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

Alexandra M. Stevens,[1] Marcos J. Ruiz,[1] Robert B. Gerbing,[2] Todd A. Alonzo,[3] Alan S. Gamis,[4] and Michele S. Redell[1]

[1]Division of Pediatric Hematology/Oncology, Baylor College of Medicine, Houston, TX; [2]Children's Oncology Group, Monrovia, CA; [3]Department of Preventive Medicine, University of Southern California, Los Angeles, CA; and [4]Children's Mercy Hospitals and Clinics, Kansas City, MO, USA

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).

Correspondence: amsteven@txch.org
doi:10.3324/haematol.2015.131508

Supplementary Methods:

Cells

Prior to cryopreservation, bone marrow cells were enriched for mononuclear cells by density centrifugation. Paired samples from the same patient were processed simultaneously. Viably frozen patient samples were thawed by gentle agitation in a 37° water bath then transferred into Iscoves modified Dulbecco medium (IMDM; HyClone) with 20% Fetal Bovine Serum (FBS; Invitrogen) and 100 units/mL penicillin and 100 μg /mL streptomycin (pen/strep; Invitrogen), washed once with the same medium, then resuspended in serum-free StemSpan H3000 (Stem-Cell Technologies) and rested for 2 hours. After the rest period, cell number and viability were assessed by Trypan blue dye exclusion. One of 25 sample pairs was not included in this analysis due to post-thaw viability <80% in the diagnostic sample. In total, 24 pairs (48 samples) were suitable for analysis. All patients / guardians provided informed written consent, in accordance with the Declaration of Helsinki, for the use of their tissue for research purposes. These studies were approved by the Institutional Review Board of Baylor College of Medicine. Characteristics of relapsed patients that were included on study were similar to those patients who could not be included due to the unavailability of paired viably frozen samples (*Supplementary Table 1*).

Control Cells

The human AML cell line Kasumi-1 was used as the positive control for ligand stimulation.[1] Kasumi-1 cells were obtained from ATCC, and grown in RPMI (ATCC) supplemented with 10% FBS and 100 units/mL penicillin and 100 μg/mL streptomycin. Kasumi-1 cells were grown in a humidified 37°C incubator with 5% CO_2. Kasumi-1 cells were processed

simultaneously along with every patient sample in 4 aliquots, including isotype control, unstimulated, 100 ng/mL G-CSF, and 50/100 ng/mL IL-6/soluble IL-6 Receptor alpha (sIL-6Rα). Responses for control cells were consistent across experiments and with previously published data.[1, 2]

Flow Cytometry

Cells were aliquoted into 9 wells at 1-2x10^5 cells/well. Five wells were left unstimulated for the measurement of isotype controls, constitutive pSTATs, receptor expression, total STAT3, and pY418-SRC. Two wells were stimulated with 10 or 100 ng/mL G-CSF (Filgrastim, Amgen), and two wells were stimulated with 5 or 50 ng/mL IL-6 (BD Pharmingen) + 10 or 100 ng/mL sIL-6Rα (R&D Biosystems), respectively. Incubation, fixing, and permeabilization were carried out as previously described.[1, 2] Cells were washed in flow buffer (phosphate buffered saline with 900 mg/L sodium azide and 2g/L bovine serum albumin) and stained for 30 minutes at room temperature with the appropriate fluorochrome-conjugated antibodies. After staining, cells were washed and analyzed on an LSRII (BD Biosciences). Data were collected using Diva (BD Biosciences) and subsequently analyzed using FCS Express4 (DeNovo). For all samples, Side Scatter (SSC) vs. CD45 was used to gate on the blast population and exclude residual lymphocytes or monocytes (*Supplementary Figure 1A*). Constitutive pY-STAT3, pS-STAT3, pY-STAT5, and phospho-tyrosine SRC (pY418-SRC) were measured in unstimulated AML cells, as well as total STAT3, the G-CSF receptor (G-CSFR), and the interleukin-6 receptor transmembrane subunit (gp130). After gating on the blast population, the percentage of positive events was measured for each antigen, using gates set to include <1% of events in the isotype control

condition (*Supplementary Figure 1B*). The differences between relapse and diagnosis were calculated by subtracting the percent positive at diagnosis from the percent positive at relapse.

