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Letters to the Editor

BCL-2 expression is mainly regulated by JAK/STAT3 pathway in human CD34$^+$ hematopoietic cells

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Dear Editor,

The maintenance and self-renewal of hematopoietic stem cells (HSC) in culture is of great importance, both for clinical transplantation therapy and basic research. While interleukin-6 (IL-6) and its signaling receptor gp130 play an important role in HSC self-renewal, as suggested by the finding of reduced numbers of primitive hematopoietic cells in IL-6$^{-/-}$[1] and in gp130 null mice,[2] the role of IL-3 is controversial, as it has been reported to negatively affect[3] or not affect[4] *in vitro* HSC self-renewal.

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway is widely used by many cytokines involved in hematopoiesis.[5] To date, IL-6 leads to STAT3 phosphorylation by JAK kinases, whereas, IL-3 binding to IL-3 receptor leads to JAK/STAT5 activation. However, whereas STAT3 is essential in early embryonic development and for embryonic stem cell self-renewal,[6] STAT5 seems to be dispensable as STAT5ab−/− mice are viable and can contribute to hematopoiesis after transplantation.[7]

HSC survival and self-renewal are likely regulated by secreted factors within the bone marrow niche, but little is known about the intracellular signal transduction pathways necessary for proliferation and self-renewal divisions of HSC. Recently, we have reported that IL-6 was able to increase long-term culture-initiating cell numbers and their derived myeloid progenitors and also to prevent the loss of telomerase activity induced by IL-3 in short-term serum-free human CD34$^+$ cell cultures.[8] Additionally, it has been demonstrated that gp130 activation seems to be not required for either survival or initial cell division of murine HSC, but amplifies stem cell yields.[9] Although it might be expected that highly proliferative HSC and hematopoietic progenitor cells would exhibit an antiapoptotic fate, there is little published work on apoptosis-related genes induction in primitive hematopoietic cells by IL-6 and IL-3.

We hypothesized that the beneficial effects of IL-6 on human CD34$^+$ cells in culture could be attributed to a different expression of apoptosis-related genes. Isolated CD34$^+$ cells from human cord blood were cultured in serum-free medium, and the effect of IL-6 and IL-3 on bcl-2, bax and bcl-xL genes in expanded cells expressing CD34 antigen was investigated. We demonstrate that CD34$^+$ cells expanded with IL-6 are more antiapoptotic than those generated with IL-3.

We first assessed gene expression following 7-day culture and found that both IL-6 and IL-3 enhanced bcl-2 expression induced by stem cell factor, thrombopoietin and flt3-ligand, named in the text STF, the minimum cytokine combination needed to recruit CD34$^+$ cells into cell cycle and to maintain their viability.[8,10] Moreover, bax was downregulated and bcl-xL expression was not affected (Figure 1a). In spite of the greater bclx/bax and bcl-2/bax values exhibited by IL-6-derived CD34$^+$ cells (Figure 1a), no differences in cell survival were observed between IL-3- and IL-6-treated cultures. In fact, cell viability ranged between 96 and 98% and apoptotic cells between 2 and 3%, as assessed by 7AAD/Annexin V staining (figure not shown).

Then, we analyzed CD133 expression on CD34$^+$ expanded cells, as CD34$^+$CD133$^+$ cell subset is enriched in primitive and high proliferative hematopoietic cells.[11] As shown in Figure 1b, bcl-2/bax values were found to decrease linearly as a function of the proportion of CD34$^+$ cells expressing CD133, approaching values obtained for freshly isolated CD34$^+$ cells. Importantly, in the linear range, CD34$^+$CD133$^+$ cells derived from IL-6-containing cultures were more antiapoptotic than those generated with IL-3.

To better understand the early effects of IL-6 and IL-3 on gene expression, freshly isolated CD34$^+$ cells were induced to cell cycling with STF, and then cytokine-stimulated. As early as in 2 h, IL-6 was able to induce a dramatic increase in bcl-2 expression, unlike the moderate two-fold increase induced by IL-3. However, both IL induced a similar decrease in bax expression (Figure 1c).

To evaluate whether this bcl-2 increase resulted in a greater resistance to apoptosis, STF-treated cells were grown with IL-3 or IL-6 for 24 h and then cytokine deprived. Twenty-four hours after growth factors withdrawal, apoptotic and dead cells showed 3.3 ± 0.6- and 3.2 ± 0.6-fold increase, respectively, in IL-3-derived cultures, but only 1.6 ± 0.1- and 1.6 ± 0.3-fold increase, respectively, in IL-6-treated cultures. Therefore, IL-6 gives human CD34$^+$ cells a more antiapoptotic fate than IL-3.

