Chapter 9.

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Conversion to complete donor chimerism is an indicator for allo-reactivities causing graft-versus-leukemia/lymphoma (GvL) effects and graft-versus-host disease (GvHD) after allogeneic stem cell transplantation (SCT). Minor histocompatibility antigens (mHags) are driving these allo-reactivities in the human leukocyte antigen (HLA)-matched setting. The mHag HA-1 is only expressed by normal and malignant hematopoietic cells. Thus, HA-1 specific immunotherapy might boost the GvL effect and complete donor chimerism without inducing severe GvHD. Here, we studied the relationship between HA-1 specific cytotoxic T-cells (HA-1 CTLs) and the long-term kinetics of host hematopoietic chimerism after allogeneic HLA-matched, HA-1 mismatched SCT. Host chimerism was measured by highly sensitive mHag specific quantitative PCR. Functional HA-1 CTLs were detectable in 6/10 patients lysing host-type hematopoietic cells in vitro. HA-1 CTL^{pos} patients largely showed an inverse correlation between peripheral blood HA-1 CTL numbers and host hematopoietic chimerism levels. Conversely, HA-1 CTL^{neg} patients had persistently high host chimerism levels. Co-incubation of human host type CD34^{+} cells with HA-1 CTLs largely prevented progenitor and cobblestone area forming cell growth in vitro and human hematopoietic engraftment in immunodeficient mice. Our data suggest that CTLs against a single mHag HA-1 are sufficient to control chimerism by eliminating the host hematopoiesis on stem/progenitor cell level.

INTRODUCTION

Allogeneic stem cell transplantation (SCT) is a curative treatment for hematological malignancies[1] but is still associated with a considerable risk for relapse and graft versus host disease (GvHD) [2]. Thus, boosting the graft-versus-leukemia/lymphoma (GvL) effect without risking GvHD [1,2] is crucial for further improvements of the SCT outcome. Minor histocompatibility antigens (mHags), i.e. immunogenic polymorphic peptides presented in HLA-molecules [3], are the major targets of both the GvL and GvHD reactivity after HLA-matched allogeneic SCT [4]. The key observation indicating that the GvL effect can be separated from GvHD was the finding that mHags display a differential tissue distribution [5].

Ubiquitously expressed mHags are the prime in situ targets of GvHD [6]. Thus, immunotherapeutic targeting of mismatched hematopoiesis-restricted mHags may evoke strong GvL effects with low risk of GvHD [4].

Among the known hematopoiesis-restricted mHags, HA-1 appears particularly suitable for immunotherapeutic purposes since it is highly immunogenic [7] and its expression is shared by virtually all hematopoietic cells including normal [8] and leukemic progenitors [9], lymphoma [10] and multiple myeloma cells [11]. The HA-1 protein comprises two alleles resulting from a single amino acid (aa) polymorphism (Histidin (H) <-> Arginin (R)) [12]. Only the HA-1^{H} (but not the HA-1^{R}) allele forms immunogenic T-cell epitopes (in HLA-A2 and –B60) [4]. Thus, HLA-matched/HA-1 mismatched SCT results in strong T-cell responses of the HA-1^{RR} donor against hematopoietic cells of the HA-$1^{HR/HH}$ patient. The in vivo relevance of HA-1 in the GvL effect is documented by several observations. First, HA-1 specific cytotoxic T-lymphocytes (HA-1 CTLs) are capable of eradicating human leukemia cells in immunodeficient mice [13]. Second, HA-1 CTLs emerging after donor-lymphocyte infusions (DLI) subsequent to T-cell depleted SCT coincide with molecular remissions of relapsed CML and multiple myeloma [14]. Finally, the relapse risk of chronic myeloid leukemia (CML) is lower in those patients with GvHD subsequent to HA-1 mismatched compared to HA-1 matched allogeneic SCT [15]. Two approaches are currently investigated to translate these observations into HA-1 specific immunotherapy, namely adoptive transfer of in vitro generated HA-1 CTLs [16] and HA-1 peptide vaccination [17].

However, the broad applicability of HA-1 as immunotherapeutic target in nearly all hematological malignancies challenges the definition of uniform endpoints in clinical studies. Namely, the large variety of disease specific markers (e.g. various molecular markers for leukemia, paraprotein for myeloma or computer tomography for lymphoma) complicates the comparison of clinical responses to immunotherapy in different diseases. Moreover, absence of such markers particularly in cytogenetically normal hematopoietic malignancies hamper a disease specific response monitoring [18,19]. The HA-1 expression in all hematopoietic cells may provide a solution for the determination of clinical responses to HA-1 specific immunotherapy, since HA-1 CTLs eliminate both malignant and non-malignant host hematopoietic cells [8-11]. Therefore, we hypothesized that the suppression of the residual host hematopoiesis could serve as a universal marker for the clinical efficacy of HA-1 specific immunotherapy. However, the relationship between the long-term HA-1 specific immune response after T-cell replete SCT on the kinetics and profoundness of the suppression of host hematopoietic chimerism has not been studied. Moreover, it is unknown whether the HA-1 specific immune response can eradicate the residual host hematopoiesis, including host stem cells, or whether a persistent immunological pressure of donor HA-1 CTLs is needed to control the residual host hematopoiesis after allogeneic SCT.

In this study, we prospectively monitored the long-term kinetics of the HA-1 specific immune response in relation to the kinetics of host chimerism detected by highly sensitive techniques in patients after T-cell replete HLA-matched, HA-1 mismatched SCT. Moreover, we studied in vitro and in NOD/SCID mouse repopulation experiments whether HA-1 CTLs can eliminate not only host hematopoietic progenitor but also stem cells.

MATERIALS AND METHODS

Subjects studied

All HLA-A2^{+} patients receiving an allogeneic 10/10 HLA-matched (on a high-resolution level for exon 2+3 for HLA-A, B, C and for exon 2 for HLA-DRB1 and -DQB1 according to the actual European Federation for Immunogenetics guidelines) SC graft between May 2005 and August 2006 at the Hannover Medical School were typed for the mHags HA-1 and HA-2 [20]. Patients included in the study were mismatched for the mHag HA-1 (donor HA-1^{RR}, patient HA-1^{H}) and compatible for the mHag HA-2 (donor and patient HA-2^{V}) with their donors. Patients were treated according to SCT protocols approved by the Institutional Review Board. Patients and donors gave written informed consent in accordance with the declaration of Helsinki. Details are provided in table 1 and supplementary information. Blood samples were obtained in EDTA, peripheral blood mononuclear cells (PBMCs) were isolated by ficoll gradient, frozen and stored in liquid nitrogen. Sample analysis was performed at the Leiden University Medical Center with approval of the Institutional Review Board.

Monitoring of HA-1 CTLs

Allophycocyanin (APC)-conjugated HA-1^{A2} and HA-2^{A2} tetramers were generated as described [21]. PBMCs were thawed and incubated overnight in 20% human serum (HS) in Iscove's Modified Dulbecco's Medium (IMDM, Biowhittaker, Verviers, Belgium) at 37°C, 5% CO2. Then, PBMCs were stained with HA-1^{A2} or HA-2^{A2} tetramer dilutions, CD3-FITC and CD8-PE (Becton Dickinson) antibodies and propidium iodide (PI) as described [22] and analyzed with a FACS Calibur (Becton Dickinson) flow cytometer. Lymphocytes were gated in a forward/sideward scatter, viable PI^{-}/CD3^{+} T-cells were selected and presented in a plot with CD8 and HA-1^{A2} (or HA-2^{A2}) tetramer. HA-2^{A2} tetramers were used to determine background staining in every sample. Absolute CD8^{+}/HA-1^{A2} tetramer^{pos} cells/ml peripheral blood were calculated as follows: white blood cells/ml x %lymphocytes x (%CD3^{+}/CD8^{+}/HA-1^{A2} tetramer^{pos} cells in the PI^{-} lymphocyte gate – % CD3^{+}/CD8^{+}/HA-2^{A2} tetramer^{pos} cells in the PI^{-} lymphocyte gate).

