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Regression of renal cell carcinoma by T cell receptor-engineered T cells targeting a human endogenous retrovirus

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ABSTRACT

Background We discovered a novel human endogenous retrovirus (CT-RCC HERV-E) that was selectively expressed in most clear cell renal cell carcinomas (ccRCC) and served as a source of antigens for T cell-mediated killing. Here, we described the cloning of a novel T cell receptor (TCR) targeting a CT-RCC HERV-E-derived antigen specific to ccRCC and characterized antitumor activity of HERV-E TCR-transduced T cells (HERV-E T cells).

Methods We isolated a $CD8^+$ T cell clone from a patient with immune-mediated regression of ccRCC post-allogeneic stem cell transplant that recognized the CT-RCC-1 HERV-E-derived peptide in an HLA-A11-restricted manner. We used 5'Rapid Amplification of cDNA Ends (RACE) to clone the full length HERV-E TCR and generated retrovirus encoding this TCR for transduction of T cells. We characterized HERV-E T cells for phenotype and function in vitro and in a murine xenograft model. Lastly, we implemented a good manufacturing practice-compliant method for scalable production of HERV-E T cells.

Results The HLA-A11-restricted HERV-E-reactive TCR exhibited a CD8-dependent phenotype and demonstrated specific recognition of the CT-RCC-1 peptide. $CD8^+$ T cells modified to express HERV-E TCR displayed potent antitumor activity against HLA-$A11^+$ ccRCC cells expressing CT-RCC HERV-E compared with unmodified T cells. Killing by HERV-E T cells was lost when cocultured against HERV-E knockout ccRCC cells. HERV-E T cells induced regression of established ccRCC tumors in a murine model and improved survival of tumor-bearing mice. Large-scale production of HERV-E T cells under good manufacturing practice conditions generated from healthy donors retained specific antigen recognition and cytotoxicity against ccRCC.

Conclusions This is the first report showing that human ccRCC cells can be selectively recognized and killed by TCR-engineered T cells targeting a HERV-derived antigen. These preclinical findings provided the foundation for evaluating HERV-E TCR-transduced T cell infusions in patients with metastatic ccRCC in a clinical trial (NCT03354390).

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Adoptive transfer of TCR-engineered T cells is an emerging modality for cancer treatment. However, its use in solid tumors has often been limited by off-tumor effects due to target antigen expression in normal tissues.

WHAT THIS STUDY ADDS

⇒ We cloned a novel TCR targeting a human endogenous retrovirus type E (HERV-E)-derived antigen specific to clear cell renal cell carcinoma (ccRCC). We demonstrate that arming T cells with this TCR empowers them with the ability to recognize and kill HERV-E expressing ccRCC tumors in vitro and in a murine model.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study offers the first data showing tumor cells can be selectively killed by TCR-engineered T cells targeting a HERV-derived antigen. The preclinical data presented here provided the foundation for a clinical trial evaluating HERV-E TCR-transduced T cell infusions in patients with advanced ccRCC (NCT03354390).

BACKGROUND

Although treatment options for kidney cancer have recently expanded, metastatic clear cell renal cell carcinoma (ccRCC) is generally incurable.[1] For decades, ccRCC was viewed as immunogenic based on rare reports of spontaneous remissions[2] and occasional durable responses to a high-dose IL-2.[3] More recently, improvements in disease-free survival associated with the upfront use of immune checkpoint inhibitors have further established the susceptibility of this tumor to the human immune system.[4] Adoptive cell therapy with gene-engineered T cells that

express chimeric antigen receptors (CARs) or T cell receptors (TCRs) is a promising investigational concept for treating solid tumors.[5–8] However, in kidney cancer, the lack of known tumor-specific antigens has limited the application of this approach, with substantial toxicities occurring in the first reported CAR T cell trial for this malignancy.[9]

Recently, there has been increasing evidence that antigens derived from human endogenous retroviruses (HERVs) could serve as actionable targets for cellular therapy.[10] HERVs represent remnants of ancient retroviral infections of the germline, and it is estimated that 8% of the human genome is composed of these retroviral signatures.[11] Due to the accumulation of mutations and epigenetic silencing, most HERVs are inactive and unable to replicate. However, some HERVs still have open reading frames with the potential for protein expression.[11 12] Indeed, a growing number of HERVs have been found to be aberrantly expressed in different cancers, leading investigators to pursue research aimed at exploring whether HERV-derived antigens can serve as targets for immunotherapy,[13–19] prognostic markers,[20] or biomarkers of response to immune checkpoint inhibitors.[21]

A novel HERV, named CT-RCC HERV-E, was discovered by our group and has been proposed to be a potential target for adoptive cell therapy in ccRCC.[22–24] From a patient with ccRCC who had prolonged immune-mediated tumor regression following an allogeneic hematopoietic stem cell transplant, we identified a $CD8^+$ T cell clone that showed tumor-specific killing of the patient's tumor cells in vitro. The target antigen of this clone was determined to be an HLA-A11-restricted peptide named CT-RCC-1 (ATFLGSLTWK), derived from CT-RCC HERV-E.[22] We first established that the CT-RCC HERV-E is expressed in most ccRCC tumors but not in normal tissues or other cancers.[22–25] We then described three critical events that regulate the selective expression of this HERV-E in ccRCC: Von Hippel-Lindau (VHL) tumor suppressor inactivation, HIF-2α transcriptional factor overexpression, and hypomethylation of the CT-RCC HERV-E 5' long terminal repeat.[24] Importantly, *VHL* inactivation followed by HIF-2α upregulation is an early driver of ccRCC tumorigenesis,[26] which likely accounts for the almost universal expression of CT-RCC HERV-E in ccRCC.

Here, we present data characterizing a TCR that recognizes HERV-E-derived CT-RCC-1 antigen. We cloned the genes that encode this TCR into a retroviral vector and evaluated the ability of HERV-E TCR-transduced T cells (HERV-E T cells) to selectively kill ccRCC tumors in vitro and in a murine xenograft model. Lastly, we describe the implementation of a good manufacturing practice (GMP)-compliant method to mass-produce highly purified populations of HERV-E-reactive T cells. Our findings provide the foundation for a first-in-human clinical trial evaluating the safety and antitumor activity of HERV-E T cells in patients with metastatic ccRCC.

