Scientific Category: Hematopoiesis

**Cell Intrinsic Defects in Cytokine Responsiveness of STAT5-Deficient Hematopoietic Stem Cells**

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Running title: A major role for STAT5 in stem cell signaling

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ABSTRACT

Secreted growth factors are integral components of the bone marrow (BM) niche and can regulate survival, proliferation, and differentiation of committed hematopoietic stem cells (HSCs). However, downstream genes activated in HSCs by early acting cytokines are not well characterized. To better define intracellular cytokine signaling in HSC function, we have analyzed mice lacking expression of both STAT5a and STAT5b (STAT5ab-/-). These studies specifically avoided possible autoimmune and/or splenomegaly disease-mediated indirect effects on HSC function by using two independent approaches; 1) crossing onto the C57Bl/6 RAG2-/- background and 2) generation of wild-type transplanted chimeric mice reconstituted with STAT5ab-/- BM cells. These experiments demonstrated that STAT5-deficient HSCs have cell autonomous defects in competitive long-term repopulating activity. Furthermore, in the chimeric mice, injected wild-type BM cells showed a progressive multilineage competitive repopulating advantage in vivo, demonstrating that steady-state hematopoiesis was also highly STAT5-dependent. Consistent with the in vivo repopulating deficiency, when Sca-1+c-kitlin- (KLS) cells were isolated and stimulated with growth factors in vitro, up to a 13-fold reduced expansion of total nucleated cells was observed in response to cocktails containing interleukin (IL)-3, IL-6, stem cell factor (SCF), Flt3 ligand, and thrombopoietin. Notably, a 10-fold reduction in expansion was observed with IL-3 and SCF. However, STAT5 activation was not required for regeneration of the KLS pool in vivo following transplant or for secondary repopulating ability. These studies support a major role for STAT5 activation as a cellular determinant of cytokine-mediated HSC repopulating potential but not self-renewal capacity.
INTRODUCTION

Hematopoietic stem cells (HSCs) have tremendous proliferative potential and are required to generate differentiated progeny cells of all hematopoietic lineages in response to short-term myelosuppression. In addition, HSCs are defined clonally by their ability to both differentiate and self-renew. Regulation of HSC commitment is not well defined but is believed to be stochastic and not regulated by the microenvironment\(^1\)\(^,\)\(^2\). On the other hand, HSC survival and self-renewal are likely regulated by secreted factors within the bone marrow (BM) niche\(^3\). Following commitment, early acting hematopoietic growth factors can drive cell cycle activation and promote differentiation of HSCs. Growth factor signals can promote HSC survival and proliferation during in vitro stimulation leading to enhanced oncoretroviral-mediated gene transfer\(^4\). However, little is known about the intracellular signal transduction pathways that are necessary for proliferation and differentiation or for self-renewal division of HSCs.

Our studies have focused on activation of Janus kinase (JAK) and the latent signal transducer and activator of transcription (STAT) transcription factor pathway. The JAK/STAT signaling axis is a conserved pathway involved in diverse aspects of development. Recent studies in Drosophila have highlighted the role of the lone STAT protein in self-renewal of germ cells during spermatogenesis\(^5\)\(^,\)\(^6\). Furthermore, murine STAT3 knockout leads to early embryonic lethality\(^7\) and its activation is essential for embryonic stem cell self-renewal in vitro\(^8\)\(^,\)\(^9\). In contrast, STAT5 expression is first evident during differentiation of embryonic stem (ES) cells\(^10\). HSCs from mice lacking expression of both STAT5a and STAT5b (STAT5ab\(^{-/-}\)) can contribute to hematopoiesis at both short-term and long-term time points following transplant\(^11\) but STAT5-deficient HSCs are non-competitive with wild-type HSCs\(^11\)\(^,\)\(^12\). Despite a multilineage competitive repopulating defect, STAT5-deficient BM does not show a reduction in the number of cells with the HSC phenotype\(^11\)\(^,\)\(^12\) or a reduced homing ability following transplant\(^12\).

