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Phenotypic and functional characterization of the CD6-ALCAM T-cell co-stimulatory pathway after allogeneic cell transplantation

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Supplemental Materials for

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SUPPLEMENTAL METHODS

Cell processing for *in vitro* functional assays

For functional *in vitro* assay, PBMC were isolated from freshly drawn blood samples by density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare) and cryopreserved in RPMI 10% DMSO before being utilized. For *in vitro* culture of PBMC or T cells complete medium (CM) was used. CM was made using DMEM (Cat 11995-065 Gibco), 10% FBS (Heat Inactivated, Gibco 10437-028), 1% Penicillin-Streptomycin (Gibco, 100X, 15140122), HEPES 10mM (Gibco, 1M, 15630080), 1% MEM Non-ess A.A. (Gibco, 100X, 11140050) and 2-Mercaptoethanol 0.05mM (Sigma-Aldrich, 14.3M, M3148).

Functional activity of itolizumab *in vitro*

Frozen PBMC obtained from patients who developed aGVHD after transplant (Supplemental Table S1) were stimulated using a combination of antiCD3/CD2/CD28 beads (Treg Suppression Inspector human 2.5 ml 5x10'7 Anti-Biotin MACSiBead particles preloaded with biotinylated CD2/CD3/CD28 antibodies, Miltenyi 130-092-909) at a bead:cell ratio of 1:2 in the presence of itolizumab 10 μg/ml (Biocon Ltd, Bangalore, India) or isotype control 10 μg/ml (cetuximab Erbitux NDC 66733-948-23, LOT c1800010). To detect T cell proliferation, PBMC were stained with CFSE (Invitrogen Thermo Fisher Scientific) as per product instruction. T cell proliferation, activation and maturation were evaluated after 72 hours of culture using the flow cytometry panel in Supplemental Table S3 and analyzed using FlowJo software. An example of the gating strategy used in this analysis is shown in Supplemental Figure S3.

Complement dependent cytotoxicity (CDC), antibody direct cytotoxicity (ADC)

After thawing HD PBMC, $CD3^+$ T cells were isolated using EasySep Human T-Cell isolation Kit (STEMCELL Cat 17951 Lot 17F79887) as per manufacturer's instructions. Purity of isolated $CD3^+$ T cells was assessed using flow cytometry with FITC-conjugated anti-CD3 antibody (Ab) (BioLegend, clone UCHT1, cat 300406). After T cell isolation, we obtained a median percentage of 96.9%, $CD3^+$ cells (range 90.5-98.1%). After 15 minutes pre-incubation with itolizumab (Biocon Ltd, Bangalore, India), positive control alemtuzumab (10 μg/ml, Campath-1H, anti-CD52, human IgG1, BioVision A1105-200, Lot 4M13A11050) or negative control cetuximab (10 μg/ml, Erbitux NDC 66733-948-23, LOT c1800010), isolated $CD3^+$ T cells were cultured at a concentration of 1 x 10^6/ml in the presence of CM with (for CDC) or without (for ADC) 25% human serum. Cells were incubated at 37'C, 5% CO2 for 6 or 24 hours. At the end of cell culture, the cell plate was kept on ice to prevent

further complement activity. Harvested cells were stained with 7-AAD (BD, 51-68981E' sold as 559925) and PE-conjugated Annexin V (BD, 51-65875X, sold as 556422) as per manufacturer's instructions and acquired on Fortessa LSR flow cytometer (BD). Each sample was acquired at medium velocity for 180 seconds. Analysis was performed using FlowJo software. An example of gating strategy is shown in Supplemental Figure 6. Percentage lysis was calculated by the average of % of 7-AAD and/or Annexin V positive cells. To detect complement-mediated cell death (CDC), percent specific lysis was calculated by subtracting the value obtained in conditions with Ab alone from the value obtained in the presence of AB+ human serum (HS). ADC activity was calculated by subtracting the values obtained in culture with medium alone from the values obtained in cultures with medium + antibody. CDC and ADC were assessed after 6 and 24 hours of culture.

Antibody dependent cellular cytotoxicity (ADCC) assay

Thawed HD PBMC were cultured at a final concentration of 1 x 10^6/ml in the presence of CM and specific ab at a concentration of 10 μg/ml. Cells were incubated at 37'C, 5% CO2 for 6 hours. Harvested cells were stained with FITC-conjugated anti-CD3 Ab (BioLegend, clone UCHT1, cat 300406), BV510-conjugated anti-CD56 Ab (BioLegend, clone HCD56 NCAM, cat 318340) and APC-conjugated anti-CD107a LAMP-1 (BioLegend, clone H4A3, cat 328620) and incubated for 30 minutes. Cells were washed two times and then stained with 7-AAD (BD, 51-68981E' sold as 559925) and PE-conjugated Annexin V (BD, 51-65875X, sold as 556422) as per manufacturer's instructions and acquired on Fortessa LSR flow cytometer (BD). Each sample was acquired at medium velocity for 180 seconds. Analysis was performed using FlowJo software. To set the positive threshold for CD107a expression an APC-conjugated isotype mouse IgG1 (BioLegend, clone MOPC-21, cat 400122) was used. An example of gating strategy is shown in Supplemental Figure 6.