METHODS

Cell lines

Cell lines were cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1% GlutaMAX (Gibco), and 1% Penicillin/Streptomycin (Gibco). Human ccRCC cell lines (RCC1-RCC13) were established from surgically resected tumors procured at the National Institutes of Health (NIH) Clinical Center. These cell lines were authenticated by HLA typing and phenotype analysis. Non-RCC cell lines: T2 (TAP-deficient lymphoblast), T24 (bladder cancer), 293T (embryonic kidney), LS174T (colon cancer), HT1080 (sarcoma), and HS1299 (lung cancer) were obtained from and authenticated by ATCC; these cells were used for transduction with a pBABE retrovirus (Addgene) encoding an HLA-A11 or HLA-A31 molecule either alone or in combination with a plasmid encoding the HERV-E derived HLA-A11 restricted CT-RCC-1 peptide. All cells were regularly tested for *Mycoplasma* contamination using the Universal Mycoplasma Detection Kit (ATCC).

Generation of HERV-E knockout and β_2 microglobulin knockout ccRCC cell lines using CRISPR/Cas9

A CRISPR dual sgRNA targeting strategy was used to delete the entire CT-RCC HERV-E genomic region (approximately 8.4 kb long) or to disrupt the β_2 microglobulin (β2m) gene from the genome of a ccRCC tumor cell line that expressed the CT-RCC HERV-E (RCC1 WT).[27] For knocking out HERV-E, sgRNAs upstream and downstream of the CT-RCC HERV-E region (5'-CCUAGGCAAAAUCGGCAUAG-3' and 5'-ACUGUUCCUGCUACACCUUC-3', respectively) along with Cas9 (Synthego) were introduced into the RCC1 WT ccRCC cell line using electroporation through 4D-Nucleofector System (Lonza). Gene-edited cell pools were then cloned by single-cell sorting into 96-well plates using BD FACSMelody cell sorter. A ccRCC tumor cell clone in which CT-RCC HERV-E was confirmed to be knocked out was expanded and subsequently validated to be derived from the original ccRCC cell line by HLA typing and *VHL* gene sequencing as described elsewhere.[24] CT-RCC HERV-E expression was quantified by qRT-PCR as previously described.[23]

Determination of the HERV-E TCR nucleotide sequence

Total RNA was extracted from a $CD8^+$ $V\beta7^+$ T cell clone (derived from a patient who had prolonged immune-mediated regression of ccRCC after an allogeneic stem cell transplant[22]) that was shown to recognize the CT-RCC-1 peptide and killed ccRCC cells in an HLA-A11-restricted fashion. Recovered RNA was used for cDNA synthesis, followed by PCR amplification using a 5' Rapid Amplification of cDNA Ends (RACE) kit and primers specific for conserved regions of α and β TCR chains sequences (Invitrogen). PCR products were extracted from the gel using a QIAquick gel extraction kit (Qiagen), cloned into TOPO-TA pCR4 vectors and used to transform One Shot TOP10 competent *Escherichia coli* (Thermo Fisher).

Clones containing amplicons with lengths from 0.5 kb up to 1 kb were selected for plasmid extraction using the Plasmid Miniprep kit (Thermo Fisher) and sequencing.

Retroviral construct for expression of the HERV-E TCR

SAMEN CMV/SRα retroviral vector was used for the expression of the TCR α and β chains (online supplemental item 1). This vector was introduced into the PG13 packaging cell line to isolate high titer retroviral producer clones for further use, including clinical use as previously reported.[28 29] This PG13 clone and a batch of high titer virus were GMP qualified for use in IND-enabling experiments for a future clinical trial intended to treat patients with metastatic ccRCC with autologous HERV-E TCR-transduced T cells.

Manufacturing of non-clinical grade HERV-E TCR-transduced T cells

Peripheral blood mononuclear cells (PBMC) were isolated from deidentified buffy coats of healthy donors procured at the NIH Department of Transfusion Medicine by Ficoll-Paque density gradient centrifugation. T cells were isolated using Pan T Cell Isolation Kit (Miltenyi), cultured in T cell media (TexMacs Medium (Miltenyi) supplemented with 3% heat-inactivated human AB serum (Sigma-Aldrich), 1% Penicillin-Streptomycin-Glutamine (Gibco), rhIL-7 (10 ng/mL) (PeproTech) and rhIL-15 (5 ng/mL) (PeproTech)] and activated using ImmunoCult Human CD3/CD28/CD2 T Cell Activator (StemCell Technologies). Three days post-activation, $CD4^+$ T cells were depleted using CD4 MicroBeads (Miltenyi). The next day (4 days post-activation), retroviral spinoculation was performed with a retrovirus encoding the HERV-E TCR at the multiplicity of infection of 4 as described elsewhere.[29] On day 7 post-transduction, TCR-transduced T cells were enriched using CD34 MicroBeads (Miltenyi) (figure 1A). Enriched cells were expanded until desired cell numbers were reached, aliquoted, and frozen. Before use, T cells were thawed and rested in T cell media for 24 hours.

Manufacturing of clinical-grade HERV-E TCR-transduced T cells

Apheresis products from deidentified healthy donors were purchased from Key Biologics (Lowell, MA). PBMCs were isolated by Ficoll and activated with anti-hCD3 monoclonal antibody (OKT3) in Aim-V culture media containing human AB Serum, 300 IU/mL rhIL2, and 100 ng/mL rhIL15. Prior to retrovirus transduction, activated T cells were enriched for $CD8^+$ T cells by depleting $CD4^+$ T cells (CliniMACS cell selector). Supernatants from the PG13 retroviral producer clone were used to transduce CD8-enriched T cells. Approximately 2×10^6 cells were added to retronectin-coated 24-well plate wells along with 300 IU/mL IL-2, 100 ng/mL IL-15, and the SAMEN HERV-E TCR vector. After spinoculation at 2000 g for 2 hours at 32°C, cells were collected and cultured for 2–3 days before enriching for transduced T cells using CD34 selection. Transduction conditions were optimized to limit transgene copy numbers to an average of <5 integrated copies per cell as mandated by the US Food and Drug Administration (FDA). HERV-E TCR-transduced T cells were purified using CD34 immunomagnetic beads and the CliniMACS cell selector from bulk T cell cultures. After CD34 selection, cells were subjected to the rapid expansion protocol (REP) as follows: 1×10^6 TCR-transduced T cells were stimulated with anti-OKT3 mAb (30 /mL) in media supplemented with 300 IU/mL IL-2, 100 ng/mL IL-15, and 200×10^6 feeder cells, composed of pooled irradiated (50 Gy) PBMC collected from at least 3 different healthy human donors. Five days after the beginning of REP, cells were transferred to a WAVE bioreactor for continued cell expansion. 8–12 days later (18–21 days after the cell culture was first initiated), cells were packaged and cryopreserved.

