Ligand-induced STAT3 signaling increases at relapse and is associated with outcome in pediatric acute myeloid leukemia: a report from the Children's Oncology Group

Signal Transducer and Activator of Transcription 3 (STAT3) responses to ligands correlate with outcome in pediatric acute myeloid leukemia (AML),[1] suggesting a possible relationship between chemotherapy response and the STAT3 pathway. We hypothesized that consistently altered changes in STAT3 signaling between diagnosis and relapse represent adaptations that promote chemotherapy resistance in relapsed pediatric AML. We examined diagnosis-relapse pairs from pediatric AML patients and found increased ligand-induced STAT3 responses at relapse compared with diagnosis in most patients. Importantly, increased interleukin-6-induced STAT3 was associated with especially poor prognosis.

Aberrant activation of STAT proteins is well described in myeloid malignancies.[1-4] STATs are critical signaling intermediates in hematopoietic cells and are activated by growth factors and cytokines in the bone marrow (BM), including granulocyte-colony stimulating factor (G-CSF) and interleukin-6 (IL-6). G-CSF stimulation results in phosphorylation of STAT3 and STAT5. Phosphorylation of STAT3 can occur on tyrosine 705 (pY-STAT3) and serine 727 (pS-STAT3), and for STAT5 on tyrosine 694 (pY-STAT5). In contrast, IL-6 signals primarily through pY-STAT3. Phosphorylated STATs (pSTATs) dimerize, translocate to the nucleus, bind DNA promoter sequences and regulate transcription. In general, pY-STAT3 and pY-STAT5 induce pro-survival gene expression changes.[5] Importantly, pY-STAT5 can have a regulatory effect on cell survival, dampening the pro-survival effects of other pSTATs.[6]

To test our hypothesis, we studied 24 pairs of samples, from diagnosis and relapse. Cryopreserved BM cells were obtained from the Children's Oncology Group (COG) AML Reference Lab. All patients were treated on the AAML0531 trial and relapsed after chemotherapy without stem cell transplantation (SCT). As the

Table 1. Patient data by Unique Patient Number.

UPN	Age/Sex	Cytogenetics	Ligand response at diagnosis	Ligand response at relapse	Change in ligand induced ΔMFI at relapse *vs.* diagnosis	Clinical Outcome
1	1.1/F		RR	RR	I	DD
2	1.5/M		RR	RR	N	A
3	8.96/M	t(8;21)	SS	SS	IG	A
4	14.4/M		SR	SR	I	DD
5	10.4/F	t(8;21)	RR	SR	G	A
6	15.9/F		RR	SS	IG	DD
7	1.8/M		RR	RR	I	DD
8	1.8/F		SR	SR	N	A
9	12.1/M	t(8;21)	RR	RR	G	A
10	10.6/M		RR	RR	G	A
11	4.6/F	t(8,21)	SR	SR	IG	TD
12	2.1/F	+8	RR	SR	G	A
13	1.7/F	11q23 NOS	RR	SS	IG	A
14	16.5/M	t(8;21)	RR	SR	N	A
15	0.8/M	11q23 NOS	RR	RR	N	A
16	18.1/F	t(9:11)	RR	RR	N	TD
17	2.4/F		RR	SR	IG	DD
18	18.2/M		RR	RR	N	TD
19	3.4/M	t(9:11)	RR	RR	I	DD
20	0.6/F	inv(16)	RR	RR	N	A
21	9.0/F	11q23 NOS	RR	RR	G	A
22	16.6/F	11q23 NOS	RR	SR	N	DD
23	1/M	inv(16)	RR	SS	IG	TD
24	16.9/F		RR	RR	N	DD

