T cell receptor gene-modified T cells with shared renal cell carcinoma specificity for adoptive T cell therapy

Matthias Leisegang* (1), Adriana Turqueti-Neves* (2), Boris Engels[$] (1), Thomas Blankenstein (1, 3), Dolores J. Schendel (2, 4), Wolfgang Uckert[§] (1, 5) and Elfriede Noessner[§] (2)

(1) Max-Delbrück-Center for Molecular Medicine, Berlin, Germany

(2) Helmholtz Zentrum München, Institute of Molecular Immunology, Munich, Germany

(3) Free University of Berlin, Institute of Immunology, Berlin, Germany

(4) Helmholtz Zentrum München, Clinical Cooperation Group "Immune Monitoring", Munich, Germany

(5) Humboldt-University of Berlin, Institute of Biology, Berlin, Germany

([$]) present address: University of Chicago, Department of Pathology and Committee on Immunology, Chicago, Illinois, USA

([*,§]) These authors contributed equally to this work.

Correspondence to:

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

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

Running title: RCC-reactive TCR with shared antigen specificity

Key words: RCC, adoptive therapy, TCR gene transfer, retrovirus vector, indicator cell line

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 more than 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.

ABSTRACT

Purpose: Adoptive therapy with genetically engineered T cells carrying redirected antigen specificity is a new option to treat 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 an 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 demonstrated tumor-specific HLA-A2-restricted effector activites (IFN-γ, TNF-α, IL-2, MIP-1β, 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 more than 60% of RCC and 25% of tumor lines of other histology, while 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.

INTRODUCTION

The power of adoptive T cell therapy in tumor eradication is best demonstrated in chronic myeloid leukemia where allogeneic donor lymphocyte infusion following hematopoietic stem cell transplantation (HSCT) can result in patient cure (1). Other successful applications include the treatment of Epstein Barr Virus (EBV)-associated post-transplantation 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 non-viral 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 over 50% were achieved following the infusion of *ex vivo* expanded tumor-infiltrating lymphocytes (TIL) that were selected for their anti-tumor 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 break-through has not yet occurred (7), although clinical data, including spontaneous remissions and the response to cytokine therapy (8-10), suggest that these tumors are susceptible to immune-mediated effector mechanisms. Furthermore, T cells were found in tumors and blood of patients that 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 HSCT, but this therapy is linked with severe graft-versus-host disease due to the limited tumor specificity of infused cells ((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, even though it recognizes RCC in an unknown non-classical manner ((27), and [1]).

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 demonstrated that transfer of the TCR genes endowed normal lymphocytes with the same RCC-associated specificity (23). While this demonstrated 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.

Now 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 lymphocytes (PBL) transferred 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 non-malignant primary cells, thus featuring hallmarks suitable for clinical development.

[1] www.clinicaltrials.gov (NCT00870389)

MATERIAL AND METHODS

Cells

Cell lines (Table 1 and Table 2) were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin-streptomycin, 1 mM L-glutamine, 1 mM sodium pyruvate and 1% non essential 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 mM L-glutamine, 1 mM sodium pyruvate, 1% non essential amino acids, and cytokines when indicated). Cells were incubated at 37°C and 6.5% CO_2 in a humidified chamber. Primary normal kidney cells (NKC) are 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 (ATCC: CRL-11268, American Type Culture Collection, Manassas, Virginia, USA), B3Z (29) and Jurkat 76 cells (30) were cultured as described (31, 32). Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous blood of healthy donors and RCC patients by Ficoll-Hypaque (Biochrom, Berlin, Germany) 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 ($5x10^3$, 100 Gy), allogeneic irradiated feeder PBL mixture ($5x10^4$, 50 Gy) and B-LCL cells ($1.5x10^4$, 100 Gy) in TCM supplemented with 100 U/ml rIL-2 and 2 ng/ml IL-7 (PromoCell GmbH, Heidelberg, Germany). After 20 days, T cell clones were transferred to 24-well plates and restimulated

with $6x10^4$ IL-2-expressing RCC-53 cells, allogeneic irradiated feeder PBL ($2x10^6$), and B-LCL cells ($1x10^5$). Unless otherwise indicated, all cell culture reagents were purchased from Invitrogen (Karlsruhe, Germany). 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, Maryland, USA) and used as *in vitro* transcription template to produce RNA using the mMESSAGE mMACHINE T7 kit (Ambion, Austin, Texas, USA). Cells ($2 x10^6$) 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 performed with Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, California, USA) 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% CO_2. Cell surface expression was determined by flow cytometry using an 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, Cincinnati, Ohio, USA). Applying repertoire analysis (34) in-frame TCRVα3.1, Vα19 and Vβ20 were identified (Arden nomenclature (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 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, Regensburg, Germany (38)). This murinized and codon-optimized vector was designated MP71-TCR53mc. All constructs were verified by sequence analysis (MWG Biotech AG, Martinsried, Germany). 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). 48 h 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-Vβ20-PE- (Immunotech, Marseille, France) and anti-CD8α-FITC-labeled antibodies (BD Pharmingen, Heidelberg, Germany). Ecotropic MLV-pseudotyped retroviruses were produced by transfection of Plat-E packaging cells (28) with MP71-TCR53m or Bullet-CD8α vector plasmids ((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-CD8α. Expression of TCR53 and human CD8α was analyzed using TCR53-specific anti-Vβ20-PE- and anti-CD8α-FITC-labeled antibodies. 24 h co-cultures with target cell lines were done in 96-well round bottom plates (BD Falcon) using $1x10^5$ B3Z-TCR53 cells (E:T = 1:1). B3Z-TCR53 cells were incubated with 1 µM ionomycin (Calbiochem, Darmstadt, Germany) and 5 ng/ml phorbol-12-myristate-13-acetate (PMA; Promega, Mannheim, Germany) for TCR-independent stimulation. Supernatants of co-cultures were harvested and IL-2 content was determined by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocol (*e*Bioscience, San Diego, California, USA). Measurement of intracellular β-galactosidase activity was performed as described (32).

