Targeting CD38 with isatuximab and a novel CD38/CD3xCD28 trispecific T-cell engager in older patients with acute myeloid leukemia

Running head: The role of CD38 as a potential target in AML

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Supplemental Methods

CD38 expression in older patients with acute myeloid leukemia (AML)

CD38 expression was measured in the surface of leukemic cells from a series of 241 newly-diagnosed older patients (median age 75, range 65 – 90) enrolled in the phase 3 PETHEMA FLUGAZA clinical trial (NCT02319135)[1]. Briefly, patients were randomized to receive three induction cycles with fludarabine and cytarabine (FLUGA) followed by six consolidation cycles with reduced intensity of FLUGA, *vs* three induction cycles with 5-azacitidine (AZA) followed by six consolidation cycles with AZA. After consolidation, patients continued with the same treatment if measurable residual disease (MRD) ≥0.01%, or stopped if MRD <0.01%. At diagnosis, the EuroFlow AML panel was used to identify leukemia-associated immunophenotypes (LAIPs)[2]. When complete response was achieved, bone marrow (BM) samples were stained with 8-color combinations conserving the same design as that at diagnosis, selecting markers based on previously identified maturation arrest, lineage commitment and LAIPs to monitor MRD.

Multidimensional flow cytometry (MFC) was used to evaluate CD38 expression in the surface of leukemic cells. EDTA-anticoagulated BM samples were processed within 24 hours after collection following the EuroFlow lyse-wash-and-stain sample preparation protocol adjusted to 10^6 nucleated cells[3]. Blasts were identified based on their pattern of CD34, CD45, CD117 and HLADR expression, and the mean fluorescence intensity (MFI) and percentage of $CD38^+$ leukemic blasts were quantified for each patient, based on the fifth monoclonal antibody (mAb) combination of the EuroFlow AML panel[3]: Pacific Blue-HLADR, OC515-CD45, FITC-CD15, PE-NG2, PerCP-Cy5.5-CD34, PECy7-CD117, APC-CD22 and APCH7-CD38. Mature nucleated red blood cells (erythroblasts) were used as negative control for CD38 expression. Data acquisition was performed in a FACSCanto II flow cytometer [Beckton Dickinson Biosciences (BDB), San Jose, CA] using the FACSDiva 6.1 software (BDB). The Infinicyt software (Cytognos SL, Salamanca, Spain) was used to analyze data.

Effector-cell abundance in BM samples from older AML patients

The presence and relative frequency of NK- and T-cells was determined in BM samples from the series of patients described above, using the first mAb combination of the EuroFlow AML panel[3]: Pacific Blue-HLADR, OC515-CD45, FITC-CD16, PE-CD13, PerCP-Cy5.5-CD34, PECy7-CD117, APC-CD11b and APCH7-CD10. Data was analyzed with a semi-automated pipeline that performs batch-analyses of flow cytometry data to avoid variability intrinsic to manual analysis, and unveils full cellular diversity based on unbiased clustering[4]. Briefly, this strategy allowed the systematic identification and quantification of a variable number of cell clusters, including NK cells ($CD10^-$, $CD13^-$, $CD16^{++}$, $CD11b^{+/-}$, $CD34^-$, $CD45^+$, $CD117^-$, low FSC/SSC) and T cells ($CD10^-$, $CD13^-$, $CD16^-$, $CD34^-$, $CD45^+$, $CD117^-$, low FSC/SSC), which grouped according to the similarity of antigen expression profiles by using the bioinformatic algorithm FlowSOM.

Antibody-dependent cellular cytotoxicity (ADCC) in AML cell lines

To determine isatuximab-induced (70 nM) killing of AML cell lines through ADCC, peripheral blood mononuclear cells (PBMC) from healthy adults were isolated by Ficoll-paque PLUS density gradient (GE Healthcare, Chicago, IL). Human NK cells were further purified by negative magnetic cell sorting in an AutoMACS Pro using the NK Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's protocol. Purified NK cells were cocultured in an effector to target cell (E:T) ratio of 1:1 with KG-1, MOLM-13 and OCI-AML3 AML cell lines. After overnight incubation, cells were stained with the mAb panel 1 indicated in the Supplemental Table 1. AML cell lysis was measured as the frequency of Annexin-V^+ AML cells.