Flow Cytometry Antibodies

Intracellular flow cytometry antibodies were purchased from BD Biosciences, including pY-STAT3-PE, pS-STAT3-AlexaFluor 488, pY-STAT5-AlexaFluor 647, pY418-SRC-Alexa Fluor 488 (Clone K98-37), and total STAT3-PE (Clone M59-50). Fluorochrome conjugated antibody to gp130, (CD130-AlexaFluor 488) was purchased from Abcam. The remainder of fluorochrome conjugated antibodies to surface receptors, and all necessary isotype controls, were purchased from BD Biosciences and included G-CSF receptor (CD114-PE), CD45-APC-H7 (Clone 2D1), CD33-PerCP-Cy5.5.

Statistical Analysis

The significance of observed differences between matched pairs was analyzed by the Wilcoxon Signed-Rank test. Spearman correlations were performed to evaluate for patterns of relationship between the various parameters. Clinical data for patients enrolled on AAML0531 were provided by COG and analyzed as of March 31, 2014. Data on bone marrow response to salvage chemotherapy was not available for this cohort, but survival and cause of death were available. Observed differences in proportions of characteristics were tested by the χ^2 test comparing groups of patients. Fisher's exact test was used when data were sparse. The Kruskal-Wallis test was used to compare differences in medians of groups. To identify whether changes in signaling parameters were predictive of the likelihood of

achieving prolonged remission of relapsed AML, clinical outcomes were compared between groups. Outcome oriented cut-point analysis converts a continuous variable to a binary variable to allow for outcome comparisons between groups. The cut point is identified based on log rank statistics. Overall Survival (OS) is defined as the time from first relapse to death. Toxic deaths (deaths not primarily attributable to the underlying disease) are frequent in patients undergoing hematopoietic stem cell transplant, and we identified 4 toxic deaths in our cohort of 24 patients (two due to infection/multiorgan failure and two due to cardiomyopathy). As deaths due to toxicity do not reflect the disease status at the time of death, these patients were censored when comparing for differences in survival between groups. Estimates of OS are reported with corresponding Greenwood standard errors. Groups were compared for significant differences by the log-rank test.

AAML0531 Treatment

Treatment on AAML051 consisted of up to 5 blocks of standard cytotoxic chemotherapy with randomization to ± gemtuzumab ozogamicin (GO).[3] Patients were also eligible for HSCT in first complete remission (CR1) based on their risk group. Patients who were eligible for HSCT in CR1 went to transplant after completing 3 of the 5 planned blocks of standard cytotoxic chemotherapy ± GO. Intrathecal cytarabine was administered on day 1 of every cycle (more frequently if patient was found to be CNS positive at diagnosis). Cycles lasted a minimum of 28 days before proceeding to the next cycle. The chemotherapy administered during each cycle is as follows:

Cycle 1(Induction 1): ADE (10+3+5) - Cytarabine 100 mg/m^2/dose q12h x 20 doses on Days 1-10. Daunorubicin 50 mg/m^2/dose on Days 1, 3, 5. Etoposide 100 mg/m^2/dose daily on Days 1-5 ± GO 3mg/m^2/dose given on Day 6.

Cycle 2 (Induction 2): ADE (8+3+5) - Cytarabine 100 mg/m^2/dose q12h x 16 doses on Days 1-8. Daunorubicin 50mg/m^2/dose on Days 1, 3, 5. Etoposide 100 mg/m^2/dose daily on Days 1-5.

Cycle 3 (Intensification 1): AE - Cytarabine 1 g/m^2/dose q12h on Days 1-5. Etoposide 150 mg/m^2/dose daily on Days 1-5.

