$CD56^{bright}CD16^{-}$ natural killer cells as an important regulatory mechanism in chronic graft-*versus*-host disease

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Supplementary material

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Supplementary Methods

Antibody clones

CD45-PB, Clone: (BioLegend, San Diego, CA, USA)

CD3-PE, Clone: (BioLegend, San Diego, CA, USA)

CD19-PE, Clone: (BioLegend, San Diego, CA, USA)

CD14-PE, Clone: (BioLegend, San Diego, CA, USA)

CD66-PE, Clone: (BioLegend, San Diego, CA, USA)

CD235-PE, Clone: (BioLegend, San Diego, CA, USA)

CD56-APC, Clone: (BioLegend, San Diego, CA, USA)

CD16-FITC, Clone: (BioLegend, San Diego, CA, USA)

CD56-PE, Clone: REA196 (Miltenyi Biotec, Auburn, CA, USA)

CXCR3-FITC, Clone: REA232 (Miltenyi Biotec, Auburn, CA, USA)

Perforin-BV785, Clone: Delta G9 (Miltenyi Biotec, Auburn, CA, USA)

CD16-APC Fire 750, Clone: 3G8 (BioLegend, San Diego, CA, USA)

Granzyme K-AF647, Clone: GM26E7 (BioLegend, San Diego, CA, USA)

GPR183-BV421, Clone: SA313E4 (BioLegend, San Diego, CA, USA)

KIR/CD158-APC (KIR2DL2, 2DL3, 2DS2, and 2DS4), Clone: HP-MA4 (BioLegend, San Diego, CA, USA)

CD25-PE, Clone: BC96 (BioLegend, San Diego, CA, USA)

NKp44(CD336)-APC, Clone: P44-8 (BioLegend, San Diego, CA, USA)

NKp46(CD335)-BV421, Clone: 9E2 (BioLegend, San Diego, CA, USA)

CD116(GM-CSFR)-PE/Cy7, Clone: 4H1 (BioLegend, San Diego, CA, USA)

CD3-BUV805, Clone: UCHT1 (BD Biosciences, Franklin Lake, NJ, USA)

Granzyme B-PE CF594, Clone: GB11 (BD Biosciences, Franklin Lake, NJ, USA)

CD127-APC R700, Clone: HIL-7R-M21 (BD Biosciences, Franklin Lake, NJ, USA)

CD62L-BUV737, Clone: DREG-56 (BD Biosciences, Franklin Lake, NJ, USA)

Immune cell populations in HSCT patients associated with cGvHD

HSCT patient peripheral blood mononuclear cells (PBMC) were utilized from a multi-centre pediatric cohort (Applied Biomarkers in Late Effects (ABLE)/PBMTC 1202 trial) (previously described[7,15,16]) (supplementary table 1) for the identification of immune cell populations associated with cGvHD. Twenty-seven pediatric transplant centers enrolled 302 patients. Patients were placed into 5 cohorts [no late aGvHD and no cGvHD (n = 132), late aGvHD (n = 30), cGvHD (including overlap syndrome; n = 41), active aGvHD day +100 to 114 (n = 28), or active cGvHD before day +114 (n = 10)].

For performing the statistical analysis, we were interested in whether CD56bright and CD56dim NK cells show differential expression for each group (cGvHD, aGvHD, and controls) as well as whether each of these cell subtypes show differential expression between controls and GvHD subjects. To test for differential expression between CD56bright and CD56dim NK cells of each gene, we applied a paired t-test on samples from each group. To test for group differences of each gene, we applied a Student's t-test on samples from each cell subtype. The expression value of each gene was normalized by dividing its value by the l2-norm of expression values of house-keeping genes (PGK1 and GAPDH) prior to analysis. For the cell subtype contrast, we declared a gene as relevant if its p-value is <0.05 and its effect ratio is >5 or <0.2, where effect ratio was defined as the mean expression of CD56bright over the mean expression of CD56dim NK cells for each group. For the group contrast, we declared a gene as relevant if its p-value is <0.05 and its effect ratio is >2 or <0.5, where effect ratio was defined as the mean expression of controls over the mean expression of GvHD subjects for each cell subtype. Given the low number of samples, we opted to use a nominal p-value threshold of 0.05 to get a more encompassing view of the differential expression patterns. Since numerous tests were conducted, the probability of a Type I error likely exceeded 0.05 but would be moderated by the additional effect ratio criteria.

We further estimated the Pearson's correlation between CD56bright Perforin negative and 74 cellular markers using measurements of all subjects collected at 3-, 6-, and 12-months post HSCT. Confounding factors including graft type (peripheral blood stem cells, bone marrow, or cord), donor source (sibling or unrelated), HLA mismatch, sex (donor and recipient), ABO mismatch, reduced intensity conditioning or Myeloablative conditioning, serotherapy (including anti-thymocyte globulin - ATG and alemtuzumab), recipient age, the use of total body irradiation, and number of days post HSCT to sample collection as well as binary variables encoding groups 1 to 6 were regressed out from the marker values prior to estimating Pearson's correlation. To combine correlation values across the three time points, we simply took the average. To combine p-values across the three time points, we performed aggregated Cauchy association test. Significant correlation was declared at an α of 0.05 with Bonferroni correlation.

NanoString study sample collection, cell sorting, RNA extraction, and analysis

Day 100 HSCT archived patient peripheral blood mononuclear cells (PBMC) from three separate cohorts [cGvHD (N = 6), late aGvHD $\geq$114 days after HSCT (N = 6), or no late aGvHD or cGvHD (N = 6)] were utilized from a well characterized multi-centre pediatric cohort (Applied Biomarkers in Late Effects (ABLE)/PBMTC 1202 trial) as previously described[7,15,16] (supplementary table 1). Samples were selected from this cohort based on the number of cells (samples with the highest cell number were selected). PBMC were isolated within 24hrs after blood collection in DB Sodium Heparin tubes by Lymphoprep (StemCell Technologies, Vancouver, BC, Canada) density gradient centrifugation and cryopreserved in the vapor phase of liquid nitrogen. Note that one sample (34-001-3) did not have enough CD56bright NK cells for the analysis (but analysis on the CD56dim NK cells for this sample was completed).

