Identification of 4 novel HLA-B*40:01 restricted minor histocompatibility antigens and their potential as targets for graft-*versus*-leukemia reactivity

Marieke Griffioen, M. Willy Honders, Edith D. van der Meijden, Simone A.P. van Luxemburg-Heijs, Ellie G.A. Lurvink, Michel G.D. Kester, Cornelis A.M. van Bergen, and J.H. Frederik Falkenburg

Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands

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Online Supplementary Design and Methods

Retroviral transduction

EBV-B cells were transduced with a retroviral vector encoding HLA-B*40:01 (MP71-HLA-B*40:01-IRES-NGFR) as previously described.[1] EBV-B cells with transduction efficiencies more than 20% based on marker gene expression were included in the panel for WGAs. Cells with less than 20% transduction efficiencies were enriched for nerve growth factor receptor (NGFR) marker gene expression after staining with PE- or APC-labeled anti-NGFR antibodies (BD Biosciences) by flowcytometry or magnetic beads (Miltenyi Biotec). For each T-cell clone, a total number of 60 EBV-B cells expressing HLA-B*40:01 endogenously or after retroviral transduction were included for WGAs.

Isolation and culture of primary hematopoietic cells

Patient PBMC obtained prior to alloSCT were stained with FITC-conjugated anti-CD14, PE-conjugated anti-CD3 and APC-conjugated anti-CD19 antibodies (BD Biosciences), and monocytes, T cells and B cells were isolated by flowcytometry based on expression of CD14, CD3 and CD19, respectively. In addition, PB and BM samples from patients with CML, AML, ALL, CLL and MM were stained with APC-conjugated anti-CD34, anti-CD33 or anti-CD19, and PE-conjugated anti-CD5 or anti-CD38 antibodies (BD Biosciences). $CD34^+$ CML, $CD33^+$ AML, $CD19^+$ ALL, $CD19^+CD5^+$ CLL and $CD38^+$ MM cells were subsequently isolated by flowcytometry. Isolated $CD34^+$ CML progenitor cells were modified into leukemic APC,[2] and isolated monocytes were cultured to immature and mature dendritic cells (DC)[3] as previously described.

Enzyme linked immunosorbent assay

Stimulator cells (3×10^4 cells/well) were co-incubated with $CD8^+$ T-cell clones (5×10^3 cells/well) overnight at 37°C in U-bottom 96-well plates. Peptide pulsing was performed by incubating donor EBV-B cells (1×10^6 cell/mL) for 2 h with synthetic peptides in IMDM with 2% FBS. Peptide-pulsed donor EBV-B cells were washed twice and subsequently used as stimulator cells. Release of IFN-γ was measured in 50 µL culture supernatants by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Sanquin, Burton upon Trent, UK).

Chromium release assay

Target cells were labeled for 1 h at 37°C with 100 µCi (3.7 MBq) $Na_2{}^{51}CrO_4$ (Amersham, Uppsala, Sweden). After washing, target cells (1×10^3 cells/well) were incubated with $CD8^+$ T-cell clones at different effector-to-target (E:T) ratios for 4 or 10 h. Release of ^{51}Cr was analyzed in 25 µL supernatants. The percentage of specific lysis was calculated with the following formula:

[experimental release (cpm) – spontaneous release (cpm)]/[maximal release (cpm) – spontaneous release (cpm)] x 100%.

Maximal release was induced by 1% Triton (Sigma-Aldrich).

Colony forming cell assay

BM samples were incubated with irradiated (20 Gy) $CD8^+$ T-cell clones at E:T ratios of 3:1. After overnight pre-incubation, single cell suspensions were cultured at 2×10^4 target cells/mL in 30 mm culture dishes containing IMDM with methylcellulose supplemented with growth factors (GM-CSF, stem cell factor, IL-3, erythropoietin and other supplements; MethoCult, STEMCELL technologies SARL, Grenoble, France). As controls, single cell suspensions containing 2×10^4 target cells/mL and irradiated T cells at E:T ratios of 3:1 were seeded without pre-incubation. After 14 days of culture, numbers of colony forming units (CFU) for granulocyte/monocyte (CFU-GM) and erythroid lineages were scored.

