Genetic engineering of virus-specific T cells with T-cell receptors recognizing minor histocompatibility antigens for clinical application

Marieke Griffioen,[1] H.M. Esther van Egmond,[1] Helen Barnby-Porritt,[2] Menno A.W.G. van der Hoorn,[1] Renate S. Hagedoorn,[1] Michel G.D. Kester,[1] Nikolai Schwabe,[2] Roel Willemze,[1] J.H. Frederik Falkenburg,[1] and Mirjam H.M. Heemskerk[1]

[1]Laboratory of Experimental Hematology, Leiden University Medical Center, Leiden, The Netherlands and [2]ProImmune Ltd., The Magdalen Centre, Oxford Science Park, Oxford, UK

ABSTRACT

Background

Donor lymphocyte infusion is an effective form of adoptive immunotherapy for hematologic malignancies after allogeneic stem cell transplantation. Graft-versus-host disease, however, often develops due to recognition of ubiquitously-expressed minor histocompatibility antigens. Transfer of T-cell receptors recognizing hematopoiesis-restricted minor histocompatibility antigens to virus-specific T cells may be a powerful anti-tumor therapy with a low risk of graft-versus-host disease. The purpose of this study was to develop an optimal T-cell receptors-encoding multi-cistronic retroviral vector and an efficient method for generating T-cell receptors-engineered virus-specific T cells.

Design and Methods

Retroviral vectors encoding the T-cell receptors for the hematopoiesis-restricted minor histocompatibility antigen HA-2 with and without selection markers were compared for T-cell receptors surface expression and HA-2-specific lysis. In addition, two different methods, i.e. peptide stimulation of $CD8^+$ cells and Pro5® MHC pentamer-based isolation of antigen-specific T cells, were investigated for their efficiency to generate T-cell receptors-transduced virus-specific T cells.

Results

Bi-cistronic vectors without selection markers most efficiently mediated T-cell receptors surface expression and HA-2-specific lysis. Furthermore, both methods were useful for generating gene-modified cells, but the purity of virus-specific T cells was higher after pentamer isolation. Finally, the capacity of gene-modified cells to express the transgenic T-cell receptors at the cell surface markedly differed between virus-specific T cells and was correlated with lysis of relevant target cells.

Conclusions

Our data support T-cell receptors gene transfer to pentamer-isolated virus-specific T cells using bi-cistronic retroviral vectors and illustrate the relevance of selection of gene-modified T cells with appropriate transgenic T-cell receptors surface expression for clinical gene therapy.

Key words: allogeneic stem cell transplantation, immunotherapy, gene therapy, T cell receptor.

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Correspondence:
Marieke Griffioen, Department of Hematology, C2-R, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands.
E-mail: m.griffioen@lumc.nl

Introduction

Patients with relapsed hematologic malignancies after allogeneic stem cell transplantion (SCT) can be successfully treated with donor lymphocyte infusions (DLI).[1-3] The graft-versus-leukemia (GvL) effect of DLI is mediated by T cells of donor origin that recognize minor histocompatibility antigens (mHag) on malignant cells. GvL reactivity is, however, often accompanied by graft-versus-host disease (GvHD) due to recognition of ubiquitously-expressed mHag. Several HLA class I-associated mHag have been identified as targets for $CD8^+$ T cells in patients who entered complete remission after treatment with allogeneic SCT and DLI.[4-6] In some patients, the appearance of $CD8^+$ T cells recognizing hematopoiesis-restricted mHag HA-1 and HA-2 was associated with anti-tumor responses with only mild GvHD[4], indicating that specific treatment with T cells directed against these mHag may selectively induce GvL reactivity without GvHD.

T-cell receptor (TCR) gene transfer is an attractive strategy for rapid *in vitro* generation of high numbers of antigen-specific T cells.[7] Functional T cells with redirected anti-leukemic reactivity have been generated by HA-1 or HA-2 TCR gene transfer to donor lymphocytes.[8,9] In contrast to unmodified DLI, early administration of cytomegalovirus (CMV)-specific T cells after allogeneic SCT does not lead to the development of GvHD and, therefore, genetic engineering of donor T cells recognizing viral antigens with TCR specific for hematopoiesis-restricted mHag are not expected to induce GvHD. We previously demonstrated that retroviral gene transfer of the HLA-A*0201 restricted HA-2 TCR to CMV-specific T cells isolated from HLA-A*0201+ and HA-2- donors or HLA-A*0201- donors led to the successful generation of anti-leukemic T cells with dual specificity for CMV pp65 as well as HA-2, whereas non-hematopoietic cells were not recognized.[3]

Since HA-1 (59-74%) and HA-2 (70-95%) are frequently expressed in various ethnic populations,[11] the numbers of $mHag^+$ patients who can be treated with TCR-transduced virus-specific T cells from mHag- donors after HLA-matched allogeneic SCT will be limited. Based on the minimized GvHD potential, however, adoptive transfer of TCR-modified virus-specific T cells also allows treatment of $mHag^+$ patients after HLA-mismatched allogeneic SCT. Other advantages of TCR gene transfer to virus-specific T cells are the reduced risk of undesired reactivities caused by chimeric TCR due to the limited TCR repertoire of virus-specific T cells, and selective transduction of T cells with memory or effector phenotypes, but not regulatory or anergic T cells with suppressive properties.

