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Selection and characterization of antibody clones are critical for accurate flow cytometry-based monitoring of CD123 in acute myeloid leukemia

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Acute myelogenous leukemia (AML) is a deadly disease characterized by high relapse rates even in patients who initially achieve complete remission. Standard therapy for AML has remained largely unchanged during the last three decades and novel treatments are urgently needed [1,2]. CD123, the trans-membrane alpha chain of the interleukin-3 receptor (IL3RA) has been shown to be over-expressed in AML blast cells and leukemia stem cells (LSCs) populations [3]. Several novel agents targeting CD123 in AML are currently under development, including: CD123 fused to Diphtheria toxin (NCT02113982); a recombinant chimeric anti-CD123 monoclonal antibody (MoAb) (NCT02472145); an antibody-drug conjugate (NCT028482480); a CD123 × CD3 duobody (NCT02715011); a CD123 × CD3 Dual Affinity Re-Targeting agent (DART) (NCT02152956); Bi-Specific Antibody CD3/CD123 T cell engagers (Amphivena, Inc., San Francisco, CA); autologous engineered T cells that express anti-CD123 chimeric antigen receptors (CARs) [4] and allogeneic anti-CD123 CAR T cells (Cellectis SA, Paris, France).

A specific challenge of targeted therapies is identification of an optimal assay for accurate detection of the target of interest, which is often used for patient selection and disease monitoring in clinical trials. Thus, we sought to evaluate five different commercially available antibodies used to detect CD123 in routine immunophenotyping. We characterized and compared the cell surface expression patterns of CD123 in 53 AML samples using the five different antibody clones. We compared the ability of the different antibodies to discriminate between normal CB and AML cells and we correlated CD123 expression with *IL3RA* transcript levels using a quantitative polymerase chain reaction (qPCR) assay.

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Primary AML cells ($n = 55$ patients) were obtained with informed consent and approval of the Institutional Review Board of Weill Cornell Medical College-New York Presbyterian Hospital. Normal cord blood mononuclear cell (MNC) samples ($n = 7$) were purchased from the NY Blood Center. Cells were stained with five different commercially available phycoerythrin (PE)-conjugated moAbs against CD123 [7G3 (BD Biosciences, Franklin Lakes, NJ), 6H6 (BioLegend, San Diego, CA), 9F5 (BD Biosciences, Franklin Lakes, NJ), AC145 (Miltenyi Biotec, Bergisch Gladbach, Germany), and FAB301P (R&D Systems, Minneapolis, MN)]. Cells were also stained with CD45 (APC-H7, 2D1, BD Pharmingen, San Diego, CA) and CD5 (APC, UCHT2, BD Biosciences, Franklin Lakes, NJ) for evaluation by multi-parameter flow cytometry. Isotype mouse IgG1-PE (BD Pharmingen, San Diego, CA) was used as negative control. Cells (600,000) were stained for 20 minutes in the dark at room temperature. DAPI (4',6-diamidino-2-phenylindole) was used to exclude dead cells. Samples were evaluated using a BD LSR-Fortessa Cytometer and data were analyzed using FlowJo software (Ashland, OR). The gating strategy was defined to evaluate the expression on CD123 in blasts cells and lymphocytes for AML samples and total MNCs for normal cells, with the objective to capture all cells expressing CD123 both in malignant and healthy specimens. Lymphocytes and blast cells were gated by their side scatter (SSC) and CD45 characteristics within the DAPI-negative gate, CD123 cells were gated within the blast/CD5- populations. RNA extraction was performed with the Quick-RNA™ MiniPrep (Plus) protocol from Zymo Research, cDNA was obtained with the SuperScript® Double-Stranded cDNA Synthesis Protocol from Thermo Fisher Scientific (Waltham, MA), and qPCR was performed with the TaqMan® Gene Expression Master Mix and protocol from Thermo Fisher Scientific (Waltham, MA). The following TaqMan® assays were used: *IL3RA* (Hs00608141_m1) and *ACTB* (Hs01060665_g1). Statistical analyses and graphs were generated in R [5–8]. Optimal %CD123+ cutoffs for distinguishing normal and AML cells were defined and identified as described using Youden's index [5].

