STAT3 positively regulates an early step in B-cell development

Wei-Chun Chou, David E. Levy, and Chien-Kuo Lee

Introduction

B lymphopoiesis involves the expression of multiple factors, including cytokine receptors and transcription factors.1-3 Among the cytokine receptors, Flt3 and IL-7R are known to be critical for early B-cell development. Targeted deletion of Flt3 or Flt3 ligand impairs the development of pro-B and pre-B cells.4,6 Similarly, genetic ablation of the IL-7 or the IL-7Rα chain results in a developmental block in CLP and pro-B stages.7-9 Mice lacking both Flt3L and IL-7Rα completely lose B lymphocytes in the BM, suggesting that these 2 molecules do not have completely overlapping functions in B lymphopoiesis.10 Several transcription factors are also indispensable for B-cell development and function in a sequential and an interdependent manner to instruct the generation of B-cell precursors from hematopoietic progenitors.11 For instance, PU.1 was first proposed to initiate B-cell differentiation by promoting the expression of IL-7R and EBF, another key transcription factor.11,12 However, more thorough studies indicated that PU.1 is not strictly required for B lymphopoiesis because mice lacking PU.1 in the B-cell lineage do not display abnormalities in early B-cell development. Nonetheless, abnormalities occur at more mature stages, including a shift of conventional B2 cells to B1 cells in the spleens of the mutant mice.13-15 The upregulation of EBF in conjunction with E2A further facilitates the formation of pro-B cells, where B-cell–specific genes such as Pax5, Igα, Igβ, λ5, VpreB, RAG1, and RAG2 are subsequently induced. Although the specification of B cells requires EBF and E2A, commitment and maintenance require Pax5.2

Although IL-7R signaling activates STAT1, STAT3, and STAT5, it is generally believed that STAT5 plays the major role in IL-7–mediated B lymphopoiesis.16-20 The expansion of pro-B cells in transgenic mice expressing constitutively active STAT5 correlated with an increase in cyclin D2, pim-1, and Bcl-xL expression, suggesting that STAT5 directly affects the proliferation and survival of pro-B cells.19 Overexpression of this form of STAT5 also restored B-cell differentiation in the IL-7R–deficient mice, including Vh immunoglobulin rearrangement and appearance of immature and mature B cells.19 In addition, a severe deficiency in B lymphopoiesis was observed in the absence of STAT5a/b, resulting in combined immunodeficiency similar to that caused by lack of IL-7 signaling.17 STAT5 also regulates germline transcription, histone acetylation, and DNA recombination of distal Vh gene segments in an IL-7–dependent manner. In the absence of STAT5a/b, these events are greatly impaired.18 Although STAT1 affects the survival and proliferation of T lymphocytes, contributes to the development of adaptive immune responses, and has a differential effect on the expression of MHC class I in T and B cells, it has not been shown to play a direct role in lymphopoiesis.21,22

The functions of STAT3 in vivo have been extensively investigated using conditional gene targeting in mice. These studies showed that STAT3 has multiple functions in different tissues, contributing to survival, apoptosis, migration, and gene expression.23 In addition, STAT3 has been implicated in oncogenesis. Activated STAT3 is observed in many malignancies in humans, and overexpression of a constitutively active form of STAT3 results in the transformation of fibroblasts.24 It is also required for the development of B-cell lymphoma in transgenic mice and for the growth and survival of human and mouse NPM-ALK–transformed
B and T cells.\textsuperscript{25} We have previously shown that STAT3 can be a negative regulator of granulocyte–colony-stimulating factor (G-CSF)–mediated granulopoiesis. Enhanced proliferation and increased granulocyte development were observed in the STAT3-deficient BM.\textsuperscript{26} However, the role of STAT3 in normal B lymphopoiesis remains to be elucidated.

