/ΔN} and STAT5ab^{null/null} FL cells (Fig. 3B). Southern blots were performed using EcoR1 digested genomic DNA and a mouse β-globin intervening sequence probe to distinguish Hb_s and Hb_d genotype as previously described [17]. To the right is shown wild-type HW80 (Hb_d) vs. wild-type C57BL/6 (Hb_s) which resulted in roughly equal contribution. To the left is shown STAT5ab^{null/null} FL (Hb_s) that was outcompeted by wild-type HW80 (Hb_d). In the middle lanes is shown the head-to-head competition where there was some competition from STAT5ab^{null/null} FL cells but this was reduced relative to STAT5ab^{ΔN/ΔN}.

To further examine HSC frequency in STAT5ab^{ΔN/ΔN} and STAT5ab^{null/null} FL compared with wild-type FL, we performed limiting dilution assays using a common CD45.1 BM competitor (Table 1). Wild-type FL had a stem cell frequency of 1 in 7,721 whereas STAT5ab^{ΔN/ΔN} FL

had a stem cell frequency of 1 in 99,047 and STAT5ab^{null/null} FL had a stem cell frequency of 1 in 115,785. These 13- to 15-fold reductions relative to wild-type were not significantly different from either mutant. As a measurement of HSC quality, calculation of the functional RU values from the highest cell dose relative to wild-type ($110 \pm 50/1 \times 10^6$ cells; N=10) showed a 183-fold reduction in STAT5ab^{ΔN/ΔN} RU ($0.6 \pm 0.3/1 \times 10^6$ cells; N=5) compared to a 367-fold reduction in STAT5ab^{null/null} RU ($0.3 \pm 0.1/1 \times 10^6$ cells; N=10). The 2-fold reduction in RU for STAT5ab^{null/null} HSC was significant (P=0.01) and was strikingly similar in magnitude to the non-erythroid head-to-head competition results of Fig. 3B.

Retroviral complementation of STAT5ab^{null/null} FL with STAT5aΔN restores only partial B lymphocyte development following FL transplantation

Although the STAT5ab^{ΔN/ΔN} mice have serendipitously provided a tool for understanding the role of the STAT5 N-domain in STAT5-mediated activities, there are limitations. It is not possible to assess STAT5ΔN expression in primitive hematopoietic cell fractions and therefore it is important to perform overexpression studies to assess HSC repopulating function. We have used MSCV-based retroviral vectors expressing either full-length STAT5a or STAT5a lacking the first 136 amino acids (referred to as STAT5aΔN). As a vector control we have transduced FL cells with MSCV-IRGFP. To document retroviral STAT5a or STAT5aΔN protein expression we used FL derived mast cell cultures derived from STAT5ab^{null/null} hosts lacking endogenous STAT5 (Fig. 4A). Both vectors were capable of reconstitution of STAT5a expression at similar levels (Fig. 4B). The initial transduction efficiencies obtained in the STAT5ab^{null/null} FL varied from experiment to experiment with the ranges indicated for IRGFP (4–61%), STAT5a (7–54%), and STAT5aΔN (9–55%). Three independent mast cell cultures were generated, the two earliest of which were in the 4.5–7% transduction range. Our third and most recent experiment using higher amounts of retrovirus achieved 54–58% transduction efficiency. However, due to a selective reconstitution of growth in the transduced mast cells, all mast cells expressing STAT5a or STAT5aΔN became >95% GFP⁺ at the time of Western blot analysis (data not shown).

To examine *in vivo* complementation of STAT5a using retroviral vectors expressing STAT5a (N=3), STAT5aΔN (N=5), or IRGFP control (N=5), we were able to test the true extent of STAT5aΔN function in hematopoiesis following transplantation *in vivo*. A total of 3 independent transductions were performed for STAT5a and STAT5aΔN, and 4 independent transductions were performed for IRGFP control. Regardless of the initial transduction efficiency, the %GFP⁺ cells were uniformly high in mice expressing STAT5a and considerably lower in mice expressing STAT5aΔN. Fig. 5 shows representative examples of the flow cytometry analyses from mice analyzed for the percentage of reconstitution of cells with lineage marker expression and GFP. A very consistent selective advantage for GFP positive cells was observed in all hematopoietic lineages for STAT5a (GFP⁺ percentage range 87–90%). In contrast, STAT5aΔN was only capable of providing a greatly attenuated selection (GFP⁺ percentage range 5–44%). The control IR-GFP vector showed the absence of donor B and T cells (CD45.2) and no evidence for a vector selective advantage in any lineage.

In order to determine whether the selective advantage was capable of significantly correcting the cytopenias associated with STAT5 deficiency in transplanted mice, we analyzed mice for total peripheral blood leukocyte counts and performed flow cytometry for lineage markers. The absolute number of reconstituted peripheral blood leukocytes is shown in Fig. 6. Wild-type STAT5a was capable of correcting absolute T cell (12-fold) and B cell (151-fold) counts when calculated based on the total peripheral blood cellularity. In contrast, STAT5aΔN provided up a 7-fold increase in absolute B cell count in 4 out of 5 recipients but had no impact upon T cell count. The effects on Gr-1 levels were the opposite, with STAT5a reducing Gr-1⁺ cells back to normal levels, whereas STAT5aΔN was not effective.

DISCUSSION

Gene expression through internal AUG usage has been described for a number of genes [27]. STAT92E Δ N in drosophila, was found to be a naturally occurring dominant negative isoform [28]. Tetramerization is mediated by the amino terminus of STATs, and with STAT4 [29,30] and STAT6 [31] this region is essential for nuclear localization and transactivation ability. STAT4 lacking the N-terminal 51 amino acids could not be phosphorylated by IL-12, whereas STAT6 with a 110 amino acid N-terminal deletion was a hypomorph, possessing reduced DNA binding and IL-4 responsive transcriptional activity. The STAT3 N-domain is dispensable for SOCS3 expression [32]. In contrast, the amino termini of STAT1 [33] and STAT5 [34] have the potential to negatively regulate dephosphorylation, resulting in increased nuclear accumulation. However, due to an important role of the N-domain in transactivation, the STAT1 N-domain deletion results in hypomorphic overall activity [35].

STAT5 Δ N is capable of nuclear localization and has strong DNA binding ability even without cytokine stimulation [8]. The increased DNA binding ability is also associated with hypomorphic activity on luciferase constructs indicating that dimerization ability remained intact (Bunting and Moriggl, unpublished observations). Since it was not possible to assess STAT5 Δ N levels in HSC from STAT5ab ^{Δ N/ Δ N} mice, it was not possible to rule out that STAT5 Δ N is expressed poorly or not expressed at all in STAT5ab ^{Δ N/ Δ N} HSC. Therefore, we also tested STAT5 N-domain deletion *in vivo* using a retroviral overexpression approach. Expression of STAT5a Δ N in STAT5ab^{null/null} FL cells gave similar results as use of STAT5ab ^{Δ N/ Δ N} FL cells for transplantation. The correlation between these two approaches provides strong evidence that the STAT5a Δ N mutant is functional but impaired in promoting specific aspects of hematopoiesis.

Consistent with two recent studies [22,23], we have observed striking defects in lymphopoiesis following transplant of STAT5ab^{null/null} FL cells. Therefore STAT5 N-domain mediated signals are especially important for lymphocyte development. Although STAT5ab ^{Δ N/ Δ N} mice have circulating T lymphocytes, we found that their competitive reconstitution ability in irradiated recipients was greatly impaired when STAT5a Δ N was expressed either endogenously in STAT5ab ^{Δ N/ Δ N} cells or through retroviral overexpression. In the B lymphocyte lineage, STAT5a Δ N was much more effective at promoting hematopoietic maturation, indicating that the role of the N-domain is less critical in B lymphocyte than T lymphocyte differentiation.

In contrast to the profound defects in T/B lymphocyte development in mice lacking STAT5 or the STAT5 N-domain, myeloid lineage engraftment (Gr-1⁺) was surprisingly normal when using fresh FL (Fig. 1) and significantly increased when using 2-day pre-stimulated FL (Fig. 5) using cytokines that promote myelopoiesis. In the absence of a competitor HSC we have always observed good myeloid engraftment with either STAT5ab ^{Δ N/ Δ N} or STAT5ab^{null/null} FL cells, even in secondary transplant recipients. While it is possible that a bias in hematopoiesis shifts development toward the granulocyte-macrophage progenitor (GMP) at the expense of the megakaryocyte-erythroid progenitor (MEP), to test this would require further study with prospectively isolated myeloid progenitor fractions. Gr-1⁺ and B220⁺ cells share the same BM niche [36] and can be reciprocally regulated, thus restored B cell development above a threshold may be sufficient to normalize Gr-1⁺ levels. However, STAT5ab ^{Δ N/ Δ N} HSC may not have been able to engraft to sufficient levels to support normalization. In these studies we also observed elevated Gr-1⁺ cell counts in transplanted mice from the IRGFP control group. Interestingly, we observed a correction in the Gr-1⁺ level between STAT5ab^{null/null} FL cells transduced with STAT5a but not STAT5a Δ N. Therefore, STAT5 expression and N-domain dependent functions are a critical determinant of lymphoid vs. myeloid lineage differentiation. This finding is superficially similar to a recent study

implicating STAT5 in regulation of emergency granulopoiesis [37], however that study identified a cell extrinsic mechanism involving liver endothelial cell production of granulocyte colony-stimulating factor (G-CSF). The strong selection for GFP⁺ cells in the Gr-1⁺ lineage for STAT5a in our study was cell intrinsic and can be viewed as an indicator of HSC selection. It is important to point out that retroviral transduction in the adult BM STAT5ab^{ΔN/ΔN} background did not provide the same selective advantage (data not shown). It is likely that expression of endogenous functional but hypomorphic STAT5ΔN limited the ability of retroviral STAT5a to promote HSC reconstitution.

Although we did not find striking reductions in erythroid CFU-C frequency, we noted that 2/5 STAT5ab^{null/null} FL examined had 1.4- to 2.2-fold increases CFU-GM frequency resulting in the large standard deviation. However, we have consistently observed decreased Ter119⁺ cells which also depend on stem cell factor and erythropoietin. Also, since defects in red blood cell development were much more severe in STAT5ab^{null/null} head-to-head competition, it is likely that STAT5ΔN can compensate for erythroid defects following HSC transplantation.

A striking aspect of these studies is that the number of HSC as determined by competitive limiting dilution assay was very dependent on the STAT5 N-domain. A very large percentage of the dramatic HSC level defects observed in STAT5ab^{null/null} FL could be accounted for in STAT5ab^{ΔN/ΔN} FL, with only a 2-fold difference observed between the two mutants in comparison to a two log difference relative to wild-type. Considering that the ability of STAT5ab^{ΔN/ΔN} to make lymphocytes is still intact, the Southern blot data may be shifted more in favor of STAT5ab^{ΔN/ΔN} competition over STAT5ab^{null/null} because of this lineage differentiation difference.

Our overexpression studies recapitulated the hematopoiesis defects and indicated that critical functions of the N-domain regulate STAT5-mediated functions critical for normal hematopoiesis. Our studies have previously demonstrated an important role for the STAT5 N-domain in leukemogenesis [8]. Therefore, further dissection of the mechanism by which STAT5 utilizes the N-domain to control gene expression may yield valuable insight into the pathogenesis of leukemias and myeloproliferative disorders. The N-domain tryptophan 37 is critical for tetramerization [5], and the N-domain includes binding sites for cAMP element response binding protein (CREB) binding protein (CBP) [38] and the glucocorticoid receptor [39]. Since STAT5 activation is downstream of many leukemic retroviral tyrosine kinases, it represents a central molecule upon which to develop therapeutic strategies for hematologic malignancies. Toward these goals, our recent studies have uncovered a key role for the N-domain in Bcl-2 expression and survival signaling (Li and Bunting, manuscript in preparation). Future studies using the retroviral complementation method may help to sub-define the STAT5 N-domain and its important role in hematopoiesis.

Acknowledgements

This work was supported by NIH R01HL073738 and R01DK059380 (K.D. Bunting), R01 HL073284 (D. Wang), the intramural program of NIDDK/NIH (L. Hennighausen), and through FWF grant SFB F28 (R. Moriggl). We thank Heath Bradley and Christine Couldrey for expert technical support. This research was also supported by the Flow cytometry and Radiation Resources Core Facilities of the Case Comprehensive Cancer Center (P30 CA43703) and by the Center for Stem Cell and Regenerative Medicine, Cleveland, OH.

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Abbreviations

STAT	signal transducer and activator of transcription
HSC	hematopoietic stem cell
FL	fetal liver
BM	bone marrow

GFP

green fluorescent protein

RU

repopulating unit

Fig. 1. Repopulation of lymphocyte and erythrocyte lineages is most severely reduced in the complete absence of STAT5 ΔN

Wild-type, STAT5ab $\Delta N/\Delta N$, or STAT5ab null/null FL cells were non-competitively transplanted into lethally-irradiated CD45.1 recipient mice and analyzed 12 weeks after transplantation for (A) peripheral blood hematology, or (B) the level of donor chimerism in peripheral blood leukocytes. The absolute cell count of donor lineage positive cells was calculated by multiplying double positive (CD45.2 positive plus either: Gr-1, CD4, B220, or Ter119) cells by the total white blood cell counts per microliter of whole blood. *P* values were calculated by a paired *t* test comparing relative to wild-type fetal liver transplant and *P*<0.05 is marked with an asterisk.

Fig. 2. Secondary repopulation of irradiated hosts with BM cells from primary FL transplant recipients

Mice that were transplanted 16 weeks earlier were euthanized and the BM cells collected. The total BM population was injected at a 1 donor to 5 recipient ratio into lethally-irradiated secondary hosts. Peripheral blood hematopoietic repopulation was analyzed in the secondary recipient mice 8 weeks following transplant. Total white blood cell counts were determined using a Coulter counter and the percentages of lineage positive cells were determined by flow cytometry. The absolute cell count of donor lineage positive cells was calculated by multiplying double positive (CD45.2 positive plus either: Gr-1, CD4, B220, or Ter119) cells by the total white blood cell counts per microliter of whole blood. P values were calculated by a paired t test comparing relative to wild-type FL transplant and $P < 0.05$ is marked with an asterisk.

Fig. 3. Direct head-to-head competitive repopulation between any two of wild-type, STAT5ab^{ΔN/ΔN} and STAT5ab^{null/null} donor fetal liver donor grafts
 HW80 background FL cells with either wild-type or STAT5ab^{ΔN/ΔN} genotype were competed 1:1 with C57BL/6 background fetal liver cells in 2 separate competitive repopulation experiments. **A.** Mice that received transplants were analyzed 12 weeks later for the relative contribution of Hb_s and Hb_d in circulating peripheral red blood cells. Hb_s controls (CD45.1 and C57BL/6/CD45.2) as well as Hb_d controls (HW80/CD45.2) are shown on the left. **B.** At the time of euthanasia, BM and spleen tissues were collected, genomic DNA was prepared and EcoR1 digested and analyzed by Southern blot using a probe that recognizes a β-globin fragment. A C57BL/6 (Hb_s) control was included to the left.

Fig. 4. Western blot analysis of retrovirally-expressed STAT5a protein *in vitro*
Hematopoietic cells were isolated from E14.5 wild-type or STAT5a^{null/null} FL. FL cells were then transduced with retroviral vectors expressing either STAT5a or STAT5aΔN. Mast cell cultures were generated by growing FL cells in IL-3 and SCF containing medium for greater than 3 weeks. After this culture, all mast cells reconstituted with either STAT5a or STAT5aΔN were >95% GFP⁺ as determined by flow cytometry. **A, B.** Total cellular protein was isolated from transduced primary mast cell cultures and analyzed by Western blot for the expression of the re-introduced STAT5a.

Fig. 5. Retroviral complementation of STAT5aΔN *in vivo* provides a selective growth advantage and restores B lymphocyte but not T lymphocytes

FL cells (CD45.2) were isolated from STAT5a^{null/null} embryos and retrovirally-transduced *ex vivo* for 2 days in concentrated retroviral supernatants from ecotropic producer cells. The FL cells were transplanted into lethally-irradiated CD45.1 recipient mice. Engraftment was determined by flow cytometry of peripheral blood leukocytes 8 weeks following transplant. Notably, STAT5aΔN was able to partially reconstitute B-lymphocytes but could not rescue T-lymphocytes. The only T-lymphocytes present in recipients of STAT5aΔN transduced FL cells were host derived. As a control, wild-type STAT5a was able to restore both T and B lymphocytes.

Fig. 6. Retroviral complementation of STAT5a ΔN *in vivo* increases the absolute number of B lymphocytes in transplant mice but is ineffective in other lineages

FL cells (CD45.2) were isolated from STAT5ab^{null/null} embryos and retrovirally-transduced *ex vivo* for 2 days in concentrated retroviral supernatants from ecotropic producer cells. The FL cells were transplanted into lethally-irradiated CD45.1 recipient mice. Engraftment was determined by flow cytometry of peripheral blood leukocytes 8 weeks following transplant. Peripheral blood leukocyte counts were determined using a Coulter counter and the absolute number of peripheral blood cells expressing Gr-1, Ter119, B220, or CD4 was calculated per microliter of whole blood. Shown are vertical dot plots with each dot representing a unique transplanted mouse. Genotypes for all panels are indicated below. Normal untransplanted wild-type GFP transgenic mice (+/+) are shown for reference.

Table 1
Competitive limiting dilution of wild-type, STAT5ab^{ΔN/ΔN} and STAT5ab^{null/null} FL HSC

Genotype of donor HSC	Cell dose injected	% Donor CD45.2 ⁺ engraftment in total peripheral blood leukocytes of individual recipient mice	# Mice engrafted/# mice injected	HSC frequency(+/- 95% confidence interval)
Wild-type	1 × 10 ⁵	74, 85, 75, 91, 91, 79, 85, 84, 83, 84	10/10	1 / 7,721 (4,083–14,601)
	5 × 10 ⁴	48, 84, 82, 81	4/4	
	4 × 10 ⁴	38, 12	2/2	
	2 × 10 ⁴	71, 56, 70, 39	4/4	
	4 × 10 ³	0.1, 0.1, 0.8, 3.4, 0.4, 0.3, 20, 1.0, 0.7	1/9	
STAT5ab ^{ΔN/ΔN}	1 × 10 ⁶	36, 16, 22, 29, 14	5/5	1 / 99,047 (46,640 – 210,340)
	4 × 10 ⁵	29, 18, 22, 25, 20	5/5	
	2 × 10 ⁵	10, 6, 10, 10	4/4	
	1 × 10 ⁵	8, 5, 5	1/3	
	4 × 10 ⁴	4, 1.4	0/2	
STAT5ab ^{null/null}	1 × 10 ⁴	1.7, 0.5, 5, 1.7	1/4	1 / 115,785 (53,778 – 249,285)
	1 × 10 ⁶	7, 12, 13, 9, 17, 19, 14, 15, 10, 15	10/10	
	5 × 10 ⁵	11, 25, 8, 12, 6	5/5	
	1 × 10 ⁵	6, 4, 8, 4, 5, 9, 11, 5	5/8	
	2 × 10 ⁴	1.5, 1.1, 1.3, 0.1, 2.2	0/5	

E14.5 FL cells were isolated from heterozygote matings and were transplanted into lethally-irradiated CD45.1 competitor BM cells. Shown is cumulative data from 3 separate transplant experiments. Donor engraftment was determined by flow cytometry on peripheral blood leukocytes. Since STAT5ab^{null/null} HSC do not produce lymphocytes, donor engraftment in the myeloid lineage (Gr-1⁺) ≥ 5% defined mice that were scored as positive for engraftment. For wild-type and STAT5ab^{ΔN/ΔN} HSC engraftment was defined as > 5% in myeloid Gr-1⁺ and lymphoid B220⁺ lineages. Included in columns under each genotype is the number of mice engrafted as a fraction of the number of mice injected. Some cell doses were not determined (n.d.) for each genotype. The cell dose at which 37% of mice tested were negative for donor multilineage engraftment at 16 weeks was used to calculate the frequency of CRU based on Poisson statistics using L-Calcul software (StemCell Technologies).