UPN: Unique Patient Number. Age: given in years at the time of initial diagnosis. NOS: fusion partner not reported. Ligand response at diagnosis and relapse columns define response as was defined in our prior analysis of diagnostic samples for ease of comparison.[1] Those patients whose MFI was ≥2 in response to 5 ng/mL IL-6 or 10 ng/mL G-CSF were considered to be sensitive to the respective ligand, while those who did not were considered resistant. RR: resistant to G-CSF and IL-6; SR: sensitive to only G-CSF; RS: sensitive to only IL-6; SS: sensitive to both G-CSF and IL-6. Change in ligand-induced MFI at relapse versus diagnosis column provides data on how individual patient's MFI to G-CSF and IL-6 changed between diagnosis and relapse. As defined by cut-point analyses, an increase in G-CSF-induced pY-STAT3 was defined as an increase in MFI ≥ 1 in response to the 100 ng/mL dose of G-CSF, while an increase in IL-6-induced pY-STAT3 was defined as an increase in MFI ≥ 0.3 in response to the 5 ng/mL dose of IL-6. IG: change in MFI ≥ 1 with G-CSF and ≥ 0.3 with IL-6; N: increased ligand induced pY-STAT3 in response to neither ligand (change in MFI < 1 with G-CSF and < 0.3 with IL-6); I: increased response to IL-6 only (change in MFI < 1 with G-CSF and ≥ 0.3 with IL-6; G: increased response to G-CSF only (change in ΔMFI ≥ 1 with G-CSF and < 0.3 with IL-6). Clinical outcome column indicates status at last contact. DD: death due to disease; A: alive at time of last follow up; TD: toxic death.

AAML0531 study advised SCT for patients with high-risk cytogenetics,[7] our study population is enriched for patients with low-risk cytogenetics (Table 1 and *Online Supplementary Table S1*). Detailed methods and statistical analyses are provided in the *Online Supplementary Appendix*.

The levels of total STAT3, constitutive pY-STAT3/pS-STAT3, and constitutive pY-STAT5 were not significantly changed between diagnosis and relapse (*data not shown*; see *Online Supplementary Figure S1* for gating). There was a slight increase in pY418-SRC at relapse, largely due to one outlying sample (1.27±0.3% mean±SEM, n=24 to 2.58±0.8, *P*=0.044) (*Online Supplementary Figure S2*). For receptors, both the G-CSFR and gp130 were significantly increased at relapse compared to diagnosis (G-CSF receptor: 74.4±3.6% to 83.1±3.3%, *P*=0.019; gp130: 52.2±6.6% to 61.6±6.1%, *P*=0.022) (*Online Supplementary Figure S3*).

Phospho-STAT responses to two ligand doses were tested: for G-CSF, 10 and 100 ng/mL; for IL-6 + soluble IL-6 receptor (sIL-6Rα), 5 ng/mL + 10ng/mL, and 50 ng/mL + 100 ng/mL. Ligand-induced responses were