T cell stimulation assay using ALCAM-Fc and anti-CD3 Ab

Recombinant Human ALCAM Fc Chimera (ALCAM-Fc, R&D Systems, cat 7187-AL-100) 10 μg/ml and anti-CD3 Ab 5 μg/ml (Invitrogen Thermo Fisher Scientific, clone OKT3, cat 16-0037-85) were resuspend in PBS (Gibco 10010-023, ph7.4) overnight at 4'C in flat bottom 96 well plate. Plate was washed 2 times with PBS before adding cells. After thawing HD PBMC, $CD3^+$ T cells were isolated through negative selection using the EasySep Human T-Cell isolation Kit (STEMCELL Cat 17951 Lot 17F79887) as per manufacturer's instructions. Purity of isolated CD3 T cells was assessed using flowcytometry with FITC-conjugated anti-CD3 antibody (Ab) (BioLegend, clone UCHT1, cat 300406), median $CD3^+$ cells of 98.15%, range 94.7-99.2%. Isolated T cells were cultured in complete medium (CM) with itolizumab (10 μg/ml) or cetuximab (10 μg/ml) at 37'C, 5% CO2 for 96 hours.

Cells were analyzed using flow cytometry (supplemental Table 4) and analyzed using FlowJo software. An example of gating strategy used is shown in Supplemental Figure 3.

Statistical analysis

Data analysis was primarily descriptive using the Wilcoxon rank-sum test for unpaired group comparison and the Wilcoxon signed-rank test for paired comparison. All tests were 2-sided at the significance level of 0.05 and multiple comparisons were not considered. Statistical analysis was performed using SAS version 9.2 (SAS Institute, Inc., Cary, NC) and R version 3.3.2 (the CRAN project) and Prism software (GraphPad). All graphs were made using Prism software (GraphPad). Heatmap was generated using GENE-E (http://www.broadinstitute.org/ cancer/software/GENE-E).

EXP	SEX	AGE	DX	DONOR	GVHD PROPHY	GVHD GRADE	SKIN	LIVER	GUT	AGVHD TX
1	F	37	NHL	MUD	Siro/Tac/MTX	3	3	0	0	Pred/ECP
2	M	65	NHL	MUD	Siro/Tac/MTX	2	3	0	1	Pred
3	M	42	NHL	MRD	Siro/Tac/MTX	2	3	0	0	Pred
4	F	52	MM	MUD	Siro/Tac/MTX	2	0	1	1	Pred
5	F	49	NHL	MUD	Tac/MTX	1	2	0	0	Pred
6	M	71	MF	MUD	Tac/MTX	2	0	0	1	Pred
7	F	52	NHL	MUD	Tac/MTX	2	3	0	0	Pred
8	M	71	MDS	MUD	Tac/MTX	4	0	2	4	Pred /Ruxolitinib
9	M	61	AML	MUD	Tac/MTX	2	3	0	0	Pred

Table S1. Clinical characteristics of patients with aGVHD used for *in vitro* functional assays. EXP, experiment number; DX, diagnosis; GVHD PROPHY, Graft versus host disease prophylaxis; aGVHD TX, acute GVHD treatment; NHL, non-Hodgkin lymphoma; MM, multiple myeloma, MF, Myelofibrosis; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; MUD, matched unrelated donor; MRD, matched related donor; Siro, sirolimus; Tac, tacrolimus; MTX, methotrexate; Pred, prednisone > 1 mg/Kg; ECP, extracorporeal photopheresis. All patients received a reduced intensity conditioning based on busulfan and fludarabine combination and a peripheral blood stem cell graft.

TUBE	TARGET	FLUOROCHROME	CLONE	COMPANY	PRODUCT
T CELL TUBE	CD45RA	BV605	HL100	BioLegend	304133
	CD279 PD-1	PE	J105	Invitrogen	12-2799-42
	CD127	APC	eBioRDR5	Invitrogen	17-1278-42
	CD25	FITC	m-A251	BioLegend	356105
	CCR7	BV711	G043-H7	BioLegend	353227
	CD4	BV510	RPA-T4	BioLegend	300545
	CD3	BV786	UCHT1	BioLegend	300472
	CD8	Alexa 700	RPA-T8	BioLegend	301027
	CD95	PeCF594 (PE Dazzle)	DX2	BioLegend	305633
	CD6	BV650	M-T605	BD	743448
APC TUBE	CD45	APC-Cy7	2D1	BD	560178
	LINEAGE:	PE-Cy7			
	CD3		SK7	BD	557851
	CD19		SJ25C1	BD	341093
	CD56		B159	BD	557747
	CD14		M5E2	BD	557742
	HLA-DR	BV510	G46-6	BD	563083
	CD11c	PE-Cy5	B-LY6	BD	551077
	CD123	FITC	AC145	Miltenyi Biotec	130-090-897
	CD166 ALCAM	BV421	3A6	BD	562936
	PD-L1	BV786	MIH1	BD	563739
	CD14	BV605	M5E2	BioLegend	301833