Flow cytometry

Fluorescently conjugated antibodies were purchased from Biolegend (anti-CD4 PB, anti-CD8 FITC, anti-CD34 APC, anti-CD45RO PE, anti-CCR7 PeCy7, anti-CD95 BV421, anti-TIM3 BV605, anti-CTLA4 PE, anti-PD1 PeCy7, anti-LAG3 BV711, anti-IFNγ BV421, anti-TNFα BV605, anti-CD107a PE, anti-IgG APC) or BD Biosciences (anti-CD3 BUV395). The HLA-A11:01/CT-RCC-1 dextramer (PE) was purchased from Immudex. Dead cells were excluded with LIVE/DEAD Fixable Yellow Dead Cell Stain (Invitrogen). For HLA-A11 surface density quantification, we used unconjugated anti-HLA-A11 antibody (MyBioSource) and QIFIKIT (Agilent) per manufacturer's protocol. Data were acquired with a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software.

T cell functional assays

For T cell degranulation and cytokine production assays, effector T cells were cocultured with an equal number of target cells in 96 well U-bottom plates. Reactivity against CT-RCC-1 peptide or HLA-A11-restricted KRAS G12D7-16 (VVVGADGVGK) peptide used as a control was determined by pulsing T2-HLA-A11-transduced cells with 10 µL/mL of peptide (Genscript) for 2 hours prior to coculture (5 hours). In some experiments, immediately after seeding effector and target (E:T) cells, anti-CD107a antibody was added to the wells. The BD Cytofix/Cytoperm kit was used for intracellular cytokine staining per the manufacturer's protocol. Percentages of $CD107a^+/IFN\gamma^-/TNF\alpha^-$ (lytic only), $CD107a^-/IFN\gamma^+/TNF\alpha^+$ (cytokine secreting only) and $CD107a^+/IFN\gamma^+/TNF\alpha^+$ (polyfunctional) were determined by flow cytometry.

For measuring IFNγ in the coculture supernatants, we cocultured 2×10^5 effector T cells with 2×10^4 tumor cells in a final volume of 0.2 mL in round-bottom 96-well plates overnight at 37°C. After 16–18 hours, coculture supernatants were harvested and diluted 10 times in PBS to assure assay linearity. The concentrations of human IFNγ in coculture supernatants were measured by Human

