

# Reprogramming of Virus-specific T Cells into Leukemia-reactive T Cells Using T Cell Receptor Gene Transfer

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## Abstract

T cells directed against minor histocompatibility antigens (mHags) might be responsible for eradication of hematological malignancies after allogeneic stem cell transplantation. We investigated whether transfer of T cell receptors (TCRs) directed against mHags, exclusively expressed on hematopoietic cells, could redirect virus-specific T cells toward antileukemic reactivity, without the loss of their original specificity. Generation of T cells with dual specificity may lead to survival of these TCR-transferred T cells for prolonged periods of time in vivo due to transactivation of the endogenous TCR of the tumor-reactive T cells by the latent presence of viral antigens. Furthermore, TCR transfer into restricted T cell populations, which are nonself reactive, will minimize the risk of autoimmunity. We demonstrate that cytomegalovirus (CMV)-specific T cells can be efficiently reprogrammed into leukemia-reactive T cells by transfer of TCRs directed against the mHag HA-2. HA-2-TCR-transferred CMV-specific T cells derived from human histocompatibility leukocyte antigen (HLA)-A2<sup>+</sup> or HLA-A2<sup>-</sup> individuals exerted potent anti-leukemic as well as CMV reactivity, without signs of anti-HLA-A2 alloreactivity. The dual specificity of these mHag-specific, TCR-redirected virus-specific T cells opens new possibilities for the treatment of hematological malignancies of HLA-A2<sup>+</sup> HA-2-expressing patients transplanted with HLA-A2-matched or -mismatched donors.

Key words: T cell receptor • gene transfer • minor histocompatibility antigen • virus-specific T cells • leukemia reactive

## Introduction

Donor lymphocyte infusion (DLI) into patients with a relapse of their leukemia or myeloma after allogeneic stem cell transplantation has been proven to be a successful treatment strategy (1–3). The beneficial graft versus leukemia effect of donor lymphocytes, however, is often accompanied by GVHD. Adoptive transfer of antigen-specific T cell lines may eradicate the relapsed hematological malignancy and can separate the antileukemic effect from GVHD (4). The main drawback of adoptive therapy is the difficulty to produce sufficient quantities of antigen-specific T cells. In addition, the specificity of the infused T cells is difficult to control.

An alternative strategy might be to equip donor T cell populations with a TCR of defined antitumor specificity. Dembic et al. (5) were the first to show that transfer of the TCR into recipient T cells resulted in redirected antigen specificity of these T cells. Since then several groups have

demonstrated that transfer of virus-specific or tumor antigen-specific TCRs resulted in T cell populations with redirected antigen specificity (6–12). Transfer of TCRs specific for class I-restricted antigens into CD8<sup>+</sup> T cells and transfer of TCRs of class II-restricted specificity into CD4<sup>+</sup> T cells leads to functional redirected T cells, exerting antigen-specific cytolytic activity as well as antigen-specific cytokine secretion. Stanislawski et al. (11) demonstrated that TCR gene transfer can be used to circumvent self-tolerance of autologous T cells to tumor-associated antigens by transferring a humanized MDM2-specific TCR selected from HLA-A201 transgenic mice. Furthermore, TCR transfer studies have demonstrated that retroviral transfer of high avidity TCRs into T lym-

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*Abbreviations used in this paper:* ΔNGF-R, truncated form of the nerve growth factor receptor; CML, chronic myeloid leukemia; DLI, donor lymphocyte infusion; EBV-LCL, EBV-transformed B cell; eGFP, enhanced green fluorescent protein; HPC, hematopoietic progenitor cell; MFI, mean fluorescence intensity; mHag, minor histocompatibility antigen; MSC, mesenchymal stem cell; PHA, phytohemagglutinin; PIA, progenitor cell inhibition assay.

phocytes is accompanied by the maintenance of the parental T cell avidity and that TCR-transferred T cells exhibit no change in the peptide-fine specificity in comparison to the parental T cell clones (12, 13). Recently, the feasibility of cellular therapy using TCR-modified T cells was demonstrated in a mouse model system (14). In this study it was demonstrated that T cells that were redirected by TCR gene transfer can be activated *in vivo*, home to effector sites, and contribute to tumor clearance.

Although the approach of TCR transfer appears to be feasible, various potential problems may occur. First, the pairing of the retroviral-introduced TCR chains with the endogenously expressed TCR chains can lead to the formation of new TCR complexes with undesired specificities. Modification of highly polyclonal T cell populations will result in a diverse set of new specificities. This may lead to the production of T cells that have self-reactive TCRs that may lead to autoimmunity. Second, TCR transfer of ignorant self-specific T cells may lead to autoaggressive T cells after triggering through the exogenous TCR. In addition, if antigen-specific TCRs are transferred into unselected primary T cells not only might a potentiating immune response develop, but also suppressive T cell reactivity may develop, due to TCR transfer into naive T cells or suppressor T cells. Furthermore, end-stage effector cells with limited proliferative capacity that will be transduced with the exogenous TCR may only exhibit short-term cytolytic activity.

Based on these various reasons, we propose to redirect the specificity of virus-specific T cells toward antileukemic reactivity using TCR gene transfer. T cells directed against viruses with a latent persistence *in vivo* like CMV and EBV would be attractive candidates to use. These virus-specific T cells will have an additive advantage because they will also be triggered by the endogenous virus-specific TCR and therefore we hypothesize that these T cells will survive for prolonged periods of time *in vivo*.

In this study we redirected virus-specific T cells into leukemia-reactive T cells by the transfer of TCRs derived from T cell clones specific for the minor histocompatibility antigen (mHag) HA-2. mHags are derived from genetically polymorphic proteins that can be differentially expressed between donor and recipient (15). Because the HA-2 mHag is exclusively expressed on cells of the hematopoietic lineages (16), T cells directed against HA-2 can be used to treat hematological malignancies relapsing after allogeneic stem cell transplantation when the patient is HA-2<sup>+</sup> and the donor is HA-2<sup>-</sup> (17). T cells directed against HA-2 will eliminate the hematopoietic cells from the patient, including the malignant cells, without affecting donor-derived hematopoiesis or directly affecting nonhematopoietic tissue of the patient.

Because a high percentage of the human population express the HA-2 (18), this mHag is not frequently useful for induction of HA-2-specific T cells in transplantations between HLA-identical individuals. However, in HLA-A2 mismatch transplants in which patient hematopoietic cells

are HLA-A2<sup>+</sup> and express the mHag HA-2, whereas the donor cells do not express the HLA-A2 restriction molecule, donor T cell responses might be generated against mHag HA-2 peptide presented in HLA-A2. Several studies have shown the *in vitro* generation of antigen-specific T cells that are restricted by nonself HLA molecules (19–23). However, stimulation with nonself HLA molecules also induced undesired allo-HLA-specific T cells, which can cause damage of all HLA-A2<sup>+</sup> hematopoietic and nonhematopoietic tissues (20, 24). Transfer of mHag-specific TCRs into virus-specific T cells from the donor would be a strategy to circumvent the undesired induction of allo-HLA-specific T cells. Because the mHag-specific TCRs were isolated from T cells exhibiting an immune response in an HLA-identical setting, the risk of transfer of TCRs that not only have mHag specificity but also anti-HLA-A2 reactivity will be minimized.

In this study we explored the possibility to transfer HA-2-specific TCRs into various HLA-restricted virus-specific T cells derived from HLA-A2<sup>+</sup> and HLA-A2<sup>-</sup> individuals. We demonstrated that HA-2-TCR-transduced virus-specific populations exerted significant virus-specific as well as antileukemic reactivity without the appearance of anti-HLA-A2 reactivity.

