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Supplemental Information

Improved hematopoietic stem cell transplantation upon inhibition of natural killer cell-derived interferon-gamma

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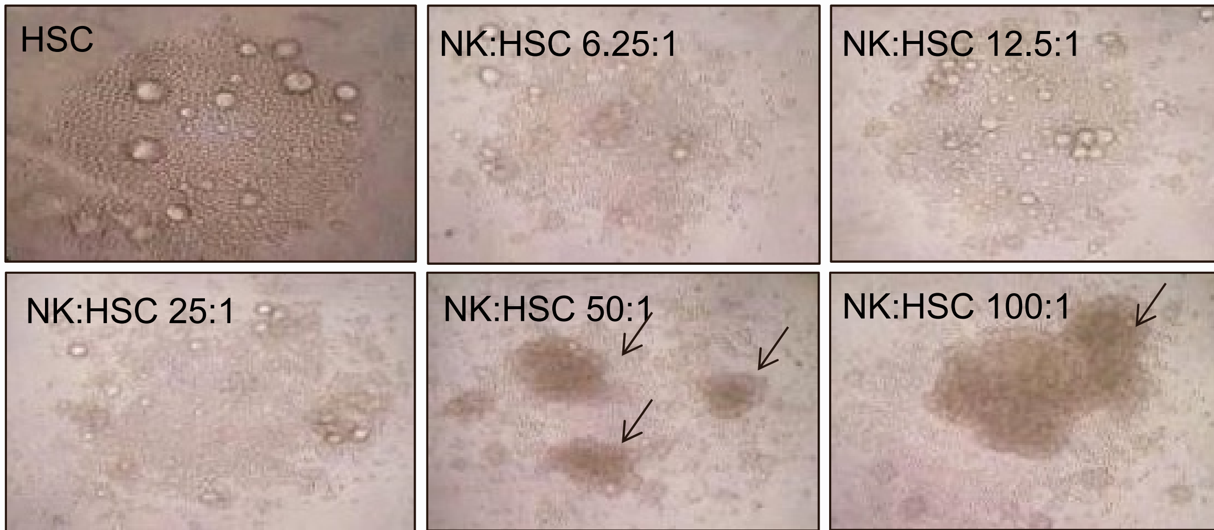
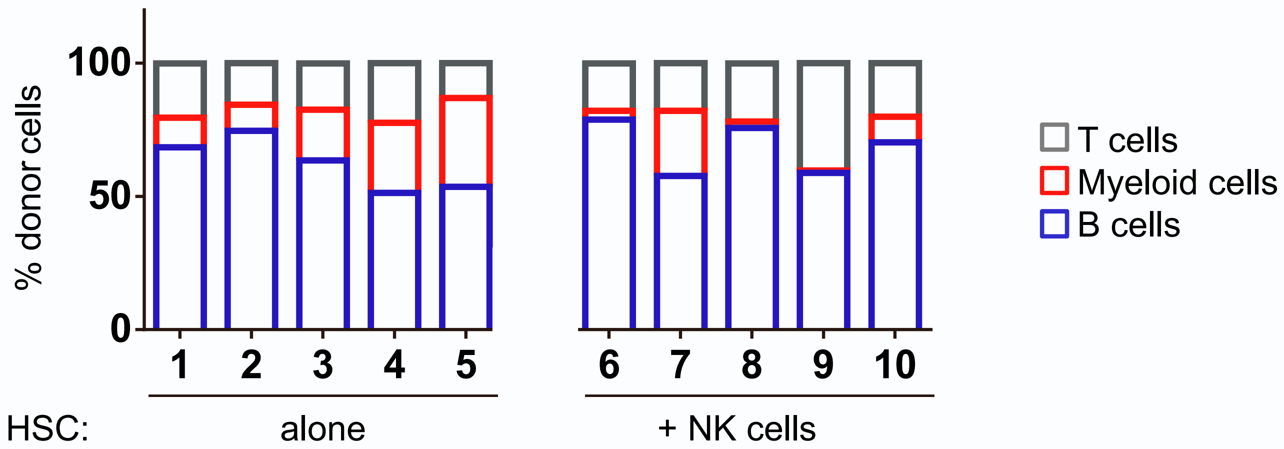


Figure S1. NK cells reduce HSC maintenance and frequency in culture. Related to figure 1. Images of HSC:NK 4-day co-cultures in the presence of IL-2. Pictures were taken by microscopy of the tissue culture plates (100 X). HSC:NK cell ratio is indicated. Arrows indicate non-viable colonies. Notice the reduction in colonies with increasing amounts of NK cells in culture.