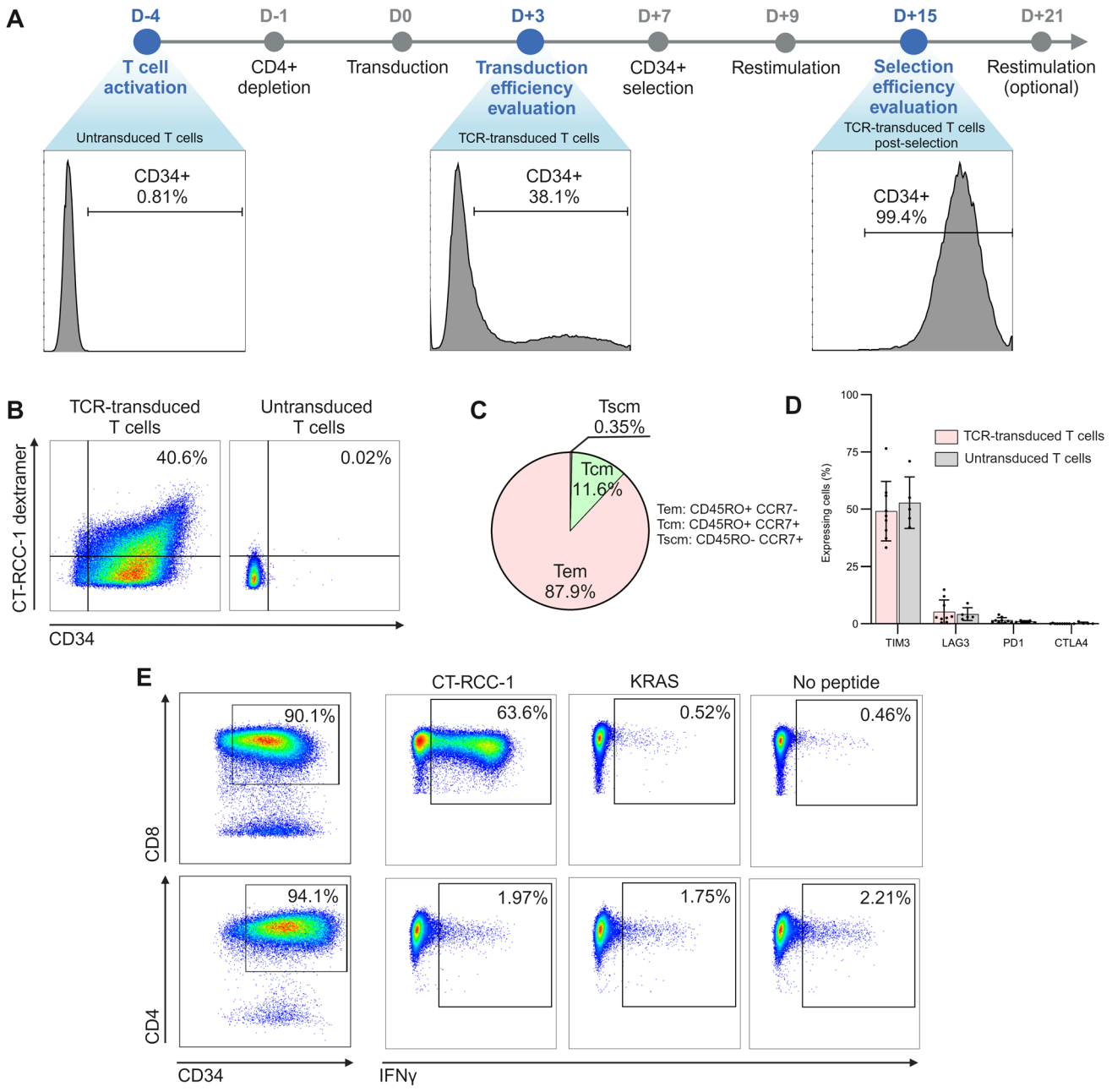

Figure 1 Characterization of HERV-E TCR-transduced T cells. (A) The preclinical method used to generate and expand HERV-E TCR-transduced CD8$^+$ T cells. Histograms show a representative example of transduction efficiency (n=7 donors) before and after CD34 enrichment as determined by CD34 surface expression by flow cytometry. (B) Representative plot of flow cytometry analysis of HLA-A11:01/CT-RCC-1 dextramer binding of HERV-E TCR-transduced T cells (n=7 donors). Untransduced T cells, which underwent the same treatment as retroviral-transduced T cells, were used as a negative control for dextramer binding. (C) A pie chart depicting the T cell subpopulation composition of TCR-transduced T cells (n=7 donors, values shown are median). (D) Surface expression of select inhibitory markers on HERV-E TCR-transduced T cells 2 weeks post-transduction (mean±SD, n=7 donors). (E) Intracellular staining for IFNγ shows HERV-E TCR-transduced CD8$^+$ T cells but not HERV-E TCR-transduced CD4$^+$ T cells recognize T2-A11$^+$ cells pulsed with the CT-RCC-1 HERV-E peptide. T2-A11$^+$ cells were peptide-pulsed with either CT-RCC-1 HERV-E peptide, a mock peptide (HLA-A11-restricted KRAS G12D7-16), or were not pulsed. HERV-E, human endogenous retrovirus type E; RCC, renal cell carcinoma.

IFN-gamma Quantikine ELISA Kit (R&D Systems) per manufacturer's instructions.

To evaluate direct cytotoxicity of HERV-E T cells, target cells were seeded in 96-well assay microplates and cultured at 37°C in 5% CO_2 for 48 hours before coculturing with effector T cells at different E:T ratios for 4 hour. We used an imaging cytometry-based method (Celigo Image Cytometer, Nexcelom Bioscience) and lactate dehydrogenase (LDH) release assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega). For

imaging cytometry-based assays, target cells were labeled with 5 µM calcein AM for 30 min in a culture medium, then washed twice before coculturing with T cells. The formula was used to calculate the percentage of specific killing: % specific lysis=(1–Calcein AM $Count_{treated}$/Calcein AM $Count_{control}$)×100%. The following formula was used to calculate the percentage of specific killing by LDH release assay: % specific lysis=[(experimental release–effector spontaneous release–target spontaneous release)/(target maximum release–target spontaneous release)×100].

To correlate the percentage of specific killing relative to HLA-A11 and CT-RCC HERV-E mRNA expression for each ccRCC line, we created a multiplicative measure designated as the HERV-E/HLA-A11 expression index. This index was calculated using the following formula: [(HLA-A11 surface density in molecules/cell)×(CT-RCC HERV-E mRNA expression relative to ACTBx10^5)/10^6].

Targeted mass spectrometry-based identification of CT-RCC-1 peptide in the context of HLA class I presentation on the surface of ccRCC cells

HLA-peptide complexes were immunopurified from ccRCC cell lysates with anti-HLA class I antibodies (W6/32) produced in-house and cross-linked to Protein A Sepharose beads following a previously established protocol.[30] In short, 10^8 cells per sample were lysed at 4°C for 1 hour with PBS containing 0.25% sodium deoxycholate, 0.2 mM iodoacetamide, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 1% octyl-beta-D-glucopyranoside, and a 1:200 protease inhibitors cocktail (all purchased from Millipore Sigma). The lysates were then cleared by ultracentrifugation and supernatants used for immunoprecipitation. Peptides were separated from HLA molecules by Sep-Pak tC18 columns and used for parallel reaction monitoring (PRM) mass spectrometry (MS) analysis on an Orbitrap Fusion Lumos nano liquid chromatography-mass spectrometry (LC-MS) system (Thermo Fisher). Peptides were separated on a 50 cm long EasySpray PepMap RSLC C18 column (Thermo Fisher Scientific) with gradient of 5%–30% acetonitrile delivered in 40 min. Precursor ions were surveyed at 375–1500 Da mass range at 120 k resolution and 4e5 automatic gain control (AGC). CT-RCC-1 peptide was isolated with an isolation window of 2.0 m/z prior to ion activation by higher-energy collisional dissociation at 20% energy, and targeted spectra were recorded at 30 k resolution and 5e4 AGC. The PRM data were processed and analyzed by Skyline using an ion mass tolerance of 0.02 m/z.[31]

Treatment of established human ccRCC tumors in a murine xenograft tumor model

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) male mice aged 8–10 weeks were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). The human HLA-A11 expressing HERV-E positive ccRCC cell line RCC1 was transduced to express firefly luciferase with a GFP lentiviral vector, with GFP-positive cells subsequently purified by FACS. NSG mice were inoculated with 3×10^6 luciferase-expressing RCC1 WT cells subcutaneously. A total of n=40 animals with established tumors were randomized using the random numbers generator into three groups: (a) mice to receive HERV-E T cells, (b) mice to receive untransduced T cells, or (c) mice not to receive T cells. T cell infusion was initiated on day 14 following tumor cell inoculation and consisted of a single intravenous injection of 10×10^6 HERV-E T cells or untransduced T cells. Mice receiving T cells also received IL-2 (200,000 IU, Teceleukin, Roche) administered daily for 3 days starting on the day of the T cell infusion. To monitor tumor burden, mice were injected with D-luciferin i.p. (150 mg/kg; Gold Biotechnology, St. Louis, Missouri, USA) and in vivo bioluminescence imaging (BLI) was performed using IVIS Spectrum In Vivo Imaging System. The imaging data were analyzed using Living Image software (PerkinElmer, Waltham, Massachusetts, USA).

