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Redirecting Human T Lymphocytes Toward Renal Cell Carcinoma Specificity by Retroviral Transfer of T Cell Receptor Genes

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ABSTRACT

Adoptive T cell therapy of renal cell carcinoma (RCC) is limited by the difficulty in generating sufficient numbers of RCC-reactive T cells in vitro. To circumvent this problem, we cloned T cell receptor (TCR) α and β chains from a tumor-infiltrating lymphocyte clone specific for an RCC tumor antigen and transferred the TCR into human T cell lines and primary T lymphocytes. Efficient TCR expression in primary T lymphocytes was obtained only with a mouse myeloproliferative sarcoma virus (MPSV)-based retroviral vector, not with a Moloney murine leukemia virus (MLV)-based vector, although both viral supernatants were similar in titer, as shown by analysis of copy number integration in transduced T cells. Reverse transcription-polymerase chain reaction analysis revealed a higher amount of TCR-encoding transcripts when T cells were transduced with the MPSV vector in comparison with the MLV vector, indicating that high TCR expression levels can be achieved by appropriate cis-regulatory vector elements. The biological activity of the transferred TCR was shown by specific lysis of RCC cells (51Cr release assay) and by interferon γ and tumor necrosis factor α release (enzyme-linked immunosorbent assay) in an antigen-specific and HLA-A*0201-restricted fashion. Comparison of the redirected T lymphocytes with the original tumor-infiltrating lymphocyte clone revealed similar killing and cytokine secretion capabilities. The functional activity of TCR-redirected T lymphocytes was stable over time. The results demonstrate that use of an optimized retroviral vector yielded a high TCR transduction efficiency and stable and high TCR expression in primary human T lymphocytes and redirected their specificity toward RCC cells.

OVERVIEW SUMMARY

The therapeutic efficacy of adoptively transferred cytotoxic T lymphocytes (CTLs) has been demonstrated in clinical trials. It is, however, limited by the difficulty in generating sufficient amounts of CTLs in vitro. Gene therapy approaches include the genetic engineering of T cell specificity by T cell receptor (TCR) gene transfer. It was demonstrated that the number of TCR molecules on the T cell is important for its function. An efficient transfer system that yields high transduction efficiency and strong and stable transgene expression is a prerequisite to achieve effector function for redirected T cells. We used an improved retroviral vector and transferred a renal cell carcinoma (RCC)-reactive TCR. The redirected peripheral blood lymphocytes of healthy donors gained specificity for an RCC tumor antigen. The reprogrammed T cells showed cytokine secretion and tumor cell killing similar to the original tumor-infiltrating lymphocyte clone.

INTRODUCTION

RENAL CELL CARCINOMA (RCC) is considered to be one of the few immunogenic tumor entities. In some patients, par-
nal or complete remission was observed after treatment with various forms of immunotherapy. Studies on allogeneic stem cell transplantation have shown partial success in treating metastatic RCC (Childs et al., 2000; Ueno et al., 2003). Adoptive T cell therapy has proved effective for the treatment of cytomegalovirus (CMV) disease, Epstein–Barr virus-positive (EBV+) lymphoma, and leukemia (Kolb et al., 1990; Rooney et al., 1995; Walter et al., 1995). Preliminary studies indicate that adoptive T cell therapy also has potential for melanoma treatment (Dudley et al., 2002). Treatment of other cancer entities via adoptive transfer is difficult for several reasons. The poor immunogenicity of many tumors is one reason, as it leads to difficulty in isolating tumor-reactive cytotoxic T lymphocytes (CTLs) from tumors. This occurs because of low numbers of tumor-infiltrating lymphocytes (TILs) or their poor state of reactivity (tolerogenic T cells). For adoptive transfer, TILs need to be amplified to large numbers, which is often not feasible. The genetic redirection of patient peripheral blood lymphocytes (PBLs) toward a desired reactivity is a promising approach to overcome these problems. The successful modification of PBL specificity through retroviral transfer of T cell receptor (TCR) or chimeric antibody receptor genes has been shown (reviewed in Eshhar, 1997; Hombach et al., 2002; Schumacher, 2002). With regard to human tumor-associated antigens, however, only TCRs recognizing melanoma or a minor histocompatibility antigen have so far been transferred into human PBLs (MART-1 [Clay et al., 1999], gp100 [Schaff et al., 2003], and mHag HA-2 [Heemskerk et al., 2003]). Here we describe the generation of RCC-specific CTLs by TCR α and β chain transfer. The TCR is derived from a cloned TIL line isolated from an RCC patient (Schendel et al., 1993). It recognizes a yet unknown RCC tumor antigen in an HLA-A*0201-restricted manner (Jantzer and Schendel, 1998). For transfer, the TCR was cloned into two different retroviral vectors: a Moloney murine leukemia virus (MLV) vector and a vector optimized for gene transfer into T lymphocytes (MP71) (Engels et al., 2003). The LXSN-based vector is a MLV-derived vector (Miller and Rosman, 1989) that has been used as a basis for several more advanced MLV vectors (MFG [Dranoff et al., 1993]). The optimized vector MP71 contains several cis-regulatory elements that drive high-level and long-lasting transgene expression in primary T lymphocytes. Strong transgene expression is mandatory in many settings where the amount of expressed protein directly correlates with function, as, for example, in the case of TCRs (Labrecque et al., 2001; Heemskerk et al., 2004). In this study, we show successful expression of a TCR recognizing an as yet unidentified RCC antigen on retrovirally transduced PBLs. The redirected T lymphocytes express sufficient amounts of the TCR to mount effector functions on specific stimulation, which are similar in magnitude to those seen with the original TIL clone.