To differentiate NK cell subpopulations, PBMC samples were stained by CD45-PB, CD3-PE, CD19-PE, CD14-PE, CD66-PE, CD235-PE, CD56-APC, and CD16-FITC (BioLegend, San Diego, CA, USA) (clones mentioned in supplemental methods) conjugated antibodies for 30min at 4°C in the dark. After two washes with 2% FBS PBS (Gibco, Grand Island, NY, USA) 7-AAD was added (BioLegend, San Diego, CA, USA), and samples were sorted using FACS Aria IIu (BD Biosciences). Dead cells, monocytes, and T and B lymphocytes were gated out, and NK cell subpopulations were sorted based on the differential CD56 and CD16 expression.

Total RNA was extracted from NK cell subpopulations using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). We determined the quantity of RNA samples by using a NanoDrop 1000 Spectrophotometer. The quality and

quantity of RNA samples were tested using the Agilent 2100 Bioanalyzer system at the Centre of Molecular Medicine and Therapeutics core facility at the University of British Columbia (Vancouver, BC, Canada). The average RNA Integrity Numbers were 9.4+/- 0.7 and 9.1 +/- out of 10 for the CD56dim and CD56bright subpopulations, respectively.

To determine gene expression analysis in NK Cells, the nCounter Immunology Panel was used. NanoString data was obtained utilizing the automated Prep Station followed by data collection on a nCounter FLEX Digital Analyzer. The resulting data was analyzed by the nSolver 4.0 Analysis Software System (nanoString, Seattle).

For performing the statistical analysis, we were interested in whether CD56bright and CD56dim NK cells show differential expression for each group (cGvHD, aGvHD, and controls) as well as whether each of these cell subtypes show differential expression between controls and GvHD subjects. To test for differential expression between CD56bright and CD56dim of each gene, we applied a paired t-test on samples from each group. To test for group differences of each gene, we applied a Student's t-test on samples from each cell subtype. Expression value of each gene was normalized by dividing its value by the l2-norm of expression values of house-keeping genes (PGK1 and GAPDH) prior to analysis. For the cell subtype contrast, we declared a gene as relevant if its p-value is <0.05 and its effect ratio is >5 or <0.2, where effect ratio was defined as the mean expression of CD56bright over the mean expression of CD56dim for each group. For the group contrast, we declared a gene as relevant if its p-value is <0.05 and its effect ratio is >2 or <0.5, where effect ratio was defined as the mean expression of controls over the mean expression of GvHD subjects for each cell subtype. Given the low number of samples, we opted to use a nominal p-value threshold of 0.05 to get a more encompassing view of the differential expression patterns. Since numerous tests were conducted, the probability of a Type I error likely exceeded 0.05 but would be moderated by the additional effect ratio criteria.

Characterization of CD56bright NK cells according to phenotype

Peripheral whole blood samples were collected into BD Vacutainer Sodium Heparin tubes (Becton, Dickinson, Franklin Lake, NJ, USA) from consenting healthy donors (Human Ethics ID's H16-00533 and H18-00022). Blood samples were processed within 24hrs of collection. From blood samples, PBMC were isolated by Ficoll Hypaque (StemCell Technologies, Vancouver, BC, Canada) density gradient centrifugation. PBMCs were stained for protein expression identification of NK cells using: [CD56-PE, CXCR3-FITC, Perforin-BV785 (Miltenyi Biotec, Auburn, CA, USA)], [CD16-APC Fire 750, Granzyme K-AF647, GPR183-BV421, KIR/CD158-APC (KIR2DL2, 2DL3, 2DS2, and 2DS4), CD25-PE, NKp44(CD336)-APC, NKp46(CD335)-BV421, CD116(GM-CSFR)-PE/Cy7 (BioLegend, San Diego, CA, USA)], [CD3-BUV805, Granzyme B-PE CF594, CD127-APC R700, CD62L-BUV737 (BD Biosciences, Franklin Lake, NJ, USA)]. Cell surface antibodies were added, the samples incubated for 20min at 4^{o}C in the dark, then were washed, followed by the Invitrogen IC Fixation Buffer procedure (Invitrogen, Waltham, MA, USA). Next, the cells were washed and FACS permeabilizing solution 2 (BD Biosciences, Franklin Lake, USA) was added according to manufactures procedures. The phenotypic expression of the NK cells was investigated through the BD FACSymphony flow cytometer. The total NK cell population was gated by selecting the CD56+ and CD3- cells.

NK cell isolation for all functional evaluations

Peripheral whole blood samples were collected and processed for PBMC. PBMC samples were enriched for total NK cells using the NK Cell Enrichment Kit (StemCell Technologies, Vancouver, BC, Canada) according to manufacturer's procedures. To differentiate the CD56brightCD16- NKreg cells from CD56dimCD16+ NK cells, enriched cells were stained using CD56-PE, CD3-APC, and CD16-FITC conjugated antibodies (BioLegend, San Diego, CA, USA) for 20min at 4°C in the dark. After two washes, 7-AAD (BioLegend, San Diego, CA, USA) was added, and the samples were sorted using sterile Beckman Coulter Astrios FACS sorting. Dead cells and CD3+ lymphocytes were gated out, and the NK cell subpopulations were sorted based on the differential expression of CD56 and CD16.

Evaluation of NKreg cell suppression

Peripheral whole blood samples were collected and processed for PBMC. CD4+ T cells were isolated using the EasySep™ Human CD4+ T Cell Isolation Kit (StemCell Technologies, Vancouver, BC, Canada), stained using Cell Proliferation Dye eF450 (eBioscience, Mississauga, ON, Canada), and activated at the time of co-culture via the ImmunoCult™ Human CD3/CD28 T Cell Activator (StemCell Technologies, Vancouver, BC, Canada) according to manufacturer's procedures. The CD56brightCD16- NK cells and CD56dimCD16+ NK cells were sorted. The CD56brightCD16- and CD56dimCD16+ NK cells were co-cultured with allogeneic activated CD4+ T cells in a round-bottom 96-well plate (Sarstedt Inc, Newton, NC, USA) at the ratios 1:1 (100K:100K), 1:2 (50K:100K), 1:4 (25K:100K), and 1:8 (12.5K:100K) NK cells to T cells, respectively, in a volume of 200µl. Control wells included activated CD4+ T cells, and non-activated CD4+ T cells. On day zero, BD LSRII Flow Cytometric analysis was completed to determine the initial cell proportion, cell viability, and baseline for the day-4 proliferation analysis. The co-culture was incubated at 37°C and 5% CO for 96hrs with complete DMEM medium (10% FBS, 1% glutamine, 1% Penicillin/Streptomycin) (Gibco, Grand Island, NY, USA). The NKreg and CD56dimCD16+ suppression experiments were repeated five times using cells derived from five different healthy donors. For the Treg versus CD4+ T cell control assay, the above procedures were performed identically using Treg cells as the suppressor population, which were provided by collaborators (previously sorted, expanded, and cryopreserved). Prior to use, the Treg cells were thawed and cultured overnight with Immunocult medium (StemCell Technologies, Vancouver, BC, Canada). The Treg suppression experiment was repeated twice using cells derived from two different healthy donors. Further, we investigated the suppressive capacity of NKreg cells towards allogeneic CD8+ T cells at the 1:1 ratio. The CD8+ T cells were isolated using the EasySep™ Human CD8+ T Cell Isolation Kit (StemCell Technologies, Vancouver, BC, Canada), and all other steps were identical to the CD4+ T cell suppression assay.