TNF-α quantification by WEHI cytotoxicity assay

TIL ($3x10^3$ cells/100 µl) in TCM supplemented with 30 U/ml rIL-2 were added to adherent target cells ($1.5x10^4$/well) in 96-well flat bottom plates (BD Falcon). Where indicated, 4 µg of an anti-HLA-A2 antibody (ATCC: HB-54) was added. TIL and target cells cultivated alone were used to determine background 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

Co-cultures of TCR53 gene-modified PBL and target cells were performed as described (31) but using $1.5x10^4$ target cells (E:T = 5:1). 24 h supernatants were harvested and analyzed for their IFN-γ content using ELISA (BD Biosciences). 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 µCi sodium chromate-51 (Hartmann GmbH, Braunschweig, Germany) and incubated with TCR53-, GFP- or non-modified PBL using E:T ratios from 20:1 to 2.5:1 by employing $2x10^3$ 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 ± mean deviation.

Multi-parameter flow cytometry

Cytokine secretion and degranulation analysis were performed using $2x10^5$ PBL incubated 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 performed with anti-CD45-Amcyan and anti-CD4-APC-A750 in FACS buffer (PBS, 2% FCS, 2 mM EDTA) for 20 min at 4°C, followed by addition of 7-amino-actinomycin-D (Sigma Aldrich, Seelze, Germany, 10 µg/ml, 20 min). Cells were fixed with 1% of paraformaldehyde (Merck, Darmstadt, Germany) for 20 min followed by permeabilization using two consecutive washes with 0.1% and 0.35% saponin (Sigma Aldrich). Intracellular staining was performed with anti-mouse-TCRβ-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, Ashland, Oregon, USA).

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 demonstrated by blocking with an antibody directed against HLA-A2. Importantly, normal kidney cultures were not recognized by TIL-53 (Fig. 1A, B). T cell receptor repertoire analysis of the TIL-53 population after three *in vitro* stimulations with autologous IL-2 secreting RCC-53 revealed oligoclonality with nine rearranged TCRVα and fourteen TCRVβ 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 TCRβ sequence Vβ20 (Arden nomenclature (35)), which in accordance to the IMGT data base (41) is TCRBV30*01-J2-5*01-D2*01 (CDR3: CAWSTLGYRETQYF) and two in-frame TCRα sequences Vα3.1 (TCRAV17*01-J21*01; CDR3: CDPNFNKFYF) and Vα19 (TCRAV41*01-J13*02; CDR3: CAAFFSGGYQKVTF).

Enhanced surface expression and functional performance of modified TCR53 chain genes

Since the sequence analysis of the TIL-53.29 cDNA revealed two TCRα and one TCRβ sequence, each TCRα-chain gene was combined with the TCRβ-chain gene in the retroviral vector MP71-PRE and used to transduce TCR-deficient Jurkat 76 cells. Here, only

the combination of TCRVα19 and TCRVβ20 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 performed. 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% (MFI 79) of $CD8^+TCRV\beta20^+$ cells when the non-modified TCR53 was applied, while TCR53m and TCR53mc yielded 19% (MFI 95) and 38% (MFI 228) of $CD8^+TCRV\beta20^+$ 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 multi-functionality

To further evaluate that the 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 Figure 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 non-transduced 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 MIP-1β. All four cytokines were detected in supernatants of PBL-TCR53 co-cultured with TCR53 p:MHC-ligand positive tumor cells, but were absent in co-cultures with TCR53 p:MHC negative cells or PBL-GFP (Fig. S1), thus demonstrating specificity and a strong pro-inflammatory 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 multi-chromatic 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 panels, 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 panels, green population). Non-transduced PBL showed very low background activity (Fig. 3A, right panels). Boolean gating analysis of the responding CD45$^+$CD8$^+$TCR53$^+$ T cell population (Fig. 3B, C) revealed high polyfunctionality with 55% of the responding TCR53$^+$ T cells displaying 2 or more functions, including the secretion of IL-2. The multi-functional

response of PBL-TCR53 was similar to PBL which were transgenic for an HLA-A2-restricted tyrosinase$_{369-377}$ peptide-specific TCR (TCR-D115, (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 (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 immuno- and chemotherapeutics, PBL were efficiently transduced with TCR53 (Fig. S2A), displayed tumor-specific lytic activity (Fig. S2A, B) and multifunctionality profiles that were indistinguishable from those of PBL-TCR53 of healthy donors (Fig. 3, Fig. S2C). TCR53-transduced PBL of healthy donors and patients could be successfully expanded *ex vivo* following the protocol utilized for clinical application ((6), 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 CD8α chain and the TCR53 chains were introduced into B3Z cells. Transduced cells (B3Z-TCR53) showed stable expression of human CD8α and TCR53Vβ20 (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 co-culture 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 CD8α, recognition of the antigenic p:MHC complex is CD8 dependent (Fig. 4B and 4C).

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 non-transformed cell lines of normal tissue. Twelve of 19 (63%) HLA-A2$^+$ RCC cell lines tested were found to stimulate IL-2 secretion after co-culture 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 co-culture 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, (43), (44) and personal communication); 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 VHL-wild-type after stable transfection ((44), Table 1). Among the 15 known ccRCC, four 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, seven PBMC and one endothelial cell culture. Only one of the normal cells (NKC-33) induced marginal IL-2 secretion (Table 1 and Table 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 perform functional testing of recognition of tumor and non-tumor 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 TCRα- 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 multi-parameter 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, while cells of normal tissues were not recognized. Since 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 than PBL-TCR53 of heathy 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 co-expression of human CD8α 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 of each prostate carcinoma, and breast carcinoma, and 2 pancreatic carcinoma, as well as 20 primary cultures of normal kidney tissues, 7

hematopoietic and 1 endothelial cells. This screening recapitulated the original TIL-53 specificity and firmly established: (i) HLA-A2 restriction, (ii) shared expression of the TCR53 p:MHC ligand in 63% of RCC and 25% of other tumor entities, and (iii) tumor selectivity with only one 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 (5/13, 38%), whereas there was no evidence of expression in sarcomas, 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 TC53-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 over-expressed 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 (48)) was

negative. Additionally, the TCR53 recognition pattern of RCC cell lines did not coincide with HERV-E expression (defined by PCR, (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 multi-functional response, that 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 non-classical RCC recognition (27), offering the therapeutic option to target RCC tumors through MHC-restricted and non-restricted 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 up-regulation 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 practised therapy of RCC that achieves approx. 15% response rates (10), to improve clinical benefit.

ACKNOWLEDGEMENTS

We thank A. Brandl, J. Hauchwitz, K. Hummel, U. Fischer for excellent technical assistance, and R. Oberneder (Urology Clinic Dr. Castringius, Munich-Planegg, Germany) and A. Buchner (Urology Clinic, LMU, Munich, Germany) for providing kidney samples. We like to thank C.D. Gerharz (Institute of Pathology, Bethesda-Johanniter Clinic, Duisburg, Germany) and W. Kaelin (Dana-Farber-Cancer Institute, Boston, USA) for providing RCC lines. This work was supported by Deutsche Forschungsgemeinschaft (SFB-TR36, SPP1230), EU (FP6 Program ATTACK) and the Initiative and Networking Fund of the Helmholtz Association within the Helmholtz Alliance on Immunotherapy of Cancer. The authors have no conflicting interests.

REFERENCES

1. Kolb HJ, Schmid C, Barrett AJ, Schendel DJ. Graft-versus-leukemia reactions in allogeneic chimeras. Blood 2004; 103:767-76.

2. Gottschalk S, Heslop HE, Rooney CM. Adoptive immunotherapy for EBV-associated malignancies. Leuk Lymphoma 2005; 46:1-10.

3. Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr Opin Immunol 2009; 21:233-40.

4. Uckert W, Schumacher TN. TCR transgenes and transgene cassettes for TCR gene therapy: status in 2008. Cancer Immunol Immunother 2009; 58:809-22.

5. Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 2006; 314:126-9.

6. Johnson LA, Morgan RA, Dudley ME, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood 2009; 114:535-46.

7. Shablak A, Hawkins RE, Rothwell DG, Elkord E. T Cell-Based Immunotherapy of Metastatic Renal Cell Carcinoma: Modest Success and Future Perspective. Clin Cancer Res 2009.

8. Lokich J. Spontaneous regression of metastatic renal cancer. Case report and literature review. Am J Clin Oncol 1997; 20:416-8.

9. Vogelzang NJ, Stadler WM. Kidney cancer. Lancet 1998; 352:1691-6.

10. Reeves DJ, Liu CY. Treatment of metastatic renal cell carcinoma. Cancer Chemother Pharmacol 2009; 64:11-25.

11. Belldegrun A, Muul LM, Rosenberg SA. Interleukin 2 expanded tumor-infiltrating lymphocytes in human renal cell cancer: isolation, characterization, and antitumor activity. Cancer Res 1988; 48:206-14.

12. Finke JH, Rayman P, Alexander J, et al. Characterization of the cytolytic activity of CD4+ and CD8+ tumor-infiltrating lymphocytes in human renal cell carcinoma. Cancer Res 1990; 50:2363-70.

13. Caignard A, Guillard M, Gaudin C, Escudier B, Triebel F, Dietrich PY. In situ demonstration of renal-cell-carcinoma-specific T-cell clones. Int J Cancer 1996; 66:564-70.

14. Gaugler B, Brouwenstijn N, Vantomme V, et al. A new gene coding for an antigen recognized by autologous cytolytic T lymphocytes on a human renal carcinoma. Immunogenetics 1996; 44:323-30.

15. Schendel DJ, Oberneder R, Falk CS, et al. Cellular and molecular analyses of major histocompatibility complex (MHC) restricted and non-MHC-restricted effector cells recognizing renal cell carcinomas: problems and perspectives for immunotherapy. J Mol Med 1997; 75:400-13.

16. Jantzer P, Schendel DJ. Human renal cell carcinoma antigen-specific CTLs: antigen-driven selection and long-term persistence in vivo. Cancer Res 1998; 58:3078-86.

17. Ronsin C, Chung-Scott V, Poullion I, Aknouche N, Gaudin C, Triebel F. A non-AUG-defined alternative open reading frame of the intestinal carboxyl esterase mRNA generates an epitope recognized by renal cell carcinoma-reactive tumor-infiltrating lymphocytes in situ. J Immunol 1999; 163:483-90.

18. Dorrschuck A, Schmidt A, Schnurer E, et al. CD8+ cytotoxic T lymphocytes isolated from allogeneic healthy donors recognize HLA class Ia/Ib-associated renal carcinoma antigens with ubiquitous or restricted tissue expression. Blood 2004; 104:2591-9.

19. Tykodi SS, Warren EH, Thompson JA, et al. Allogeneic hematopoietic cell transplantation for metastatic renal cell carcinoma after nonmyeloablative conditioning: toxicity, clinical response, and immunological response to minor histocompatibility antigens. Clin Cancer Res 2004; 10:7799-811.

20. Geiger C, Nossner E, Frankenberger B, Falk CS, Pohla H, Schendel DJ. Harnessing innate and adaptive immunity for adoptive cell therapy of renal cell carcinoma. J Mol Med 2009; 87:595-612.

21. June CH. Adoptive T cell therapy for cancer in the clinic. J Clin Invest 2007; 117:1466-76.

22. Ueno NT, Cheng YC, Rondon G, et al. Rapid induction of complete donor chimerism by the use of a reduced-intensity conditioning regimen composed of fludarabine and melphalan in allogeneic stem cell transplantation for metastatic solid tumors. Blood 2003; 102:3829-36.

23. Engels B, Noessner E, Frankenberger B, Blankenstein T, Schendel DJ, Uckert W. Redirecting human T lymphocytes toward renal cell carcinoma specificity by retroviral transfer of T cell receptor genes. Hum Gene Ther 2005; 16:799-810.

24. Gouttefangeas C, Stenzl A, Stevanovic S, Rammensee HG. Immunotherapy of renal cell carcinoma. Cancer Immunol Immunother 2007; 56:117-28.

25. Sato E, Torigoe T, Hirohashi Y, et al. Identification of an immunogenic CTL epitope of HIFPH3 for immunotherapy of renal cell carcinoma. Clin Cancer Res 2008; 14:6916-23.

26. Takahashi Y, Harashima N, Kajigaya S, et al. Regression of human kidney cancer following allogeneic stem cell transplantation is associated with recognition of an HERV-E antigen by T cells. J Clin Invest 2008; 118:1099-109.

27. Wang QJ, Hanada K, Yang JC. Characterization of a novel nonclassical T cell clone with broad reactivity against human renal cell carcinomas. J Immunol 2008; 181:3769-76.

28. Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther 2000; 7:1063-6.

29. Karttunen J, Sanderson S, Shastri N. Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. Proc Natl Acad Sci U S A 1992; 89:6020-4.

30. Heemskerk MH, Hoogeboom M, de Paus RA, et al. Redirection of antileukemic reactivity of peripheral T lymphocytes using gene transfer of minor histocompatibility antigen HA-2-specific T-cell receptor complexes expressing a conserved alpha joining region. Blood 2003; 102:3530-40.

31. Leisegang M, Engels B, Meyerhuber P, et al. Enhanced functionality of T cell receptor-redirected T cells is defined by the transgene cassette. J Mol Med 2008; 86:573-83.

32. Sommermeyer D, Neudorfer J, Weinhold M, et al. Designer T cells by T cell receptor replacement. Eur J Immunol 2006; 36:3052-9.

33. Schendel DJ, Gansbacher B, Oberneder R, et al. Tumor-specific lysis of human renal cell carcinomas by tumor-infiltrating lymphocytes. I. HLA-A2-restricted recognition of autologous and allogeneic tumor lines. J Immunol 1993; 151:4209-20.

34. Steinle A, Reinhardt C, Jantzer P, Schendel DJ. In vivo expansion of HLA-B35 alloreactive T cells sharing homologous T cell receptors: evidence for maintenance of an oligoclonally dominated allospecificity by persistent stimulation with an autologous MHC/peptide complex. J Exp Med 1995; 181:503-13.

35. Arden B, Clark SP, Kabelitz D, Mak TW. Human T-cell receptor variable gene segment families. Immunogenetics 1995; 42:455-500.

36. Engels B, Cam H, Schuler T, et al. Retroviral vectors for high-level transgene expression in T lymphocytes. Hum Gene Ther 2003; 14:1155-68.

37. Cohen CJ, Zhao Y, Zheng Z, Rosenberg SA, Morgan RA. Enhanced Antitumor Activity of Murine-Human Hybrid T-Cell Receptor (TCR) in Human Lymphocytes Is Associated with Improved Pairing and TCR/CD3 Stability. Cancer Res 2006; 66:8878-86.

38. Scholten KB, Kramer D, Kueter EW, et al. Codon modification of T cell receptors allows enhanced functional expression in transgenic human T cells. Clin Immunol 2006; 119:135-45.

39. Willemsen R, Ronteltap C, Heuveling M, Debets R, Bolhuis R. Redirecting human CD4+ T lymphocytes to the MHC class I-restricted melanoma antigen MAGE-A1 by TCR alphabeta gene transfer requires CD8alpha. Gene Ther 2005; 12:140-6.

40. Espevik T, Nissen-Meyer J. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J Immunol Methods 1986; 95:99-105.

41. Brochet X, Lefranc MP, Giudicelli V. IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. Nucleic Acids Res 2008; 36:W503-8.

42. Wilde S, Sommermeyer D, Frankenberger B, et al. Dendritic cells pulsed with RNA encoding allogeneic MHC and antigen induce T cells with superior antitumor activity and higher TCR functional avidity. Blood 2009; 114:2131-9.

43. Gerharz CD, Ramp U, Dejosez M, et al. Resistance to CD95 (APO-1/Fas)-mediated apoptosis in human renal cell carcinomas: an important factor for evasion from negative growth control. Lab Invest 1999; 79:1521-34.

44. Iliopoulos O, Kibel A, Gray S, Kaelin WG, Jr. Tumour suppression by the human von Hippel-Lindau gene product. Nat Med 1995; 1:822-6.

45. Almeida JR, Price DA, Papagno L, et al. Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. J Exp Med 2007; 204:2473-85.

46. Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol 2008; 8:247-58.

47. Vissers JL, De Vries IJ, Schreurs MW, et al. The renal cell carcinoma-associated antigen G250 encodes a human leukocyte antigen (HLA)-A2.1-restricted epitope recognized by cytotoxic T lymphocytes. Cancer Res 1999; 59:5554-9.

48. Buchner A, Pohla H, Willimsky G, et al. Phase I Trial of an Allogeneic Gene-modified Tumor Cell Vaccine (RCC-26/CD80/IL-2) in Patients with Metastatic Renal Cell Carcinoma. Hum Gene Ther 2009.; DOI: 10.1089/hum.2008.192

49. Griffiths RW, Gilham DE, Dangoor A, et al. Expression of the 5T4 oncofoetal antigen in renal cell carcinoma: a potential target for T-cell-based immunotherapy. Br J Cancer 2005; 93:670-7.

50. Hole N, Stern PL. A 72 kD trophoblast glycoprotein defined by a monoclonal antibody. Br J Cancer 1988; 57:239-46.

TABLE 1

	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

	HLA-A2	HLA-A2 transfection	B3Z-TCR53 IL-2 (pg/ml)
renal cell carcinoma cells			
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
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

TCR53 p:MHC ligand prevalence among RCC and NKC

B3Z-TCR53 were co-cultured 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.* (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. (§) 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, (43)) induced similar amounts of IL-2 than its VHL-/- parental line 786-0 (46 pg/ml; not shown). NKC are short-term cultures of cells of tumor-free kidney cortices. HLA-A2 transfection was performed using RNA. The HLA-A2 status of all cell lines and cultures was confirmed by flow cytometry. (*) RPTEC 2814-3 is a primary renal proximal tubular epithelial cell purchased from BioWhittaker, Walkersville, Maryland, USA.

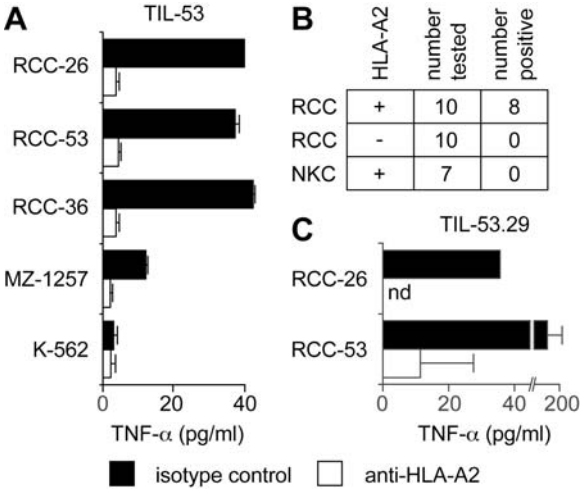
TABLE 2

		HLA-A2	HLA-A2 transfection	B3Z-TCR53 IL-2 (pg/ml)
other tumor cells (origin)				
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
other tumor cells (origin)				
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**
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

TCR53 p:MHC ligand prevalence among tumor and normal cells other than RCC or NKC

B3Z-TCR53 were co-cultured 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 performed 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, (16) peripheral blood mononuclear cells (§ of seven different donors).

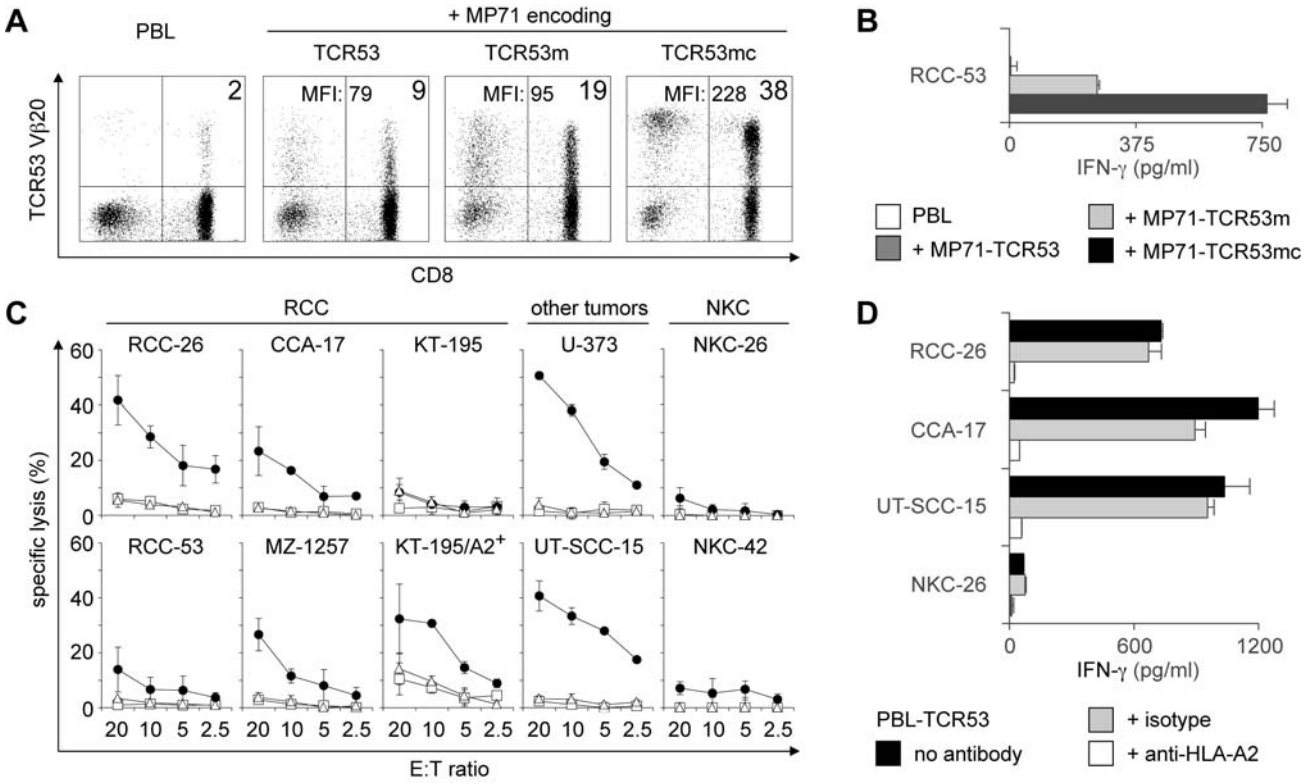
FIGURE 1



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 co-cultured with indicated tumor cells 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 ± mean deviation of triplicates. nd = not done. (B) Shown is the summarized screen of renal cell carcinoma (RCC) cell lines and normal kidney cells (NKC) by using TIL-53.

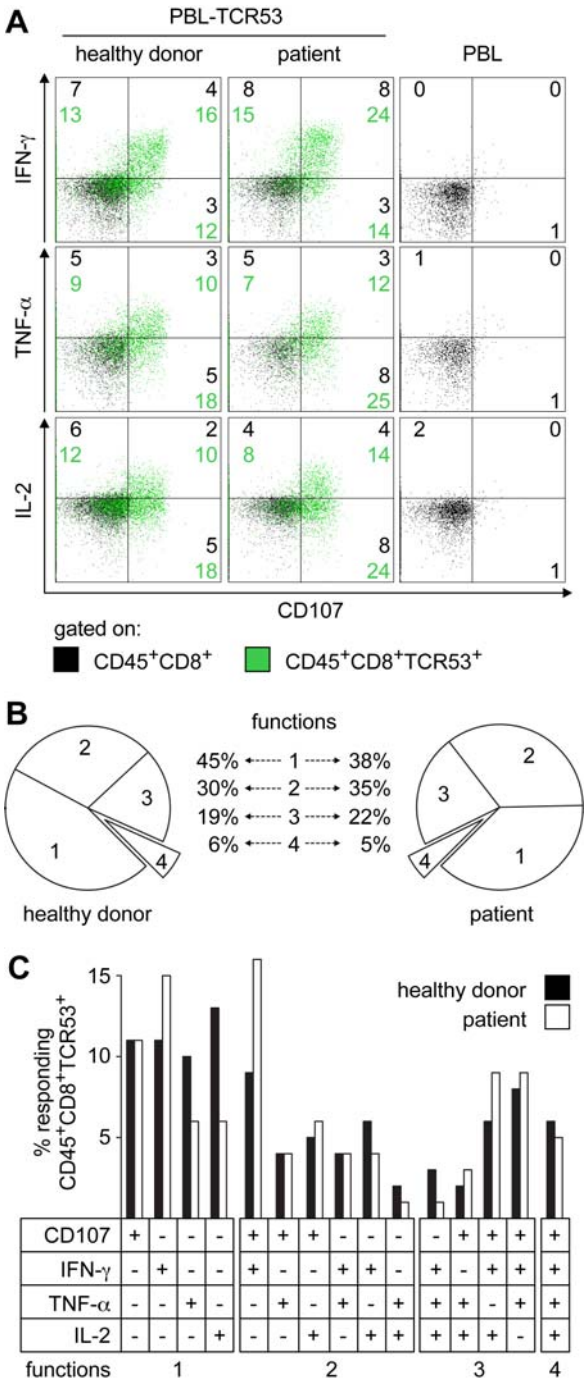
FIGURE 2



Enhanced cell surface expression of optimized TCR53 genes and functionality of TCR53-transduced PBL

(A) FACS analysis of untransduced PBL (PBL) or PBL transduced with the retrovirus vector MP71-PRE encoding TCRα- and β-chain cDNAs linked by the P2A element using wild-type TCR53 genes (TCR53), murinized (TCR53m) or murinized and codon optimized (TCR53mc) variants. TCR53β-chain expression was determined using an anti-Vβ20-specific antibody. Numbers represent the percentage of $V\beta20^+CD8^+$ T cells, MFI values refer to the Vβ20 intensity of these cells. Shown is one representative experiment of three. (B) PBL, PBL transduced with MP71-TCR53, MP71-TCR53m or MP71-TCR53mc were co-cultured with RCC-53 and IFN-γ release was determined by ELISA. Bars are the mean of duplicates ± mean deviation. Shown is one representative experiment of three. (C) Cell-mediated cytotoxicity of titered numbers of MP71-TCR53mc-transduced PBL (circles, transduction efficiency: 20%), GFP-transduced PBL (triangle) or untransduced PBL (squares) against $HLA\text{-}A2^+$ 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)), $HLA\text{-}A2^+$ normal kidney cells (NKC-26, NKC-42), $HLA\text{-}A2^-$ (KT-195) RCC tumor lines and HLA-A2-transfected KT-195 cells ($KT\text{-}195/A2^+$). Percent specific lysis values are means of duplicates ± 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 represent mean values of duplicates ± mean deviation. Experiments were repeatedly done with PBL of four different donors. One set of representative data is shown.

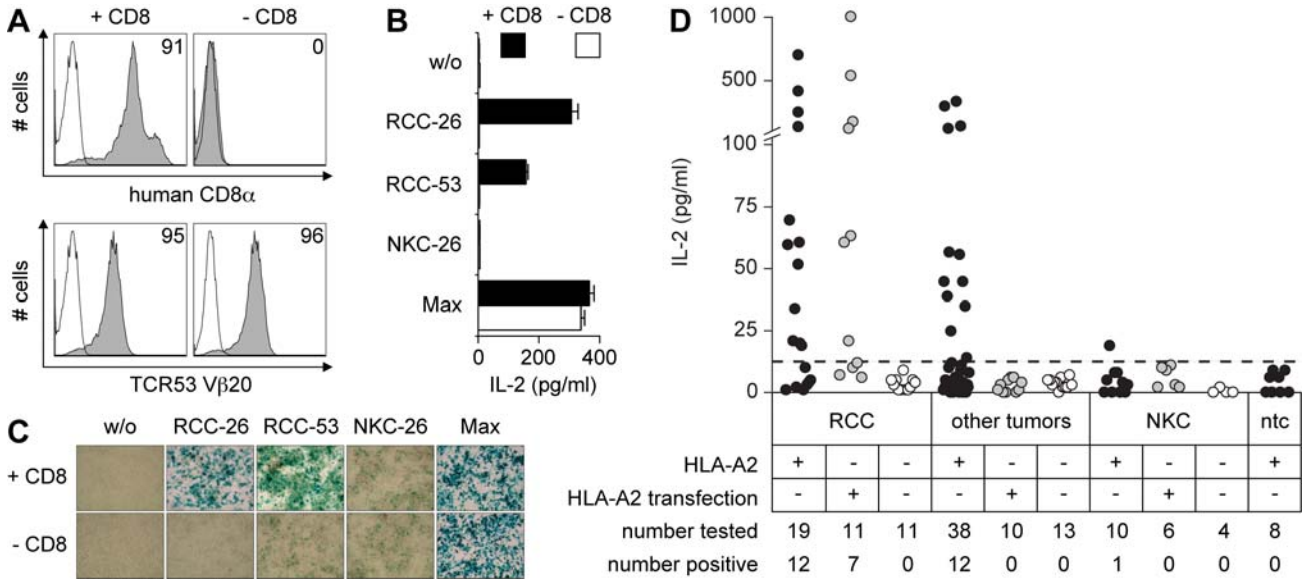
FIGURE 3



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 non-transduced 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 indicate the percentage of cells within the respective quadrant. (B, C) Results of boolean gating analysis for the responding CD45^{+}CD8^{+}TCR53^{+} T cells after co-culture with RCC-26 are shown. The pie chart (shown in 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 (Fig. S2) and when using other TCR53 p:MHC^{+} tumor cell lines for stimulation (not shown).

FIGURE 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

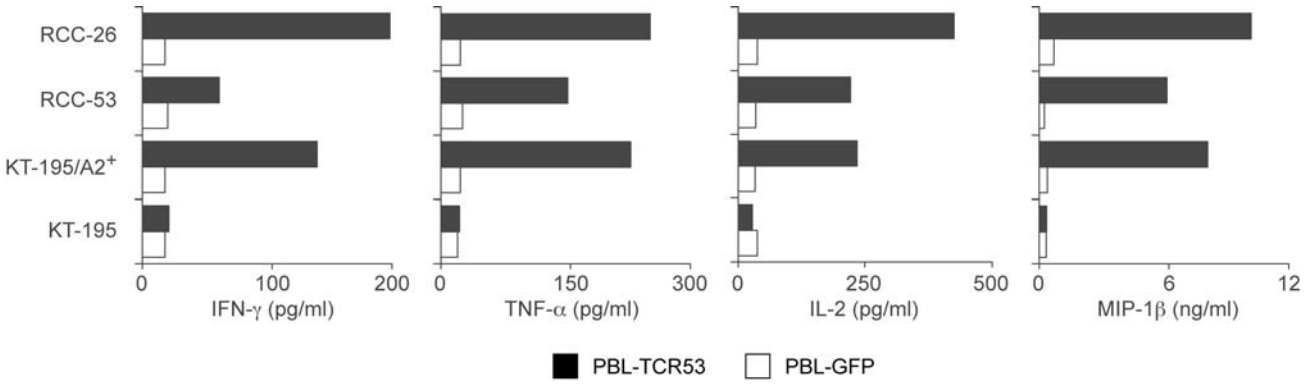
(A) Surface expression of human CD8α and TCR53Vβ20 on B3Z-TCR53 cells was detected by flow cytometry. Numbers indicate the percentage of $V\beta20^+$ or human $CD8\alpha^+$ B3Z cells (grey curves). (B) B3Z-TCR53 cells transduced or non-transduced with human CD8α were co-cultured alone (w/o) or with cell 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 represent the mean of duplicates ± mean deviation. (C) B3Z-TCR53 cells were co-cultured as in (B), fixed, and β-galactosidase was detected by X-Gal staining. (D) B3Z-TCR53 cells were co-cultured with indicated tumor cell lines and cells of normal tissues for 24 h and IL-2 production was quantified by ELISA. The dashed line indicates IL-2 background level produced by the indicator cell line alone (12 pg/ml). Each dot represents the IL-2 value of B3Z-TCR53 cells after co-culture with one specific cell line (see Table 1 and Table 2) of the group: RCC, renal cell carcinoma (n = 41); other tumors (n = 61); NKC, normal kidney cells (n = 20); ntc: non-tumor cell lines (n = 8). For each group, $HLA\text{-}A2^+$ (closed), HLA-A2-transfected (grey), and $HLA\text{-}A2^-$ (open) cells are shown. For individual results see Table 1 and Table 2.

SUPPLEMENTARY METHODS

Multiplex cytokine assay

The supernatants from cytotoxicity assays (E:T = 10:1) were measured for cytokine content using a custom-mixed human Multiplex bead assay system and Luminex xMAP Technology (Bio-Plex System, Bio-Rad, München, Germany) allowing simultaneous detection of cytokines IFN-γ , TNF-α, IL-2 and MIP-1β. Data analysis was done using the Bio-Rad Array Operation System and applying five parameter logistic regression algorithms.

SUPPLEMENTARY FIGURE 1



TCR53 gene-modified PBL show pro-inflammatory cytokine response

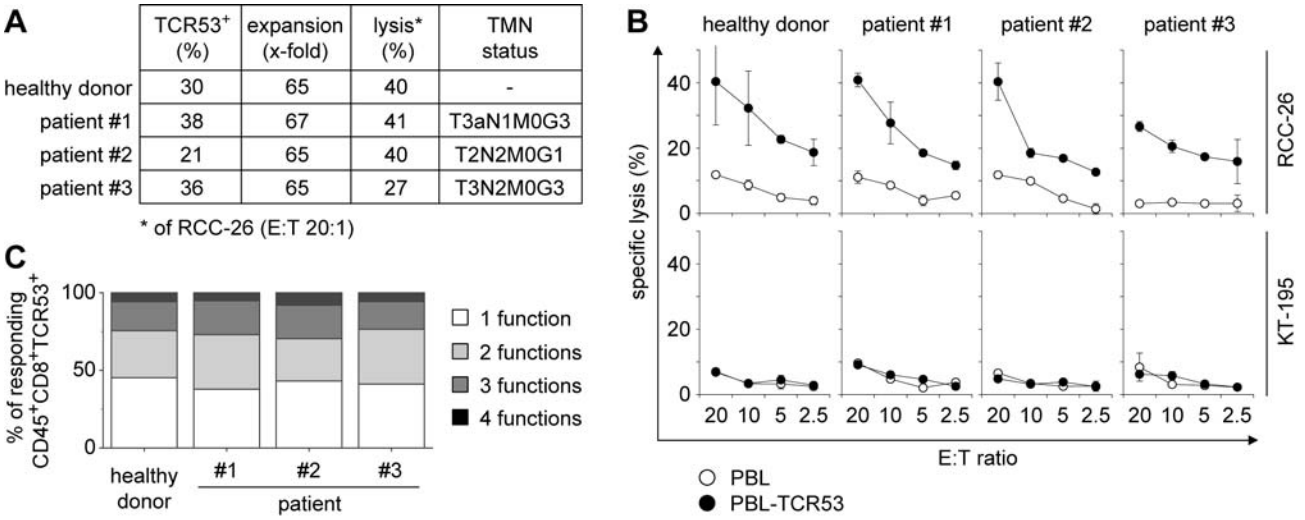
PBL-TCR53 were co-cultured with indicated tumor cell lines and supernatants were tested for their cytokine content using Bio-Plex cytokine assay allowing simultaneous detection of IFN-γ , TNF-α, IL-2 and MIP-1β. A set of representative data is shown.

SUPPLEMENTARY FIGURE 2

A

	TCR53$^+$ (%)	expansion (x-fold)	lysis* (%)	TMN status
healthy donor	30	65	40	-
patient #1	38	67	41	T3aN1M0G3
patient #2	21	65	40	T2N2M0G1
patient #3	36	65	27	T3N2M0G3

* of RCC-26 (E:T 20:1)



PBL-TCR53 of healthy donors and RCC-patients show equivalent T cell expansion and functional response

PBL were obtained from a healthy donor and three RCC-patients who had progressive metastatic disease. For transduction, PBL of the healthy donor and patients were defrosted from liquid nitrogen, stimulated and transduced as described (see Material and Methods, main manuscript). Transduced PBL were expanded with anti-CD3 (ATCC: CRL-8001 (OKT 3), kindly provided by E. Kremmer) and anti-CD28 antibodies (Becton Dickinson) in TCM containing 300 U rIL-2/ml (Cetus, Emeryville, USA). Cells were used for functional assays on day 9 and 20 (not shown) after transduction, with similar results achieved on both time points. (A) Summary of TCR53 transduction efficiency, T cell expansion, lytic response and clinicopathologic patient characteristics (TMN status). Patient #1 (detailed shown in Fig. 3, main manuscript) was enrolled in an immuno-chemotherapy treatment program and the PBL were harvested after the patient had received 5 cycles of IFN-α and 5-fluorouracil. The transduction efficiency (% TCR53$^+$) is expressed as the percentage of mTCR$^+$ PBL determined on day 4 after transduction. The fold-expansion is the relative increase in cell number between day 0 (1x10^6 cells) and day 9. The lysis refers to the cytotoxic value against RCC-26 at an E.T ratio of 20:1. (B) Shown is the cell-mediated cytotoxicity of untransduced and TCR53-transduced PBL against RCC tumor lines RCC-26 (p:MHC$^+$) and KT-195 (HLA-A2$^-$). Percent specific lysis values are means of duplicates ± mean deviation. E:T ratio refers to the total CD3 cell number. (C) The graph depicts the percentage of responding T cells showing 1, 2, 3, or 4 functional responses simultaneously. The values were obtained through boolean gating analysis gated on the CD45$^+$CD8$^+$TCR53$^+$ cells from 8-color flow cytometry as described in Material and Methods (main manuscript). Dot plots and details of the polyfunctional response of healthy donor and patient #1 are shown in Fig. 3 (main manuscript).