Immunophenotyping

Immunophenotyping of HA-1^{A2} tetramer positive cells in PBMCs was performed on an LSR II flow cytometer (BD Biosciences) after staining CD3-Pacific-Blue, CD8-Alexa-700, CD27-PE, CD28-FITC and CD45RO-PE-Cy7 antibodies (Beckman Coulter Corporation, Miami, Fl, USA) and PI. Analysis gates were set using a CD45RO^{+}/CD27^{-}/CD28^{-} reference HA-1 CTL clone. Data analysis was performed using FlowJo (Treestar, Ashland, USA).

Isolation of mHag-specific CTL clones.

HA-1^{A2} tetramer^{+} CD8^{+} cells in PBMCs were sorted at one cell per well by using a FACSVantage cell sorter (Becton Dickinson) into a 96 well plate containing 10% HS in IMDM, irradiated allogeneic PBMCs, EBV LCLs, 1% phytohemagglutinin and 120 IU /ml interleukin-2 (IL-2, Chiron, Amsterdam, The Netherlands). CTLs were cultured until sufficient HA-1 CTLs could be harvested for functional assays.

Chromium release assay

Cytotoxicity was tested in a standard chromium release assay as described [16].

Quantification of host chimerism

Detection of Y-chromosome^{pos} and HA-1^{H} chimeric cells was performed on PBMCs as described earlier [23]. In short, the DNA was isolated with the QIAamp DNA blood minikit (Qiagen, Leusden, The Netherlands). Y-chromosome specific chimerism was analyzed using a one-step real-time PCR protocol. A second PCR reaction detecting the human Hematopoietic Cell Kinase gene (HCK) was carried out in parallel to standardize the data. Detection of HA-1^{H} chimeric cells was performed using a nested PCR protocol. Amplification data were collected and analyzed with the MyiQ^{TM} Single-Color real-time PCR detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands). The data are expressed as normalized fold expression, which was calculated according to the corresponding HCK levels. All samples were tested in at least two separate tests. A titration series of a mHag^{pos} EBV LCL Cell Line diluted in mHag^{neg} EBV-LCL was used as reference for linear regression analysis.

CD34^{+} and CD133^{+} cell purification

Mononuclear cells were isolated from CB or BM using a ficoll density gradient. CD34^{+} or CD133^{+} cells were isolated by magnetic cell separation using the direct CD34^{+} or CD133^{+} progenitor cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), checked for purity by flow cytometry and frozen at -80°C until use. The CD34^{-} or CD133^{-} fraction was used for HA-1 typing.

In vitro progenitor and stem cell assays

5000 CB or BM derived CD34^{+} or CD133^{+} cells were co-incubated overnight with irradiated (3000 rad) CTLs (CMV CTL clone 5D5 [13], HA-1 CTL clone 3HA15 [5] or allo-A2 CTL clone MBM13 (kindly provided by Prof. Fred Falkenburg, LUMC, The Netherlands)) at an effector to target ratio of 7:1 in a 96 round bottom plate in 10% HS in IMDM. In total, 6 wells per condition were plated. The next day, cells were washed twice with CAFC medium (IMDM supplemented with 3.2% inactivated fetal calf serum (FCS), 3.2% HS, 2.3 mM glutamine (Gibco, Breda, The Netherlands), 3x10^{2} U/ml penicillin (Bio-Whittaker) and 3x10^{2} µg/ml streptomycin (Bio-Whittaker), 7.2x10^{-3}mM hydrocortisone (Sigma, Zwijndrecht, The Netherlands) and 7.2 mM □-mercapto-ethanol (Sigma)). Viable CD34^{+} cells were quantitatively determined by flow cytometry with CD45-FITC, CD34-PE, 7AAD and Flow-Count™ fluorospheres (all Beckman Coulter, Mijdrecht, The Netherlands). Cells of 1 well per condition were subjected to a liquid human progenitor cells (HPC) assay and cells of 4-5 wells per condition were subjected to a cobblestone area-forming cell (CAFC) assay.

Liquid HPC and HALO assays

HPC assays were performed as described by the manufacturer (StemCell Technologies Inc., Grenoble, France). In short, 1000 BM- or 200 CB-derived CD34^{+} or CD133^{+} cells (supplemented with CTLs or not) were cultured in 1.1 ml Methocult dispensed in 35-mm dishes (Greiner, Alphen a/d Rijn, The Netherlands) for 14 days at 37°C, 5% CO_2. The number of erythroid, granulocyte and monocyte colonies was expressed as number of colonies formed per plate using an inverted light microscope. The HALO-96 human assay [24] was used to detect human progenitor cell proliferation in mouse BM as described by the manufacturer (HemoGenix, Colorado, USA). Briefly, 1,25x10^{5} BM WBC/well were plated in a 96-wells plate and incubated at 37°C, 5% CO_2 for 6 days with HPC specific cytokine mix (HemoGenix). Intracellular ATP content served as substrate for a luciferin/luciferase reaction was

measured as relative light units (RLU) (Centro LB 960 luminometer, Berthold Technologies, Vilvoorde, Belgium) and was determined exactly based on an ATP standard curve included into the assay.

Cobblestone area forming cell assay

Irradiated (500 rad) $2.7x10^4x10^4$ NIH3T3 mouse fibroblasts in IMDM supplemented with 10% FCS were added to a 96 well flat bottom plate pre-coated with 1% gelatin. The next day, fibroblasts were overlain with the $CD34^+$ cells obtained from the overnight incubation with CTLs or medium. Subsequently, the cells were cultured at 37°C in 5% CO_2; medium was replenished weekly by exchanging half of the medium with fresh CAFC medium. After 5 weeks, wells were scored positive if at least one phase-dark hematopoietic clone (a cobblestone area of at least 4 cells) was observed.

NOD/SCID repopulation experiments

8-10 weeks old female NOD.CB17-prkdc<scid>/J (NOD/SCID) mice (Charles River, France) were used [25] after ethical approval by the Leiden University Medical Center. 24 hours after sublethal irradiation with 3.5 Gy, NOD/SCID mice received a tail injection with $2x10^5$ $CD34^+$ cells pre-incubated overnight in a sterile FACS tube with irradiated CTLs (5D5, 3HA15 and MBM13) at an effector to target ratio of 7:1. Three UCBs were used with 3-4 mice per group for each UCB. Mice were sacrificed after 14 and 16 weeks. Cell suspensions (BM and spleen) and peripheral blood were prepared and analyzed by flow cytometry as described [26].

Statistical Analysis

Groups were compared using a two-tailed Student's t-test. A p-value < 0.05 was considered statistically significant.

RESULTS

Kinetics of HA-1 CTL responses post transplant

Ten patients transplanted with SC grafts of HLA-matched, HA-1 mismatched donors were included in this study. The patient characteristics are shown in table 1 and detailed clinical information is provided in the Supplementary information 1.

