SUPPLEMENTARY APPENDIX

CD123 expression patterns and selective targeting with a CD123-targeted antibody-drug conjugate (IMGN632) in acute lymphoblastic leukemia

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 doi:10.3324/haematol.2018.205252

Received: August 23, 2018.

Accepted: October 22, 2018.

Pre-published: October 25, 2018.

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CD123 Expression Patterns and Potential of IMGN632, a CD123-Specific Antibody-Drug Conjugate, in Acute Lymphoblastic Leukemia

SUPPLEMENTARY METHODS

Study group

We identified retrospectively patients with B-ALL or T-ALL treated at our institution between January 1, 2013 and January 1, 2017. The selection of this timeframe was based on the inclusion of CD123 assessment in our multicolor/multiparameter flow cytometry (MFC) immunophenotyping panel for acute leukemia. All patients fulfilled the diagnostic criteria of B-ALL and T-ALL according to WHO classification criteria. A cutoff of <25% bone marrow (BM) blasts was used to define lymphoblastic lymphoma (LBL) in patients who presented with an extramedullary mass; unless otherwise specified, the ALL designation in this manuscript includes LBL patients. Patients with mixed phenotype or acute leukemia with ambiguous lineage, and those with blastic transformation of chronic myeloid leukemia (CML) were excluded.

Multicolor/multiparameter flow cytometry (MFC) analysis

Analysis of CD123 expression in leukemic blasts was performed using MFC on FACS Canto II analyzers (BD Biosciences, Mountain View, CA) on bone marrow aspirate or peripheral blood samples collected in EDTA in most patients. Analysis was performed on pleural effusion fluid in 2 patients with T-LBL and on a lymph node fine needle aspiration sample in 1 patient with extramedullary B-ALL relapse. Details of the MFC panel and gating strategies were described previously.(1) The panel of monoclonal antibodies included the following: CD2, CD3 (cytoplasmic and surface), CD4, CD5, CD7, CD13, CD14, CD15, CD19, CD22, CD25, CD33,

CD34, CD36, CD38, CD41, CD45, CD56, CD64, CD117, CD123, HLA-DR, MPO, TDT. At least 10,000 events per antibody were acquired. Data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA).

Cell lines

B-ALL cells were dispensed during the exponential growth phase into 96-well sterile assay plates in culture medium. Serially diluted IMGN632 or a non-binding ADC (Ab-DGN149) were added to the seeded cells. Each assay plate included the following controls: wells with media only, and wells with untreated cells. The assay plates were incubated for 5-7 days at 37°C, 6% CO_2, in a 100% humidified incubator after which the water-soluble tetrazolium salt 8 (WST-8) (Dojindo Molecular Technologies) or alamarBlue (Invitrogen) reagents were added to the wells and light absorbance was read on a SPECTRA Max M2 plate reader (Molecular Devices). Cell viability was determined from background-corrected absorbance, with the surviving fractions calculated by dividing the viability value for each treatment by the viability value in the control (untreated) wells and plotting against the test article concentrations to estimate IC_{50} values. All assays were performed in triplicate.

***In vitro* evaluation of primary B-ALL samples**

Bone marrow mononuclear cells from B-ALL patients were thawed, stained with markers of lymphocytes and leukemic blasts (CD10, CD19, CD34, CD38, CD45, CD123, HLA-DR) and analyzed by MFC on a FACS Canto II analyzer (BD Biosciences). Remaining cells were resuspended in StemSpan H3000 culture media (STEMCELL Technologies) supplemented with 1 µM of human IgG to block Fc receptors, and 50 ng/ml each of IL-3, stem cell factor, and Flt-3 (Humanzyme). Cells were seeded into wells of a 24-well tissue culture plate (approximately $3x10^5$ cells per sample), dosed with serial dilutions of IMGN632, and incubated at 37°C in 6% CO_2 in a humidified incubator. Untreated cells were included as a control for each sample. Five

days later, the cells were collected from the assay wells, washed, Count Bright™ absolute counting beads (Life Technologies) were added to each sample, and the samples were analyzed using MFC. Blasts and lymphocytes in each sample were identified based on CD45 expression and side-scatter characteristics and additional markers (CD10, CD19, CD34, CD38, CD123, HLA-DR), quantified, and normalized to the number of counting beads. The normalized numbers of blasts and lymphocytes in each treated sample were divided by the cell number in the untreated sample to determine the IMGN632 effect. Samples were considered evaluable if the untreated controls contained at least 20% viable cells (FVS620 gate) and there was evidence of cell proliferation.

