



## Adult and cord blood T cells can acquire HA-1 specificity through HA-1 T-cell receptor gene transfer

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**Background and Objectives.** Minor histocompatibility antigen (mHag)-specific graft-versus-leukemia reactivities are observed following unselected donor lymphocyte infusion for the treatment of relapse after HLA-matched mHag-mismatched stem cell transplantation (SCT). Adoptive transfer of donor-derived *ex vivo*-generated HA-1-specific oligoclonal T cells or HA-1 peptide patient vaccination are currently being explored as curative tools for stem cell based immunotherapy of hematologic malignancies. Another treatment modality to eradicate residual leukemic cells after SCT is the transfer of the HA-1 hematopoietic-specific T-cell receptor (TCR) into cells from the stem cell donor. This strategy would be particularly useful in case of relapse after cord blood transplantation (CBT) and is explored in this study.

**Design and Methods.** HLA-A2<sup>neg</sup> adult peripheral blood and cord blood mononuclear cells were transduced with the genes encoding the HA-1 $\alpha$  and  $\beta$  TCR chains derived from established HA-1 specific cytotoxic T lymphocyte clones.

**Results.** The T cells transduced with HA-1 TCR $\alpha\beta$  showed consistent marker gene expression, but low staining with HLA-A2/HA-1 tetrameric complexes. They did, however, show hematopoietic-restricted cytolytic activity against HLA-A2<sup>pos</sup>/HA-1<sup>pos</sup> target cells, including leukemic cells.

**Interpretation and Conclusions.** The low level of HA-1-specific tetramer staining of HA-1 TCR $\alpha\beta$  transduced T cells may be caused by hybrid TCR formation of the transferred TCR $\alpha$  and  $\beta$  chains with endogenous TCR $\alpha$  and  $\beta$  chains. This may cause unwanted alloreactivity and requires attention. The HA-1 TCR $\alpha\beta$  transduced T cells show that the HA-1 TCR can be functionally transferred into donor mononuclear cells, which can be exploited in immunotherapeutic settings of SCT and CBT for hematologic malignancies.

**Key words:** T cell receptor, gene transfer, minor histocompatibility antigen, immunotherapy

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The minor histocompatibility antigen (mHag) HA-1 is a polymorphic antigen that is presented in the context of HLA-A2.<sup>1</sup> The tissue distribution of HA-1 is restricted to hematopoietic cells and carcinomas.<sup>2,3</sup> It can, therefore, function as a tumor target antigen for stem cell based immunotherapy of malignancies. In the setting of HLA-matched HA-1-mismatched stem cell transplantation (SCT) for hematologic malignancies, T cells from the HA-1<sup>neg</sup> stem cell donor can recognize HA-1 expressed by the patient's leukemic cells.<sup>4</sup> *In vivo* and *in vitro* generation of HLA-A2-restricted HA-1-specific cytotoxic T lymphocytes (CTL) has previously been reported.<sup>5,6</sup> T cells expressing the HA-1-specific T-cell receptor (TCR) can be monitored by staining peripheral blood mononuclear cells with HLA-A2/HA-1 tetramers<sup>7</sup> and by TCRBV spectratyping.<sup>8</sup> Both *in vitro* and *in vivo* generated HA-1-

specific T-cell clones analyzed so far exclusively use the TCR BV7S9 variable domain in combination with different TCR BD, BJ, TCRAV and AJ regions.<sup>9,9</sup> The CDR1 region of the TCRBV does not, however, seem to play a major role in the interaction with the HLA-A2/HA-1 ligand.<sup>10</sup>

Donor lymphocyte infusion (DLI) with HA-1-specific CTL generated from adult or cord blood donor cells provides a feasible treatment for relapsed HLA-A2<sup>pos</sup>/HA-1<sup>pos</sup> leukemia patients.<sup>6,11</sup> *Ex vivo* CTL induction and expansion for adoptive immunotherapy is, however, time-consuming and not successful in all stem cell donors. Gene transfer of the HLA-A2-restricted HA-1-specific TCR into donor T cells may provide an alternative treatment strategy. Several studies have described the transmission of various antigenic specificities by TCR transfer.<sup>12-15</sup> We earlier