## Materials and Methods

*T Cell Clones and Cell Lines.* The HLA-A2-restricted T cell clones HA2.5 and HA2.27 specific for the mHag HA-2 were isolated from the peripheral blood of a patient with chronic myeloid leukemia (CML) during an ongoing graft versus leukemia response after DLI (17). The allo-HLA-A2-specific T cell clone MBM13 was derived from a mixed lymphocyte reaction with peripheral blood from haploidentical siblings by limiting dilution analysis (9). The HLA-A2<sup>+</sup> HFF fibroblast cell line was derived from the American Type Culture Collection (ATCC). COS and 293 EBNA cell lines (ATCC) were transduced with a retroviral vector encoding for the HLA-A2 molecule (9). Mesenchymal stem cells (MSCs) were derived from the bone marrow of CML patients by culturing the adherent cells for several weeks on DMEM supplemented with L-alanyl-L-glutamine, sodium pyruvate, 1 mg/ml glucose, pyridoxine (GIBCO BRL), and 10% FBS.

*Generation of Retroviral Vectors and Virus Supernatant.* By RT-PCR, using primers that cover the complete repertoire of known TCR chains (25), the TCR  $\alpha$  and  $\beta$  usage of the mHag HA-2-specific HA2.5 was determined. The T cell clone expressed two in-frame gene transcripts, TCR AV15S1 and TCR BV18S1. The different TCR  $\alpha$  and  $\beta$  chains of the T cell clone were individually cloned into retroviral vectors. The Moloney murine leukemia virus-based retroviral vector LZRS and packaging cells  $\phi$ -NX-A were used (26). Two bicistronic retroviral vectors were constructed in which the multiple cloning site is linked to the downstream internal ribosome entry sequence and the marker gene enhanced green fluorescent protein (eGFP; reference 27) or truncated form of the nerve growth factor receptor ( $\Delta$ NGF-R; reference 28). The TCR  $\alpha$  chain was cloned into the retroviral vectors in combination with eGFP and the TCR  $\beta$  chain was cloned in combination with the  $\Delta$ NGF-R. Retroviral vectors encoding eGFP or  $\Delta$ NGF-R alone were used as control vectors in the experiments. In addition, the gene product of the lower

matrix protein pp65 of HCMV AD169 (provided by E. Wiertz, Leiden University Medical Center, Leiden, Netherlands) was cloned into the retroviral vector in combination with eGFP. The constructs were transfected into  $\phi$ -NX-A cells using calcium phosphate (Life Technologies) and 2 d later 2  $\mu$ g/ml puromycin (CLONTECH Laboratories, Inc.) was added. 10–14 d after transfection,  $6 \times 10^6$  cells were plated per 10-cm Petri dish in 10 ml IMDM supplemented with 10% FBS without puromycin. The next day the medium was refreshed and the day after retroviral supernatant was harvested, centrifuged, and frozen in aliquots at  $-70^\circ\text{C}$ .

**Retroviral Transduction of Virus-specific T Lymphocytes and Selection of Transduced T Cells.** Virus-specific T cells were isolated from the peripheral blood of healthy individuals. After informed consent PBMCs were harvested and labeled with tetrameric complexes for 2 h at  $4^\circ\text{C}$  in RPMI without phenol, supplemented with 2% FBS, washed three times, and sorted at  $4^\circ\text{C}$  using the FACS Vantage<sup>TM</sup> (Becton Dickinson). Tetramer<sup>+</sup> T cells were stimulated with either 800 ng/ml phytohemagglutinin (PHA; Murex Diagnostics) and 100 IU/ml IL-2 (Chiron Corp.), or with virus peptide-pulsed autologous PBMCs and 100 IU/ml IL-2. The autologous PBMCs were pulsed with 10  $\mu$ M CMV peptide for 1 h, irradiated (30 Gy), and washed, and  $10^6$  peptide-loaded autologous PBMCs were added to each well of a 24-well plate containing  $2 \times 10^5$  virus-specific T cells. After 2 d of culture, the T cells were transduced with retroviral supernatant. The transduction procedure used for the peripheral blood T cells was based on the use of recombinant human fibronectin fragments CH-296 (29) and has been described (27). In brief,  $10^6$  T cells were cultured on CH-296-coated 24-well nonculture-treated plates (Falcon) together with 1 ml thawed retroviral supernatant for 6 h or overnight at  $37^\circ\text{C}$ , washed, and transferred to 24-well tissue culture plates. The transduction efficiency as measured by the expression of the markers eGFP and  $\Delta$ NGF-R was analyzed by flow cytometry 3–5 d after transduction. In addition, flow cytometric analyses were performed with the relevant tetrameric complexes. HA-2-TCR-transduced virus-specific T cells were FACS<sup>®</sup> sorted on bases of marker gene expression or HA-2 tetramer<sup>+</sup> staining, and cultured at 1 cell/well (clones) or 25 cells/well (cell lines) in IMDM supplemented with 10% human serum. Sorted T cells were nonspecifically stimulated every 2 wk with feeder cell mixtures containing irradiated allogeneic PBMCs (30 Gy), irradiated EBV-transformed B cells (EBV-LCLs; 50 Gy), 800 ng/ml PHA, and 100 IU/ml IL-2, or stimulated with virus peptide-pulsed irradiated autologous PBMCs (30 Gy) and 100 IU/ml IL-2.

**Tetrameric HLA Class I/Peptide Complexes, Flow Cytometric Analyses, and FACS<sup>TM</sup>Sort.** PE- or APC-conjugated tetrameric complexes were constructed as previously described (30) with minor modifications. Tetrameric HLA-A2 molecules in complex with CMV pp65-derived peptide NLVPMVATV (CMV<sup>A2</sup> tetramer) and HA-2-derived peptide YIGEVLVSV (HA-2<sup>A2</sup> tetramer) were constructed. In addition, tetrameric HLA-B7 molecules in complex with CMV pp65-derived peptide TPRVTGGGAM (CMV<sup>B7</sup> tetramer) were constructed. For flow cytometric analyses as well as FACS<sup>TM</sup>Sort experiments, cells were labeled with tetrameric complexes for 2 h at  $4^\circ\text{C}$  in RPMI without phenol, supplemented with 2% FBS, and washed three times. During the last 30 min, mAbs directed against the various cell surface molecules were added. The mAbs used were anti-CD4 (FITC), anti-CD8 (FITC or PE-Cy5), anti-CD40 (FITC), and anti-NGF-R (PE; all from Becton Dickinson).

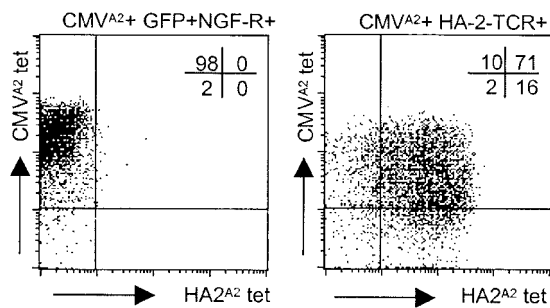
**Cytotoxicity Assay.** Target cells were harvested, labeled with 50  $\mu$ Ci Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> for 60 min at  $37^\circ\text{C}$ , washed three times, and

added to various numbers of effector T cells in a final volume of 150  $\mu$ l IMDM supplemented with 10% FBS in 96-well U-bottomed microtiter plates. HA-2<sup>+</sup> and HA-2<sup>-</sup> EBV-LCLs, HFF, COS, 293 EBNA cells, and leukemic cells harvested from patients after informed consent were used as target cells. In addition, EBV-LCLs transduced with a retroviral vector encoding for the gene product of the lower matrix protein pp65 of HCMV AD169 were used (EBV-Z pp65). In some experiments, <sup>51</sup>Cr-labeled target cells were loaded by preincubation for 1 h at  $37^\circ\text{C}$  with different concentrations of synthetic peptides, washed, and added to the effector cells. After 4 or 18 h of incubation of target and effector cells at  $37^\circ\text{C}$  and 5% CO<sub>2</sub>, 25  $\mu$ l supernatant was harvested and measured in a luminescence counter (Topcount-NXT; Packard Instrument Co.). The mean percentage of specific lysis of triplicate wells was calculated as follows: specific lysis = [(experimental release – spontaneous release)/(maximal release – spontaneous release)]  $\times$  100.

**Liquid Hematopoietic Progenitor Cell Inhibition Assay (PIA).** The PIA was performed as previously described (31, 32). T cells at a concentration of  $10^4$  cells/well were cocultured with  $10^4$  cells/well of cell suspensions containing malignant hematopoietic progenitor cells (HPCs) in 96-well U-bottomed microtiter plates in a volume of 200  $\mu$ l IMDM supplemented with 10% FBS and multiple hematopoietic growth factors. T cells were irradiated with 15 Gy before use, preventing their proliferation in the PIA. HPCs collected from different CML patients were used as target cells. After 5 d of culture, the wells were pulsed with 1  $\mu$ Ci [<sup>3</sup>H]thymidine for 18 h. Cells were harvested and the incorporated [<sup>3</sup>H]thymidine was measured by luminescence counter to determine the capacity of the T cell clones to inhibit the growth of the HPCs. The mean percentage of growth inhibition of triplicate wells was calculated as follows: [(1 – experimental cpm/mean cpm HPC only)  $\times$  100%].

## Results

**HA-2-TCR Transfer into CMV pp65-specific T Cells Directly Isolated from Peripheral Blood.** To determine whether virus-specific T cells could be isolated, retrovirally transduced and expanded HLA-A2-restricted CMV pp65-specific T cells (CMV<sup>A2</sup>-specific T cells) were isolated from the peripheral blood of HLA-A2<sup>+</sup> HA-2<sup>-</sup> individuals by FACS<sup>TM</sup>Sort using CMV<sup>A2</sup> tetrameric complexes, and T cells were subsequently stimulated with PHA, IL-2, and allogeneic feeder cells. 2 d after stimulation, the T cells were transduced with the HA-2-TCR complex derived from the HA-2-specific HA2.5 T cell clone, and sorted at day 5 on the basis of HA-2<sup>A2</sup> tetramer positivity. In addition, T cells were transduced with the control retroviral vectors and sorted on the basis of marker gene expression. T cells were sorted in bulk or single cell and expanded by nonspecific stimulation. Fig. 1 shows the HA-2<sup>A2</sup> and CMV<sup>A2</sup> tetramer staining of the HA-2-TCR-transduced and control-transduced CMV<sup>A2</sup>-specific T cell lines 1 wk after sorting. The results demonstrated that the CMV<sup>A2</sup>-specific T cells transduced with the HA-2-TCR and sorted on the basis of HA-2<sup>A2</sup> tetramer staining expressed both TCR complexes at the cell surface. The mean fluorescence intensity (MFI) of the CMV<sup>A2</sup> tetramer staining was lower on the HA-2-TCR-transduced T cells compared with the control-trans-



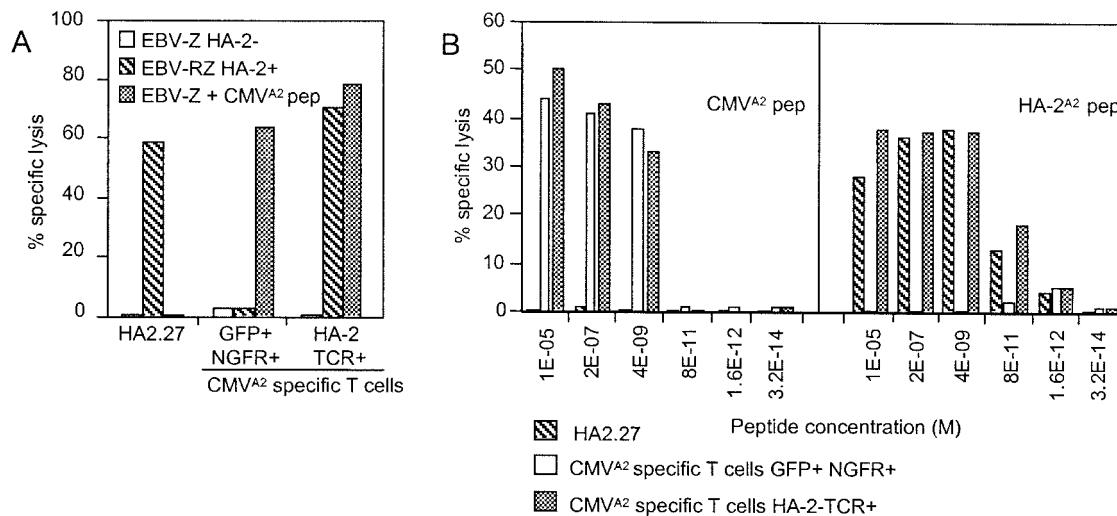
**Figure 1.** Tetramer staining of CMV<sup>A2</sup>-specific T cells transduced with the HA2.5-TCR or control vectors. The sorted HA-2-TCR- and control GFP-NGF-R-transduced T cells were labeled with PE-conjugated HA-2<sup>A2</sup> tetramers and APC-conjugated CMV<sup>A2</sup> tetramers for 2 h at 4°C.

duced T cells. The MFI decreased from 265 on control-transduced to 86 on HA-2-TCR-transduced CMV-specific T cells, indicating competition between the two TCR complexes for cell surface expression.

*The HA-2-TCR-redirected CMV<sup>A2</sup>-specific T Cells Exert HA-2<sup>A2</sup> as well as CMV<sup>A2</sup> Reactivity.* To determine whether the HA-2-TCR-transduced CMV<sup>A2</sup>-specific T cells were able to recognize the HA-2 mHag as well as CMV pp65 antigen, we analyzed the cytotoxicity against a panel of different target cells. As demonstrated in Fig. 2 A, the HA-2-TCR-modified CMV<sup>A2</sup>-specific T cells exerted cytolytic activity against HA-2-expressing EBV-LCLs as well as CMV<sup>A2</sup> peptide-loaded EBV-LCLs. In contrast, control-transduced CMV<sup>A2</sup>-specific T cells exerted only cytolytic activity against CMV<sup>A2</sup> peptide-loaded target cells. By peptide titration on T2 cells we illustrated that the CMV<sup>A2</sup> peptide reactivity of the HA-2-TCR-modified T cell populations did not significantly differ from the

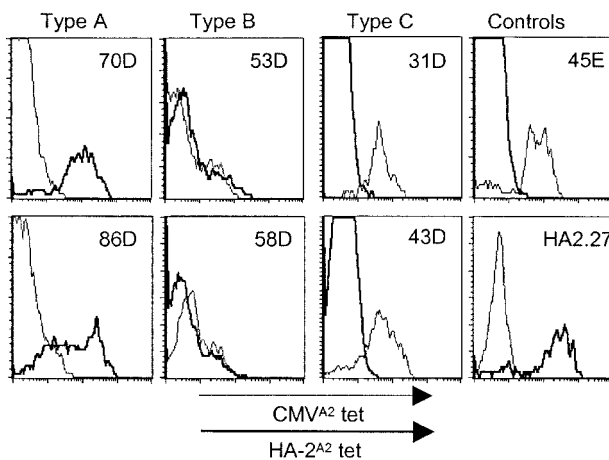
CMV<sup>A2</sup> peptide reactivity of the control-transduced T cells (Fig. 2 B). The HA-2-TCR-transferred T cells displayed similar cytolytic efficiencies toward HA-2 peptide-loaded target cells as the original HA-2-specific T cell clone HA2.27. These results demonstrated the dual specificity of the HA-2-TCR-modified CMV<sup>A2</sup>-specific T cells.

In addition to the HA-2-TCR-modified T cell lines, we generated HA-2-TCR-modified CMV-specific T cell clones to analyze the expression of the two TCR complexes in more detail, and to correlate this with the functional activity of the individual TCR-transferred T cell clones. As demonstrated in Fig. 3, the HA-2-TCR-modified CMV-specific T cell clones exhibited differences in HA-2<sup>A2</sup> and CMV<sup>A2</sup> tetramer staining. Some HA-2-TCR-modified T cell clones preferentially expressed the HA-2-specific TCR (type A), whereas other T cell clones preferentially expressed the CMV-specific TCR (type C). Interestingly, the majority of the T cell clones showed intermediate expression of both TCR complexes (type B). Although the HA-2<sup>A2</sup> and CMV<sup>A2</sup> tetramer staining in the TCR-transferred T cell clones varied, the overall TCR  $\alpha$  and  $\beta$  expression was similar, indicating that the composition of cell surface TCRs containing the endogenous and introduced TCR chains was variable in the different T cell clones, and not the absolute numbers of TCRs expressed. These results indicated that the different TCR complexes competed for cell surface expression. Importantly, the expression of the HA-2-TCR and CMV-TCR complexes on the different T cell clones as measured by HA-2<sup>A2</sup> and CMV<sup>A2</sup> tetramer staining, respectively, was stable during a culture period of 2 mo. Furthermore, as illustrated in Table I, which shows representative examples of each clone type, the lytic activity of the dual-specific T cell clones correlated



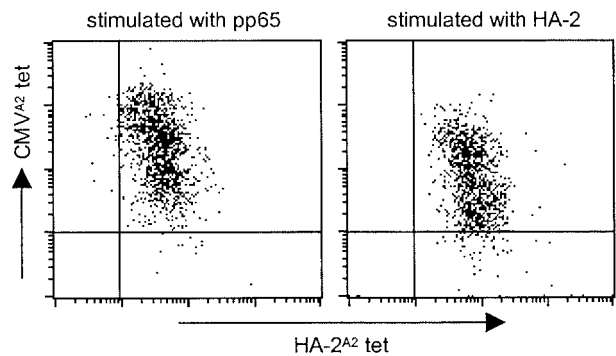
**Figure 2.** HA-2-TCR-modified CMV<sup>A2</sup>-specific T cells exert dual specificity. (A) HA-2-TCR-modified CMV<sup>A2</sup>-specific T cells, control-transduced CMV<sup>A2</sup>-specific T cells, and the original HA-2-specific T cell clone HA2.27 were tested against EBV-LCLs expressing the HA-2 mHag (EBV-RZ), EBV-LCLs negative for HA-2 (EBV-Z), and EBV-Z loaded with 10  $\mu$ M of the CMV<sup>A2</sup> peptide at an E/T ratio of 10:1, illustrating dual recognition by the HA-2-TCR-modified CMV<sup>A2</sup>-specific T cells. (B) The different effectors were tested against T2 cells loaded with different concentrations of HA-2<sup>A2</sup> peptide or CMV<sup>A2</sup> peptide. T2 cells were preincubated with peptides for 1 h at 37°C, washed, and added to the T cells at an E/T ratio of 10:1. The results demonstrated that the virus-specific reactivity did not significantly differ between the two transduced T cell populations.





**Figure 3.** Tetramer staining of the HA-2-TCR-modified CMV<sup>A2</sup>-specific T clones. Representative HA-2-TCR-transferred T cell clones were labeled with either PE-conjugated HA-2<sup>A2</sup> tetramers (bold line) or CMV<sup>A2</sup> tetramers (thin line). As controls, the control-transduced CMV<sup>A2</sup>-specific T cell clone 45E and the original HA-2-specific T cell clone HA2.27 are shown. Three different types of HA-2-TCR-modified CMV<sup>A2</sup>-specific T clones were identified.

in general with the TCR expression. Type A and type B T cell clones exerted efficient HA-2-specific cytolytic activity directed against HA-2 peptide-loaded as well as endogenous HA-2-expressing target cells. Importantly, these type A and type B T cells were CMV specific because CMV pp65 peptide-loaded target cells as well as target cells endogenously expressing the CMV pp65 protein were recognized by these TCR-transduced T cell clones (Table I), demonstrating the dual specificity of these T cell clones. In agreement with the bright CMV<sup>A2</sup> tetramer staining, type C T cells exerted efficient cytolytic activity directed against the CMV pp65<sup>+</sup> target cells. Although only minimal HA-2-TCRs could be visualized by HA-2<sup>A2</sup> tetramers on these type C T cell clones, T2 cells loaded with HA-2 peptides could be lysed by these T cells (Table I).



**Figure 4.** No loss of HA-2-TCR expression on HA-2-TCR-transduced CMV<sup>A2</sup>-specific T cells after CMV pp65-specific stimulation. HA-2-TCR-transduced CMV<sup>A2</sup>-specific T cells were sorted on basis of HA-2<sup>A2</sup> tetramer positivity and expanded for 3 wk. The HA-2-TCR-transduced T cells were then split and stimulated twice with either CMV pp65-expressing EBV-LCLs cells or HA-2-expressing EBV-LCLs over a period of 30 d. T cells were labeled with PE-conjugated HA-2<sup>A2</sup> tetramers and APC-conjugated CMV<sup>A2</sup> tetramers for 2 h at 4°C.

To predict whether stimulation in vivo with CMV-infected cells would lead to the stimulation and expansion of all three types of TCR-transferred virus-specific T cells, HA-2-TCR-transferred CMV-specific T cells were stimulated twice in vitro with CMV pp65-expressing stimulator cells over a period of 30 d. As demonstrated by tetramer staining shown in Fig. 4, the total population of HA-2-TCR-transduced CMV-specific T cells expressed after 30 d marked levels of CMV-TCRs as well as HA-2-TCRs at the cell surface. Stimulation of the HA-2-TCR-transferred CMV-specific T cells with stimulator cells endogenously expressing the HA-2 mHag also resulted in expansion of the TCR-transduced CMV-specific T cells. Tetramer staining demonstrated the marked expression of both TCRs at the cell surface, with a tendency of lower levels of CMV-TCR expression. In parallel we stimulated control-transduced CMV-specific T cells with stimulator cells either expressing the CMV or the HA-2 antigen. Stimulator

**Table I.** HA-2<sup>A2</sup> and CMV<sup>A2</sup> Tetramer Staining and Cytolytic Activity of the HA-2-TCR-transduced CMV-specific T Cell Clones

T cell clones	Type	HA-2 <sup>A2</sup> tet	CMV <sup>A2</sup> tet	EBV-Z HA-2-	EBV-Z HA-2- pp65 <sup>+</sup> a	EBV-RZ HA-2 <sup>+</sup>	T2 HA-2-	T2 + CMV <sub>pp65</sub> (6E-10 M)	T2 + HA-2 <sub>pp65</sub> (6E-10 M)
		(MFI)	(MFI)	(% lysis)	(% lysis)	(% lysis)	(% lysis)	(% lysis)	(% lysis)
70D	A	146 ± 78	17 ± 12	0	15	37	0	47	27
58D	B	39 ± 17	29 ± 17	0	24	28	0	57	30
31D	C	9 ± 2	82 ± 47	0	45	5	0	67	16
45E	—	6 ± 3	126 ± 9	0	46	0	0	44	0
HA2.27	—	226 ± 73	7 ± 2	0	0	31	0	0	35

HA-2<sup>A2</sup> and CMV<sup>A2</sup> tetramer staining (indicated as MFI) and cytolytic activity at an E/T ratio of 3:1 of representative HA-2-TCR-transduced (D clones) and control-transduced (E clone) CMV-specific T cell clones and the original HA-2-specific T cell clone HA2.27. The tetramer stainings were performed at different time points of culture, at least 7 d after restimulation during a period of 2 mo (*n* = 4).

<sup>a</sup>EBV-Z retrovirally transduced with the CMV pp65 protein.

cells endogenously expressing the HA-2 mHag were unable to stimulate and expand the control-transduced CMV-specific T cells, whereas stimulator cells expressing CMV pp65 induced expansion of the control-transduced CMV-specific T cells. In a cytotoxicity assay we demonstrated that the HA-2-TCR-transferred T cells stimulated with CMV pp65-expressing stimulator cells exerted, comparable to the HA-2-TCR-transferred T cells stimulated with HA-2-expressing stimulator cells, specific cytolytic activity directed against HA-2-expressing target cells as well as CMV pp65-expressing target cells (Table II), illustrating that the dual specificity of the TCR-transferred virus-specific T cells is preserved after stimulation of either the endogenous or the introduced TCR.

**HA-2-TCR-transduced CMV<sup>A2</sup>-specific T Cells Exert Antileukemic Reactivity.** To determine whether the mHag HA-2 specificity of the HA-2-TCR-modified CMV-specific T cells could be translated into antileukemic reactivity, the TCR-transferred T cells were tested against a panel of CML cells in a cytotoxicity assay. The HA-2-TCR-modified CMV-specific T cell clones were able to exert cytolytic activity against HA-2-expressing CML cells and not against HA-2<sup>-</sup> CML cells (Fig. 5 A). The cytolytic activity of the TCR-transferred T cells was for most TCR-transferred T cell clones as efficient as the original HA-2-spe-

**Table II.** Cytolytic Activity of the HA-2-TCR-transduced CMV-specific T Cells after Expansion by Stimulation with Either CMV pp65 or HA-2

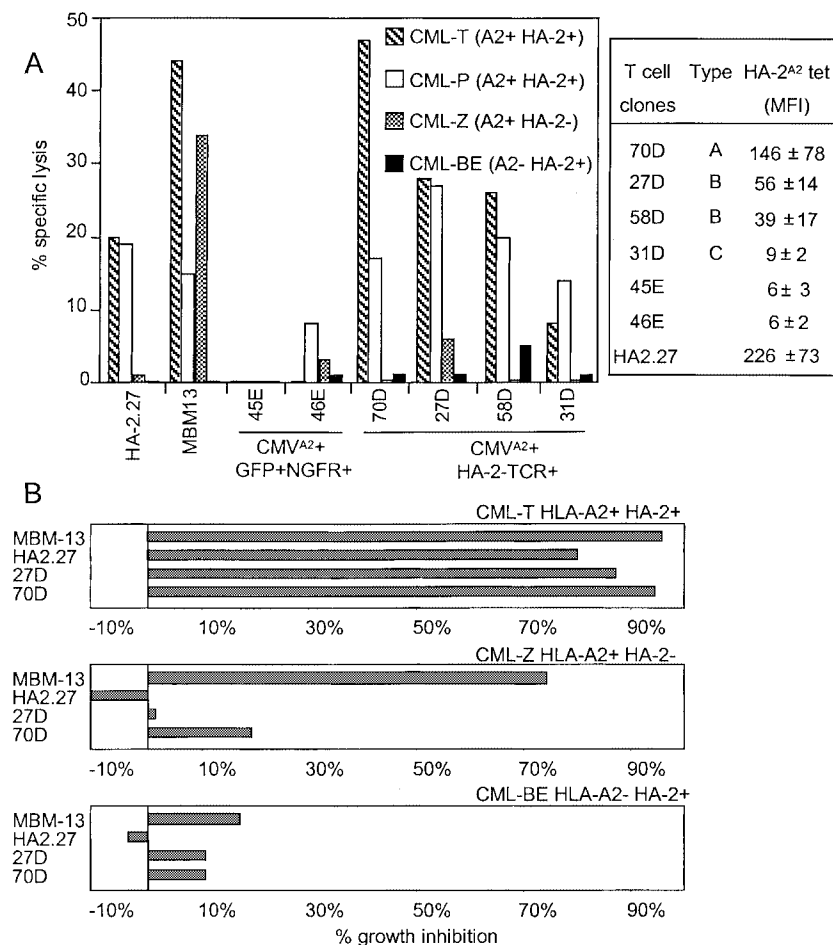
T cells	Stimulation <sup>a</sup>	EBV-Z		
		HA-2-	HA-2- pp65 <sup>+b</sup>	EBV-RZ HA-2 <sup>+</sup>
		(% lysis)	(% lysis)	(% lysis)
Control transduced	pp65	5	46	0
Control transduced	HA-2	n.e. <sup>c</sup>	n.e.	n.e.
HA-2-TCR transduced	pp65	3	27	19
HA-2-TCR transduced	HA-2	0	17	22

<sup>a</sup>CMV<sup>A2</sup>-specific T cells transduced with control vectors or the HA-2-TCR were sorted on the basis of HA-2<sup>A2</sup> tetramer positivity or marker gene expression and expanded for 3 wk. The HA-2-TCR-transduced T cells were then split and stimulated twice over a period of 30 d with stimulator cells expressing the antigen indicated. The cells were tested at an E/T ratio of 3:1 in a 4-h cytotoxicity assay.

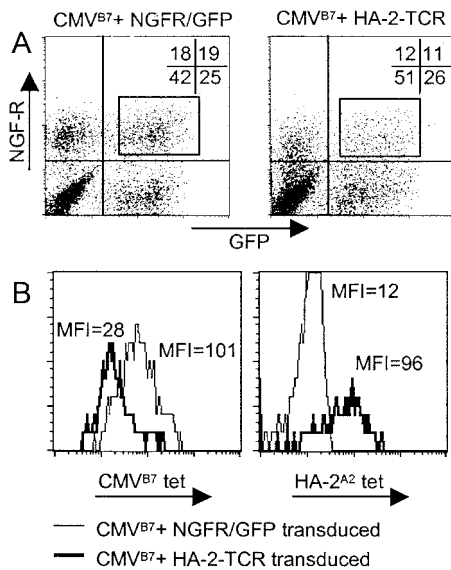
<sup>b</sup>EBV-Z retrovirally transduced with the CMV pp65 protein.

<sup>c</sup>No expansion of cells to test the reactivity.

cific T cell clone HA2.27. The HA-2-specific TCR density, measured by HA-2<sup>A2</sup> tetramer staining, correlated with the ability of the TCR-modified T cell clones to lyse the



**Figure 5.** Antileukemic reactivity of HA-2-TCR-transferred CMV<sup>A2</sup>-specific T cell clones. (A) HA-2-TCR-transduced (D clones), control-transduced (E clones) CMV<sup>A2</sup>-specific T cell clones, the original HA-2-specific T cell clone HA2.27, and the allo-HLA-A2-specific T cell clone MBM13 were tested in an 18-h cytotoxicity assay against a panel of different CML cells at an E/T ratio of 3:1. The MFI of the HA-2<sup>A2</sup> tetramer staining of the different T cell clones is indicated in the table on the right. (B) Two HA-2-TCR-modified CMV<sup>A2</sup>-specific T cell clones, 27D and 70D, and the control clones HA2.27 and MBM13 were tested in a liquid hematopoietic PIA against different CML cells at an E/T ratio of 1:1.

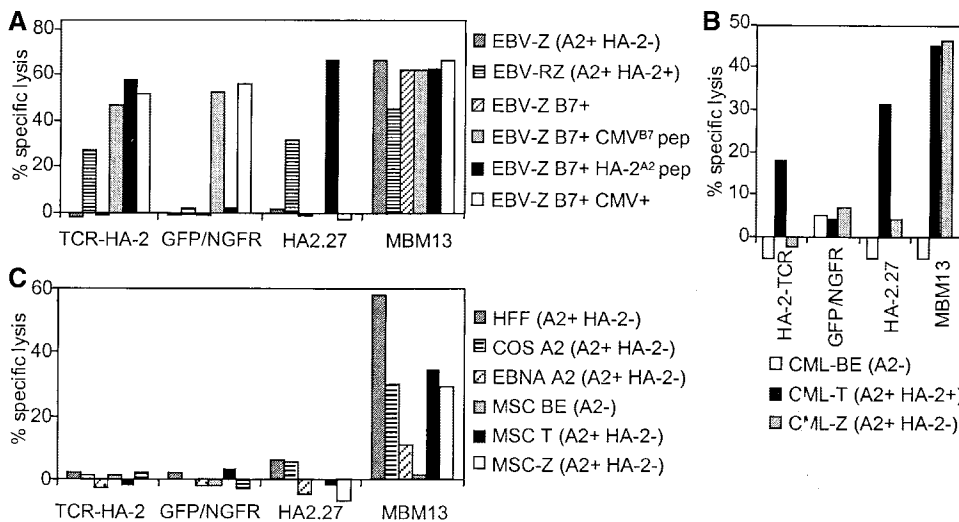


**Figure 6.** Efficient HA-2-TCR transfer of CMV<sup>B7</sup>-specific T cells. The CMV<sup>B7</sup> tetramer<sup>+</sup> sorted T cells transduced with either control retroviral vectors or with the retroviral vectors encoding for the HA-2-TCR were labeled with PE-conjugated anti-NGF-R in combination with either APC-conjugated CMV<sup>B7</sup> or HA-2<sup>A2</sup> tetrameric complexes. The CMV<sup>B7</sup> and HA-2<sup>A2</sup> tetramer stainings of the gated double marker gene<sup>+</sup> cells of the control-transduced (thin line) and the HA-2-TCR-transduced (bold line) CMV<sup>B7</sup>-specific T cells are shown as histogram plots. The MFI of the CMV<sup>B7</sup> and HA-2<sup>A2</sup> tetramer stainings on the two gated T cell populations are indicated in the histogram plots.

HLA-A2<sup>+</sup> HA-2<sup>+</sup> leukemic cells. In addition, we tested two HA-2-TCR-modified CMV-specific T cell clones in the liquid hematopoietic PIA, and demonstrated that these T cell clones were able to specifically inhibit the growth of HLA-A2<sup>+</sup> CML progenitor cells expressing the HA-2 mHag, and not the growth of HLA-A2<sup>+</sup> HA-2<sup>-</sup> or HLA-

A2<sup>-</sup> CML progenitor cells (Fig. 5 B). The specific growth inhibition of the HA-2-TCR-transferred CMV-specific T cells was as efficient as the original T cell clone HA2.27.

*HA-2-TCR-transduced CMV<sup>B7</sup>-specific T Cells Derived from HLA-A2<sup>-</sup> Individuals Are Cytotoxic to Leukemic Cells, without Signs of Anti-HLA-A2 Reactivity.* To explore the possibility of an alternative strategy to treat hematological malignancies of HLA-A2<sup>+</sup> HA-2-expressing patients transplanted with HLA-A2<sup>-</sup> donors, we isolated CMV-specific T cells from an HLA-A2<sup>-</sup> individual and transferred these T cells with HA-2-TCRs. For this purpose CMV-specific HLA-B7-restricted T cells (CMV<sup>B7</sup>-specific T cells) were FACS<sup>®</sup> sorted using CMV<sup>B7</sup> tetramers stimulated with CMV<sup>B7</sup> peptide-loaded, irradiated autologous PBMCs and transduced with the HA-2-TCR derived from the HA-2-specific T cell clone HA2.5, or with control retroviral vectors. By FACS<sup>®</sup> analyses we demonstrated that a high percentage of CMV<sup>B7</sup>-specific T cells was transduced (49–58%) and that the majority of HA-2-TCR  $\alpha$  and  $\beta$  chain transduced T cells stained with the HA-2<sup>A2</sup> tetramer (Fig. 6). Similar to the HA-2-TCR-transduced CMV<sup>A2</sup>-specific T cells, the CMV<sup>B7</sup> tetramer staining was decreased on the HA-2-TCR-transduced CMV<sup>B7</sup>-specific T cells compared with the control-transduced CMV<sup>B7</sup>-specific T cells. The double marker gene<sup>+</sup> CMV<sup>B7</sup>-specific T cells transduced with either the HA-2-TCR or with the control vectors were sorted and cultured for an additional 10 d in IMDM supplemented with 10% human serum and 100 IU/ml IL-2. The expanded HA-2-TCR-transferred CMV<sup>B7</sup>-specific T cells were tested in a cytotoxicity assay against a panel of target cells. As can be seen in Fig. 7 A, HA-2-TCR-transduced T cells were able to efficiently lyse the HA-2-expressing EBV-LCLs (EBV-RZ) and not the HA-2-negative EBV-LCLs (EBV-Z). To investigate whether the HA-2-TCR-transduced CMV<sup>B7</sup>-specific T cells exerted dual



**Figure 7.** Antileukemic reactivity of HA-2-TCR-transferred CMV<sup>B7</sup>-specific T cells without anti-HLA-A2 reactivity. HA-2-TCR-transduced CMV<sup>B7</sup>-specific T cells, control-transduced CMV<sup>B7</sup>-specific T cells, the original HA-2-specific T cell clone HA2.27, and the allo-HLA-A2-specific T cell clone MBM13 were tested against a panel of different target cells at an E/T ratio of 5:1. (A) T cells were tested against HLA-A2<sup>+</sup> HA-2<sup>-</sup> EBV-LCLs (EBV-Z) and HLA-A2<sup>+</sup> HA-2<sup>+</sup> EBV-LCLs (EBV-RZ), EBV-Z transduced with HLA-B7 and loaded with either 10  $\mu$ M CMV<sup>B7</sup> peptide (EBV-Z B7+ CMV<sup>B7</sup> pep) or HA-2<sup>A2</sup> peptide (EBV-Z B7+ HA-2<sup>A2</sup> pep) for 1 h at 37°C, or not loaded (EBV-Z B7+) and HLA-B7<sup>+</sup> EBV-Z transduced with the pp65 gene (EBV-Z B7+ CMV+) in a 4-h cytotoxicity assay. (B) T cells were tested against HLA-A2<sup>-</sup> CML cells (CML-BE), HLA-A2<sup>+</sup> HA-2<sup>+</sup> CML cells (CML-T), and HLA-A2<sup>+</sup> HA-2<sup>-</sup> CML cells (CML-Z) in an 18-h cytotoxicity assay. (C) T cells were tested against HLA-A2<sup>+</sup> HA-2<sup>-</sup> HFF cells, COS and 293 EBNA cells transduced with HLA-A2, and MSCs negative for HLA-A2 (MSC-BE), HLA-A2<sup>+</sup> HA-2<sup>-</sup> MSCs (MSC-T), and HLA-A2<sup>+</sup> HA-2<sup>-</sup> MSCs (MSC-Z) in an 18-h cytotoxicity assay.

B7+ CMV+) in a 4-h cytotoxicity assay. (B) T cells were tested against HLA-A2<sup>-</sup> CML cells (CML-BE), HLA-A2<sup>+</sup> HA-2<sup>+</sup> CML cells (CML-T), and HLA-A2<sup>+</sup> HA-2<sup>-</sup> CML cells (CML-Z) in an 18-h cytotoxicity assay. (C) T cells were tested against HLA-A2<sup>+</sup> HA-2<sup>-</sup> HFF cells, COS and 293 EBNA cells transduced with HLA-A2, and MSCs negative for HLA-A2 (MSC-BE), HLA-A2<sup>+</sup> HA-2<sup>-</sup> MSCs (MSC-T), and HLA-A2<sup>+</sup> HA-2<sup>-</sup> MSCs (MSC-Z) in an 18-h cytotoxicity assay.

HA-2 and CMV specificity, similar to the HA-2-TCR-transferred CMV<sup>A2</sup>-specific T cells, HLA-B7-transduced EBV-Z were loaded with the CMV<sup>B7</sup> peptide or were additionally transduced with the pp65 gene of CMV. As can be seen in Fig. 7 A, the HLA-B7<sup>+</sup> EBV-Z target cells either loaded with CMV<sup>B7</sup> peptide or endogenously expressing the pp65 protein of CMV were efficiently lysed by the control-transduced as well as the HA-2-TCR-transduced CMV<sup>B7</sup>-specific T cells, illustrating that the HA-2-TCR-transduced CMV<sup>B7</sup>-specific T cells exerted dual specificity. In addition, HLA-A2<sup>+</sup> CML cells expressing the HA-2 mHag (CML-T) were efficiently lysed by the HA-2-TCR-modified T cells (Fig. 7 B). In contrast, HLA-A2<sup>+</sup> CML cells negative for HA-2 or HLA-A2<sup>-</sup> CML cells were not lysed by the HA-2-TCR-modified T cells. No anti-HLA-A2 alloreactivity was observed because HLA-A2<sup>+</sup> target cells negative for HA-2, including the HLA-A2<sup>+</sup> HFF fibroblast cell line, HLA-A2-transduced COS and EBNA cells, and the HLA-A2<sup>+</sup> MSCs derived from the patients with CML (MSC-T and MSC-Z), were not lysed by the HA-2-TCR-transduced CMV<sup>B7</sup>-specific T cells (Fig. 7 C). In contrast, the allo-HLA-A2-restricted control T cell clone MBM13 lysed all of these HLA-A2-expressing target cells efficiently. In agreement with the expression pattern of the HA-2 mHag, the MSCs of patient T were not lysed by the HA-2-specific T cells because MSCs in contrast to the cells derived from the hematopoietic lineage do not express the HA-2 mHag. These results illustrate that reprogramming of virus-specific T cells into antileukemic reactive T cells by transfer of mHag-specific TCRs without the occurrence of allo-HLA reactivity is feasible.

## Discussion

In this study we demonstrated the efficient reprogramming of virus-specific T cells into leukemia-reactive T cells using TCR gene transfer. HLA-A2-restricted as well as HLA-B7-restricted CMV-specific T cells derived from HLA-A2<sup>+</sup> and HLA-A2<sup>-</sup> individuals transferred with the HA-2-TCR were able to exert efficient antileukemic reactivity, without signs of anti-HLA-A2 alloreactivity. The virus-specific T cells were isolated by tetrameric complexes, expanded by specific or aspecific stimulation, and transferred with the genes encoding for the TCR directed against the mHag HA-2 in the context of HLA-A2. The CMV-specific T cells isolated by CMV<sup>A2</sup> or CMV<sup>B7</sup> tetrameric complexes were >95% pure, could be efficiently transduced (40–60%), and expanded vigorously. The HA-2-specific cytolytic activity of most HA-2-TCR-transferred T cell populations was similar to the original HA-2-specific T cell clones and in addition, the TCR-redirected virus-specific T cells exerted efficient cytolytic activity against CMV peptide-loaded target cells or target cells that endogenously express the CMV pp65 protein, demonstrating the dual specificity of the HA-2-TCR-transferred CMV-specific T cells.

No anti-HLA-A2 alloreactivity was observed because HLA-A2<sup>+</sup> nonhematopoietic cell lines, HA-2<sup>-</sup> HLA-A2<sup>+</sup>

EBV-LCLs, and HLA-A2<sup>+</sup> MSCs of patients from which the HA-2<sup>+</sup> CML cells were derived were not recognized by the HA-2-TCR-transferred T cells. The HA-2-TCR-transferred T cells only exerted specific CMV as well as leukemia reactivity. Therefore, we speculate that these T cells will be useful in HLA mismatch transplantations because after these transplantations administration of unmodified HLA class I disparate DLI has a high risk of inducing GVHD.

Regardless of whether the HA-2-TCR-transferred virus-specific T cells were stimulated with HA-2 or CMV, no selective decrease in the HA-2-TCR expression was observed. Based on these results and the latent persistence of herpes viruses like CMV and EBV in vivo, we hypothesize that the TCR-transferred dual specific T cells exerting both leukemia as well as virus specificity will be triggered continuously with low doses of viral antigens via their endogenous virus-specific TCR in vivo. Therefore, we speculate that due to this low dose triggering of these TCR-transferred dual specific T cells, the T cells will survive for prolonged periods of time in vivo. Furthermore, we hypothesize that by virus-specific peptide vaccinations we will be able to promote the survival of these leukemia-reactive, TCR-transferred virus-specific T cells in vivo.

During CMV reactivation, there might be preferential proliferation of TCR-transduced T cells with the highest activity against CMV. Although the TCR-transduced CMV-specific T cells with the highest HA-2 activity may not expand as vigorously during CMV disease, we speculate that these T cells will persist in the patient and during reappearance of the malignancy, these T cells with the highest HA-2-specific avidity are likely to preferentially expand.

Because in vivo the compartments in which specific viral antigens are expressed may not be identical to the site of the malignancy, the homing characteristics of the engineered T cells might be a relevant factor. By selecting for TCR transfer T cells specific for not only CMV but also for other target antigens like EBV, reprogrammed T cells might be directed to several tissues in search of the malignancy.

An advantage of using virus-specific T cells for TCR gene transfer is that these polyclonal T cell responses consist of a restricted TCR repertoire. The formation of mixed TCR dimers due to pairing of the endogenous TCR chains with the introduced TCR chains that may have unpredictable specificities possibly leading to harmful immune reactions, will therefore be limited. In addition, TCR transfer of virus-specific T cells will minimize the induction of autoaggressive T cells that may develop due to triggering of ignorant self-specific T cells through their introduced TCR.

Furthermore, if antigen-specific TCRs are transferred into unselected primary T cells not only might a potentiating immune response develop, but also suppressive T cell reactivity. If naive T cell populations that are transduced with the specific TCR encounter their stimulatory antigen in vivo in the context of nonprofessional antigen-presenting cells, the T cell response may abort or antigen-specific suppression may even occur. In addition, if circulating suppressor T cells are transduced with the specific TCR, suppression of the de-



sired T cell response may develop. Furthermore, end-stage effector cells with limited proliferative capacity that will be transduced with the exogenous TCR may only exhibit short-term cytolytic activity. In contrast, virus-specific memory T cells are likely to expand rapidly *in vivo*, and are probably destined to undergo multiple cell divisions. Therefore, as host cells for TCR gene transfer, it may be desirable to select for memory T cell populations that apparently have been successfully triggered in the past by professional antigen-presenting cells, and are capable of further large scale expansion in response to specific activation.

Transfer of TCR  $\alpha$  and  $\beta$  chains into T cells results in cell surface expression of the introduced TCR, the endogenous TCR, and mixed TCR dimers, which consist of endogenous and exogenous TCR chains. If the individual TCR  $\alpha$  and  $\beta$  chains pair with similar affinity to each other and no competition for cellular components between the individual TCR  $\alpha$  and  $\beta$  chains occurs, the introduced TCR and endogenous TCR density on individual T cells would be  $\sim 25\%$  of the total TCR expression for each TCR complex. In addition,  $\sim 50\%$  of the total TCR  $\alpha\beta$  expression would consist of mixed TCR dimers. The majority of the HA-2-TCR-transferred T cell clones demonstrated intermediate expression of both the virus- and the HA-2-specific TCR complexes, representative for the type B T cell clones (Fig. 3). Because the TCR  $\alpha\beta$  cell surface expression on these HA-2-TCR-transferred T cell clones is similar to the TCR  $\alpha\beta$  expression of the type A and type C TCR-transferred T cell clones, these results indicated that formation of chimeric TCR complexes is occurring. Furthermore, we demonstrated that the cell surface expression of the introduced HA-2-TCR complex in relation to the endogenous CMV-specific TCR varied between T cells. This might be due to differences in expression levels of the retroviral vectors encoding for the TCR  $\alpha$  and  $\beta$  chains or due to intrinsic properties of the TCR-transferred T cells. Preferential pairing of particular TCR  $\alpha$  and  $\beta$  chains might be responsible for the variations in TCR expression. Hence, further selection of certain virus-specific T cells with a particular TCR  $\alpha$  and  $\beta$  chain usage unable to pair with the introduced mHag-specific TCR would then be relevant.

In conclusion, these results demonstrated that it is possible to redirect the specificity of virus-specific T cells toward antileukemic reactivity. Based on the dual specificity of these TCR-redirected virus-specific T cells and the minimization of autoimmunity, TCR transfer of virus-specific T cells will open new possibilities for the treatment of hematological malignancies of HLA-A2<sup>+</sup> HA-2-expressing patients transplanted with HLA-A2-matched or -mismatched donors.