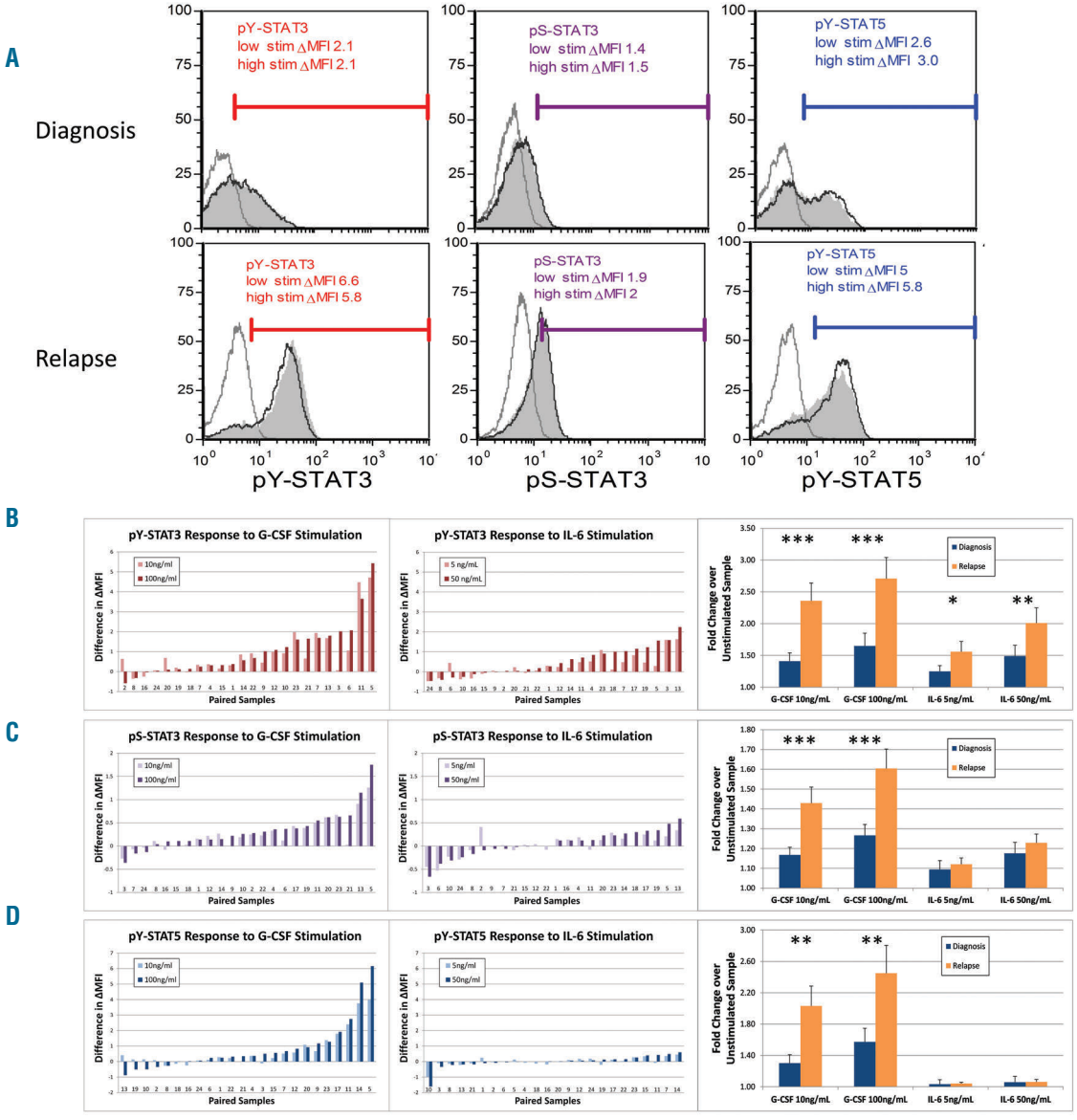

Figure 1. Difference in ΔMFI of pSTATs between diagnosis and relapse in response to ligand stimulation. (**A**) Representative sample with increased response to G-CSF. The top row depicts response to G-CSF stimulation at initial diagnosis, and the bottom row shows the response at relapse. Light gray unshaded curves depict the p-STAT level of the unstimulated sample; solid gray and black line curves depict the responses to 10 ng/mL and 100 ng/mL doses of G-CSF, respectively. Horizontal bars indicate gates that were created to include <1% of events in the isotype control. The difference in pY-STAT3 ΔMFI in response to 10 ng/mL of G-CSF for this sample was 6.6-2.1=4.5. Data from UPN 11. (**B-D**) Waterfall plots demonstrate the differences in ΔMFI for pY-STAT3 (**B**), pS-STAT3 (**C**) and pY-STAT5 (**D**). Samples are ordered according to the response to the higher dose of G-CSF (left) or IL-6 (center) and each is identified by a UPN. Bar graphs (right) show the mean ± SEM for each stimulation, at diagnosis *versus* relapse (n=24). **P*>0.05, ***P*<0.01, ****P*<0.001: ΔMFI= MFI of stimulated sample/MFI of unstimulated sample. Difference in ΔMFI= ΔMFI at relapse - ΔMFI at diagnosis.

expressed as the fold change in mean fluorescence intensity (MFI) between stimulated and unstimulated conditions (ΔMFI=stimulated MFI /unstimulated MFI). Using ΔMFI≥2 as the definition of a response,[1] 4 of 24 (17%) diagnostic samples responded to 10 ng/mL G-CSF with pY-STAT3, and 2 of 24 (8%) responded with pY-STAT5. One of 24 (UPN 3; 4%) responded to 5 ng/mL IL-6 with pY-STAT3. At relapse, 12 of 24 (50%) samples responded to G-CSF with pY-STAT3, and 8 of 24 (33%) responded with pY-STAT5. Four of 24 relapse samples (17%) responded to IL-6 with pY-STAT3 (Table 1).