**MATERIALS AND METHODS**

**Cell lines and primary cells**

293-10A1 packaging cells (Farson et al., 1999) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS; Greiner, Frickenhausen, Germany). The human T cell line HuT78 (TIB161; American Type Culture Collection [ATCC], Manassas, VA), a CD4+ cutaneous T cell lymphoma line, was cultured in RPMI 1640 medium with 10% FCS, 10 mM HEPES, and 2 mM glutamine. The proerythroblastemic cell line K-562 (CCL-243; ATCC) and the Burkitt’s lymphoma cell line Daudi (CCL-213; ATCC) were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, and penicillin–streptomycin (100 U/ml) (“standard growth medium”). The following cell lines were established from RCC patients and cultured in standard growth medium: NKC-26, SV40 large T antigen-transformed normal kidney cells of patient 26; LCL-26, an EBV-transformed B-lymphoblastoid cell line of patient 26; and RCC-26 and RCC-53, renal cell carcinoma tumor cells of patients 26 and 53, respectively. TIL-26, a tumor-infiltrating lymphocyte clone of patient 26 (Schendel et al., 1993), was cultured in RPMI 1640 medium supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 7.5% heat-inactivated FCS, 7.5% heat-inactivated pooled human serum, and recombinant human interleukin 2 (rIL-2; 50 U/ml) (Roche Applied Science, Mannheim, Germany). TIL-26 cells were stimulated bimonthly with irradiated (100 Gy, using α137Cs source a gammacell 40, Atomic Energy of Canada, Ottawa, ON, Canada) IL-2-secreting autologous RCC-26 cells (Pohla et al., 2000), irradiated (50 Gy) pooled allogeneic peripheral blood mononuclear cells (PBMCs), and irradiated (150 Gy) EBV-transformed autologous B-LCL. Fresh medium was given biweekly and TILs were split when cells were confluent. For functional assays measuring antigen-specific cytotoxicity or induction of cytokine secretion TILs were used on days 7 and 14, respectively, after the last stimulation. Human peripheral blood lymphocytes (PBLs) were isolated from healthy donors by Ficoll gradient centrifugation (Seromed-Biochrom, Berlin, Germany) and stimulated for 72 hr with rIL-2 (50 U/ml) and immobilized anti-CD3 and anti-CD28 monoclonal antibodies (mAbs; BD Biosciences Pharmingen, San Diego, CA) as described (Uckert et al., 2000). PBLs were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 10 mM HEPES, penicillin–streptomycin (100 U/ml), and rIL-2 (10 U/ml), if not stated differently. For monthly restimulation 2 × 10⁵ PBLs were seeded into a well of a 24-well plate. PBMCs (3.2 × 10⁶) from two donors, irradiated with 30 Gy, rIL-2 (50 U/ml), and anti-CD3 mAb (30 ng/ml), were added to the wells. All tissue culture media and additives were purchased from GIBCO (Karlsruhe, Germany).