Table 1. Patient characteristics

NO	Disease	Age	Disease status at SCT	Condition-ing	Gender D→P	Sib	GvHD prophy-laxis	Graft	CD34+ cells (10^6/kg)	Neutro >500/ul on day	Outcome
1	AML	41	CR2	FLAMSA/ TBI/Cy/ATG	F → F	No	CsA/MMF	PBSC	6.2	26	Relapse d149 Death d391
2	PMF	58	Dup.sc. interm.	Flu/Bu/ATG	F → F	Yes	CsA/MTX	PBSC	3.7	31	CR Alive > d1701
3	ALL	18	CR1	TBI/VP-16/Thy	F → M	Yes	CSA/Pred	PBSC	5.5	15	CR Death d275
4	AML	39	PR1	FLAMSA/ TBI/Cy/ATG	F → M	Yes	CsA/MMF	PBSC	6.7	28	CR Alive >d1651
5	AML	59	PR1	FLAMSA/ TBI/Cy/ATG	F → M	No	CsA/MMF	PBSC	7.15	16	Relapse d453 Death d654
6	sAML	51	CR2	FLAMSA/ TBI/Cy/ATG	M → M	No	CsA/MMF	PBSC	4.5	20	Relapse d503 Death d984
7	CLL	48	PR2	TBI/Flu/Cy/ Cam	M → F	No	CsA	BM	1.45	17	PD Death d290
8	SAA	37	PD	TBI/Flu/Cy	F → F	Yes	CsA	BM	1.9	22	well Alive>d1319
9	CLL	41	PR2	Flu/Cy	M → M	Yes	CsA/MTX	PBSC	7.4	15	PD Alive >d1306
10	PMF	46	Dup.sc. interm.	Flu/Bu/ATG	M → M	No	CsA/MTX	PBSC	21.6	24	CR Alive >d1763

Abbreviations: AML, acute myeloid leukemia; sAML, secondary AML; ALL, acute lymphoblastic leukemia; PMF, primary myelofibrosis; CLL: chronic lymphoblastic leukemia; SAA, severe aplastic anemia; Sib: matched sibling donor; PR: partial remission; CR: complete remission; PD: progressive disease; Dup.Sc.interm: Dupriez score intermediate; Bu, Busulphan; Cy, Cyclophosphamide; Flu, Fludarabin; TBI, Total Body Irradiation; VP-16, Etoposide; the FLAMSA protocol consisted of Fludarabine, Cytosine-Arabinoside and Amsacrine; D, donor; P, patient; M, male; F, female; MMF, Mycophenolate Mofetil; MTX, Methotrexate; CsA, Cyclosporine A; Pred, prednisolone; ATG, Anti-Thymocyte Globulin; Thy, Thymoglobuline; Cam, Campath; PBSC, peripheral blood stem cells; BM, bone marrow

The median observation time post transplant was 613 days (range 125-1162). Presence of HA-1 CTLs was analyzed in donor PBMCs obtained prior to SC collection and in all available patient PBMC samples before and after allogeneic SCT. None of 8 evaluable donors showed HA-1 CTLs in the peripheral blood before SCT. Post-transplant patient's samples collected at 18 (median; range 9-36) post-transplant time points per patient with a median interval of 15 days (median; range 5-250) were analyzed. HA-1 CTLs were detectable in 6/10 patients (patients 1-6) after SCT with frequencies between 0.02% and 0.14% of the viable $CD3^+/CD8^+$ T-cells. HA-1 CTLs were first detected 72 days (median; range 28-195) after allogeneic SCT (Figure 1). Increment of HA-1 CTLs was detectable 26 days (median; range 11-70) after discontinuation of CsA, MMF or prednisolon (patient 1,2, 4-6), 19 (median; range 0-69)

days after onset of acute or chronic GvHD (patients 3-6) or 46 days after the last DLI (patient 5). Additional HA-1 CTL peaks were found during the course of GvHD (patient 3). HA-1 CTLs were observed until day 516 (median; range 125-699) after SCT. Overall, HA-1 CTLs were detectable and largely persisting in half of the patients analyzed in this study.

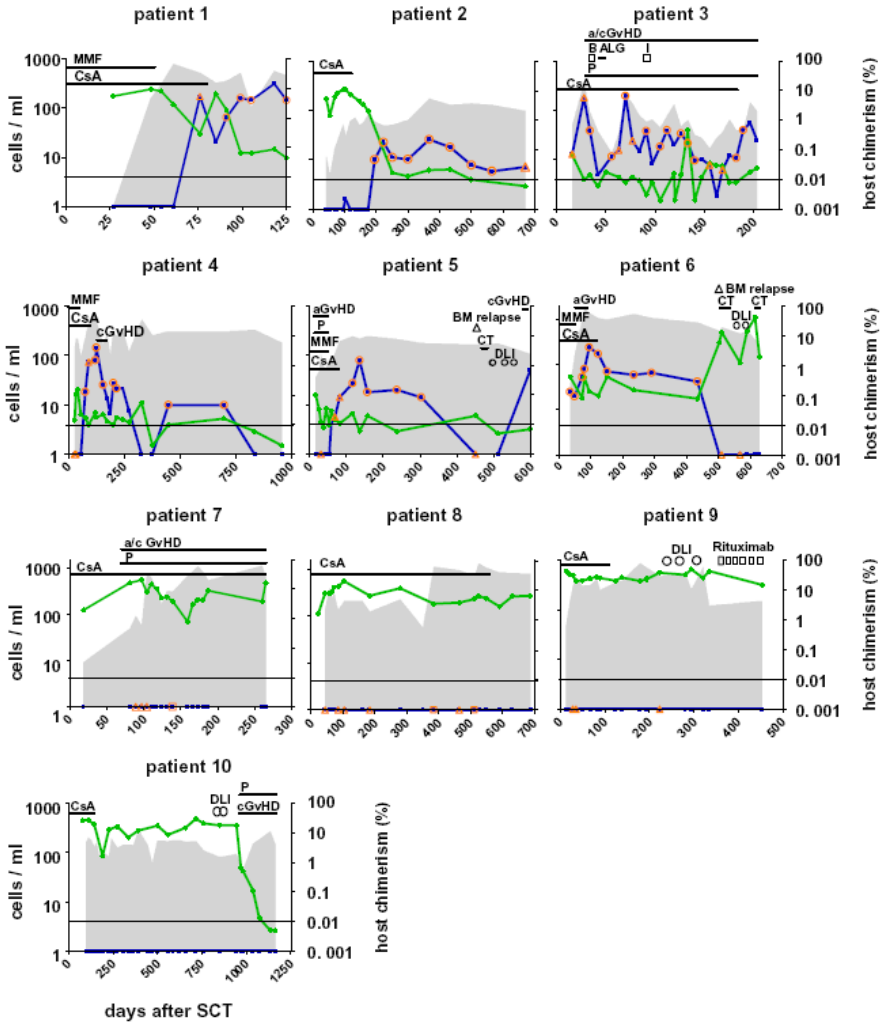

Figure 1. Kinetics of the donor HA-1 CTL response in relation to host chimerism in the peripheral blood. Depicted are the results of 10 patients undergoing allogeneic HLA-matched, HA-1 mismatched SCT; x-axis: days after allogeneic SCT; left y-axis, blue squares and line: T-cells in absolute numbers per ml peripheral blood detected and calculated as described in Materials and Methods; open circles: samples from which HA-1 CTLs could be isolated capable of lysing Epstein-Barr virus/lymphoid cell lines (EBV LCLs) expressing the HA-1^{H} natural ligand; open triangles: samples with unsuccessful attempts to isolate HA-1^{A2} tetramer staining cells; shaded areas: $CD8^{+}$ T-cells in 10^{3} cells per ml peripheral blood. right y-axis, green diamonds and line: host chimerism determined by highly sensitive mHag HA-1 (patient 1-2, 6-10) or H-Y (patient 3-5) specific quantitative PCR. The lower detection limit is 1 host in 10^{4} donor cells for HA-1^{H} allele specific PCR and 1 host in 10^{5} donor cells for H-Y specific PCR; horizontal black bars indicate times of GVHD, immunosuppression (CsA, MMF, P, ALG, B and I) or CT, black open circles indicate DLI, black open triangles indicate relapse, black open squares indicate rituximab treatment, Abbreviations: CsA, cyclosporine A; MMF, mycophenolate mofetile; P, prednisone; B, basiliximab; I, infliximab; ALG: anti-lymphocyte globulin; CT: chemotherapy.