Though many samples had ΔMFI<2, and thus did not meet our previous definition of a response,[1] the majority of pairs demonstrated increased ligand-induced pSTAT activity at relapse compared to diagnosis (Figure 1). These consistent increases were significant for both doses of each ligand. At the lower dose, the G-CSF-induced pY-STAT3 ΔMFI increased from 1.41±0.13 to 2.36±0.28 (P<0.001). At the higher dose, ΔMFI increased from 1.65±0.20 to 2.71±0.33 (P<0.001). For IL-6, at the lower dose, the pY-STAT3 ΔMFI increased from 1.25±0.09 to 1.56±0.16 (P=0.017), and at the higher dose, ΔMFI increased from 1.49±0.17 to 2.01±0.24 (P=0.005). Similarly, significant increases in pS-STAT3 ΔMFI between diagnosis and relapse occurred in response to both doses of G-CSF. At the lower dose, ΔMFI increased from 1.17±0.04 to 1.43±0.08 (P=0.001). At the higher dose, ΔMFI increased from 1.27±0.06 to 1.60±0.10 (P<0.001). Significant increases in ΔMFI of pY-STAT5 between diagnosis and relapse were seen in response to both doses of G-CSF. At the lower dose, ΔMFI increased from 1.30±0.11 to 2.03±0.25 (P=0.001). At the higher dose, ΔMFI increased from 1.57±0.17 to 2.45±0.35 (P=0.007).

Spearman correlations between signaling parameters revealed insights into signaling biology. We found a positive relationship between change in G-CSF receptor