Construction and screening of a plasmid cDNA expression library

A cDNA library was constructed as previously described.[4] $Poly(A)^+$ mRNA was isolated from patient EBV-B cells, and converted to cDNA using an oligo-d(T) primer. The cDNA was size-fractionated using column chromatography and ligated into pCR3.1 (Invitrogen, Breda, The Netherlands). Ligation products were transformed into *E. Coli* Top10 bacteria, and ampicillin resistant clones were divided into pools of approx. 50 different cDNA. Pools of cDNA were transfected into COS-7 cells stably expressing HLA-B*40:01. Transfection and screening of the cDNA library was performed using different mixtures of MiHA specific $CD8^+$ T-cell clones.

Whole genome association scanning

WGAs were performed as previously described.[5] Briefly, a panel of 60 EBV-B cell lines stably expressing HLA-B*40:01

endogenously or after retroviral transduction (>20% marker gene expression) was tested for T-cell recognition in IFN-γ ELISA. For each T-cell clone, EBV-B cell lines were divided into MiHA positive and negative groups based on a threshold of 5-fold the background production of IFN-γ. All EBV-B cells included in the panel were genotyped for more than 1.1 million SNPs by Human 1M-duo arrays (Illumina, Inc., San Diego, CA, USA). WGAs analysis was performed by combining T-cell recognition and SNP genotyping data using Plink software (*http://pngu.mgh.harvard.edu/purcell/plink/ version 1.03*), and the significance of association between both patterns was calculated using Fisher's exact test.

Genotyping for single nucleotide polymorphisms

Genomic DNA was isolated by the Gentra Systems PureGene genomic isolation kit (Biocompare, San Francisco, CA, USA). SNP rs1049232 in the *TRIP10* gene was analyzed using forward and reverse primers for amplification and two TaqMan MGB probes labeled with VIC and FAM dyes to detect the different alleles (Applied Biosystems, Foster City, CA, USA). Genotyping for SNP rs1049229 (TRIP10), rs13047599 (SON DNA binding protein), rs415895 (SWAP70) and rs1065674 (NUP133) was performed using allele-specific primers labeled with VIC and FAM dyes according to the manufacturer's instructions (KBioScience, Hoddesdon, UK).

Microarray gene expression analysis

Malignant cells were isolated from PB and BM samples from patients with CML, AML, ALL, CLL and MM by flowcytometry based on expression of CD34, CD33, CD19, CD19/CD5 and CD38, respectively. Non-malignant B cells, T cells and monocytes were isolated from PBMC based on expression of CD19, CD3 and CD14, respectively. Hematopoietic stem cells (HSC) were isolated from G-CSF mobilized PB based on expression of CD34. Non-hematopoietic cells included skin-derived FB and KC, and PTEC cultured with and without IFN-γ (100 IU/mL). Total RNA was isolated using small and micro scale RNAqueous isolation kits (Ambion, Inc., Austin, TX, USA), and amplified using the TotalPrep RNA amplification kit (Ambion). After preparation using the whole-genome gene expression direct hybridization assay (Illumina), cRNA samples were dispensed onto Human HT-12 v3 Expression BeadChips (Illumina). Hybridization was performed in the Illumina hybridization oven for 17 h at 58°C. Microarray gene expression data were analyzed using Rosetta Resolver 7.2 software.

In vivo *T-cell monitoring*

PB samples were stained with a mixture of FITC-conjugated antibodies against CD4, CD14, CD19 and CD56 as well as a mixture of APC- and PE-conjugated tetramers for the HLA-B*40:01 restricted MiHA or a mixture of an APC-conjugated HA-2 tetramer and PE-conjugated HA-1 tetramer. For each tetramer, a minimum of 5×10^5 PBMC were stained. Tetramers were constructed as previously described[6] with minor modifications.