**Construction of retroviral vectors and production of viral supernatants**

TCR α and β chains were derived from a TIL clone isolated from RCC patient 26 (TIL-26; Vα20-Jα22 [TRAV4*01-TRAJ22*01] and Vγ22-Jγ2.7 [TRBV2*03-TRBJ2-7*01], respectively; Jantzer and Schendel, 1998). Total RNA was isolated with RNA-Bee (Tel-Test, Friendswood, TX). After the generation of cDNA by random primers and Moloney murine leukemia virus reverse transcriptase (M-MLV-RT; USB, Cleveland, OH), the chains were amplified with oligonucleotides Vα20-sense (5′-GTC GTC GAC CAC CAT GAG GCA AGT G-3′), Cεxho antisense (5′-CAC TCG ATG CAG CTG GAC CAC AGC-3′), Vγ22-sense (5′-CGT GCC GCC GCC ACC
ATG GAT ACC TGG C-3'), and Cβxba antisense (5'-GCA TCT AGA CTA GCC TCT GTA ATC TCT CTT G-3'). They were subsequently cloned into the plasmid pPBS, containing the poliovirus internal ribosomal entry site (IRES, kindly provided by W.F. Anderson) (Morgan et al., 1992), to obtain pPBSβIREsα. The βIREsα fragment including the Kozak sequence was then cloned into two retroviral vectors. The vector plasmid pβIREsα (pL-tcr, 7,235 bp) is a derivative of pLXSN (Miller and Rosman, 1989). For the construction of pβ-tcr the simian virus 40 promoter (S) and the neomycin resistance gene (N) were excised from pLXSN by HpaI and Nael digestion (all restriction enzymes were purchased from Amersham Biosciences/GE Healthcare, Freiburg, Germany) and replaced by the βIREsα fragment, obtained by Xhol restriction of pPBSβIREsα, followed by incubation with Klenow fragment (USB) and a final NolI digestion. The vector plasmid pMP71βIREsαPRE (pMP-tcr, 7,893 bp) is based on pMP71GPbRE (Engels et al., 2003). For the development of pMP-tcr, pMP71GPbRE was partially digested with EcoRI, in order to maintain the posttranscriptional regulatory element (PRE). After incubation with Klenow fragment the vector was digested with NolI and the βIREsα fragment, excised as described above, was inserted into the vector. The green fluorescent protein (GFP)-encoding vector pMP71GPbRE was used as a mock control throughout this study, and is named pMP-gfp.

Stable producer cell clones were generated by cotransfection of 293-10A1 packaging cells with either of the retroviral vector plasmids and the plasmid pWLAeo (Stratagene, Heidelberg, Germany), using calcium phosphate precipitation (Amersham Pharmacia Biotech) (Uckert et al., 2000). High-titer virus vector particle-containing supernatant (viral supernatant) was produced in 175-cm² tissue culture flasks (Corning Costar, Bochum, Germany) at 37°C and stored in aliquots at −80°C.

Transduction of T cells

HuT78 cells and stimulated PBLs were transduced in 24-well non-tissue culture plates coated with RetroNectin (Takara Bio, Otsu, Japan) as described (Uckert et al., 2000). HuT78 cells (1 × 10⁶ per well, and per ml) or PBLs (2 × 10⁶ per well, and per ml) were transduced for 24 hr with 1 ml of viral supernatant supplemented with protamine sulfate (4 μg/ml; Sigma-Aldrich Chemie, Munich, Germany). For PBLs, rhIL-2 (50 U/ml) was added. Cells were then transferred to culture flasks and cultured as described above.

Flow cytometric analysis and magnetic cell sorting

Cells were stained with fluorescein isothiocyanate (FITC-) or phycoerythrin (PE-) conjugated anti-TCR chain Vγ22 mAb (Serotec, Oxford, UK) or with anti-CD3, anti-CD4, or anti-CD8 mAb (BD Biosciences Pharmingen). DNA analysis and analysis was performed on a FACSCaliber (BD Biosciences Immunocytometry Systems, San Jose, CA) using CellQuest Pro software.

To enrich for Vγ22-positive cells, MP-tcr-transduced PBLs were stained with PE-conjugated anti-Vγ22 mAb and subsequently with anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The stained cells were thereafter purified with an autoMACS separator (Miltenyi Biotec) according to the manufacturer’s instructions.

Genomic DNA polymerase chain reaction

Genomic DNA (gDNA) was isolated from L-tcr-, MP-tcr-, and nontransduced PBLs on days 11 and 32 posttransduction, using a DNase-free tissue kit (Qiagen, Hilden, Germany), and subsequently treated with DNase-free RNase (Roche Applied Science). One hundred nanograms and 10 ng of gDNA were employed for each sample. Forward primer fvb-c (5'-GCA GAA CCC CCG CAA CCA CT-3') and reverse primer rIRES (5'-CGA CAT CAC CGG GGA AAC AG-3') were used to amplify a vector-specific 450-bp product. As loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers were used to generate a 451-bp product (forward primer, 5'-ACC ACA GTC CAT GCC ATC AC-3'; reverse primer, 5'-TCC ACC CTG TTG CTG TA-3'). PCRs were run in a T personal thermocycler (Biometra, Göttingen, Germany) using Taq DNA polymerase (Invitrogen, Karlsruhe, Germany). PCR conditions were 2 min at 94°C; 25, 30, or 35 cycles of 94°C for 30 sec; 68°C for 30 sec and 72°C for 1 min; and a final 10-min incubation at 72°C for the virus-specific reaction, and with an annealing temperature of 55°C for GAPDH. PCR products were analyzed by 1% agarose gel electrophoresis, ethidium bromide staining, and quantification with the Lumi-Imager F1 and Lumi-Analyist software (both from Roche Applied Science).

Reverse transcription-polymerase chain reaction

Total RNA was isolated from PBLs on days 11 and 32 posttransduction with either L-tcr or MP-tcr according to Chomczynski and Sacchi (1987). RNA concentration was determined spectrophotometrically after RNase-free DNase I digestion (Roche Applied Science). cDNA was synthetized using 1 μg of total RNA, random primers, and reverse transcriptase (RT; both from Invitrogen) in a 20-μl reaction mix. A 0.5- or 0.1-μl volume of cDNA was used per PCR. As negative controls no-template and no-RT samples were included. Reactions were run in a Pernalson thermocycler using Taq DNA polymerase. The same primers and PCR conditions as for gDNA PCR were used. PCR products were analyzed by 1% agarose gel electrophoresis, ethidium bromide staining, and quantification with the Lumi-Imager F1 and Lumi-Analyist software.

Cytokine release assay

Target cells (1.5 × 10⁶) were cocultured with 1.0 × 10⁴ PBLs or 3.0 × 10⁴ TIL-26 cells in 96-well round-bottom plates for 24 hr. The supernatant was removed and tested for interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) content by enzyme-linked immunosorbent assay (ELISA) (sensitivity, 4 pg/ml; Biowiscience, San Diego, CA) according to the manufacturer’s protocol. Inhibition assays were performed by adding MA2.1 (anti-HLA-A2 mAb, 4 μg/well; ATCC HB-54), W6/32 (anti-MHC class I Ab, 2 μg/well; ATCC HB-95), or MOPC21 (mouse IgG1 isotype control, 4 μg/well; Sigma) during cocultivation. K-562 and Daudi cells, which lack MHC class I molecules, were used as negative controls for stimulation, and anti-CD3 mAb (BD Biosciences Pharmingen)-coated wells (5 μg/ml) were used as a positive control. The data are given as mean values with standard deviations of duplicates, derived from one representative experiment. At least two independent experiments were performed.
Cytotoxicity assay

Cell-mediated lysis of target cells by T cell clone TIL-26 and TCR gene-modified PBLs was quantified in a 4-hr ⁵¹Cr release assay. Effector-to-target cell ratios ranged from 20:1 to 1.25:1, employing 2.0 × 10³ target cells. Spontaneous release was determined by incubating target cells alone, and the maximal release was determined by directly counting labeled cells. The percentage of cytotoxicity was calculated as: percent specific lysis = [(cpm_{experimental} − cpm_{spontaneous})/(cpm_{maximal} − cpm_{spontaneous})] × 100. Duplicate measurements of four-step titration of effector cells were used for all experiments. K-562 and Daudi cells were used as controls. Results shown represent mean values and standard deviations from one of two independent experiments.

RESULTS

Expression of RCC-specific TCR in T cell lines and primary human T lymphocytes is superior with MPSV-based vector

Rearranged TCR α and β chains from TIL-26 were cloned into MLV (pLXSN)- and MPSV (pMP71)-based vectors and named L-tcr and MP-tcr, respectively (Fig. 1A). Viral supernatants containing either vector were used to transduce HuT78 T cells and primary T lymphocytes of three different donors.

Surface expression of the exogenous TCR was analyzed by flow cytometry using an anti-TCR chain Vβ22 mAb. Both the L-tcr and MP-tcr vectors led to transgene expression in HuT78 cells (Fig. 1B). However, a difference could be seen in the percentage of Vβ22-expressing T cells and also in the level of TCR surface expression, measured as mean fluorescence intensity (MFI). Forty-six percent of L-tcr-transduced T cells expressed Vβ22 with an MFI of 44, whereas 83% of MP-tcr-transduced T cells expressed Vβ22 with an MFI of 74. Such an expression pattern was observed in several experiments (data not shown).