Antibody-dependent cellular phagocytosis (ADCP) in AML cell lines

To determine isatuximab-mediated ADCP, human $CD14^+$ monocytes were isolated from healthy adults' PBMC with CD14 MicroBeads Human kit (Miltenyi Biotech,

Bergisch Gladbach, Germany). Cell sorting was performed in an AutoMACS Pro and purified monocytes were resuspended in complete medium (RPMI1640, 10% fetal bovine serum (FBS), 1% L-Glutamine and 1% Penicillin/Streptomycin) supplemented with macrophage colony-stimulating factor (10 ng/mL), and seeded in 6-well plates at a density of 1.5 x 10^6 cells/mL. Cells were kept in culture for 7 days, and medium with growth factors was replaced every 2 days. Differentiated macrophages were detached with 10 mM PBS-EDTA and cultured in 24-well plates in complete medium to allow macrophages to adhere to the plate. After overnight incubation, the culture medium was removed and RPMI1640 medium without FBS was added for two hours. KG-1, MOLM-13 and OCI-AML3 cell lines were labeled with violet proliferation dye (VPD) 450 (BD Horizon™, BDB, San Jose, CA) and treated with or without isatuximab (70 nM) for 30 minutes, before culturing with macrophages in a 1:2 E:T ratio. After 2 hours at 37 ºC, cells were collected with 10 mM PBS-EDTA and labeled with mAb panel 2 (Supplemental Table 1). Isatuximab-mediated phagocytosis was determined as the frequency of $CD14^+$, $CD300e^+$ and VPD^+ triple positive macrophages after exclusion of dead cells using sytox green staining. All samples were evaluated in a FACSCanto II flow cytometer using the FACSDiva 6.1 software (BDB, San Jose, CA). Data analysis was performed with the Infinicyt software (Cytognos SL, Salamanca, Spain).

Ex vivo efficacy of isatuximab in older AML patients

We measured ADCC in primary samples from 17 patients with AML (median age 69, range 34 – 81) upon treatment with 70 nM of isatuximab for 24 hours, and using the panel 3 of mAbs (Supplemental Table 1). Live leukemic cells were identified after exclusion of debris and doublets based on their unique scatter characteristics, expression of CD34 and CD117, plus negative staining for Annexin-V. Untreated samples were used as control and depletion of leukemic cells was determined according to the following formula: [(% leukemic cells in untreated - % of leukemic cells in treated sample) / % of leukemic cells in untreated] * 100. Data acquisition was performed in a

FACSCanto II flow cytometer using the FACSDiva 6.1 (BDB, San Jose, CA) and the Infinicyt softwares (Cytognos SL, Salamanca, Spain) to analyze data.

Analysis of the mechanisms of action triggered by isatuximab and the CD38/CD3xCD28 trispecific TCE (CD38-TCE)

To compare CD38 regulation, KG-1, MOLM-13 and OCI-AML3 cell lines were treated with isatuximab or the CD38-TCE (both at 70 nM) for 96 hours, and CD38 expression was analyzed every 24 hours by flow cytometry with a non-competing anti-CD38 antibody (clone 11B6, kindly provided by Sanofi).

MOLM-13 cells were stained with VPD and pre-treated with isatuximab or the CD38-TCE (700 pM) and their corresponding isotype controls for 30 minutes. Then, MOLM-13 cells were co-cultured with PBMC at a 10:1 E:T ratio for 24 and 48 hours. Alternatively, T- and NK- cells were further purified in an AutoMACS Pro using magnetic-bead assays for negative selection (Miltenyi Biotech, Bergisch Gladbach, Germany). Purified effector cells were co-cultured with VPD-labeled and drug pre-treated MOLM-13 cells at a 2:1 E:T ratio for 24 and 48 hours. At the indicated time-points, samples were harvested and labeled with the panel 4 of mAbs (Supplemental Table 1), to determine leukemic cell lysis, effector NK- and T-cell activation, as well as regulation of CD38 expression in MOLM-13 cells, as detailed above. Samples were acquired in an Omnicyt flow cytometer, and analyzed using the Infinicyt software (both from Cytognos SL, Salamanca, Spain).