Table S2. Conjugated monoclonal antibodies in the flow cytometry immune monitoring panel. One tube was used for the characterization of CD6 expression on T cells (T CELL TUBE) and another tube for the characterization of ALCAM expression on monocytes and antigen presenting cells (APC TUBE).

TARGET	FLUOROCHROME	CLONE	COMPANY	PRODUCT
CD3	AF700	UCHT1	BD	557943
CD4	BV510	SK3	BD	562970
CD8	PerCP/Cy5.5	RPA-T8	BioLegend	301032
CD25	PE-CF594	M-A251	BD	562403
CD45RO	BV786	UCHL1	BD	564290
CD14	PE-Cy7	M5E2	BioLegend	301814
CD166	BV421	3A6	BD	562936
ANNEXIN V	PE	/	BD	556422
CFSE	CFSE	/	Invitrogen Thermo Fisher Scientific	C34554
ZOMBIE NIR	APC-Cy7	/	BioLegend	423105

Table S3. Conjugated monoclonal antibodies used in the T cell activation assay (Figure 4)

TARGET	FLUOROCHROME	CLONE	COMPANY	PRODUCT
CD4	BV510	SK3	BD	562970
CD8	PerCP/Cy5.5	RPA-T8	BioLegend	301032
CD25	PE-CF594	M-A251	BD	562403
CD45RO	BV786	UCHL1	BD	564290
CD166	BV421	3A6	BD	562936
CFSE	CFSE	/	Invitrogen Thermo Fisher Scientific	C34554
ZOMBIE NIR	APC-Cy7	/	BioLegend	423105

Table S4 Conjugated monoclonal antibodies used for ALCAM and T cell activation assay (Figure 6).

	Day 30 T cell chimerism at 1 month after HCT								
	<95				95-100				
	N	**Median**	**Q1**	**Q3**	**N**	**Median**	**Q1**	**Q3**	**p-value**
%CD6$^+$ Treg	56	74.25	68.11	82.68	11	69.9	58.83	80.45	0.26
%CD6$^+$ Tcon	56	98.08	97.07	98.84	11	97.42	95.98	98.1	0.25
%CD6$^+$ CD8$^+$ T cells	56	87.04	82.92	91.7	11	76	70.91	94.49	0.62
%ALCAM in CD14$^+$ monocytes	75	80.44	72.83	85.79	12	83.78	81.16	85.91	0.16
%ALCAM$^+$ mDC	75	18.74	13.51	28.61	12	16.73	10.01	21.91	0.37
%ALCAM$^+$ pDC	75	95	92.05	97.97	12	94.11	88	99.13	0.71
CD6 MFI in CD8 + T cells	56	8886	7225.8	10488	11	9149.6	6234.4	10784	0.77
CD6 MFI in Treg	56	6507.4	5936	7969.9	10	6508.4	4721.9	8022.3	0.49
CD6 MFI in Tcon	56	17057	14328	18599	11	16107	13399	17949	0.44
ALCAM MFI in CD14+ monocytes	75	10131	8379.5	11385	12	12141	8668.4	13496	0.22
ALCAM MFI in mDCs	75	3609.5	3043.6	4314.3	12	3209.7	2914.9	4103.2	0.37
ALCAM MFI in pDCs	75	18307	15962	22602	12	21176	16139	25528	0.30

Table S5. Expression of CD6 and ALCAM in patients with different levels of donor chimerism.