T cells recognizing CMV and Epstein-Barr virus (EBV) antigens are ideal target cells for TCR gene transfer because of the high frequencies of these T cells in the majority of human individuals due to latent persistence of CMV (~50%) and EBV (~90%). Moreover, it can be speculated that CMV- and EBV-specific T cells are frequently triggered via endogenous TCR due to persistent antigen presentation and may, therefore, show prolonged survival *in vivo*. The *in vivo* survival and efficacy of CMV- and EBV-specific T cells have been demonstrated by a number of human studies using cellular immunotherapy to treat CMV infection or lymphoproliferative disease after allogeneic SCT or solid organ transplantation.[12-14]

Recently, patients with advanced melanoma have been treated by adoptive transfer of lymphocytes genetically modified with the TCR for MART-1/Melan-A.[15] Although the clinical response rate was low and optimization of the treatment regime to increase clinical efficacy is still required, the results support the therapeutic potential of TCR gene-modified lymphocytes as an anti-tumor treatment. Clinical application of virus-specific T cells transferred with TCR for hematopoiesis-restricted mHag requires development of an optimal TCR-encoding multicistronic retroviral vector and an efficient method to generate TCR-modified virus-specific T cells. In this study, retroviral vectors were constructed containing the AV15 and BV18 genes of the HA-2 TCR linked by an internal ribosomal entry site (IRES) or 2A-like sequence derived from insect virus *Thosea asigna* (T2A). The IRES mediates internal entry of the ribosome upstream of a translation initiation codon, and allows coordinate expression of multiple transgenes by a single retroviral transcript, whereas the T2A sequence needs to be translated in the same open reading frame as that of as the TCR α and β chains for stoichiometric expression by protein cleavage.[16] IRES elements are much larger than 2A peptides and cleavage of 2A peptides, in contrast to IRES-dependent transcription, does not appear to be influenced by external factors. Moreover, since multiple IRES elements may lead to competition for translation factors and/or homologous recombination, the use of a 2A peptide for linkage of the TCR α and β genes allows additional inclusion of the human nerve growth factor receptor (NGFR) selection marker gene by an IRES element. The various HA-2 TCR-encoding retroviral vectors were introduced into human T cells and compared for TCR surface expression and HA-2 specific lysis to select an optimal vector for clinical use. In addition, we explored two different methods, i.e. peptide stimulation of isolated $CD8^+$ cells and Pro5® MHC pentamer-based isolation of antigen-specific T cells, for their efficiency to generate TCR-engineered virus-specific T cells.

Design and Methods

Cell culture

Peripheral blood mononuclear cells (PBMC) were collected from healthy individuals containing serum antibodies against CMV and EBV after approval by the Leiden University Medical Center institutional review board and informed consent according to the Declaration of Helsinki. PBMC were isolated by Ficoll gradient centrifugation, and frozen in liquid nitrogen. Patient RZ is an HLA-A*0201^+ and $HA\text{-}2^+$ subject with

chronic myeloid leukemia transplanted from an HLA-matched HA-2$^-$ sibling donor Z.[4] Phytohemagglutinin-stimulated T cell blasts (PHA-blasts) were cultured in Iscove's modified Dulbecco's medium (IMDM, Cambrex, Verviers, Belgium) with 5% fetal bovine serum (FBS), 5% human ABO serum and 100 IU/mL of interleukin-2 (IL-2, Chiron, Amsterdam, The Netherlands). The TCRα/β-deficient Jurkat T-cell clone 76[9] was cultured in IMDM with 8% FBS.

Construction of HA-2 TCR-encoding retroviral vectors

The genes encoding enhanced green fluorescent protein (EGFP) and woodchuck hepatitis response element were removed from MP71-EGFP-bWPRE[17] and a new multiple cloning site was inserted. A fragment comprising the human NGFR gene linked to an IRES was obtained from LZRS-IRES-NGFR[8,10] and inserted into the new multiple cloning site, leading to MP71-IRES-NGFR. MP71-AV15-T2A-BV18-IRES-NGFR was constructed by inserting AV15-T2A-BV18 into MP71-IRES-NGFR. AV15-T2A-BV18, containing the T2A sequence,[16] was obtained by two-step polymerase chain reactions (PCR). AV15 sequences were amplified from LZRS-AV15-IRES-EGFP[8,10] using EcoRI-AV15-fw and T2A-AV15-rv primers and BV18 sequences were amplified from LZRS-BV18-IRES-NGFR[8,10] using T2A-BV18-fw and XhoI-BV18-rv primers (primers are shown in Table 1). AV15-T2A-BV18 was amplified from a mixture of AV15 and BV18 fragments using EcoRI-AV15-fw and XhoI-BV18-rv primers. MP71-AV15-T2A-BV18 was obtained by removing IRES-NGFR from MP71-AV15-T2A-BV18-IRES-NGFR. MP71-AV15-IRES-BV18 was generated by replacing IRES-NGFR from MP71-AV15-IRES-NGFR with IRES-BV18. MP71-AV15-IRES-NGFR was constructed by inserting the AV15 cDNA from LZRS-AV15-IRES-EGFP into MP71-IRES-NGFR. IRES-BV18 was obtained by two-step PCR. IRES sequences were amplified from MP71-IRES-NGFR using XhoI-IRES-fw and BV18-IRES-rv primers and BV18 sequences were amplified from MP71-AV15-T2A-BV18 using IRES-BV18-fw and NotI-BV18-rv primers. IRES-BV18 was amplified from a mixture of IRES and BV18 fragments using XhoI-IRES-fw and NotI-BV18-rv primers. PCR reactions were performed using a program of 2 min at 95°C, 25 cycles of 30s at 95°C, 1 min at 55°C, 1 min at 72°C, and 7 min at 72°C. HA-2 TCR fragments were also cloned into the LZRS vector. LZRS-AV15-T2A-BV18-IRES-NGFR was constructed by cloning AV15-T2A-BV18 from MP71-AV15-T2A-BV18-IRES-NGFR into LZRS-IRES-NGFR. LZRS-AV15-T2A-BV18 was constructed by removing IRES-NGFR. LZRS-AV15-IRES-BV18 was constructed by cloning IRES-BV18 from MP71-AV15-IRES-BV18 into LZRS-AV15-IRES-EGFP (after removal of IRES-EGFP). All constructs were confirmed by DNA sequencing.

Table 1. Primers for the construction of HA-2 TCR-encoding retroviral vectors.

Primer	*Sequence (5'-3')*
EcoRI-AV15-fw	TAGAGAATTCACCATGAAGACATTTGCTGGATTTT[a]
T2A-AV15-rv	CCACGTCACCGCATGTTAGAAGACTTCCTCTGCCCTCGCTGGACC ACACCGCAGCGT[b,c]
T2A-BV18-fw	TCTAACATGCGGTGACGTGGAGGAGAATCCCGGCCCTATG GACACCAGACTACTCTGCTG[b]
XhoI-BV18-rv	TATACCGCTCGAGCTAGCCTCTGGAATCCTTTCTCTT[d]
XhoI-IRES-fw	GGCCTCGAGAAATTCCGCCCCTCTCCCTC[d]
BV18-IRES-rv	CAGAGTAGCCTGGTGTCCATGGTGCCCATATTATCATCGTGTTTTT-[e]
IRES-BV18-fw	AAAAACACGATGATAATATGGGCACCATGGACACCAGGCTACTCTG[f]
NotI-BV18-rv	TATACCGGCGGCCGCCTAGCCTCTGGAATCCTTTCTCTT[g]

The following sequences are underlined: [a]EcoRI site; [b]T2A sequences; [c]removal of the stop codon of the TCR AV15 gene; [d]XhoI site; [e]TCR BV18 sequences; [f]IRES sequences; [g]NotI site.

Production of MP71 and LZRS retroviral supernatants

Packaging Φ-NX-A cells were seeded the day before transfection at a density of 3×10^6 cells per 75 cm^3 tissue culture flask in IMDM with 8% FBS. Cells were transfected with 10 mg LZRS vector or 10 μg MP71 vector and 10 μg M57 plasmid encoding gag and pol by the calcium phosphate precipitation method (Invitrogen, Breda, The Netherlands). Two days after transfection, MP71 vector-containing supernatants were harvested, and frozen at -80°C. Φ-NX-A cells transfected with LZRS vectors were seeded in 175 cm^3 tissue culture flasks in IMDM with 8% FBS and 2 mg/mL puromycin (CLONTECH Laboratories, Mountain View, CA, USA). Puromycin-resistant cells were seeded at a density of 20×10^6 cells per 175 cm^3 tissue culture flask. Supernatants were harvested after 48 h, and frozen at -80°C.

Tetramer staining and flow cytometry

Phycoerythrin (PE)- and allophycocyanin (APC)-conjugated CMV and EBV tetramers were constructed as previously described[18] with minor modifications. Tetramers were constructed for HLA-A*0101 CMV pp50 VTEHDTLLY (pp50-VTE) and pp65 YSEHPTFTSQY (pp65-YSE), HLA-A*0201 CMV pp65 NLVPMVATV (pp65-NLV), HLA-B*0702 CMV pp65 TPRVTGGGAM (pp65-TPR) and RPHERNGFTVL (pp65-RPH) and HLA-B*0801 EBV BZLF1 RAKFKQLL (BZLF1-RAK), EBNA3A FLRGRAYGL (EBNA3A-FLR) and QAKWRLQTL (EBNA3A-QAK) and CMV IE1 ELRRKMMYM (IE1-ELR) and QIKVRVDMV (IE1-QIK).[19-21] Cells were labeled with tetramers for 2 h at 4°C in RPMI 1640 without phenol with 2% FBS. During the last 30 min, fluorescent isothiocyanate (FITC)- or PE-labeled antibodies against CD8 or NGFR were added. For each tetramer, T cells were demonstrated to specifically lyse peptide-pulsed target cells in ^{51}Cr-release assays (*data not shown*).

Retroviral transduction of peptide-stimulated CD8$^+$ cells

CD8$^+$ cells were isolated from PBMC using magnet-

ic beads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) and incubated in T-cell medium (IMDM with 10% human ABO serum and 50 IU/mL of IL-2) containing viral peptides (each at 1 μg/mL), as described for tetramer construction. Peptide-stimulated CD8$^+$ cells were harvested on day 3, and seeded at a density of 1×10^6 cells/well in non-tissue culture-treated 24-well plates (Greiner Bio-One, Longwood, FL, USA) coated with fibronectin CH-296 (Takara Shuzo, Otsu, Shiga, Japan) for retroviral transduction.[8,10] After incubation overnight with retroviral supernatants, cells were resuspended in T-cell medium, and transferred to 24-well tissue culture plates. The T-cell medium was refreshed every 2-3 days.

Retroviral transduction of Pro5® MHC pentamer-isolated antigen-specific T cells

PBMC (10-50×10^6 cells) were washed with washing buffer (PBS with 0.1% sodium azide and 0.1% bovine serum albumin), and incubated with 2 μL biotin-labeled CMV- or EBV- specific Pro5® MHC Pentamers (ProImmune Limited, Oxford, United Kingdom) in 100 μL washing buffer for 50 min at 4°C. After washing, cells were incubated with 10 μL streptavidin or anti-biotin beads (Miltenyi Biotec) in 90 μL washing buffer for 20 min at 4°C, and isolated according to the manufacturer's instructions. Following pentamer isolation, no virus-specific T cells were detected in the negative fractions after staining with streptavidin-PE or -APC. Pentamer-isolated cells were transduced on day 3 in 96-well plates as described for peptide-stimulated CD8$^+$ cells. HA-2 TCR-transduced pentamer-isolated cells were restimulated on days 8-10 with peptide-pulsed irradiated autologous PBMC. T-cell medium was refreshed every 2-3 days.

^{51}Cr-release assays

Target cells were labeled with 50 μCi $Na_2{}^{51}CrO_4$ for 60 min at 37°C and washed either directly or after pulsing with peptides for 1 h at 37°C. Target cells were seeded in 96-well U-bottomed plates together with effector cells at different ratios in 150 μL IMDM with 10% FBS. After 4 h of incubation, 25 μL of supernatant were harvested and measured in a luminescence counter (Topcount-NXT; Packard Instrument Co.). The mean percentage of specific lysis of triplicate wells was calculated according to the following formula: [(experimental release – spontaneous release)/(maximal release – spontaneous release)] x 100.

Results

Comparison of HA-2 TCR-encoding multi-cistronic retroviral vectors

Various multi-cistronic LZRS and MP71 retroviral vectors were constructed containing the AV15 and BV18 chains of the HLA-A*0201 restricted HA-2 TCR linked by an IRES or T2A element with or without the human NGFR selection marker gene (Figure 1) and introduced into Jurkat T cells lacking endogenous expression of TCR α and β chains. High TCR surface

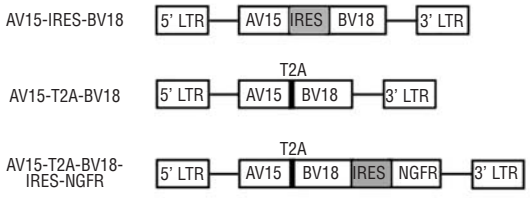

Figure 1. Schematic drawing of HA-2 TCR-encoding multi-cistronic retroviral vectors. LZRS and MP71 retroviral vectors contain the AV15 and BV18 genes of the HA-2 TCR linked by an internal ribosomal entry site (IRES) or 2A-like sequence derived from insect virus *Thosea asigna* (T2A). In addition, LZRS and MP71 vectors were constructed with AV15 and BV18 genes linked by T2A as well as the human nerve growth factor receptor (NGFR) selection marker gene linked by an IRES.

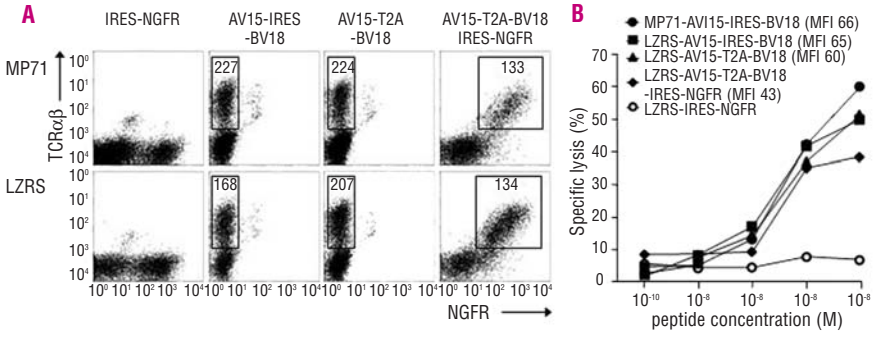

Figure 2. Comparison of HA-2 TCR-encoding multi-cistronic retroviral vectors. (A) TCRα/β-deficient Jurkat T cells were transduced with various HA-2 TCR-encoding MP71 (upper) and LZRS (lower) retroviral vectors. Four days after transduction, Jurkat-T cells were analyzed by flow cytometry. The mean fluorescent intensities (MFI) of anti-TCRαβ-PECy5 staining are shown. (B) CD8$^+$ cells were isolated from donor CBS, and stimulated with a mixture of CMV and EBV peptides (pp50-VTE, IE1-ELR, IE1-QIK, BZLF1-RAK, EBNA3A-FLR). Peptide-stimulated CD8$^+$ cells were transduced with various HA-2 TCR-encoding vectors and tested against EBV-transformed B lymphoblastoid cells from donor Z (HLA-A*0201$^+$ HA-2$^-$) pulsed with titrated HA-2 peptide concentrations on day 9 in 4-h ^{51}Cr release assays. The MFI of HA-2 tetramer staining and percentages of specific lysis at effector:target ratios of 10:1 are shown. Bi-cistronic retroviral vectors without the NGFR selection marker mediated TCR surface expression and HA-2 specific lysis most efficiently.

expression (mean fluorescence intensity: 168-227) was observed in TCRα/β-deficient Jurkat T cells transduced with MP71 and LZRS vectors containing AV15 and BV18 linked by an IRES or T2A element without the selection marker gene (Figure 2A). TCR surface expression was slightly reduced (MFI, 134-136) upon linkage of the NGFR gene. No difference in TCR surface expression was noted between LZRS and MP71 vectors.

The multi-cistronic retroviral vectors were also compared for HA-2-specific lysis after transfer to virus-specific T cells. $CD8^+$ cells were isolated from a healthy individual and stimulated with a mixture of EBV and CMV peptides. Peptide-stimulated $CD8^+$ cells were transduced with TCR-encoding retroviral vectors on day 3, and analyzed for cytolytic activity against EBV-transformed B lymphoblastoid cells from donor Z (HLA-A*0201$^+$ HA-2$^-$) pulsed with titrated HA-2 peptide concentrations on day 9 in a 4-h ^{51}Cr-release assay (Figure 2B). Transfer of TCR-encoding vectors without the selection marker gene led to similar levels of HA-2-specific lysis. Slightly reduced, but still significant HA-2-specific lysis was recorded when the NGFR gene was included, whereas virus-specific T cells transduced with empty vector did not mediate HA-2-specific lysis. As in TCRα/β-deficient Jurkat T cells, HA-2 TCR surface expression after transfer of TCR-encoding retroviral vectors with NGFR gene (MFI, 43) was lower than that after transfer of vectors without the NGFR gene (MFI, 60-66). These data demonstrate that bi-cistronic retroviral vectors containing the HA-2 TCR α and β chains without the selection marker gene mediated TCR surface expression and HA-2-specific lysis more efficiently and may, therefore, be preferable for clinical applications.

Generation of HA-2 TCR-transduced virus-specific T cells

T-cell lines with multiple distinct virus specificities were generated by stimulating isolated $CD8^+$ cells with a mixture of CMV and EBV peptides. $CD8^+$ cells were transduced with a retroviral vector encoding the HA-2 TCR as well as NGFR selection marker (LZRS-AV15-T2A-BV18-IRES-NGFR) on day 3, and analyzed for virus tetramer$^+$ T cells by flow cytometry and lytic activity in 4-h ^{51}Cr-release assays on days 8-12 (Figure 3). Despite variation in the expansion of T cells with different specificities (Figure 3A), all peptide-stimulated $CD8^+$ cells contained significant cumulative percentages of virus-specific T cells (22-80%, Figure 3B) as well as NGFR$^+$ cells (10-37%, Figure 3C). The percentages of virus-tetramer$^+$ T cells correlated with specific

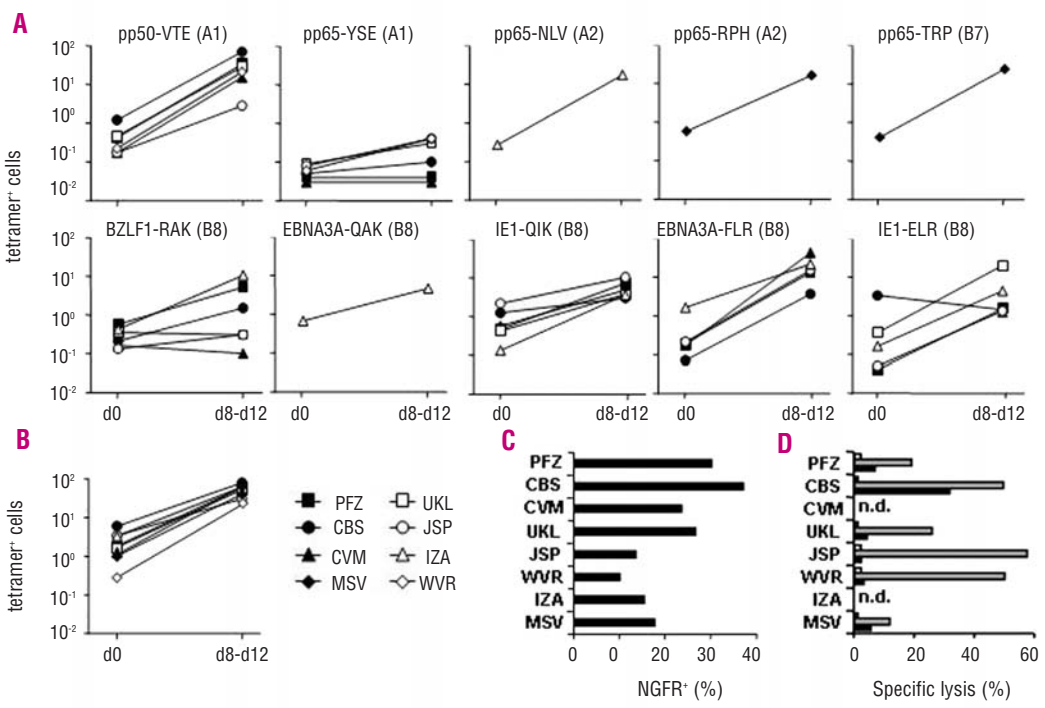

Figure 3. HA-2 TCR gene transfer to peptide stimulated $CD8^+$ cells. $CD8^+$ cells were stimulated with CMV and EBV peptides, and transduced with LZRS-AV15-T2A-BV18-IRES-NGFR. (A) Frequencies of virus-tetramer$^+$ T cells in non-stimulated PBMC (day 0) and peptide-stimulated $CD8^+$ cells (days 8-12) are shown for donors PFZ, CBS, CVM, UKL and JSP (HLA-A*0101$^+$ HLA-B*0801$^+$ HA-2$^+$), WVR (HLA-A*0101$^+$ HA-2$^+$), IZA (HLA*0201$^+$ HLA-B*0801$^+$ HA-2$^-$) and MSV (HLA-B*0702$^+$ HA-2$^+$). (B) Cumulative frequencies of virus-tetramer$^+$ T cells are shown for non-stimulated PBMC (0.3-8.6%) and peptide-stimulated $CD8^+$ cells (22-80%). Peptide-stimulated $CD8^+$ cells contained high cumulative numbers of T cells with multiple distinct virus specificities. (C) Percentages of NGFR$^+$ cells are shown. Peptide-stimulated $CD8^+$ cells were efficiently transduced with retroviral vectors. (D) TCR-transduced peptide-stimulated $CD8^+$ cells were tested against non-pulsed (open bars) and HA-2 peptide-pulsed (gray bars) PHA-blasts from donor Z (HLA-A*0201$^+$ HA-2$^-$) and patient RZ (HLA-A*0201$^+$ HA-2$^+$) (closed bars) in 4-h ^{51}Cr-release assays at an effector: target ratio of 10:1. Percentages of specific lysis are shown (n.d.: not determined). TCR-transduced peptide-stimulated $CD8^+$ cells specifically lysed peptide-pulsed target cells, but lysis of target cells with endogenous HA-2 expression was often low.

lysis of autologous PHA-blasts pulsed with viral peptides (*data not shown*). Moreover, all HA-2 TCR-transduced peptide-stimulated CD8$^+$ cells were shown to specifically lyse HA-2 peptide-pulsed, but not non-pulsed, PHA-blasts from donor Z (HLA-A*0201$^+$ HA-2$^-$). Lysis of PHA-blasts from patient RZ (HLA-A*0201$^+$ HA-2$^+$) with endogenous HA-2 expression was, however, often low (Figure 3D).

In addition, gene-modified virus-specific T cells were generated by isolating T cells with specificity for a single viral antigen using pentamer-coated magnetic beads. Pro5® MHC pentamers contain five MHC-peptide complexes that are multimerized by a self-assembling coiled-coil domain, and biotinylated pentamers therefore allow isolation of antigen-specific T cells by streptavidin or anti-biotin beads. PBMC from healthy carriers were incubated with biotinylated CMV or EBV pentamers followed by isolation using streptavidin or anti-biotin beads. Pentamer-isolated cells were transduced with mock or HA-2 TCR-encoding LZRS vectors on day 3 and analyzed for tetramer$^+$ T cells by flow cytometry and cytolytic activity in ^{51}Cr-release assays on days 8-12 or 18-22 after a single restimulation with irradiated autologous PBMC pulsed with viral peptides. Table 2 shows that pentamer isolation, in the absence of any additional exogenous stimulus, led to highly-purified virus-specific T cells (>85%) with significant transduction efficiencies (20-53% NGFR$^+$ cells). Despite similar transduction efficiencies, variable surface expression of the transgenic HA-2 TCR, as measured by tetramer staining, was observed on different virus-specific T cells (Table 3). As illustrated for representative donors CBS and CVO in Figure 4A, T cells specific for CMV pp50-VTE (donor CBS) and IE1-QIK (donor CVO) contained 20% and 25% of HA-2 tetramer$^+$ cells, respectively, whereas no tetramer staining was observed after transfer of the HA-2 TCR to EBNA3A-FLR specific T cells from both donors. Figure 4B shows that HA-2 tetramer$^+$ virus-specific T cells efficiently lysed PHA-blasts from patient RZ with endogenous HA-2 expression, whereas lysis of these target cells by HA-2 tetramer$^-$ virus-specific T cells could not be measured. Virus-specific T cells with and without HA-2 tetramer staining were both capable of lysing HA-2 peptide-pulsed target cells, although the cytolysis mediated by tetramer$^-$ cells was reduced as compared to that mediated by tetramer$^+$ cells. Finally, HA-2 tetramer$^+$ virus-specific T cells were shown to lyse HLA-A*0201$^+$ and HA-2$^+$ acute lymphoblastic leukemia cells, but not HLA-A*0201$^-$ acute lymphoblastic leukemia cells, as efficiently as a parental HA-2-specific cytotoxic T lymphocyte clone (Figure 4C).

In conclusion, we demonstrated the successful use of both peptide stimulation of isolated CD8$^+$ cells and pentamer-based isolation of antigen-specific T cells for generating gene-modified virus-specific T cells, although the purity of virus-specific T cells was higher after pentamer isolation. Furthermore, large differences in surface expression of the transgenic HA-2 TCR and lysis of HA-2$^+$ target cells were observed between virus-specific T cells, emphasizing the relevance of selection of virus-specific T cells with appropriate surface expression of the transgenic TCR for adoptive immunotherapy.

Discussion

TCR gene transfer to virus-specific T cells may be an effective adoptive anti-tumor therapy after allogeneic SCT, with a low risk of GvHD. In this study, various HA-2 TCR-encoding multi-cistronic retroviral vectors were compared for TCR surface expression and HA-2 specific lysis. In addition, we explored two different methods, i.e. peptide stimulation of isolated CD8$^+$ cells

Table 2. Gene-modified virus-specific T cells generated by Pro5® MHC pentamer-coated beads.

Donor	*Pentamer*[a]		*NGFR*[b]	*Virus tetramer*
PFZ	pp50-VTE	CMV-A1	29	95
	EBNA3A-FLR	EBV-B8	26	94
UKL	pp50-VTE	CMV-A1	35	95
JSC	pp65-TPR	CMV-B7	53	94
MSV	pp65-TPR	CMV-B7	37	96
JSP	pp50-VTE	CMV-A1	25	85
	EBNA3A-FLR	EBV-B8	29	88
CBS	pp50-VTE	CMV-A1	38	95
	EBNA3A-FLR	EBV-B8	20	90
CVO	EBNA3A-FLR	EBV-B8	22	91
	IE1-QIK	CMV-B8	39	97

[a]CMV- and EBV-specific T cells were isolated from PBMC (10×10^6 cells) by the indicated biotinylated pentamers and streptavidin or anti-biotin beads. [b]Retroviral transduction with LZRS-IRES-NGFR mock vector was performed on day 3 after isolation. On days 8-10, mock-transduced pentamer-isolated cells were stained with anti-NGFR antibodies and virus-tetramers. The percentages of cells that were NGFR$^+$ or virus-tetramer$^+$ are indicated.

Table 3. HA-2 TCR-engineered virus-specific T cells generated by Pro5® MHC pentamer-coated beads.

Donor	*Pentamer*[a]		*HA-2 tetramer*[b]
UKL	pp50-VTE	CMV-A1	28
	IE1-ELR	CMV-B8	11
	IE1-QIK	CMV-B8	1
	EBNA3A-FLR	EBV-B8	0
MRJ	pp65-NLV	CMV-A2	6
UGW	pp50-VTE	CMV-A1	11
	IE1-ELR	CMV-B8	11
	BZLF1-RAK	EBV-B8	0
CBS	pp50-VTE	CMV-A1	20
	EBNA3A-FLR	EBV-B8	0
CVO	pp50-VTE	CMV-A1	16
	IE1-ELR	CMV-B8	0
	IE1-QIK	CMV-B8	25
	BZLF1-RAK	EBV-B8	8
	EBNA3A-FLR	EBV-B8	0

[a]Virus-specific T cells were isolated from PBMC (10×10^6 cells) by pentamer-coated beads and transduced on day 3 with the HA-2 TCR-encoding vectors LZRS-AV15-T2A-BV18 or LZRS-AV15-IRES-BV18. In parallel, cells were transduced with LZRS-IRES-NGFR, showing percentages of NGFR$^+$ cells between 17-51%. [b]TCR-transduced pentamer-isolated cells were stained with HA-2 tetramers on days 8-10. The percentages of virus-specific T cells that were HA-2 tetramer$^+$ are indicated.

and Pro5® MHC pentamer-based isolation of antigen-specific T cells, and demonstrated that both methods efficiently generated gene-modified cells, but that the purity of virus-specific T cells was higher after pentamer isolation.