Comparison of the *ex vivo* efficacy of isatuximab and the CD38-TCE in AML

We measured tumor-cell killing in primary samples from eight patients with AML (median age 73, range 44 – 84), incubated for 48 hours in the presence of isatuximab or the CD38-TCE (7 – 700 pM) and their respective isotypes as controls. Before treatment, samples were labeled with two combinations of mAbs to determine the frequency of

leukemic blasts and other immune cell subsets (Supplemental Table 1, panel 5), and to characterize baseline immunophenotype of effector cells (Supplemental Table 1, panel 6). After treatment in 48-well plates, samples from each well were splitted into two aliquots and labeled with two combinations of mAbs to determine the effect on leukemic cells (Supplemental Table 1, panel 5) and other immune cell types (Supplemental Table 1, panel 7). Live leukemic cells were identified as detailed above. Samples treated with the isotype of either isatuximab or the CD38-null trispecific TCE were used as control, and depletion of leukemic cells was determined according to the following formula: [(% leukemic cells in isotype - % of leukemic cells in treated sample) / % of leukemic cells in isotype] * 100. Data acquisition was performed in a CytoFlex LX [Beckman Coulter (BC) Life Science, Indianapolis, IN] using the CytExpert v2.3 (BC) and the Infinicyt softwares (Cytognos SL, Salamanca, Spain) to analyze data.

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Supplemental Tables

Supplemental Table 1. Description of the antibodies and reagents used in this study.

Panel	Flow cytometer	Antigen / Reagent	Label	Clone	Cat #	Company
1	FACS Canto II (BDB)	CD4	Pacific Blue		B49197	BC
		CD56	BV510	NCAM16.2	563041	BDB
		CD38	FITC	Multi-epitope[1]	CYT-38F2	Cytognos
		CD13	PE	L138	347406	BDB
		CD45	PerCP-Cy5.5	HI-30	304028	Biolegend
		Annexin V	APC		ANXVDY-200T	Immunostep
		CD3	APCH7	SK7	641415	BDB
2	FACS Canto II (BDB)	Violet proliferation dye 450	BV421		C34571	Thermo Fisher Scientific
		Sytox green	FITC		S7020	Thermo Fisher Scientific
		CD14	PerCP-Cy5.5		550787	BDB
		CD300e	APC		IREM2A-100T	Immunostep
3	FACS Canto II (BDB)	CD45	Pacific Blue			

		CD38	FITC	Multi-epitope[1]	CYT-38F2	Cytognos
		Annexin V	PerCP-Cy5.5			
		CD117	PE-Cy7		IB49221	BC
		CD34	APC	8G12	345804	BDB
4	Omnicyt (Cytognos)	Violet proliferation dye 450	BV421		C34571	Thermo Fisher Scientific
		CD56	BV510	NCAM16.2	563.041	BDB
		CD38	FITC	11B6[2]	Kindly provided by Sanofi	
		CD45	PerCP-Cy5.5	HI30	304028	Biolegend
		CD69	PE-Cy7	FN50	310912	Biolegend
		Annexin V	APC		ANXVDY-200T	Immunostep
		CD3	APCH7	SK7	641415	BDB
5	CytoFlex XL (BC)	CD33	BUV395	P676	745709	BDB
		CD28	BUV496	L293	749972	BDB
		CD38	FITC	11B6[2]	Kindly provided by Sanofi	
		HLA-DR	Pacific Blue	L243	307633	Biolegend
		CD16	BV480	3G8	566108	BDB
		CD3	BV605	SK7	563219	BDB
		CD13	BV650	L138	745350	BDB
		CD34	BB700	8G12	746104	BDB
		CD11b	PE	ICRF44	301306	Biolegend