Isolation and functional characterization of HA-1 CTLs

HA-1 CTLs were isolated from 57 of 71 HA-1^{A2} tetramer^{pos} PBMC samples after allogeneic SCT by single-cell sorting and expansion on allogeneic feeder cells from patients 1-6. 60 (median; range 34-142) HA-1 CTL clones per patient were isolated and expanded in vitro. 13 (median; range 8-25) HA-1 CTL clones per patient were tested in a ^{51}Cr-release assay for cytotoxicity against HA-1^{H} (natural ligand of host type), HA-1^{RR} (natural ligand of donor type) and HA-1^{H} peptide loaded HA-1^{RR} target cells. From an additional 15 (median; range 7-18) HA-1 CTL clones per patient, sufficient cell numbers were only available for testing lysis of HA-1^{H} natural ligand and of HA-1^{H} peptide loaded HA-1^{RR} target cells. In summary, 82% (median; range: 69-100%) of all HA-1 CTL clones analyzed recognized target cells with the HA-1^{H} natural ligand and 2% (median; range: 0-25%) of the HA-1 CTL clones did not recognize the HA-1^{H} natural ligand but did recognize HA-1^{H} peptide loaded target cells. None of the HA-1 CTL clones tested against target cells expressing the HA-1^{RR} of the donor showed cytotoxicity (Figure 2). All clinical samples wherein natural HA-1^{H} ligand specific HA-1 CTL clones were successfully isolated are indicated in Figure 1 (open circles). PBMCs without detectable HA-1^{A2} tetramer^{pos} cells from six patients (patient 4-9) were used as control for the validity of the HA-1^{A2} tetramer staining to detect functional HA-1 CTLs (open triangles). None of the HA-1^{A2} tetramer^{neg} PBMC samples did contain HA-1 CTL clones recognizing the natural HA-1^{H} ligand. Overall, most HA-1^{A2} tetramer^{pos} PBMCs samples contained HA-1 CTLs specifically lysing hematopoietic cells expressing the natural HA-1^{H} ligand of the host.

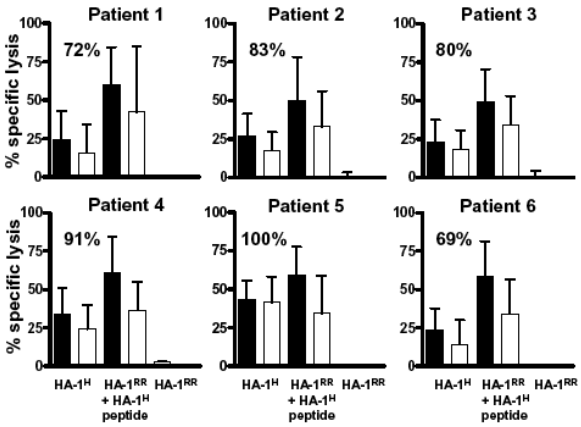

Figure 2. Cytotoxicity of isolated HA-1 CTLs. (A) HA-1 CTL clones were isolated from peripheral blood of patients 1-6 at multiple time-points and expanded in vitro. Median 13 (range: 8-25) HA-1 CTL clones per patient were tested in a 4h ^{51}Cr-release assay for HA-1 specific recognition of target cells in vitro. Left upper corner: percentage of HA-1 CTL clones lysing HA-1^{H} naturally expressing EBV LCLs; x-axis: HA-1^{H} naturally expressing EBV LCLs, HA-1^{RR} EBV LCLs loaded with HA-1^{H} peptide, HA-1^{RR} EBV LCLs, effector to target ratio: back: 10:1, white: 1:1; y-axis: % specific lysis.

Phenotyping of HA-1 CTLs

The PBMCs containing HA-1^{A2} tetramer^{pos} cells were phenotypically characterized in PBMCs by CD45RO staining to distinguish memory T-cells from naïve T-cells and by CD27/CD28 staining to determine the level of differentiation [27;28]. The analysis was performed longitudinally on most samples containing HA-1^{A2} tetramer^{pos} cells (shown in Figure 1) using an established CD45RO^{+}CD27^{-}CD28^{-} HA-1 CTL clone to standardize gating. HA-1^{A2} tetramer^{pos} cells were largely CD45RO^{+} in all investigated samples indicating the antigen-experienced status of the HA-1 CTLs (Figure 3A-C: patient 3,6,5; Supplementary Figure S1A-C: patient 2,4,1). HA-1^{A2} tetramer^{pos} cells were predominantly CD27^{+} throughout the observation period. The CD28 staining revealed either a transition from CD28^{+} to CD28^{-} in patient 3 (Figure 3A), 2 and 4 (Supplementary Figure S1A, B) during the post-transplant period,

consistently a CD28^{-} population in patient 6 (Figure 3B), consistently a CD28^{+} population in patient 5 (Figure 3C) or consistently a mixed CD28$^{+/-}$ population in patient 1 (Supplementary Figure S1C). The CD27 staining became largely negative during the observation period only in patient 6 (Figure 3B). Overall, the HA-1 CTLs were largely antigen-experienced and underwent a variable degree of differentiation after allogeneic SCT.

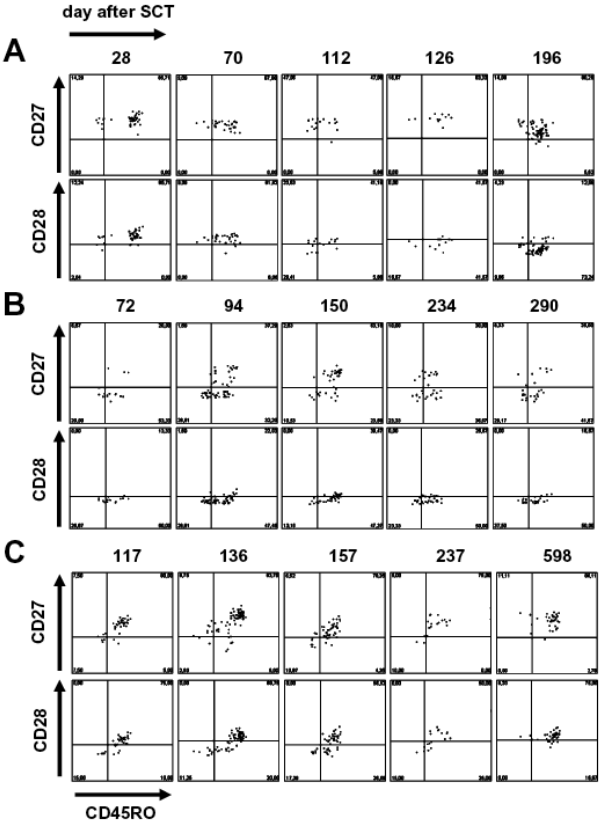

Figure 3. Longitudinal analysis of the HA-1 CTL phenotype in PBMCs after allogeneic SCT. (A-C) HA-1^{A2} tetramer^{pos} CD3^{+}/CD8^{+} cells in patient PBMCs after allogeneic SCT were phenotypically characterized by CD45RO, CD27 and CD28 staining. An established CD45RO^{+}CD27^{-} CD28^{-} HA-1 CTL clone was used to standardize gating (not shown). Depicted are selected results of patients 3(A), 6(B) and 5(C).