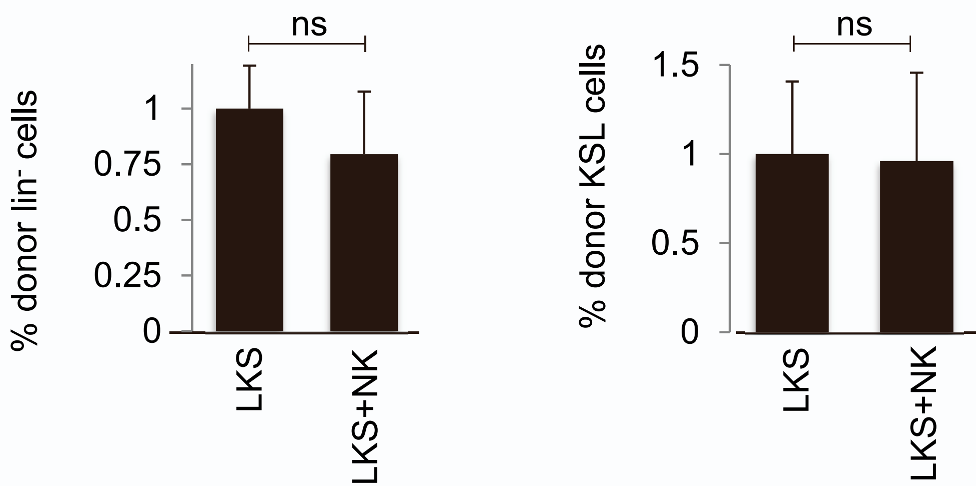
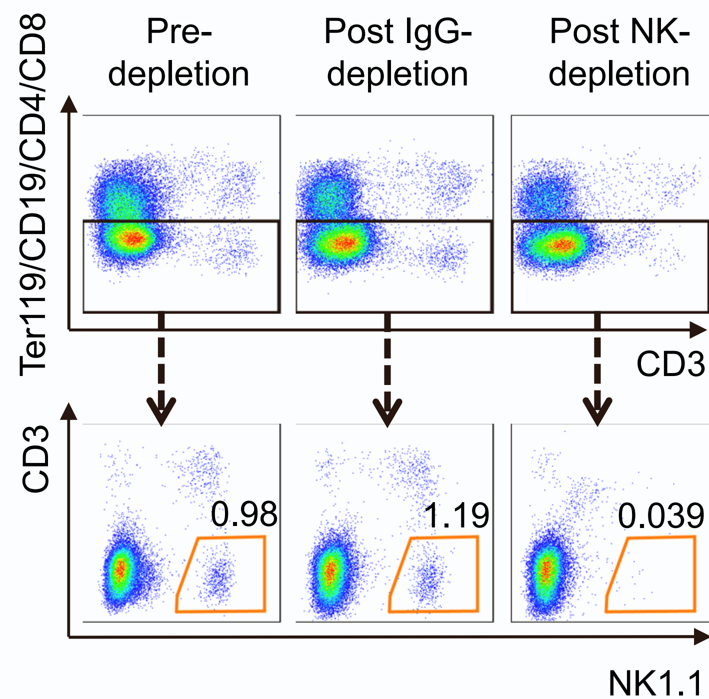


Figure S2. Donor cell contribution to production of T,- B-, myeloid cells and homing assays. Related to figure 2. (A) Blood sample analysis nine weeks post-transplantation. The bars show the average percentages of donor CD45.1/2 cells expressing myeloid (Gr-1 and CD11b), B- (B220), or T- (CD4/8) cell lineage markers. 1-5 indicates mice transplanted with HSC, and 6-10 indicates mice transplanted with HSC exposed to NK cells. (B) Lineage^{-} c-Kit^{+} Sca-1^{+} (LKS) cells (CD45.1) were injected into sublethally irradiated CD45.1/2 mice (n=4 per group) in the presence or absence of NK cells (CD45.2). Bone marrow was obtained from recipients 30 hours after transplant and the percentage of both CD45.1/2 lineage negative cells and LKS cells was measured by flow cytometry analysis. The addition of NK cells to the donor cell preparations did not affect homing of progenitor cells, lineage negative as well as LKS donor cells were quantified, Two-tailed Student's t-test was used to assess statistical significance, ns: not significant ($p \geq 0.05$). Results are shown by normalization of the percentages of recovered cells in the LKS^{+} NK group to the LKS injected group.