Statistics

Statistical analyses were performed with GraphPad Prism V.9 software. Multiple unpaired t-tests with Welch correction were used to calculate the statistical significance of the differences between the specific lysis of ccRCC targets by HERV-E T cells and untransduced T cells from the same donor. Spearman correlation with simple linear regression was used to describe the relationship between specific lysis and the HERV-E/HLA-A11 expression index. The differences in reactivity and killing of clinical-grade HERV-E T cells against different ccRCC cell lines were evaluated using a two-way analysis of variance multiple comparisons test. Statistical analysis of the mouse survival data was done using log rank (Mantel-Cox) test. A $p<0.05$ was considered statistically significant.

RESULTS

Characterization of HLA-A11-restricted TCR that targets CT-RCC-1 peptide and phenotyping of TCR-transduced T cells

The TCR from an HLA-A11-restricted HERV-E reactive $CD8^+$ T cell clone had α chain AV22S1/AJ45/AC and the β chain BV7S3/BJ1S4/BC1. PCR primers were designed to clone the full-length HERV-E TCR chains and build the subsequent SAMEN retroviral vector encoding the TCR and CD34t marker gene (online supplemental item 1).

With this construct, we transduced T cells from the PBMC of seven healthy donors. Transduction efficiency was determined by surface CD34 expression and varied from 32.6% to 51.6% (median 42.5%). Eight days following CD34 selection, transduced T cells' purity was greater than 95% in all cultures (figure 1A). 32%–40.6% (median: 37%) of CD34-expressing T cells were found to bind an HLA-A11:01/CT-RCC-1 dextramer (figure 1B).

Transduced T cells predominantly exhibited the $CD45RO^+/CCR7^-$ effector memory T cell phenotype (Tem) (median 87.9%, range 74.2%–95.1%). In contrast, only a minority of HERV-E T cells had a $CD45RO^+/CCR7^+$ central memory (Tcm) phenotype (median 11.6%,

range 4.7%–25.5%). The frequency of cells showing a CD45RO$^-$/CCR7$^+$ stem cell memory (Tscm) phenotype was <1% (figure 1C). An analysis of inhibitory receptors showed that HERV-E T cells had low CTLA-4, PD-1 and LAG-3 surface expression and relatively high expression of Tim-3 (median 47.06%, range 33.2%–76.3%). The profile of inhibitory receptor surface expression in TCR-transduced T cells was not significantly different compared with untransduced cells from the same donors (p>0.05) (figure 1D).

Co-culture experiments were conducted between HERV-E TCR-transduced T cells and T2 cells transduced with HLA-A11 molecule (T2-A11$^+$ cells) and pulsed with the HERV-E CT-RCC-1 peptide. IFNγ secretion was only observed in CD8$^+$ T cells expressing the HERV-E TCR, and not CD4$^+$ T cells (figure 1E). These data are consistent with the HERV-E TCR being CD8-dependent.

TCR-transduced T cells specifically recognize and kill HLA-A11$^+$ HERV-E-expressing ccRCC cells in vitro

To assess the CT-RCC-1-specific reactivity of HERV-E T cells, we performed intracellular cytokine and degranulation assays using TCR-transduced cells from three healthy donors. HERV-E T cells were cocultured with ccRCC cell lines with different HLA-A11 and CT-RCC HERV-E expression profiles including (a) an HLA-A11$^+$ ccRCC line that naturally expressed CT-RCC HERV-E (RCC1 WT), (b) a subclone of this ccRCC line that had either CT-RCC HERV-E or β2m knocked out using CRISPR (RCC1 HERV-E knockout [KO] and RCC1 b2m KO, respectively), (c) another wild-type HLA-A11$^+$/CT-RCC HERV-E expressing cell line (RCC2), (d) an HLA-A11$^-$/CT-RCC HERV-E expressing cell line (RCC3), (e) the HERV-E expressing RCC3 cell line transduced with HLA-A11 (RCC3 A11$^+$) and (f) a native ccRCC cell line that expressed neither HLA-A11 nor CT-RCC HERV-E (RCC4) (figure 2A). Lytic activity of transduced T cells was assessed using CD107a while cytokine-secreting activity was assessed by measuring intracellular IFNγ and TNFα by flow cytometry. Cells stained for CD107a and IFNγ/TNFα were considered polyfunctional. When the results from all three donors were combined, we observed the following: (a) untransduced T cells had minimal CD107a expression and cytokine secretion when cocultured with ccRCC lines regardless of CT-RCC HERV-E expression, (b) TCR-transduced T cells had significantly greater CD107a expression and cytokine secretion when cocultured with HLA-A11$^+$ ccRCC cells expressing CT-RCC HERV-E compared with non-transduced controls (p<0.001), (c) tumor recognition was lost when TCR-transduced T cells were cocultured with the CT-RCC HERV-E KO or β2m KO subclones generated from a ccRCC cell line that initially expressed the CT-RCC HERV-E (RCC1 WT), and (d) tumor recognition by TCR-transduced T cells could be induced in a CT-RCC HERV-E expressing HLA-A11 negative cell line after transducing it with HLA-A11.

We next assessed the capacity of TCR-transduced T cells to kill ccRCC cells using an imaging cytometry-based cytotoxicity assay. TCR-transduced T cells generated from healthy donors (n=3) were highly cytotoxic to ccRCC cells expressing both HLA-A11 and CT-RCC HERV-E. Lysis of HLA-A11$^+$/CT-RCC HERV-E$^+$ ccRCC was significantly higher (p<0.0001) with TCR-transduced T cells compared with non-transduced T cells from the same donor. Non-transduced T cells showed a very low level of tumor killing (figure 2B). Minimal tumor killing was observed when TCR-transduced T cells were cocultured with either HLA-A11$^+$/HERV-E$^-$ or HLA-A11$^-$/HERV-E$^+$ ccRCC cells. Importantly, tumor killing of the RCC1 cell line (HLA-A11$^+$/CT-RCC HERV-E$^+$) was abrogated when either CT-RCC HERV-E or β2m were knocked out using CRISPR. Similarly, tumor killing was induced in the RCC3 cell line (HLA-A11$^-$/CT-RCC HERV-E$^+$) on transducing it with the HLA-A11 gene.