Kinetics of host chimerism post transplant

Host chimerism was determined in all post-transplant samples of patients 1-2 and 6-10 by HA-1 specific qPCR (detection of 1 host in 10^4 donor cells). In three male patients with female donors (patients 3-5), host chimerism was determined by H-Y specific qPCR (detection of 1 host in 10^5 donor cells). Host chimerism data were compared with the number of HA-1^{A2} tetramer^{pos} cells for each same sample. In HA-1 CTL^{+} patients (patients 1-6), emergence of HA-1 CTLs was associated with a rapid decline of host chimerism (patient 1 and 2; Figure 1). Moreover, persistence of HA-1^{A2} tetramer^{pos} cells was associated with host chimerism levels remaining just above the detection limit (patient 2-5; Figure 1). Fluctuations of HA-1 CTLs were mostly associated with a reverse kinetics of host chimerism (patients 1,3,5,6; Figure 1). Finally, loss of functional HA-1 CTLs was followed by leukemia relapse in the bone marrow (patient 5; Figure 1) and rapid re-emergence of host (leukemia) cells in the peripheral blood (patient 6; Figure 1).

The four HA-1 CTL$^-$ patients (patient 7-10) had persistent host chimerism levels >1% almost throughout an observation period of up to 900 days (patient 7-10; Figure 1). Overall, these data suggest that the suppression of host chimerism was mostly associated with the presence of functional HA-1 CTLs. However, one HA-1 CTL$^-$ patient with persistent high host chimerism levels of more than 1% showed a rapid decline of host chimerism to undetectable levels after day 900 subsequent to two DLIs (patient 10; Figure 1).

HA-1^H CTLs eliminate human hematopoietic stem/progenitor cells in vitro

Next, the capacity of HA-1 CTLs to eliminate human hematopoietic stem/progenitor cells (HSPCs) in vitro was investigated. CD34$^+$ cells were isolated from HLA-A2$^+$/HA-1^H or HA-1^{RR} UCBs and co-incubated overnight with medium, a control CMV CTL clone 5D5, the well defined HA-1 CTL clone 3HA15 [5] and an allo HLA-A2 specific CTL clone MBM13.

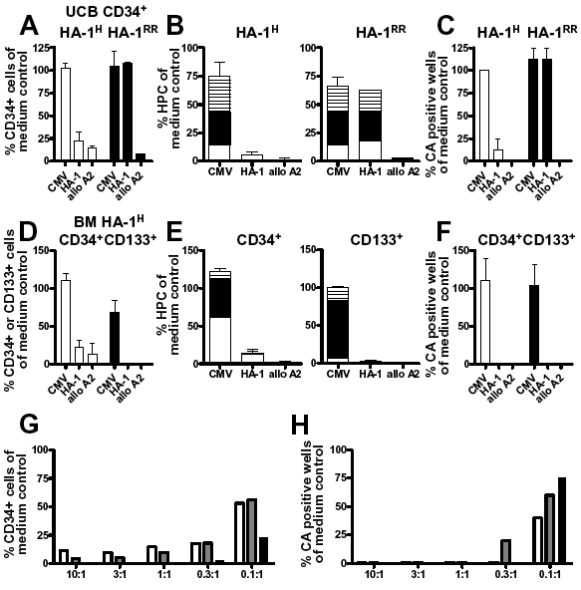

Figure 4. HA-1^H specific elimination of human umbilical cord blood and bone marrow derived hematopoietic progenitor and stem cells by HA-1 CTLs studied in vitro. (A-F) Umbilical cord blood (UCB) derived CD34+ and bone marrow (BM) derived CD34$^+$ and CD133$^+$ cells from HLA-A2/HA-1^H or HLA-A2/HA-1^{RR} donors were incubated with medium, an irradiated control CMV CTL clone, an HA-1 CTL clone or an anti-HLA-A2 specific CTL clone overnight. Depicted are the mean values +/- standard deviation of three independent experiments, respectively. The results of the medium control were set as 100%. (A, D) % viable CD34$^+$ or CD133$^+$ cells compared to the medium control directly after over-night co-incubation with CTLs. (B, E) % colony forming cells compared to the medium control as determined after 14 days; colony forming unit-macrophage (CFU-M; striped bar), CFU-Granulocyte (CFU-G; black bar), blast forming unit (BFU-E; white bar). (C, F) % of cobblestone area positive wells in a CAFC assay compared to the medium control as determined after 5 weeks. (G-H) An HA-1 CTL clone was titrated to BM derived CD34+ cells (white bars), UCB derived CD34+ cells (grey bars) and to BM derived CD133$^+$ cells (black bars) from HLA-A2/HA-1^H donors and co-incubated over night. x-axis: HA-1 CTL to CD34 or CD133 cell ratio. Y-axis: % viable CD34$^+$ or CD133+ cells compared to the medium control (G) and % of cobblestone area positive wells in a CAFC assay compared to the medium control as determined after 5 weeks (H).

Co-incubation of HA-1^{RR} (i.e. donor type) CD34$^+$ cells with the HA-1 CTL clone resulted in no significant differences in viable CD34$^+$ cell numbers, HPC colonies and CAFC colonies compared to the medium and CMV CTL control (Figure 4A-C). In contrast, co-incubation of HA-1^H (i.e. patient type) CD34$^+$ cells with the HA-1 CTL clone resulted in a considerable reduction of viable CD34$^+$ cells in the flow cytometry, a considerable reduction of particularly CFU-G and –M growth in the HPC assays and in a considerable reduction in the colony formation in the CAFC assay compared to the medium and CMV

CTL control ($p<0.01$, for all assays) (Figure 4A-C). Similar results were obtained after co-incubation of BM derived HA-1^H $CD34^+$ and $CD133^+$ cells with HA-1 CTLs (Figure 4D-F). These data indicate that HA-1 CTLs can eliminate UCB and BM derived HA-1^H HSPC and that HA-1 CTL mediated HSPC killing is specific for the presence of HA-1^H. Finally, the HA-1 CTL clone was titrated to human UCB derived $CD34^+$ cells, BM derived $CD34^+$ and BM derived $CD133^+$ cells from HLA-$A2^+$/HA-1^H donors. Co-incubation with HA-1 CTLs resulted in a comparable reduction of $CD34^+$ (from UCB and BM) and $CD133^+$ cells in the flow cytometry and a comparable suppression of CAFC colonies (Figure 4G,H). The minimum required effector to target cell ratio ensuring complete suppression of CAFC colonies was 0.3-1:1. In all experiments, the allo-HLA-A2 specific control CTLs largely deleted CD34+ or CD133+ cells, suppressed HPC growth and entirely prevented colony formation in CAFC assays. In summary, HA-1 is expressed on human HPCs and on hematopoietic stem cells and functions as target for HA-1 CTLs.