Then, we extended the study to explore at the molecular level these effects. As JAK-regulated STAT tyrosine phosphorylation is a prerequisite for STAT dimerization, nuclear translocation and gene transcription,[12] we first examined the effect of IL-6 and IL-3 on STATs activation, and then, how the JAK inhibitors tyrphostin AG490 and curcumin inhibited IL-mediated STAT activation. With only 15 min exposure to IL-6 or IL-3, STAT3 and STAT5 became phosphorylated, respectively (Figure 1d). Both JAK inhibitors inhibited IL-6-mediated STAT3 phosphorylation, but curcumin was more potent, showing nearly complete inhibitory effect at 50 μM (Figure 1d). However, at the doses tested, both compounds were less potent in inhibiting IL-3-induced STAT5 phosphorylation (Figure 1d). To assess whether inhibition of STATs

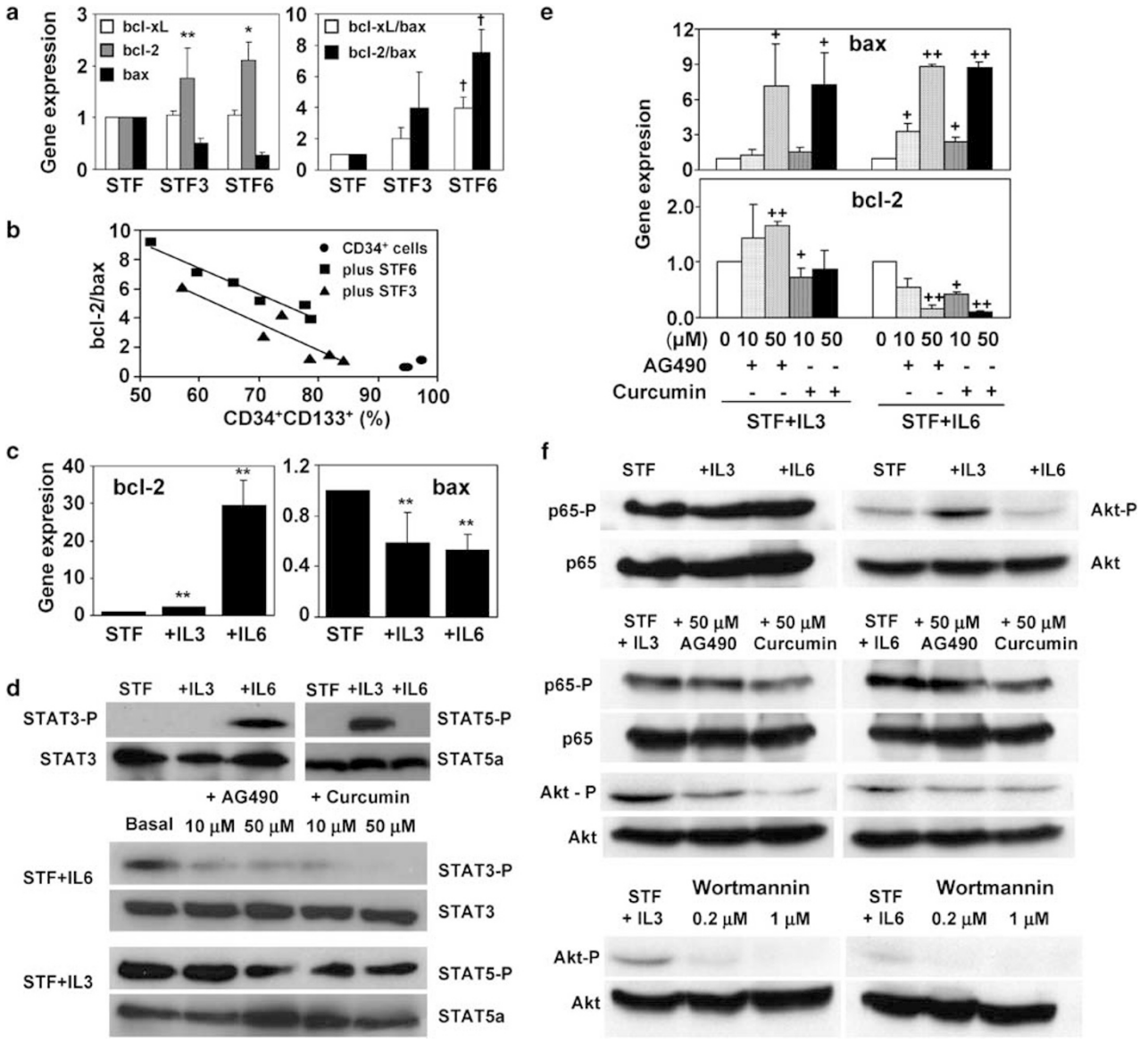

Figure 1 IL-6 and IL-3 induce changes in bcl-2 and bax transcription in human CD34$^+$ cells via the JAK/STAT pathway. Freshly isolated CD34$^+$ cells from human cord blood were cultured in serum-free medium[8] containing STF: 50 ng/ml stem cell factor, 50 ng/ml flt3-ligand and 10 ng/ml thrombopoietin; STF6: STF plus 20 ng/ml IL-6; or STF3: STF plus 20 ng/ml IL-3 (all cytokines supplied by R&D Systems Inc., Minneapolis, MN, USA). After 7-day culture, CD34$^+$ cells were isolated by MACS system (Milteny Biotec, Bergisch Gladbach, Germany), analyzed by flow cytometry, using anti-CD34-APC (Becton-Dickinson, Heidelberg, Germany) and used for RT-PCR analysis. cDNA from CD34$^+$ cells was obtained using the cells-to cDNA kit (Ambion, Austin, TX, USA) and quantitative end-point PCR performed using the Amplifluor Gene Systems for amplification and detection of bcl-2, bcl-xL and bax-alpha (Intergen, Serologicals Corp., Norcross, GA, USA). Copy numbers were calculated with relative standard curves made with known DNA amounts of the selected genes supplied with the kits and gene expression normalized by glyceraldehyde-6-phosphate dehydrogenase cDNA levels. Values of copy numbers of bcl-2, bcl-xL and bax as well as their ratios were normalized against values obtained for STF-treated cells (**a**) and a very good correlation was found between normalized bcl-2/bax values and the percentage of CD34$^+$ cells expressing CD133 antigen (anti-CD133/2-PE from Milteny Biotec) ($r^2 = 0.95$ for both lines, $P < 0.001$ between STF6 and STF3) (**b**). In short-time experiments, freshly isolated CD34$^+$ cells were cultured during 3 days with STF, then stimulated for 2 h with