reported on successful gene transduction of the TCR specific for the mHag HA-2 into peripheral T lymphocytes.<sup>16,17</sup> Since the hematopoietic-specific HA-1 antigen is additionally expressed on a series of epithelial carcinomas,<sup>3,18</sup> we studied the feasibility of HA-1 TCR gene transfer into peripheral blood cells derived from adult or cord blood donors. We chose to transfer the HA-1 TCR specificity into HLA-A2<sup>neg</sup> donor T cells which enables usage of the HA-1-specific immunotherapy in HLA-mismatched SCT settings. The HA-1 TCR $\alpha$  and  $\beta$  genes used for transduction were derived from two established HA-1-specific HLA-A2-restricted T-cell clones, 3HA15 and 5W38, previously isolated from different patients after HLA-identical SCT.<sup>5</sup> Both clones expressed the same TCR BV7S9, but different TCR AV chains. The individual genes encoding the  $\alpha$  and  $\beta$  TCR chains of both CTL clones were cloned into retroviral vectors and the specificity and functionality of the TCR-transduced adult and cord blood CD8<sup>+</sup> T cells were studied.

## Design and Methods

### Construction of retroviral vectors and generation of retroviral supernatant

Total RNA from the mHag HA-1-specific HLA-A2-restricted T cell clones 3HA15 and 5W38 was extracted using Trizol (Gibco, Carlsbad, CA, USA). The mRNA was reverse transcribed into single-strand cDNA by reverse transcriptase using oligo dT primers (Pharmacia, Uppsala, Sweden). Using primers that cover the complete repertoire of known TCR chains, TCR $\alpha$  and  $\beta$ , usage of the two clones was determined. Both T cell clones expressed the TCR BV7S9, as previously described.<sup>8,9</sup> The 5W38 T-cell clone expresses the TCR AV10S1; clone 3HA15 expresses two in-frame TCR $\alpha$  chains, TCR AV32 and TCR AV3S1 (*data not shown*). Pilot experiments revealed that TCR AV3S1, in combination with TCR BV7S9, forms the functional HA-1-specific HLA-A2-restricted TCR of CTL clone 3HA15. Two bicistronic retroviral vectors based on the pLZRS backbone<sup>19</sup> were used, containing an internal ribosome entry site (IRES) and the marker gene enhanced green fluorescent protein (pLZRS-eGFP)<sup>20</sup> or a truncated form of the nerve growth factor receptor (pLZRS-dNGF-R).<sup>21</sup> The individual genes encoding the  $\alpha$  and  $\beta$  TCR chains were amplified by polymerase chain reaction using primers containing relevant restriction sites and cloned into the pLZRS-vectors. The 5' forward primer sequences used were ATTGAATTCAGAAGAATG-GAACTCTC containing the EcoRI restriction-site for the TCR AV3S1 chain, CGCGGATCCACCATG-

GTCCTGAAATTCTCCG containing the BamHI restriction-site for the TCR AV10S1 chain, TATGGATC-CCTGCCATGGGCACCAG containing the BamHI restriction-site for the 3HA15 TCR BV7S9 chain and TAGAGAATTCCACCATGGGCACCAGTCTCC-TATGC containing the EcoRI restriction-site for the 5W38 TCR BV7S9 chain. The 3' reverse primer sequences used were TATCTCGAGATAAATT-CGGGTAGGATC containing the XhoI restriction-site for both TCR AV chains, GGTGTGCGACTGG-GATGGTTTTGGAG containing the Sall restriction-site for the 3HA15 TCR BV7S9 chain and CCG-GAATTCAGAAATCCTTTCTCTGACC containing the EcoRI restriction-site for the 5W38 TCR BV7S9 chain (Eurogentec, Seraing, Belgium). The TCR AV3S1 chain of 3HA15 was cloned into the pLZRS-dNGF-R vector, while the 3HA15 TCR BV7S9 chain was cloned into the pLZRS-eGFP vector. The TCR AV10S1 chain of clone 5W38 was cloned into the pLZRS-eGFP vector, whereas its TCR BV7S9 chain was cloned into the pLZRS-dNGF-R vector. Retroviral vectors encoding eGFP or dNGF-R without additional inserts were used as control (mock) vectors in the experiments. Control cycle sequencing was performed after which the constructs were transfected (0.66  $\mu$ g/mL) into the amphotropic phoenix packaging cell line (kindly provided by G. Nolan, Stanford University School of Medicine, Stanford, CA, USA) using the calcium phosphate transfection kit (Life Technologies, Gaithersburg, MD, USA). The phoenix cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum. Two days following transfection, 2  $\mu$ g/mL puromycin (Clonotech Laboratories, Palo Alto, CA, USA) was added and 10 to 14 days later  $20 \times 10^6$  cells were plated per 150 cm<sup>2</sup> culture flask (Beckton Dickinson, San José, CA, USA) in fresh medium without puromycin. The following day the medium was refreshed and 24 hours thereafter retroviral supernatant was harvested, centrifuged, and frozen in aliquots at -70°C.