		CD117	PE-Cy7		B49221	BC
		CD56	APC-AF700		B92446	BC
		CD45	APC-Cy7	2D1	368516	Biolegend
		Maleimide / AnnexinV	IR885/ APC		129585 / ANXVDY-200T	Sigma / Immunostep
6	CytoFlex XL (BC)	HLA-DR	BUV395	TU39	740302	BDB
		CD8	BUV496	HIT8A	741161	BDB
		TIM3	BB515	7D3	565568	BDB
		CD107a	BV421	H4A3	328626	Biolegend
		CD16	BV480	3G8	566108	BDB
		CD3	BV605	SK7	563219	BDB
		TIGIT	BV650	741182	747940	BDB
		CD137	PE	4B4-1	561701	BDB
		CD57	PCF594	NK-1	562488	BDB
		CD69	PE-Cy7	FN50	310912	Biolegend
		CD38	APC		555462	BDB
		CD56	APC-R700		B92446	BC
		CD45	APC-Cy7	2D1	368516	Biolegend
		Maleimide	IR885		129585	Sigma
7	CytoFlex XL (BC)	CD69	BUV395	FN50	564364	BDB
		CD8	BUV496	HIT8A	741161	BDB
		TIGIT	BV421	A15153G	372709	Biolegend
		CD3	BV480	SK7	746437	BDB

PD1	BV650	EH12	564104	BDB
TIM3	BB515	7D3	565568	BDB
CD137	PE	4B4-1	561701	BDB
CD25	PE-Cy7	M-A251	557741	BDB
HLA-DR	APC	L243	307610	Biolegend
CD56	APC-R700		B92446	BC
CD45	APC-Cy7	2D1	368516	Biolegend

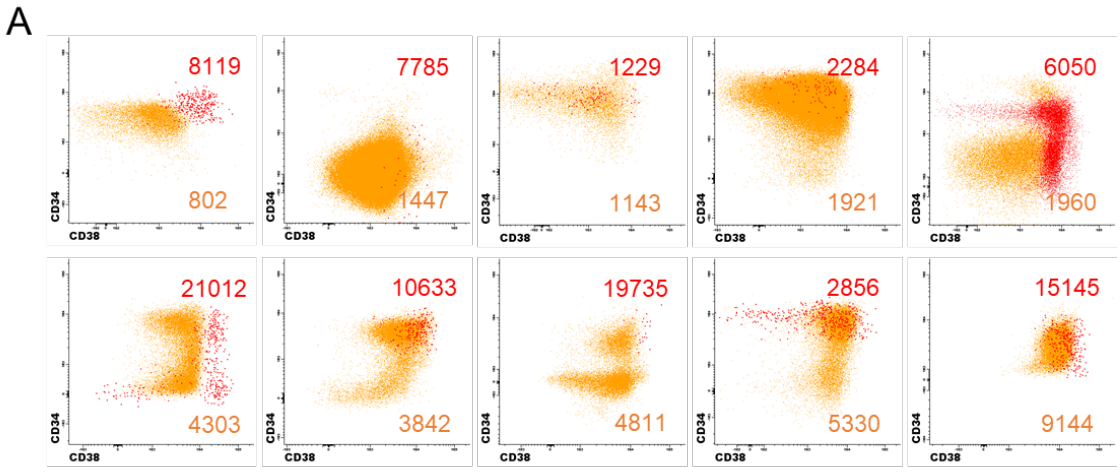
BC: Beckman Coulter (Indianapolis, IN); BDB: Beckton Dickinson Biosciences (San Jose, CA); Biolegend (San Diego, CA); Cytognos SL (Salamanca, Spain); Immunostep SL (Salamanca, Spain); Thermo Fisher Scientific (Walthman, MA); Sigma-Aldrich (St Louis, MO).

[1] This anti-CD38 antibody is multi-epitope and thus less likely to compete with isatuximab or the CD38-TCE for the CD38-binding domain

[2] This anti-CD38 antibody did not compete with isatuximab or the CD38-TCE for the CD38-binding domain.

Supplemental Figures

Figure S1. CD38 expression in older patients with acute myeloid leukemia (AML). **(A)** Bivariate CD34 *vs* CD38 dot-plots with merged, patient-matched leukemic cells at diagnosis (in yellow) and at complete response after three induction cycles [i.e., measurable residual disease (MRD)] (in red). CD38 median fluorescence intensity (MFI) in blasts at both stages is indicated with numerical values. **(B)** CD38 expression in patient-matched leukemic cells at diagnosis and relapse. In this population-band dot-plot graphical representation, each cell is represented by a colored dot (dark-orange: diagnosis; dark-brown: relapse), whereas the MFI is represented by circles and indicated with numerical values.