Percentage of CD123 and MFI for CD123 within the blast population for 55 primary AML patient samples was evaluated with each of the five antibodies and isotype controls (Table 1). The percentage of cells expressing surface CD123 was determined for each AML and normal MNC sample using each of the five antibodies, along with appropriate isotype controls. We found that the percentages of CD123-positive cells in any given sample varied significantly depending on the antibody tested (Figure 1(a); $p < .0001$, two-way ANOVA). As shown in Figure 1(b), while clones 9F5 and 6H6 successfully separated normal samples from AML samples by CD123+ cell percentage, other antibodies were less effective. Significant antibody-to-antibody variation was noted in the percent of CD123+ cells scored for each sample ($p < .0001$; two-way ANOVA), though the extent of this variation was patient-dependent. Receiver operating characteristic (ROC) analysis was used to systematically identify optimal %CD123+ cutoffs that could distinguish normal from AML samples for each antibody (Figure 1(c)). For clones 9F5 and 6H6, clear cutoffs were established to distinguish normal from AML samples based on CD123+ cell percentages (9F5: 42.9%; 6H6: 32.5%) achieving AUC = 1.00, thus indicating excellent sensitivity and specificity. In contrast, significant overlap between normal and AML samples was noted using 7G3, AC145, and FAB301. With these antibodies, AUCs ranged from 0.87 to 0.95,

suggesting that normal and AML samples could not be readily distinguished based on CD123 positivity.

Given these differences, we next sought to evaluate the extent to which the CD123 mean fluorescence intensity (MFI) produced by each antibody correlated with the *IL3RA* transcript level for each sample (Figure 2). Spearman's rank correlation (ρ) between CD123 MFI determined by flow cytometry and *IL3RA* transcript level assessed using qPCR was essentially identical for 9F5 and 6H6 ($\rho = 0.49$; $p = .0009$, Bonferroni corrected). In contrast, the other antibody clones demonstrated much weaker or negative correlations that did not attain significance. Similar results were noted using Pearson's correlation (r). Thus, the 9F5 and 6H6 antibody clones were superior in discriminating between AML and normal samples, and in correlating with underlying levels of *IL3RA* transcript.

Several novel therapeutic modalities targeting CD123 in AML have already entered early stage clinical trials. Thus, accurate, quantitative assessment of CD123 expression is of critical importance to investigate its potential importance in patient selection for clinical trials, disease monitoring, and as a predictor of response to therapy. We found significant differences in CD123 expression as measured by standard, commercially available antibody clones. Such discrepancies may alter patient selection and create difficulties in interpreting responses after exposure to CD123 targeted therapies. Our data show that assessments of CD123 expression using the 9F5 and 6H6 antibody clones were more predictive of the malignant phenotype and better correlated with *IL3RA* transcript levels compared to other commercially available clones. We suggest that these findings be taken into consideration for drug development and clinical trials involving CD123 targeted agents.

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Figure 1.

Percent CD123+ cells varies when scored by different anti-CD123 antibody clones in the same sample. (a) Percent CD123+ cells are indicated for each antibody (colored dot). Horizontal axis indicates %CD123+ cells. Each row indicates different sample ($N = 53$ AML samples; $N = 7$ normal MNCs). (b) Percent cells expressing CD123 in AML (red circles) vs. normal MNCs (blue triangles) for each antibody clone. The optimal %CD123+ cells cutoff for separating AML samples and normal MNCs is indicated by the black crossbar. The median %CD123+ cells in AML (red crossbar) and normal MNCs (blue

crossbar) are shown. (c) ROC curves indicated the optimal cutoff and AUC with the 95% C.I. indicated in parentheses. The vertical axis indicates true positive fraction and the horizontal axis indicates the false positive fraction.

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Figure 2.