Cycle 4 (Intensification 2): MA – Mitoxantrone 12 mg/m^2/dose daily on Days 3-6. Cytarabine 1 g/m^2/dose q12h on Days 1-4 ± GO 3mg/m^2/dose on Day 7.

Cycle 5 (Intensification 3): Capizzi - Cytarabine 3 g/m^2/dose q12h on Days 1, 2, 8, 9. E. coli L-Asparaginase 6000 IU/m^2/dose on Days 2 and 9.

AAML0531 Risk Classification:

On AAML0531, patients that were classified as high risk (due to -7, -5/5q-, FLT3-ITD with high Allelic Ratio, or >15% blasts in the BM at the end of Induction 1) were assigned to receive best available HSCT after 3 cycles of chemotherapy. Patients that were classified as low risk (inv(16)/t(16;16), t(8;21)) were assigned to receive 5 blocks of chemotherapy and were not eligible for HSCT unless they relapsed at a later date. A third risk group, intermediate risk, included patients not meeting high or low risk criteria. Intermediate risk patients were assigned to HSCT after 3 cycles of chemotherapy if they had a matched related donor available. Those rare patients who did not have an M1 marrow at the end of cycle 2 or 3 were removed from study for salvage therapy. Since we excluded patients that

underwent HSCT in first remission, to maintain the uniformity of our cohort, we did not have high risk AML patients in our study.

Supplemental Table 1. Characteristics of Relapsed Patients on Study

	Patients Studied (n=24)		Patients Not Included on Study(n=207)		
	N	%	N	%	p-value*
Sex					
Male	11	46%	108	52%	0.556
Female	13	54%	99	48%	
Age, yr olds					
Median (range)	6.8	(0.61 - 18.2)	10.5	(0.01- 29.8)	0.224
0-2 y	10	42%	45	22%	**0.030**
3-10 y	6	25%	62	30%	0.614
11-23 y	8	33%	100	48%	0.164
Race					
White	20	91%	140	76%	0.175
Black or African American	2	9%	28	15%	0.748
Other	0	0%	17	9%	0.226
Unknown	2		22		
Ethnicity					
Hispanic or Latino	6	26%	41	21%	0.594
Not Hispanic or Latino	17	74%	154	79%	
Unknown	1		12		
WBC x10^3 µL - median (range)	82	(4.2 - 439.2)	26.8	(0.2 - 519)	**0.021**
BM Blasts %	74	(35.4 - 99)	71	(0 - 100)	0.183
FAB Classification^					
M0	1	5%	8	5%	1.000
M1	6	27%	22	13%	0.102
M2	4	18%	32	19%	1.000
M4	5	23%	50	29%	0.514
M5	4	18%	37	22%	1.000
M6	0	0%	4	2%	1.000
M7	1	5%	4	2%	0.460
Other	1	5%	13	8%	1.000
Unknown	2		37		
Cytogenetics					
Normal	1	4%	40	20%	0.088
t(8;21)	5	21%	18	9%	0.077
inv(16)	2	8%	34	17%	0.385
t(9;11)/11q23	7	29%	49	24%	0.589
t(6;9)(p23;q34)	0	0%	4	2%	1.000
monosomy 7 or del(7q) or -5/5q-	0	0%	6	3%	1.000
+8	1	4%	16	8%	1.000
Other	8	33%	36	18%	0.097
Unknown	0		4		
FLT3/ITD, high AR (Lab results)					
ITD +, high AR	0	0%	14	8%	0.377
ITD - or ITD+, low AR	23	100%	163	92%	
Unknown	1		30		
CEBPα / NPMmutation					
CEBPα only	1	4%	5	3%	0.526
NPM only	1	4%	5	3%	0.526
Neither	21	91%	166	94%	0.634
Unknown	1		31		
Risk Group†					
Standard	15	63%	127	63%	0.995
Low	9	38%	60	30%	0.424
High	0	0%	16	8%	0.230
Unknown	0		4		
Treatment Arm					
Arm A	15	63%	108	52%	0.337
Arm B	9	38%	99	48%	
Course 1 response					
CR	16	70%	167	81%	0.272
Not in CR	7	30%	40	19%	
Not evaluable	1		0		