HA-1^H is expressed on human NOD/SCID mouse repopulating hematopoietic stem cells

Next, we investigated whether HA-1 CTLs can eliminate human non-obese diabetic / severe combined immune deficiency (NOD/SCID) mouse repopulating hematopoietic stem cells. UCB derived HLA-$A2^+$/HA-1^H $CD34^+$ cells were co-incubated with control CMV CTLs, HA-1 CTLs and allo-HLA-A2 specific CTLs (3-4 mice per group) overnight prior to transplantation into NOD/SCID mice. Human hematopoietic engraftment was determined 14 or 16 weeks after transplantation in three independent experiments. All mice transplanted with $CD34^+$ cells co-incubated with control CMV CTL showed considerable human $CD45^+$ cell engraftment in the peripheral blood (week 14-16: mean 8.5%, range 0.3 - 40.8), spleen (week 14-16: mean 2.0%, range 0.1-8.2) and BM (week 14-16: mean 20.1%, range 1.8-58.0) (Figure 5). Sub-analysis of the human $CD45^+$ cells in the bone marrow revealed presence of $CD34^+$ (week 14-16: mean 3.7%, range 0.4-8.3), $CD33^+$ (week 14-16: mean 4.1%, range 0.2-12.4) and $CD19^+$ cells (week 14-16: mean 9.3%, range 2.2-20.5). In contrast, no human $CD45^+$ cells were detectable in the peripheral blood, spleen or BM of mice transplanted with CD34+ cells co-incubated with HA-1 CTLs or with allo HLA-A2 specific CTLs (Figure 5). Finally, BM cells of mice sacrificed after 16 weeks were further investigated for the presence of human cells capable of proliferation in progenitor cell assays. In the HALO assay, BM from mice transplanted with CMV CTL treated $CD34^+$ cells showed human cell proliferation, while BM from mice transplanted with HA-1 CTL or with allo-HLA-A2 CTL treated $CD34^+$ cells were negative for human cell proliferation (Supplementary Figure S2). These data suggest that HA-1 is expressed on human hematopoietic stem cells with the potential of long-standing engraftment in NOD/SCID mice.

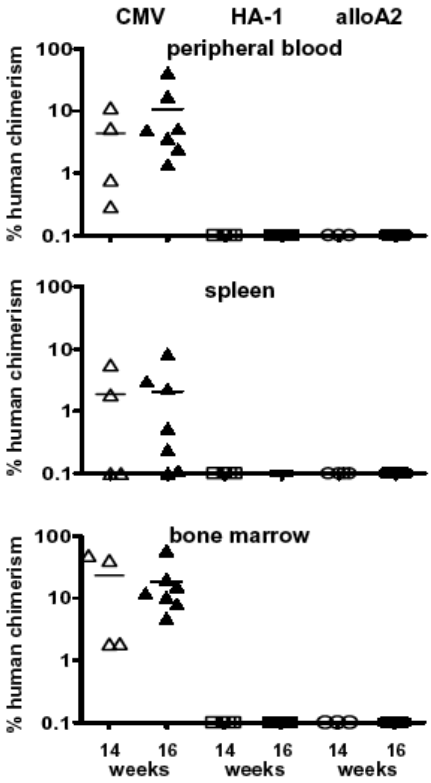

Figure 5. Elimination of NOD/SCID mouse repopulating human stem cells by HA-1 CTLs. UCB derived $CD34^+$ cells from three different HLA-A2/$HA\text{-}1^H$ donors were incubated with an irradiated control CMV CTL clone (triangles), HA-1 CTL clone (boxes) or anti-HLA-A2 CTL clone (circles) and transplanted into sublethally irradiated NOD/SCID mice. After 14-16 weeks, mice were sacrificed. Depicted is the % human hematopoietic chimerism in the peripheral blood, spleen and bone marrow of the individual mice after 14 (white symbols) and 16 (black symbols) in three independent experiments. The bar represents the mean of the group.

DISCUSSION

This study for the first time describes that donor-derived CTLs against a single mHag HA-1 are sufficient after allogeneic SCT to control chimerism by eliminating the host hematopoiesis on the level of stem/progenitor cells. This knowledge provides important guidance for the definition of suitable clinical and immunological endpoints of studies aiming at boosting the GvL effect by HA-1 specific immunotherapy.

Long-term monitoring of the HA-1 specific immune response

HA-1 CTLs were detectable in 6/10 patients after T-cell replete HLA-matched, HA-1 mismatched SCT. Increasing HA-1 CTLs were measurable subsequent to discontinuation of immune suppression, GvHD or DLI. While HA-1 CTLs during GvHD [22] and subsequent to DLI [14] were described earlier, detection of HA-1 CTLs subsequent to the cessation of immune suppression might have been missed due to T-cell depleted SC grafts in earlier studies. Remarkably, HA-1 CTLs not only shortly peaked but mostly persisted after emergence for months or years at low levels in our study. Thus, HA-1 CTLs can create a long lasting immunity after allogeneic SCT. Interestingly, HA-1 CTLs could be isolated and expanded to numbers sufficient for functional testing from almost all HA-1^{A2} $tetramer^{pos}$ PBMC samples. This analysis revealed that HA-1 CTLs emerging after allogeneic SCT largely consist of high avidity, i.e. HA-1^{H} ligand specific CTLs. Four out of 10 patients analyzed did not show HA-1 CTLs in the entire post-transplant period regardless of the termination of immune suppression (patients 8-10), GvHD (patients 7,10) or DLI (patients 9,10). Thus, neither HA-1 mismatched SCT itself nor the described clinical events are sufficient to evoke detectable HA-1 CTLs. It remains unclear, which factors determine HA-1 specific responses after allogeneic SCT. Pre-immunizations of healthy donors may have an impact [29], since both mHag sensitization and tolerization can occur through pregnancy in mothers and children [30,23]. However, the donor PBMCs in our study did not contain HA-1 CTLs. Additional studies are required to determine the predictive value of the donor immunization for HA-1 CTL emergence in allo-transplanted patients.

HA-1 CTLs in reverse correlation with host chimerism

A series of factors has been described influencing the donor cell engraftment kinetics, such as conditioning intensity [31] and graft composition [32,33]. The impact of the transplanted donor immune system on donor SC engraftment is well documented by the negative effects of T-cell depletion [34] and the positive effects of DLI [35,36]. Our data for the first time show that CTLs directed against host hematopoietic mHags are important mediators of the conversion to complete donor chimerism in the HLA-matched setting. Namely, HA-1 CTL^{+} patients showed low to undetectable host chimerism levels during HA-1 CTLs presence. Moreover, HA-1 CTL increase in the peripheral blood was mostly accompanied by a decline of host chimerism and vice versa. Importantly, the detection of the reverse correlation between the HA-1 specific immune response and host chimerism required the high sensitivity of mHag allele specific quantitative PCR. Namely, the long-term fluctuation of chimerism largely occurred at levels below the detection levels (10^{-2}) of the clinically used chimerism tests (particularly patient 3,5,6). Finally, HA-1 CTL^{-} patients showed persistent host chimerism at high levels throughout the post SCT period. Certainly, the host hematopoiesis is not solely suppressed by CTLs specific for the hematopoiesis-restricted mHag HA-1. Although we detected anti-HY responses only in 1/3 patients transplanted with a gender mismatch (data not shown), also immune responses against other known and unknown mismatched mHags are expected to contribute to the conversion to full donor chimerism. This

assumption is supported by the observation in patient 10 who converted to complete donor chimerism after DLIs without detectable HA-1 CTLs.