In this study, we have demonstrated that STAT3 is required for efficient B lymphopoiesis. Mice lacking STAT3 in the BM displayed impaired B-cell development and reduced B-cell lineages, including pre-B, pre-B, immature B, and mature B cells. IL-7–mediated proliferation of BM cells was also significantly reduced, which correlated with a decreased number of IL-7–responsive precursors in the BM. These results indicate that STAT3 is critical for normal early B-cell development and that it acts in conjunction with IL-7 and STAT5 to facilitate the transition from pre–pro-B cells.

### Materials and methods

#### Mice

Generation of mice with a conditional \textit{STAT3} allele has been described previously.\textsuperscript{27} These mice were mated with a transgenic line bearing Cre recombinase driven by the IFN-inducible Mx promoter.\textsuperscript{28} Littermate mice cells were seeded in complete medium with methylcellulose (MethoCult antibodies, as indicated: anti–B220-PE (RA3-6B2), anti–IL-7R

#### Genotyping for targeted alleles

Genomic DNA was prepared by boiling tail samples in 300 μL NaOH 50 mM for 2 hours at 95°C followed by neutralization with Tris-Cl, pH 7.5, to final 100 mM. Two microliters of DNA solution was subjected to PCR with L NaOH 50

#### Flow cytometric analysis

Single-cell suspensions were prepared from bone marrow, spleen, and peripheral blood after erythrocyte depletion. Cells were stained with antibodies, as indicated: anti–B220-PE (RA3-6B2), anti–IL-7Rα-PE (A7R34), anti–CD24-PE-Cy5 (M1/69), anti–BrdU-FITC and streptavidin—

#### Statistical analysis

All statistical comparisons were validated using the Student \( t \) test.

#### Colony-formation assay

 Colony-formation assay was performed as described.\textsuperscript{26} Briefly, 2 \( \times 10^5 \) BM cells were seeded in complete medium with methylcellulose (MethoCult M3630; StemCell Technologies, Vancouver, BC, Canada) with or without exogenously added rmIL-7 (PeproTech, Rocky Hill, NJ) at 10 and 100 ng/mL for 7 days, followed by enumerating pre-B CFUs by light microscopy.

### In vitro proliferation assay

Proliferation assay was performed with either \(^{[3]H}\)-thymidine incorporation or BrdU incorporation assay. For \(^{[3]H}\)-thymidine incorporation, BM cells were seeded at 2 \( \times 10^5 \) cells per well in 96-well plates and were incubated in the absence or presence of rmIL-7 at 5 or 50 ng/mL or in the absence or presence of 1 ng/mL rmIL-3 (all from PeproTech) for 48 hours. Then 1 μCi (0.037 MBq) \(^{[3]H}\)-thymidine was added during the final 16 hours before harvest. For BrdU incorporation assays, 2.5 \( \times 10^5 \) BM cells without or with rmIL-3 (5 ng/mL) or IL-7 (10 or 50 ng/mL) for 38 hours were incubated with 10 μM BrdU (Sigma) for the final 3 hours. Labeled cells were stained with anti-B220, anti-CD43, and anti-BrdU, followed by FACS analysis. Stimulation index was calculated as percent cytokine stimulated + percent medium alone.

### In vitro apoptotic assay

Single-cell suspensions were prepared from BM after erythrocyte depletion and were incubated in vitro in complete medium without cytokines for 0 hours and 3 hours. BM cells were then stained with anti-B220, anti-CD43, and annexin V, and then FACS analysis was performed.

### BM transplantation

Five million BM cells from control or STAT3KO mice were transplanted intravenously into RAG1KO mice that were previously irradiated with 950 rads (9.5 Gy). Two months later, cells from BM or spleens of chimeric mice were stained with anti-IgM, anti-B220, and anti-CD43 (for BM cells) or anti-IgM and anti-IgD (for splenocytes), and then FACS analysis was performed.

### Intracellular staining of STAT3

BM cells were surface stained with antibodies to B220, CD43, and IgM; this was followed by fixation and permeabilization (Fix&Perm, Caltag, Burlingame, CA) and staining with rabbit anti-STAT3 or phospho-STAT3 antibodies (Cell Signaling, Beverly, MA). Washed cells were incubated with FITC-conjugated secondary antibody to rabbit antibody and then underwent FACS analysis.