The difference in transgene expression by the two vectors was even more pronounced in transduced primary T lymphocytes, where only MP-tcr led to strong TCR expression. In the experiment shown in Fig. 1B, transduction of PBLs of donor 1 with MP-tcr yielded 52% Vβ22-positive cells, compared with 7% obtained with L-tcr. This finding was confirmed in several experiments performed at different times with three different donors (Table 1). Looking at the expression of Vβ22 in L-tcr-transduced PBLs in more detail, an increase of only low Vβ22-expressing cells can be observed. The 7% of Vβ22-positive PBLs include the 3.5% endogenously Vβ22-expressing cells, as seen for the nontransduced PBLs (thin lines in Fig. 1B).

In MP-tcr-transduced primary T lymphocytes many cells expressed the transgenic TCR at lower levels compared with the endogenous TCR; however, a substantial number of cells expressed the TCR at similar levels. Compared with L-tcr-transduced PBLs, MP-tcr-transduced cells showed significantly higher expression lev-
els (MFI, 27 versus 14). We cannot exclude the potential formation of mixed hybrid TCRs of TCR-26 chains with endogenous TCR chains for both transduced Hut78 cells and PBLs. This would lead to a lower amount of TCR-26-positive compared with Vβ22-positive cells. A vector control (MP-gfp) did not increase Vβ22 expression (data not shown).

Both vectors transduce PBLs at similar rates

PBLs were transduced with viral supernatants harvested from producer cell clones selected for high viral titer production. As neither of the vectors contains a selectable marker and therefore titration by transduction of indicator cells was not possible, the relative titer was determined through analysis of copy number integrations. Hence gDNA was prepared from L-tcr-, MP-tcr-, and nontransduced PBLs of two donors on days 11 and 32 posttransduction. PCRs specific for the viral genomes were performed, and the bands were subsequently quantified. The amount of gDNA employed per reaction (100 and 10 ng) was confirmed by GAPDH-specific amplification. Furthermore, PCR samples were analyzed after 25, 30, and 35 cycles of amplification, altogether giving comparable results. In Fig. 2A donor 1 samples amplified for 30 cycles are shown. Bands were quantified and Boehringer light units (BLU) were plotted (Fig. 2B). Similar band intensities were obtained for cells transduced with either vector, demonstrating similar numbers of integrated viral genomes. No signals were obtained when nontransduced PBLs and template-negative controls were analyzed. This clearly shows that the T cells were transduced at similar rates, indicating comparable viral titers of L-tcr and MP-tcr.

To further analyze the discrepancy between similar viral copy integration and differing TCR expression in L-tcr- and MP-tcr-transduced PBLs, we performed RT-PCR analysis. Total RNA was isolated from L-tcr-, MP-tcr-, and nontransduced PBLs of two donors on days 11 and 32 posttransduction. For RT-PCR we employed two different cDNA amounts, using the same virus- and GAPDH-specific primers described above. Unlike the gDNA PCR, the RT-PCR showed a clear difference in the amount of TCR transcripts between L-tcr- and MP-tcr-transduced PBLs. One experiment representative of two for donor 1 is shown in Fig. 2C and the respective bar diagram is shown in Fig. 2D. For days 11 and 32 posttransduction more TCR-encoding transcript could be detected in MP-tcr-transduced PBLs compared with L-tcr-transduced PBLs. On the basis of GAPDH-corrected virus band intensities, a 4-fold higher amount of TCR transcripts was found for MP-tcr-transduced

### Table 1. Transduction Rates for PBLs of Different Donors in Independent Experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
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<td>1</td>
<td>32/3</td>
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<td>16/6</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>52/7</td>
<td>—</td>
<td>35/—</td>
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*2.5–3.5% Vβ22+ cells represent endogenous Vβ22 expression.

bFunctional data and molecular analysis are shown for donor 1.

c—, not determined, as transduction was not performed.
dData shown in Fig. 1B.
PBLs at both time points. Nontransduced cells led to no virus-specific signal. No signals were amplified when either template or reverse transcriptase were omitted from the PCR (data not shown). These results confirm the data obtained for TCR protein expression analysis by flow cytometry and indicate that the strong cis-regulatory elements in the MPSV-based retroviral vector are responsible for the higher TCR expression.