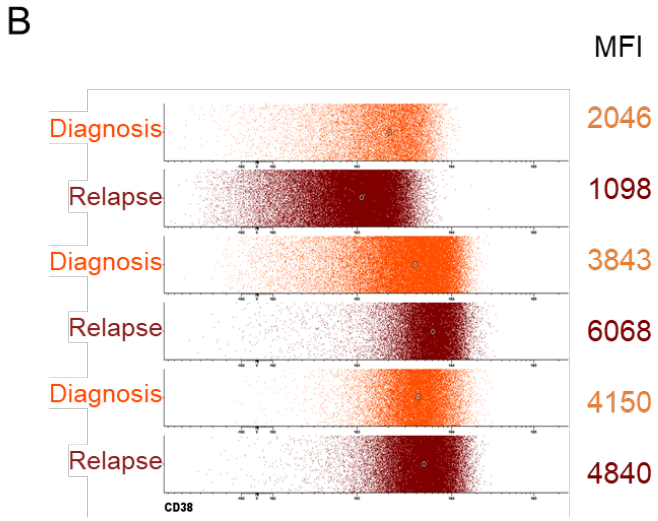


Figure S2. Effector-cell activation by the CD38/CD3xCD28 trispecific TCE (CD38-TCE). MOLM-13 cells were pretreated with the CD38-TCE for 30 minutes and cocultured with isolated T- or NK-cells from healthy donors (n=3). After 48 hours, CD69 positivity was assessed by flow cytometry as a readout of effector-cell activation.

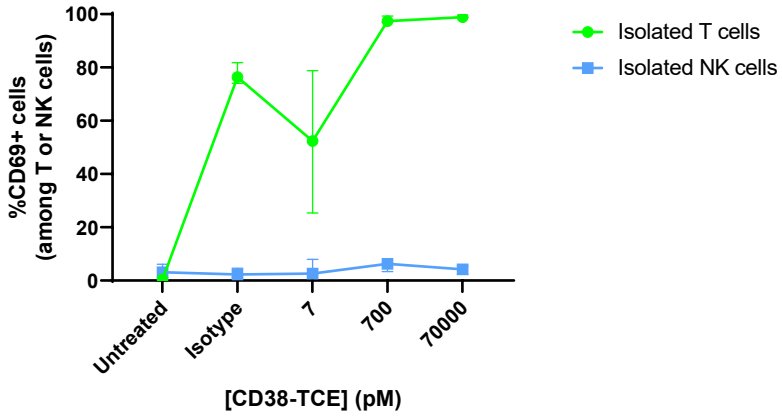


Figure S3. Relationship between CD38 expression and drug-mediated leukemic blast lysis and target regulation. **(A)** Basal CD38 expression in leukemic blasts was measured by flow cytometry in both groups of responders and non-responders to the CD38/CD3xCD28 trispecific TCE (CD38-TCE) and isatuximab. *, $P < .05$ **(B)** $CD38^{lo}$ (KG-1) and $CD38^{hi}$ (MOLM-13 and OCI-AML3) AML cell lines were treated with isatuximab or the CD38-TCE (70 pM). After 24, 48, 72 and 96 hours, CD38 levels were measured and mean fluorescence intensity (MFI) values were normalized to those at baseline.