HERV-E T cell-mediated tumor killing correlates with the HLA-A11 surface expression and CT-RCC HERV-E mRNA expression levels in ccRCC cells

To screen the cytotoxic potential of HERV-E T cells against a larger panel of ccRCC cell lines, we performed an imaging cytometry-based cytotoxicity assay screen against 13 different ccRCC tumor cell lines with varying levels of (a) HLA-A11 surface density and (b) CT-RCC HERV-E mRNA expression. Using TCR-transduced T cells generated from two healthy donors, we observed a significant positive correlation between the percentage of specific tumor killing and the HERV-E/HLA-A11 expression index (Spearman correlation r=0.82 (p<0.001): figure 3A–C). Additionally, HLA-A11$^+$/CT-RCC HERV-E$^+$ cell line (RCC1 WT) was killed at low E:T ratio (figure 3D). We also assessed the functional avidity of HERV-E TCR in an assay showing that HERV-E T cells recognized their cognate peptide and lysed target cells with EC50 of 2.2 nM (figure 3E).

HERV-E TCR-transduced T cells specifically recognize CT-RCC-1 cognate antigen through HLA-A11 molecule

We verified the TCR used in our experiments recognized the CT-RCC-1 peptide in an HLA-A11-dependent fashion. Non-RCC human cell lines T24 (bladder cancer), 293T (embryonic kidney), LS174T (colon cancer), HT1080 (fibrosarcoma), and HS1299 (lung cancer) were transduced with a retroviral vector encoding for HLA-A11 or HLA-A31 molecule either alone or in combination with a plasmid encoding the CT-RCC-1 peptide. HERV-E T cells were observed to only recognize cells expressing both HERV-E CT-RCC-1 and HLA-A11, as determined by IFNγ release. In contrast, HERV-E T cells did not recognize HERV-E-negative non-RCC cells expressing HLA-A11 alone, HLA-A31 alone, or HLA-A31 and CT-RCC-1 peptide (figure 4).

CT-RCC-1 peptide is exclusively derived from the CT-RCC HERV-E region

Since HERVs are highly repetitive sequences, we also explored whether the source of the CT-RCC-1 peptide