References

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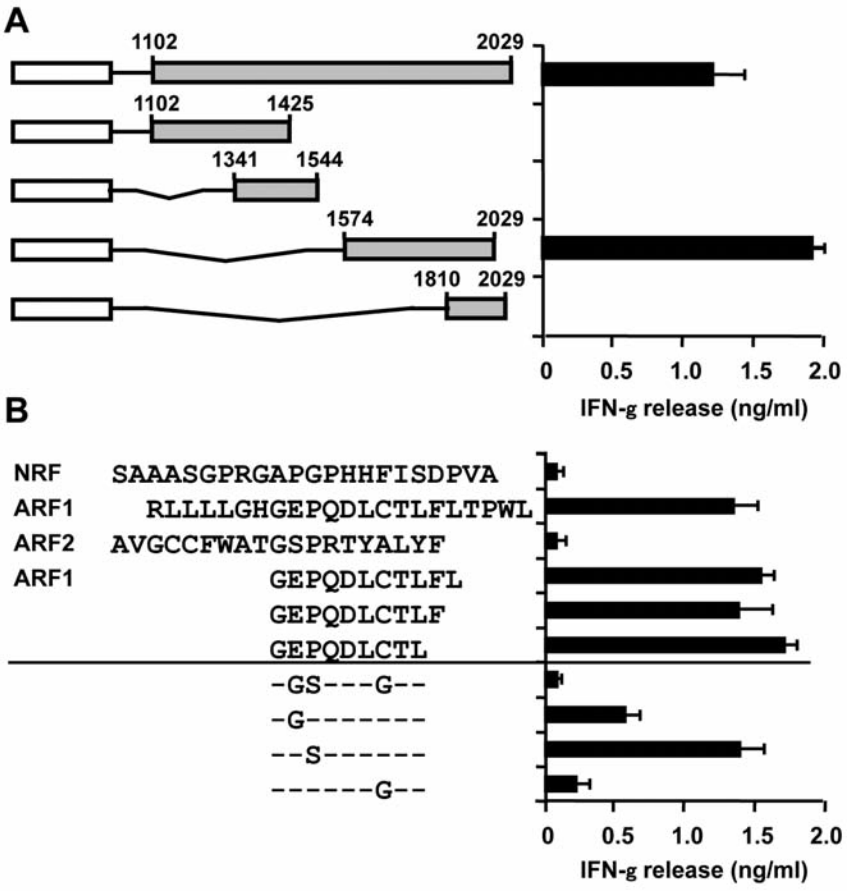
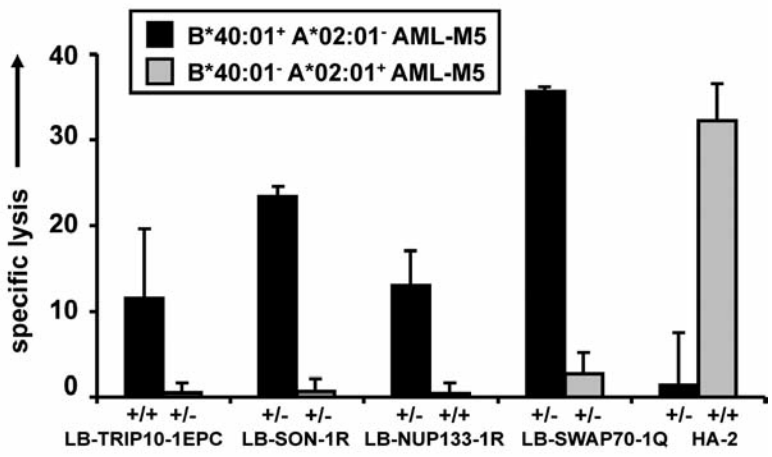
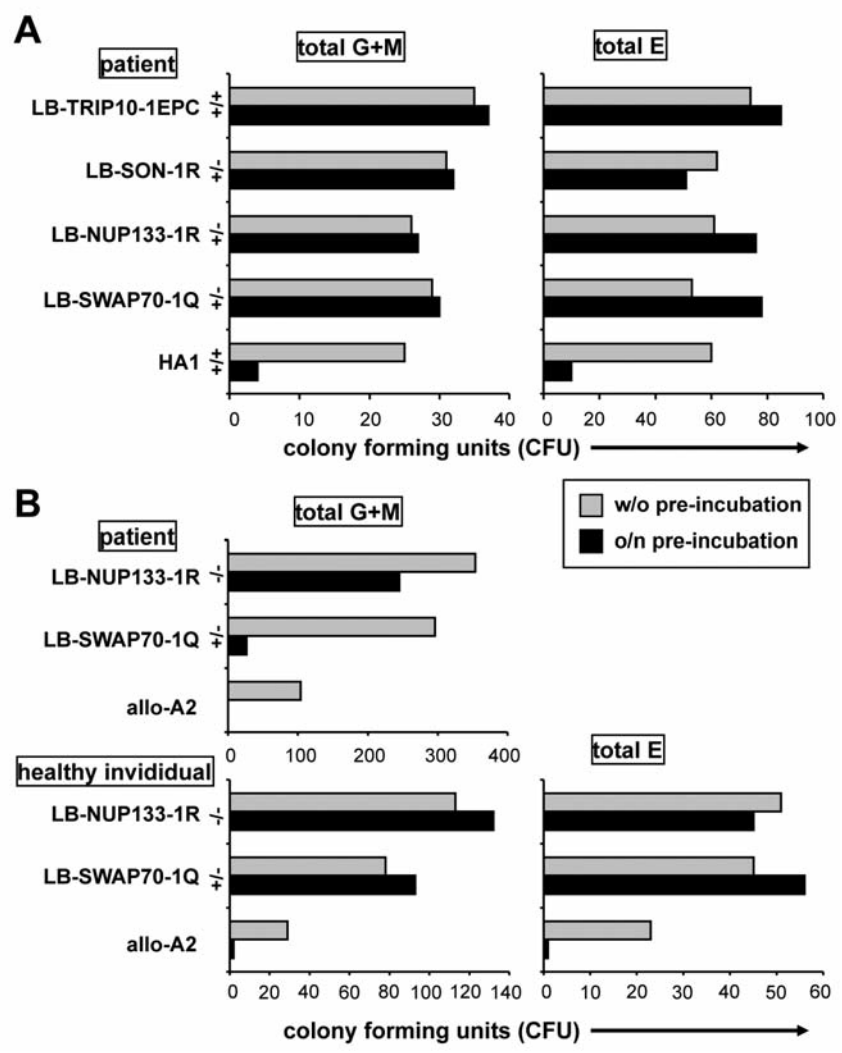