Comparison of multi-cistronic retroviral vectors revealed that transfer of vectors encoding the HA-2 TCR as well as the NGFR selection marker mediated slightly reduced TCR surface expression as compared to constructs without the selection marker gene. Since decreased expression of the transgenic TCR indicates a lower avidity and diminished functional activity of the T cell, TCR-encoding vectors without the selection marker gene may be preferable for clinical applications. No difference in TCR expression and HA-2 specific lysis was noted between the LZRS vector, which was used in our previous TCR gene transfer studies,[8,10] and the MP71 vector, which has been designed for increased safety in medical applications.[22] The data also showed comparable TCR expression and HA-2 specific lysis mediated by constructs containing the TCR α and β genes linked by an IRES or T2A element. The 590 bp IRES mediates internal entry of the ribosome upstream of a translation initation codon, and allows comparable expression of multiple transgenes by a single transcript. Although the IRES does not encode a known functional protein, the presence of multiple ATG start sites does not exclude formation of cryptic immunogenic peptides. The 54 bp T2A sequence needs to be translated in the same open read-

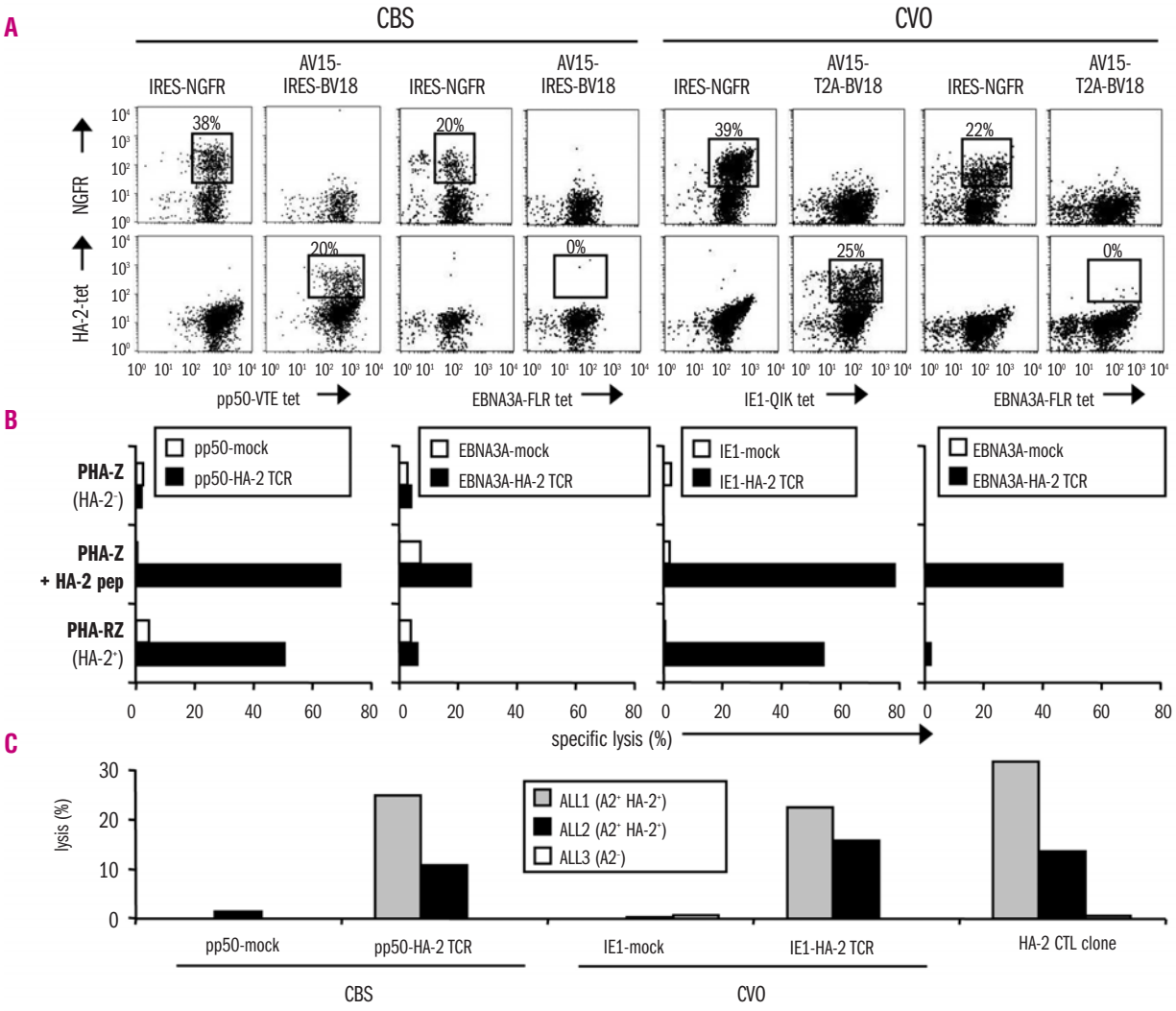

Figure 4. HA-2 TCR gene transfer to pentamer-isolated virus-specific T cells. (A) T cells specific for CMV pp50-VTE and EBV EBNA3A-FLR from donor CBS and T cells specific for CMV IE1-QIK and EBV EBNA3A-FLR from donor CVO were isolated using Pro5® MHC pentamer-coated beads. Pentamer-isolated cells were transduced with LZRS-AV15-IRES-BV18 or LZRS-AV15-T2A-BV18 on day 3, restimulated with peptide-pulsed irradiated autologous PBMC on days 8-10 and analyzed by flow cytometry on days 18-20. The percentages of cells that were NGFR$^+$ (upper) or HA-2 tetramer$^+$ (lower) are indicated. Virus-specific T cells markedly differed in capacity to express the transgenic HA-2 TCR at the cell surface. (B) Retrovirally-transduced pentamer-isolated cells were analyzed against non-pulsed and HA-2 peptide-pulsed PHA-blasts from donor Z (HLA-A*0201$^+$ HA-2$^-$) and patient RZ (HLA-A*0201$^+$ HA-2$^+$) in 4-h ^{51}Cr-release assays. Percentages of specific lysis at an effector:target ratio of 30:1 are shown. HA-2 tetramer$^+$ virus-specific T cells specifically lysed target cells with endogenous HA-2 expression. (C) Retrovirally-transduced pentamer-isolated cells as well as a parental HA-2 specific CTL clone were analyzed against HLA-A*0201$^+$ and HA-2$^+$ acute lymphoblastic leukaemia samples (ALL1 and ALL2) as well as HLA-A*0201- ALL cells in a 6-h ^{51}Cr-release assay. Percentages of specific lysis at an effector:target ratio of 10:1 are shown. HA-2 tetramer$^+$ virus-specific T cells specifically lysed primary leukemic cells.