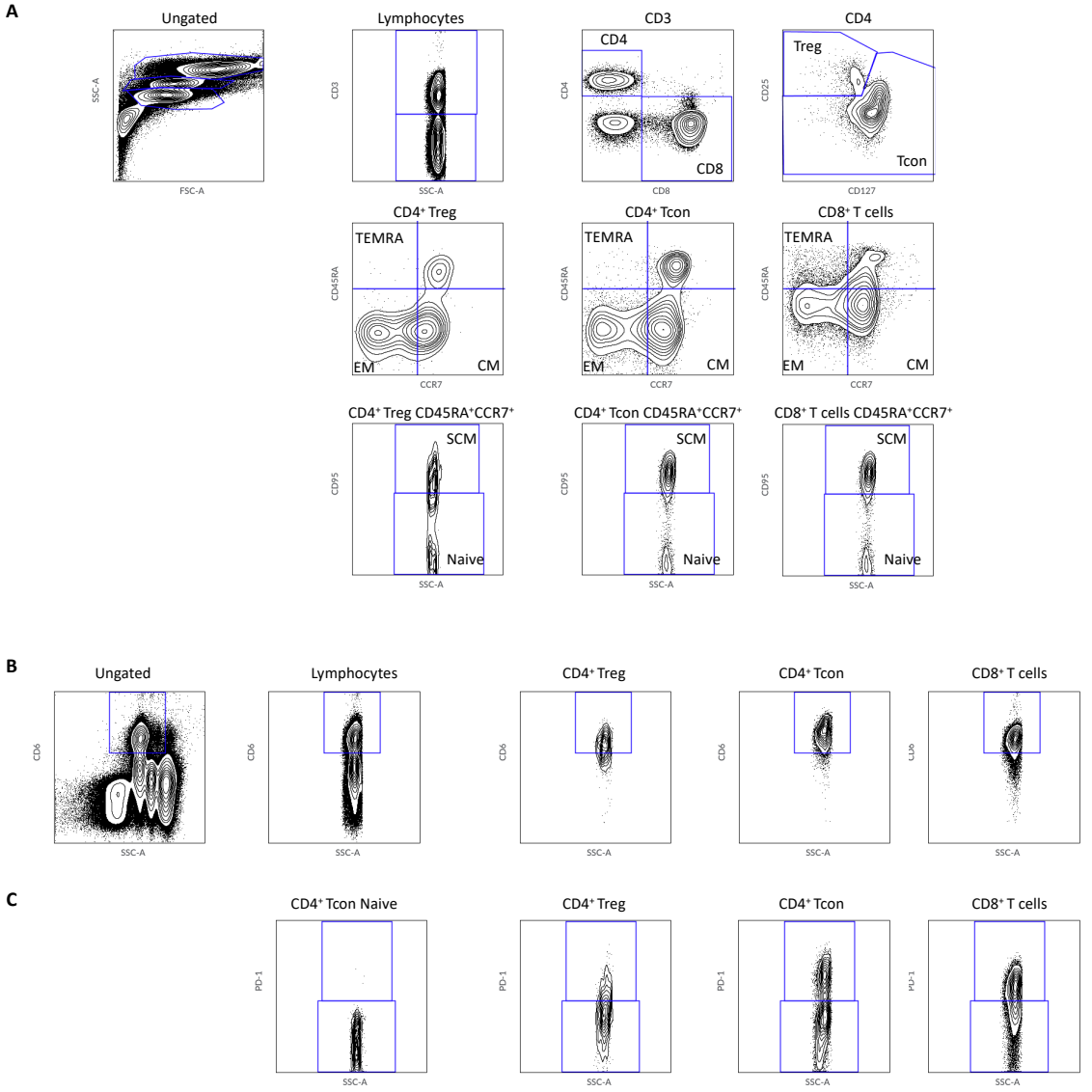

Figure S1. **Gating strategy for T cell subset analysis**. (A) Three major T cell populations, CD4Treg, CD4Tcon, and CD8 T cells, were defined as $CD3^+CD4^+CD8^-CD25^+CD127^-$, $CD3^+CD4^+CD8^-$ non-Treg cells and $CD3^+CD4^-CD8^+$, respectively. Within each T cell population, subsets were defined as follows: central memory (CM, $CD45RA^-CCR7^+$), effector memory (EM, $CD45RA^-CCR7^-$), terminally differentiated effector memory (TEMRA, $CD45RA^+CCR7^-$), T cells naïve ($CD45RA^+CCR7^+$ $CD95^-$) and stem cell memory (SCM, $CD45RA^+CCR7^+$ $CD95^+$). (B) The threshold of CD6 gate on T cell subsets was made using monocytes as internal negative control. (C) PD-1 expression was monitored on each T cell subset, using Tcon naïve cells as internal negative control. 265 peripheral blood samples were tested with this panel: n=9 HD; n=67 at 1 mo; n=76 at 2 mo; n=78 at 3 mo; n=35 at 6mo.