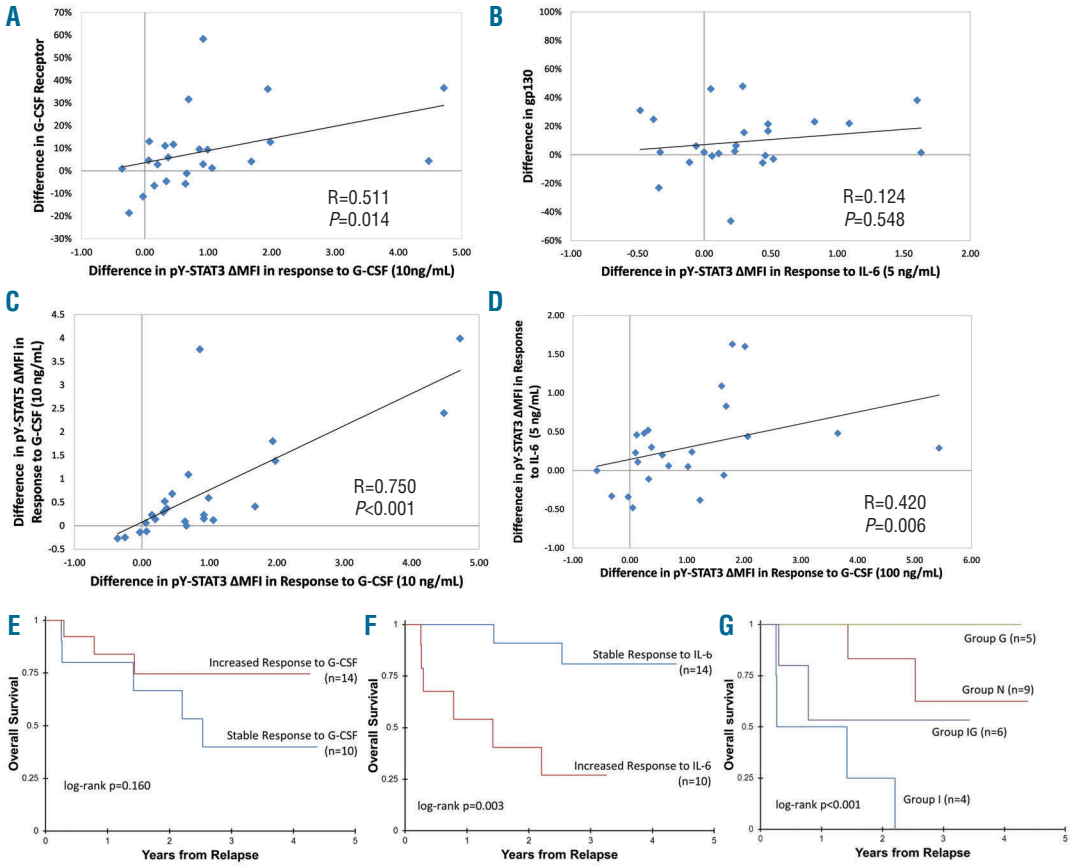

Figure 2. Bivariate correlations between STAT pathway parameters and overall survival (OS) stratified by change in pY-STAT3 response to G-CSF or IL-6 between diagnosis and relapse. (A) There was a positive relationship between change in G-CSF receptor expression and G-CSF-induced pY-STAT3 at the 10 ng/mL dose. (B) There was no significant relationship between IL-6 receptor expression and IL-6-induced pY-STAT3 at the 5 ng/mL dose. (C) There was a positive relationship between pY-STAT5 and pY-STAT3 responses to G-CSF at the 10 ng/mL dose, and (D) a positive relationship between IL-6- (5 ng/mL dose) and G-CSF- (100 ng/mL dose) induced pY-STAT3. R = Spearman correlation coefficient. (E) Cut-point analysis for the difference in pY-STAT3 ΔMFI in response to G-CSF revealed that at the 10ng/mL dose, a difference in pY-STAT3 ΔMFI ≥0.45 divided patients into roughly equal groups with a trend towards improved survival in those patients whose blasts became more responsive to G-CSF. (F) Cut-point analysis for the difference in pY-STAT3 ΔMFI in response to IL-6 revealed that at the 5 ng/mL dose, a difference in pY-STAT3 ΔMFI ≥0.3 divided patients into two roughly equal groups with a significantly inferior survival for those patients whose blasts became more responsive to IL-6. (G) Patients were divided into groups based on the response to G-CSF (100 ng/mL with a difference in ΔMFI Cut-point of ≥1) and IL-6 (5 ng/mL with a difference in ΔMFI cut point of ≥0.3). Group I included patients whose blasts demonstrated increased pY-STAT3 in response to IL-6 but stable response to G-CSF. These patients had the worst outcome. Group N had stable response to G-CSF and IL-6 at relapse and had intermediate survival. Group IG had increased response to both IL-6 and G-CSF at relapse and had intermediate survival. Group G included patients that had increased response to G-CSF at relapse but stable response to IL-6 at relapse and had 100% overall survival at the time of last follow up. *P*-values determined by log rank test. Toxic deaths (n=4) were censored.

expression and change in G-CSF-induced pY-STAT3 [*P*=0.014 for 10 ng/mL, (Figure 2A) R=0.483, *P*=0.020 for 100 ng/mL (*data not shown*)]. This suggests that the increased responsiveness to G-CSF at relapse may be partly attributable to increased receptor expression. Bivariate analysis revealed no significant relationship between the change in gp130 expression and change in IL-6-induced pY-STAT3 [*P*=0.548 for 5 ng/mL (Figure 2B); R=0.181, *P*=0.379 for 50 ng/mL (*data not shown*)]. There were no significant correlations between changes in total STAT3 and changes in constitutive or induced pSTATs (*data not shown*). These results are consistent with our previous findings with diagnostic samples,[1] and further support the idea that the pY-STAT3 response to G-CSF is partly regulated by receptor expression, while the pY-STAT3 response to IL-6 is independent of surface gp130 levels, and instead may be regulated by downstream factors.[8]

We found a positive relationship between changes in pY-STAT3 and pY-STAT5 responses to G-CSF at both doses [10 ng/mL (Figure 2C); 100 ng/mL: R = 0.437, *P*=0.0358 (*data not shown*)]. We also found a positive relationship between changes in G-CSF- and IL-6-induced pY-STAT3 (Figure 2D), suggesting that increased activity in the STAT3 pathway can be driven by G-CSF or IL-6, and conversely, that cells that fail to respond to one ligand often fail to respond to the other. These relationships are consistent with our previous study.[1]

To determine if changes in ligand responses between diagnosis and relapse were associated with outcome, we performed cut-point analyses. At the lower G-CSF dose, a difference in pY-STAT3 ΔMFI ≥0.45 divided patients into two roughly equal groups with a trend towards improved overall survival (OS) in patients with increased responses to G-CSF at relapse (Figure 2E). Conversely, at the lower IL-6 dose, a difference in ΔMFI ≥0.3 identified patients with significantly worse survival, compared to those with a difference in ΔMFI less than 0.3. Patients with increased responses to IL-6 at relapse compared to diagnosis had a 3-year OS from relapse of 27±18%, compared to 81±8% in the group with stable IL-6-induced pY-STAT3 (*P*=0.003) (Figure 2F). Therefore, increased pY-STAT3 response to IL-6 at relapse was a negative prognostic marker in this cohort.

