

Original Article

STAT5 N-domain deleted isoforms are naturally occurring hypomorphs partially rescued in hematopoiesis by transgenic Bcl-2 expression

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Received July 11, 2014; Accepted August 5, 2014; Epub September 5, 2014; Published September 15, 2014

Abstract: Signal transducer and activator of transcription 5 (STAT5) is a critical regulator of normal and leukemic lympho-myeloid development through activation downstream of early-acting cytokines, their receptors, and JAKs. Truncation of STAT5 can be mediated through alternative translation initiation from an internal start codon giving rise to N-terminally deleted isoforms. To determine whether these isoforms could be detected naturally in normal murine tissues, Western blot analyses were performed on heart, lung, brain, spleen, liver, and kidney. Relative expression of full-length to truncated STAT5 was variable among tissues. Since we have previously demonstrated that STAT5ab Δ N lacks the ability to effectively upregulate pro-survival signals and bcl-2 expression, we used a transgenic mouse approach to next determine whether constitutive expression of human Bcl-2 in STAT5ab^{ΔN/ΔN} mouse hematopoietic cells could restore normal hematopoiesis. Transgenic H2K-Bcl-2 expression in hypomorphic STAT5ab^{ΔN/ΔN} mice largely rescued peripheral B and T lymphocyte numbers whereas multilineage donor contribution was only rescued to levels about 10% of normal. At the hematopoietic stem cell level, direct competitive repopulation with H2K-Bcl-2/STAT5ab^{ΔN/ΔN} against STAT5ab^{ΔN/ΔN} competitor showed a corrective effect of Bcl-2 expression whether the STAT5ab^{ΔN/ΔN} genotype was competed as the donor or as the host versus H2K-Bcl-2/STAT5ab^{ΔN/ΔN} genotype bone marrow cells. Therefore, STAT5ab Δ N isoforms are heterogeneously expressed and lack key functional activities that can be partially rescued by adding back Bcl-2.

Keywords: Cytokine signaling, JAK/STAT, hematopoiesis, apoptosis, Bcl-2

Introduction

Signal transducer and activator of transcription 5 (STAT5) is a critical regulator of normal and leukemic lympho-myeloid development through activation downstream of early-acting cytokines, their receptors, and janus kinases (JAKs) [1-5]. STAT5-mediated regulation of bcl-2 in hematopoietic cells has been reported both in mouse [6] and human hematopoietic cells [7]. In our previous study, we showed that STAT5ab^{ΔN/ΔN} mast cells had a reduced level of both bcl-2 and bcl-X_L expression. Furthermore, we demonstrated that bcl-2 is a bone fide direct target of STAT5 by chromatin immunoprecipitation (ChIP) - polymerase chain reaction (PCR) analysis and that STAT5 requires the N-domain for suppression of miR15/16, induction of bcl-

2, and induction of survival signaling in mast cells and in myeloproliferative neoplasms (MPNs) [8].

Hematopoietic homeostasis is maintained through the balance between decisions on life and death and self-renewal and differentiation. Apoptosis plays a key role in regulating hematopoietic cell numbers [9]. Overexpression of bcl-2 increases both the stem cell number as well as the repopulation potential [10-12]. A Bcl-2 transgenic mouse in which human Bcl-2 is overexpressed under control of the mouse H-2K^b promoter and combined with the Moloney murine leukemia virus LTR expressed high levels of Bcl-2 in hematopoietic stem cells and many other tissues [11]. The anti-apoptotic function of bcl-2 has been demonstrated in this

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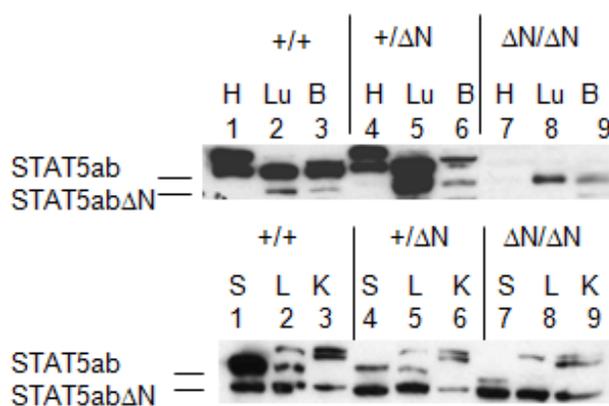