On day 4, the proliferation and viability of the T cells in co-culture were evaluated via cell proliferation dye dilution, 7-AAD dye (BioLegend, San Diego, CA, USA), and cell counting beads (Invitrogen, Waltham, MA, USA) using BD LSRII flow cytometry, and the data was analysed using Flowjo software (version 10.7.1). The flow cytometry analysis included gating for eF450 positive and 7-AAD negative cells (live T cells). The proliferation modeling function was used to determine the division index of gated live T cells. Percentage of suppression = 1 – (division index of target cells co-cultured with suppressor cells / division index of target cells cultured without suppressor cells) x 100%.

To determine if the suppressive capacity of CD56brightCD16- NKreg cells on CD4+ T cells was contact dependent the above procedures were followed using a 96-well transwell plate (Corning, Corning, NY, USA). The NK cells were inserted to the lower chamber at a volume of 200µl and the CD4+ T cells were inserted to the upper chamber at a volume of 75µl. To verify this finding, we removed the supernatant from a 96hr 1:1 NKreg versus CD4+ T cell suppression assay and cultured the supernatant with fresh allogeneic activated CD4+ T cells for 72hrs and analysed the CD4+ T cell proliferation as described above. The transwell suppression assay experiment was repeated five times using cells derived from five different healthy donors.

To determine if the suppressive mechanism of NKreg cells is reliant on NKp44, NKp46, or GPR183 receptors the above procedures were followed for preparing and analyzing the suppression assay. The soluble antagonists were added separately to a 1:1 NKreg:CD4+ condition at the following concentrations (previously reported to be optimal for *in vitro* blocking): 1ug/ml Ultra-LEAF™ Purified anti-human NKp46 (BioLegend, San Diego, CA, USA)10, 1ug/ml Ultra-LEAF™ Purified anti-human NKp44 (BioLegend, San Diego, CA, USA)10, and 25nM NIBR18920 (Sigma Aldrich, Oakville, CA, USA)18. The suppression assay with blocker experiments were repeated three times using cells derived from three different healthy donors.

Evaluation of NKreg cell induction of apoptosis

The FITC Annexin V apoptosis detection kit (BioLegend, San Diego, CA, USA) was utilized according to manufacturers procedures. After 96hrs, the suppression assay co-cultured cells were harvested. To phenotypically identify the two cell populations, the NK cells were stained with CD56-APC and the T cells were stained with CD4-

PB (BioLegend, San Diego, CA, USA). BD LSRII flow cytometry and Kaluza software (version 2.1) was utilized for acquiring and analysing the data. Annexin V and 7-AAD may be used to determine the proportion of apoptotic CD4+ T cells after co-culture [early apoptosis (7-AAD-, Annexin V+) and late apoptosis (7-AAD+, Annexin V+)]. The apoptosis detection assay was repeated three times using cells derived from three different healthy donors.

Further, this kit was utilized to determine the cytolytic effect of FACS sorted NKreg cells towards MOLT¬-4 (ATCC® CRL¬1582™), and Jurkat, Clone E6¬1 (ATCC® TIB¬152™) cell lines. The NK and leukemic cells were co-cultured for 72hrs at 37^{o}C and 5% CO_2 at the 1:1 (50K:50K cells), and 10:1 (50K:5K cells) ratios, respectively. After 72hrs, the co-cultured cells were harvested. To phenotypically identify the NK cells, the samples were stained with CD56-APC (BioLegend, San Diego, CA, USA). BD LSRII flow cytometry and Kaluza software (version 2.1) was utilized for acquiring and analysing the data. Annexin V and 7-AAD may be used to determine the proportion of apoptotic leukemic cells after co-culture [early apoptosis (7-AAD-, Annexin V+) and late apoptosis (7-AAD+, Annexin V+)]. The apoptosis detection assay was repeated two times using NK cells derived from two different healthy donors.

Evaluation of NKreg cell induction of K562 cell killing

Peripheral whole blood samples were collected and processed for PBMC. Sorted NKreg cells and CD56dim NK cells were co-cultured with D-luciferin K562 cells (Gold Biotechnology, St Louis, MO, USA) at the ratios 2:1 and 1:1 (NK:K562) in a black 96-well plate (Corning, Corning, NY, USA) and conditioned medium [MACS NK medium (Miltenyi Biotec, Auburn, CA, USA), 25ng/ml IL-2 (BioLegend, San Diego, CA, USA), 1/5000 anti-CD2 and anti-CD335 (Biolegend, San Diego, CA, USA), and 10% human AB serum (Corning, Corning, NY, USA)]. Luciferin was added at a 1/1000 dilution. The proportion of live K562 cells after incubation at 37^{o}C and 5% CO_2 for 24hrs was determined using Spectral Instruments luminescence imaging (Ami-X). This experiment was repeated twice using cells derived from two different healthy donors.

Functional assay statistical analysis

Kaluza Analysis version 2.1 software (Beckman Coulter, Mississauga, ON, Canada) was used for FACs analysis and creation of flow cytometry dot plots. For the suppression assay experiments, analysis was performed using Flowjo software version 10.7.1 (proliferation modeling function) (BD Biosciences, Franklin Lake, USA). Statistical analyses were performed using Microsoft Excel version 2110 and a two-tailed T test – two-sample assuming unequal variance. $P < 0.05$ = *, $P < 0.005$ = **, $P < 0.0005$ = ***, NS = not significant.