A difficulty in the characterization of HSC defects in adult STAT5ab\(^{-/-}\) mice has been the expansion of erythroid (Ter119\(^+\)) precursor cells in the spleen\(^13\)\(^,\)\(^14\) and accumulation of activated phenotype T lymphocytes\(^13\). The splenomegaly and possible autoimmune phenotypes reduce the life span of homozygous double mutant C57Bl/6 background mice. The etiology of this disease is complex and it was not the goal of this study to explore the disease phenotype. However, demonstration that adult STAT5-deficient mice show greater cell cycle activation in BM Sca-1\(^+\)lin\(^-\) hematopoietic progenitors\(^12\) suggested that microenvironmental factors might increase cell cycle status resulting in decreased long-term engraftment following transplant. Such a phenotype could be due to an observed age-dependent increase in T lymphocytes accumulating in the adult BM (unpublished observations). It is important to note that STAT5-deficient mice share some common phenotypes with the IL-2 receptor β chain knockout mouse, which has an activated T cell phenotype that moderately reduces repopulating activity\(^15\). To specifically avoid these potentially confounding features of the STAT5-deficient mouse, we have utilized two independent approaches. First, the STAT5 mutations were crossed onto the RAG2\(^{-/-}\) background. This provided a model for testing the cell intrinsic repopulating ability of the graft in the absence of adult T lymphocytes. Second, the ability of STAT5-deficient HSCs to fully reconstitute lethally-irradiated recipient mice avoided possible cell non-autonomous effects by reconstituting STAT5-deficient HSCs into a wild-type microenvironment.
METHODS

Mice genotyping and drug injections – The STAT5 mouse colony (C57Bl/6; Ly-5.2/Hb) was maintained by crossing heterozygote STAT5ab+/− mice to yield viable STAT5ab−/− pups that were genotyped by PCR as previously described 11;16. The congenic mice B6.C-H1b/By (HW80) and B6.SJL-Ptprc+/−Pep3b/BoyJ (Ly-5.1) were obtained from The Jackson Laboratory (Bar Harbor, ME). These congenic mouse strains have polymorphic changes at the hemoglobin locus (Hb) and the CD45 (Ly5) locus that facilitate tracking of transplanted donor cells. C57Bl/6 RAG2−/− mice (B6.SJL-Ptprc+/−BoCrTac-[KO]Rag2N10) were purchased from Taconic Labs (Germantown, NY). Clinical grade 5-fluorouracil (5-FU; ICN Pharmaceuticals, Inc., Costa Mesa, CA) was diluted 1:5 from the 50 mg/ml stock solution in sterile PBS to 10 mg/ml for intraperitoneal injection at a dose of 150 mg/kg.

Bone marrow transplant and peripheral blood analyses – BM was harvested from both hind limbs (tibias and femurs) of either STAT5ab−/− or littermate wild-type mice. For competitive repopulation assays, the cell mixtures were injected via the lateral tail-vein into lethally irradiated (1100 rads) recipient mice. The minimum cell dose injected was 2 x 10⁶ cells for each BM graft. For generation of chimeric mice, equal donor equivalents of BM cells were injected for each primary recipient mouse. For some experiments, BM was harvested from primary recipients at times 12 to 16 weeks following transplant and injected via the lateral tail-vein into lethally-irradiated secondary recipients (1100 rads). In other experiments, the secondary transplants were a competitive repopulation of Ly-5.1/wild-type vs. Ly-5.2/STAT5ab−/− BM cells from primary transplanted mice. Beginning at 8 weeks post-transplant, peripheral blood was obtained following puncture of the retroorbital venous sinus using a microcapillary tube. Microcapillary tubes were spun in a microcentrifuge (Stat-Spin Inc., Norwood, MA) and hematocrits were read manually. Hemoglobin patterns were analyzed from packed peripheral red blood cells by electrophoresis on cellulose acetate gels. To calculate the relative proportions of single and diffuse donor hemoglobin in peripheral blood from reconstituted mice, the hemoglobin gels were digitized using a ScanJet IIcx/T scanner (Hewlett Packard, Palo Alto, CA). Data files were quantitated by densitometry using ImageQuant software (Molecular Dynamics). For experiments where donor grafts were competed using the Ly-5.1/Ly-5.2 system, mice were analyzed by flow cytometry as described below.