^FAB Classification was determined by central pathology review

†Risk Group is determined by cytogenetics and presence or absence of FLT3/ITD high allelic ratio, CEBPα, or NPM1 mutation

*χ^2 or Fisher exact test for comparisons with small numbers, includes only patients who relapsed after chemotherapy alone

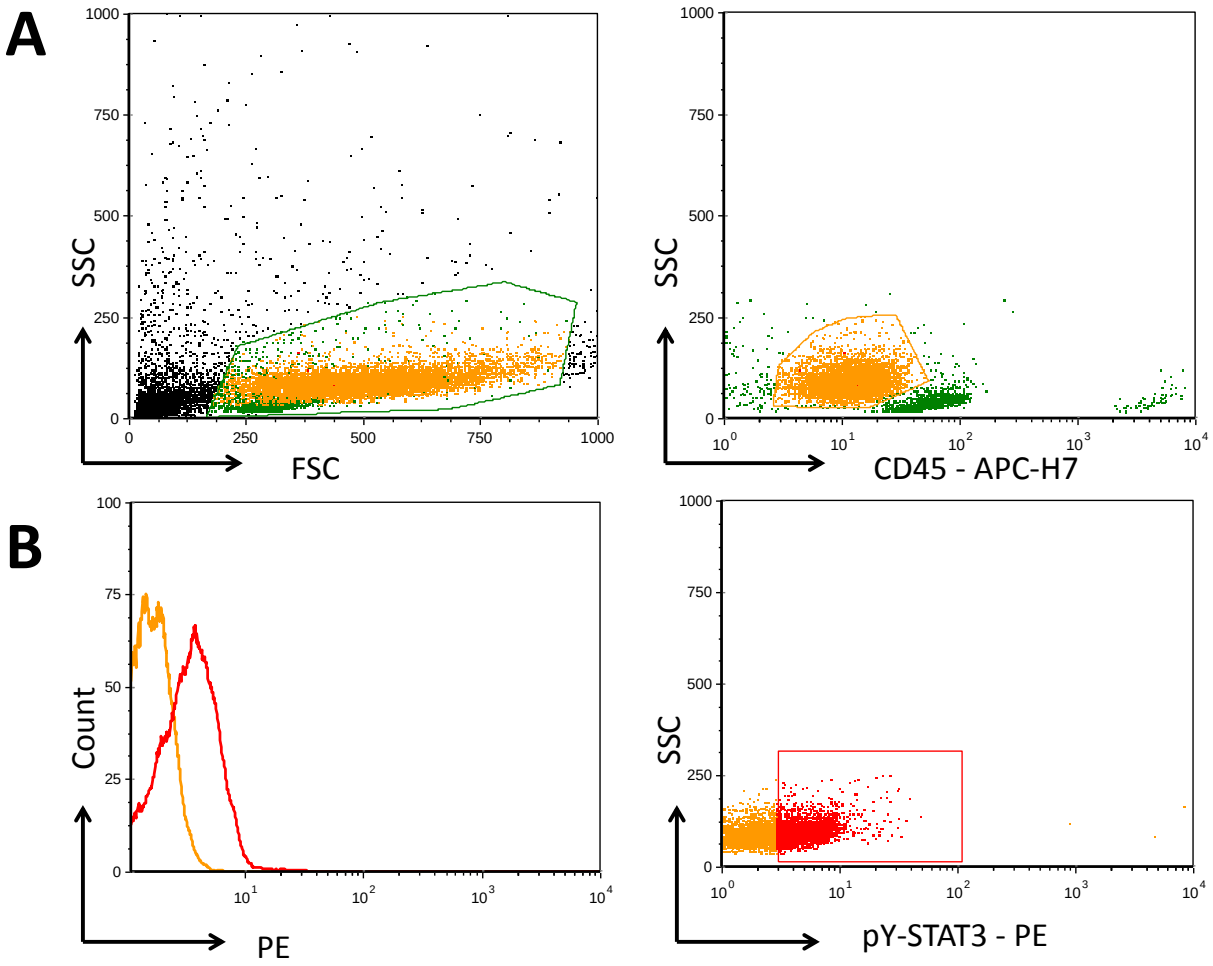
Supplementary Figure Legends:

Supplementary Figure 1. FACS analysis of unstimulated primary AML cells (A) Representative example of gating on blasts. (Left) Gating on intact cells by FSC and SCC. (Right) Gating on the blast population by CD45 vs SSC allowed exclusion of residual lymphocytes and/or monocytes when appropriate. (B) (Left) Gates were set based on the appropriate isotype control to include <1% of isotype stained blasts, orange=isotype, red=pY-STAT3-PE (Right) Representative sample with 37% of unstimulated pY-STAT3-positive blasts present within the gate.

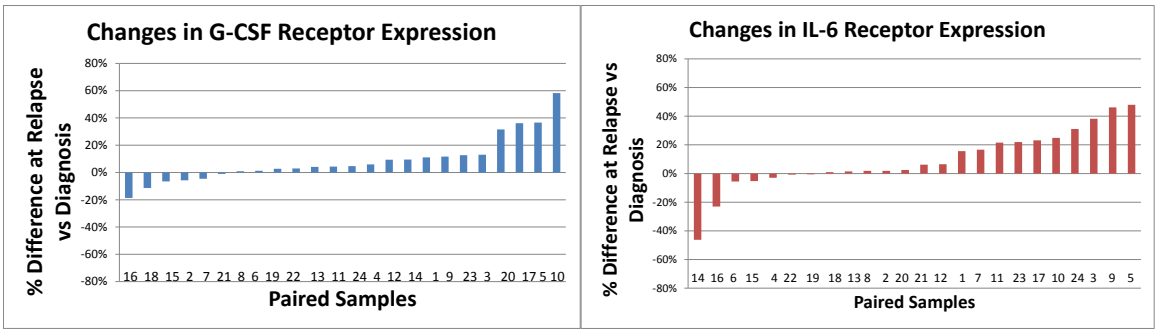
Supplementary Figure 2. Changes in G-CSF Receptor and gp130 expression between diagnosis and relapse. Waterfall plots illustrate distributions of changes between diagnosis and relapse for G-CSF Receptor (left) and the transmembrane IL-6 receptor subunit (gp130) (right). Each sample is identified by a UPN. Bar graph shows mean ± SEM for diagnosis and relapse samples (n=24). * $p<0.05$

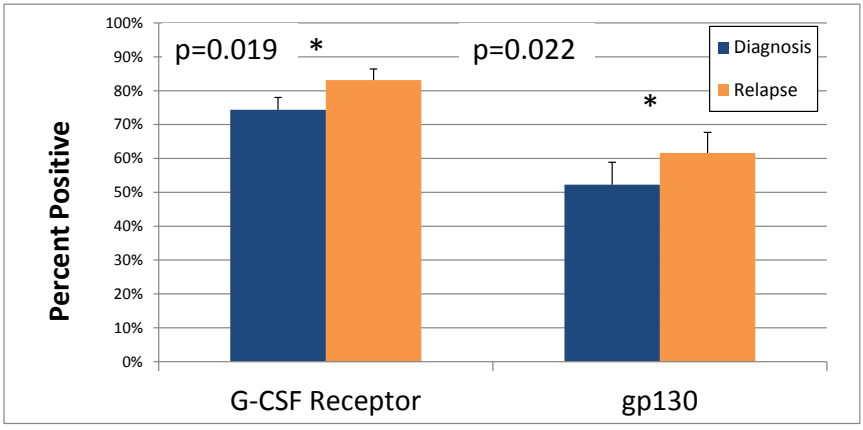
Supplementary Figure 3. Changes in Total STAT3 and constitutive pY418-SRC levels between diagnosis and relapse. Waterfall plots illustrate distributions of changes in values of additional baseline STAT pathway parameters, including Total STAT3 (left) and pY418-SRC (right). Each paired sample is identified by their Unique Patient Number (UPN). Means and SEM are presented for diagnosis and relapse in the bar graph (n=24). * indicates $p<0.05$

Supplementary Figure S1

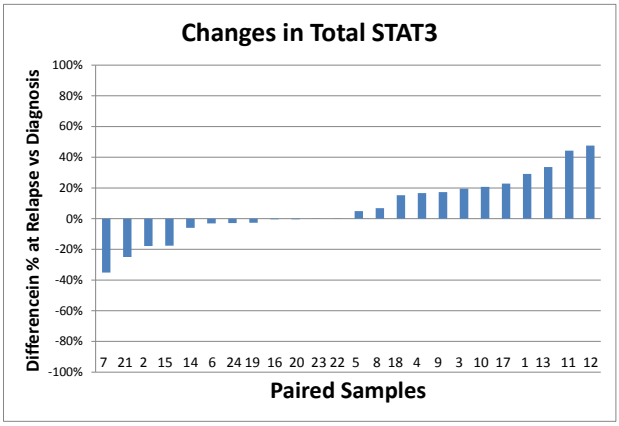


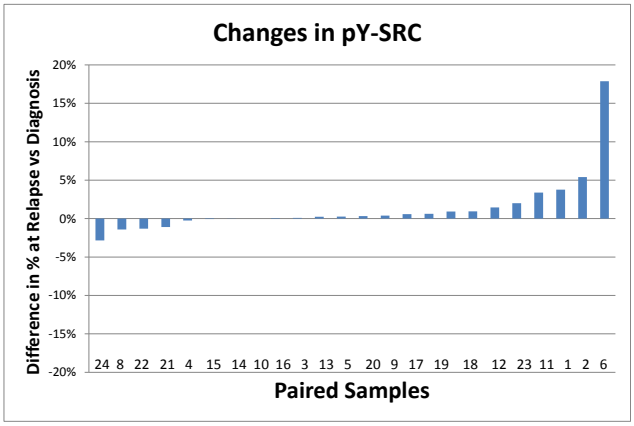
Supplementary Figure S2

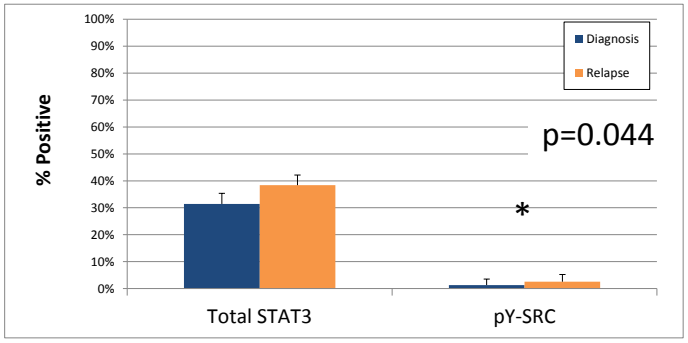




Supplementary Figure S3







Supplementary Material References

1. Redell MS, Ruiz MJ, Gerbing RB, et al. FACS analysis of Stat3/5 signaling reveals sensitivity to G-CSF and IL-6 as a significant prognostic factor in pediatric AML: a Children's Oncology Group report. Blood. 2013;121(7):1083-1093.
2. Redell MS, Ruiz MJ, Alonzo TA, Gerbing RB, Tweardy DJ. Stat3 signaling in acute myeloid leukemia: ligand-dependent and -independent activation and induction of apoptosis by a novel small-molecule Stat3 inhibitor. Blood. 2011;117(21):5701-5709.
3. Gamis AS, Alonzo TA, Meshinchi S, et al. Gemtuzumab Ozogamicin in Children and Adolescents With De Novo Acute Myeloid Leukemia Improves Event-Free Survival by Reducing Relapse Risk: Results From the Randomized Phase III Children's Oncology Group Trial AAML0531. J Clin Oncol. 2014;32(27):3021-3032.