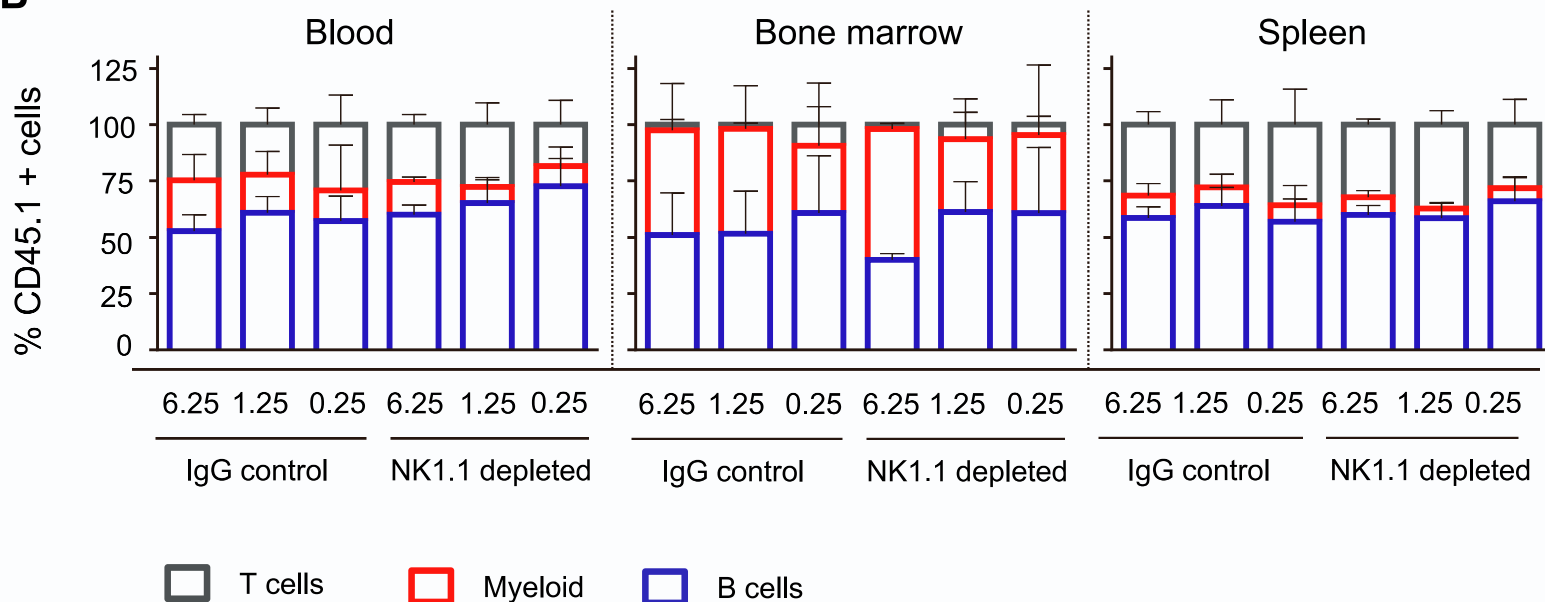


Figure S3. NK cell depletion from murine BM grafts improves hematopoietic recovery of recipient mice. Related to figure 3. (A) Flow cytometry analysis of BM donor samples prior to depletion (pre-depletion), IgG control depleted (post IgG-depletion), and NK cell depleted (post NK-depletion). Upper dot plots indicate Ter119/CD19/CD4/CD8 staining versus CD3 staining. Black box indicates gating used for further analysis shown in lower panels. Lower dot plots show CD3 and NK1.1 staining. Orange box indicates percentage of NK cells ($CD3^{-}NK1.1^{+}$) from alive cells. (B) Lineage contribution of $CD45.1^{+}$ donor cells to blood formation in recipient mice. Y axes indicate the percentage of $CD45.1^{+}$ cells to each lineage. X axes indicate the different groups based on IgG control and NK1.1 depletion. Cell doses used for transplantation are indicated as 6.25 (6.25 x 10^{6} cells), 1.25 (1.25 x 10^{6} cells) and 0.25 (0.25 x 10^{6} cells). Gray boxes indicate the percentage of T cells, red boxes the percentage of myeloid cells, and blue boxes the percentage of B cells. Each column represents average values and standard deviation for at least 5 mice/group.

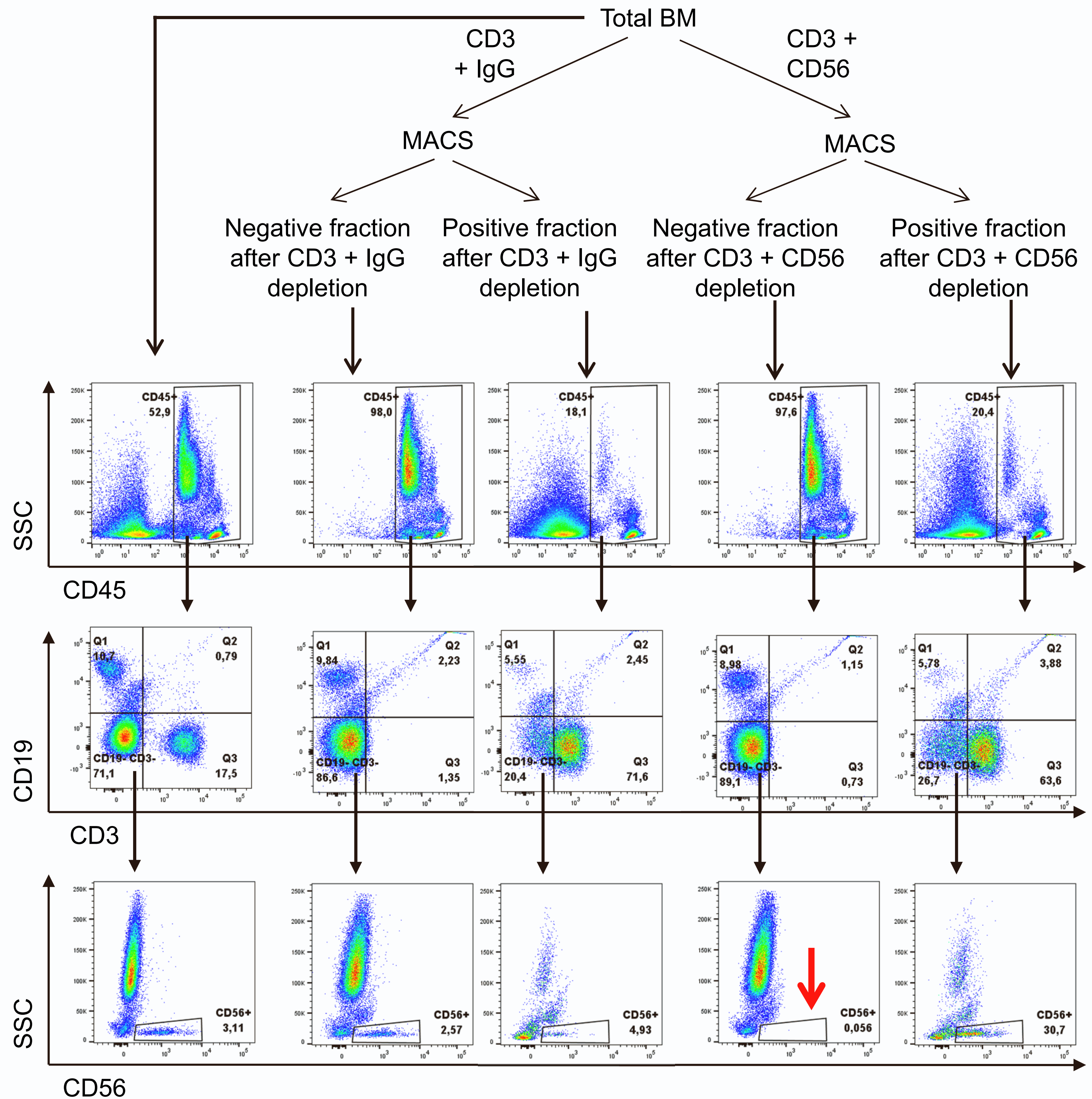


Figure S4. Human bone marrow fractionation. Related to figure 4. Upper scheme: graphical representation of the procedure. BM cells were fractionated based on CD3 and IgG expression or CD3 and CD56 expression. After MACS separation negative and positive fractions were recovered. Lower flow cytometric plots: Separated BM populations were stained using antibodies against CD45, CD19, CD3, and CD56. Red arrow points to the efficiently deleted NK cells.

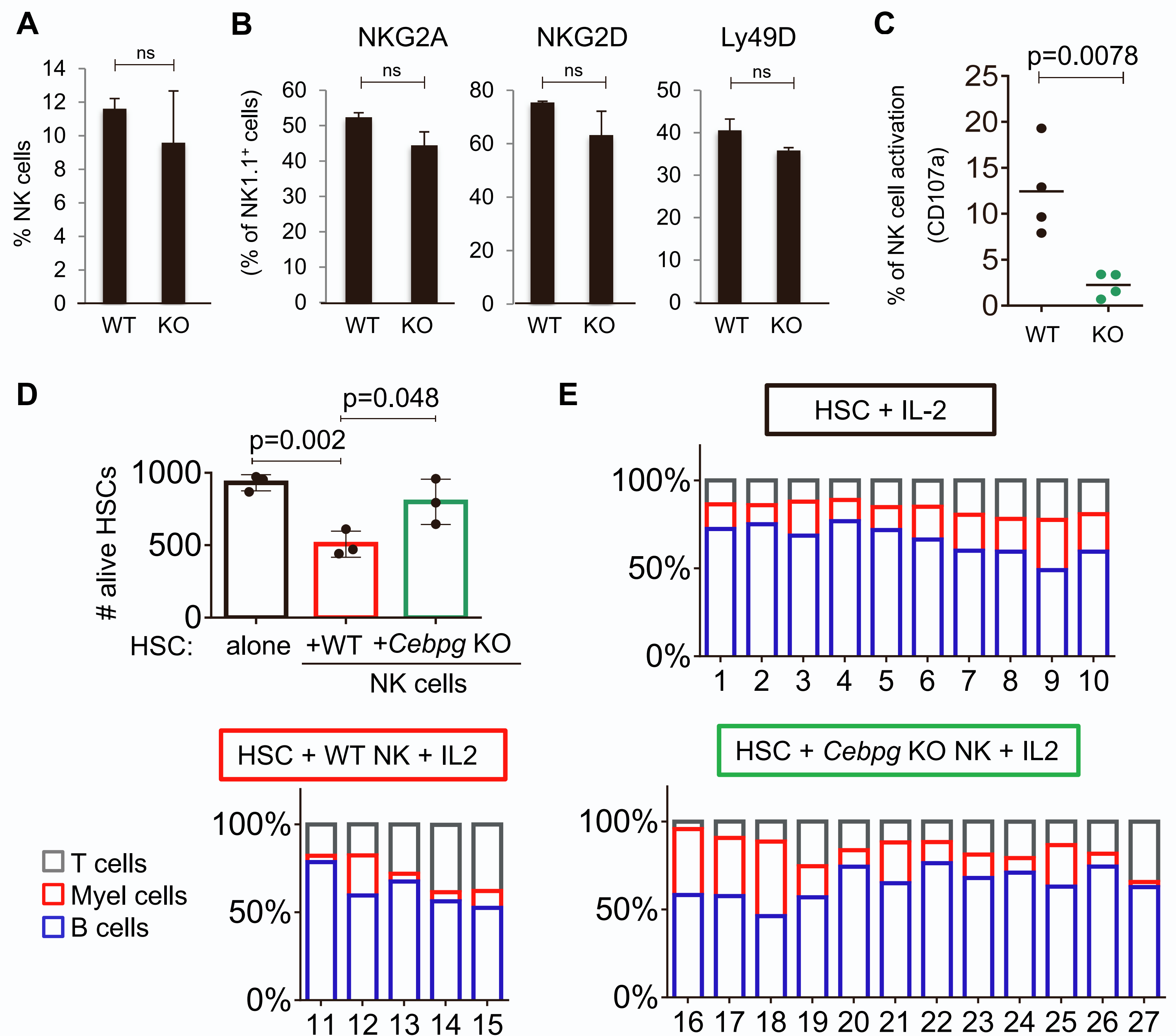


Figure S5. Characterization and function of *Cebpg* KO NK cells in comparison to WT NK cells. Related to figure 5. (A) Percentage of NK cells determined by flow cytometry analysis in WT and *Cebpg* KO spleen cells. NK cells were determined by expression of NK1.1 antigen in the absence of Ter119, CD4, CD8, CD19 and CD3. (B) Expression of the receptors NKG2A, NKG2D and Ly49H in WT and *Cebpg* KO NK cells determined by flow cytometry (n=4 per group). Y axes indicate the expression of each receptor among NK1.1$^+$ gated cells. (C) NK cell cytotoxicity against the murine YAC-1 MHC-I-deficient lymphoma cell line determined by the detection of CD107a by flow cytometry after five hours of a 10:1 ratio of NK:YAC-1 co-culture. Y axis illustrates the percentage (%) of CD107a among NK1.1$^+$ gated cells. (D) Number of HSCs assessed by FACS. Y axis indicates number of alive Hoescht 33258$^-$ HSCs after overnight incubation. X axis indicates culture conditions. (E) Flow cytometry analysis of peripheral blood samples from recipient mice 16 weeks post-transplant. Donor CD45.1/2 cells were analyzed for expression of T (CD3, grey box), myeloid (Gr-1 and CD11b, red box) or B (B220, blue box) cell lineage markers. Y axes indicates percentage of the distinct populations. X axes indicate individual animals: 1-10 transplanted with HSC alone, 11-15 transplanted with HSC co-cultured with WT NK cells, 16-27 transplanted with HSC co-cultured with *Cepbg* KO NK cells, all co-cultures were kept in media with IL-2. Two-tailed Student's t-test was used to assess statistical significance (p values are indicated, ns: not significant).

TABLE S1. List of genes belonging to the NK cell signature. Related to Figure 6.

TABLE S2. Differential pathway enrichment analysis in WT and *Cebpg* KO NK cells. Related to Figure 6.

Index	Name	P-value	Z-score	Combined Score
1	toll like receptor signaling pathway	0.002136	-1.90	3.08
2	apoptosis	0.003123	-1.85	3.00
3	b cell receptor signaling pathway	0.003992	-1.75	2.84
4	vegf signaling pathway	0.01860	-1.82	1.05
5	aminoacyl trna biosynthesis	0.02612	-1.55	0.89
6	pancreatic cancer	0.02696	-1.69	0.97
7	ubiquitin mediated proteolysis	0.02886	-1.28	0.73
8	chronic myeloid leukemia	0.03096	-1.61	0.93
9	c21 steroid hormone metabolism	0.03397	0.75	-0.43
10	cytokine cytokine receptor interaction	0.04043	-1.53	0.87
11	MAPK signaling pathway	0,04184	-1.54	0,87

Results of KEGG pathway analysis of WT versus *Cebpg* KO NK cells with the threshold of $p<0.05$.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Co-cultures with OP9 stromal cells
Co-cultures of HSCs (lin$^-$ c-Kit$^+$ Sca-1$^+$ CD48$^-$ CD150$^+$) and NK cells (Ter 119$^-$ CD19$^-$ CD8$^-$ CD4$^-$ CD3$^-$ NK1.1$^+$) in different ratios were performed using serum-free stem cell medium and cytokines (100 ng/mL SCF, 100 ng/mL FLT3-ligand, 10 ng/mL IL-3, 10 ng/mL IL-6, 25 ng/mL TPO, murine IL-2 1000 U/mL) over a confluent layer of mitomycin C treated OP9 stromal cells. 4 days after culture cells were stained for HSC markers (lin$^-$ c-Kit$^+$ Sca-1$^+$ CD48$^-$ CD150$^+$), enumerated by flow cytometry analysis, and colonies were visualized by microscopy.

Murine colony-forming unit (CFU) assays
Co-cultures of HSCs and NK cells were performed using StemSpan serum-free stem cell medium and cytokines (100 ng/mL SCF, 100 ng/mL FLT3-ligand, 10 ng/mL IL-3, 10 ng/mL IL-6, 25 ng/mL TPO, IL-2 1000 U/mL) in 96-well round bottom plates overnight. When indicated, aforementioned media was supplemented with 10 ug/mL neutralizing anti-IFN gamma monoclonal Antibody (clone XMG1.2, eBioscience) or Rat IgG1 kappa Isotype Control (eBRG1, eBioscience). After overnight incubation, cells were plated in methylcellulose-based medium MethoCult GF M3434 (Stemcell Technologies) supplemented with murine IL-2 1000 U/mL. 100 murine HSCs alone or 100 HSCs co-cultured with 1000 or 5000 NK cells were plated in duplicates in 12-well plate. Number of colonies was assessed 10 days after plating.

Human CFU assays
Human CD34$^+$ cells and NK cells (CD19$^-$ CD8$^-$ CD4$^-$ CD3$^-$ CD56$^+$) were isolated from adult bone marrow (BM) obtained from femoral heads removed during hip replacement surgeries. First, mononuclear cells were separated by density gradient centrifugation using Ficoll-Paque PREMIUM (GE Healthcare). One part of the mononuclear cell suspension was frozen and later human NK cells were sorted from thawed material by using BD FACSAriaIIu. Another part was immediately used to obtain CD34$^+$ cells by magnetic separation using human CD34$^+$ MicroBead kit (Miltenyi Biotec) and autoMACS Separator (Miltenyi Biotec). CD34$^+$ cells were frozen as well. Thawed human CD34$^+$ cells were cultured alone or with NK cells from the same patient (ratio 1:10) in serum-free stem cell medium and human cytokines (100 ng/mL SCF, 50 ng/mL G-CSF, 100 ng/mL FLT3-ligand, 25 ng/mL IL-3, 20 ng/mL TPO, IL-2 1000 U/mL) in 96-well round bottom plate overnight. After overnight incubation, cell suspensions of 500 or 1000 CD34$^+$ cells alone or co-cultured with 5000 or 10000 NK cells, respectively, were plated in duplicates in 12-well plate and number of colonies was assessed 10 days after plating. CFU assays were performed using MethoCult H4535 (Stemcell Technologies) supplemented with human IL-2 1000 U/mL (Gibco).

Gene expression analysis
Total RNA was isolated from NK cells sorted from WT and *Cebpg* KO murine spleens cultured in RPMI with 10% FBS overnight in the presence of 1000 U/ml IL-2 (PeproTech) or vehicle. RNA extraction was performed using RNeasy Plus Mini Kit (Qiagen). RNA integrity was analyzed by Agilent Bioanalyzer 2100 (Agilent), and only samples with intact RNA profiles were used for expression profiling (RIN > 7). Four biological replicates were used for each phenotype and treatment. 600 pg RNA was amplified with PicoSL WTASystem V2 (NuGEN), labeled with Encore Biotin IL Module (NuGEN) and 750 ng of labeled RNA was hybridized on MouseRef-8 v2.0 Expression BeadChip (Illumina) according to the manufacturer procedure. Raw data were processed using the beadarray package of Bioconductor and analyzed as described previously(Melenovsky et al., 2011). Gene set enrichment analysis (GSEA) was performed using the Enrichr gene analysis tool (http://amp.pharm.mssm.edu/Enrichr)(Chen et al., 2013). Microarray data were deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5604.

Cytokine assay
A total of 250.000 WT or *Cebpg* KO purified NK cells were cultured in RPMI1640 supplemented with 10% FBS and 1000 U/mL of IL-2 for 24 hours. Then, supernatants were collected, and IL2, IL4, IL6, IL10, IL17A, TNFα and IFNγ cytokine levels were measured by flow cytometry analysis using Cytometric Bead Array technology (BD Biosciences) according to manufacturer's instructions.

Murine BM transplantation
1500 HSCs, defined as lin$^-$ c-Kit$^+$ Sca-1$^+$ CD48$^-$ CD150$^+$ cells, were sorted from mice (CD45.1/2) and cultured with or without IL-2 and/or NK cells (CD3$^-$ NK1.1$^+$) in a ratio of 10^5 NK cells to 1.5 x 10^3 HSCs per mouse. The 100:1.5 NK:HSC ratio was chosen to roughly mimic the proportions of these two cell subtypes in a normal BM (approximately 1% of NK cells and 0.01% of HSCs). Then, the content of individual wells was mixed together with 0.5 × 10^6 BM cells as a support (CD45.1) and injected through the tail vein into lethally irradiated (6 Gy)

CD45.1 recipients (3 independent experiments were performed) For engraftment analysis, cells were stained with anti-CD45.1 and CD45.2 antibodies to distinguish donor-derived cells from the host cells, as well as with lineage-specific antibodies Mac1, Gr1, B220 and CD3 to identify myeloid, B and T lineages, respectively. Engraftment analysis was performed after 9 and 16 weeks of transplant.
For murine limiting dilution assays, total BM cells (CD45.1) were submitted to NK1.1 or IgG depletion and then different numbers injected into lethally irradiated (6 Gy) CD45.2 recipients (n=6 or 11 per group, 2 experiments). Peripheral blood was collected from each mouse at 8 and 16 weeks, and BM and spleen were obtained at 16 weeks for final chimerism analysis. For this assay, a recipient mouse was considered positive if >0.1% CD45.1^{+} cells were detected and three lineages were reconstituted (>1% of CD3^{+}, Gr1^{+}Mac1^{+} or B220^{+} cells from CD45.1^{+} cell population).

NK depletion from murine and human BM
After red blood cells lyse, cells from murine whole BM were stained with 0.1 µg per 10^{6} cells (in a 1:50 dilution) of anti-mouse IgG2a (MOPC-173) or NK1.1 (PK136) biotinylated antibodies (Biolegend) for 30 minutes, washed and stained with anti-biotin Microbeads Ultrapure as recommended by manufacturer, and then submitted to immunomagnetic depletion using an auto MACS separator (Miltenyi Biotec). After depletion, cell suspensions were prepared in sterile PBS for mice injection. Depletion efficiency was tested in parallel in all experiments by staining samples before depletion and after IgG or NK depletion with other lineage markers (Ter119, CD19, CD4, CD8), CD3 and NK1.1. NK cells were gated as viable, lineage negative, CD3 negative and NK1.1^{high}. For human NK cells depletion, BM was obtained from healthy volunteers after informed consent and in accordance to Institutional policies (1st Department of Medicine - Department of Haematology, First Faculty of Medicine, Charles University in Prague and General University Hospital, Prague, Czech Republic). Fresh BM cells were submitted to mononuclear cell isolation by Ficoll Hypaque density gradient, washed with sterile PBS/20% FBS, stained with 0.25 µg per 10^{6} cells of anti-human biotinylated CD3 (MEM-57) and IgG2a (MOPC-173) or CD56 (MEM-188), and then taken to immunomagnetic separation with streptavidin beads. T-cell depletion with CD3 was performed in both IgG and CD56 depleted experimental arms in order to avoid GVHD and improve survival of the recipient mice. In human experiments, CD56 depletion efficiency was tested by staining pre-depletion and post-depletion samples with CD45, CD19, CD3, CD56 (Figure S4). Serial cell dilutions were prepared in sterile PBS for injection into mice.

Xenotransplantation
NK cell-depleted (CD56) or non-depleted (IgG) human grafts were transplanted into sublethally irradiated (200 cGy) NOD.Cg-*Prkdc^{scid}Il2rg^{tm1Wjl}*/SzJ (NSG) mice at different doses. The percentages of human CD45^{+} cells in the murine blood (8 and 14 weeks) and BM (14 weeks) after transplantation were determined.

Mobilized blood transplantation and IFNγ treatment
NOD.Cg-*Prkdc^{scid}Il2rg^{tm1Wjl}*/SzJ (NSG) mice were transplanted with 2 x 10^{6} human mobilized blood cells containing an equivalent of 3.6 x 10^{5} CD34+ cells. Mice were treated at day 0, 3, 7, 10, and 14 after transplantation with 100 µg murine anti-human IFNγ neutralizing monoclonal antibody (clone NIB42, Exbio, Czech Republic) or murine isotype control IgG1 monoclonal antibody (clone PPV-06, Exbio, Czech Republic). Engraftment was determined at day 17 after transplantation by assessing the percentage of human CD45+ and human CD34+ cells in the BM.

SUPPLEMENTAL REFERENCES

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