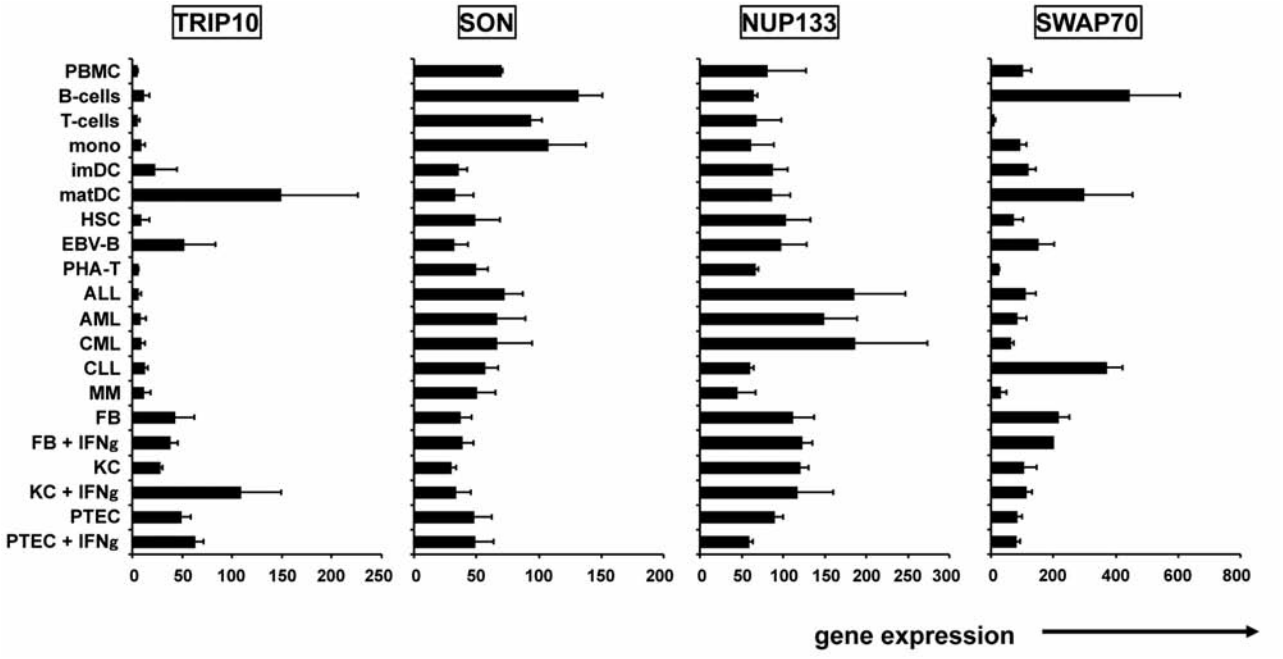
Online Supplementary Figure S1. Identification of LB-TRIP10-1EPC by screening a plasmid cDNA library. Transfection and screening of a cDNA plasmid library generated from mRNA from patient derived EBV-B cells in COS-7 cells expressing HLA-B*40:01 (COS-B*40:01) revealed isolation of a 1102 – 2029 bp TRIP10 cDNA recognized by T-cell clone ZRZ16. (A) Deletion variants of the 1102 – 2029 bp TRIP10 cDNA were constructed and tested for T-cell recognition after transfection into COS-B*40:01 cells in IFN-γ ELISA. Indicated is the mean release of IFN-γ (ng/mL) in 50 μL culture supernatants of duplicate wells. The results show that the MiHA is encoded by a 1574 - 1810 bp region which contains the stop codon of the TRIP10 coding sequence at 1704 bp. (B) Peptides comprising 3 patient type AA encoded by SNP rs1049229, rs1049230, and rs1049232 that are predicted to bind to HLA-B*40:01 were synthesized and tested for T-cell recognition in IFN-γ ELISA. The mean release of IFN-γ (ng/mL) in 50 μL culture supernatants of duplicate wells is indicated. A 9-mer peptide (GEPQDLCTL) encoded in the 3' untranslated region (3' UTR) in an alternative reading frame (ARF1) as the upstream encoded TRIP10 protein was recognized by clone ZRZ16. The 9-mer donor type peptide (GGSQDLGTL) was not recognized. Analysis of patient type peptides containing single AA of donor origin demonstrated that the E and C most significantly contributed to T-cell recognition.