Supplementary figure legend

Supplemental figure 1: Cell surface and intracellular characteristics of the NK cell population.
Healthy donor PBMC were stained for NK cell surface and intracellular expression of select markers and analyzed by flow cytometry. Lymphocytes were first gated using FSC and SSC. Single cells were gated using FSC-H and FSC-W. NK cells were gated as $CD56^{+}CD3^{-}$ cells. $CD56^{bright}$ NK cells have low expression of CD16, perforin, and Granzyme B, unlike the $CD56^{dim}$ NK cells. However, $CD56^{bright}$ NK cells show high expression of GPR183R, CD127 (IL7R), CD62L (SELL), Granzyme K, CXCR3, CD25, NKp44, and NKp46. The data is from a single experiment representative of 3 experiments.

Supplemental figure 2: KIR (polyclonal antibody) expression on the NK cell population.
Phenotyping of $CD56^{+}CD3^{-}$ NK cells was completed using the BD FACSymphony flow cytometer. $CD56^{bright}$ NK cells have low expression KIR, unlike the $CD56^{dim}$ NK cells. Phenotyping was completed with use of three differing fresh healthy donor blood samples.

Supplemental figure 3: Sorting strategy for $CD56^{bright}CD16^{-}$ NK_{reg} cells, and the perforin and Granzyme B expression of sorted NK_{reg} cells.
$CD56^{bright}CD16^{-}$ NK_{reg} cells were sorted from healthy donor peripheral blood. Enriched NK cells were stained with the conjugated antibodies CD56-PE, CD3-APC, CD16-FITC, and 7-AAD. A) With use of FSC-A and SSC-A, lymphocytes were gated (gate A) and using FSC-H and FSC-W single cells were gated (gate B). After, live NK cells were gated by selecting $7\text{-}AAD^{-}$ and $CD3^{-}$ cells (gate C). Lastly, the NK_{reg} cells were gated as the $CD56^{bright}CD16^{-}$ population, and for control assays, the $CD56^{dim}CD16^{+}$ NK cells were also gated and both NK cell subsets were sorted. Note that the percentages shown are representative of the percentage of cells within the specified gate from the total initial cell population taken for sorting. B) Sorted $CD56^{bright}CD16^{-}$ NK cells are 95% Granzyme B^{-} and $perforin^{-}$. The data is from a single experiment representative of all sorting experiments performed (26 experiments).

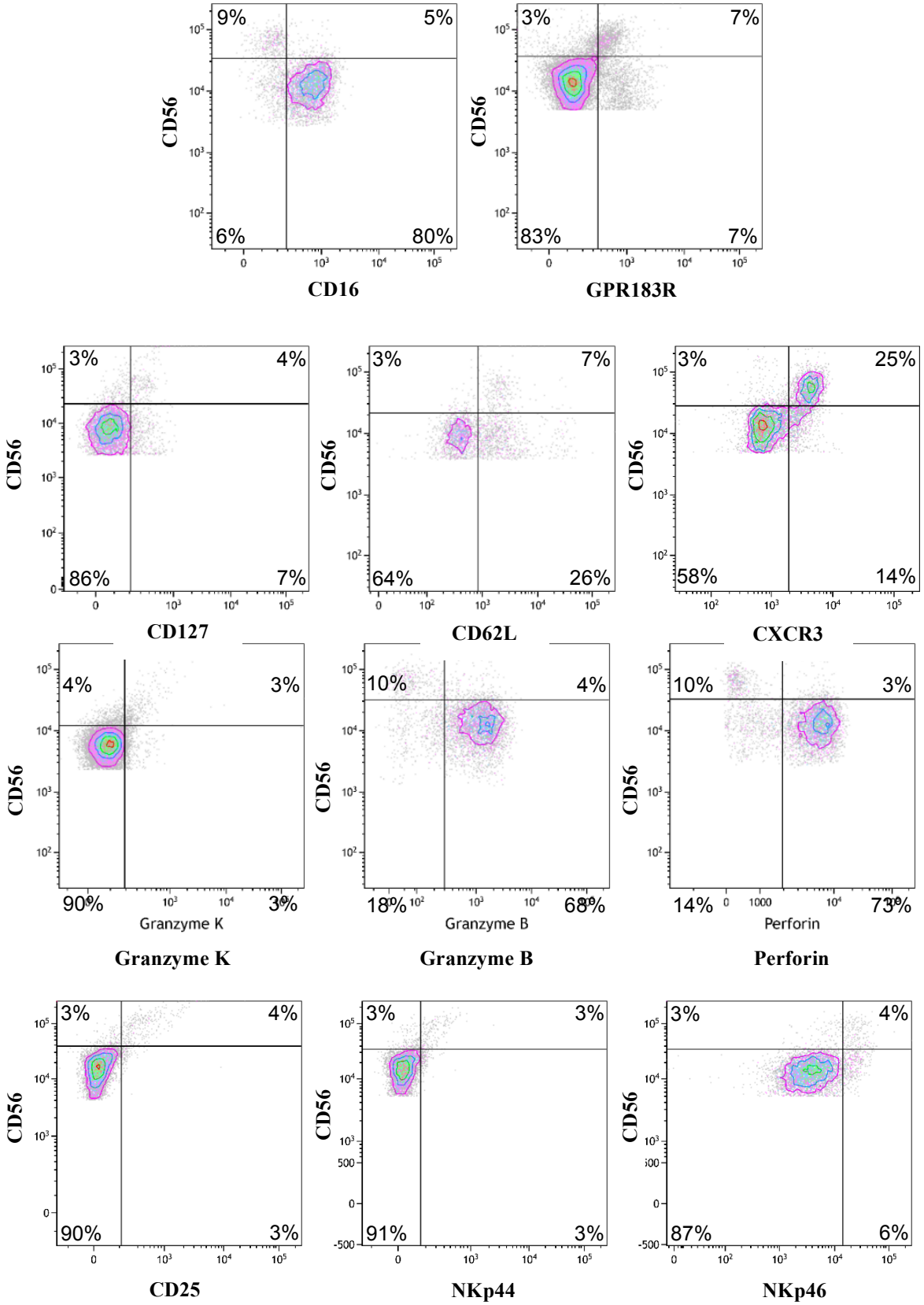
Supplemental figure 4: $CD56^{bright}CD16^{-}$ NK_{reg} cell induction of K562 cell killing.
Cytotoxic effect of sorted $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells towards K562 cells at the ratios 2:1 and 1:1 over a 24-hr period. The percentage of live K562 cells in the K562 cell control as opposed to the K562 cells co-cultured with NK_{reg} cells or NK^{dim} cells are shown (mean +/- SD). $P < 0.05$ = *. Statistical analyses were performed using Microsoft Excel version 2110 and a two-tailed T test – two-sample assuming unequal variance. The data is representative of 2 experiments.