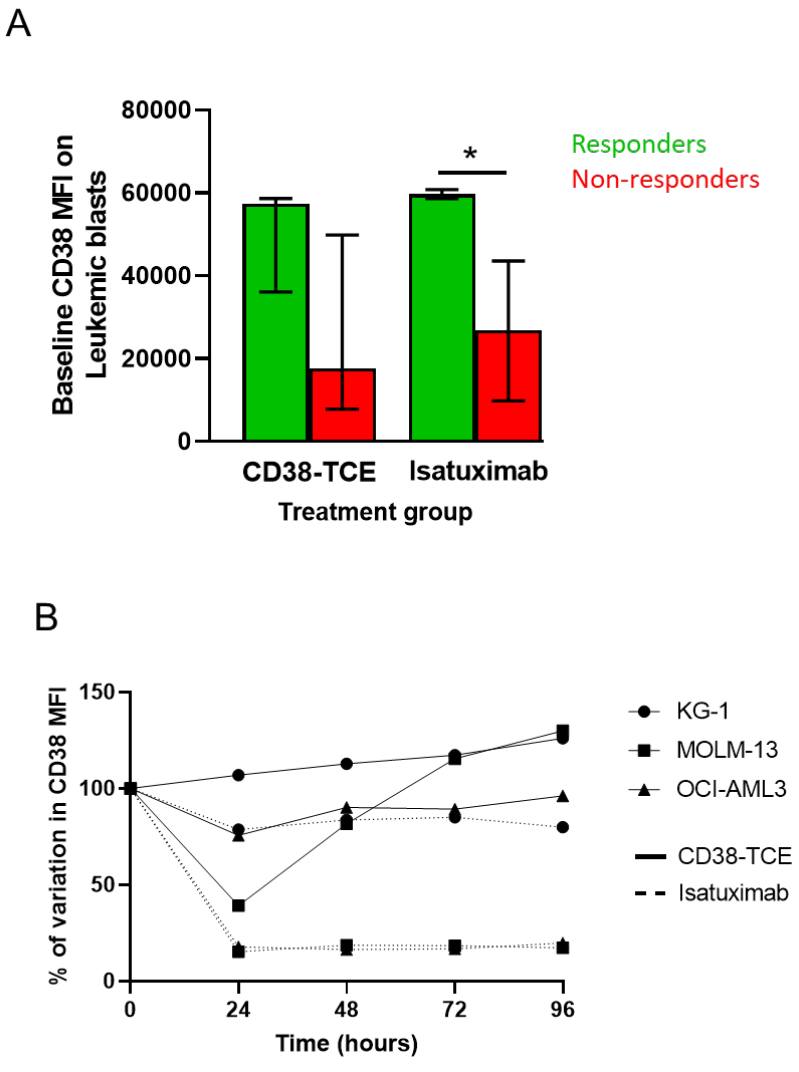


Figure S4. Correlation between tumor lysis and immune features. Baseline immune features were correlated with tumor lysis induced ex vivo after 48 hours of treatment with 700 pM isatuximab or the CD38/CD3xCD28 trispecific TCE (CD38-TCE). Red, blue and white colors represent positive, negative and no correlation, respectively (Spearman's coefficient). Asterisks indicate a significant correlation (P <.05).