Online Supplementary Figure S2. T-cell mediated lysis of primary leukemic blasts. $CD8^+$ T-cell clones specific for LB-TRIP10-1EPC, LB-SON-1R, LB-SWAP70-1Q and LB-NUP133-1R were investigated for cytolytic activity against primary leukemic blasts in 10 h ^{51}Cr-release assays at E:T ratios of 30:1. Mean specific lysis of triplicate wells is shown for an HLA-B*40:01 positive and A*02:01 negative AML-M5 sample positive for all 4 HLA-B*40:01 associated MiHA, and an HLA-B*40:01 negative and A*02:01 positive AML-M5 control sample.



Online Supplementary Figure S3. T-cell mediated lysis of hematopoietic progenitor cells. Lysis of hematopoietic progenitor cells was measured in CFC assays. BM cells ($2x10^4$ cells/mL) were seeded as single cell suspensions in medium with methylcellulose supplemented with growth factors after overnight pre-incubation with irradiated T-cell clones at E:T ratios of 3:1. (A) The T-cell clones for LB-TRIP10-1EPC, LB-SON-1R, LB-SWAP70-1Q and LB-NUP133-1R and a T-cell clone for HA-1 were analyzed for specific lysis of the malignant hematopoietic progenitor cells from the patient with CML from whom the T-cell clones were isolated. For this purpose, a BM sample was selected at the time of relapse after alloSCT prior to DLI. (B) The T-cell clone for LB-SWAP70-1Q and an HLA-A*02:01 specific allo-reactive T-cell clone were tested for lysis of hematopoietic progenitor cells from an HLA-B*40:01 and LB-SWAP70-1Q positive patient with transformed juvenile CML (upper graph) and 2 HLA-B*40:01 and LB-SWAP70-1Q positive healthy individuals. Representative data are shown for one healthy individual (lower graph). Indicated are the numbers of CFU for granulocytes and monocytes (CFU-GM) and erythroid cells as scored after 14 days of culture of BM cells after overnight pre-incubation with the irradiated T-cell clones (black bars) and, as controls, for BM cells and irradiated T-cells seeded as single cell suspensions without overnight pre-incubation (gray bars).



Online Supplementary Figure S4. Expression patterns of the genes encoding the HLA-B*40:01 restricted MiHA. Expression as measured by microarray gene expression analysis is shown for the genes encoding TRIP10, NUP133, SON, and SWAP70 in non-malignant hematopoietic cells (PBMC, B cells, T cells, monocytes, immature and mature DC and hematopoietic stem cells), malignant hematopoietic samples (CML, ALL, AML, CLL and MM), and non-hematopoietic cell types (FB, KC and PTEC pre-treated with and without IFN-γ).