Correlation of transcript levels of *IL3RA* with CD123 surface expression using different antibody clones. The x-axis indicates the $\log_{10}$ mean fluorescence intensity (MFI) for CD123 expression determined by flow cytometric analysis and the y-axis represents the $-\Delta Ct$. Each dot in the graph represents an AML sample ($N=53$). CD123 surface expression analysis was evaluated with antibody clones 9F5, 6H6, 7G3, FAB301, AC145, and isotype control (clockwise beginning in upper left corner). Linear regression is shown with the 95% confidence interval bands. Spearman's rank correlation (ρ) and Pearson's correlation (r) are indicated for each comparison.

Table 1

Primary AML samples.

AML sample	Blast (%)	Isotype	CD123 (MFI)					CD123 (%)					
			9F5	7G3	6H6	FAB301	AC145	Isotype	9F5	7G3	6H6	FAB301	AC145
AML76	36.6	491	15,432	46,587	14,328	25,428	75,084	1.45	55.5	45.3	48	56.8	55.2
AML55	94.3	364	7133	23,247	6689	10,177	44,724	1.23	89.3	82.5	82.9	91.1	93.3
AML2	97.4	819	28,249	29,398	32,640	13,913	36,584	6.8	99.3	82.4	99.3	97.9	97.8
AML79	87.3	480	14,720	19,472	17,372	10,168	20,738	2.35	96.8	66.4	95.2	95.3	92.5
AML14	54.1	315	9523	12,204	9366	3703	8870	0.069	96.9	24.3	92.2	71.7	72.5
AML61	94.2	368	15,491	16,563	19,930	7109	28,774	0.1	93.7	49.3	94.2	89.9	72
AML3	64.9	354	20,784	21,916	24,808	9405	29,867	1.81	92.7	74.7	94.3	96.2	95.3
AML4	60.8	409	52,689	57,646	65,765	27,470	66,940	2.03	98.4	93.2	98.4	98.2	97.7
AML5	67.2	404	28,807	26,500	32,004	12,501	30,941	0.93	96.4	78.2	94.9	96.3	96.6
AML54	98.1	401	9574	13,460	11,833	5365	13,214	1.16	91	33	88.1	82.8	83.8
AML8	12.4	363	5469	11,648	6413	2218	6955	2.54	90.6	14.7	91.1	47.1	69.6
AML72	36.2	411	12,812	21,068	11,799	11,703	29,718	1.14	94.5	78	90.8	98.3	97.5
AML34	67.3	342	20,193	24,602	20,340	10,345	34,248	0.095	93.6	76.9	89.8	95.6	95.8
AML75	16.2	471	13,392	14,230	14,908	6591	21,779	1.38	67.4	45.8	60.4	64.6	86.9
AML73	96.6	123	5212	12,748	8710	2526	5333	0.013	95.7	84.3	88.2	56.1	30.7
AML95	97.5	187	15,126	27,099	19,041	8310	16,950	0.0065	98	89.7	96	87.1	75.9
AML10	98.4	79.3	6141	11,788	7406	3457	5308	0.01	98.3	68.3	91.5	71.9	25.8
AML17	96.2	108	21,625	37,885	27,698	11,528	27,215	0.013	99.9	99.5	99.7	99.5	99
AML15	95.3	174	19,986	51,303	30,806	12,800	38,541	0.046	90.2	89.8	90.4	90	86.8
AML33	84.6	85.9	23,624	37,489	26,943	11,442	24,256	0.016	93.3	83.8	92.8	84.9	53.4
AML99	98.3	107	4794	8524	6434	2305	6645	0.017	95.7	81.4	93	59.1	50
AML20	86.2	72	3172	5238	3550	2283	4000	0.032	74.5	31.2	47.2	46.4	16.4
AML37	94.3	125	9251	25,615	13,573	8259	17,561	0.067	89.6	92.5	86.1	92.2	85.5
AML1	71.9	87.6	20,251	26,428	15,308	6950	17,939	0.042	70.7	57.7	54.6	40.6	48.3
AML7	91.3	126	10,336	30,758	14,014	7490	20,206	0.054	95	95.9	90.5	91	70.1
AML105	15.9	92.3	1010	2828	1215	685	2655	0.23	79.2	52.8	61.3	63.8	72.7
AML115	74.5	185	3845	6726	5066	1898	4163	0.14	82.2	64.5	79.5	49.3	42.6