ing frame as that of the TCR α and β chains to mediate coordinate expression by protein cleavage.[16] The T2A sequence does not contain ATG sites and, when inserted between AV15 and BV18 of the HA-2 TCR, does not contain peptides with predicted binding to common HLA alleles and may, therefore, be less immunogenic *in vivo*.

In a clinical X-SCID gene therapy trial, several patients developed lymphomas after treatment with autologous CD34$^+$ stem cells transduced with a retroviral vector encoding the common γ cytokine receptor subunit.[23] In contrast to hematopoietic stem cells, retroviral vector integration has no consequence on the biology and function of transplanted T cells, as demonstrated by long-term engraftment of donor lymphocytes genetically engineered with the suicide gene thymidine kinase of herpes simplex virus after allogeneic SCT.[24,25]

Peptide stimulation of isolated CD8$^+$ cells and pentamer-based isolation of antigen-specific T cells are both methods that can be used to obtain significant numbers of gene-modified virus-specific T cells. Pentamers allow isolation of T cells with specificity for a single viral antigen present at low frequencies, whereas stimulation of CD8$^+$ cells with a mixture of viral peptides requires high cumulative frequencies of virus-specific T cells with distinct specificities. Pentamer isolation demonstrated that virus-specific T cells markedly differ in capacity to express the transgenic TCR at the cell surface. We previously showed variable surface expression of the introduced HA-2 TCR in T cells expressing different endogenous TCR with the same CMV-pp65-NLV specificity.[26] In this previous study, CMV pp65-specific T cells that efficiently expressed the transgenic HA-2 TCR at the cell surface were shown to contain an endogenous TCR, which is poorly expressed after transfer to γδ T cells and, vice versa, CMV pp65-specific T cells with a poor capacity to express the HA-2 TCR at the cell surface displayed a TCR that is efficiently expressed on γδ T cells after retroviral transduction. These findings provide evidence that transgenic and endogenous TCR compete for cell surface expression in favor of the TCR-CD3 complex with the best pairing properties.

In the present study, we demonstrate that variable surface expression of the transgenic TCR is not restricted to CMV pp65-NLV specific T cells, but a broad phenomenon in T cells with different virus specificities. The data also show that surface expression of the introduced TCR is required for appropriate recognition of endogenous antigens on relevant target cells. Therefore, although stimulation of CD8$^+$ cells with a mixture of viral peptides leads to efficient expansion and transduction of virus-specific T cells with distinct specificities, lysis of target cells with endogenous mHag expression may be hardly measurable due to low overall numbers of virus-specific T cells with detectable surface expression of the transgenic TCR. To improve mHag specific lysis, virus-specific T cells with appropriate surface expression of the transgenic TCR may be purified after retroviral transduction or, alternatively, peptides or pentamers may be selected for preferential stimulation and transduction of permissive virus specific T cells that allow expression of the transgenic TCR at the cell surface.

In the present study, CMV- and EBV-specific T cells displayed different central memory or effector memory phenotypes, dependent on the epitope and individual tested (*data not shown*). It has recently been demonstrated that virus-specific T cells with central memory phenotypes persisted long-term *in vivo*, whereas effector memory T cells survived for only a short duration after adoptive transfer,[27] suggesting that mHag TCR-engineered virus-specific T cells with central memory phenotypes are preferable for clinical applications.

Recently, a clinical TCR gene transfer study was performed using peripheral blood lymphocytes retrovirally-transduced with the MART-1/Melan-A-specific TCR for adoptive transfer to patients with advanced melanoma after lymphodepleting pre-conditioning regimens.[15] Although the clinical response rate was low, with only two of 15 patients showing objective regression of metastatic melanoma lesions, high sustained levels of >20% of circulating gene-marked cells were observed in all patients. Numbers of circulating MART-1/Melan-A tetramer$^+$ T cells were, however, low, with >1% of tetramer$^+$ cells in only four of 15 patients, including the two patients with tumor regression.

Several approaches are currently under investigation to increase the efficiency of TCR surface expression after retroviral gene transfer, including increased transcription by selecting optimal retroviral vectors,[28] enhanced translation by codon optimization,[29] selection of TCR with higher affinities[30,31] and prevention of chain mispairing by modification of the TCR constant regions.[32,33] Our findings, however, indicate that adoptive transfer of lymphocytes selected for appropriate surface expression of the transgenic TCR, either by selection of permissive lymphocytes prior to retroviral transduction or by purification of lymphocytes with transgenic TCR surface expression after retroviral transduction, may be an effective alternative approach to improve the clinical efficacy of TCR gene therapy.

Authorship and Disclosures

MG designed and performed the research, analyzed the data and wrote the manuscript; HMEvE performed research and analyzed the data; HB-P wrote the manuscript; MAWGvdH, RSH, MGDK performed the research; NS, RW wrote the manuscript; JHFF designed research, analyzed the data and wrote the manuscript; MHMH designed research, analyzed the data and wrote the manuscript. All authors approved the final version of the manuscript for publication. The authors reported no potential conflicts of interest.

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