**Redirected PBLs are stimulated by RCC-26 cells**

To assess the function of virally expressed TCR, we performed stimulation assays. L-tcr-, MP-tcr-, and nontransduced PBLs of three different donors were cocultured with the tumor cell line RCC-26, which expresses the peptide–MHC ligand for the transgenic TCR. Secretion of IFN-γ reflected stimulation of the T cells by RCC-26 cells. Shown are representative results obtained for PBLs of donor 1 (Fig. 3). MP-tcr-transduced PBLs were clearly activated by RCC-26 cells (IFN-γ, 1421 pg/ml). In contrast, L-tcr-transduced PBLs secreted less IFN-γ, in fact only slightly more than did nontransduced PBLs (304 versus 244 pg/ml). The general ability of all cells to be activated and to secrete cytokines was proven by unspecific, redirected stimulation (anti-CD3 mAb, data not shown). The marginal increase in IFN-γ secretion by L-tcr-transduced PBLs, compared with nontransduced cells, may indicate that the TCR expression by L-tcr is not sufficient to trigger TCR-mediated T cell stimulation. The MP-tcr-encoded tumor-specific TCR was, however, expressed at levels sufficient to induce activation of the transduced T cells on cocultivation with the RCC-26 cell line. Referring to the flow cytometric analysis, the higher percentage of Vβ22-positive cells allowed activation of the MP-tcr-transduced PBL population. As hardly any TCR-related activity was detected for L-tcr-transduced PBLs, all further experiments were conducted with MP-tcr-transduced T cells only.

**Redirected PBLs are stimulated by target cells as efficiently as the original TIL clone**

The comparison of MP-tcr-transduced PBLs with the original TIL-26 cells, both carrying the same TCR, revealed similar responses to RCC-26 cells. In this assay, the number of effector cells was adjusted on the basis of the percentage of Vβ22-positive cells. According to flow cytometry analysis on the day of assay, one-third of the transduced PBLs were Vβ22 positive, compared with 100% of the TIL-26 cells (data not shown). Therefore, we employed 1 × 10^5 MP-tcr-transduced PBLs or 3 × 10^5 TIL-26 cells per well. The production of IFN-γ and TNF-α on stimulation of the cells of three different donors showed that TCR-26 gene-modified PBLs developed a similar cytokine response as TIL-26 cells, which express the same TCR endogenously (Fig. 4). The MP-tcr-transduced PBLs of donor 1 secreted, per milliliter, 1790 pg of IFN-γ and 230 pg of TNF-α, whereas TIL-26 cells secreted 1850 pg of IFN-γ and 260 pg of TNF-α (Fig. 4A and B). The similar amount of both cytokines secreted by each Vβ22-positive T cell population argues for physiological TCR transgene expression in the transduced PBLs. MP-gfp- and nontransduced PBLs did not respond to RCC-26 cell stimulation. All cells, however, responded similarly to redirected stimulation with anti-CD3 mAb (data not shown).

Using a set of different target cells, the specificity of redirected PBLs was investigated. MP-tcr-transduced PBLs were not stimulated by the allogeneic renal cell carcinoma cell line RCC-53, the autologous normal kidney cell line NKC-26, or the autologous lymphoblast cell line LCL-26, which are all negative for the peptide-MHC ligand for the transgenic TCR, or by Daudi cells, which are MHC class I negative (Fig. 4C and D, solid columns). This indicated antigen-specific recognition by the transgenic T cells. Furthermore, the redirected PBLs showed the same specificity as seen for the TIL-26 clone (Fig. 4C and D, open columns).

**Antigen-specific recognition by redirected PBLs is HLA-A2 restricted**

To further confirm specific TCR peptide–MHC interaction resulting in cytokine secretion, we performed blocking assays with various antibodies (Fig. 5). Using the HLA-A2-specific mAb MA2.1 as a blocking reagent, 81% less IFN-γ was produced by RCC-26 cell-stimulated MP-tcr-transduced PBLs of donor 1. When the anti-MHC class I antibody W6/32 was used, 95% less IFN-γ was secreted. The isotype control mAb (MOPC21) did not reduce the amount of IFN-γ secretion. A similar pattern of blocking was also observed for coculture of RCC-26 cells with additional TCR-transduced PBLs of two donors and the TIL-26 clone that expresses the TCR naturally (data not shown). These data demonstrate that the stimulation of transduced PBLs was based on TCR-mediated recognition of an HLA-A2-restricted RCC tumor antigen, as has been shown for the original TIL-26 cells (Schendel et al., 1997; Jantzer and Schendel, 1998).

**Redirected PBLs and TIL-26 cells show similar lytic activity against RCC-26 cells**

Next we evaluated the cytotoxicity of redirected PBLs of the three different donors. For a comparison of tumor cell lysis by MP-tcr-transduced PBLs and TIL-26 cells on a cell-to-cell basis, we enriched MP-tcr-transduced PBLs for Vβ22-positive T cells by MACS, which yielded an 86% Vβ22-
positive cell population for donor 1 (Fig. 6A). Enriched MP-tcr-transduced PBLs and the original TIL-26 clone lysed RCC-26 cells to similar extent (Fig. 6B). Thus, as has been observed for the cytokine response, MP-tcr-transduced PBLs had antitumor activity comparable to that of TIL-26 cells. Mock-transduced and nontransduced PBLs showed similar low background lysis.