Figure 1. STAT5ab Δ N proteins are naturally occurring isoforms expressed heterogeneously in murine tissues. Whole cell lysates were prepared and proteins were separated on SDS-PAGE gels from wild type (+/+), STAT5ab +/ Δ N (+/ Δ N) and STAT5ab Δ N/ Δ N (Δ N/ Δ N) mouse tissues (H: Heart; Lu: lung; B: brain; S: spleen; L: liver; K: Kidney) homogenized in RIPA buffer. Representative Western blots results are shown.

transgenic mouse which has an increased hematopoietic stem cell pool and is resistant to irradiation. In our studies, Bcl-2 overexpression in Ku70-deficient hematopoietic stem cells almost completely rescued the impaired quiescence, repopulation, and bone marrow niche occupancy capacities but without restoration of DNA repair capacity [13]. In a persistently active STAT5a (STAT5a^{S711F}) driven MPN mouse model, we found that STAT5 lacking the N-domain is deficient at MPN induction because of an intrinsic inability to maintain hematopoietic stem cell survival *in vivo* [8]. This deficient survival function could be restored by adding back Bcl-2 with H2K-Bcl-2 transgenic mice. The mice expressing STAT5a Δ N^{S711F} plus Bcl-2 recapitulated the disease of STAT5a^{S711F}. STAT5 tetramer formation requires the N-domain in promoter recruitment through protein-protein interactions [14]. These higher order interactions also appear to be closely associated with leukemogenesis [15] and may be a potential therapeutic target for translational approaches to hematologic malignancies [16]. However, whether the STAT5 N-domain mediated regulation of bcl-2 plays a role in normal multi-lineage hematopoiesis is not well understood. Here we report that different STAT5 isoforms missing the N-domain could be detected in various normal tissues. STAT5ab^{ΔN/ΔN} mice crossed with Bcl-2 transgenic mice largely rescued peripheral B and T lymphocyte numbers but conferred

only about 10% restoration of multi-lineage repopulation ability. This correction was further tested in novel head-to-head competitive repopulation assays of STAT5ab^{ΔN/ΔN} hematopoietic stem cells with or without transgenic Bcl-2 expression.

Materials and methods

Mice

The C57BL/6 (CD45.2), the congenic strains B6.SJL-Ptprc^aPep3^b/BoyJ (CD45.1) and GFP transgenic mice (CD45.2) were obtained from the Jackson Laboratory (Bar Harbor, ME). STAT5ab^{+/ Δ N} mice were obtained from Jim Ihle (St. Jude) [17]. H2K-Bcl-2 transgenic mice were obtained from Jos Domen [11] (Medical College of Wisconsin). All mice unless indicated otherwise are on C57BL/6 background by backcrossing more than 9 generations and housed in a specific pathogen-free environment. All mouse studies were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University (Cleveland, OH) and at Emory University (Atlanta, GA).

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., Norwood, MA) and hematocrits were read manually. Hemoglobin electrophoresis was performed as described previously [1]. For red and white blood cell counts, Cells were diluted in isotonic saline solution and analyzed using Coulter counter (Beckman Coulter Inc., Miami, FL).

Bone marrow cell transplantation

Bone marrow competitive and non-competitive transplants were performed as previously described [18, 19]. To analyze donor engraftment, the bone marrow and peripheral blood of the recipient mice were stained with CD45.2 and subjected to FACS analysis. For head-to-head competitive repopulation between STAT5ab^{ΔN/ΔN} (C57BL/6) or H2K-Bcl-2/STAT5ab^{ΔN/ΔN} (C57BL/6) versus STAT5ab^{ΔN/ΔN} (HW80) mice [20] bone marrow cells from both HW80 and C57BL/6 background mice were mixed thor-