Loss of HA-1 specific CTLs in relation to relapse

Presence of HA-1 CTLs early after allogeneic SCT is not sufficient to prevent relapse at a later time point. Namely, patients 1, 5 and 6 relapsed despite detectable levels of HA-1 CTLs early after allogeneic SCT. The observation that relapse was associated with a loss of functional HA-1 CTLs in patients 5 and 6 indicates the relevance of HA-1 CTL persistence to control the host hematopoiesis including the malignant cells. To evaluate whether loss of functional HA-1 CTLs is associated with progressive HA-1 CTL differentiation after allogeneic SCT, we performed an extensive phenotypic analysis of the HA-1^{A2} tetramer^{pos} CTLs in PBMCs. HA-1 CTLs had predominantly a memory phenotype, were largely $CD27^+/CD28^+$ at first detection and showed a progressive reduction of CD28 during the post-transplant period. Interestingly, patient 6, i.e. the patient with the highest host chimerism levels during the presence HA-1 CTLs, was the only patient with $CD28^-$ HA-1 CTLs in all samples (Figure 3B). Together with the additional loss of CD27 shortly before relapse in this patient, these data may suggest that the continuous stimulation of HA-1^H host cells in the peripheral blood had induced an early terminal differentiation and, ultimately, loss of HA-1 CTLs. However, patient 5, i.e. a patient with very low host chimerism levels in the peripheral blood in the entire observation period and loss of HA-1 CTLs at BM relapse showed no signs of terminal HA-1 CTL differentiation (Figure 3C). Therefore, other factors than persisting antigen stimulation may limit HA-1 CTL persistence after allogeneic SCT. Particularly emergence of regulatory T-cells [37] or host-anti-graft immune responses [38,39] need to be considered and require further studies.

Functional HA-1 expression on host hematopoietic stem/progenitor cells

The persistence of host chimeric cells in most of the patients throughout the post transplant period raised the question whether HA-1 specific immunotherapy would be ultimately capable of definitely eradicating the host hematopoiesis. This question becomes increasingly relevant particularly with the progressive use of reduced intensity conditioning frequently resulting in a slower conversion to donor chimerism compared to myeloablative regimens [31]. We showed earlier that HA-1 CTLs can eliminate normal and malignant hematopoietic progenitor cells [8,9]. In the current study, we evaluated whether HA-1 is also functionally expressed on normal hematopoietic stem cells serving as a model for the residual host hematopoiesis. In contrast to previous studies [8,9], HSPCs were derived not only from BM but also from UCB and were further refined by surface expression of CD34 and CD133. Co-incubation of UCB and BM derived $CD34^+$ or $CD133^+$ stem cells with an HA-1 CTL clone isolated earlier from an allo-transplanted patient [5] largely abrogated HPC and CAFC colony formation in vitro. Most importantly, HA-1 CTLs eliminated NOD/SCID mouse repopulating stem cells for at least 16 weeks post transplant indicating that also HSPC with the potential of long-standing engraftment are eliminated by HA-1 CTLs. Of note, our study solely focused on the eradication of normal hematopoietic stem cells as model for the residual host hematopoiesis. However, also the residual malignant hematopoiesis contributes to host chimerism in leukemia patients. The HLA-B8 restricted mHag UTY [40] and the HLA-B*2705 restricted mHag DDX3Y [41] are described to be expressed on leukemia stem cells. However, although HA-1 is expressed on virtually all hematological cancers [9-11], the formal proof of functional HA-1 expression by malignant stem cells is very cumbersome due to the controversial or still absent characterization of these

cells in the various malignancies [42-45]. Nevertheless, our data indicate that an HA-1 CTL to stem cell ratio of at least 0.3-1:1 within in the stem cell niche would be necessary for the eradication of the host hematopoiesis. It remains to be determined whether HA-1 specific immunotherapy is capable of enriching HA-1 CTLs in the BM compartment at these quantities.

Conclusions

Our data help to define suitable clinical and immunological endpoints of HA-1 specific immunotherapy studies. First, suppression of host chimerism appears to be a generally applicable marker for the clinical response to HA-1 specific immune reactivities. Nevertheless, disease related indicators remain necessary to complete the information on the GvL efficacy of immunotherapy. Second, the inverse correlation between host hematopoiesis suppression and HA-1 specific immune responses indicates that HA-1 specific immunotherapy optimally aims at a maximal ratio between HA-1 CTLs and host hematopoietic cells. This implies that HA-1 specific immunotherapy should start pre-emptively to eradicate the residual host cells before relapse can occur. Third, although HA-1 seems to be functionally expressed on HSPCs, the efficacy of immunotherapy to generate high HA-1 CTL numbers in the stem cell niche is still unknown. Therefore, immunotherapy should aim at generating long-lasting HA-1 specific immune responses.

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SUPPLEMENTARY INFORMATION 1

Overall, this study included 10 patients having received reduced-intensity (n=8) or myeloablative conditioning (n=2: patients 3,7) before transplantation of G-CSF-mobilized peripheral blood stem cells (PBSC, n=8) or bone marrow (BM, n=2: patients 7,8). Four patients were transplanted from HLA-matched sibling donors (MSD) and six patients were transplanted from HLA-matched unrelated donors (MUD). Three male patients (patients 3-5) received a sex-mismatched graft. GvHD prophylaxis consisted of cyclosporin A (CsA), methotrexate (MTX), mycophenolic acid (MMF), steroids and antibodies. Immune suppression was stopped in 8 patients (patient 1,2,4-6,8-10) during the observation period, 4 patients (patient 3,5-7) had episodes of acute GvHD, 3 patients (patient 4,5,10) had newly occurring chronic GvHD timely separated from acute GvHD and 5 patients (patients 5,6,9,10) received DLIs.

Patient 1

A 41 year old woman with AML (FAB M4/M5, complex karyotype) in second complete remission (CR2) received SCT from a female MUD after FLAMSA/TBI/Cy/ATG conditioning. GvHD prophylaxis consisted of CsA and MMF. Immune suppression ended on day 80 and blood collection ended on patient's request on day 125. On day 142 she received a prophylactic DLI ($5x10^5$ CD3+ cells/kg). Meningeal relapse was diagnosed on day 149 which was treated with chemotherapy, radiation and additional DLIs ($1x10^6$ CD3+ cells/kg on day 257, $5x10^6$ CD3+ cells/kg on day 296 and $5x10^6$ CD3+ cells/kg on day 380). The patient died on day 391.

Patient 2

A 58 year old woman with PMF received SCT from her sister after Fludarabin/Busulphan/ATG conditioning. GvHD prophylaxis consisted of CsA and MTX. The post-transplant period was complicated by a veno-occlusive disease responding to Prociclide. Sample analysis ended on day 670. The patient is alive and well.

Patient 3

A 18 year old man with ALL (pro-B ALL, normal karyotype) in CR1 received SCT from his sister after TBI/etoposide/thymoglobuline conditioning. GvHD prophylaxis consisted of CsA and steroids. The post-transplant period was complicated by GvHD IV^o with gut involvement starting on day 29. Regression of symptoms occurred after high dose steroids (500mg/d), Basiliximab and a course of anti-lymphocyte globulin on day 43. Steroids were increased and infliximab was started on day 90 resulting in a temporary reduction of symptoms. On day 126 ileus symptoms started together with candida sepsis. On day 149 the bowel was depressurized by an ileostoma. On day 183 onset of psychotic symptoms associated with cerebral lesions in the magnetic resonance tomography. Regressive GvHD symptoms allowed tapering of immune suppression. Sample analysis ended on day 203. On day 248, stereotactic brain biopsy revealed cerebral fungus infection. The patient died on day 275.

Patient 4

A 39 year old man with AML (FAB M5, t(9;11)) in first partial remission (PR1) received SCT from his sister after FLAMSA/TBI/Cy/ATG conditioning. GvHD prophylaxis consisted of CsA and MMF. The post-transplant period was uncomplicated. A discrete chronic GvHD I-II^o of skin and mouth was

diagnosed on day 120 which disappeared without specific treatment. Sample analysis ended on day 958. The patient is alive and well.