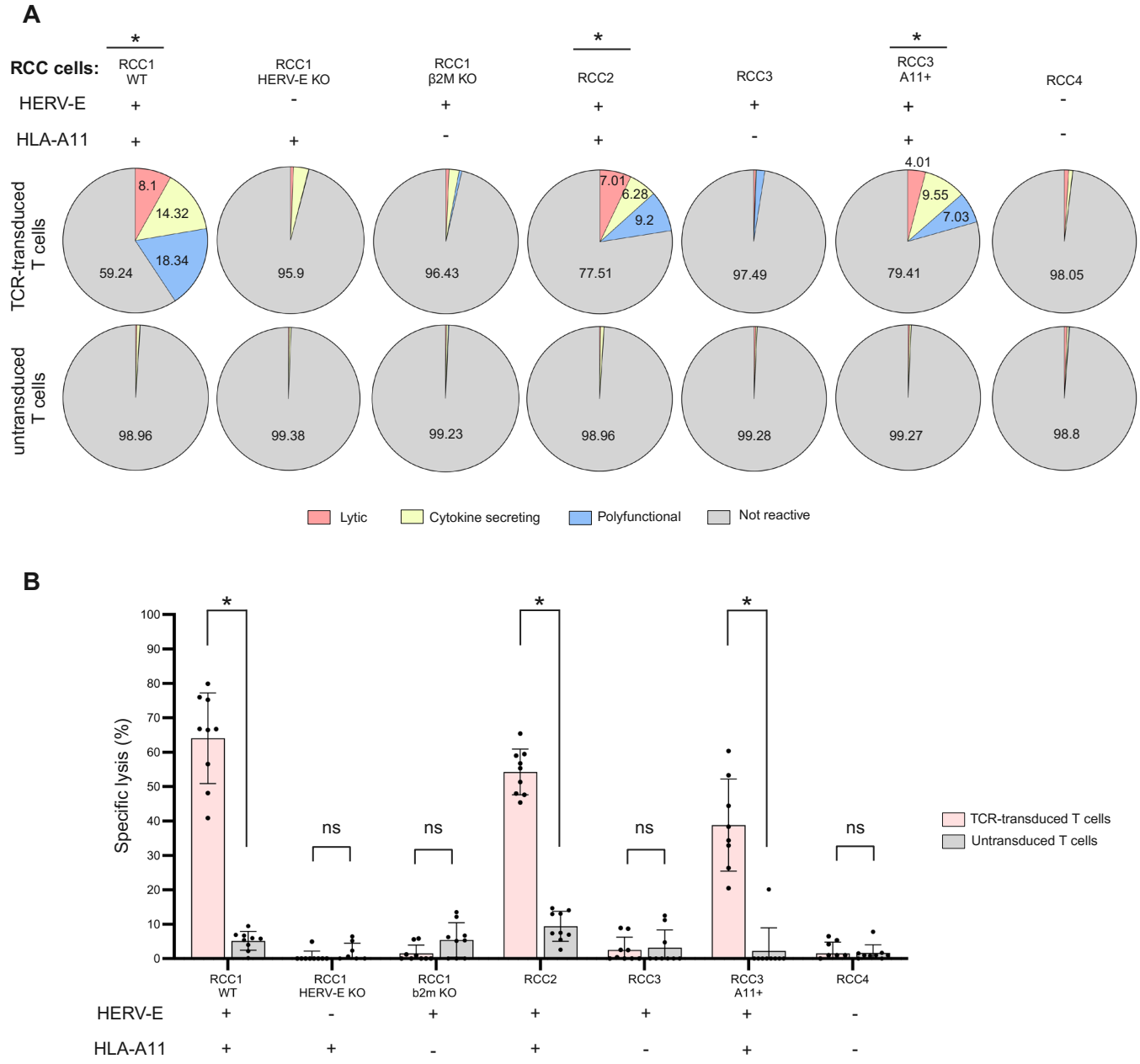

Figure 2 Characterization of the HERV-E T cell function versus ccRCC cells in vitro. (A) The lytic and cytokine-producing ability of HERV-E T cells and untransduced T cells from the same donors are shown when cocultured with ccRCC cells with different HLA-A11 and CT-RCC HERV-E expression profiles. The target cell name and the expression of CT-RCC HERV-E and HLA-A11 are indicated above each pie chart group. Pie chart percentages are median values (n=3 donors), represented as $CD107a^+/IFN\gamma^-/TNF\alpha^-$ (lytic), $CD107a^-/IFN\gamma^+/TNF\alpha^+$ (cytokine secreting) and $CD107a^+/IFN\gamma^+/TNF\alpha^+$ (both lytic and cytokine secreting, polyfunctional). Each experiment was done in technical triplicates. *p<0.001. (B) HERV-E T cell-mediated cytolysis of ccRCC tumor lines in vitro, as determined by Celigo Imaging Cytometry (E:T 10:1). The target cell name and the expression of CT-RCC HERV-E and HLA-A11 are indicated under each cell line name. The data displayed are representative of three healthy donors. Each experiment was done in technical triplicates. *p<0.001, ns, not significant. ccRCC, clear cell renal cell carcinoma; E:T, effector to target; HERV-E, human endogenous retrovirus type E.

could have potentially originated from regions other than CT-RCC HERV-E. Using the immunopeptidomic approach to isolate peptides presented by HLA class I molecules, we performed targeted mass spectrometry (MS) and detected the CT-RCC-1 peptide fingerprint in the RCC1 WT cell line that expressed HERV-E but not in subclones of this cell line that had HERV-E or β2m knocked out using CRISPR (figure 5A). Comparison of synthetic and native CT-RCC-1 peptide fingerprints further confirms the presence of the endogenous peptide in ccRCC cells (figure 5B,C). This suggests that the CT-RCC-1 peptide recognized by TCR-transduced T

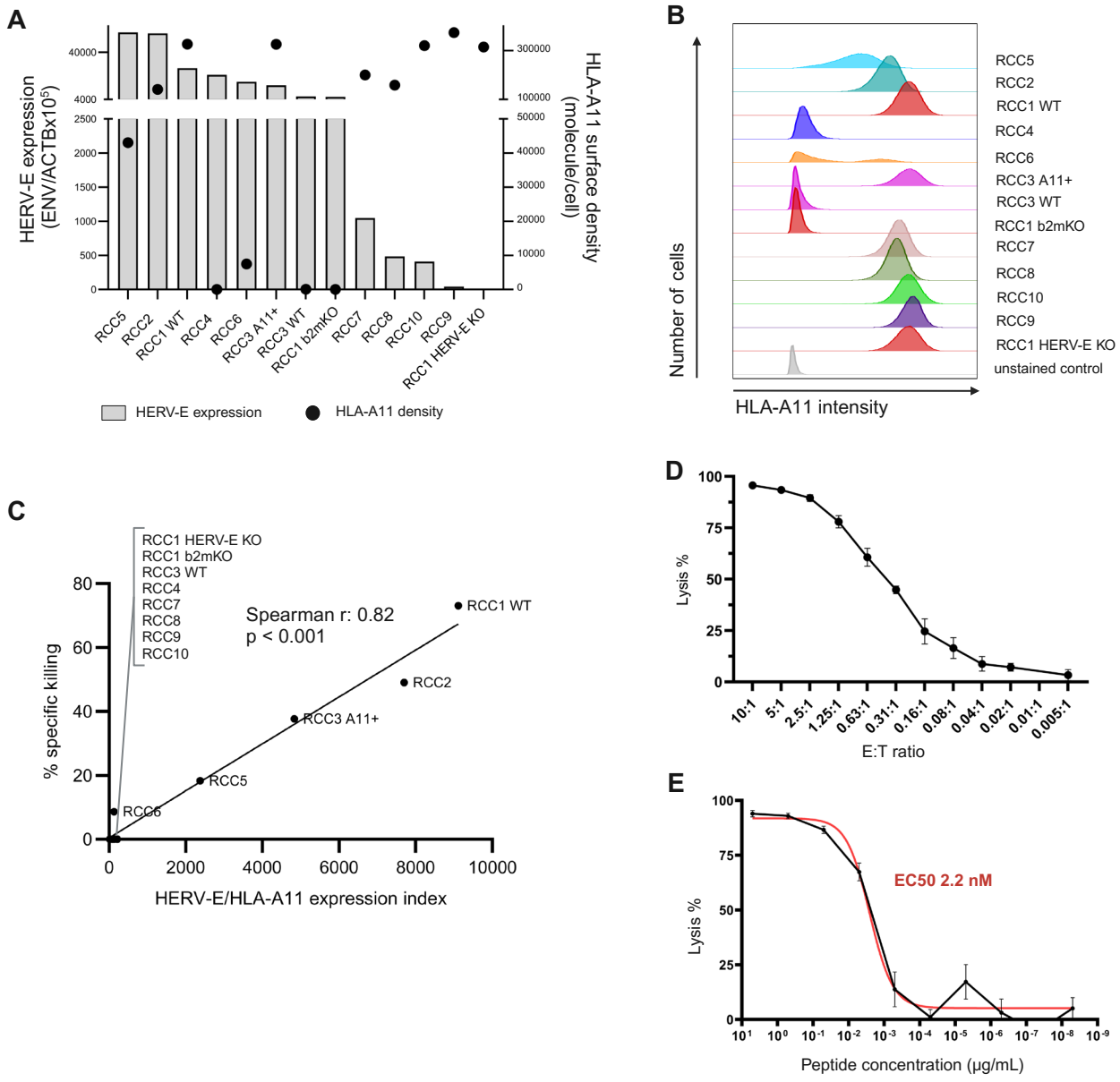

Figure 3 The cytotoxic potential of HERV-E T cells is dependent on HLA-A11 surface density and CT-RCC HERV-E mRNA expression levels in ccRCC tumor cells. (A) HLA-A11 surface density and CT-RCC HERV-E mRNA expression levels relative to the ACTB house-keeping gene in 13 different ccRCC cell lines used in cytotoxicity experiments. HLA-A11 surface density was determined by quantitative flow cytometry and CT-RCC HERV-E mRNA expression by qRT-PCR. Each assay was done in triplicate. (B) HLA-A11 surface expression flow cytometry profiles of the cell lines used in this experiment. (C) The cytotoxic activity of HERV-E T cells expressed as % specific killing correlates with the HERV-E/HLA-A11 index (r=0.82, p<0.001). HERV-E/HLA-A11 index is a multiplicative measure incorporating both HERV-E expression and HLA-A11 surface density as one value. Data show HERV-E T cells generated from two healthy donors targeted against 13 different ccRCC tumors (all assays were done in triplicates, E:T 10:1). (D) HERV-E T cells cocultured with an RCC1 WT (HLA-A11$^+$/CT-RCC HERV-E$^+$) ccRCC tumor at different E:T ratios show specific lysis of target cells even at low E:T ratios. (E) The avidity of HERV-E T cells for CT-RCC-1 antigen. A functional avidity assay with the % target cell lysis in a 4-hour coculture as readout. The target cells are T2-A11$^+$ cells pulsed with CT-RCC-1 peptide at different concentrations. Error bars represent the mean of three technical replicates. ccRCC, clear cell renal cell carcinoma; E:T, effector to target; HERV-E, human endogenous retrovirus type E.