Supplemental figure 5: Evaluation of $CD56^{bright}CD16^{-}$ NK_{reg} cell supernatant suppression of $CD4^{+}$ T cell proliferation.
Suppressive effect of $CD56^{bright}CD16^{-}$ NK_{reg} cell supernatant towards allogeneic $CD4^{+}$ T cells when co-cultured for 72 hrs. A) Proliferation of $CD4^{+}$ T cells in the absence of NK_{reg} cell supernatant (control), B) proliferation of $CD4^{+}$ T cells co-cultured with NK_{reg} cell supernatant. On the histograms, the x-axis displays the cell proliferation dye (CPD) eF450 and the y-axis displays the cell count. Percentage of suppression = 1 – (division index of target cells cultured with supernatant / division index of target cells cultured without supernatant) x 100%.

Supplemental figure 6: Evaluation of NKp44, NKp46, and GPR183R dependence for $CD56^{bright}CD16^{-}$ NK_{reg} cell suppression.
Suppressive effect of $CD56^{bright}CD16^{-}$ NK_{reg} cells towards allogeneic $CD4^{+}$ T cells when cells are co-cultured for 96 hrs at the ratio 1:1 with the NKp44 antibody blocker, NKp46 antibody blocker, or GPR183R antagonist. A) Proliferation of $CD4^{+}$ T cells (control), B) proliferation of $CD4^{+}$ T cells co-cultured with NK_{reg} cells with no soluble blocker/antagonist, C) proliferation of $CD4^{+}$ T cells co-cultured with NK_{reg} cells with NKp44 antibody blocker, D) proliferation of $CD4^{+}$ T cells co-cultured with NK_{reg} cells with NKp46 antibody blocker, E) proliferation of $CD4^{+}$ T cells co-cultured with NK_{reg} cells with GPR183R antagonist. On the histograms, the x-axis displays the cell proliferation dye (CPD) eF450 and the y-axis displays the cell count. F) Proliferation of $CD4^{+}$ T cell control compared to $CD4^{+}$ T cells co-cultured with NK_{reg} cells with or without the NKp44, NKp46, or GPR183R soluble antibody blockers/antagonists (mean +/- SD). NS = not significant. Statistical analyses were performed using Microsoft Excel version 2110 and a two-tailed T test – two-sample assuming unequal variance. The data is from a single experiment representative of 3 experiments.

Supplementary figures

Supplemental figure 1:



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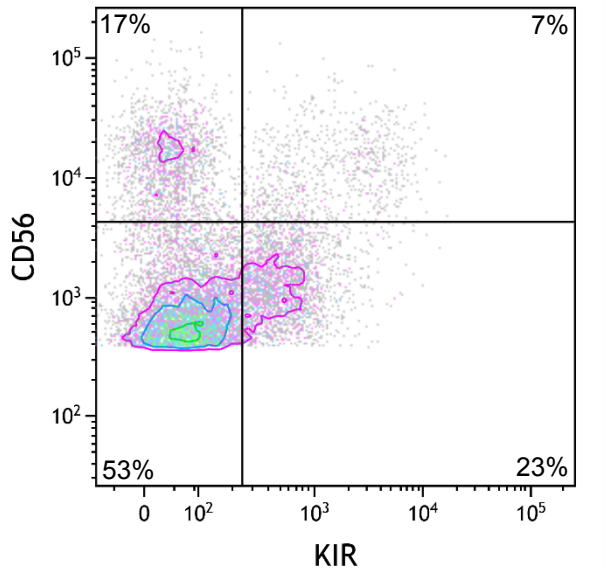
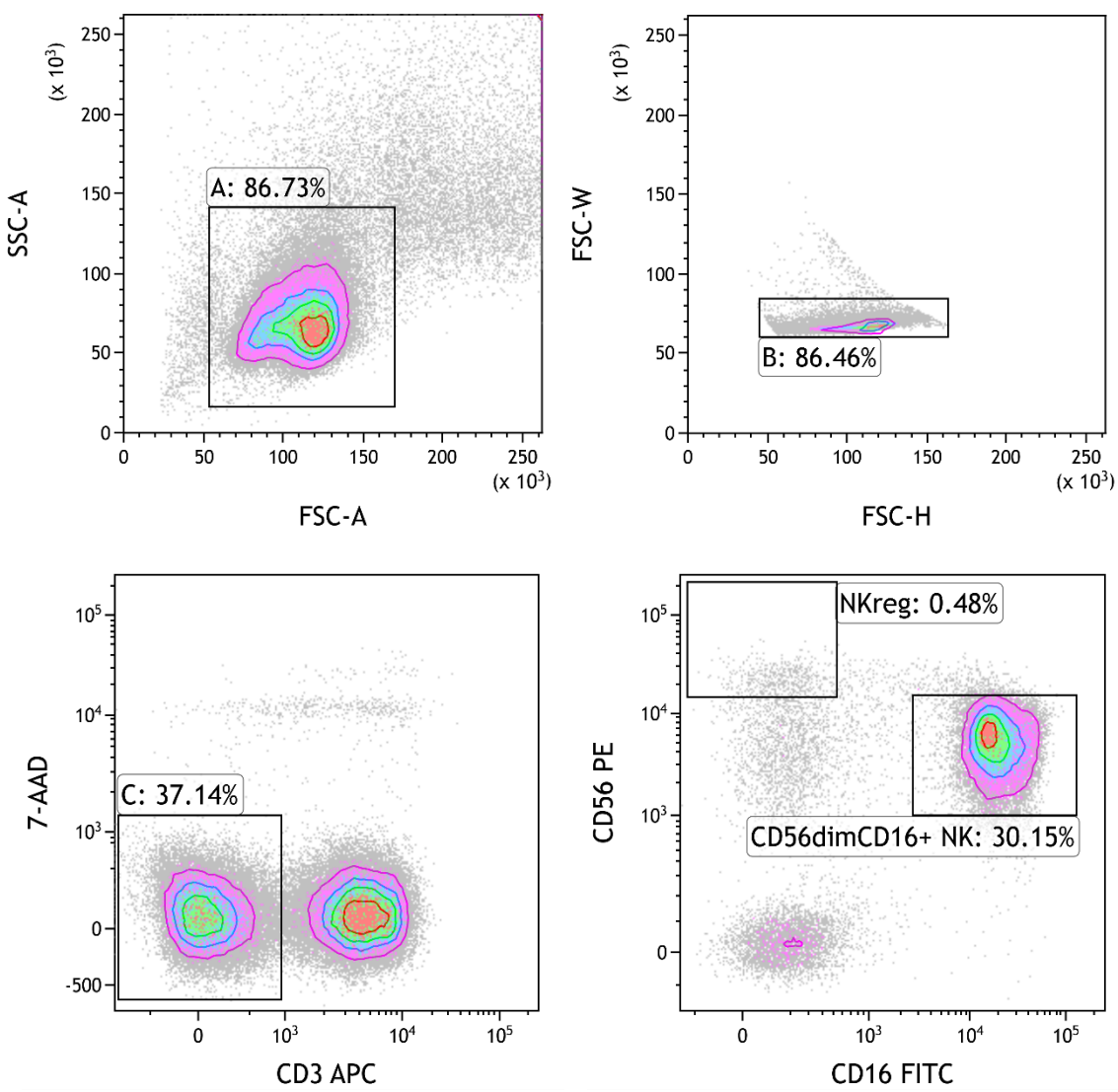
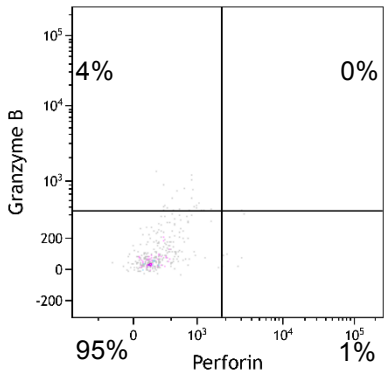