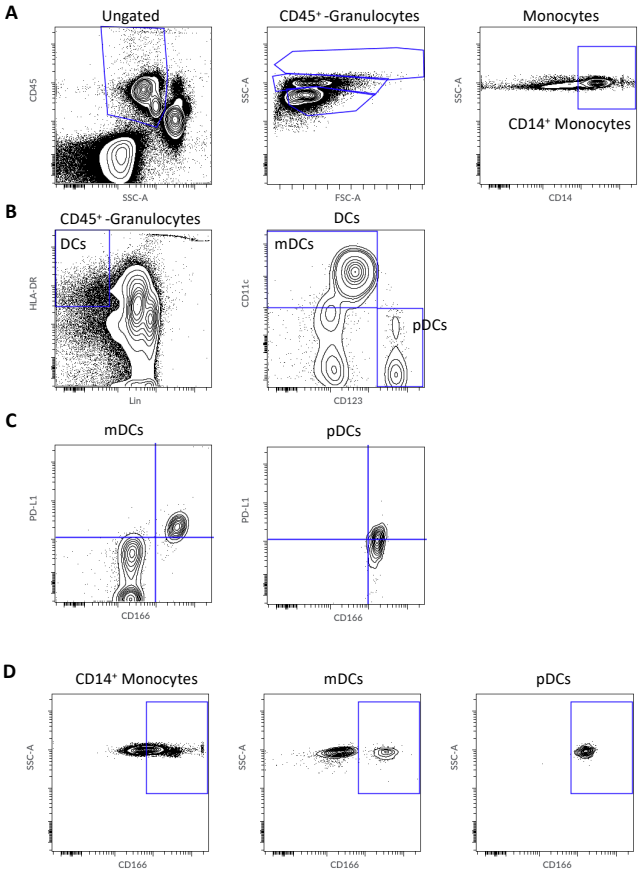

Figure S2. **Gating strategy for dendritic cell subsets and monocyte analysis**. (A) Monocyte were defined within the CD45$^+$ cells, after excluding granulocytes, based on the SSC-A, FSC-A and CD14 expression. (B) Dendritic cells (DCs) were defined within the CD45+ cells, after excluding granulocytes, based on the positivity of HLA-DR and negativity of lineage markers (CD14, CD3, CD19 and CD56). DC subsets were defined as follows: myeloid DCs (mDCs, CD11c$^+$CD123$^-$) and plasmacytoid DCs (pDCs, CD11c$^-$CD123$^+$). (C) PD-L1 and CD166 (ALCAM) expression on both mDCs and pDCs. (D) CD166 (ALCAM) expression on CD14$^+$ monocytes, mDCs and pDCs. Samples with less than 1000 DC events were excluded from the analysis. 236 peripheral blood samples were tested with this panel: n=9 HD; n=62 at 1 mo; n=66 at 2 mo; n=69 at 3 mo; n=30 at 6mo.

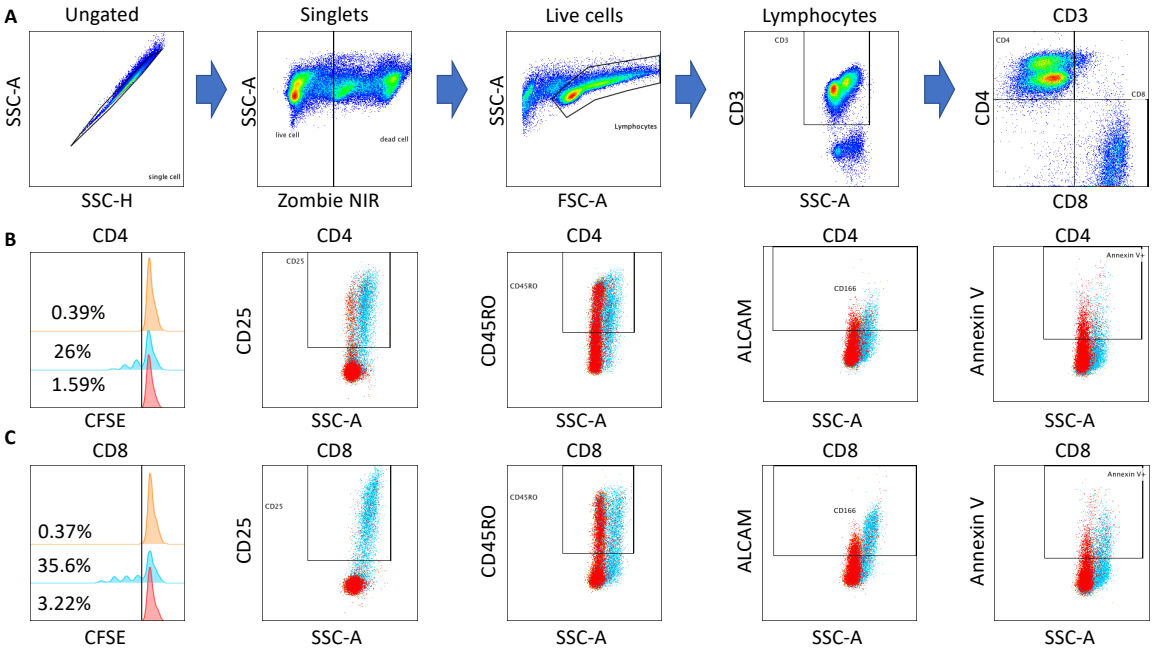

Figure S3. **Gating strategy for *in vitro* functional assays**. (A) Example of the gating strategy of $CD4^+$ and $CD8^+$ T cells, after exclusion of doublets and dead cells. Gating strategy on CD4+ T cells (B) and CD8+ T cells (C) for CFSE dilution (T cell proliferation), CD25 (T cell activation), CD45RO (T cell maturation), ALCAM and Annexin V (T cell apoptosis) expression. Cell alone (orange), cells+beads+cetuximab (blue), cells+beads+itolizumab (red).