Next, we divided patients into four groups based on their combined G-CSF and IL-6-induced pY-STAT3 profiles. We based group assignment on the changes in pY-STAT3 in response to the doses of ligand that demonstrated strongly correlated pY-STAT3 responses (100 ng/mL for G-CSF and 5 ng/mL for IL-6) (Figure 2D). Group N demonstrated stable responses to both ligands (n=9, 37%); Group IG had increased responses to both ligands (n=6, 25%); Group G had increased responses to G-CSF only (n=5, 21%); and Group I demonstrated increased responses to IL-6 only (n=4, 17%). While the number of patients in each group is low, this analysis suggested two notable subgroups (Figure 2G). Group G patients all responded to chemotherapy sufficiently to undergo SCT, and all were alive at last contact (median follow up 4.2 years). This suggests that the pattern of isolated increased G-CSF-induced pY-STAT3 is a potential favorable prognostic factor in relapsed pediatric AML. In contrast, the 4 patients in Group I all died of relapsed/refractory leukemia, suggesting that isolated increased IL-6-induced pY-STAT3 is a potential unfavorable prognostic factor.

The range of signaling responses to G-CSF may in part explain the spectrum of clinical responses to salvage regimens with G-CSF priming,[9] in which G-CSF is used to drive quiescent AML cells into cycle, theoretically increasing sensitivity to chemotherapy.[10] We propose that patients with robust G-CSF-induced pY-STAT3 at relapse may achieve superior responses to G-CSF-containing salvage chemotherapy.[9,11] Future studies may determine whether differences in G-CSF-induced pY-STAT3 account for the differences in response to G-CSF-containing regimens.

Increased pY-STAT3 response to IL-6 at relapse was a marker of poor prognosis. This is consistent with our earlier results, where sensitivity to IL-6 but not G-CSF identified a subset of patients with a 5-year event-free survival and OS of 29%.[1] Our findings are congruent with reports of increased IL-6 in the tumor environment, even secreted by blasts themselves, promoting tumor aggressiveness and chemotherapy resistance.[2,12-14] Understanding how IL-6-induced STAT3 activation contributes to poor outcomes in pediatric AML may provide an opportunity to target this pathway and improve chemotherapy response rates.

It is interesting to consider why pY-STAT3 induced by G-CSF could be favorable, while pY-STAT3 induced by IL-6 is clearly unfavorable. While increased proliferation from G-CSF-induced pY-STAT3 *versus* increased apoptosis resistance from IL-6-induced pY-STAT3 may in part account for the difference in outcomes, it is unlikely to fully explain our results.[15] Recent work in breast cancer suggests that the difference in outcomes may also be due to concurrent pY-STAT5 induced by G-CSF but not IL-6.[6]

In conclusion, we have shown the STAT3 pathway to be more responsive to ligand stimulation at relapse compared with diagnosis in over half the patients studied. Our results may contribute to refining risk stratification and help identify those patients most likely to benefit from targeted agents.

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Acknowledgments: the authors would like to thank the Cytometry and Cell Sorting Core at Baylor College of Medicine, which has funding from the NIH (P30 AI036211, P30 CA125123, and S10 RR024574) and the expert assistance of Dr. Amos Gaikwad and Tatiana Gotslova. Collection and storage of human specimens by the Children's Oncology Group was supported through funding from the NIH (U24CA114766), and the COG Statistics and Data Center is also supported through funding from the NIH (1U10CA180899-01).

Funding: this work was supported by grants from the Children's Cancer Research Fund (MSR), Thrasher Research Fund Early Career Award (AMS), and National Institutes of Health, National Heart, Lung, and Blood Institute, and National Cancer Institute Grants K08HL085018 (MSR) and K12CA090433 (AMS).

The online version of this letter has a Supplementary Appendix.

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doi:10.3324/haematol.2015.131508

Key words: acute myeloid leukemia, pediatric, relapse, STAT3, ligand stimulation.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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