### Retroviral transduction of TCR $\alpha\beta$ deficient Jurkat cells

Prior to transduction of donor T cells, Jurkat clones deficient for TCR $\alpha$  ( $\alpha^{-}$ ) or TCR $\beta$  ( $\beta^{-}$ ) or for both chains ( $\alpha\beta^{-}$ ), clone 3, clone 4, and clone 76 respectively,<sup>16</sup> were transduced using the various viral supernatants according to the procedure described below. At day three, correct expression of transduced TCR $\alpha$  and/or - $\beta$  chains was confirmed by measuring the TCR $\alpha\beta$  expression by FACS analysis, using PEcy5 conjugated anti-TCR $\alpha\beta$  monoclonal antibody (Pharmingen, San Diego, CA, USA).

### Isolation and retroviral transduction of T cells derived from adult- or cord blood

Peripheral blood mononuclear cells isolated from HLA-A2<sup>neg</sup> adult or cord blood donors were stimulated with 800 ng/mL phytohemagglutinin and 25 U/mL interleukin-2 (Cetus, Emeryville, CA, USA) in IMDM containing 10% human serum at a concentration of  $1 \times 10^6$ /mL. After 2 days of culture, T cells were transduced with retroviral supernatant using recombinant CH-296 human fibronectin fragments<sup>22</sup> (Retronectin, Takara, Otsu, Japan). Briefly,  $0.5 \times 10^6$  T cells per well were cultured overnight at 37°C together with 0.25 mL TCR $\alpha$ - and 0.25 mL TCR $\beta$  retroviral supernatant and 0.5 mL of fresh IMDM containing 10% fetal calf serum and 25 U/mL interleukin-2 in non-tissue culture treated CH-296-coated 24-well plates.<sup>16</sup> Next, the cells were washed and transferred to tissue culture treated 24-well plates at a concentration of  $0.5 \times 10^6$  cells per well in IMDM containing 10% human serum and 25 U/mL interleukin-2.

### Flow cytometric analysis and fluorescence-activated cell sorting

Transduction efficiencies were measured 3-5 days after transduction by the expression of the markers eGFP and dNGF-R. T cells positive for both markers and negative for CD4 were sorted at 1 or 25 cells/well by fluorescence-activated cell sorting (FACS) using a FACSVantage (Becton Dickinson). PE-conjugated and PECy5-conjugated antibodies (Pharmingen) were used to detect dNGF-R expression and CD4 expression, respectively. FACS sorted cells were restimulated with randomly selected peripheral blood mononuclear cells irradiated with 30 Gy, HLA-A2<sup>pos</sup>/HA-1<sup>pos</sup> EBV-LCL irradiated with 50 Gy, 25 U/mL interleukin-2 and 800 ng/mL phytohemagglutinin.

### Tetramer staining and cytotoxicity assay

Expression of the TCR specific for HLA-A2/HA-1 complexes was measured by staining the cells with PE-conjugated HLA-A2/HA-1 tetrameric complexes (HA-1<sup>A2</sup>), either in combination or not with APC-conjugated CD8 antibodies (BD Biosciences, Amsterdam, The Netherlands). Tetramers were generated and validated as previously described.<sup>7</sup>

CTL activity was measured in a chromium release assay. HLA-A2<sup>pos</sup> EBV-LCL either positive or negative for HA-1 were used as target cells. Peripheral blood or bone marrow containing more than 95% morphologically recognizable malignant cells derived from HLA-A2<sup>pos</sup> chronic myeloid leukemia (CML) patients were used as leukemic target cells. HLA-A2<sup>pos</sup> fibroblasts derived from a HA-1<sup>pos</sup> donor were used to test hematopoietic-restricted specificity of HA-1 TCR $\alpha\beta$  transduced T cells. Fibroblasts were treated for 24

hours with 250 U/mL interferon- $\gamma$  and tumor necrosis factor- $\alpha$  before incubation with effector cells. Target cells were pulsed with 1  $\mu$ M HA-1<sup>II</sup> peptide (VLHDDLLEA) where indicated. Chromium labeled target cells were added to various numbers of effector cells and were cultured for 4 or 18 hours at 37°C. Supernatant was harvested and measured in a luminescence counter (Topcount-NXT; Packard, Meriden, CT, USA). The mean percentage of specific lysis in triplicate wells was calculated using the following formula:

$$\frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \times 100\%$$

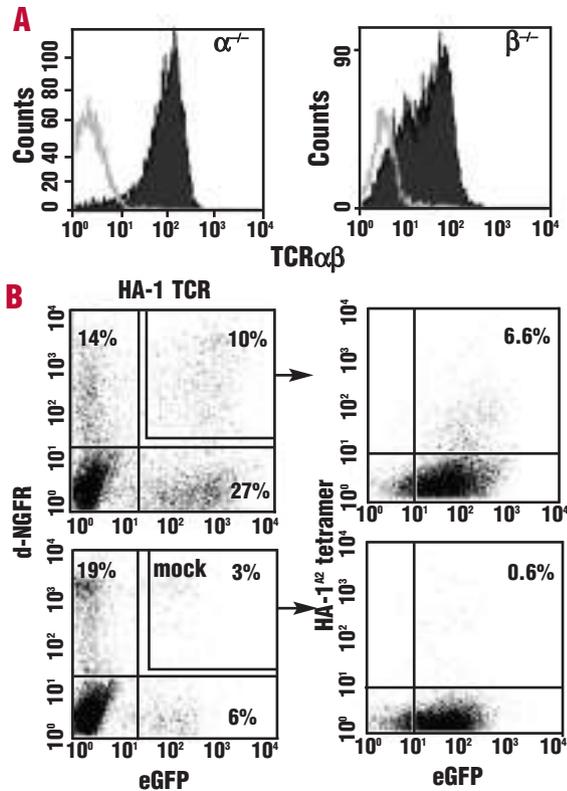
## Results

### Cell surface expression of TCR $\alpha\beta$ following retroviral transduction into Jurkat cells

Retroviral vectors encoding the different TCR $\alpha$  or  $\beta$  chains derived from HA-1-specific CTL clones 3HA15 or 5W38 were transduced into various Jurkat cells. The cell surface expression of the transduced TCR $\alpha\beta$  gene products was analyzed. The TCR $\alpha$ -deficient Jurkat cells (*see Design and Methods*) transduced with the HA-1 TCR  $\alpha$  chain from either HA-1-specific CTL clone 3HA15 (Figure 1A) or 5W38 (*data not shown*) expressed TCR $\alpha\beta$  complexes at the cell surface. Similarly, the TCR $\beta$ -deficient Jurkat cells (*see Design and Methods*) transduced with the HA-1 TCR $\alpha\beta$  chain from either HA-1 CTL clone 3HA15 or 5W38 expressed TCR $\alpha\beta$  complexes at the cell surface. These data demonstrate that the retrovirally transduced HA-1 TCR $\alpha$  chains as well as the HA-1 TCR $\beta$  chains can pair with the endogenous Jurkat TCR chains, respectively. However, some TCR $\beta$ <sup>-/-</sup> Jurkat cells transduced with HA-1 TCR $\beta$  chains did not express the HA-1 TCR or expressed TCR at a low level. Transduction of both the HA-1 TCR $\alpha$  and  $\beta$  chains of the two different HA-1 CTL clones into TCR $\alpha\beta$ -deficient Jurkat cells resulted in intact HA-1 TCR $\alpha\beta$  cell surface expression (*data not shown*). Thus, the individual HA-1 TCR chains pair with each other and are able to form stable TCR complexes at the cell surface.

### Transduction of the HA-1 TCR $\alpha\beta$ chains into adult peripheral blood-derived T cells

The TCR $\alpha$  and  $\beta$  chain derived from the HA-1-specific CTL clones 3HA15 and 5W38 were transduced into peripheral blood mononuclear cells isolated from various HLA-A2<sup>neg</sup> adult donors. The transduction efficiency of the HA-1 TCR $\alpha$  chains varied between 14-23%, whereas the transduction efficiency of the HA-1 TCR $\beta$  chains varied between 27-35%. T cells isolated from adult peripheral blood

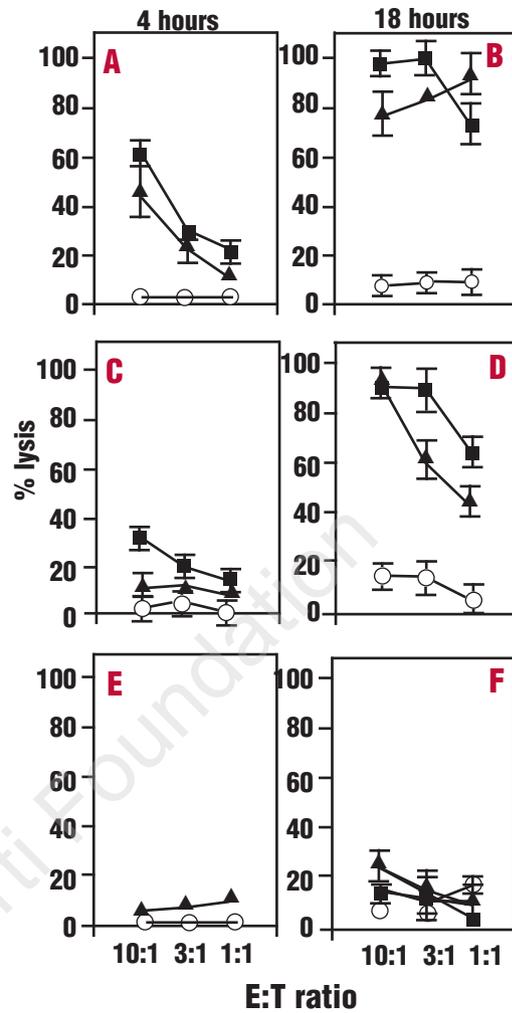


**Figure 1.** Cell surface expression of TCR after transfer of genes encoding HA-1 TCR $\alpha$  and TCR $\beta$  chains into Jurkat cells and adult T cells. TCR $\alpha$  or  $\beta$  deficient Jurkat cells (A) or peripheral blood-derived adult T cells (B) were transduced with HA-1 TCR $\alpha$  and  $\beta$  chains from CTL clone 3HA15. Intact TCR $\alpha\beta$  expression is shown as filled histogram plots. Open histograms represent mock-transduced Jurkat cells positive for the marker gene. HA-1 TCR $\alpha\beta$  or mock-transduced adult T cells double positive for eGFP and dNGFR expression were FACS sorted (indicated by arrow), expanded and stained with HA-1<sup>A2</sup> tetrameric complexes.

mononuclear cells that were negative for CD4 and expressed both marker genes (6-10%) were FACS sorted and expanded (see *Design and Methods*). Despite stable expression of the marker genes eGFP and dNGFR (*data not shown*), only low numbers of HA-1 TCR $\alpha\beta$  transduced adult T cells stained specifically with HA-1<sup>A2</sup> tetramers (Figure 1B).

**Functional analysis of HA-1 TCR $\alpha\beta$  transduced adult peripheral blood-derived T cells**

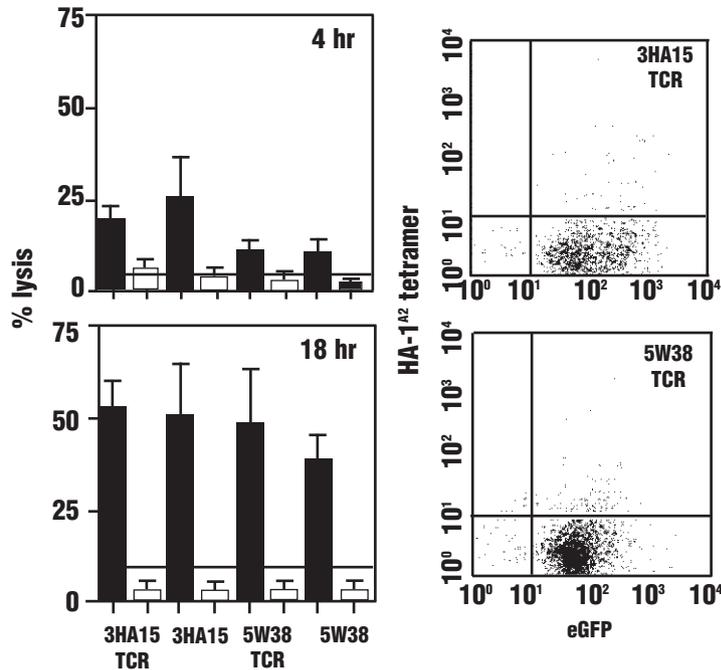
The HA-1 TCR $\alpha\beta$  transduced T cells were subsequently analyzed for their functional activity and specificity. The cytolytic activities of 3HA15 TCR $\alpha\beta$  transduced T cells from one representative donor are depicted in Figure 2. The 3HA15 or 5W38 TCR $\alpha\beta$  transduced T cells specifically lysed HLA-A2<sup>pos</sup>/HA-1<sup>neg</sup> EBV-LCL target pulsed with HA-1 peptide and, importantly, the natural ligand expressing HLA-A2<sup>pos</sup>/HA-1<sup>pos</sup> EBV-LCL target cells. Compared with the original CTL clone 3HA15 analyzed in parallel, the HA-1 TCR $\alpha\beta$  transduced T cells required a longer incubation time to lyse



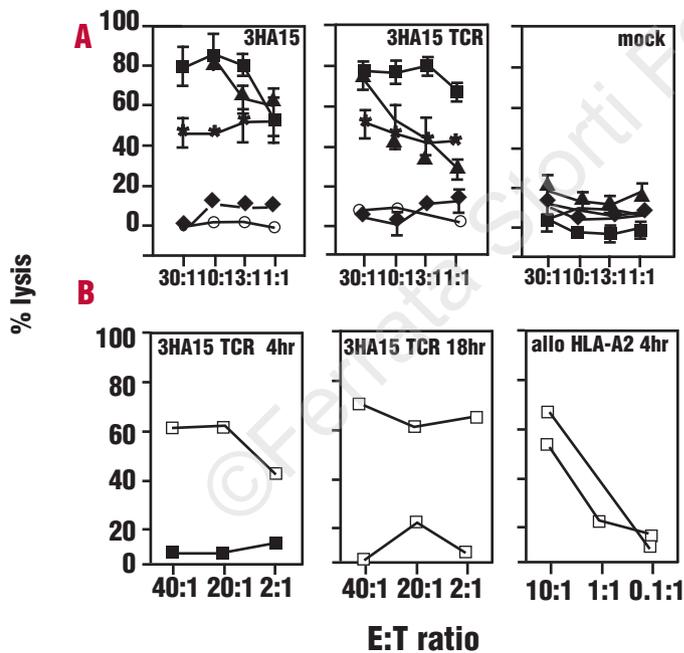
**Figure 2.** Functional analysis of HA-1 transduced adult T cells. The HA-1-specific cytotoxic activity of HA-1 TCR $\alpha\beta$  transduced T cells (C,D) and mock-transduced T cells (E,F) after 4 and 18 hours of incubation is shown. The original CTL clone 3HA15 was tested in parallel (A,B). Target cells: HA-1<sup>neg</sup> EBV-LCL (open dots), HA-1<sup>neg</sup> EBV-LCL pulsed with HA-1 peptide (filled squares) and HA-1<sup>pos</sup> EBV-LCL (filled triangles).

target cells expressing HA-1. After 4 hours of incubating effector and target cells together, HA-1 TCR $\alpha\beta$  transduced T cells showed specific lysis of target cells pulsed with HA-1 peptide, but no lysis on the natural ligand. After 18 hours of incubation, HA-1 TCR $\alpha\beta$  transduced T cells displayed strong and specific lytic capacities on both the peptide loaded and the natural ligand target cells comparable to that of the original CTL clones. The mock-transduced bulk population did not lyse any of the target cells.

Next, we tested the lysis of leukemic targets by 3HA15 or 5W38 TCR $\alpha\beta$  transduced T cells (Figure 3). Short-term expanded HA-1 TCR $\alpha\beta$  transduced T cells lysed leukemic cells expressing HA-1, but not HA-1<sup>neg</sup> leukemic cells, after 4 hours of incubation. Specific and much stronger lysis was observed after 18 hours of



**Figure 3.** Recognition patterns of HA-1 TCR $\alpha\beta$  transduced adult T cells against leukemic cells. HA-1-specific lysis of two representative HA-1 TCR $\alpha\beta$  transduced T-cell clones was measured after 4 or 18 hours of effector/target cell incubation at an E:T ratio of 10:1. The original HA-1-specific CTL clones 3HA15 and 5W38 were analyzed in parallel. The horizontal lines in the left figures represent the background lysis by mock-transduced T cells tested in parallel. Target cells: HA-1<sup>pos</sup> chronic myeloid leukemia cells (CML, filled bars), HA-1<sup>neg</sup> CML cells (open bars). Corresponding HA-1<sup>A2</sup> tetramer staining of the 3HA15 and 5W38 HA-1 TCR $\alpha\beta$  transduced T-cell clones is shown.



**Figure 4.** Functional analysis of HA-1 TCR $\alpha\beta$  transduced cord blood T cells. **A.** HA-1 specific lysis by HA-1 TCR $\alpha\beta$  transduced cord blood T cells and by mock-transduced cord blood T cells after 18 hours of incubation is shown. The original HA-1-specific CTL clone 3HA15 was tested in parallel. Target cells: HA-1<sup>neg</sup> EBV-LCL (open dots), HA-1<sup>neg</sup> EBV-LCL pulsed with HA-1 peptide (filled squares), HA-1<sup>pos</sup> EBV-LCL (filled triangles), HA-1<sup>neg</sup> CML cells (open diamonds) and HA-1<sup>pos</sup> CML cells (asterisks). **B.** Hematopoietic-restricted lysis by HA-1 TCR $\alpha\beta$  transduced cord blood T cells after 4 and 18 hours of effector/target cell incubation is shown. A control allo HLA-A2-specific CTL clone was tested in parallel. Target cells: fibroblasts (filled squares) and fibroblasts pulsed with HA-1 peptide (open squares). The fibroblasts were derived from an HLA-A2<sup>pos</sup>/HA-1<sup>pos</sup> donor.