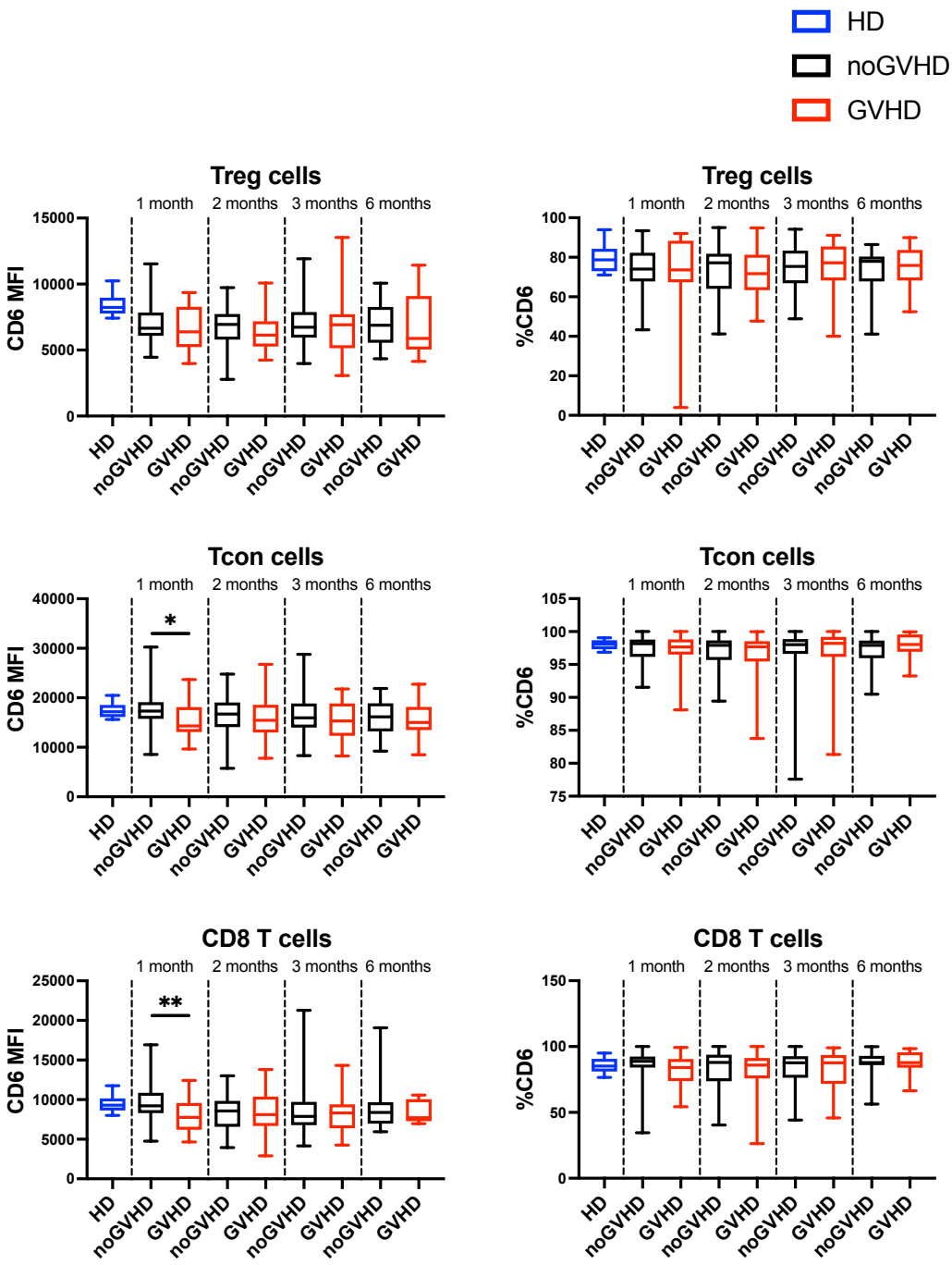

Figure S4. Comparison of CD6 expression on Treg, Tcon and CD8 T cells in patients with and without GVHD at specific time points after transplant. CD6 MFI was lower in GVHD group compared noGVHD group at 1 month after transplant in Tcon and CD8 T cells. Statistically significant differences are noted: * P< 0.05; ** P< 0.01. Wilcoxon rank-sum test. HD, n=9; noGVHD month 1, n=41; GVHD month 1, n=26; noGVHD month 2, n=40; GVHD month 2, n=36; noGVHD month 3, n=45; GVHD month 3, n=33; noGVHD month 6, n=23; GVHD month 6, n=12.