The specificity of the lysis reaction was shown by incubating the same effector cells with different target cells (Fig. 6C). Again, no reactivity toward autologous control cells, that is, NKC-26 and LCL-26 cells, was measured, nor were RCC-53 cells lysed. The MHC class I-negative K-562 line, which is a control for natural killer cell activity, was also not lysed. Mock-transduced and nontransduced PBLs exhibited some degree of lytic activity against RCC-26 cells. Because a similar level of activity was observed against MHC class I-negative Daudi cells (data not shown) it is most likely that this activity is nonspecific, induced by exposing PBLs to IL-2 and anti-CD3/anti-CD28 mAbs before retroviral transduction. The pattern of lytic activity, in particular the clear distinction of RCC-26 cells from normal kidney cells, demonstrated that the transduced PBLs specifically lysed cells that present the tumor epitope specific for the transgenic TCR.

**MP-tcr-driven TCR expression is stable over time**

After expression and function of the transferred TCR-26 cells were shown for MP-tcr-transduced PBLs, the duration of transgene expression was analyzed. In two independent experiments, each with two donors, PBLs transduced with the MP-tcr or the L-tcr vector were cultured *in vitro* for up to 111 days and V\(_{\beta}22\) expression was monitored over time. To maintain the cultures, transduced cells were unspcecifically restimulated with irradiated feeder cells and anti-CD3 mAb every 30 days. MP-tcr-, L-tcr-, and nontransduced PBLs proliferated similarly at each round of stimulation (data not shown). Flow cytometry data obtained for transduced PBLs of donor 1 on days 5 and 111 post-transduction show 18 and 15% for MP-tcr, respectively, and 6 and 3% for L-tcr, respectively, of V\(_{\beta}22\)-positive cells (Fig. 7A). In Fig. 7B V\(_{\beta}22\) expression is plotted against time. After 111 days, 83% of the initial V\(_{\beta}22\)-positive MP-tcr transduced PBLs still expressed TCR-26, whereas only 26% of L-tcr transduced
104 RCC-26 cells were cocultivated for 24 hr. Various anti-fails to express the TCR at high and constant levels. expression over more than 100 days, whereas the L-tcr vector. Taken together, the MP-tcr vector guarantees high transgene similar to those of cells used 14 days posttransduction (Fig. 3). showed that TCRs were still functionally active on day 55 post-transduction. Assay was performed on day 18 posttransduction. PBLs of three donors were analyzed, altogether giving similar results. Assay was performed on day 18 posttransduction.

cells retained TCR-26 expression. These data clearly indicate that transgene expression by MP-tcr is stable over time, whereas it decreases when using L-tcr.

Further analysis of the long-term in vitro-cultured cells showed that TCRs were still functionally active on day 55 post-transduction as indicated by IFN-γ release (data not shown), similar to those of cells used 14 days posttransduction (Fig. 3). Taken together, the MP-tcr vector guarantees high transgene expression over more than 100 days, whereas the L-tcr vector fails to express the TCR at high and constant levels.