incubation. Similar results were obtained when HA-1-expressing EBV-LCL target cells were used (*data not shown*). In line with the results on bulk HA-1 TCR $\alpha\beta$  transduced T cells, short-term expanded HA-1 TCR $\alpha\beta$  transduced T cells stained low but specifically with HA-1<sup>A2</sup> tetramers.

**Transduction of the HA-1 TCR TCR $\alpha\beta$  chains into cord blood-derived T cells**

The TCR $\alpha$  and  $\beta$  chains derived from the HA-1 specific CTL clone 3HA15 were transduced into peripher-

al blood mononuclear cells isolated from various HLA-A2<sup>neg</sup> cord blood donors. The transduction efficiencies of both the TCR $\alpha$  and the TCR $\beta$  chains were in the same range as observed for the peripheral blood mononuclear cells from adults (15-40%). HA-1 TCR $\alpha\beta$  transduced cord blood-derived T cells (4-20%) were FACS sorted (depleted for CD4<sup>+</sup> T cells) and expanded. HA-1 TCR $\alpha\beta$  transduced cord blood T cells displayed low HA-1<sup>A2</sup> tetramer staining comparable to that of the HA-1 TCR $\alpha\beta$  transduced adult T cells described above (*data not shown*).

### Functional analysis of HA-1 TCR $\alpha\beta$ transduced cord blood-derived T cells

The HA-1 TCR $\alpha\beta$  transduced cord blood T cells were subsequently analyzed for their hematopoietic-specific lytic capacities (Figure 4). HA-1 TCR $\alpha\beta$  transduced cord blood T cells lysed HLA-A2<sup>pos</sup>/HA-1<sup>pos</sup> leukemic cells and EBV-LCL target cells that were either pulsed with HA-1 peptide or naturally expressed HA-1. Similar to the results obtained with the HA-1 TCR $\alpha\beta$  transduced adult T cells, specific and strong lysis of target cells expressing HA-1 required prolonged incubation of effector and target cells. The original HA-1-specific CTL clone 3HA15 and the mock-transduced T cells were analyzed in parallel. Mock-transduced T cells did not lyse any of the target cells.

Besides the recognition of the relevant EBV-LCL and leukemia cells, the HA-1 TCR $\alpha\beta$  transduced cord blood T cells were analyzed for their hematopoietic-restricted specificity (Figure 4B). HA-1 TCR $\alpha\beta$  transduced cord blood T cells did not lyse fibroblasts derived from an HLA-A2<sup>pos</sup>/HA-1<sup>pos</sup> donor, whereas these target cells were recognized by an allo HLA-A2-specific T-cell clone tested in parallel. The same fibroblasts pulsed with HA-1 peptide were efficiently lysed by HA-1 TCR $\alpha\beta$  transduced cord blood T cells. Herewith, the recognition pattern of the HA-1 TCR $\alpha\beta$  transduced cord blood T cells is indicated to be restricted to cells specific to the hematopoietic system.

Collectively, these results show that HA-1 TCR $\alpha\beta$  transfer into HLA-A2<sup>neg</sup> adult or into HLA-A2<sup>neg</sup> cord blood T cells results in functional cytotoxic T cells that display specific reactivity against HLA-A2<sup>pos</sup> HA-1-expressing target cells including leukemic cells.

## Discussion

We studied the feasibility of transferring the genes encoding HA-1 TCR $\alpha$  and  $\beta$  chains into HLA-A2<sup>neg</sup> adult and into HLA-A2<sup>neg</sup> cord blood T cells and analyzed the HA-1 TCR $\alpha\beta$  transduced T cells for their antigen-specific lytic potential. We showed that these HA-1 TCR $\alpha\beta$  transduced HLA-A2<sup>neg</sup> adult and cord blood T cells can indeed acquire HA-1 specific and lytic activity. The feasibility of transferring functional HA-1 TCR $\alpha\beta$  encoding genes into HA-1 TCR negative cells lays the basis for a potential broad spectrum of applications in stem cell based immunotherapy of hematologic malignancies and solid tumors. It is worthwhile mentioning that besides the hematopoietic-restricted specificity, HA-1 is also expressed on epithelial cancer cells. Moreover, our results set the stage for broadening the use of the immunodominant and hematopoietic-specific mHag HA-1 to the setting of HLA-mismatched SCT. It should be noted however that although the specific functional activity of the HA-1 TCR can indeed be

transferred, significant improvements in transduction efficiency, HA-1 TCR avidity for its ligand and relevant expansion of HA-1 TCR TCR $\alpha\beta$  transduced T cells need to be established before HA-1 TCR transduced T cells can be therapeutically applied.