AML sample	Blast (%)	Isotype	CD123 (MFI)					CD123 (%)				
			9F5	7G3	6H6	FAB301	AC145	9F5	7G3	6H6	FAB301	AC145
AML117	88.2	148	5479	25,586	6954	5671	24,431	95.5	98.5	94.1	97.4	98.9
AML118	57.6	185	6230	20,966	8092	3812	20,521	86.4	85.8	83.6	84.3	77.4
AML94	96.3	172	33,560	75,919	53,652	22,578	60,391	98.3	98.4	98.4	98.3	97.4
AML22	88.8	169	11,070	32,687	13,666	9519	25,814	86.9	91.1	81.3	85.4	81.3
AML119	94	80.8	19,908	32,318	25,403	9624	15,475	0.00787	99.6	97.1	99.4	98.4
AML120	93.5	173	4155	32,956	4141	5804	28,689	0.047	64.4	54.6	32.5	52.1
AML121	50.9	176	3130	7097	3125	1989	6092	0.026	63.1	49	35.6	35.7
AML122	95.8	102	8084	14,644	10,778	4026	6546	0.055	76.1	66.7	76.5	58
AML81	93.5	203	22,424	56,115	24,868	25,460	50,726	0.04	93.9	94.4	92.5	95
AML123	96.8	115	9746	16,955	11,523	6154	8841	0.025	91.1	83.4	92.7	86.8
AML19	94.7	121	21,428	55,663	37,039	16,135	36,791	0.004	98.5	98.3	99	98.4
AML124	8.08	66	659	6437	1546	1141	6142	2.57	58.4	60.9	48.6	59.5
AML127	89.2	222	9821	20,734	11,989	6687	17,051	1.72	53.6	32.8	47.6	55.5
AML30	86.7	281	22,503	45,633	32,786	12,833	35,103	1.29	95.3	96.3	95.5	95.5
AML116	81	468	11,597	31,331	14,566	8196	28,387	1.24	65.5	85.1	67.8	77.2
AML125	47.7	362	2854	27,091	3988	4924	29,592	4.3	73.5	94.1	56.8	81.1
AML55	98.5	219	4540	21,403	5602	5259	22,828	2.15	87.5	96.4	84.5	91.9
AML34	92.7	245	18,762	47,102	23,930	12,262	44,547	1.72	96.4	96.5	95.4	96.6
AML126	64.9	353	4397	21,557	5129	6266	22,364	1.39	42.9	65.5	54.9	72.6
AML104	20.3	194	1998	9126	2620	1692	8026	0.14	75	71.8	61.7	75.5
AML40	82.3	270	8248	19,121	12,349	4969	14,043	1.17	88.2	75.4	83.7	72.2
AML114	9.96	78.9	1786	5612	2144	1089	3427	0.31	84	89.7	68.4	90
AML19	87.1	165	24,414	53,461	35,781	13,970	36,465	0.039	99.4	99.5	99.3	98.8
AML83	94.2	186	9476	20,526	13,708	4938	11,694	0.092	99.2	99.2	99.5	98
AML21	87.9	322	6316	11,522	6243	3125	6170	0.27	92.4	84.9	89.7	76.8
AML90	93.8	133	4376	14,172	7790	2826	4793	0.048	95.4	97.3	99	83.6
AML41	44.7	463	55,445	100,394	37,938	33,043	78,607	2.96	95.5	97.3	94.2	96.2
AML92	47.4	298	18,266	37,875	27,386	10,450	25,522	1.07	89.7	98.2	95	95.9

Percentage of CD123 and mean fluorescent intensity (MFI) for CD123 within the blast population for 55 primary acute myeloid leukemia (AML) patient samples was evaluated with each of the five antibodies (9F5, 7G3, 6H6, FAB301 and AC145) and isotype controls.