

T-Cell Receptor Gene–Modified T Cells with Shared Renal Cell Carcinoma Specificity for Adoptive T-Cell Therapy

Matthias Leisegang¹, Adriana Turqueti-Neves⁴, Boris Engels¹, Thomas Blankenstein^{1,2}, Dolores J. Schendel^{4,5}, Wolfgang Uckert^{1,3}, and Elfriede Noessner⁴

Abstract

Purpose: Adoptive therapy with genetically engineered T cells carrying redirected antigen specificity is a new option for the treatment of cancer. This approach is not yet available for metastatic renal cell carcinoma (RCC), due to the scarcity of therapeutically useful reagents. We analyzed tumor-infiltrating lymphocytes (TIL) from RCC to identify T-cell specificities with shared tumor-specific recognition to develop T-cell receptor (TCR)-engineered T lymphocytes for adoptive therapy of RCC.

Experimental Design: We established a T-cell clone from TIL that recognized a human leukocyte antigen (HLA)-A2–restricted tumor antigen. The TCR α - and β -chain genes were isolated, modified by codon optimization and murinization, and retrovirally transduced into peripheral blood lymphocytes (PBL). A TCR-expressing indicator line (B3Z-TCR53) was established to screen for antigen prevalence in RCC, other malignancies, and normal cell counterparts.

Results: TCR53-engineered PBL recapitulated the specificity of the TIL and showed tumor-specific HLA-A2–restricted effector activities (IFN- γ , tumor necrosis factor- α , interleukin-2, macrophage inflammatory protein-1 β , cytotoxicity). PBL-TCR53 of healthy donors and RCC patients exhibited similar transduction efficiency, expansion, and polyfunctional profile. Using B3Z-TCR53 cells, 130 tumor and normal cells were screened and shared TCR53 peptide: MHC expression was found in >60% of RCC and 25% of tumor lines of other histology, whereas normal tissue cells were not recognized.

Conclusions: To date, TCR53 is the only TCR with shared HLA-A2–restricted recognition of RCC. It fulfills the criteria for utilization in TCR gene therapy and advances T cell–based immunotherapy to patients with RCC and other malignancies expressing the TCR ligand. *Clin Cancer Res*; 16(8): 2333–43.

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The power of adoptive T-cell therapy in tumor eradication is best shown in chronic myeloid leukemia in which allogeneic donor lymphocyte infusion following hematopoietic stem cell transplantation can result in patient cure (1). Other successful applications include the treatment of

EBV-associated posttransplantation lymphoproliferative disease and EBV-associated cancers (2). The clinical success achieved in these malignancies is based on the expression of specific antigens by tumor cells, including minor histocompatibility antigens or tumor-associated viral antigens, which are targets for the adoptively transferred T lymphocytes. Recent data of three consecutive clinical trials of metastatic melanoma patients (3) give hope that solid tumors expressing nonviral antigens can also be successfully targeted by adoptive T-cell therapy if T cells with appropriate tumor-antigen specificity are identified. In these trials, remarkably high response rates of >50% were achieved following the infusion of *ex vivo* expanded tumor-infiltrating lymphocytes (TIL) that were selected for their antitumor reactivity. Recent advances in genetic engineering now allow normal lymphocytes to be endowed with selected T-cell receptor (TCR) genes (4), thus overcoming limitations due to poor proliferative capacity and poorly defined specificity of TIL. First clinical trials using TCR-engineered T cells attested to the feasibility of this approach (5, 6).

In renal cell carcinoma (RCC), a similar breakthrough has not yet occurred (7), although clinical data, including spontaneous remissions and the response to cytokine

Authors' Affiliations: ¹Max-Delbrück-Center for Molecular Medicine, ²Free University of Berlin, Institute of Immunology, and ³Humboldt-University of Berlin, Institute of Biology, Berlin, Germany; ⁴Institute of Molecular Immunology and ⁵Clinical Cooperation Group "Immune Monitoring", Helmholtz Zentrum München, Munich, Germany

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M. Leisegang, A. Turqueti-Neves, W. Uckert, and E. Noessner contributed equally to this work.

Current address for B. Engels: University of Chicago, Department of Pathology and Committee on Immunology, Chicago, Illinois, USA.

Corresponding Authors: Elfriede Noessner, Helmholtz Zentrum München, Institute of Molecular Immunology, Marchioninistraße 25, 81377 Munich, Germany. Phone: 49-89-7099303; Fax: 49-89-7099300; E-mail: noessner@helmholtz-muenchen.de and Wolfgang Uckert, Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Straße 10, 13092 Berlin, Germany. Phone: 49-30-94063196; Fax: 49-30-94063306; E-mail: wuckert@mdc-berlin.de.

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Translational Relevance

Recent achievements in T-cell immunology and molecular T-cell receptor (TCR) engineering have enabled new approaches to utilize adoptive T-cell transfer in clinical medicine. Despite evidence that renal cell carcinoma (RCC) is immunosensitive, the lack of therapeutically applicable T cells has hindered such advances for these patients. The TCR that we have identified has an exquisite specificity recognizing an antigen that is shared by >60% of RCC and 25% of other malignancies, while sparing normal cell counterparts. A transfer vector encoding the TCR sequences can be used as a generic reagent to imbue patient lymphocytes with antitumor function, including tumor-specific cytotoxicity and polyfunctional cytokine secretion. The identification of this new TCR specific for a broad panel of tumors expedites T cell-based immunotherapy to patients with RCC and other malignancies expressing the TCR ligand.

therapy (8–10), suggest that these tumors are susceptible to immune-mediated effector mechanisms. Furthermore, T cells were found in the tumors and blood of patients who had reactivity against RCC lines when tested *ex vivo* (11–20). Infusion of lymphokine-activated killer cells or TIL, alone or in combination with interleukin-2 (IL-2), provided benefit for a few patients (21). Improved response rates were seen with allogeneic hematopoietic stem cell transplantation, but this therapy is linked with severe graft-versus-host disease due to the limited tumor specificity of infused cells (ref. 22 and refs. in 7). It is conceivable that clinical benefit could be further improved if better suited T cells can be identified. However, there are few suitable T-cell specificities to spur clinical development, as most RCC-reactive T cells proliferate poorly, recognize antigens expressed only by a small set of tumors, or use infrequent MHC-restriction elements (23–26). This paucity is reflected by the fact that the only known TCR with broad RCC recognition is now undergoing clinical evaluation, although it recognizes RCC in an unknown nonclassical manner [ref. 27, and www.clinicaltrials.gov (NCT00870389)].

We used TIL as a source to isolate T cells that recognized RCC lines *ex vivo* (15). These T cells recognized naturally expressed RCC-associated antigens. For one T-cell specificity, we also showed that transfer of the TCR genes endowed normal lymphocytes with the same RCC-associated specificity (23). Although this showed proof of principle that transgenic TCR could be applied in RCC, the selected TCR recognized a unique ligand expressed only by the autologous tumor cells and, therefore, was not suitable for clinical development.

Thus, we characterized a new TIL reactive against several RCC lines and isolated the TCR genes from one derived T-cell clone. TCR expression in peripheral blood lympho-

cytes (PBL) transferred human leukocyte antigen (HLA)-A2-restricted cytotoxicity and cytokine release. Further, by use of a TCR-engineered indicator cell line, we provide evidence that this TCR recognizes a broadly expressed ligand present on tumor cell lines, but not expressed by nonmalignant primary cells, thus featuring hallmarks suitable for clinical development.

Materials and Methods

Cells. Cell lines (Tables 1 and 2) were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/mL penicillin-streptomycin, 1 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 1% nonessential amino acids (CM). PBL and TIL were cultured in T-cell medium (TCM; RPMI 1640 supplemented with 10% human serum, 100 U/mL penicillin-streptomycin, 1 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1% nonessential amino acids, and cytokines when indicated). Cells were incubated at 37°C and 6.5% CO₂ in a humidified chamber. Primary normal kidney cells were short-term cultures (passage 2 to 4) of cells from tumor-free kidney cortices obtained from RCC patients undergoing complete nephrectomy (Table 1). They were maintained in CM supplemented with 1% insulin-transferrin-selenium-X. Plat-E packaging cells (28), 293T (CRL-11268, American Type Culture Collection), B3Z (29) and Jurkat 76 cells (30) were cultured as described (31, 32). Peripheral blood mononuclear cells were obtained from heparinized venous blood of healthy donors and RCC patients by Ficoll-Hypaque (Biochrom) density gradient centrifugation.

TIL-53 was isolated from a primary clear cell (cc) RCC tumor (T2N1MxG2-3, 6 cm) following the published procedure (33). TIL-53 was restimulated once with irradiated (100 Gy) autologous IL-2-expressing RCC-53 (see ref. in 33). On day 10 after stimulation, TIL-53 was cloned by single-cell dilution using IL-2-expressing RCC-53 cells (5×10^3 , 100 Gy), allogeneic irradiated feeder PBL mixture (5×10^4 , 50 Gy), and B-LCL cells (1.5×10^4 , 100 Gy) in TCM supplemented with 100 U/mL rIL-2 and 2 ng/mL IL-7 (PromoCell GmbH). After 20 d, T-cell clones were transferred to 24-well plates and restimulated with 6×10^4 IL-2-expressing RCC-53 cells, allogeneic irradiated feeder PBL (2×10^6), and B-LCL cells (1×10^5). Unless otherwise indicated, all cell culture reagents were purchased from Invitrogen. The collection of blood and patient material was approved by the local ethics committee, and donors gave informed consent.

Transfection of HLA-A2 RNA into tumor and normal cells. The plasmid pCDM8-HLA-A2 encoding HLA-A*0201 cDNA (kindly provided by E. Weiß, Munich, Germany) was linearized with *NotI* (Fermentas) and used as *in vitro* transcription template to produce RNA using the mMES-SAGE mMACHINE T7 kit (Ambion). Cells (2×10^6) were resuspended in OptiMEM I medium (Invitrogen), placed in a 0.4-cm electroporation cuvette, and briefly incubated on ice. RNA (15 µg, 200 µL) was added and electroporation was done with Gene Pulser Xcell (Bio-Rad

Table 1. TCR53 p:MHC ligand prevalence among renal cell carcinoma and normal kidney cells

	HLA-A2	HLA-A2 transfection	B3Z-TCR53 IL-2 (pg/mL)
Renal cell carcinoma cells			
786-O*	–	+	63
A-498*	+	–	52
CCA-1	–	–	≤12
CCA-1	–	+	191
CCA-7	–	–	≤12
CCA-8	–	–	≤12
CCA-8	–	+	≤12
CCA-9	–	–	≤12
CCA-9	–	+	102
CCA-13	–	–	≤12
CCA-17	+	–	433
CCA-23	–	–	≤12
CCA-23	–	+	21
CCA-29	+	–	≤12
KT-2	+	–	20
KT-13	+	–	34
KT-15	+	–	152
KT-30	+	–	19
KT-53	+	–	21
KT-111	+	–	≤12
KT-187	–	–	≤12
KT-187	–	+	≤12
KT-195	–	–	≤12
KT-195	–	+	137
MZ-1257	+	–	61
MZ-2175	–	–	≤12
MZ-2175	–	+	555
RCA-1770	–	–	≤12
RCC-1.11	+	–	≤12
RCC-1.24	+	–	70
RCC-1.26	+	–	≤12
RCC-26	+	–	267
RCC-36	+	–	720
RCC-53	+	–	60
SKRC-12	–	–	≤12
SKRC-12	–	+	61
SKRC-17	+	–	≤12
SKRC-28	–	+	≤12
SKRC-38	–	+	≤12
SKRC-44	+	–	≤12
SKRC-59	+	–	≤12
Normal kidney cells			
NKC-2	+	–	≤12
NKC-3	+	–	≤12
NKC-4	+	–	≤12
NKC-6	–	–	≤12
NKC-7	+	–	≤12
NKC-26	+	–	≤12

Table 1. TCR53 p:MHC ligand prevalence among renal cell carcinoma and normal kidney cells (Cont'd)

	HLA-A2	HLA-A2 transfection	B3Z-TCR53 IL-2 (pg/mL)
NKC-32	–	–	≤12
NKC-33	+	–	19
NKC-36	–	+	≤12
NKC-37	+	–	≤12
NKC-38	–	+	≤12
NKC-39	–	+	≤12
NKC-40	–	–	≤12
NKC-40	–	+	≤12
NKC-41	–	+	≤12
NKC-42	+	–	≤12
NKC-43	+	–	≤12
NKC-47	–	–	≤12
NKC-49	+	–	≤12
RPTEC [†]	–	+	≤12

NOTE: B3Z-TCR53 were cocultured with indicated cells and IL-2 values are listed (12 pg/mL was the background value of B3Z-TCR53 cells alone). Cell lines were collected through laboratory exchanges or generated locally. CCAs (provided by Gerharz et al., ref. 42), as well as 786-0, A498, MZ-1257, RCC-1.11, RCC-1.24, RCC-1.26, RCC-26, RCC-36, and RCC-53 are of known cc histology. NKC are short-term cultures of cells of tumor-free kidney cortices. HLA-A2 transfection was done using RNA. The HLA-A2 status of all cell lines and cultures was confirmed by flow cytometry.

*Indicates RCC with described VHL-inactivating mutations. A variant of 786-0 expressing the wild-type VHL after transfection (786-0 VHLwt8, provided by W. Kaelin, ref. 43) induced similar amounts of IL-2 with those of its VHL^{–/–} parental line 786-0 (46 pg/mL; not shown).

[†]RPTEC 2814-3 is a primary renal proximal tubular epithelial cell purchased from BioWhittaker.

Laboratories) at 400 V and 5 ms. Immediately after electroporation, cells were returned to culture medium and incubated for 24 h at 37°C and 6.5% CO₂. Cell surface expression was determined by flow cytometry using a HLA-A2-specific antibody (ATCC: HB-54, see ref. in 23).

Isolation of TCR53 cDNA and construction of retroviral vectors. Total cellular RNA was isolated from T-cell clone TIL-53.29 using TRI Reagent (Molecular Research Center). Applying repertoire analysis (34) in-frame TCRVα3.1, Vα19, and Vβ20 were identified (Arden nomenclature; ref. 35). TCR53Vα19- and Vβ20-chain genes were amplified using gene-specific oligonucleotides, linked by a P2A peptide element to yield the transgene cassette 5'-TCRβ-P2A-TCRα-3', and integrated into the retrovirus vector MP71-PRE (31, 36). This vector was designated MP71-TCR53. To enhance TCR surface expression, the constant

Table 2. TCR53 p:MHC ligand prevalence among tumor and normal cells other than renal cell carcinoma or normal kidney cells

Other tumor cells (origin)		HLA-A2	HLA-A2 transfection	B3Z-TCR53 IL-2 (pg/mL)
624.38	(1)	+	−	≤12
93.04-A12	(1)	+	−	≤12
A-375	(1)	+	−	≤12
A-673	(2)	+	−	≤12
BLM	(1)	+	−	56
BOE	(3)	−	−	≤12
BOE	(3)	−	+	≤12
CCL-121	(2)	−	−	≤12
COLO-205	(4)	+	−	≤12
COLO-357	(5)	−	−	≤12
CRL-1543	(2)	+	−	≤12
CRL-1544	(2)	+	−	≤12
D-283	(6)	−	−	≤12
D-458	(6)	−	−	≤12
DU-145	(7)	−	+	≤12
EWING-AK	(2)	−	−	≤12
FADU	(8)	−	−	≤12
FADU	(8)	−	+	≤12
GRANTA-519	(3)	+	−	350
HBL-2	(3)	−	+	≤12
HT-29	(4)	−	+	≤12
HTC-116	(4)	+	−	≤12
JEKO-1	(3)	+	−	≤12
JVM-2	(3)	−	+	≤12
K-562	(9)	−	−	≤12
K-562	(9)	−	+	≤12
KARPAS-422	(3)	+	−	≤12
KELLY	(10)	−	−	≤12
L-428	(3)	−	−	≤12
LCL-1	(11)	+	−	57
LCL-2	(11)	+	−	≤12
LCL-3	(11)	+	−	≤12
LCL-4	(11)	+	−	25
LCL-26	(11)	+	−	≤12
LNCAP	(7)	+	−	≤12
MACA-1	(12)	−	−	≤12
MACA-1	(12)	−	+	≤12
MCF-7	(12)	+	−	≤12
MG-63	(2)	−	−	≤12
NALM-6	(3)	+	−	49
PANC-TU1	(5)	+	−	136
PC-3	(7)	−	+	≤12
PCI-1	(8)	+	−	≤12
SAO-S2	(2)	+	−	≤12
SK-23	(1)	+	−	≤12
SK-MEL25	(1)	+	−	≤12
SK-MEL29	(1)	−	−	≤12
SK-MEL29	(1)	−	+	≤12
SK-NSH	(10)	+	−	39
SKW-6	(3)	+	−	35

Table 2. TCR53 p:MHC ligand prevalence among tumor and normal cells other than renal cell carcinoma or normal kidney cells (Cont'd)

Other tumor cells (origin)		HLA-A2	HLA-A2 transfection	B3Z-TCR53 IL-2 (pg/mL)
SW-480	(4)	+	–	≤12
SW-620	(4)	+	–	≤12
TC-71	(2)	+	–	≤12
THP-1	(9)	+	–	13
U2-OS	(2)	+	–	≤12
U-87	(13)	+	–	≤12
U-251MG	(14)	+	–	45
U-373	(13)	+	–	155
UT-SCC-15	(8)	+	–	313
WM-115	(1)	+	–	≤12
WM-226.4A	(1)	+	–	≤12
Other normal cells (origin)				
BMEC	(15)	+	–	≤12
PBMC1-7*	(16)	+	–	≤12

NOTE: 3Z-TCR53 were cocultured with indicated cells and IL-2 values are listed (12 pg/mL was the background value of B3Z-TCR53 cells alone). Cell lines were collected through laboratory exchanges. HLA-A2 transfection was done using RNA. HLA-A2 status was determined by flow cytometry. The numbers in parenthesis after the cell name indicate the histologic origin: (1) melanoma, (2) sarcoma, (3) B-lymphocytic leukemia/lymphoma, (4) colon carcinoma, (5) pancreas carcinoma, (6) medullablastoma, (7) prostate carcinoma, (8) squamous carcinoma, (9) myelocytic leukemia, (10) neuroblastoma, (11) EBV-B-lymphoblastoid cell line, (12) breast carcinoma, (13) glioblastoma, (14) astrocytoma, (15) brain microvascular endothelial cells, and (16) peripheral blood mononuclear cells (PBMC).

*1-7 indicates PBMC of seven different donors.

regions of both TCR chain genes were exchanged by their mouse counterparts (37) and ligated into MP71-PRE as indicated above (MP71-TCR53m). In addition, the transgene cassette of MP71-TCR53m was optimized for most frequent human codon usage, while avoiding cryptic splice sites and RNA instability motifs (GENEART; ref. 38). This murinized and codon-optimized vector was designated MP71-TCR53mc. All constructs were verified by sequence analysis (MWG Biotech AG). Primer sequences will be provided on request.

Production of TCR retroviruses and transduction of cells. Amphotropic mouse leukemia virus (MLV)-pseudotyped retrovirus particles were produced by transient transfection of 293T cells using plasmids encoding Moloney-MLV-gag-pol, MLV-10A1-env, and MP71-TCR53, MP71-TCR53m, or MP71-TCR53mc, as described (31). Forty-eight hours after transfection, retroviral supernatant was harvested, filtrated, and used for transduction of activated

human PBL as described (31). TCR53 surface expression was determined at day 10 after transduction using anti-V β 20-phycoerythrin (PE; Immunotech) and anti-CD8 α -FITC-labeled antibodies (BD Pharmingen). Ecotropic MLV-pseudotyped retroviruses were produced by transfection of Plat-E packaging cells (28) with MP71-TCR53m or Bullet-CD8 α vector plasmids (ref. 39, kindly provided by R. Debets, Rotterdam, the Netherlands) and applied to transduce B3Z cells.

Generation of TCR53-expressing mouse B3Z indicator cells. A TCR53-expressing mouse B3Z indicator cell line (B3Z-TCR53), which contains the bacterial β -galactosidase gene under the control of the minimal human IL-2 promoter with binding sites for the nuclear factor of activated T cells (29), was established by successive transduction with the retrovirus vectors MP71-TCR53m and Bullet-CD8 α . Expression of TCR53 and human CD8 α was analyzed using TCR53-specific anti-V β 20-PE- and anti-CD8 α -FITC-labeled antibodies. Twenty-four-hour cocultures with target cell lines were done in 96-well round bottom plates (BD Falcon) using 1×10^5 B3Z-TCR53 cells [effector to target (E:T) ratio = 1:1]. B3Z-TCR53 cells were incubated with $1 \mu\text{mol/L}$ ionomycin (Calbiochem) and 5 ng/mL phorbol-12-myristate-13-acetate (PMA; Promega) for TCR-independent stimulation. Supernatants of cocultures were harvested and IL-2 content was determined by ELISA following the manufacturer's protocol (eBioscience). Measurement of intracellular β -galactosidase activity was done as described (32).

Tumor necrosis factor- α quantification by WEHI cytotoxicity assay. TIL (3×10^3 cells/100 μL) in TCM supplemented with 30 U/mL rIL-2 was added to adherent target cells (1.5×10^4 /well) in 96-well flat bottom plates (BD Falcon). Where indicated, $4 \mu\text{g}$ of an anti-HLA-A2 antibody (ATCC: HB-54) was added. TIL and target cells cultivated alone were used to determine background tumor necrosis factor- α (TNF- α) secretion. After 24 h, supernatants were collected and assayed for TNF- α content by measuring the cytotoxicity against WEHI 164 clone 13 as described (40).

Cytokine release assay and HLA-A2 blocking. Cocultures of TCR53 gene-modified PBL and target cells were done as described (31) but using 1.5×10^4 target cells (E:T = 5:1). Twenty-four-hour supernatants were harvested and analyzed for their IFN- γ content using ELISA (BD Bio-sciences). Antibody blocking of HLA-A2 (ATCC: HB-54) was done as described (23).

Cytotoxicity. Cell-mediated lysis of target cells by TCR53 gene-modified PBL was determined by a standard 4-h chromium release assay. Target cells were labeled for 1 h with $50 \mu\text{Ci}$ sodium chromate-51 (Hartmann GmbH) and incubated with TCR53-, GFP- or nonmodified PBL using E:T ratios from 20:1 to 2.5:1 by employing 2×10^3 target cells, as described (23). All measurements were done as duplicates and K-562 and Daudi cells were used as controls. Data are shown as mean values $\pm$ mean deviation.

Multiparameter flow cytometry. Cytokine secretion and degranulation analysis were done using 2×10^5 PBL in-

cubated with target cells at a ratio of 1:1 for 12 h. After 1 h, GolgiStop and Brefeldin-A (BD Biosciences) were added. Degranulation was detected by the addition of anti-CD107a-FITC and anti-CD107b-FITC during the stimulation phase. Membrane staining was done with anti-CD45-Amcyan and anti-CD4-APC-A750 in FACS buffer (PBS, 2% FCS, 2 mmol/L EDTA) for 20 min at 4°C , followed by addition of 7-amino-actinomycin-D ($10 \mu\text{g/mL}$, 20 min; Sigma Aldrich). Cells were fixed with 1% of paraformaldehyde (Merck) for 20 min followed by permeabilization using two consecutive washes with 0.1% and 0.35% saponin (Sigma Aldrich). Intracellular staining was done with anti-mouse-TCR β -PE (detecting the mouse constant region of TCR53 β -chain), anti-CD8-PacificBlue, anti-IFN- γ -PE-Cy7, anti-IL-2-APC, and anti-TNF- α -A700 antibodies for 30 min. All antibodies were purchased from BD Pharmingen. Data acquisition was done with LSRII (BD Pharmingen) and data were analyzed using FlowJo (TreeStar).

Results

RCC-infiltrating T cells show broad tumor specificity and HLA-A2 restriction. TIL, isolated from a primary ccRCC tumor of patient 53 (TIL-53, T2N1MxG2-3), displayed specificity for an antigenic epitope that is shared among RCC tumors and restricted by the MHC class I allotype HLA-A2 as shown by blocking with an antibody directed against HLA-A2. Importantly, normal kidney cultures were not recognized by TIL-53 (Fig. 1A and B). T-cell receptor repertoire analysis of the TIL-53 population after

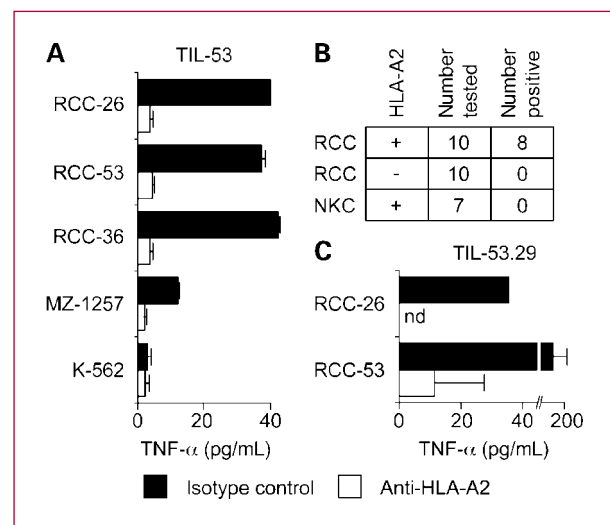


Fig. 1. HLA-A2-restricted recognition of RCC by TIL-53 and T-cell clone TIL-53.29. TIL-53 (A) or TIL-53.29 cells (C) were cocultured with indicated tumor cell lines in the presence of HLA-A2 blocking antibody or an isotype control antibody for 24 h. Recognition of target cells was determined in a WEHI assay. Results are expressed as TNF- α $\pm$ mean deviation of triplicates. nd = not done. B, summarized screen of renal cell carcinoma (RCC) cell lines and normal kidney cells (NKC) by using TIL-53.

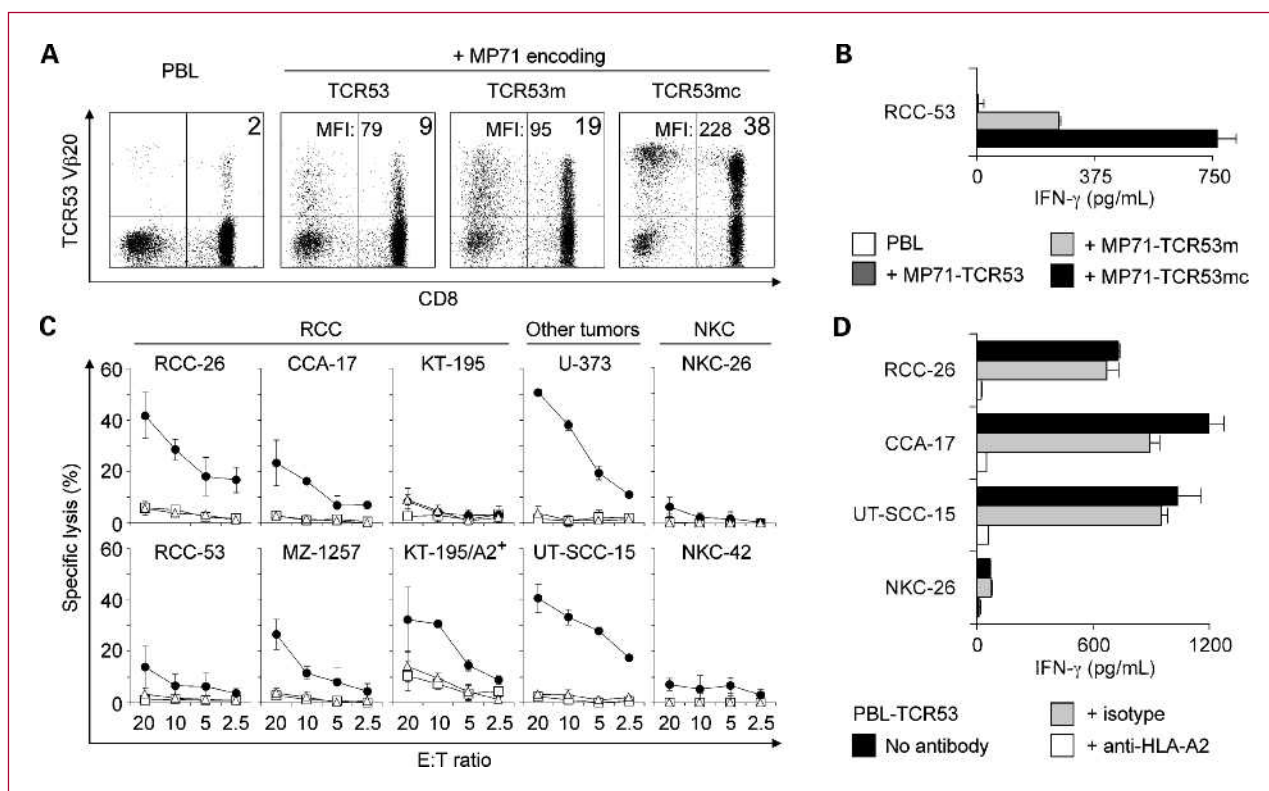


Fig. 2. Enhanced cell surface expression of optimized TCR53 genes and functionality of TCR53-transduced PBL. **A**, FACS analysis of nontransduced PBL (PBL) or PBL transduced with the retrovirus vector MP71-PRE encoding TCR α - and β -chain cDNAs linked by the P2A element using wild-type TCR53 genes (TCR53), murinized (TCR53m) or murinized and codon optimized (TCR53mc) variants. TCR53 β -chain expression was determined using an anti-V β 20-specific antibody. Numbers are the percentage of V β 20⁺CD8⁺ T cells, MFI values refer to the V β 20 intensity of these cells. Shown is one representative experiment of three. **B**, PBL, PBL transduced with MP71-TCR53, MP71-TCR53m, or MP71-TCR53mc were cocultured with RCC-53 cells and IFN- γ release was determined by ELISA. Bars, mean of duplicates $\pm$ mean deviation. Shown is one representative experiment of three. **C**, cell-mediated cytotoxicity of titrated numbers of MP71-TCR53mc-transduced PBL (●; transduction efficiency, 20%), GFP-transduced PBL (Δ), or nontransduced PBL (□) against HLA-A2⁺ RCC tumor cell lines (RCC-26, RCC-53, CCA-17, MZ-1257), cell lines of other tumor histology [glioblastoma (U-373), squamous cell carcinoma (UT-SCC-15)], HLA-A2⁺ normal kidney cells (NKC-26, NKC-42), HLA-A2⁻ (KT-195) RCC tumor line, and HLA-A2-transfected KT-195 cells (KT-195/A2⁺). Percent specific lysis values are means of duplicates $\pm$ mean deviation. E:T ratio refers to the total CD3 cell number. **D**, IFN- γ secretion using PBL-TCR53 in the presence or absence of HLA-A2 blocking antibody or an isotype control antibody. Bars, mean values of duplicates $\pm$ mean deviation. Experiments were repeatedly done with PBL of four different donors. One set of representative data is shown.

three *in vitro* stimulations with autologous IL-2 secreting RCC-53 cells revealed oligoclonality with 9 rearranged TCRV α and 14 TCRV β gene segments (not shown). Using limited dilution cloning, 106 T-cell clones were recovered and 14 showed RCC-53 recognition. T-cell clone TIL-53.29 recapitulated HLA-A2 restriction and the TIL-53 reactivity pattern when tested against selected target cells (Fig. 1C). Poor cell growth limited further detailed characterization of the parental TIL-53 and the derived T-cell clone. TCR sequences of TIL-53.29 revealed one in-frame TCR β sequence V β 20 (Arden nomenclature; ref. 35), which in accordance with the ImMunoGeneTics (IMGT) data base (41) is TCRBV30*01-J2-5*01-D2*01 (CDR3: CAWSTLGYRETQYF), and two in-frame TCR α sequences V α 3.1 (TCRAV17*01-J21*01; CDR3: CDPNFKFYF) and V α 19 (TCRAV41*01-J13*02; CDR3: CAFFSGGYQKVF).

Enhanced surface expression and functional performance of modified TCR53 chain genes. Because the sequence analysis of the TIL-53.29 cDNA revealed two TCR α and

one TCR β sequence, each TCR α -chain gene was combined with the TCR β -chain gene in the retroviral vector MP71-PRE and used to transduce TCR-deficient Jurkat 76 cells. Here, only the combination of TCRV α 19 and TCRV β 20 genes resulted in surface expression of TCR53 (not shown). In primary human T cells, sufficient expression of this combination was only achieved when additional optimizations of the TCR sequences were done. Replacement of human constant regions by mouse counterparts (murinization, TCR53m) or murinization and codon optimization (TCR53mc) yielded improved TCR expression. Transduction of primary human T cells using titer-equilibrated MP71-TCR53 variants resulted in 9% [mean fluorescence intensity (MFI) 79] of CD8⁺TCRV β 20⁺ cells when the nonmodified TCR53 was applied, whereas TCR53m and TCR53mc yielded 19% (MFI 95) and 38% (MFI 228) of CD8⁺TCRV β 20⁺ cells, respectively (Fig. 2A). Importantly, TCR53 sequence optimization clearly improved functionality of engineered PBL, as seen by superior

target-specific cytokine response of PBL transduced with MP71-TCR53mc (Fig. 2B).

TCR53 gene-modified PBL show tumor-associated multifunctionality. To further evaluate whether TCR53 antigen specificity could be transferred, PBL of healthy donors were transduced with MP71-TCR53mc and the specificity of TCR53 gene-modified T cells was determined in a cytotoxicity assay using different HLA-A2⁺ or HLA-A2⁻ tumor cell lines or primary kidney cultures. As shown in Fig. 2C, TCR53-transduced PBL (PBL-TCR53) showed specific lysis of HLA-A2⁺ RCC lines, exemplarily shown for RCC-26, RCC-53, CCA-17, MZ-1257, and tumor cell lines of glioblastoma (U-373) and squamous cell carcinoma (UT-SCC-15). The specific lytic activity against the HLA-A2-transfected RCC line KT-195 (KT-195/A2⁺), but not the parental HLA-A2⁻ KT-195 cell line confirmed the HLA-A2 restriction of PBL-TCR53. Primary cultures of HLA-A2⁺ normal kidney cells (NKC) were not recognized; exemplarily shown are NKC-26 and NKC-42 (Fig. 2C). The cytotoxicity was TCR53 mediated, as GFP- and nontransduced PBL showed no lytic activity. Furthermore, PBL-TCR53 secreted IFN- γ only in response to the HLA-A2⁺ RCC-26, CCA-17, and UT-SCC-15, but not in response to the HLA-A2⁺ normal kidney line NKC-26. IFN- γ secretion was blocked by anti-HLA-A2 antibody (ATCC: HB-54), but not by an isotype control antibody, attesting the HLA-A2 restriction (Fig. 2D).

The secretion of cytokines was studied using a multiplex bead assay system that included the simultaneous measurement of IFN- γ , TNF- α , IL-2, and macrophage inflammatory protein-1 β . All four cytokines were detected in supernatants of PBL-TCR53 cocultured with TCR53 p: MHC ligand-positive tumor cells, but were absent in cocultures with TCR53 p:MHC-negative cells or PBL-GFP (Supplementary Fig. S1), thus showing a strong proinflammatory response profile of PBL-TCR53.

Using flow cytometry for cytokine secretion (IFN- γ , TNF- α , IL-2) and lytic granule exocytosis (CD107), the polyfunctional effector response of PBL-TCR53 was analyzed on a single-cell level. RCC-26 cells were used as target cells. Antibody specific for the mouse constant region of the TCR53 β -chain was included in the multichromatic staining to evaluate whether the observed effector functions were performed by T cells expressing the transgenic TCR53. Stimulation with RCC-26 caused CD8⁺ T cells to degranulate and secrete cytokines TNF- α , IFN- γ , and IL-2 (Fig. 3A, left, black and green populations). The majority of the responding CD8⁺ T cells carried the TCR53 receptor (Fig. 3A, green population). The most prominent effector activities of PBL-TCR53 were lytic granule release (CD107⁺, 28%) and IFN- γ secretion (29%) followed by IL-2 (22%) and TNF- α (19%; Fig. 3A, left, green population). Nontransduced PBL showed very low background activity (Fig. 3A, right). Boolean gating analysis of the responding CD45⁺CD8⁺TCR53⁺ T-cell population (Fig. 3B and C) revealed high polyfunctionality with 55% of the responding TCR53⁺ T cells displaying two or more functions, including the secretion of IL-2. The multifunctional

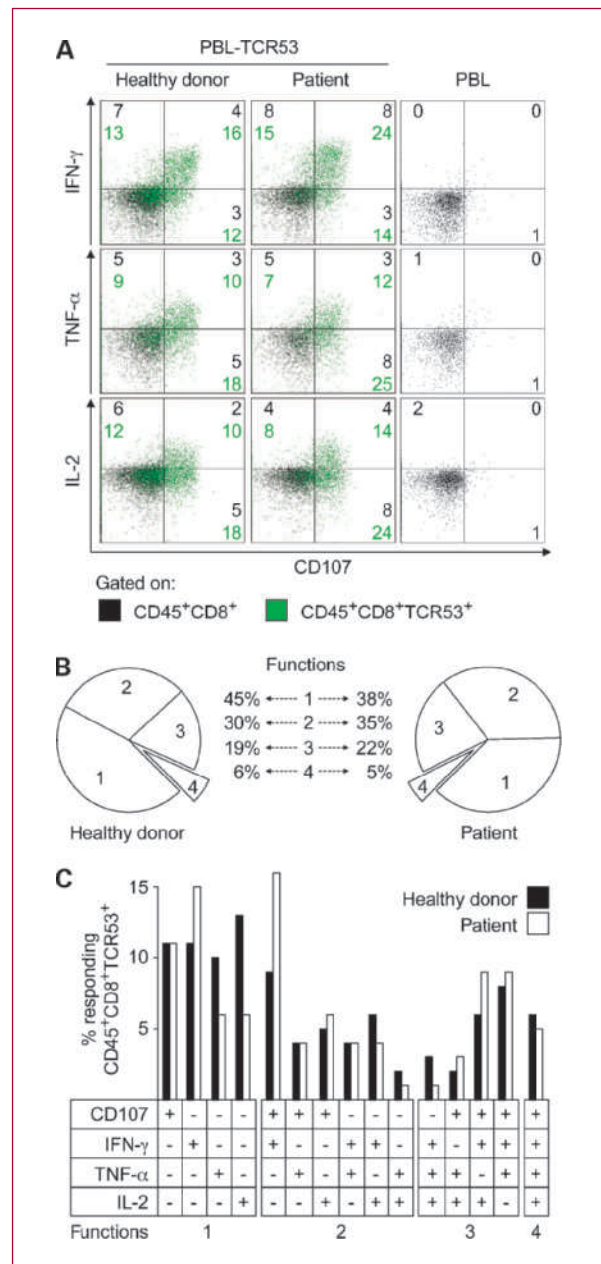


Fig. 3. TCR53 gene-modified PBL of healthy donors and RCC patients show complex effector responses against TCR53 p:MHC-expressing cell lines. **A**, TCR53 gene-modified or nontransduced PBL of one healthy donor and RCC patient 1 were used on day 9 after TCR transduction to analyze the polyfunctional response profile. Transduced PBL were incubated with TCR53 p:MHC⁺ RCC-26 and analyzed for cytokines (IFN- γ , TNF- α , IL-2) and lytic granule release (CD107) in an 8-color combination staining. T cells were selected using CD45. Dot plots of the gated CD45⁺CD8⁺ T cells (black) or CD45⁺CD8⁺TCR53⁺ T cells (green) display two functional parameters. Numbers, percentage of cells within the respective quadrant. **B**, **C**, results of boolean gating analysis for the responding CD45⁺CD8⁺TCR53⁺ T cells after coculture with RCC-26. The pie chart (**B**) summarizes the fractions of PBL-TCR53 showing one, two, three, or four functional responses. Similar results were obtained with PBL of other healthy donors and RCC patients (Supplementary Fig. S2) and when using other TCR53 p:MHC⁺ tumor cell lines for stimulation (not shown).

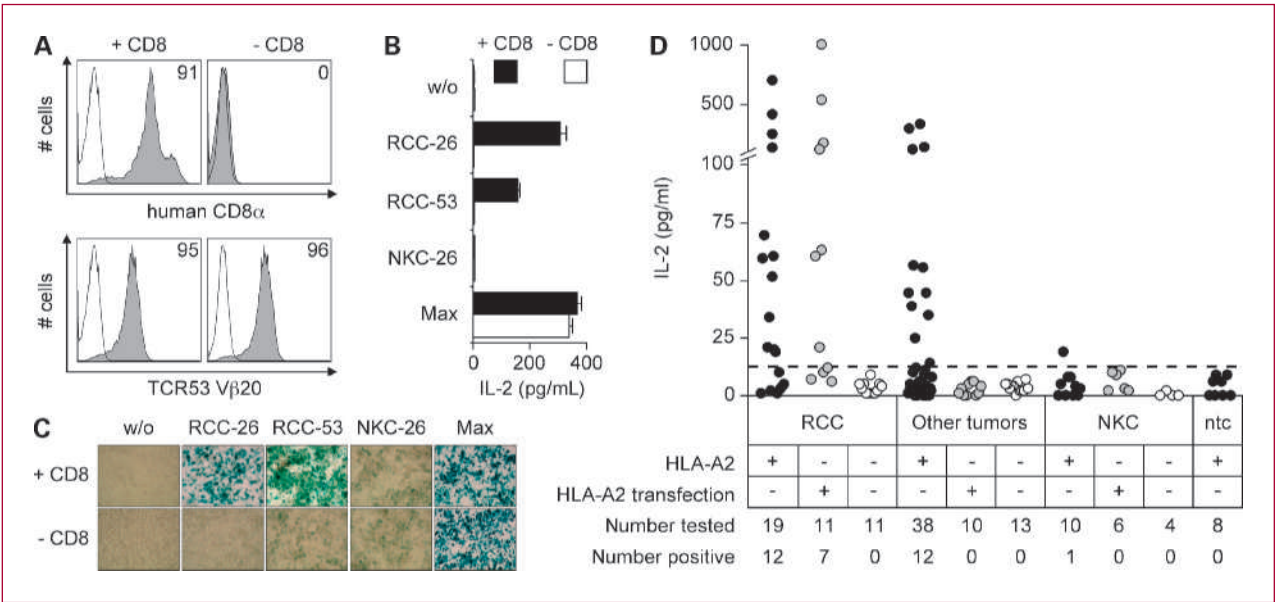


Fig. 4. Specificity of the B3Z-TCR53 indicator cell line and prevalence of the TCR53 p:MHC ligand among tumor cell lines and cells of normal tissues. A, surface expression of human CD8 α and TCR53V β 20 on B3Z-TCR53 cells was detected by flow cytometry. Numbers, percentage of V β 20 $^{+}$ or human CD8 α^{+} B3Z cells (grey curves). B, B3Z-TCR53 cells transduced or nontransduced with human CD8 α were cocultured alone (without) or with cell lines RCC-26, RCC-53, and NKC-26 for 24 h. B3Z-TCR53 cells were incubated with ionomycin and PMA for TCR-independent stimulation (Max). IL-2 concentration of supernatants was determined by ELISA. Bars, mean of duplicates $\pm$ mean deviation. C, B3Z-TCR53 cells were cocultured as in B, fixed, and β -galactosidase was detected by X-Gal staining. D, B3Z-TCR53 cells were cocultured with indicated tumor cell lines and cells of normal tissues for 24 h and IL-2 production was quantified by ELISA. Dashed line, IL-2 background level produced by the indicator cell line alone (12 pg/mL); each dot, the IL-2 value of B3Z-TCR53 cells after coculture with one specific cell line (see Tables 1 and 2) of the group. RCC, $n = 41$; other tumors, $n = 61$; NKC, $n = 20$; ntc, (nontumor cells), $n = 8$. For each group, HLA-A2 $^{+}$ (closed), HLA-A2-transfected (grey), and HLA-A2 $^{-}$ (open) cells are shown. For individual results see Tables 1 and 2.

response of PBL-TCR53 was similar to PBL which were transgenic for a HLA-A2-restricted tyrosinase₃₆₉₋₃₇₇ peptide-specific TCR (TCR-D115; ref. 42; data not shown).

TCR53 is efficiently expressed and highly functional in PBL of RCC patients. Next, we investigated the transduction efficacy and functional profile of PBL of five RCC-patients (Supplementary Fig. S2 and not shown). All patients had progressive metastatic disease, and two of them had received several applications of IFN- α and 5-fluorouracil prior to blood donation. Despite the poor clinical status and the exposure to immunotherapeutics and chemotherapeutics, PBL were efficiently transduced with TCR53 (Supplementary Fig. S2A), displayed tumor-specific lytic activity (Supplementary Fig. S2A and B) and multifunctionality profiles that were indistinguishable from those of PBL-TCR53 of healthy donors (Fig. 3, Supplementary Fig. S2C). TCR53-transduced PBL of healthy donors and patients could be successfully expanded *ex vivo* following the protocol utilized for clinical application (ref. 6; Supplementary Fig. S2A) and were highly lytic and polyfunctional on day 20 of expansion (not shown).

Specific recognition of TCR53 p:MHC ligand by the indicator cell line B3Z-TCR53. An indicator cell line, based on the mouse hybridoma B3Z, was generated to evaluate the distribution of the p:MHC ligand specifically recognized by TCR53. By retroviral transfer, the genes encoding the human CD8 α chain and the TCR53 chains were intro-

duced into B3Z cells. Transduced cells (B3Z-TCR53) showed stable expression of human CD8 α and TCR53V β 20 (Fig. 4A, grey curves). These cells secreted IL-2 after incubation with TCR53 p:MHC $^{+}$ RCC-26 and RCC-53 tumor cell lines, but not after coculture with NKC-26, which is TCR53 p:MHC $^{-}$ (Fig. 4B). This recognition profile could be confirmed by measuring the intracellular activity of β -galactosidase (Fig. 4C). As B3Z-TCR53 cells were not stimulated in the absence of human CD8 α , recognition of the antigenic p:MHC complex is CD8 dependent (Fig. 4B and C).

High incidence of TCR53 p:MHC in RCC cells and in tumor cells of other histology. B3Z-TCR53 cells were used to determine the frequency of TCR53 p:MHC ligand occurrence in RCC and tumors of other histologies, as well as in nontransformed cell lines of normal tissue. Twelve of 19 (63%) HLA-A2 $^{+}$ RCC cell lines tested were found to stimulate IL-2 secretion after coculture with B3Z-TCR53 cells above background (B3Z-TCR53 alone, 12 pg/mL; Fig. 4D, Table 1). Furthermore, HLA-A2 $^{-}$ RCC cell lines were transfected with HLA-A2 mRNA and HLA-A2 expression was confirmed by flow cytometry (not shown). After coculture of these HLA-A2-transfected cells with B3Z-TCR53 cells, 7 of 11 RCC lines (64%) induced IL-2 secretion (Fig. 4D, Table 1). Fifteen of the 30 HLA-A2 $^{+}$ RCC lines are known ccRCC (Table 1; refs. 43, 44); none of the remaining are of defined non-cc histology. B3Z-TCR53 recognition was observed for 11 (73%) of the ccRCC and 9 (60%) of the unknown RCC histology.

Cell lines 768-0 and A498 are examples of ccRCC with von Hippel Lindau (VHL)-inactivating mutations. Both cell lines stimulated B3Z-TCR53 cells to secrete IL-2, as did the 786-0 variant that expressed wild-type VHL after stable transfection (ref. 44; Table 1). Among the 15 known ccRCC, 4 were not recognized by B3Z-TCR53 cells, suggesting that the presence of the TCR53 epitope is not necessarily linked to the cc histology.

In addition, HLA-A2⁺ tumor lines of other origin (25%), like B-lymphoblastoid cells (LCL-1, LCL-4) and B-lymphocytic lymphoma (NALM-6, SKW-6, GRANTA-519), brain tumor lines (U-373, SK-NSH, U-251MG), melanoma (BLM), pancreatic adenocarcinoma (PANC-TU1), and squamous cell carcinoma (UT-SCC-15) were able to stimulate the indicator cell line (Fig. 4D, Table 2). Furthermore, 24 normal cell cultures, which either expressed HLA-A2 naturally or after mRNA transfection, were tested for TCR53 p:MHC ligand expression. These included 16 HLA-A2⁺ NKC, 7 peripheral blood mononuclear cells, and 1 endothelial cell culture. Only one of the normal cells (NKC-33) induced marginal IL-2 secretion (Tables 1 and 2).

Discussion

We characterized T cells infiltrating RCC tumors and defined various reactivity patterns, including both MHC-restricted and non-MHC-restricted specificities (15). The poor proliferative capacity of most TIL precluded their further development into clinically applicable reagents. Technological advances in TCR transfer overcome constraints of poor proliferation, and T cells with desired specificities can be generated through recovery of their TCR sequences from T-cell clones. In our studies of various RCC infiltrates, we identified one TIL population that displayed the necessary requirements for clinical application, including HLA-A2 restriction and recognition of a shared antigen expressed by RCC that was absent in normal kidney cells. The limited growth capacity of the parental TIL-53 and a derivative T-cell clone, TIL-53.29, was overcome through TCR cloning and transfer into recipient lymphocytes. Thereby, unlimited numbers of T cells became available to carry out functional testing of recognition of tumor and nontumor cell lines. The results of these extensive specificity studies revealed the potential clinical value of this HLA-A2-restricted TCR for use in adoptive T-cell therapy.

However, the wild-type TCR53 was poorly expressed in PBL, and modification of the TCR α - and β -chain sequences, particularly the use of both murinization (37) and codon optimization (38), was required for efficient cell surface expression and functionality. This is in agreement with recently published data regarding several other TCR, which were only efficiently expressed to yield TCR gene-modified T cells with high functional avidity after optimization of the TCR genes (4).

To achieve clinical efficacy in adoptive T-cell therapy, it is essential that TCR-transgenic T cells show highly specific

tumor recognition while ignoring normal cells. In addition, they should mediate multiple effector functions, including tumor cell lysis and cytokine secretion which, in turn, can activate other accessory components of the immune system. Indeed, recent studies have shown that the capacity of a T-cell population to simultaneously exert multiple effector functions is an essential requirement for effective immune responses (45, 46). The transfer of optimized TCR53 genes enabled PBL to mediate highly effective lysis of various tumor cell lines. This was accompanied by the production of T_H1 cytokines and the secretion of IL-2, parameters that were recently described as characteristic markers of optimally effective T cells (46). Further analysis by multiparameter flow cytometry revealed that the majority of T cells exhibited two or more effector functions simultaneously. The functional response of PBL-TCR53 was restricted to tumor cells, whereas cells of normal tissues were not recognized. Because TCR53 gene-modified T cells of several healthy donors achieved high functionality, this TCR can be considered as an effective reagent to redirect T-cell specificity. PBL of RCC patients were also efficiently transduced with TCR53 and had similar lytic and polyfunctional profile with PBL-TCR53 of healthy donors. All PBL-TCR53 could be expanded to high numbers *ex vivo* and maintained the multifunctional response profile, thus displaying qualities imperative to therapeutic application.

Another important parameter to judge the potential therapeutic value of a TCR is the prevalence of TCR ligand expression among tumor cells. The generation of an indicator cell line based on the coexpression of human CD8 α and TCR53 in mouse B3Z hybridoma cells allowed extensive screening of a large panel of tumor and normal cell cultures. We analyzed 130 cell lines and primary cells from different histologies, including 41 RCC, 18 hematopoietic malignancies, 10 melanoma, 9 sarcoma, 7 brain tumor lines, 5 colon carcinoma, 4 squamous carcinoma, 3 each of prostate carcinoma and breast carcinoma, and 2 pancreatic carcinoma, as well as 20 primary cultures of normal kidney tissues, 7 hematopoietic cells, and 1 endothelial cell. This screening recapitulated the original TIL-53 specificity and firmly established: (a) HLA-A2 restriction, (b) shared expression of the TCR53 p:MHC ligand in 63% of RCC and 25% of other tumor entities, and (c) tumor selectivity with only 1 of 24 HLA-A2⁺ normal cells being marginally recognized. CcRCC is the most common type of RCC and has in most cases characteristic VHL-inactivating mutations that are not found in non-ccRCC or tumors of other histologies (9, 44). In our panel, 11 of 15 HLA-A2⁺ ccRCC lines (73%) were recognized by B3Z-TCR53 cells. Due to the absence of RCC with defined non-cc histologies our panel does not allow an assumption on the TCR53 antigen prevalence in ccRCC versus RCC of non-cc histology. It is, however, very unlikely that the TCR53 epitope is derived from VHL, as RCC with mutant VHL, or transfected wild-type VHL, were equally well recognized. Moreover, other tumor cell lines and normal cells, which mostly have wild-type VHL, were generally

not recognized by TCR53. Among the non-RCC tumors, the TCR53 p:MHC ligand expression was frequently found in malignant B cell lines and B-LCL (5 of 13, 38%), whereas there was no evidence of expression in sarcomas, and prostate and breast carcinomas. Malignant T-cell lines were not included in our analysis. Treatment of tumor cells with IFN- α , but not IFN- γ , enhanced TCR53 p:MHC ligand expression. Unlike IFN- γ , IFN- α did not increase levels of HLA-A2, thus it apparently regulates TCR53 p:MHC ligand expression either by increasing antigen expression levels or by improving antigen processing independently of MHC class I (not shown).

At present, the molecular nature of the TCR53-stimulatory antigen is not known. A screen (using peptide-loaded T2 cells and ELISPOT) of several common epitopes with predicted HLA-A2 binding motifs that were found by others to be frequently overexpressed in RCC compared with normal kidney tissues, including G250 (47), Her2/neu, WT-1, MUC-1, hTERT, VEGF, various MAGE epitopes, and survivin (peptide sequences; see ref. 48) was negative. Additionally, the TCR53-recognition pattern of RCC cell lines did not coincide with HERV-E expression (defined by PCR, ref. 26), and B3Z-TCR53 cells did not recognize the cell line (HT-29), which is positive for the commonly RCC-expressed 5T4 antigen (49, 50). From these data, we conclude that the epitope recognized by TCR53 is different from those presently known for RCC. The identification of this new tumor-associated antigen is now feasible through the use of TCR-gene transfer to generate sufficient cell numbers for the screening procedure.

At present, immunotherapeutic options are limited for the treatment of metastatic RCC. For adoptive therapy of melanoma (3, 5, 6) several candidate TCR are currently under clinical evaluation. The here described TCR53 is the first TCR with HLA-A2-restricted shared recognition of RCC. With its broad tumor-specific multifunctional response, which includes the secretion of IL-2, and the restriction by the prevalent HLA-A2 class I molecule, TCR53 gene-modified T cells are well suited for adoptive

T-cell therapy. TCR53 complements the recently described TCR with a nonclassical RCC recognition (27), offering the therapeutic option to target RCC tumors through MHC-restricted and nonrestricted effector mechanisms, thereby limiting immune escape for patient benefit. In the clinical situation, the B3Z-TCR53 indicator cell line can be employed to screen biopsies to identify TCR53 p:MHC ligand expression on tumor cells, enabling individualized application of TCR53 gene-modified T cells to those patients with TCR53 p:MHC ligand-positive tumors who will most likely benefit from the therapy. The specific upregulation of TCR53 p:MHC ligand expression on tumor cells by IFN- α suggests that adoptive T-cell therapy with TCR53 gene-modified T cells could be usefully combined with IFN- α treatment, a practiced therapy of RCC that achieves approximately 15% response rates (10), to improve clinical benefit.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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