Supplemental figure 3:

a)



b) Sorted NK_{reg}



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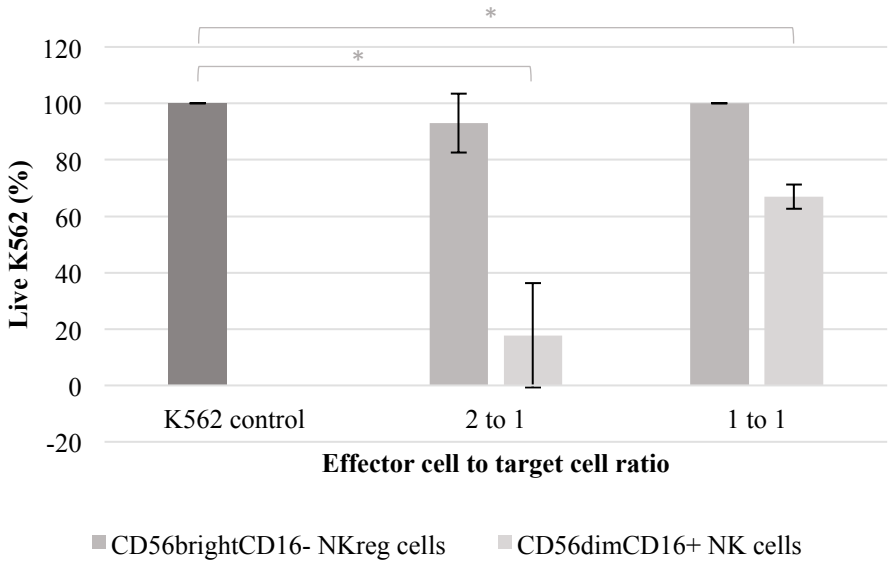

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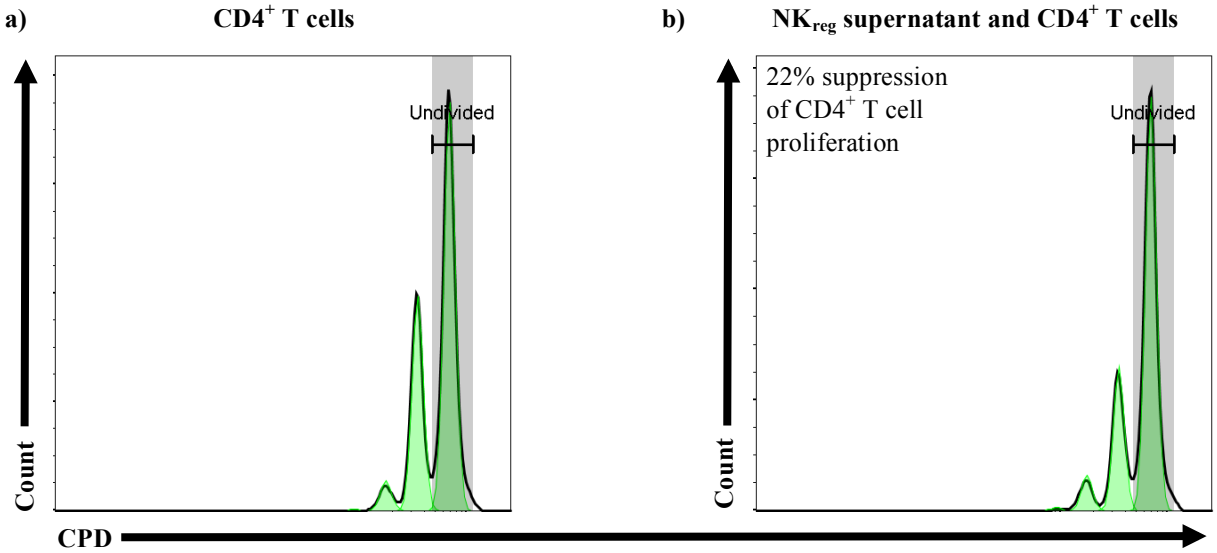