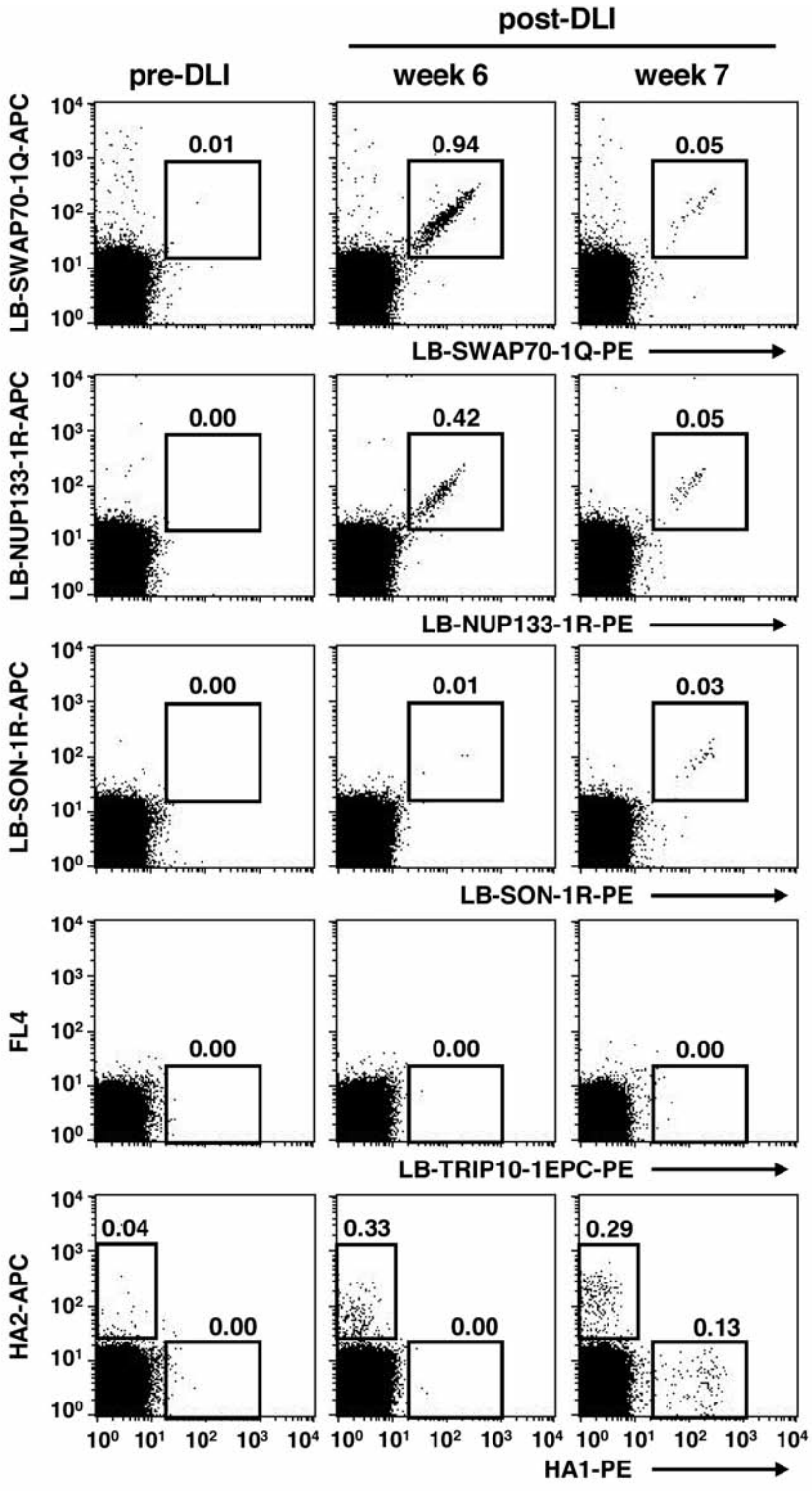


Figure 5. *In vivo* monitoring for MiHA specific T cells. Screening for MiHA specific T cells was performed for the patient with CML from whom the T-cell clones were isolated. PB samples pre-DLI and 6 and 7 weeks post-DLI were selected for *in vivo* T-cell monitoring. PBMC were stained with a mixture of FITC-conjugated anti-CD4, CD14, CD19 and CD56 antibodies and a mixture of PE- and APC-conjugated tetramers containing LB-SWAP70-1Q, LB-NUP133-1R, LB-SON-1R or LB-TRIP10-1EPC, or a mixture of PE-conjugated HA-1 and APC-conjugated HA-2 tetramers. Cells were gated for negative expression of CD4, CD14, CD19 and CD56, and mean fluorescence intensities are shown for PE- and APC-conjugated tetramers. Percentages are shown of gated cells that are double positive for PE- and APC-conjugated LB-SWAP70-1Q, LB-NUP133-1R, and LB-SON-1R tetramers, single positive for PE-conjugated LB-TRIP10-1EPC and HA-1 tetramers, and single positive for the APC-conjugated HA-2 tetramer.