Chapter 3, Engineering RCC-reactive CTLs

Redirecting human T lymphocytes toward renal cell carcinoma specificity by retroviral transfer of T cell receptor genes

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

BORIS ENGELS,[1,2] ELFRIEDE NOESSNER,[3] BERNHARD FRANKENBERGER,[3]
THOMAS BLANKENSTEIN,[2,4] DOLORES J. SCHENDEL,[3] and WOLFGANG UCKERT[1,2]

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 (^{51}Cr 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-

[1]Institute of Biology, Humboldt University Berlin, 10115 Berlin, Germany.
[2]Max Delbrück Center for Molecular Medicine, 13092 Berlin, Germany.
[3]Institute of Molecular Immunology, GSF-National Research Center for Environment and Health, 81377 Munich, Germany.
[4]Institute of Immunology, Charité Campus Benjamin Franklin, 12200 Berlin, Germany.

tial 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 transfer 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 [Schaft *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 m*M* HEPES, and 2 m*M* glutamine. The proerythroleukemic 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 m*M* glutamine, 1 m*M* 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 m*M* L-glutamine, 1 m*M* sodium pyruvate, 7.5% heat-inactivated FCS, 7.5% heat-inactivated pooled human serum, and recombinant human interleukin 2 (rhIL-2; 50 U/ml) (Roche Applied Science, Mannheim, Germany). TIL-26 cells were stimulated bimonthly with irradiated (100 Gy, using as ^{137}Cs 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 allogeneic 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 rhIL-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 m*M* glutamine, 10 m*M* HEPES, penicillin–streptomycin (100 U/ml), and rhIL-2 (10 U/ml), if not stated differently. For monthly restimulation 2×10^5 PBLs were seeded into a well of a 24-well plate. PBMCs (3.2×10^6) from two donors, irradiated with 30 Gy, rhIL-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; $V_\alpha 20$-$J_\alpha 22$ [*TRAV4*01-TRAJ22*01*] and $V_\beta 22$-$J_\beta 2.7$ [*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 Vα20sal sense (5′-GTC GTC GAC CAC CAT GAG GCA AGT G-3′), Cαxho antisense (5′-CAC TCG AGT CAG CTG GAC CAC AGC-3′), Vβ22not sense (5′-CGT GCG GCC GCC ACC

ATG GAT ACC TGG C-3′), and Cβxba antisense (5′-GCA TCT AGA CTA GCC TCT GGA ATC CTT 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 pLβIRESα (pL-tcr, 7235 bp) is a derivative of pLXSN (Miller and Rosman, 1989). For the construction of pL-tcr the simian virus 40 promoter (S) and the neomycin resistance gene (N) were excised from pLXSN by *Hpa*I and *Nae*I digestion (all restriction enzymes were purchased from Amersham Biosciences/GE Healthcare, Freiburg, Germany) and replaced by the βIRESα fragment, obtained by *Xho*I restriction of pPBSβIRESα, followed by incubation with Klenow fragment (USB) and a final *Not*I digestion. The vector plasmid pMP71βIRESαPRE (pMP-tcr, 7893 bp) is based on pMP71GPRE (Engels *et al*., 2003). For the development of pMP-tcr, pMP71GPRE was partially digested with *Eco*RI, in order to maintain the posttranscriptional regulatory element (PRE). After incubation with Klenow fragment the vector was digested with *Not*I and the βIRESα fragment, excised as described above, was inserted into the vector. The green fluorescent protein (GFP)-encoding vector pMP71GPRE 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 pWLneo (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-cm^2 tissue culture flasks (Corning Costar, Bodenheim, 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 (1×10^5 per well, and per ml) or PBLs (2×10^5 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 $V_\beta 22$ mAb (Serotec, Oxford, UK) or with anti-CD3, anti-CD4, or anti-CD8 mAb (BD Biosciences Pharmingen). Data acquisition and analysis was performed on a FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA) using CellQuest Pro software.

To enrich for $V_\beta 22$-positive cells, MP-tcr-transduced PBLs were stained with PE-conjugated anti-$V_\beta 22$ 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 DNeasy 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 AGA 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 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-Analyst 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 synthesized 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 Tpersonal 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 LumiAnalyst software.

Cytokine release assay

Target cells (1.5×10^4) were cocultured with 1.0×10^4 PBLs or 3.0×10^3 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; eBioscience, 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 ^{51}Cr release assay. Effector-to-target cell ratios ranged from 20:1 to 1.25:1, employing 2.0×10^3 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 = $[(cpm_{experimental} - cpm_{spontaneous})/(cpm_{maximal} - cpm_{spontaneous})] \times 100$. 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 $V_\beta22$ 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 $V_\beta22$-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 $V_\beta22$ with an MFI of 44, whereas 83% of MP-tcr-transduced T cells expressed $V_\beta22$ 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% $V_\beta22$-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 $V_\beta22$ in L-tcr-transduced PBLs in more detail, an increase of only low $V_\beta22$-expressing cells can be observed. The 7% of $V_\beta22$-positive PBLs include the 3.5% endogenously $V_\beta22$-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-

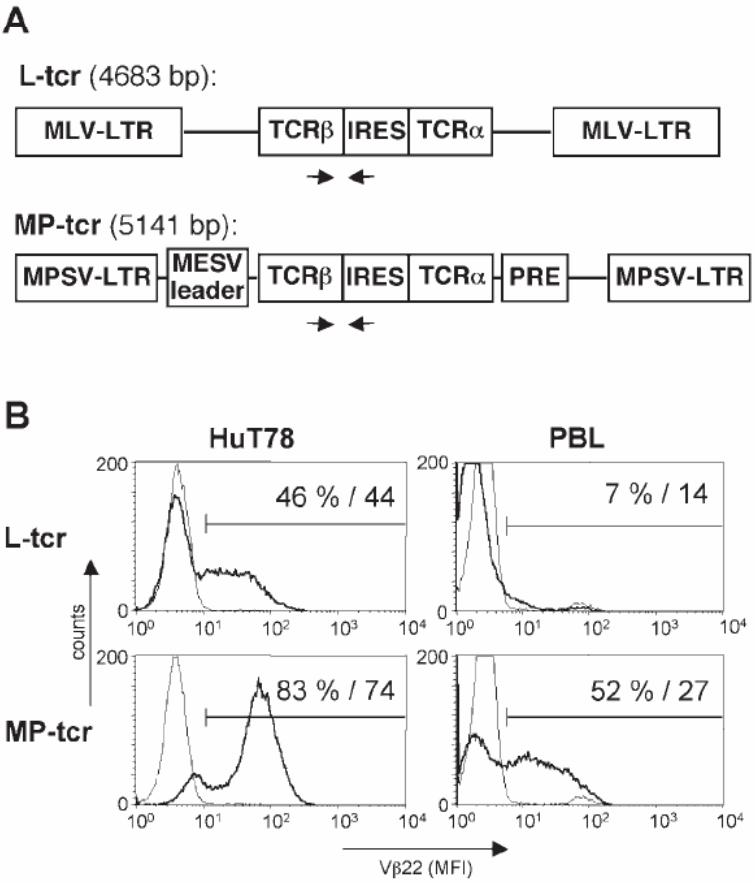

FIG. 1. The retrovirally encoded TCR-26 β chain is strongly expressed on the cell surface of MP-tcr-transduced T cells. (**A**) Schematic representation of vector constructs used in this study. The size of the retroviruses is shown. Arrows indicate the position of primers used for titration by gDNA PCR and RT-PCR analysis in Fig. 2. IRES, poliovirus internal ribosomal entry site; LTR, long terminal repeat; MLV, Moloney murine leukemia virus; MPSV, myeloproliferative sarcoma virus; MESV leader, modified murine embryonic stem cell virus leader sequence; PRE, woodchuck hepatitis virus posttranscriptional regulatory element; TCR, T cell receptor. (**B**) T cell line HuT78 and PBLs were transduced with either vector and analyzed 4 or 20 days posttransduction, respectively. TCR β chain surface expression is visualized by staining nontransduced cells (thin line) and transduced cells (thick line) with a PE-conjugated anti-$V_\beta22$ mAb. The numbers indicate the percentage of $V_\beta22$-positive cells within the whole cell population (cells were gated on live cells, of which >99% were CD3 positive) and the mean fluorescence intensity (MFI) of the cell population within the marked region. The MFI was calculated for the difference between transduced and nontransduced cells, in order to exclude endogenous $V_\beta22$-positive cells. One representative result from donor 1 is shown.

TABLE 1. TRANSDUCTION RATES FOR PBLs OF DIFFERENT DONORS IN INDEPENDENT EXPERIMENTS

	PBL donor[a] *+MP-tcr/L-tcr (% $V_\beta 22^+$)*		
Experiment	*1*[b]	*2*	*3*
1	32/3	—[c]	19/5
2	37/3	28/3	—
3	20/6	16/6	—
4	52/7[d]	—	35/—

[a]2.5–3.5% $V_\beta 22^+$ cells represent endogenous $V_\beta 22$ expression.
[b]Functional data and molecular analysis are shown for donor 1.
[c]—, not determined, as transduction was not performed.
[d]Data shown in Fig. 1B.

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 $V_\beta 22$-positive cells. A vector control (MP-gfp) did not increase $V_\beta 22$ 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 T cells. On the basis of GAPDH-corrected virus band intensities, a 4-fold higher amount of TCR transcripts was found for MP-tcr-transduced

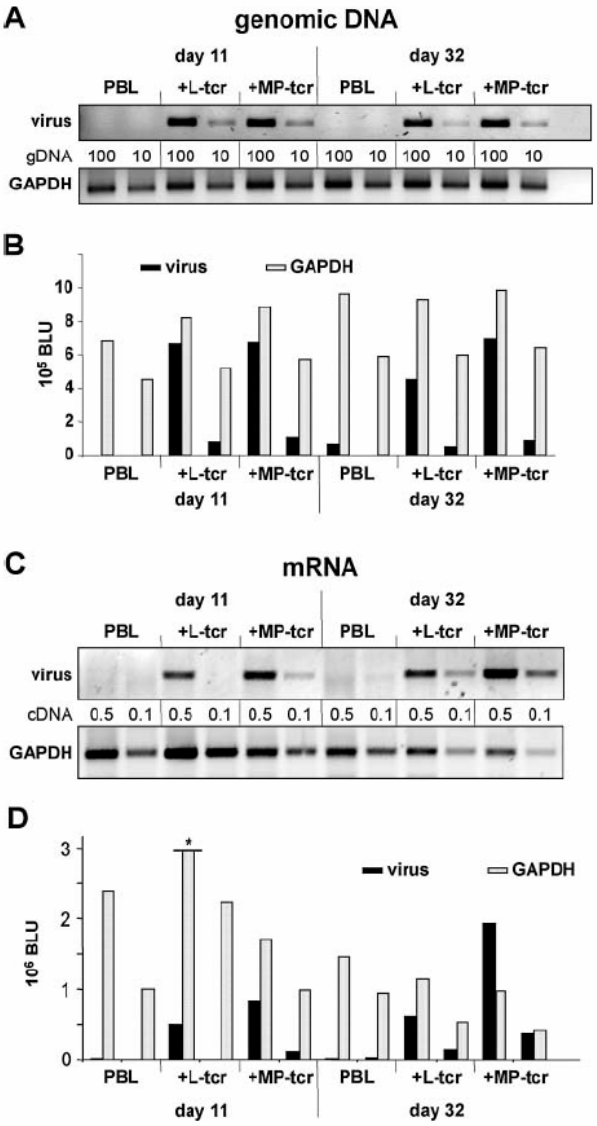

FIG. 2. MP-tcr-transduced PBLs contain similar numbers of integrated viral genomes as L-tcr-transduced PBLs, but more TCR-26 transcripts. Nontransduced, L-tcr-transduced, and MP-tcr-transduced PBLs (donor 1) from days 11 and 32 posttransduction were analyzed for integrated viral genomes and TCR transcripts. (**A**) gDNA PCR: 100 and 10 ng of gDNA were amplified with primers specific for both vectors (450 bp; see Fig. 1A) and for GAPDH (451 bp). Shown are samples analyzed after 30 cycles of amplification, which were run in a 1% agarose gel and stained with ethidium bromide. (**B**) Ethidium bromide-stained DNA bands were digitally quantified. BLU, Boehringer light units. (**C**) RT-PCR with the same primers as used in (**A**); 0.5 and 0.1 μl of cDNA (derived from a 20-μl RT reaction with 1 μg of total RNA) were amplified for 30 cycles and visualized as in (**A**). (**D**) Digital quantification as in (**B**). *4.37×10^6 BLU.

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 V_β22-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 V_β22-positive cells. According to flow cytometry analysis on the day of assay, one-third of the transduced PBLs were V_β22 positive, compared with 100% of the TIL-26 cells (data not shown). Therefore, we employed 1×10^4 MP-tcr-transduced PBLs or 3×10^3 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 V_β22-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).

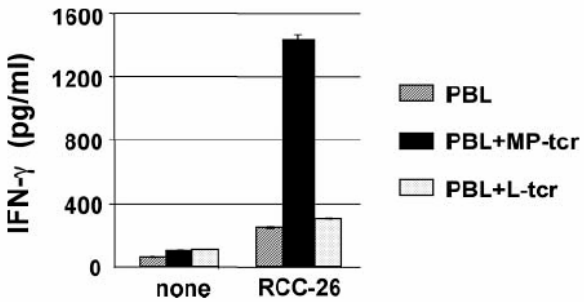

FIG. 3. MP-tcr-transduced but not L-tcr-transduced PBLs secrete IFN-γ on stimulation with RCC-26 cells. Cytokine was measured in the supernatant of stimulated PBLs. Values shown represent mean values and standard deviations of duplicates obtained by ELISA. The experiment was performed three times and PBLs of three donors were analyzed. Effector cells (1.0×10^4; MP-tcr-transduced PBLs [solid columns], L-tcr-transduced PBLs [dotted columns], or nontransduced PBLs [hatched columns]) were cultivated alone (none) or with 1.5×10^4 RCC-26 cells. Assays were performed 12 days after transduction.

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 V_β22-positive T cells by MACS, which yielded an 86% V_β22-

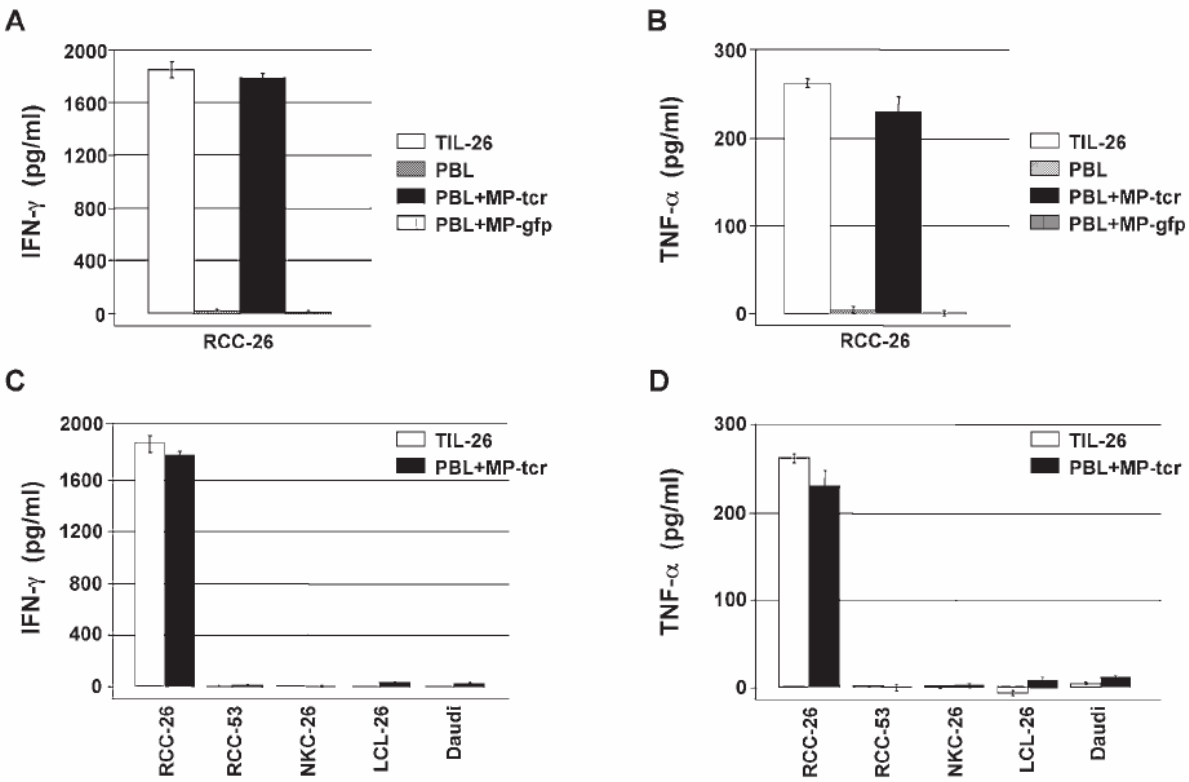

FIG. 4. MP-tcr-transduced PBLs and TIL-26 secrete similar amounts of IFN-γ and TNF-α on stimulation with RCC-26 cells. Cytokines were measured in the supernatant after cocultivation of 1.0×10^4 (PBL) or 3.0×10^3 (TIL-26) effector cells and 1.5×10^4 target cells for 24 hr. Values shown represent mean values and standard deviations of duplicates obtained by ELISA. Experiments were performed twice and PBLs of three donors were analyzed, altogether giving similar results. (**A**) IFN-γ ELISA and (**B**) TNF-α ELISA, utilizing TIL-26 cells (open columns) and MP-tcr-transduced (solid columns), MP-gfp-transduced (striped columns), and nontransduced PBLs (shaded columns) cocultivated with RCC-26 cells. Assays were performed on day 16 post-transduction. (**C**) IFN-γ ELISA and (**D**) TNF-α ELISA, utilizing TIL-26 cells (open columns) and MP-tcr-transduced PBLs (solid columns), which were cocultivated with RCC-26 [same result as shown in (**A**) and (**B**)], RCC-53, NKC-26, LCL-26, and Daudi cells as indicated.

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 unspecifically 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

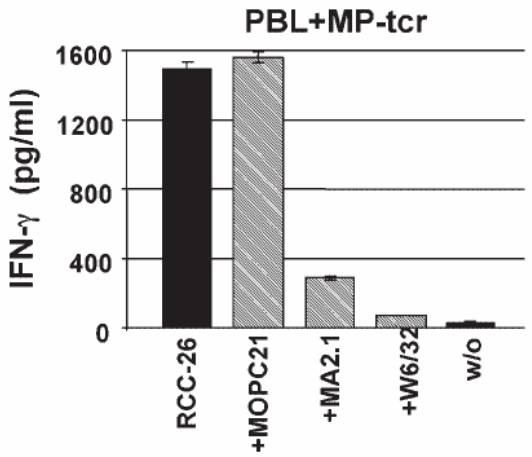

FIG. 5. Antigen recognition via the transgenic TCR is HLA-A2 restricted. MP-tcr-transduced PBLs (1.0×10^4) and 1.5×10^4 RCC-26 cells were cocultivated for 24 hr. Various antibodies were added to the coculture to block TCR peptide–MHC interactions: MA2.1 (anti-HLA-A2), W6/32 (anti-MHC class I), and MOPC21 (isotype control). w/o, no target cells. Values shown are mean values and standard deviations of duplicates obtained by ELISA. Experiments were performed twice and 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 posttransduction 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-derived vector (MP71) (Schambach *et al.*, 2000). With both, we generated MLV-10A1-pseudotyped vector particles to efficiently transduce PBLs (Uckert *et al.*, 2000). The optimized MPSV-based vector (MP-tcr) yielded strong and durable TCR expression associated with the ability to perform effector function, whereas the MLV vector (L-tcr) failed to express sufficient amounts of TCR to exert effector function.

These results confirm and extend previous data describing approximately 20-fold higher expression levels of a cytoplasmic protein (GFP) in primary human T lymphocytes transduced with MP71 (MP-gfp) in comparison with LXSN (Engels *et al.*, 2003). The stronger expression obtained with MP-gfp was not due to multiple integration events, but could clearly be ascribed to the stronger *cis*-regulatory elements of the MP-gfp vector, as demonstrated by Southern blot and RT-PCR analysis. Now we demonstrate that the MPSV vector also performs better than the MLV vector when used to express a complex multimeric protein such as a TCR, which consists of α and β chain proteins and requires additional proteins of the CD3 complex for transport to the cell surface. To exclude that the higher TCR expression level obtained with MP-tcr compared with L-tcr was due to higher viral titers and consequently multiple integrations, we employed the most objective criteria for titer estimation and analyzed the number of integrated viral copies in the transduced T cells. Both MP-tcr- and L-tcr-transduced T lymphocyte populations revealed similar amounts of integrated vector genomes, as shown by semiquantitative DNA PCR and real-time PCR (data not shown). Although similar viral titers were used, hardly any surface expression and no effector function of the transferred TCR were detected in L-tcr-transduced T lymphocytes. In contrast, the MP-tcr-transduced PBL bulk culture strongly expressed TCR-26 and responded to RCC-26 tumor cell stimulation with cytokine secretion and cytotoxicity.

The discrepancy between similar transduction efficiency and integration rates on the one hand and different transgene expression on the other is most likely ascribed to the different *cis*-regulatory elements of the two retroviral vectors. The strongest 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 [Willemsen *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-

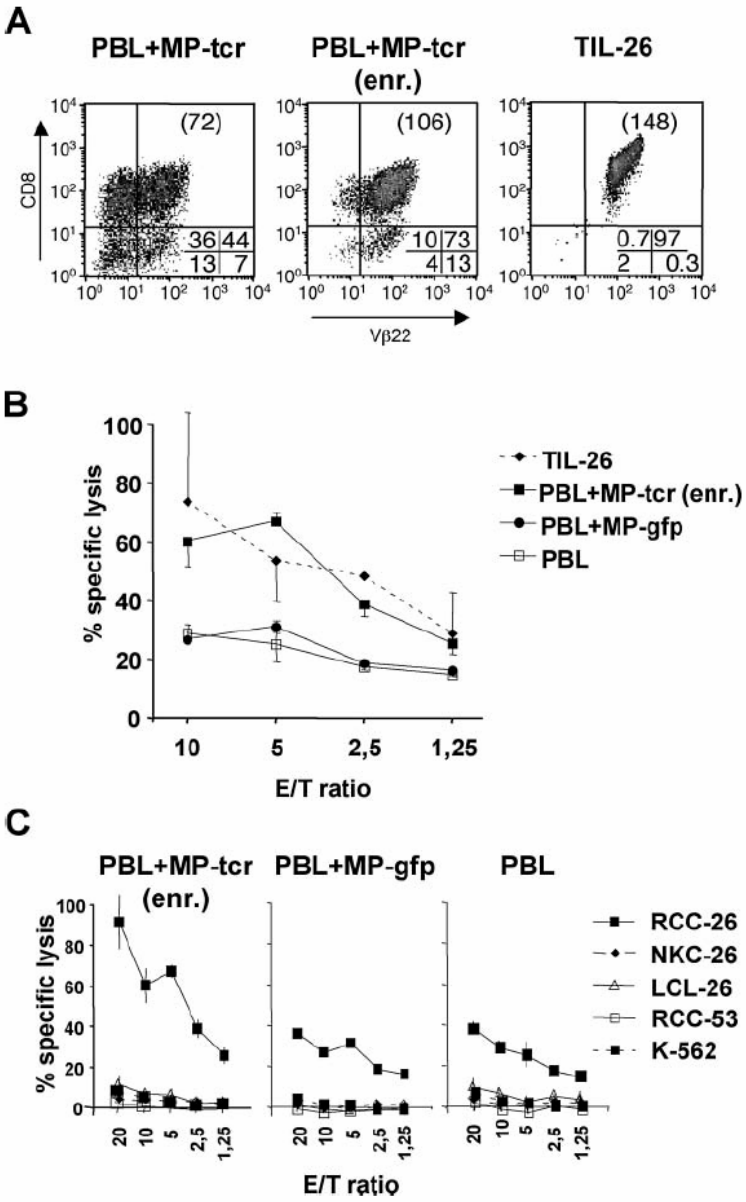

FIG. 6. MP-tcr-transduced PBLs lyse RCC-26 cells as efficiently as do TIL-26 cells. (**A**) Flow cytometric analysis of cells employed for the lysis assay. MP-tcr-transduced PBLs, $V_\beta 22$-immunoselected transduced PBLs (enr., enriched), and TIL-26 cells were stained for CD8 and $V_\beta 22$ one day after the lysis assay (cells were gated on live cells, of which >99% were CD3 positive). Numbers at bottom right of each panel indicate the percentage of cells in the quadrants; numbers in parentheses indicate the MFI for $V_\beta 22$. (**B** and **C**) Specific lysis was evaluated in a 4-hr ^{51}Cr release assay, employing 2×10^3 target cells and titered concentrations of effector cells, as indicated by the effector-to-target ratio (*E*/*T* ratio). Data shown represent mean values and standard deviations of duplicates from one experiment. Two independent experiments were performed and PBLs of three donors were analyzed on day 12 post-transduction. (**B**) Effectors, TIL-26 cells (dotted line, diamonds), MP-tcr-transduced PBLs (solid line, solid squares), MP-gfp-transduced PBLs (solid line, solid circles), and nontransduced PBLs (solid line, open squares). MP-tcr-transduced PBLs were enriched (enr.) for $V_\beta 22$-positive cells by MACS and used against RCC-26 target cells. (**C**) Effectors, MP-tcr-transduced, MP-gfp-transduced, and nontransduced PBLs, as indicated, were used against targets RCC-26 cells [solid line, solid squares; same results as shown in (**B**)], NKC-26 cells (dotted line, solid diamonds), LCL-26 cells (solid line, open triangles), RCC-53 cells (solid line, open squares), and K-562 cells (dotted line, solid squares).

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 mel-

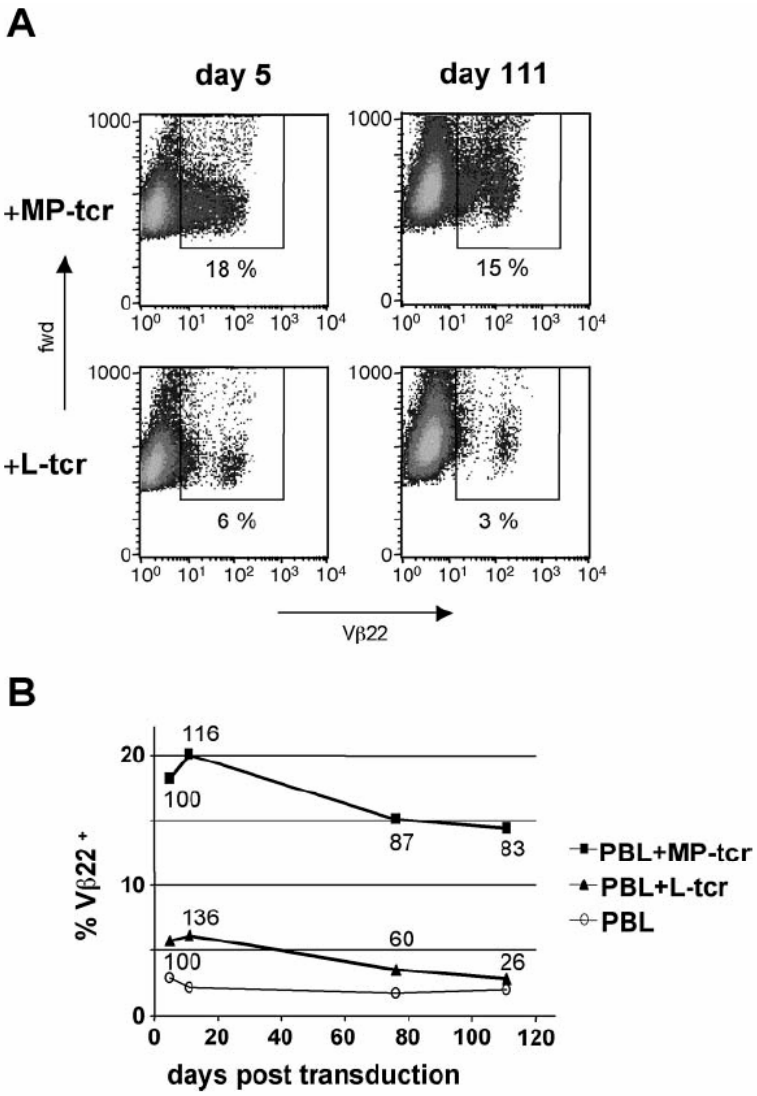

FIG. 7. TCR-26 expression is stable in MP-tcr-transduced PBLs. PBLs of two donors were transduced using either the L-tcr or the MP-tcr vector. Cells were analyzed for $V_\beta22$ expression on day 5, 11, 76, and 111 posttransduction. TCR β chain surface expression was visualized by staining with a PE-conjugated anti-$V_\beta22$ mAb. (**A**) Flow cytometry data obtained for PBLs of donor 1 transduced with L-tcr and MP-tcr from days 5 and 111 posttransduction. Dot blots of $V_\beta22$ staining against forward scatter (fwd) are shown. Regions marking $V_\beta22$-positive cells were defined according to isotype control staining. (**B**) The percentage of $V_\beta22$-positive cells is plotted against time (days posttransduction). The numbers indicate the percentage of initial $V_\beta22$ expression (based on total $V_\beta22$ expression minus endogenous $V_\beta22$ expression); numbers on day 5 posttransduction were used as reference value and set to 100%.

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-redirected 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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Address reprint requests to:
Dr. Wolfgang Uckert
Max Delbrück Center for Molecular Medicine
Robert Rössle Strasse 10
13092 Berlin, Germany

E-mail: wuckert@mdc-berlin.de

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