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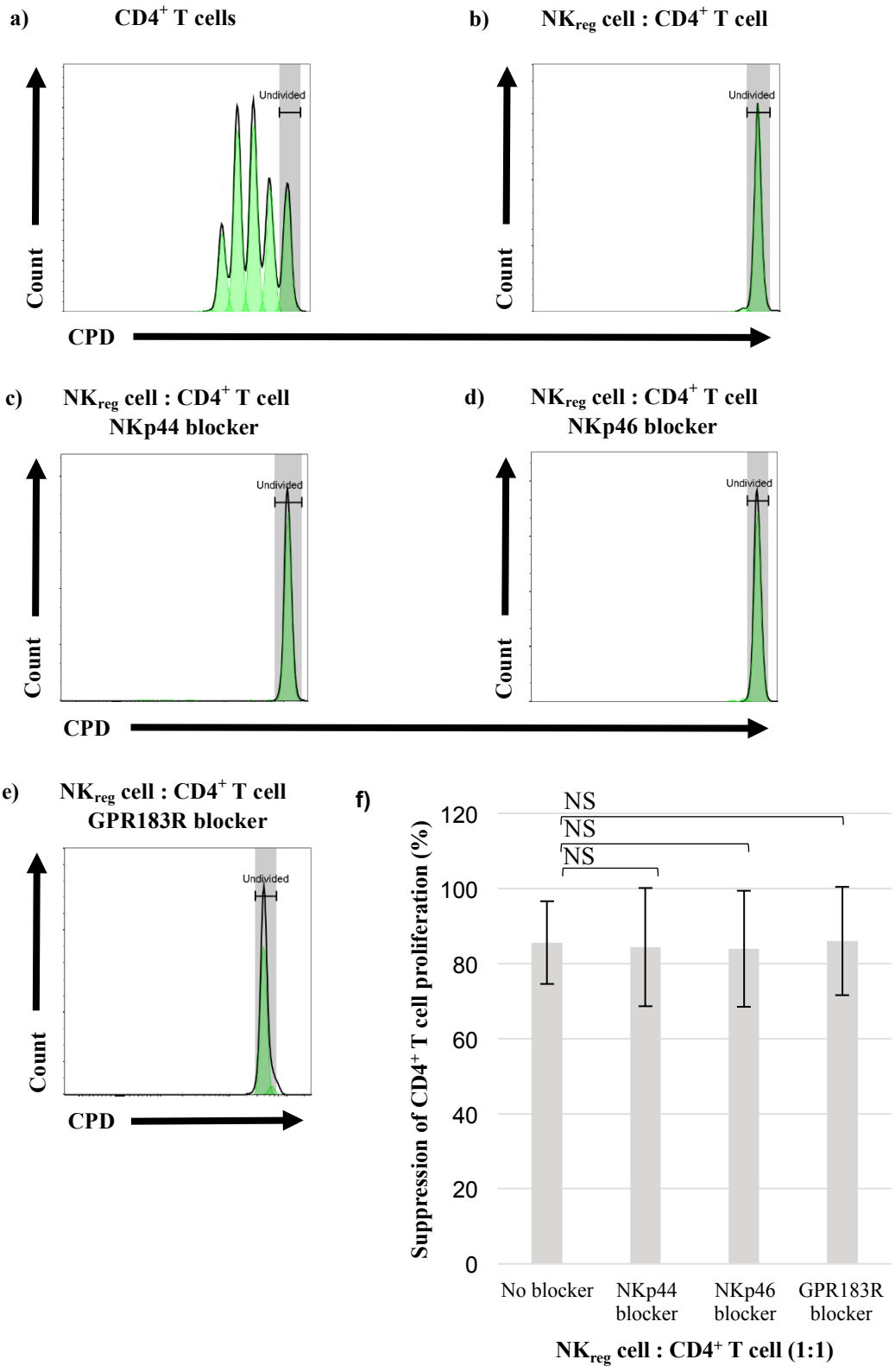

Characteristic (overall percentages of entire evaluable cohort; n = 243)	No L-aGVHD or cGVHD (n = 132)	L-aGVHD (n = 60)	cGVHD (n = 51)	Nanostring study (no cGvHD) (N=6)	Nanostring study (late aGvHD) (N=6)	Nanostring study (cGvHD) (N=6)

Supplementary Table 1. Baseline patient characteristics (adapted from Cuvelier GDE, Nemecek ER, Wahlstrom JT, et al. Benefits and challenges with diagnosing chronic and late acute GVHD in children using the NIH consensus criteria. Blood. 2019;134(3):304-316.).

Diagnoses						
Malignant (n = 168; 69.1%)	78 (59)	49 (81.7)	41 (80.4)	5 (83.3)	5 (83.3)	6 (100)
ALL, n	33	23	18	2	2	3
MDS/AML, n	37	21	15	2	3	2
Other, n	8	5	8	1	0	1
Nonmalignant (n = 75; 30.9%)	54 (41)	11 (18.3)	10 (19.6)	1 (16.7)	1 (16.7)	0 (0)
Sickle cell anemia, n	12	4	4	0	1	0
Severe aplastic anemia, n	12	1	1	1	0	0
Thalassemia, n	4	1	0	0	0	0
Primary immunodeficiency, n	12	2	1	0	0	0
Inherited marrow failure, n†	5	1	2	0	0	0
Other nonmalignant disorder, n‡	9	2	2	0	0	0
Sex						
Male (55.6%)	72 (54.5)	30 (50)	33 (64.7)	3 (50)	2 (33.3)	3 (50)
Female (44.4%)	60 (45.5)	30 (50)	18 (35.3)	3 (50)	3 (50)	2 (33.3)
N/A	0 (0)	0 (0)	0 (0)	0 (0)	1 (16.7)	1 (16.7)
Age at transplant						
Median (IQR) range), y	(0.2-17.9)	(0.9-18)	(2.0-18)	(5.2-11.5)	(6.9-14.4)	(2.0-14.6)
Donor and HLA match						
HLA-matched sibling donor (8/8)	51 (38.6)	13 (21.7)	8 (15.7)	1 (16.7)	2 (33.3)	1 (16.7)
HLA-matched nonsibling family donor	3 (2.3)	3 (5)	0 (0)	1 (16.7)	0 (0)	0 (0)
Haploidentical family donor (with PTCy)	2 (1.5)	1 (1.7)	1 (2)	0 (0)	0 (0)	0 (0)
HLA-matched unrelated donor (8/8)	48 (36.4)	21 (35)	22 (43.1)	2 (33.3)	1 (16.7)	2 (33.3)
HLA-mismatched unrelated donor (≤7/8)	9 (6.8)	9 (15)	12 (23.5)	2 (33.3)	2 (33.3)	2 (33.3)
HLA-matched cord blood (6/6)	9 (6.8)	4 (6.6)	1 (2)	0 (0)	Unk	Unk
HLA-mismatched cord blood (≤5/6)	9 (6.8)	7 (11.7)	6 (11.7)	0 (0)	Unk	Unk
Double cord blood	1 (0.8)	2 (3.3)	1 (2)	0 (0)	0 (0)	0 (0)
Stem cell source						