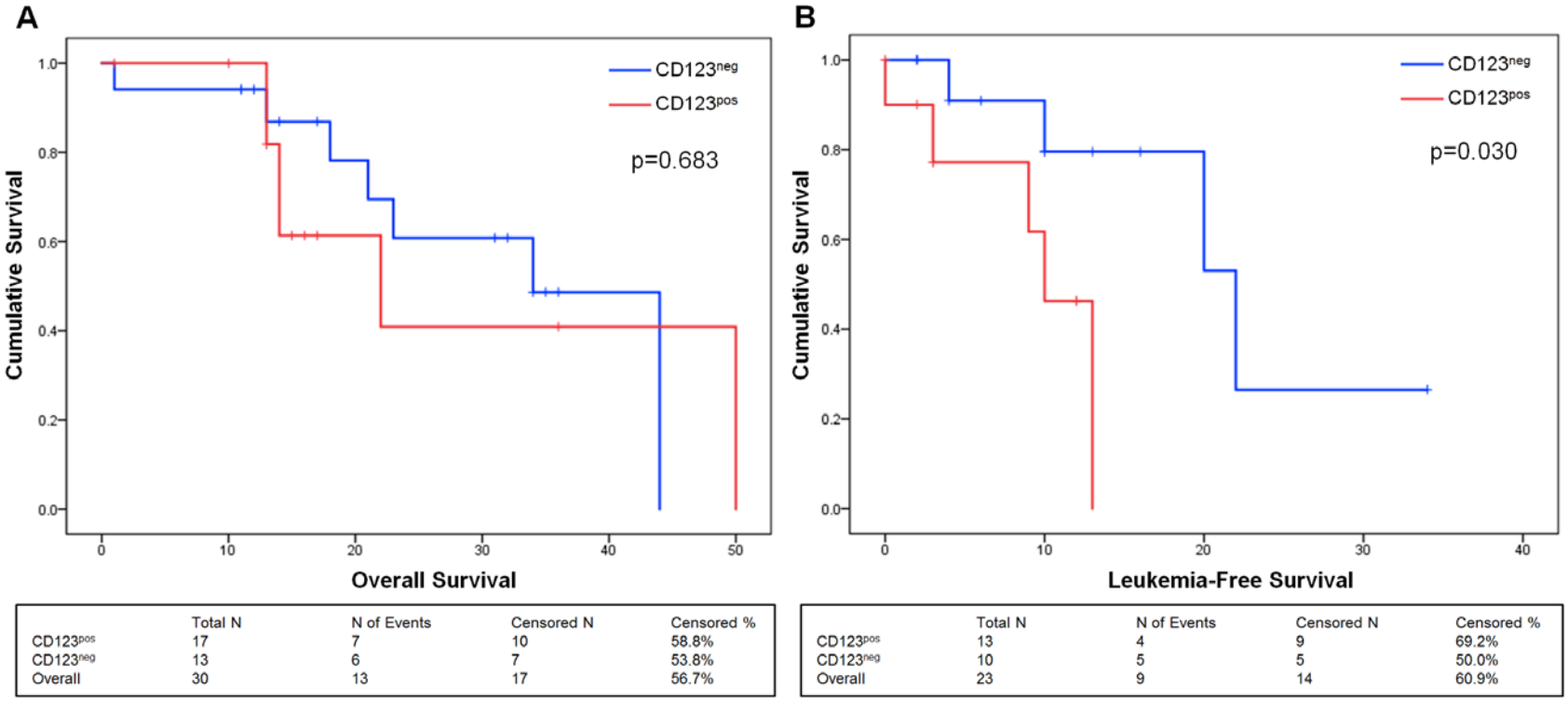
Cell proliferation for each sample was assessed by one of two methods. In one method, cells were added to wells of a 96-well assay plate in the supplemented StemSpan culture media, stained with CellTiter-Glo® (Promega) immediately after seeding, and additionally at the completion of a 5-day incubation period. At each timepoint, the plate wells were read using a SPECTRAmax M2 plate reader. An increase in the luminescence signal on day 5 indicated cell proliferation. Alternatively, freshly thawed untreated cells were washed into phosphate-buffered saline (PBS) at pH 7.2, and loaded with Cell Trace™ Violet stain (Invitrogen) for 20 minutes at 37°C, diluted in PBS with 0.5% bovine serum albumin (BSA), and separated from unreacted dye by centrifugation. An aliquot of cells loaded with the dye was additionally stained with FVS620 viability dye and CD45 and then fixed in 1% formaldehyde in PBS at pH 7.2; the remaining cells were resuspended in supplemented StemSpan H3000 medium and incubated as described above. At the completion of the incubation period, the cells were collected, washed, and stained with FVS620 viability stain and CD45 and fixed as mentioned above. Samples were then analyzed by MFC; a decrease in the Cell Trace Violet fluorescent signal in the cells incubated for 5 days indicated cell proliferation.

Statistical analysis

The associations between groups and factors of interest were assessed using the Chi-square test, Wilcoxon rank-sum test, or Kruskal-Wallis test, as appropriate. The times for OS and leukemia-free survival (LFS) were computed from the date of diagnosis to the time of last follow-up or the event of interest (relapse or death, respectively). Patients who were alive at the date of last follow-up were censored. Patients who underwent SCT were censored at the date of transplant. The Kaplan-Meier method was used to estimate OS times. Differences in survival between groups were assessed using the log-rank test. Associations between the prognostic factors of interest and OS or RFS were assessed using the Cox proportional hazards regression model. Impact of variables was expressed in hazards ratio (HR) and associated 95% confidence intervals (CI). Statistical analyses were performed using SPSS and GraphPad Prism.

SUPPLEMENTARY FIGURES AND LEGENDS

Figure S1

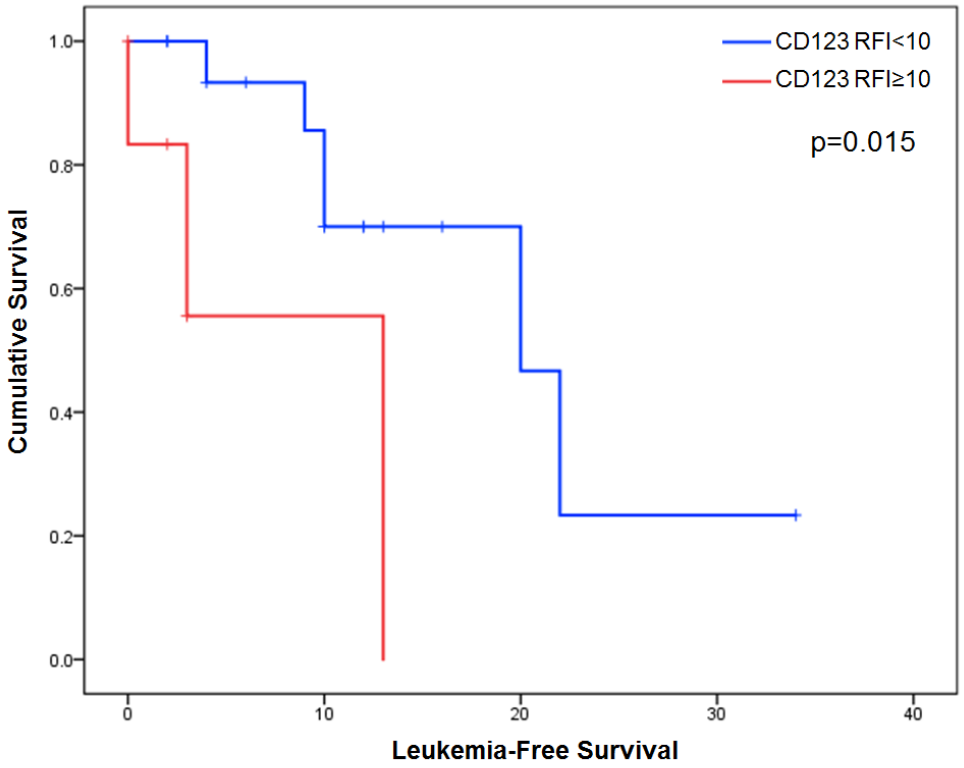


	Total N	N of Events	Censored N	Censored %
CD123pos	17	7	10	58.8%
CD123neg	13	6	7	53.8%
Overall	30	13	17	56.7%

	Total N	N of Events	Censored N	Censored %
CD123pos	13	4	9	69.2%
CD123neg	10	5	5	50.0%
Overall	23	9	14	60.9%

Figure S1. Kaplan-Meier survival curves for patients with T acute lymphoblastic leukemia/lymphoma based on CD123 expression status. (**A**) Overall survival. (**B**) Leukemia-free survival.