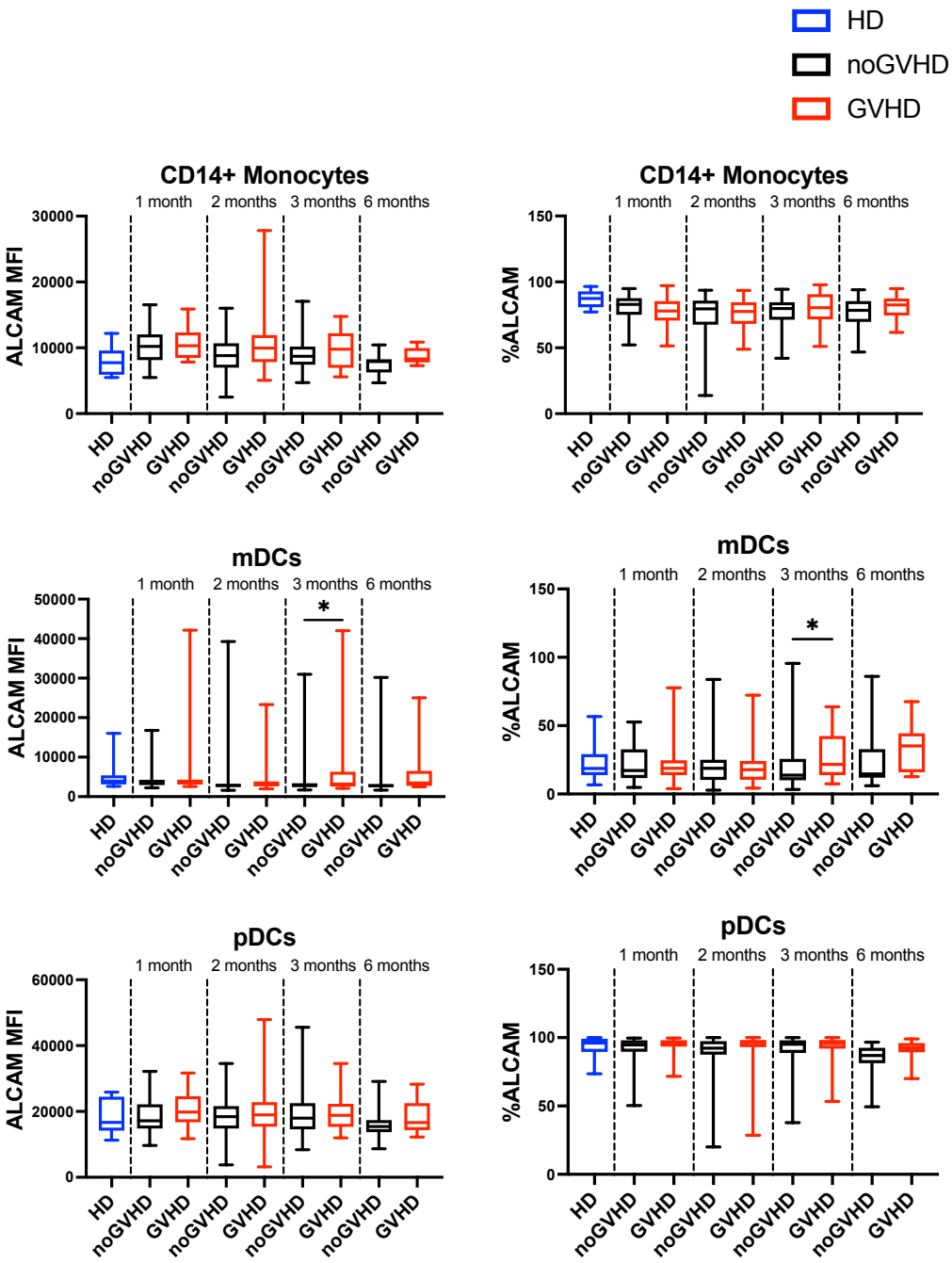

Figure S5. Comparison of ALCAM expression on CD14+ Monocytes, mDCs and pDCs in patients with and without GVHD at specific time points after transplant. ALCAM MFI and % of ALCAM positive cells on mDCs was higher in the GVHD cohort compared to noGVHD at 3 months after transplant. Statistically significant differences are noted: * P< 0.05; Wilcoxon rank-sum test. HD, n=9; noGVHD month 1, n=41; GVHD month 1, n=26; noGVHD month 2, n=40; GVHD month 2, n=36; noGVHD month 3, n=45; GVHD month 3, n=33; noGVHD month 6, n=23; GVHD month 6, n=12.