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## References

- Kolb, H.J., J. Mittermuller, C. Clemm, E. Holler, G. Lederose, G. Brehm, M. Heim, and W. Wilmanns. 1990. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood*. 76:2462–2465.
- Collins, R.H., Jr., O. Shpilberg, W.R. Drobyski, D.L. Porter, S. Giralt, R. Champlin, S.A. Goodman, S.N. Wolff, W. Hu, C. Verfaillie, et al. 1997. Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. *J. Clin. Oncol.* 15:433–444.
- Porter, D.L., R.H. Collins, Jr., C. Hardy, N.A. Kernan, W.R. Drobyski, S. Giralt, M.E. Flowers, J. Casper, A. Leahy, P. Parker, et al. 2000. Treatment of relapsed leukemia after unrelated donor marrow transplantation with unrelated donor leukocyte infusions. *Blood*. 95:1214–1221.
- Falkenburg, J.H., A.R. Wafelman, P. Joosten, W.M. Smit, C.A. van Bergen, R. Bongaerts, E. Lurvink, M. van der Hoorn, P. Kluck, J.E. Landegent, et al. 1999. Complete remission of accelerated phase chronic myeloid leukemia by treatment with leukemia-reactive cytotoxic T lymphocytes. *Blood*. 94:1201–1208.
- Dembic, Z., W. Haas, S. Weiss, J. McCubrey, H. Kiefer, H. von Boehmer, and M. Steinmetz. 1986. Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature*. 320:232–238.
- Clay, T.M., M.C. Custer, J. Sachs, P. Hwu, S.A. Rosenberg, and M.I. Nishimura. 1999. Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. *J. Immunol.* 163:507–513.
- Fujio, K., Y. Misaki, K. Setoguchi, S. Morita, K. Kawahata, I. Kato, T. Nosaka, K. Yamamoto, and T. Kitamura. 2000. Functional reconstitution of class II MHC-restricted T cell immunity mediated by retroviral transfer of the alpha beta TCR complex. *J. Immunol.* 165:528–532.
- Calogero, A., G.A. Hospers, K.M. Kruse, P.I. Schrier, N.H. Mulder, E. Hooijberg, and L.F. de Leij. 2000. Retargeting of a T cell line by anti MAGE-3/HLA-A2 alpha beta TCR gene transfer. *Anticancer Res.* 20:1793–1799.
- Heemskerk, M.H., R.A. de Paus, E.G. Lurvink, F. Koning, A. Mulder, R. Willemze, J.J. van Rood, and J.H. Falkenburg. 2001. Dual HLA class I and class II restricted recognition of alloreactive T lymphocytes mediated by a single T cell receptor complex. *Proc. Natl. Acad. Sci. USA*. 98:6806–6811.
- Orentas, R.J., S.J. Roskopf, G.P. Nolan, and M.I. Nishimura. 2001. Retroviral transduction of a T cell receptor specific for an Epstein-Barr virus-encoded peptide. *Clin. Immunol.* 98:220–228.
- Stanislowski, T., R.H. Voss, C. Lotz, E. Sadovnikova, R.A. Willemsen, J. Kuball, T. Ruppert, R.L. Bolhuis, C.J. Melief, C. Huber, et al. 2001. Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. *Nat. Immunol.* 2:962–970.
- Rubinstein, M.P., A.N. Kadima, M.L. Salem, C.L. Nguyen, W.E. Gillanders, M.I. Nishimura, and D.J. Cole. 2003. Transfer of TCR genes into mature T cells is accompanied by the maintenance of parental T cell avidity. *J. Immunol.* 170:1209–1217.
- Schaft, N., R.A. Willemsen, J. De Vries, B. Lankiewicz, B.W. Essers, J.W. Gratama, C.G. Figdor, R.L. Bolhuis, R. Debets, and G.J. Adema. 2003. Peptide fine specificity of anti-glycoprotein 100 CTL is preserved following transfer of

- engineered TCR alpha beta genes into primary human T lymphocytes. *J. Immunol.* 170:2186–2194.
14. Kessels, H.W., M.C. Wolkers, M.D. van den Boom, M.A. van der Valk, and T.N. Schumacher. 2001. Immunotherapy through TCR gene transfer. *Nat. Immunol.* 2:957–961.
  15. Falkenburg, J.H., W.M. Smit, and R. Willemze. 1997. Cytotoxic T-lymphocyte (CTL) responses against acute or chronic myeloid leukemia. *Immunol. Rev.* 157:223–230.
  16. de Bueger, M., A. Bakker, J.J. van Rood, F. Van der Woude, and E. Goulmy. 1992. Tissue distribution of human minor histocompatibility antigens. Ubiquitous versus restricted tissue distribution indicates heterogeneity among human cytotoxic T lymphocyte-defined non-MHC antigens. *J. Immunol.* 149:1788–1794.
  17. Marijt, W.A., M.H. Heemskerk, F.M. Kloosterboer, E. Goulmy, M.G. Kester, M.A. van der Hoorn, S.A. van Luxemburg-Heys, M. Hoogeboom, T. Mutis, J.W. Drijfhout, et al. 2003. Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proc. Natl. Acad. Sci. USA.* 100:2742–2747.
  18. van Els, C.A., J. D’Amaro, J. Pool, E. Blokland, A. Bakker, P.J. van Elsen, J.J. van Rood, and E. Goulmy. 1992. Immunogenetics of human minor histocompatibility antigens: their polymorphism and immunodominance. *Immunogenetics.* 35:161–165.
  19. Munz, C., R. Obst, W. Osen, S. Stevanovic, and H.G. Rammensee. 1999. Alloreactivity as a source of high avidity peptide-specific human CTL. *J. Immunol.* 162:25–34.
  20. Amrolia, P.J., S.D. Reid, L. Gao, B. Schultheis, G. Dotti, M.K. Brenner, J.V. Melo, J.M. Goldman, and H.J. Stauss. 2003. Allorestricted cytotoxic T cells specific for human CD45 show potent antileukemic activity. *Blood.* 101:1007–1014.
  21. Gao, L., I. Bellantuono, A. Elsasser, S.B. Marley, M.Y. Gordon, J.M. Goldman, and H.J. Stauss. 2000. Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. *Blood.* 95:2198–2203.
  22. Sadovnikova, E., L.A. Jopling, K.S. Soo, and H.J. Stauss. 1998. Generation of human tumor-reactive cytotoxic T cells against peptides presented by non-self HLA class I molecules. *Eur. J. Immunol.* 28:193–200.
  23. Mutis, T., E. Blokland, M. Kester, E. Schrama, and E. Goulmy. 2002. Generation of minor histocompatibility antigen HA-1-specific cytotoxic T cells restricted by nonself HLA molecules: a potential strategy to treat relapsed leukemia after HLA-mismatched stem cell transplantation. *Blood.* 100:547–552.
  24. Mutis, T., G. Gillespie, E. Schrama, J.H. Falkenburg, P. Moss, and E. Goulmy. 1999. Tetrameric HLA class I-minor histocompatibility antigen peptide complexes demonstrate minor histocompatibility antigen-specific cytotoxic T lymphocytes in patients with graft-versus-host disease. *Nat. Med.* 5:839–842.
  25. Arden, B., S.P. Clark, D. Kabelitz, and T.W. Mak. 1995. Human T-cell receptor variable gene segment families. *Immunogenetics.* 42:455–500.
  26. Kinsella, T.M., and G.P. Nolan. 1996. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum. Gene Ther.* 7:1405–1413.
  27. Heemskerk, M.H., B. Blom, G. Nolan, A.P. Stegmann, A.Q. Bakker, K. Weijer, P.C. Res, and H. Spits. 1997. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *J. Exp. Med.* 186:1597–1602.
  28. Ruggieri, L., A. Aiuti, M. Salomoni, E. Zappone, G. Ferrari, and C. Bordignon. 1997. Cell-surface marking of CD(34+)-restricted phenotypes of human hematopoietic progenitor cells by retrovirus-mediated gene transfer. *Hum. Gene Ther.* 8:1611–1623.
  29. Hanenberg, H., X.L. Xiao, D. Dilloo, K. Hashino, I. Kato, and D.A. Williams. 1996. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat. Med.* 2:876–882.
  30. Burrows, S.R., N. Kienzle, A. Winterhalter, M. Bharadwaj, J.D. Altman, and A. Brooks. 2000. Peptide-MHC class I tetrameric complexes display exquisite ligand specificity. *J. Immunol.* 165:6229–6234.
  31. Smit, W.M., M. Rijnbeek, C.A. van Bergen, W.E. Fibbe, R. Willemze, and J.H. Falkenburg. 1998. T cells recognizing leukemic CD34(+) progenitor cells mediate the antileukemic effect of donor lymphocyte infusions for relapsed chronic myeloid leukemia after allogeneic stem cell transplantation. *Proc. Natl. Acad. Sci. USA.* 95:10152–10157.
  32. van der Hoorn, M.A., S.A. van Luxemburg-Heijs, C.A. van Bergen, R. Bongaerts, R. Willemze, and J.H. Falkenburg. 2003. The progenitor cell inhibition assay to measure the anti-leukemic reactivity of T cell clones against acute and chronic myeloid leukemia. *Methods.* 31:113–119.