**DISCUSSION**

In this study we show the transfer of antitumor specificity from a TIL clone established from an RCC patient to PBLs of healthy donors. Redirected T lymphocytes stably expressed the TCR on the cell surface, were specifically activated by RCC-26 tumor cells as shown by cytokine secretion on coculture, and gained similar killing capabilities as the originally isolated TIL clone. To transfer the TCR genes, we employed two different retroviral vectors: a Moloney murine leukemia virus (MLV)-derived vector (LXSN) (Miller and Rosman, 1989) and an MPSV-based vector (MP71) (Schambach et al., 2000). The stronger activating effect in the MP71 vector can be attributed to the modified 5′-untranslated region, originating from the murine embryonic stem cell virus (MESV) (Grez et al., 1990). This leader sequence contains an artificial splice acceptor site complementing the retroviral splice donor site. In addition, all start codons have been removed from within the leader sequence. Consequently, the ATG of the transgene is the first start codon 3′ of the promoter (Hildinger et al., 1999). Moreover, the MPSV long terminal repeat (LTR) promoter and enhancer elements by themselves improve transgene expression in T lymphocytes (Schambach et al., 2000; Engels et al., 2003) and lymphohematopoietic cells (Baum et al., 1995; Hildinger et al., 1998). The MP71-based vectors differ from the related MSCV vectors (Hawley et al., 1994) in the U3 region of the 3′ LTR. The MP71 vector carries the MPSV U3 region (Schambach et al., 2000). In contrast, MSCV vectors contain the respective PCC4-embryonal carcinoma cell-passaged myeloproliferative sarcoma virus (PCMV) region (Hilberg et al., 1987; Hawley et al., 1994). Furthermore, MSCV vectors contain a hybrid leader sequence of MESV and LNCX origin, whereas the leader of MP71 vectors is solely of MESV origin. Finally, the introduction of the woodchuck hepatitis virus posttranscriptional regulatory element (PRE) into the MP71 vectors accounts for a further increase in the level of transgene expression (Zufferey et al., 1999). The LXSN vector we have used in this comparison is a basic, entirely MLV-based vector. Several advanced vectors based on LXSN have been described, including grafting of T lymphocytes with antigen receptors (MFG-based pMX [Fujio et al., 2000; Kessels et al., 2000] and pBullet [Willemse et al., 2000]). They were not included in our study. However, data on GFP expression by MP71 and pBullet revealed higher transgene expression rates in MP71-transduced cells (our unpub-
lished results). Taken together, the stronger cis-regulatory elements of MP-tcr in comparison with those of L-tcr lead to superior TCR expression, as shown at the transcriptional level by RT-PCR analysis and at the translational level by flow cytometric analysis of TCR-26 cell surface expression. The strong transgene expression by MP-tcr is of particular relevance because (1) the amount of TCR on the cell surface critically determines the functionality of the gene-modified T cell (Labrecque et al., 2001) and (2) a high level of TCR expression is achieved at a low multiplicity of infection (MOI), thereby potentially reducing the risk of insertional mutagenesis (Hacein-Bey-Abina et al., 2003; Fehse et al., 2004).

The MP-tcr vector is superior to the L-tcr vector not only regarding the level of TCR-26 expression, but also regarding the duration of TCR-26 expression. This is an indispensable prerequisite for the generation of a sufficient number of TCR-redirected T lymphocytes for adoptive transfer into patients. In contrast to the stable TCR-26 expression by MP-tcr transduction, L-tcr-transduced PBLs gradually lost expression over time. Genomic DNA PCR analysis of transduced PBLs showed that the number of integrated viral genomes remained similar for both vectors over time, indicating that the decrease in TCR-26 expression in L-tcr transduced cells is due to lower expression rates, probably induced by gene silencing through methylation of the MLV LTR (Jahner et al., 1982; Challita and Kohn, 1994; Wang et al., 1998).

So far, the majority of genetically engineered tumor-reactive T lymphocytes have been endowed with TCRs recognizing melanoma-associated antigens (Clay et al., 1999; Schaft et al., 2003). Furthermore, effector functions of TCR-redirected human PBL bulk cultures have so far been described only for melanoma.
anoma-associated gp100 specificity (Morgan et al., 2003). Here we demonstrate for the first time that RCC tumor cell-specific CTLs can be generated by the transfer of antigen-specific TCR into PBLs of healthy donors. We proved the functionality of the TCR-26-redrected PBLs, using unsorted bulk cultures of MP-tcr-transduced T lymphocytes. After TCR gene transfer the PBLs of healthy donors gained HLA-A2-restricted, RCC-specific effector functions comparable in magnitude to those of the original TIL clone. In contrast, using the L-tcr vector the expression levels were not sufficiently high to induce T effector function by RCC-26 cell stimulation. Notably, in many previous studies with MLV-derived vectors, only enriched or cloned TCR-transduced T cells were investigated, indicating a low TCR expression level by MLV vectors also in other systems (Clay et al., 1999; Cooper et al., 2000; Heemskerk et al., 2003). Similar results have been also described in a study using a chimeric antibody receptor (Weijtens et al., 2000).

In conclusion, we have demonstrated that PBLs of healthy donors can be redirected toward tumor antigens by specific TCR gene transfer. Using an optimized retroviral vector, we generated PBLs that are specific for an RCC tumor antigen and exert effector functions of similar strength and specificity as observed for the CTL clone that expresses the TCR endogenously. The antigen recognized by the transduced TCR-26 is unknown. Antigen identification requires significant numbers of T cells, which are often not achieved in RCC investigations. The described approach provides an important tool with which to identify this tumor antigen, as well as those of other TIL clones, where long-term T cell clones cannot be established. It can be envisioned that small repertoires of T cells bearing transgenic TCR to redirect their specificity toward tumors or viruses will be used in adoptive T cell therapy.

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REFERENCES


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