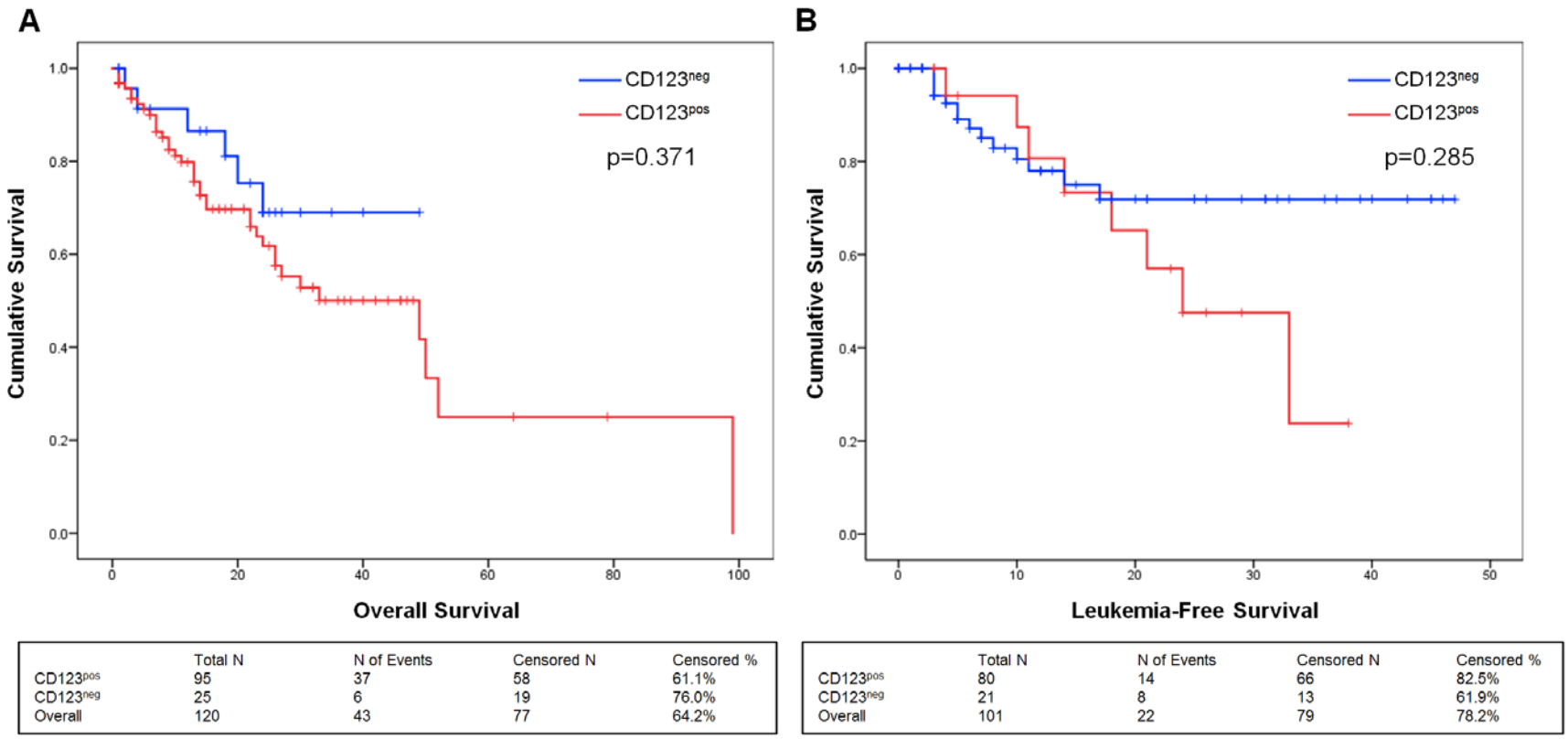
Figure S2



	Total N	N of Events	Censored N	Censored %
CD123 RFI<10	17	6	11	64.7%
CD123 RFI≥10	6	3	3	50.0%
Overall	23	9	14	60.9%

Figure S2. Leukemia-free survival comparison among patients with T acute lymphoblastic leukemia/lymphoma based on intensity of CD123 expression status (mean fluorescence intensity ratio; RFI).

Figure S3



	Total N	N of Events	Censored N	Censored %
CD123^{pos}	95	37	58	61.1%
CD123^{neg}	25	6	19	76.0%
Overall	120	43	77	64.2%

	Total N	N of Events	Censored N	Censored %
CD123^{pos}	80	14	66	82.5%
CD123^{neg}	21	8	13	61.9%
Overall	101	22	79	78.2%