Endogenous TCR, transduced TCR and hybrid TCR possibly compete for CD3 association and therewith for functional cell surface expression.<sup>23</sup> This feature may explain the lack of correlation between the intensity of double marker expression and the cell surface expression, as measured by HLA-A2/HA-1-specific tetramers, of stable HA-1-specific TCR complexes following transduction. The presence of other TCR on the cell surface may also hamper HA-1 TCR clustering, lipid raft formation<sup>24,25</sup> and rapid activation upon antigen encounter. Moreover, the granzyme depot and thus the intrinsic cytolytic capacity of the TCR-transduced T cells may be inferior to that of the non-transduced CTL clones. We also noticed that HA-1 TCR $\alpha\beta$  transduced T cells generally require more time than the original non-transduced HA-1-specific CTL clones to lyse their target cells. We encountered the same phenomenon of low tetramer staining and *slow* but antigen-specific lysis in our earlier study using HA-2-specific TCR transfer.<sup>16</sup> A single chain construct containing both the TCR $\alpha$  and  $\beta$  chains combined with strategies that can prevent the formation of hybrid TCR or suppress endogenous TCR expression<sup>23</sup> is necessary to improve functional HA-1 TCR transfer. Improved gene expression may be obtained with more effective retroviral vector systems<sup>26</sup> or a lentiviral-based transduction procedure. Hybrid pairing of the different TCR $\alpha$  and  $\beta$  chains following retroviral transduction may also result in the formation of new TCR of unknown specificities.<sup>23</sup> It is clear that the above mentioned, as yet unsolved, processes need extensive additional analyses before *in vivo* transfer with TCR-transduced T cells can be executed. Serious attention should be focused on the potential risks of graft-versus-host-disease as well as undesired autoimmune reactions that may occur upon adoptive transfer of TCR-modified T cells. Suicide gene control of the *ex vivo* HA-1 TCR $\alpha\beta$  transduced T cells may be included to potentially control undesired alloreactivity.<sup>27,28</sup>

HA-1 TCR $\alpha\beta$  transfer may be of special use in the setting of CBT. Currently, a treatment for hematologic malignancies that relapse after CBT is lacking. Cord blood is obtained anonymously and contains far too few lymphocytes for the purpose of DLI. CBT is usually performed with 1-2 HLA-mismatched grafts.<sup>29</sup> HLA disparity is not, however, significantly associated with a higher risk of graft-versus-host-disease in this transplantation modality. HA-1-specific TCR $\alpha\beta$  transfer into cryopreserved HLA-A2<sup>neg</sup> cord blood T cells may be a strategy requiring low numbers of donor cells for immunotherapeutic purposes for HLA-A2 positive patients. A universal option would be to generate *pre-*

fabricated HA-1 TCR $\alpha\beta$  transduced T cells derived from HLA-A2<sup>neg</sup> cord blood donors who have frequent HLA-homozygous haplotypes. HLA-A2<sup>pos</sup> patients who match the remaining HLA type of the cord blood donor can be treated with these off the shelf HA-1 TCR $\alpha\beta$  transduced T cells.

In summary, our results provide the proof of principle that transfer of HA-1 specificity into HA-1 TCR negative cells is feasible. Current studies focus on the generation of sufficient numbers of HA-1 TCR $\alpha\beta$  transduced cord blood T cells with high *ex vivo* expansion potential and lytic capacity.

*BM: construction of retroviral vectors, generation of retroviral supernatants, TCR transduction experiments and specificity analyses of TCR-transduced cells. BM prepared Figures 1A, 2 and 4A and participated in drafting the manuscript. AGSvH assisted in the experiments concerning TCR transduced cord blood cells, analysis and interpretation of data, preparation of Figures 1B, 3 and 4B, drafting the manuscript and its final version. JP: construction of retroviral vectors. LvdV assisted in TCR transduction experiments and cytotoxicity assays. BW participated in analysis and interpretation of data and drafting the manuscript.*

*MHMH guided the experiments, participated in analysis and interpretation of data and in drafting the manuscript. EG: conception and design of the study, interpretation of data, drafting the manuscript and correction of its final version. The authors declare that they have no potential conflicts of interests.*

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