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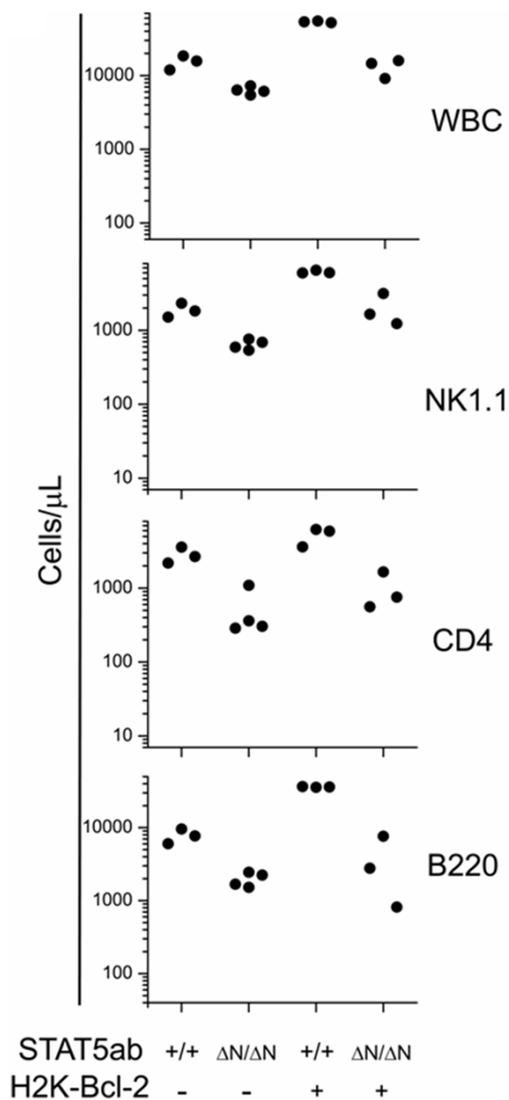


Figure 2. Peripheral blood hematology of wild-type, STAT5ab^{ΔN/ΔN}, H2K-Bcl-2 and H2K-Bcl-2/STAT5ab^{ΔN/ΔN}. The total white blood cells (WBC) were counted by Coulter counter after the lysis of red blood cells. The absolute cell count of NK1.1, CD4 and B220 was calculated by multiplying the positive (NK1.1, CD4 or B220) cells by the total white blood cell counts per microliter of whole blood. Shown are values obtained from individual mice represented as vertical dot plots.

oughly at a 50:50 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. In some

experiments GFP transgenic mouse bone marrow was used as the donor and injected into STAT5ab^{ΔN/ΔN} bone marrow chimeric mice in the absence of additional irradiation.

Statistical analysis

Student's t-test was used to compare the significance between two independent data.

Results

STAT5ab^{ΔN/ΔN} mice were initially generated by targeting exons encoding the initiation methionine but it was discovered that the mutant mice still expressed N-domain truncated STAT5 by using downstream in frame methionine(s) as the translation start site. To determine whether these truncated STAT5 isoforms were also present in tissues from normal wild-type mice, we performed Western blot analysis using antibodies to the C-terminus of STAT5 (**Figure 1**). N-domain truncated STAT5 was naturally expressed in normal wild type mice tissues including lung, brain, spleen, and kidney and the relative ratio between full length and truncated forms of STAT5 varied widely amongst the different mouse tissues. This result is consistent with a previous report showing that truncated STAT5 isoforms are detected in wild type mouse spleen [21]. Since we have recently shown that the STAT5 N-domain is critical for the survival promoting activity of STAT5, we wanted to test the extent to which a robust transgenic mouse expressing Bcl-2 could correct hematopoietic defects resulting from loss of the N-domain. We first crossed STAT5ab^{ΔN/ΔN} mice with Bcl-2 transgenic mice (H2K-Bcl-2). The Bcl-2 transgene was driven by the H2K promoter and Moloney murine leukemia virus enhancer that has proven optimal for expression throughout hematopoiesis, including hematopoietic stem cells [10-12]. We examined peripheral blood lineages from STAT5ab^{ΔN/ΔN}, H2K-Bcl-2/STAT5ab^{ΔN/ΔN} and littermate control wild-type and H2K-Bcl-2/STAT5ab^{+/+} mice. Expression of Bcl-2 significantly increased total white blood cell (WBC), and NK1.1⁺ cell counts relative to STAT5ab^{ΔN/ΔN} alone ($P < 0.05$) (**Figure 2**). Also, the B220⁺ and CD4⁺ counts were increased to levels not significantly different than normal.

To test whether Bcl-2 mediated correction of the early hematopoietic stem and progenitor

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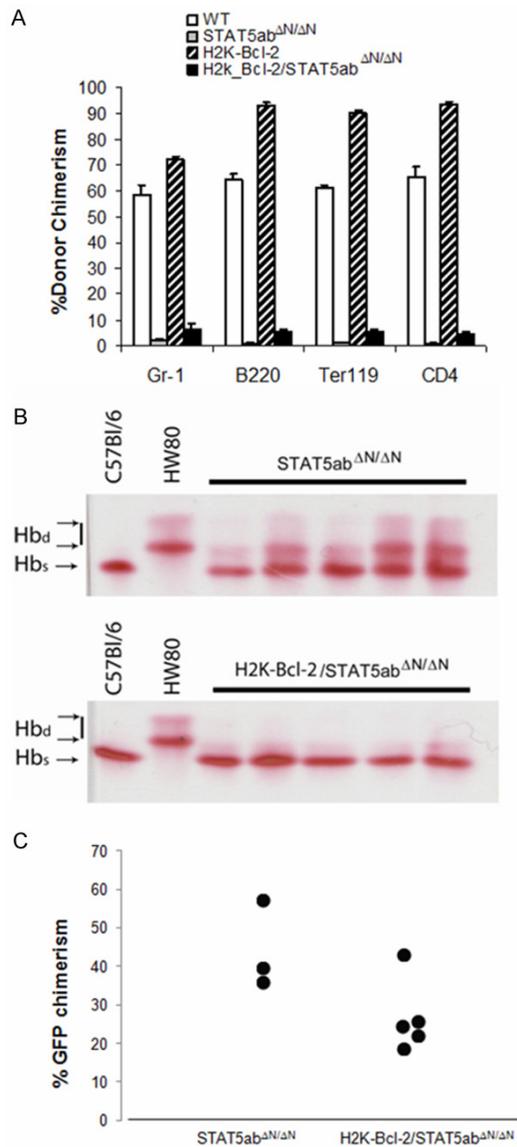


Figure 3. Transgenic Bcl-2 expression partially restores lympho-myeloid development from STAT5ab^{ΔN/ΔN} bone marrow transplant. **A:** Wild-type (WT), STAT5ab^{ΔN/ΔN}, H2K-Bcl-2, and H2K-Bcl-2/STAT5ab^{ΔN/ΔN} bone marrow cells (CD45.2+) were mixed with Boy J bone marrow cells (CD45.1+) at the ratio of 1:1, transplanted into the lethally irradiated Boy J mice at the ratio of 1 donor to 5 recipients and analyzed 12-16 weeks after transplantation for donor chimerism in peripheral blood leukocytes. The results are shown as the representative of two independent transplantation experiments. **B:** STAT5ab^{ΔN/ΔN} or H2K-Bcl-2/STAT5ab^{ΔN/ΔN} bone marrow cells on C57BL/6 background were mixed with STAT5ab^{ΔN/ΔN} on the HW80 background at the ratio of 1:1, transplanted into the lethally irradiated Boy J mice (5-8 mice per group) and hemoglobin gel electrophoresis was performed 12-16 weeks after transplantation. The results are shown as the representative of two independent transplantation experiments. **C:** STAT5ab^{ΔN/ΔN} or H2K-Bcl-2/STAT5ab^{ΔN/ΔN} bone mar-

row cells were first transplanted into lethally irradiated Boy J mice. 16 weeks after transplantation the mice were then injected with a single dose of 5 million of GFP transgenic bone marrow cells. Donor GFP engraftment was measured 16 weeks after transplantation.

defects, we performed competitive bone marrow transplantations using CD45.2 positive STAT5ab^{+/+}, STAT5ab^{ΔN/ΔN}, H2K-Bcl-2 and H2K-Bcl-2/STAT5ab^{ΔN/ΔN} bone marrow cells competed against wild-type CD45.1 bone marrow cells at the ratio of 1:1 mix. As shown in **Figure 3A**, STAT5ab^{ΔN/ΔN} bone marrow cells had severe defects. The H2K-Bcl-2 transgene markedly enhanced the competitive repopulation ability of wild type control bone marrow in multiple lineages. H2K-Bcl-2/STAT5ab^{ΔN/ΔN} did have enhanced competitive repopulation ability ($P < 0.005$) but the magnitude was small, comprising only about 10% of the deficiency. Since the correction of repopulating dysfunction was small, we confirmed the repopulating defect in direct head-to-head competitive repopulation of STAT5ab^{ΔN/ΔN} on the congenic HW80 background against H2K-Bcl-2/STAT5ab^{ΔN/ΔN} on the C57BL/6 background (**Figure 3B**). Donors were distinguished by hemoglobin type. The C57BL/6 H2K-Bcl-2/STAT5ab^{ΔN/ΔN} graft was dominant over the HW80 STAT5ab^{ΔN/ΔN} graft. This result further confirmed that add-back of Bcl-2 can partially correct STAT5ab^{ΔN/ΔN} stem cell competitive repopulation ability.

We next performed bone marrow transplantations using wild-type, STAT5ab^{ΔN/ΔN}, H2K-Bcl-2/STAT5ab^{ΔN/ΔN} as donors into lethally irradiated CD45.1 recipients. The peripheral blood was analyzed 16 weeks post-transplantation. H2K-Bcl-2/STAT5ab^{ΔN/ΔN} transplanted recipient mice had the same total white blood cell (WBC) numbers as wild-type transplanted mice, while STAT5ab^{ΔN/ΔN} bone marrow transplanted mice had a 60% decrease of total WBC count. Bcl-2 transgenic mice also had significantly enhanced numbers of B-cells (B220⁺, $P < 0.05$) and T-cells (CD4⁺, $P < 0.01$) (**Figure 4A**). The only chimerism observed was in the T-cell lineage and this was completely corrected by the Bcl-2 transgene (**Figure 4B**). When STAT5ab^{ΔN/ΔN} and H2K-Bcl-2/STAT5ab^{ΔN/ΔN} bone marrow chimeric mice were challenged with a single dose of 5 million GFP transgenic mice bone marrow cells, the level of GFP donor engraftment was lower in H2K-Bcl-2/STAT5ab^{ΔN/ΔN} chimeric mice than that in

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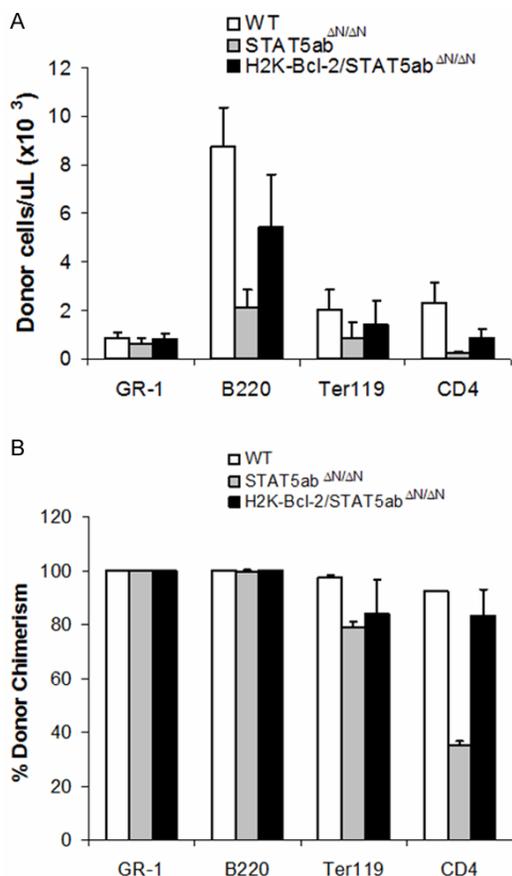


Figure 4. Transgenic Bcl-2 expression restores circulating lymphocyte levels in STAT5ab^{ΔN/ΔN} mice. Wild type (WT), STAT5ab^{ΔN/ΔN} and H2K-Bcl-2/STAT5ab^{ΔN/ΔN} bone marrow cells were transplanted into lethally irradiated Boy J recipients at the ratio of 1 donor to 5 recipients and analyzed 12-16 weeks after transplantation for the percentage of donor CD45.2 chimerism (A) or for the absolute cell count of donor lineage positive cells in peripheral blood leukocytes (B). The results are represented as the Mean ± SD from the combination of two independent transplantation experiments with 9-10 recipient mice in each group.

STAT5ab^{ΔN/ΔN} mice (Figure 3C). This result further confirmed that add-back of bcl-2 can partially increase the innate competitive ability as these H2K-Bcl-2/STAT5ab^{ΔN/ΔN} hematopoietic stem cells tended to hold their niche position better than STAT5ab^{ΔN/ΔN} hematopoietic stem cells.

Discussion

Previously we reported that STAT5ab^{ΔN/ΔN} mice lack the ability to effectively upregulate pro-survival signals and bcl-2 expression in mast cells. In this report, we show that constitutive

expression of human Bcl-2 with transgenic H2K-Bcl-2 in STAT5ab^{ΔN/ΔN} mouse hematopoietic cells could bring the total white blood cell count to a normal level the same as in wild type control mice and could largely restore peripheral B and T lymphocyte numbers. This result is very similar to the conclusion reached by Snow et al. [22] though they suggested that transgenic bcl-2 was not sufficient to rescue hematolymphoid defects in STAT5ab^{ΔN/ΔN} mice [2]. However, in our studies competitive multilineage donor contribution was rescued to levels about 10% of normal uniformly in all lympho-myeloid lineages. Furthermore, at the hematopoietic stem cell level, direct competitive repopulation between H2K-Bcl-2/STAT5ab^{ΔN/ΔN} and STAT5ab^{ΔN/ΔN} bone marrow cells showed that H2K-Bcl-2/STAT5ab^{ΔN/ΔN} bone marrow could outcompete STAT5ab^{ΔN/ΔN} bone marrow (lower panel of Figure 3B), and H2K-Bcl-2/STAT5ab^{ΔN/ΔN} chimeric mice clearly held their niche position better than STAT5ab^{ΔN/ΔN} mice when the mice were challenged with wild-type GFP bone marrow cells (Figure 3C) all indicating a significant corrective effect of Bcl-2 expression. These results indicated that white blood counts and lymphocyte homeostasis likely depend on survival signaling mediated through STAT5-Bcl-2 signaling. However, the STAT5 N-domain mediated Bcl-2 survival signaling only contributed in a modest but highly significant degree to the STAT5ab^{ΔN} competitive repopulation ability. Snow et al. used a bcl-2 transgene that was expressed from the constitutive β-actin promoter instead of H2K promoter and Moloney murine leukemia virus enhancer. The H2K-Bcl-2 expression cassette [11] is likely to be expressed a relatively higher level of the transgene in hematopoietic stem cells than the bcl-2 transgene under the β-actin promoter [22]. However, a direct comparison of intracellular staining for bcl-2 expression would be needed to fully clarify the difference.

Interestingly, Bcl-2 overexpression rescued the hematopoietic stem cell defects in Ku70-deficient mice [13] but only partially restored the competitive repopulation defect from STAT5ab^{ΔN/ΔN} mice in this study. This indicates that the STAT5 N-domain mediated bcl-2 expression and survival signaling is not a major contributor to the hematopoietic repopulating defects observed in STAT5ab^{ΔN/ΔN} mice. There are several other potential interactions with the

N-domain that may explain the hematopoietic repopulating defects. The interaction of STAT5 with the glucocorticoid receptor is mediated by the N-domain. STAT5 tetramerization is also dependent on the N-domain of STAT5 which might be critical for expression of a subset of target genes other than bcl-2. Recent studies with STAT5a-STAT5b double knockin N-domain mutant mice in which STAT5 protein form dimers but not tetramers showed that STAT5 tetramerization is critical for cytokine responses and normal immune function with defects in CD4⁺CD25⁺ T cells, NK cells, and CD8⁺ T cells [23]. Future structure-function studies are needed to better understand the role of the STAT5 N-domain and its contribution to all defects observed in hematopoietic repopulating activity of STAT5ab^{ΔN/ΔN} mice.

Acknowledgements

We gratefully acknowledge the funding support of NIH R01DK059380 and the technical support of the flow cytometry core facilities of the Case Comprehensive Cancer Center and the Emory + Children's Pediatric Research Center.

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