Antibody staining and flow cytometry --BM cells were lineage depleted using a magnetically labeled antibody kit (StemSep, Stem Cell Technologies, Vancouver, Canada) which was followed by staining with a cocktail of phycoerythrin (PE)-conjugated antibodies to lineage markers that included Ly-6G (Gr-1), CD11b (Mac-1), CD45R/B220, CD4 (L3T4), CD8 (Ly-2), Ter119/Ly-76, CD90.2 (Thy1.2), and NK1.1 (NKR-P1B and NKR-P1C). The cells were also stained with antibodies to fluorescein isothiocyanate (FITC)-conjugated Ly-6A/E (Sca-1) and biotin-conjugated CD117 (c-kit). The biotinylated c-kit antibody was detected using a secondary streptavidin-phycoerythrin Cy5 conjugate (SA-PE-Cy5). All antibodies for these studies were obtained from BD Pharmingen (San Diego, CA). Cells were then analyzed on a FACSVantage SE flow cytometer (BD Biosciences, San Jose, CA) equipped with an INNOVA 70C laser providing 488 nm of excitation, at 70 mW output power. For peripheral blood analyses, the percentage of Ly-5.2 donor engraftment in Ly-5.1 recipient mice was quantitated using a FITC-conjugated CD45.2 (anti-Ly-5.2) antibody in combination with either direct PE-conjugated or
biotinylated-PE-Cy5-conjugated lineage antibodies. Some analyses were also performed on a BD LSR (BD Biosciences, San Jose, CA). For 4-color analyses of KLS cells and Ly-5.1 on the BD LSR, allophycocyanin (APC)-conjugated antibody to c-kit and PE-Cy5-conjugated antibody to CD45.1 (Ly-5.1) were used.

**Southern blot analyses** -- Genomic DNA was prepared from BM and spleen tissues from transplanted mice as previously described. DNA was then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with 2.5 volumes of ice cold ethanol and 1/10 volume sodium acetate. Five µg of DNA was digested overnight with NcoI enzyme and separated on a 0.8% agarose gel by electrophoresis. Gels were blotted overnight onto Hybond N+ nylon membrane (Amersham, Arlington Heights, IL), UV crosslinked, and hybridized with a [32P]-labeled mouse Y-chromosome probe. Blots were washed at a final stringency of 0.5X SSC/0.5% SDS at 65°C, exposed overnight, and autoradiographic images were obtained using a Molecular Dynamics Storm phosphorimager and X-ray film (Eastman Kodak, Rochester, NY). [32P]-dCTP was obtained from Amersham.

**RESULTS**

**Long-term repopulating defects are intrinsically dependent on STAT5 activation**
To eliminate lymphocytes, the C57Bl/6 STAT5ab+/− RAG2−/− background where splenomegaly disease observed. BM cells were isolated from both hind limbs of donor C57Bl/6 (hemoglobin single, Hb_s) RAG2−/−STAT5ab+/+ or RAG2−/−STAT5ab−/− littermate mice and mixed 1:1 with congenic wild-type HW80 (hemoglobin diffuse; Hb_d) mouse BM cells using the approach previously described. Genotype was crossed onto the C57Bl/6 and autoimmune phenotypes were not shown. Lethally-irradiated BM transplant (BMT) recipients were analyzed 19 and 18 weeks later for experiment (Expt.) #1 and Expt. #2 respectively (Fig. 1).

At all time points post-transplant, no Hb_s was detectable from the RAG2−/−STAT5ab−/− graft, indicating a very severe engraftment disadvantage. As a control, RAG2 deficiency alone (RAG2−/−STAT5ab+/+) did not affect repopulating potential, resulting in levels of engraftment of 48 ± 2% in Expt. #1 and 38 ± 6% in Expt. #2. However, RAG2−/−STAT5ab−/− mice still had an
abnormally reduced lifespan and thus were not useful as an alternative to increase the yield of surviving adult C57Bl/6 STAT5ab−/− mice.