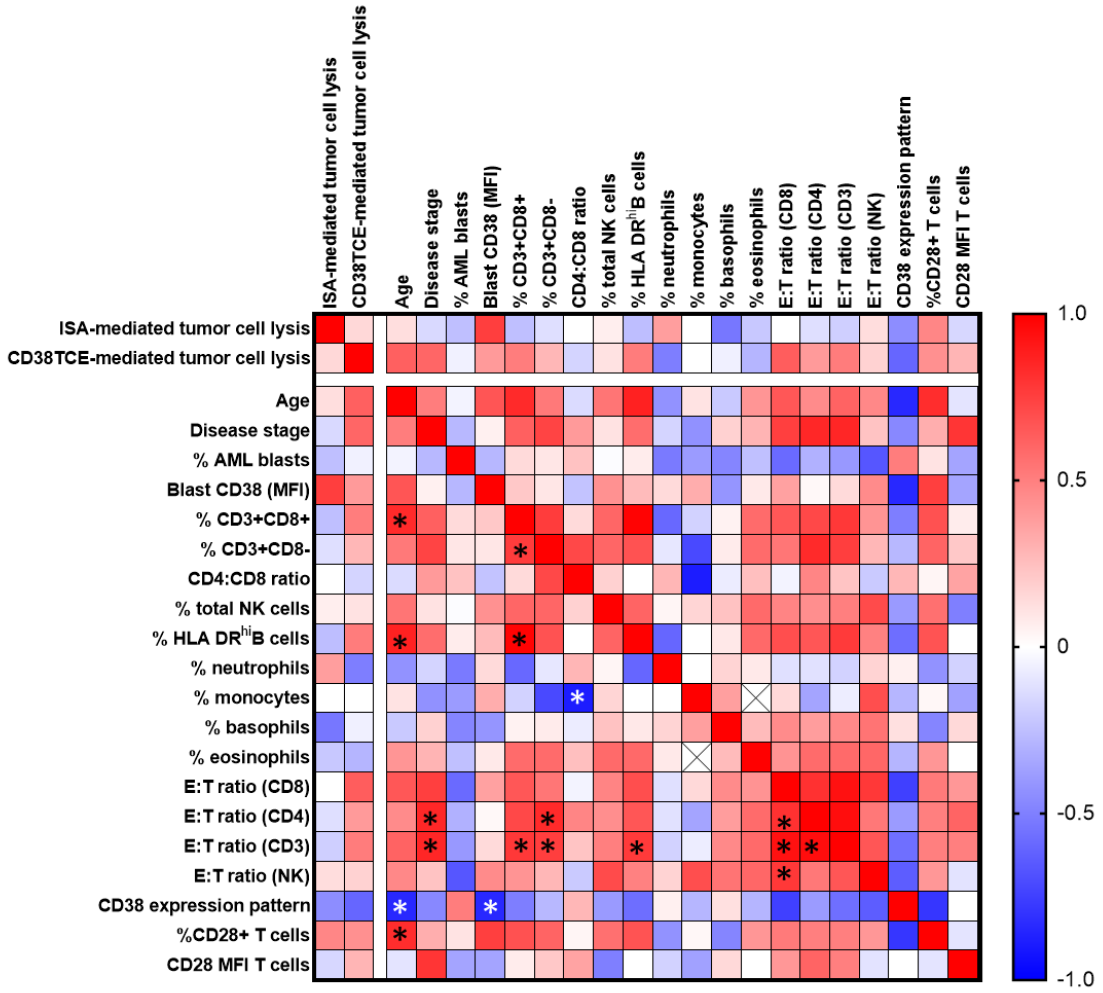


Figure S5. Lack of association between responsiveness to the CD38/CD3xCD28 trispecific TCE (CD38-TCE) and baseline frequency of CD28^{+} T cells in bone marrow microenvironment. Samples were considered as responders when AML blast lysis observed in the CD38-TCE-treated condition was higher than that observed in the CD38-null TCE-isotype condition (48 hours, 700 pM). Among eight primary samples tested, three responded to the CD38-TCE. ns, non-significant.

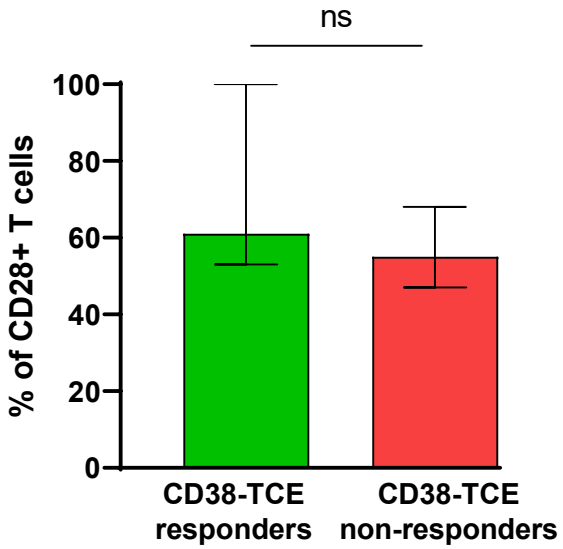


Figure S6. CD38 expression in AML blasts vs normal cell types. Using the Infinicyt software (Cytognos, Salamanca, Span), we merged the FCS files of blasts from the AML patients included in this study (N = 241) with various cell types identified in normal bone marrow aspirates from healthy adults (N = 10). The levels of CD38 expression (measured with the clone HB7 conjugated with APCH7; BD Biosciences, San Jose, CA) are represented by population-band histograms, and ordered from the lowest into the highest expression detected among various normal cell types.