### Results

Impaired B-cell development in STAT3KO mice

We previously analyzed the role of STAT3 in BM using conditional knockout mice and documented a requirement for STAT3 as a negative regulator for G-CSF–mediated granulopoiesis.\textsuperscript{26} In the same experiments, we noted reduced production of lymphocytes in the periphery, prompting us to investigate which population of lymphocytes was affected. First, we determined the degree of STAT3 deletion in the BM and periphery. Genomic DNA from BM, spleen, or peripheral blood leukocytes (PBLs) was subjected to PCR amplification for the targeted allele. As shown in Figure 1A (left panel), \textit{STAT3} was efficiently deleted in the BM following poly (I:C) injection. However, deletion was less efficient in the spleen and PBL, judging by the residual \textit{STAT3\textsuperscript{flox}} allele. Deletion of STAT3 in BM was further confirmed through Western blotting, as shown in Figure 1A (right panel). We next analyzed which population of lymphocytes was affected in the STAT3KO mice. PBLs of control or STAT3KO mice were subjected to FACS analysis with the use of anti-CD3 and anti-CD19 antibodies. As shown in Figure 1B, reduced numbers of B cells were observed in the PBLs of STAT3KO mice, whereas numbers of T cells remained
IL-7R is expressed primarily on common lymphoid progenitors and is important for the development of B and T lymphocytes.\textsuperscript{30,31}

To study whether the reduction in number of B-lineage cells in STAT3KO mice was the result of impaired responses to IL-7, BM from control or STAT3KO mice was treated with IL-7. As shown in Figure 3A, dose-dependent proliferation of control BM cells was observed in response to IL-7, whereas the STAT3KO cells displayed only minimal response. The impaired response was specific for IL-7 because IL-3-mediated proliferation was comparable between control and mutant cells (Figure 3B). To identify the IL-7 responders, BrdU was used to label proliferating cells, which, in conjunction with surface marker staining, could differentiate responses in B or non–B cells. As shown in Figure 3C, though non–B (B220\textsuperscript{+}) cells of both mice incorporated BrdU independently of IL-7 stimulation, B-lineage cells were responsive to IL-7. Interestingly, B cells of STAT3KO mice displayed less BrdU staining than did those of control mice (right panels). Moreover, the main responders to IL-7 in STAT3KO mice were B220\textsuperscript{+} BM cells, but the main responders to IL-3 were B220\textsuperscript{+} BM cells (Figure 3D-E). B220\textsuperscript{+} STAT3KO BM displayed reduced proliferation in response to IL-7, whereas the response to IL-3 in B220\textsuperscript{+} BM was almost equivalent to that of control cells, again indicating that impaired response in mutant cells was specific for IL-7.

Several mechanisms could account for the reduced response to IL-7. First, because IL-7 activates STAT proteins,\textsuperscript{16} it is possible that the loss of STAT3 results in impaired IL-7 receptor signaling, preventing STAT phosphorylation. To investigate this possibility, the kinetics of activation of STAT1 and STAT5 in response to IL-7...
Because the expression of IL-7Rα marks the development from multiple lineage progenitors to common lymphoid progenitors, it is crucial for determining the fate of B-cell lineages. Therefore, the reduced number of IL-7Rα expressers prompted us to determine whether progenitors can differentiate into pre-B cells in the absence of STAT3. BM cells were seeded in methylcellulose supplemented with different doses of IL-7, and pre-B colonies were scored. As shown in Figure 4C, the number of CFU–pre-B in the control BM cells increased with the enhanced doses of IL-7, whereas reduced numbers of CFU–pre-B were derived from mutant BM. These results indicated that IL-7–responsive progenitors existed in reduced numbers in the absence of STAT3.

Enhanced apoptosis of pro-B cells in the absence of STAT3

One of the prominent functions of STAT3 is to serve as a survival factor,32 and targeted deletion of STAT3 results in enhanced apoptosis in sensory neurons, motoneurons, and thymic epithelium.33-35 Therefore, we determined whether the reduced B lineages in STAT3KO mice were attributed to enhanced apoptosis. BM cells prepared from control or STAT3KO mice were incubated in vitro for 0 hour or 3 hours in the absence of cytokines, followed by FACS analysis for the apoptotic rate of different B-cell lineages. As shown in Figure 5, though a comparable percentage of annexin V–positive B220+ (non-B) or B220+CD43− (pre-B, immature B, and mature B) was found between control and STAT3KO mice, significantly enhanced apoptosis of B220+CD43+ (pro-B) cells at both times was observed in the absence of STAT3, suggesting a critical role of STAT3 for maintaining survival of pro-B cells.

Cell-autonomous requirement of STAT3 for normal B-cell development

To investigate whether the impaired development of B cells resulted from a cell-autonomous mechanism rather than an indirect effect of STAT3 loss elsewhere in the animal, we performed BM transplantation. Five million BM cells from control or STAT3KO mice were transplanted into lethally irradiated RAG1KO mice. Two months later, repopulated splenocytes or BM cells derived from the RAG1KO mice that had received transplants were subjected to immunoblot analysis for the expression of STAT3 protein and to FACS analysis for different lineages of B cells. As shown (Figure 6A, E), the repopulated BM or splenocytes derived from STAT3KO mice were completely devoid of STAT3. It was also clear that STAT3KO→RAG1KO chimeric mice displayed significantly reduced percentages and cellularities of pro-B, pre-B, immature B, and mature B in the BM as opposed to control→RAG1KO chimeric mice (Figure 6B-C). Interestingly, though an enhanced percentage of T1 transitional B cells (IgDlow-IgMhigh) were observed in the spleens of STAT3KO→RAG1KO chimeric mice, the percentage of T2 transitional B/mature B (IgDhighIgMintermediate-high) was only slightly reduced compared with control→RAG1KO mice (Figure 6D, F). However, the cellularity of T1 and T2/mature B cells was drastically reduced in the STAT3KO→RAG1KO chimeric mice compared with control→RAG1KO mice (Figure 6G-H), which might have reflected a poor reconstitution rate of splenocytes in the absence of STAT3. Taken together, these results further demonstrated that the impaired B lymphopoiesis in BM and spleen was the result of a cell-autonomous requirement of STAT3 in differentiating B-cell progenitors.

was measured by immunoblotting. No significant differences in STAT1 or STAT5 protein levels or phosphorylation were observed between control and mutant cells (data not shown), indicating that receptor-proximal signaling was intact. We also asked whether reduced expression of IL-7Rα or reduced numbers of IL-7 responding cells in STAT3KO mice could account for impaired IL-7 responses. BM from control or STAT3KO mice was analyzed by FACS using antibodies to IL-7Rα and B220. As shown in Figure 4A-B, IL-7Rα–expressing cells were mostly B220internaive, indicative of pro-B and pre-B populations. Interestingly, although IL-7Rα expression (MFI) in STAT3KO BM was comparable to that in control cells, the percentage of IL-7Rα–positive cells was greatly reduced. These results suggested that reduced proliferation of STAT3KO BM cells in response to IL-7 was likely caused by reduced numbers of responders instead of reduced expression or responsiveness of IL-7R.
Because the deletion of STAT3 resulted in an increased pre–pro-B population and an impaired development of pro-B, pre-B, immature, and mature B cells, we reasoned that STAT3 might be differentially expressed in different lineages of B cells in the BM. To determine the protein levels of STAT3 in different populations, intracellular staining for STAT3 in conjunction with surface staining for different B lineages of BM cells from control mice was performed. As shown in Figure 7B (upper panels), pro-B cells expressed almost twice as much STAT3 as did pre-B, immature B, or mature B cells. We next determined whether tyrosine phosphorylation of STAT3 was also enhanced in the pro-B population. Indeed, the level of phosphotyrosyl STAT3 was also increased in pro-B cells relative to other lineages of B cells (Figure 7B, lower panels), suggesting that activation of STAT3 is critical for B-cell development at an early stage.

Discussion

We have previously shown that mice lacking STAT3 in their hematopoietic progenitors developed neutrophilia and that BM cells were hyperresponsive to G-CSF stimulation, suggesting that STAT3 is a negative regulator for G-CSF–mediated granulopoiesis.26 Here, we have demonstrated that STAT3 serves as a positive regulator for B lymphopoiesis. In the absence of STAT3, B but not T cells were significantly reduced in the periphery. The decreased number of peripheral B cells resulted from the impaired development of B-cell lineages in the BM, including reduced numbers of pro-B, pre-B, immature, and mature B cells (Figures 1, 2). Interestingly, an increased percentage of pre–pro-B cells or Hardy

Figure 4. Decreased numbers of IL-7 responders in the BM of STAT3KO mice. (A) BM cells of control (left panels) or STAT3KO (right panels) mice were stained with anti-B220 and anti–IL-7Rα followed by FACS analysis. Light scatter patterns are shown in the top panels, and percentages of IL-7Rα+/B220−/H9251 and IL-7Rα+/B220−/H9252 are shown in the bottom panels. (B) Percentage (left panel) and cellularity (right panel) of B220−IL-7Rα+ cells in control (●) and STAT3KO (○) mice were measured. Cellularity was calculated by multiplying the percentage with the total number of BM cells, n = 3; *P < .05; Student t test. (C) Reduced CFU–pre-B progenitor cells in the BM of STAT3KO mice. Colony-formation assay was performed by seeding 2 × 10⁶ BM cells of control (●) or STAT3KO (○) mice in the complete medium without or with 10 or 100 ng/mL IL-7 for 7 days, followed by counting of the number of the pre-B colonies under a light microscope. Colony-formation assay is presented as mean ± SE of replicate samples.

Figure 5. Enhanced apoptosis of pro-B cells in the BM of STAT3KO mice. BM cells of control (●) or STAT3KO (○) mice were incubated in the culture medium for 0 hour or 3 hours and were stained with anti-B220, anti-CD43, and annexin V, then FACS analysis was performed. Percentages of apoptotic pro-B (CD43+/B220− annexin V−), pre-B/IMB/MB (CD43−/B220−/annexin V−), and non-B (B220−/annexin V−) were plotted (control mice, n = 8; STAT3KO mice, n = 5). Apoptosis results are presented as mean ± SE of replicate samples.

Figure 6. Impairment of B-cell development in the absence of STAT3 is cell autonomous. BM of control (●) or STAT3KO (○) mice was intravenously transplanted into lethally irradiated RAG1KO mice for 2 months. Total cell extracts prepared from BM (A) or spleen (E) were subjected to immunoblot using antibodies to STAT3 or β-actin. BM cells of chimeric mice were stained with anti-CD43, anti-IgM, and anti-B220 antibodies, followed by FACS analysis. Percentages of T1 and T2+MB are shown. Percentage (F) and cellularity (G−H) are shown. IgMhigh IgDlow represent T1 cells. IgMintermediate-high IgDhigh represent T2 or mature B (MB) cells. Results are presented as mean ± SE of replicate samples.
constitutively active in B-1, but not B-2, cells. We also noticed that STAT3 is required for Flt3-dependent development of den-

in the absence of STAT3 (Figure 2C). In addition, it has been shown that STAT3 is partial developmental arrest at the pre–pro-B stage in the absence of Flt3. It is known that Flt3 expression starts in multipotent progenitors and that Flt3 downstream molecules Bcl-2 and Mcl-1 are critical for cell survival. Therefore, one possible mechanism for the increased apoptosis is reduced expression of these antiapoptotic proteins in the absence of STAT3. It remains unclear why the pro-B population is particularly susceptible to apoptosis when STAT3 is deleted. STAT5 appears to be absolutely critical for FL-7 signaling in T and B lymphocytes, but the loss of STAT3 only impaired B lymphopoiesis. The critical role of STAT3 in early B-cell development without a corresponding requirement in T-cell development remains an enigma.

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