IL-6 or IL-3 and bcl-2 and bax expression determined (**c**) or were stimulated for 15 min and phosphorylated and non-phosphorylated STAT3 and STAT5 analyzed by Western blotting with STAT-specific antibodies (phospho-STAT3 pTyr 705 from Cell Signaling Technology, Beverly, MA, USA; STAT5a, phospho-STAT5 pTyr 694 and STAT3 from Chemicon, Serologicals Corp.) (**d**). Treatment of CD34$^+$ cells with the JAK inhibitors AG490 (Calbiochem, San Diego, CA, USA) or curcumin (LKT Laboratories, St. Paul, MN, USA) for 2 h or 30 min, respectively, before IL stimulation inhibited completely or partially IL-mediated STATs phosphorylation, without affecting their corresponding non-phosphorylated STATs (**d**) and reversed the effect on IL-mediated gene transcription (**e**). Additionally, the effect of IL-3 and IL-6 on PI3-K/Akt and NF-κB pathways was studied. IL-3 enhanced Akt phosphorylation observed in STF-treated cells, and no changes in NF-κB p65 were detected (**f**). When CD34$^+$ cells were preincubated with the JAK inhibitors, both of them slightly inhibited IL-3-induced Akt phosphorylation, but wortmannin (Sigma, St. Louis, MO, USA) inhibited completely Akt phosphorylation induced by both IL (**f**). Antibodies against Akt1/2 (H-136) and NF-κB p65 (C-20) were from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA and against phosphorylated Akt (Ser473) and NF-κB p65 (Ser536) from Cell Signaling. Data are the mean $\pm$ S.E.M. of five (**a**) or three (**c** and **e**) independent experiments performed in triplicate. STF3 or STF6 *versus* STF $^*P < 0.05$, $^{**}P < 0.01$; STF6 *versus* STF3 $\dagger P < 0.05$; AG490- or curcumin-treated cells *versus* non-treated cells $^+P < 0.05$, $^{++}P < 0.01$

phosphorylation affected cell survival, we examined the effect of these inhibitors on IL-6- and IL-3-induced cell proliferation. At 25 μM, both compounds completely inhibited cell proliferation induced by IL-6. However, 50 μM curcumin was needed to inhibit fully the proliferative effects of IL-3, whereas, at this concentration, AG490 inhibited cell growth by only 45%.

Next, we determined whether the inhibition of STATs activation reversed the effect induced by IL on gene expression. We found that both JAK inhibitors reversed the effect mediated by IL-6 or IL-3 on bax transcription and that of IL-6 on bcl-2 expression (Figure 1e). However, only the lowest concentration of curcumin was able to slightly decrease the effect induced by IL-3, and the opposite effect was observed with AG490 (Figure 1e).