Bone marrow (62.6%)	94 (71.2)	33 (55)	25 (49.0)	5 (83.3)	2 (33.3)	3 (50)
PBSC (20.6%)	19 (14.4)	13 (21.7)	18 (35.3)	0 (0)	1 (16.7)	2 (33.3)
Cord blood (15.2%)	18 (13.6)	12 (20)	7 (13.7)	1 (16.7)	3 (50)	1 (16.7)
Double cord blood (1.6%)	1 (0.8)	2 (3.3)	1 (2.0)	0 (0)	0 (0)	0 (0)
Serotherapy						
ATG (44.4%)	60 (45.4)	24 (40)	24 (47.1)	4 (66.7)	1 (16.7)	1 (16.7)
Alemtuzumab (10.7%)	20 (15.2)	4 (6.7)	2 (3.9)	0 (0)	1 (16.7)	0 (0)
None (44.9%)	52 (39.4)	32 (53.3)	25 (49)	2 (33.3)	4 (66.7)	5 (83.3)
History of aGVHD (maximal grade)						
None (44%)	87 (65.9)	12 (20)	8 (15.7)	4 (66.7)	6 (100)	6 (100)
Grade 1 (19.4%)	19 (14.4)	14 (23.3)	14 (27.4)	1 (16.7)	0 (0)	0 (0)
Grade 2 (20.2%)	17 (12.9)	17 (28.3)	15 (29.4)	0 (0)	0 (0)	0 (0)
Grade 3 (12.3%)	7 (5.3)	12 (20)	11 (21.6)	1 (16.7)	0 (0)	0 (0)
Grade 4 (4.1%)	2 (1.5)	5 (8.4)	3 (5.9)	0 (0)	0 (0)	0 (0)

Supplementary Table 2. Differences between $CD56^{bright}$ and $CD56^{dim}$ NK cells that are associated with a lack of GvHD and differences in gene expression between $CD56^{bright}$ NK cells in HSCT patients who were immune tolerant (no late aGvHD or cGvHD) versus those who developed GvHD measured on samples from day 100 post HSCT

	$CD56^{bright}$:$CD56^{dim}$ NK cells Effect ratio (p value < 0.05)			$CD56^{bright}$ NK cells Effect Ratio (p value)
Gene	**No late aGvHD or cGvHD N = 6**	**Late aGvHD N = 6**	**cGvHD N = 6**	**No GvHD (immune tolerant):cGvHD N = 6:6**
Increased expression in $CD56^{bright}$ cells of HSCT patients with no GvHD reported as mean ratios				
	ER (p value)	**ER (p value)**	**ER (p value)**	**ER (p value)**
IL7R	27.8 (0.0001)	25.5 (0.05)	20.8 (0.04)	0.51 (0.18) NS
GPR183	19.1 (0.006)	15.2 (0.004)	14.8 (0.008)	1.13 (0.67) NS
Granzyme K	16.2 (0.03)	26.0 (0.03)	11.8 (0.06) NS	0.85 (0.75) NS
TNFRSF11A (RANK)	12.1 (0.0009)	16.7 (0.001)	10.0 (0.005)	1.2 (0.33) NS
CSF2 (GM-CSFR)	7.9 (0.005)	6.8 (0.07) NS	4.3 (0.03)	1.4 (0.31) NS
TCF7	6.8 (0.001)	10.1 (0.006)	5.8 (0.0004)	1.5 (0.09) NS
IL23A	6.8 (0.007)	5.6 (0.02)	3.7 (0.06) NS	1.6 (0.15) NS
XCL1	6.2 (0.01)	14.9 (0.01)	14.0 (0.04)	0.97 (0.94) NS
SELL (CD62L)	6.2 (0.03)	5.8 (0.03)	5.4 (0.04)	1.1 (0.83) NS
TNFSF11	5.2 (0.04)	8.5 (0.05)	10.6 (0.01)	0.68 (0.29) NS
Decreased expression in $CD56^{bright}$ cells of HSCT patients with no GvHD reported as mean ratios				
FCGR3A/B	0.04 (0.03)	0.02 (0.12) NS	0.06 (0.09) NS	0.44 (0.17) NS
CCL4	0.04 (0.04)	0.09 (0.002)	0.18 (0.006)	0.59 (0.24) NS
LILRB1	0.06 (0.04)	0.17 (0.07) NS	0.23 (0.09) NS	0.18 (0.06) NS
FCGR2A/C	0.11 (0.008)	0.16 (0.09) NS	0.34 (0.15) NS	0.32 (0.29) NS
CD6	0.12 (0.03)	0.32 (0.01)	0.34 (0.05)	0.41 (0.04)
IRF4	0.14 (0.03)	0.25 (0.05)	0.57 (0.02)	0.22 (0.08) NS
B3GAT1	0.16 (0.001)	0.24 (0.20) NS	0.20 (0.06) NS	1.1 (0.83) NS
IKZF3	0.16 (0.01)	0.11 (0.009)	0.23 (0.007)	0.55 (0.39) NS

Abbreviations: IL7R (CD127) – Interleukin 7 receptor; GPR183 (EBI2) – G-protein coupled receptor 183 (Epstein-Barr virus-induced G-protein coupled receptor 2); TNFRSF11A (RANK) – Tumor necrosis factor receptor superfamily member 11A (Receptor activator of nuclear factor kappa-B); CSF2 (GM-CSFR) – Colony stimulating factor 2 (Granulocyte-macrophage colony-stimulating factor receptor); TCF7 – Transcription factor 7; IL23A – Interleukin-23 subunit alpha; XCL1 – X-C Motif Chemokine Ligand 1; SELL (CD62L) – L-selectin; TNFSF11 – Tumor necrosis factor superfamily member 11; FCGR3A/B – Low affinity immunoglobulin gamma Fc region receptor III-A (polymorphism); CCL4 – Chemokine (C-C motif) ligand 4; LILRB1 – Leukocyte immunoglobulin-like receptor subfamily B member 1; FCGR2A/C – Low affinity immunoglobulin gamma Fc region receptor III-A (polymorphism); CD6 – Cluster of differentiation 6; IRF4 – Interferon regulatory factor 4; B3GAT1 – 3-beta-glucuronosyltransferase 1; IKZF3 – Ikaros family zinc finger protein 3; *NS = not significant.
Selected for ER ≥ 5 or ≤ 0.20 and $p < 0.05$ for no late aGvHD or cGvHD patients (column 1).

Supplementary table 2: