

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Vago L, Perna SK, Zanussi M, et al. Loss of mismatched HLA in leukemia after stem-cell transplantation. *N Engl J Med* 2009;361:478-88.

SUPPLEMENTARY METHODS

CHIMERISM ANALYSIS

STR chimerism analysis was performed either by the AmpFISTR Profiler Plus kit (Applied Biosystems) or by the AmpFℓSTR Identifile PCR Amplification Kit (Applied Biosystems). These PCR kits amplify respectively nine and fifteen STR loci on different chromosomes and amelogenin as control gene. PCR products were separated using an automatic sequencing device (Automatic Sequencer 377, Perkin Elmer), and results were analyzed using the Genescan software (Applied Biosystems). HLA chimerism analysis was performed by 2-digit genomic typing of the HLA-A, B, C, DRB, DQB1 loci, using the Dynal RELI™ SSO kit (Dynal-Biotech, Invitrogen Corporation), according to the manufacturer's recommendations. Positive signals were read by scanner image capturing (Dynal-Biotech, Invitrogen Corporation) and analyzed for signal intensity by visual assessment.

LOH STUDIES

STR analysis of LOH was performed for all five patients by the use of twelve highly informative microsatellite markers spanning chromosome 6 from ~831 to ~148,731 kb (Ensembl Homo Sapiens version 51.36m; www.ensembl.org), to map the genomic rearrangement responsible for loss of the patient-specific HLA haplotype at disease relapse. Genomic DNA was PCR amplified with fluorescein-labeled primers for 28 cycles (94°C for 30''; 55°C for 30''; and 72°C for 30''), followed by separation on a 3730 DNA Analyzer (Applied Biosystems) and analysis by the GeneMapper v3.7 software (Applied Biosystems).

For UPN #7, UPN #16 and UPN #43 SNP analysis of LOH and copy number variation was performed with the Illumina Human CNV370-Quad Bead Array, according to the Illumina Infinium II assay manual ^{1, 2}. This array guarantees high genomic coverage of the SNPs from Phase I and II of the HapMap Project, and of copy number variant (CNV) regions from deCODE and the Database of Genomic Variants. In particular, chromosome 6 is mapped on HumanCNV370-Quad Bead Arrays by 26841 SNPs, 1933 of which are CNV, and the MHC locus in p21.3 is densely mapped by 4359 SNPs among which 927 are CNV. Normalized bead intensity data obtained for each sample were analyzed with the Illumina Genome Studio v1.0.2 software and genotyping quality was assessed according to the individual Call Rate, accepting only Call Rates ≥ 0.95 .

For UPN #7, UPN #13 and UPN #33 SNP analysis of LOH and copy number variation was performed with the Affymetrix Human SNP array 6.0 according to the manufacturer's instructions. This array in particular addresses 57785 SNPs and 55040 CNVs located on chromosome 6. The genotype calls of each individual were determined by Affymetrix[®] GeneChip[®] Command Console[®] Software v3.0.2, that implements robust genotyping algorithms including Birdseed v2 and a new copy number polymorphism genotyping algorithm (Canary) developed by the Broad Institute. Quality control assessment was performed using the Contrast QC (CQC) algorithm.

FUNCTIONAL READOUTS OF MLC

The cytotoxic activity of T lymphocytes was analyzed in a standard ⁵¹Cr-release assay, as described ³. Briefly, effector cells were plated in serial dilutions and to exhaust their preformed cytotoxic granule release they were incubated for 30 minutes

at 37 °C in the presence of 30.000 cells unlabelled K562 leukemic cells per well, known to be a preferential NK cell target (cold target). Subsequently, 1.000 relevant ⁵¹Cr-labelled target cells per well were added (hot target), for a final ratio of cold: hot target of 30:1. After four hours of co-incubation the supernatant was harvested and counted on a γ -counter to assess T cell-mediated specific lysis.

The frequency of T lymphocytes specifically releasing interferon- γ (IFN- γ) in response to leukemia was assessed by ELISpot assay, as described ⁴. Briefly, 25,000 effector cells and 25,000 target cells were plated together for 18-20 hours in 150 μ l of complete medium. After detection, spot-forming cells were counted by a KS Elispot Reader (Zeiss). All experiments were performed in triplicate.

To test leukemia-specific T cell proliferation, 15,000 responder cells were plated in the presence of 15,000 irradiated (3,000 rads) stimulators in a final volume of 150 μ l of complete medium. After 72 hours, cells were pulsed for 16 hours with 1 μ Ci/well of ³H-thymidine (Amersham Pharmacia Biotech), harvested and counted in a scintillation counter. All experiments were performed in triplicate.

CYTOFLUORIMETRIC ANALYSIS OF LEUKEMIA

PBMCs harvested from UPN #16 at the moment of leukemia diagnosis and at the documentation of the mutated relapse were thawed and kept over-night in X-VIVO medium (Lonza) supplemented with 5% human serum (Cambrex). Thereafter, cell surface staining with monoclonal antibodies (all from BD Pharmingen) was performed. Leukemic blasts were identified on the basis of their surface expression profile (CD45^{dim} CD117⁺ CD33⁺ CD14⁺) and expression of the markers listed in Supplementary Figure 2 was addressed on this subset. Samples were analyzed by

cytofluorimetric analysis on a FACSCanto II (Becton Dickinson) and with the FlowJo program (Tree Star, Inc.)

NK CELL DEGRANULATION ASSAY

NK cell degranulation was evaluated in a CD107a flow cytometric assay, according to a protocol adapted from Alter et al.⁵. Briefly, 2×10^5 PBMCs from UPN #16 stem cell donor, from the patient at two follow-up time points (61 days after first HSCT and 64 days after second HSCT), and from a third-party donor were plated in IMDM medium containing 10% FCS, rhIL-2 1200 IU/ml and anti-CD107a mAb 20 μ l/ml (BD Pharmingen), in 96-well round bottom plates, in the presence or absence of 2×10^4 target cells at 37°C. After 3 hours, Monensin A (Sigma) was added to the culture at the final concentration of 30 μ g/ml. Following 3 additional hours of incubation, cells were transferred to V bottom plates, washed and extracellular staining for CD3 and CD56 was performed.

MINOR HISTOCOMPATIBILITY ANTIGEN GENOTYPING

Genomic DNA from was extracted from pre-transplantation samples of the five described patients and their respective stem cell donors by QIAamp DNA Blood Mini Kit (Qiagen). Allele-specific SSP-PCR for 11 minor histocompatibility antigens (mHAgs) was performed by the Dynal AllSet Minor Histocompatibility Antigen Typing Kit (Dynal-Biotech, Invitrogen Corporation) according to the manufacturer's instruction. The kit allows the genotyping genotyping of the following mHAgs: HA-1, HA-2, HA-3, HA-8, HB-1, ACC-1, ACC-2, HwA-9 (SP110), HwA-10 (PANE1), UGT2B17 and HY^{6,7}. Results were interpreted on the basis of the of the donors' and

recipients' HLA typing using the Leiden University Medical Center Minor Histocompatibility Knowledge Database (<http://www.lumc.nl/dbminor/>).

AUTHOR CONTRIBUTIONS

L.V. performed experiments, collected and analyzed the data, and drafted the manuscript; S.K.P., M.Z., B.M., Cr.B., N.F.P., Cr.C., F.T., A.A. and M.C. performed experiments; M.T.L.S., B.F., J.P., M.B. and Co.C. provided patient care and collected the clinical data; A.B., M.F., S.R., M.G.R. and Cl.B. provided advice on the study design and critical reading of the manuscript; Ch.B. supervised experimental design, wrote the manuscript; F.C. supervised patient clinical care, wrote the manuscript; K.F. designed and supervised the study, decided to publish and wrote the manuscript.

SUPPLEMENTARY RESULTS

CELL SURFACE PHENOTYPIC PROFILE OF LEUKEMIA

Leukemic blasts harvested from UPN #16 at the moment of disease diagnosis and at the documentation of loss of the patient-specific HLA haplotype were compared for activation marker CD83, of HLA molecules (class I, HLA-DR and HLA-G) and of leukocyte adhesion ligands CD54 and CD58. As shown in Supplementary Figure 2, the cell surface phenotypic profile of the leukemic blast remained largely unchanged, and the costimulatory receptors CD80 and CD86 were even moderately upregulated in blasts taken at relapse. Thus, we confirmed that loss of the GvL effect mediated by donor-derived T cells cannot be explained by a change in the costimulatory capacity

of the blasts, as documented also by their efficient recognition by T cells from a third-party responder (Figure 4).

NK CELL ANTILEUKEMIC ACTIVITY

Loss of the patient-specific HLA haplotype did not alter the gene copy number (Figure 3), nor the cell surface expression level (Supplementary Figure 2) of HLA class I molecules, suggesting that it may not have substantially altered the susceptibility of leukemic blasts to NK cell mediated lysis. To confirm this, we tested in a standard CD107a degranulation assay NK cells harvested from the stem cell donor or from the patient at two different time-points after HSCT, against leukemic blasts taken before and after the loss of the patient-specific HLA haplotype. As shown in Supplementary Figure 3, primary leukemic blasts were recognized by less than 10% of NK cells, irrespective of whether taken at diagnosis or at relapse, consistent with their equally high level of expression of HLA Class I molecules. Conversely, at least 50% of NK cells from both donors and the patient after HSCT efficiently responded to the reference leukemia cell line K562, which is devoid of HLA class I molecules on its cell surface.

MINOR HISTOCOMPATIBILITY ANTIGEN MATCHING

The five patients in whom we documented loss of the patient-specific HLA haplotype and their stem cell donors were genotyped for the presence of the immunogenic alleles of eleven of the best to date characterized minor histocompatibility antigens. Results were interpreted on the basis of the genomic typing of leukemic blasts, which by the rearrangement we described became homozygous for the HLA haplotype shared between the donor and the patient. For only one of the five patients (UPN #43)

a predictably immunogenic mismatch could be evidenced, involving the ACC-1 minor antigen.

SUPPLEMENTARY REFERENCES

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SUPPLEMENTARY FIGURE LEGENDS

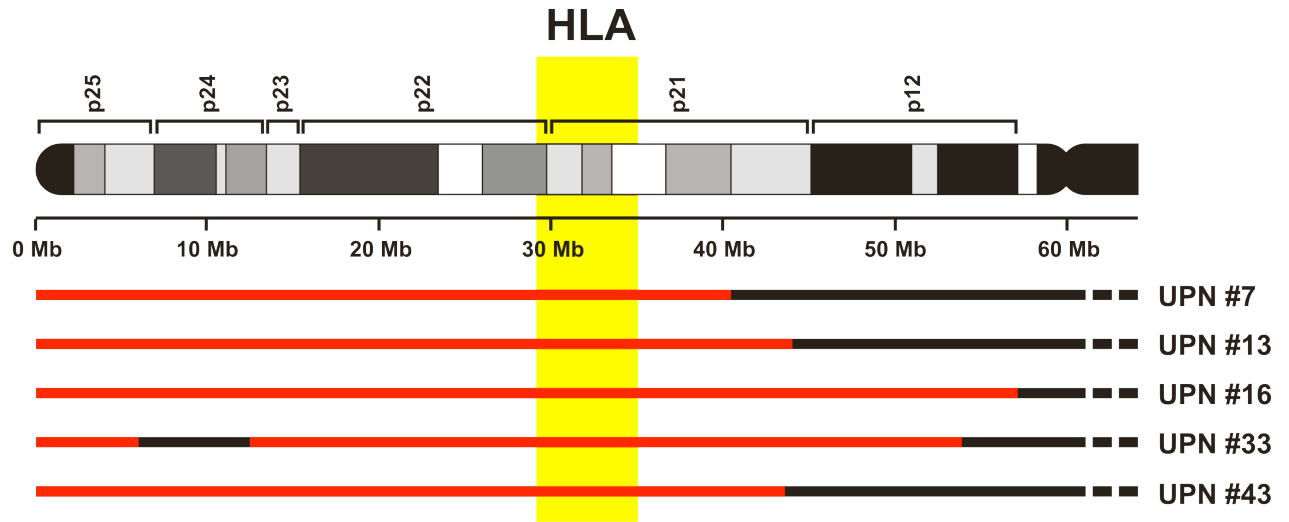
Supplementary Figure 1. Boundaries of the region of chromosome 6 acquired uniparental disomy. Ideogram representation of human chromosome 6p, with highlighted in yellow the HLA region. Below it, red lines represent the extension of the regions of chromosome 6 acquired uniparental disomy in the five patients we described, as documented by the SNP array studies.

Supplementary Figure 2. Cell Surface Phenotypic Profile of Leukemia at Diagnosis and at Relapse. Surface staining of leukemic blasts (identified as CD45^{dim} CD117⁺ CD33⁺ CD14⁻ cells) taken from UPN #16 at the moment of disease diagnosis (black profile) or at the documentation of loss of the patient-specific HLA haplotype (red profile). Light gray histograms represent staining with the appropriate histotypic control antibodies.

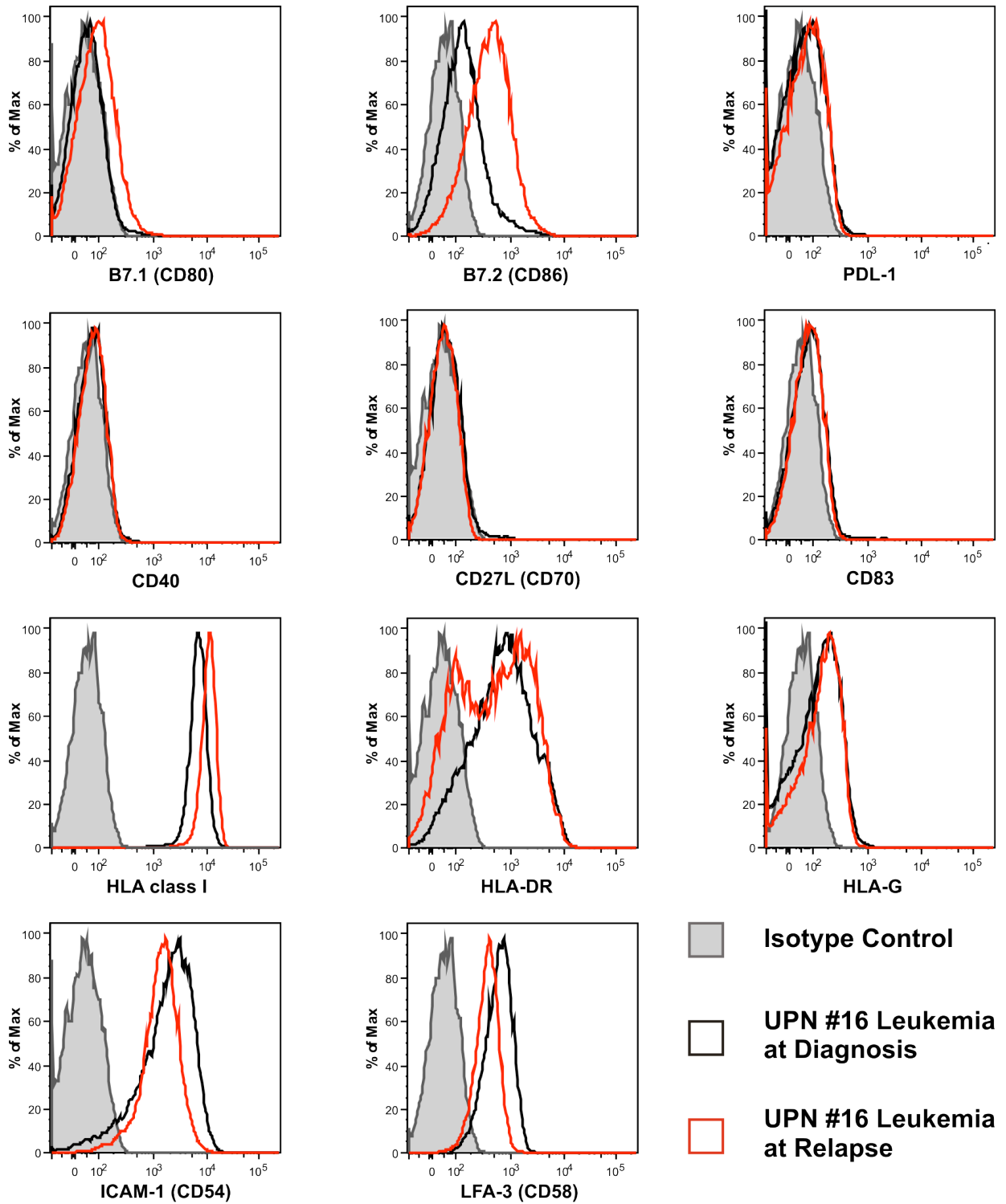
Supplementary Figure 3. NK Cell Degranulation in Response to Leukemia at Diagnosis and Relapse. PBMCs taken from UPN #16 stem cell donor, from the patient at two different post-transplantation time-points and from a healthy third-party control donor, were challenged with the HLA class I-negative K562 leukemia or primary leukemic blasts harvested from UPN #16 at leukemia diagnosis or at the loss of the patient-specific HLA haplotype. Activity of NK cells (identified as CD3⁻ CD56⁺ cells, boxed in red) was assessed after 6 hours of co-incubation as percentage of mobilization of the granule-specific marker CD107a. Quadrants in the dot plots evidence relative contribution of the CD56^{bright} (in the upper quadrants) and CD56^{dim} (in the lower quadrants) NK cell subsets to the overall antileukemic activity. Note that

in both donors and the patient only less than 10% of NK cells respond to the primary leukemic blasts, even after loss of the patient-specific alleles occurred at relapse

SUPPLEMENTARY FIGURE 1



SUPPLEMENTARY FIGURE 2



SUPPLEMENTARY FIGURE 3