Since we previously showed that STAT5-deficient HSCs could engraft transplant recipients in the absence of a competitor marrow11, a second approach was to generate chimeric mice transplanted with Ly-5.2/Hbα STAT5ab−/− or littermate Ly-5.2/Hbα wild-type BM cells into congenic HW80 (Ly-5.2/Hbδ) recipients. Control mice were simultaneously generated by transplanting wild-type Ly-5.1/Hbα BM cells into normal HW80 recipients. All sets of primary transplanted mice were generated by injection with the same donor equivalent of cells. The hemoglobin markers were used for analysis of chimerism. At 16 weeks post transplant, BM was collected from all three sets of chimeric primary transplanted mice. Secondary competitive repopulation experiments were then set up using BM cells from primary recipients: STAT5ab−/− (Ly-5.2) vs. wild-type (Ly-5.1), referred to as −/− BMT in Fig. 2A, or littermate wild-type BM cells (Ly-5.2) vs. wild-type (Ly-5.1), referred to as +/- BMT in Fig. 2A. This approach provided a normal non-hematopoietic microenvironment for all three sets of stem cells to reside in the primary recipients and allowed for comparison of secondary repopulating potential. The relative engraftment with the donor Ly-5.2 STAT5ab−/− cells was markedly reduced at 1:1 (0.4 ± 0.05%; n=4) and even at a 10:1 (4 ± 2%; n=4) mixing ratio (p<0.001), which was highly biased toward the Ly-5.2 graft. The engraftment defects were of similar magnitude in multiple lineages, as determined by antibody staining (data not shown). As expected,

![Fig. 2. Secondary transplantation of STAT5-deficient BM cells with and without competition with wild-type BM cells.](image)

Chimeric mice were first generated by primary transplant of either wild-type or STAT5-deficient BM cells into irradiated HW80 recipients. A. For the competitive secondary transplant experiment, BM was collected 16 weeks following transplant and mixed at a 1:1 ratio with Ly-5.1 BM from identically derived primary recipients. 16 weeks following secondary transplant, mice were analyzed for peripheral blood percentage of Ly-5.2 positive cells. B. For the non-competitive secondary transplant experiments, BM cells were injected into irradiated recipients and secondary engraftment was documented following transplant into either HW80 recipients using hemoglobin electrophoresis (top panels) or following transplant into Ly-5.1 recipients using FACS for multilineage reconstitution (bottom panels).

control wild-type BMT marrow competition showed roughly the input ratio of cells with 49 ± 4% at 1:1 mixing and 84 ± 4% at 10:1 mixing. In separate transplants in the absence of a competitor, STAT5ab−/− HSCs were always able to reconstitute secondary recipients at levels roughly equivalent to primary recipients (Fig. 2B). Shown are representative separate transplants into HW80 or Ly-5.1 recipients. Although increased chimerism was observed in mature red blood cells, Ter119+ erythroid progenitors, and CD4+ T lymphocytes, lymphomyeloid engraftment of B220+ B lymphocytes and Gr-1+ myeloid
cells remained complete. Additionally, the hematocrits of secondary BMT mice of 41 ± 2% for STAT5ab+/− and 49 ± 4% for wild-type transplanted mice were essentially the same as following primary transplant.11

STAT5-deficient HSCs are not 5-FU sensitive and are non-competitive during steady-state hematopoiesis

Using the BM chimera approach, engraftment was complete at all times from 8 weeks post transplant, except for the previously described STAT5ab+/− T lymphocyte chimerism. To determine the sensitivity of STAT5-deficient HSCs to a cell-cycle active drug, between 12 and 16 weeks post transplant the chimeras were treated with a single i.p. injection of 5-FU and analyzed serially for the hematocrit and the engraftment measured by hemoglobin electrophoresis (Fig. 3). Although the myelosuppression was severe in the STAT5ab+/− chimeras, the recovery from the nadir was not delayed. At 2 weeks post transplant for Expt. #1 the hematocrit of 20 ± 7% for STAT5ab+/− was lower than the 33 ± 2% for wild-type chimeras. However, in both Expt. #1 and Expt. #2, the hematocrit was able to rebound to levels that unexpectedly were not different from the wild-type chimeras. In Expt. #1 at 6 weeks the hematocrit was normal (42 ± 3% for STAT5ab+/− vs. 46 ± 2% for wild-type) and at 15 weeks the STAT5ab+/− chimeras had a hematocrit of 47 ± 3% (n=3) and the wild-type chimeras had a hematocrit of 50 ± 6% (n=4). In Expt. #2 at 13 weeks the STAT5ab+/− chimeras had a hematocrit of 42 ± 2% (n=3) and the wild-type chimeras had a hematocrit of 43% (n=2). Engraftment was always 100% at all time points as determined by hemoglobin electrophoresis (data not shown).

![Fig. 3. Recovery from myelosuppression in wild-type and STAT5-deficient chimeric mice following a single injection of 5-fluorouracil.](image)

Although the HSC pool was not ablated by 5-FU treatment in the context of the chimeric mouse, we also wanted to determine whether STAT5-deficient repopulating cells might be more 5-FU sensitive than wild-type HSCs. For these experiments, we treated both mutant and wild-type young (3-5 week old) untransplanted mice with 150 mg/kg 5-fluorouracil (5-FU) and two days...
later harvested the BM for competitive repopulation against PBS control treated BM cells. Two experiments were performed, one with male treated mice and the other with female treated mice. In both cases, the male vs. female competitive repopulation was also performed with both PBS treated mice as a control. The mice were reconstituted for 16 weeks and then sacrificed for BM collection. Pooled BM cells were then analyzed by Southern blot for the percentage of male DNA as determined using a standard curve of male DNA mixed into mouse Y-negative female DNA (Fig. 4, left panels). Toxicity from the 5-FU treatment was reflected by an increase in the reconstitution with PBS treated competitor as shown in the right panels. Modest 5-FU toxicity was observed on wild-type BM cells, however relative to the wild-type samples, the STAT5-deficient 5-FU treated samples competed on average 1.7-fold better against its own PBS treated competitor. Thus STAT5 deficiency does not increase 5-FU sensitivity but rather may decrease sensitivity even in the 3-5 week old mutant microenvironment. The average overall engraftment of the donor cell pool was used as the measure of repopulating potential to control for possible variation between individual recipients of STAT5ab-/- BM cells, which were likely limiting in total HSC activity.

In order to test whether STAT5-deficient chimeras would be favorable recipients for a wild-type BM challenge, chimeric mice were again generated. STAT5ab-/- or wild-type littermate BM cells (Ly-5.2/Hb,) were transplanted into either Ly-5.1/Hb, recipients (Expt. #1) or HW80 (Ly-5.2/Hb) primary recipients (Expt. #2). Engraftment with donor cells was documented in the lethally-irradiated hosts by either Ly-5.2 FACS (Expt. #1) or by hemoglobin electrophoresis (Expt. #2). Mice in Expt. #1 were challenged with a dose of 5 x 10^6 wild-type HW80 BM cells (Hb). Analysis of engraftment with the HW80 challenge cells was then tracked over time by hemoglobin electrophoresis. Fig. 5A,B show results of serial analysis of all mice challenged with HW80 BM cells. Mice in Expt. #2 were challenged with a dose of 5 x 10^6 wild-type Ly-5.1 cells and analysis of engraftment was by FACS. A representative example of the serial FACS analysis is shown in Fig. 5C. The average of all mice analyzed is shown in Fig. 5D (n=4). In both experiments, the donor cells had a competitive advantage in the STAT5ab-/- chimeric mice but not in the wild-type chimeric mice. Engraftment levels were observed in multiple hematopoietic lineages (Gr-1+, 86 ± 6%; B220+, 90 ± 4%; CD4+, 62 ± 48%; Ter119+, 83 ± 8%). The CD4+ T lymphocyte variability was likely due to competition with the endogenous host T
lymphocytes that were not replaced after the primary transplant. In contrast, minimal levels of engraftment were detected in challenged wild-type chimeras lineages (Gr-1+, 0.4 ± 0.2%; B220+, 1.8 ± 0.6%; CD4+, 1.4 ± 0.6%; Ter119+, 1.3 ± 0.3%) with unchallenged mice showing a background of 0.2 ± 0.1% as an average of all lineages. After 16 weeks, the mice were euthanized and the BM was collected. Ly-5.1 FACS analyses on the BM from Expt. #2 of the euthanized mice showed 81 ± 6% Ly-5.1+ cells in the STAT5ab−/− chimera but only 0.23 ± 0.03% in the wild-type chimera. Secondary transplant recipients from Expt. #1 (Fig. 5A) and Expt. #2 (data not shown) showed multilineage reconstitution with the wild-type donor cells, confirming the stem cell level engraftment advantage.

STAT5-deficient KLS cells are defective in long-term repopulating activity in vivo and have reduced cytokine responsiveness in vitro

We next wanted to determine whether the effects observed in vivo would be evident in an enriched HSC population isolated from the BM of the STAT5-deficient mice. For some experiments, Sca-1+c-kit+lin− (KLS) cells were transplanted at limiting dilution into lethally-irradiated recipient mice along with 2 x 10⁵ competitor cells (Table 1). In all three transplants with wild-type KLS cells, long-term repopulating activity was seen with cell doses as low as 250 cells. However, in two separate KLS transplants using mutant cells, no detectable engraftment...
was observed with 10- to 20-fold higher KLS cell doses. Even following injection of lineage negative non-KLS cells from Expt. #3, no engraftment was detected in three mice receiving 14,000 cells, while 1 out of 4 mice receiving 43,000 wild-type lineage negative non-KLS cells did show some engraftment (6%). This result demonstrates the complete loss of competitive long-term repopulating activity despite a normal KLS phenotype.

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<th>Table 1 - <em>In Vivo</em> Repopulating Activity of Sorted KLS Cells</th>
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BM cells were obtained from both hindlimbs of STAT5ab⁻/⁻ or littermate wild-type mice, lineage depleted, and sorted by FACS for the Sca-1⁺/c-kit⁺/lin⁻ (KLS) cells. Cells were mixed with 2 × 10⁵ HW80 competitor cells and transplanted into lethally-irradiated recipient HW80 mice (Expt. #1, #2). HW80 competition experiments were analyzed by hemoglobin electrophoresis for Hb s contribution, followed by densitometry of gels. For Expt. #3, Ly-5.1 recipient mice were used and analysis was by FACS for Ly-5.2⁺ cells in the peripheral blood. Analyses were performed at times between 12 and 16 weeks. n.a., not attempted.

In other experiments, 200 to 800 KLS cells/well were put into liquid suspension culture in the presence of cytokine cocktails to stimulate proliferation *in vitro* over a 6-day incubation period. Consistent with the defective repopulating activity *in vivo*, the KLS cells showed a reduced expansion potential *in vitro* (Table 2).

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<th>Table 2 - <em>In Vitro</em> Cytokine Stimulated Cell Expansion From Sorted KLS Cells</th>
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KLS cells were sorted by FACS and plated in 96 well plates in the presence of various cytokine cocktails. After 6 days in culture the total nucleated cell count was determined using a hemacytometer. The fold cell expansion was calculated based on the starting number of cells seeded per well. IL3, murine interleukin 3 (20 ng/ml); 6, human interleukin 6 (50 ng/ml); S, murine stem cell factor (50 ng/ml); F, murine flt-3 ligand (50 ng/ml); T, murine thrombopoietin (50 ng/ml); The results are the average ± SD for 4-6 experiments.

As expected, IL-3 supported the greatest proliferation and was required for maximal levels of cell expansion. Stimulation with cocktails containing other early-acting growth factors such as IL36S, IL3S, and IL3SFT revealed the greatest deficiency for STAT5-deficient KLS cells. These growth factor combinations are known to synergize with IL-3 to promote cell proliferation and survival during short-term *ex vivo* culture. As described previously, in all experiments the absolute number of hindlimb KLS cells was not different between wild-type and STAT5-deficient mice. Interestingly, analysis of KLS numbers from three separate experiments 16
weeks following transplant also showed equivalent numbers of KLS cells between both hindlimbs of the wild-type transplanted (7945 ± 4738), STAT5ab−/− transplanted (4367 ± 1168), and untransplanted wild-type (4703 ± 1836) mice. All KLS cells from transplanted mice were 98 ± 1% Ly-5.2+ and thus donor derived. Therefore, complete regeneration of the KLS pool following transplant did not require STAT5 activation.

DISCUSSION

This study sought to determine whether the competitive disadvantage of STAT5-deficient HSCs was primarily due to a cell intrinsic requirement for STAT5 activation or secondary to the other life-threatening phenotypes observed in these mice\textsuperscript{13,15}. Since previous reports have shown anti-apoptosis\textsuperscript{15} and cell cycle related\textsuperscript{12} defects in the adult STAT5-deficient mouse, we have clarified a critical issue regarding the role of STAT5 in long-term repopulating activity. The STAT5-deficient mouse is severely affected by many phenotypes that reduce the life span and which may likely contribute to an autoimmune-mediated effect on HSC survival within the BM cavity. The results of this study indicate that STAT5 activation is required downstream of early acting cytokines to promote cytokine-mediated repopulating activity of HSCs. The inability to rescue this defect by elimination of lymphocytes or by transplant into a “normal” wild-type microenvironment demonstrates that the primary mechanism for the defect is cell intrinsic.

We isolated KLS fractions from young STAT5ab−/− mice and tested these cells for repopulating activity in vivo and cytokine responsiveness in vitro. Consistent with the whole BM competitive repopulation experiments, the sorted KLS cells were unable to contribute to hematopoiesis even at very high numbers in the competitive repopulation assay. This was also consistent with the report by Snow et al. using sorted Sca-1+ Lin− BM cells\textsuperscript{12}. In contrast, good engraftment was observed in all three transplants with the positive control wild-type KLS cells. HSC surface phenotype can change associated with activation, resulting in c-kit downregulation\textsuperscript{17}, Mac-1 upregulation\textsuperscript{17}, or CD34 upregulation\textsuperscript{18}. Although we showed the inability of lineage negative non-KLS cells from STAT5-deficient mice to engraft, we cannot rule out that the STAT5-deficient HSCs had acquired Mac-1, since an antibody to Mac-1 was used in our lineage cocktail. However, the 5-FU studies in Figs. 3 and 4 strongly suggest that STAT5-deficient repopulating cells are not “activated” but instead may be more quiescent. Consistent with the transplant data, the in vitro cytokine stimulation was highly reduced in the STAT5-deficient KLS cells. The 10-fold deficiency in response to IL-3 and SCF, provides evidence that STAT5 may be a key intermediate in SCF signaling in primitive hematopoietic cells. Further studies will be required to determine the degree to which SCF signaling may be specifically impaired in these mice. In the BM challenge experiments, the ability of wild-type BM cells to take over the hematopoietic system also had similar kinetics with that described by others following injection into c-kit signaling deficient W/W\textsuperscript{v} mice\textsuperscript{19}. Repopulating deficiencies were therefore not due to a homing defect since injected competitor cells could take over even after prior engraftment with the primary transplant and in the absence of further host conditioning. However we cannot rule out that migration and competition for the BM niche during steady-state hematopoiesis\textsuperscript{20} might be defective. c-kit is known to interact with both JAK2 and SHP-1, which act as positive and negative regulators respectively for modulating downstream signaling\textsuperscript{21,22} and possibly modulating STAT5 activation. Some evidence exists that SCF can activate STAT5 in mast cells\textsuperscript{23} and some cell lines in vitro\textsuperscript{24}, although evidence supporting STAT5 activation downstream of SCF/c-kit in HSCs has not been previously reported.
Bromodeoxyuridine labeling studies have shown that within 30 days all murine HSCs will have gone through one cell division\textsuperscript{25; 26}. Therefore, rather than a clonal succession model with both proliferating and deeply quiescent HSC populations, HSCs are currently believed to all be slowly cycling but at any one point in time only 4-5\% of the HSC population will be in S/G2/M. Here we show by competitive repopulation that repopulating STAT5-deficient HSCs are less sensitive to the mild 5-FU effects than wild-type HSCs. This result suggests that less cell cycle activation reduced 5-FU toxicity\textsuperscript{27} or 5-FU mediated HSC activation\textsuperscript{17} which has been associated with decreased repopulating activity\textsuperscript{28}. Further studies using 5-FU in combination with SCF will be necessary to test more HSC toxic regimens\textsuperscript{27}. Possible downstream targets of STAT5 in HSCs could be the cell cycle associated genes such as the cyclins\textsuperscript{29; 30}. Cyclin D2 is expressed at high levels in long-term repopulating HSCs\textsuperscript{26}. While we were unable to obtain sufficient numbers of STAT5-deficient mice and KLS cells for cell cycle analysis by FACS, future studies on differential gene expression might identify important downstream genes that are dysregulated. STAT5 is a mediator of p210Bcr-abl signaling in transduced cells\textsuperscript{31} and chronic myelogenous leukemia is known to be a clonal stem cell proliferative disease. Constitutively activated mutants of STAT3\textsuperscript{32} and STAT5\textsuperscript{33} are also oncogenic. Therefore, the decreased cytokine responsiveness reported here for STAT5-deficient HSCs, provides further support for targeting STAT5 as a treatment for at least some leukemias. However, STAT5 is not essential for myeloproliferative disease in chronic myelogenous leukemia models, since STAT5-deficient mice could still develop disease\textsuperscript{34}. In an acute myeloid leukemia (AML) model for the Tel-JAK2 translocation, STAT5 activation was essential for leukemogenesis\textsuperscript{33} and STAT5 activation is associated with AML\textsuperscript{35; 36}. In these studies we have not determined whether anti-apoptosis gene expression is dysregulated in the absence of STAT5. This might also comprise a significant component of the deficiency, although increased apoptosis in the HSC compartment would be predicted to reduce the pool size. Survival signaling pathways mediated through c-mpl\textsuperscript{37} and/or Flt3\textsuperscript{38} might also require STAT5 activation in HSCs. Other signaling components downstream of c-kit could also compensate for the STAT5-mediated defects, possibly resulting in a similar but less severe phenotype than reported for the W/W\textsuperscript{v} mouse. Our data demonstrate that STAT5 activation is not essential for HSC self-renewal, since the KLS pool was restored to levels equivalent with wild-type mice following transplant and hematopoietic reconstitution following 5-FU treatment or secondary transplant was not severely reduced. This finding was consistent with the idea that SCF may not be essential for murine HSC self-renewal\textsuperscript{39-41} but capable of promoting cell cycle entry\textsuperscript{42}. However, differences might exist between fetal and adult HSCs in this regard\textsuperscript{43}. Direct demonstration of STAT5 activity in the specific proliferative or anti-apoptotic responses to cytokine stimulation will require further study. In summary, this study demonstrates that overall stem cell cytokine responsiveness and repopulating potential are highly STAT5 dependent and that in the STAT5-deficient background, wild-type BM cells have a dominant selective advantage in multiple lineages. Inversely, it remains to be determined whether conditional STAT5 activation might be used to confer a competitive engraftment advantage on wild-type BM cells. Recent demonstration of STAT5-dependent expansion of multipotential hematopoietic cells by signals from JAK2 and c-kit or Flt3 supports this concept\textsuperscript{44}. 

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