Figure S3. Kaplan-Meier survival curves for patients with Philadelphia-negative B acute lymphoblastic leukemia/lymphoma based on CD123 expression status. (A) Overall survival. (B) Relapse-free survival.

Figure S4

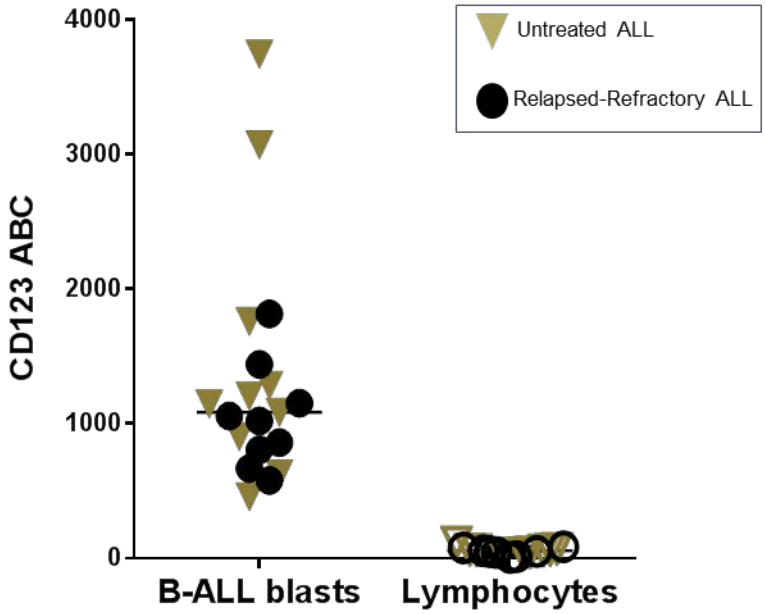


Figure S4. Number of IMGN632 antibody-binding sites (CD123 ABC) on leukemic blasts and lymphocytes from B acute lymphoblastic leukemia/lymphoma patients.

REFERENCES

1. Zhou Y, Jorgensen JL, Wang SA, Ravandi F, Cortes J, Kantarjian HM, et al. Usefulness of CD11a and CD18 in flow cytometric immunophenotypic analysis for diagnosis of acute promyelocytic leukemia. Am J Clin Pathol. 2012 Nov;138(5):744-50.