Considering that IL-6 can activate the PI3-K/Akt pathway,[13] also activated by IL-3,[14] and that curcumin inhibits NF-κB activation,[15] we analyzed by Western blot the effects of these JAK inhibitors on both survival pathways. As shown in Figure 1f, IL-6 did not modify Akt phosphorylation induced by STF, but it was increased 3.1 ± 1.0-fold ($P = 0.0002$) by IL-3, as assessed by densitometric analysis. However, NF-κB p65 activation observed in STF-treated CD34$^+$ cells was not affected by both IL. Moreover, both inhibitors were able to slightly inhibit (15–20%) Akt phosphorylation induced by IL-3 (Figure 1f), and no changes in p65 phosphorylation were observed. Nevertheless, to discard the efffect of PI3-K/Akt pathway on CD34$^+$ cell survival, CD34$^+$ cells were treated with wortmannin, a well-known inhibitor of PI3-K pathway. After 48 h culture, CD34$^+$ cell proliferation was 2.5 ± 0.2- and 1.8 ± 0.2-fold increased by IL-3 and IL-6, respectively. As expected, wortmannin inhibited IL-induced Akt phosphorylation (Figure 1f), but this effect did not correlate with a decrease in cell proliferation or cell survival. In fact, at doses ranging between 0.1 and 1 μM, CD34$^+$ cell proliferation was only decreased by 10–15% in both cultures without affecting cell survival, which was always higher than 94%.

These results suggest that in human CD34$^+$ hematopoietic cells, bax expression must be highly regulated by its upstream activator, JAK/STAT. However, other signaling pathways, including Ras/MAPK pathways,[14] must regulate IL-3-induced bcl-2 expression. Even so, we cannot discard that a crosstalk between signaling pathways exists, explaining the observed effect or that concentrations of inhibitors used are not enough to reverse the IL-3-mediated effect on bcl-2 expression. Nevertheless, IL-3 was able to induce only an early moderate bcl-2 expression, and a lesser bcl-2/bax ratio after 7-day culture, when compared with that induced by IL-6. These findings led us to suggest that STAT5 activation must be important for proliferative responses to IL-3, but not responsible for self-renewal of human CD34$^+$ cells.

These data contrast the observations where activation of STAT5 but not STAT3 in mouse CD34$^-$Sca-1$^+$c-kit$^+$lin$^-$ cells promoted *in vitro* multilineage differentiation. Interestingly, long-term repopulating capacity of CD34$^-$Sca-1$^+$c-kit$^+$lin$^-$ cells transduced with STAT3 was not seriously affected. Moreover, selective activation of STAT5 in CD34$^-$Sca-1$^+$c-kit$^+$lin$^-$ cells, but not in CD34$^+$Sca-1$^+$c-kit$^+$lin$^-$ cells, induced fatal myeloproliferative disease,[16] indicating that STAT5 is important in HSC, but not in committed progenitors, self-renewal capacity. However, previous experiments have shown that STAT5 activation is not essential for HSC self-renewal, although cytokine stimulation was highly reduced in the STAT5-deficient Sca-1$^+$c-kit$^+$lin$^-$ cells.[17] Perhaps, the different effects of STAT5 activation may reflect differences between human and murine hematopoietic cells, in culture conditions and in target population. In this respect, it is interesting to mention that constitutive activation of STAT5a in human CD34$^+$ cells resulted in enhanced HSC self-renewal and erythropoiesis, but required the co-culture on stroma.[18]

In this study, we further demonstrated that STAT3 activation provided an antiapoptotic advantage in human CD34$^+$ cells, essentially owing to the overexpression of bcl-2. It is important to note that, loss-of-function analyses involving STAT3 demonstrated that murine HSC transduced with the dn STAT3 were able to form colonies *in vivo* and *in vitro*, but exhibited a very reduced long-term lymphoid–myeloid repopulating ability.[19] Interestingly, STAT3 transcript levels were higher in the more primitive human hematopoietic CD34$^+$CD38$^-$ cells than in their counterpart CD34$^+$CD38$^+$ cells.[19] Thus, STAT3 must be dispensable for hematopoietic cell differentiation, but not for self-renewal activity, and must provide a survival advantage for hematopoietic CD34$^+$ cells.

In the present study, we have shown that activation of STAT3 by IL-6 provides an antiapoptotic advantage in human cord blood CD34$^+$ cells, essentially owing to the overexpression of bcl-2. These results can explain in part the beneficial effects of IL-6 on maintenance and self-renewal of primitive hematopoietic cells, and are useful to optimize *ex vivo* expansion cultures, when long-term engraftment in patients is required.

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