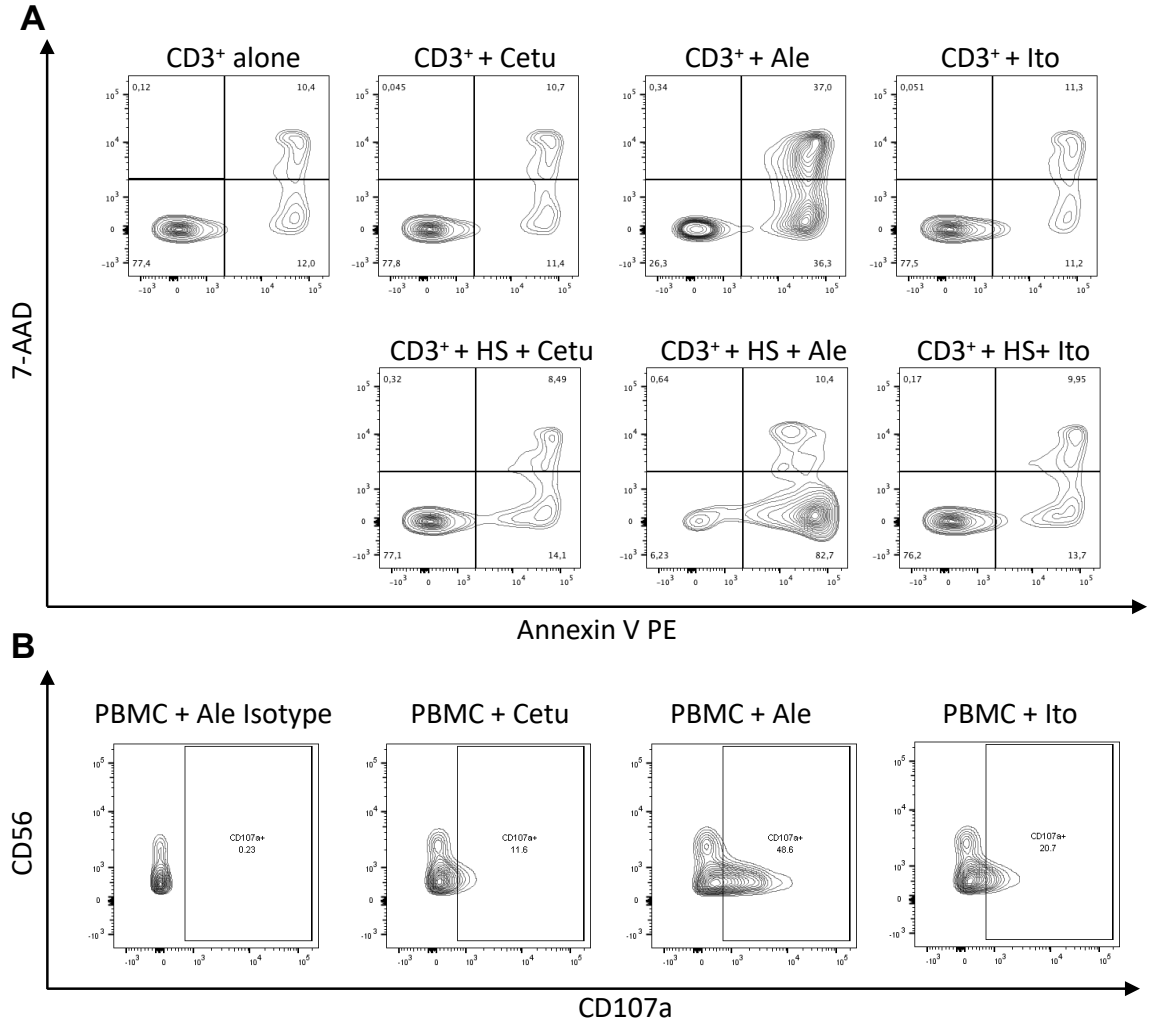

Figure S6. **Gating strategy to test itolizumab complement dependent cytotoxicity (CDC), antibody dependent cytotoxicity (ADC) and antibody direct cellular cytotoxicity (ADCC) analysis.**

(A) Representative example of percentage of cell lyses, based on the expression of 7-AAD and annexin V. (B) Representative example of CD107a expression on NK cells, as marker of ADCC. An APC-conjugated mouse isotype was used to design the gate for CD107a. Percentage of cell lysis was calculated by adding the percentage of positive cells for 7-AAD or Annexin V or both. CDC activity was calculated by subtracting the values obtained in the medium + antibody from the values obtained in the culture with medium + antibody + human serum (HS). ADC activity was calculated by subtracting the values obtained in the culture with medium alone from the values obtained in the culture with medium + antibody. For ADCC PBMC form HD were cultured in the presence of antibody for 6 hours. Both percentage of cell lyses and CD107a expression on NK cells were evaluated after 6 hours of culture.