Patient 5

A 59 year old man with AML (FAB M0, complex karyotype) in PR1 received SCT from a female MUD after FLAMSA/TBI/Cy/ATG conditioning. GvHD prophylaxis consisted of CsA and MMF. The post-transplant period was complicated by veno-occlusive disease responding to Prociclide. Skin GvHD II^o was diagnosed on day 12 which responded to steroids. The post-transplant period was uncomplicated until on day 453 bone marrow relapse was diagnosed. The patient received chemotherapy and 3 DLIs ($1x10^7$ CD3+ cells/kg on day 496, $2x10^7$ CD3+ cells/kg on day 528, $1x10^8$ CD3+ cells/kg on day 552). Mild chronic GvHD of skin, mouth and liver was diagnosed on day 579 and the patient was treated with CsA and steroids. Sample analysis ended on day 598. The patient died on day 654 due to chronic lung GvHD and multi organ failure.

Patient 6

A 51 year old man with sAML/MDS (FAB M6, complex karyotype) in CR2 received SCT from a male MUD after FLAMSA/TBI/Cy/ATG conditioning. GvHD prophylaxis consisted of CsA and MMF. The post-transplant period was complicated by a skin GvHD I^o starting on day 45. On day 503, bone marrow relapse was diagnosed and treated with chemotherapy. The patient received a 1st DLI ($1.4x10^7$ CD3+ cells/kg) on day 554 and a 2nd DLI ($5x10^7$ CD3+ cells/kg) on day 582. A new chemotherapy was started on day 619 leading to aplasia. On day 665 a second SCT from another donor was performed. Sample analysis ended on day 629. The patient died on day 984 after second relapse.

Patient 7

A 48 year old woman with CLL (complex karyotype) with PR2 received BMT from a male MUD after TBI/Fludarabin/Cyclophosphamide/Campath conditioning. GvHD prophylaxis consisted of CsA. The post-transplant period was complicated by a herpes encephalitis (onset on day 25), bacteraemias and venous thrombosis. Skin GvHD II^o started on day 67 which responded to steroids but reoccurred on day 149 after tapering of immune suppression. Fungal pneumonia was diagnosed on day 241. Chronic skin GvHD increased on day 267. Sample analysis ended on day 265. The patient died on day 290 due to sepsis.

Patient 8

A 37 year old woman with severe aplastic anemia with no response to ATG/CsA upon relapse after initially successful ATG/CsA treatment received 71 months after first diagnosis BMT from her sister after TBI/Fludarabin/Cyclophosphamide conditioning. GvHD prophylaxis consisted of CsA. The post-transplant period was uncomplicated. Sample analysis ended on day 686. The patient is alive and well.

Patient 9

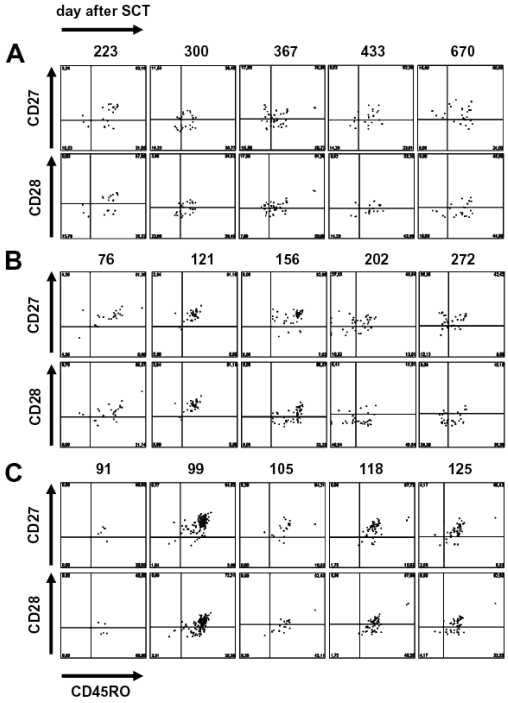
A 41 year old man with CLL (normal karyotype, non-mutated VH-Gen) in PR2 received SCT from his brother after Fludarabin/Cyclophosphamide/ATG conditioning. GvHD prophylaxis consisted of CsA and MTX. The post-transplant period was uncomplicated. Due to persistently low donor chimerism, the patient received several DLIs (1st DLI $1x10^6$ CD3+ cells/kg on day 237, 2nd DLI $5.8x10^6$ CD3+ cells/kg

on day 266, 3rd DLI 1.1x10^7 CD3+ cells/kg on day 306) without clinical response. On day 349, the presented with LN swellings. The patient received 13 courses Rituximab from day 364 to 530 resulting in an increase of donor chimerism. Sample analysis ended on day 453. Additional DLIs (4th DLI 3.1x10^7 CD3+ cells/kg on day 593, 5th DLI 5.8x10^7 CD3+ cells/kg on day 644, 6th DLI 7.9x10^7 CD3+ cells/kg on day 868) were applied without clinical response. The patient is currently treated with Rituximab.

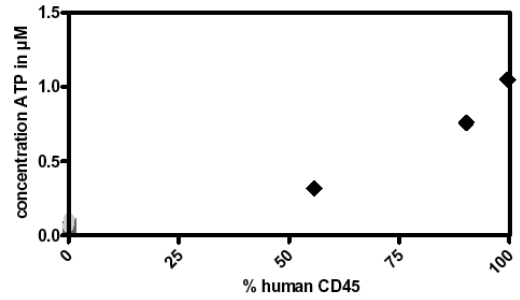
Patient 10

A 46 year old man with PMF received SCT from a male MUD after Fludarabin/Busulphan/ATG conditioning. GvHD prophylaxis consisted of CsA and MTX. The post-transplant period was uncomplicated. Due to persistently low donor chimerism, the patient received several DLIs (1st DLI 1x10^6 CD3+ cells/kg on day 834, 2nd DLI 5x10^6 CD3+ cells/kg on day 862, 3rd DLI 1x10^7 CD3+ cells/kg on day 889). Due to signs of chronic GvHD (liver) on day 958, the patient received steroids until day 1381. The patient converted to complete donor chimerism. Sample analysis ended on day 1162. The patient is alive and well.

SUPPLEMENTARY FIGURES



Supplementary Figure S1. Longitudinal analysis of the HA-1 CTL phenotype in PBMCs after allogeneic SCT. (A-C) HA-1^{A2} $tetramer^{pos}$ $CD3^{+}/CD8^{+}$ cells in PBMCs were phenotypically characterized by CD45RO, CD27 and CD28 staining. An established CD45RO+CD27-CD28- HA-1 CTL clone was used to standardize gating (not shown). Depicted are the results of patient 2(A), 4(B) and 1(C).



Supplementary Figure S2. Determination of human progenitor cell growth in the BM of NOD/SCID mice. BM of NOD/SCID mice transplanted with UCB derived CD34+ cells preincubated with CMV (black diamonds), HA-1 (red boxes) or alloA2 CTLs (green circles) was subjected 16 weeks after transplantation to an HALO progenitor cell assay and flowcytometry for human CD45. This assay determines intracellular ATP levels as measure for cell proliferation in response human cytokine stimuli. ATP is detected after 7 days of in vitro culture with bioluminscence in relative light units (RLU). Exact ATP concentrations were calculated based on the RLU in relation to a standard ATP curve. The %human CD45+ cells after 7 days of in vitro culture was determined for every HALO sample. X-axis: ATP concentration in µM; Y-axis: % human CD45% cells.