cells in the context of HLA-A11 is encoded by transcripts originated exclusively from the CT-RCC HERV-E region.

Additionally, an NCBI BLASTp query of the CT-RCC-1 peptide sequence (ATFLGSLTWK) against a six-frame translated customized database compiled from three different sources[32–34] showed no exact match between the CT-RCC-1 antigen with other peptides or potential peptides. Only three peptides without indels showed

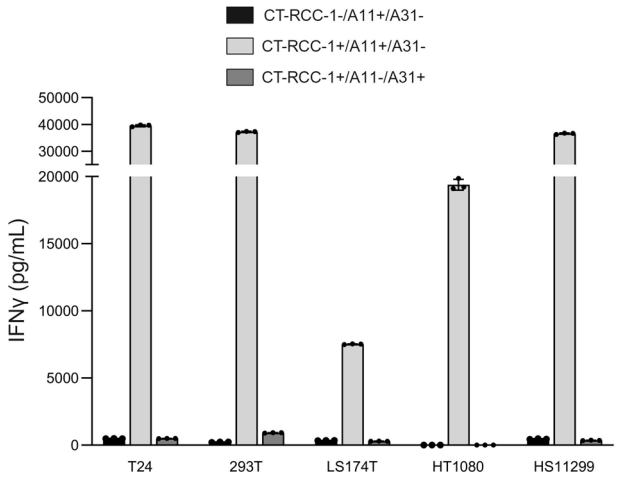

Figure 4 Testing the specificity of HERV-E T cells. Verification of HLA-A11 dependence of CT-RCC-1 peptide recognition by HERV-E T cells. Non-RCC cell lines T24 (bladder cancer), 293T (embryonic kidney), LS174T (colon cancer), HT1080 (sarcoma), HS1299 (lung cancer) encoding HLA-A11 or HLA-A31 either alone or in combination with plasmids encoding the CT-RCC-1 peptide were used as targets in an IFNγ ELISA assay. Transduced T cells only recognized target cells expressing CT-RCC-1 peptide and HLA-A11. Cells expressing either HLA-A11 or HLA-A31 alone failed to activate transduced T cells, as was the case for cells expressing HLA-A31 and the CT-RCC-1 epitope. The assay was done in triplicates. HERV-E, human endogenous retrovirus type E; RCC, renal cell carcinoma.

significant hits to the 10-mer CT-RCC-1 sequence, but the alignment length was 5–7 amino acids providing a minimum of 3 amino acid mismatches.

HERV-E T cells induce regression of human ccRCC tumors in a murine model

The ability of TCR-transduced T cells to home to and kill tumors in vivo in a mouse model may be an important predictor of their therapeutic efficacy in patients. We, therefore, established a xenograft mouse model of advanced ccRCC by engrafting human luciferase transduced HLA-A11$^+$ RCC1 tumors expressing CT-RCC HERV-E subcutaneously into immunodeficient mice. Tumor-bearing animals were randomized into three groups: (a) mice treated with HERV-E T cells from three different donors, (b) mice treated with untransduced T cells from the same three donors, or (c) control mice not receiving T cells. BLI performed 14 days after tumor inoculation and immediately before the T cell infusion showed human ccRCC tumors were established in all mice. Subsequent serial BLI imaging showed progressive tumor growth in mice that received untransduced T cells and in mice that did not receive T cells in contrast to mice receiving HERV-E T cells, where marked tumor regression was observed. Remarkably, HERV-E T cells recipients also had a significant prolongation in survival (median survival: 50 days, $p<0.001$) compared with mice that received untransduced T cells (median survival: 20 days, $p<0.001$) or did not receive T cells (median survival: 20 days, $p<0.001$) where rapid disease progression led to early mortality (figure 6). Similar results were observed in repeat experiments in mice that were inoculated with a lower initial tumor burden (2×10^6 RCC cells s.c.) that subsequently received treatment with T cells at an earlier time point (day 10 post-tumor injection; online supplemental item 2).

Large-scale expanded clinical-grade HERV-E T cells retain tumor-specific recognition and cytotoxicity

To translate our preclinical findings from the bench to the clinic, we implemented a method to produce clinical-grade HERV-E T cells using culture conditions and reagents suitable for human use. The manufacturing conditions were ramped up to full scale using 15 L apheresis collected from three healthy donors (GMP products 1, 2 and 3). The initial transduction efficiency of the three full-scale products (based on CD34 surface expression on CD8$^+$ T cells) ranged from 19.9% to 26.3%. Following CD34 enrichment of transduced cells using immunomagnetic beads, the purity of CD8$^+$ T cells expressing CD34 increased to a median of 96.5% (range 96%–97%). Immediately after thawing, TCR-transduced products contained an average of 94.6%±2.5% viable cells, with viability maintained when cells were cultured in media for an additional 4 hours (92.0%±3.0%, online supplemental item 3). Flow cytometry analysis of all three thawed products demonstrated they contained predominantly CD8$^+$ T cells (median 89.6%, range 83.5%–90.6%), with more than 95% of cells staining positive for the CD34 selection marker (online supplemental item 4).

Antigen recognition by clinical-grade HERV-E T cells showed they produced IFNγ only when cocultured with ccRCC cells expressing both HLA-A11 and CT-RCC HERV-E ($p<0.05$) (figure 7A). Further, all three clinical-grade TCR-transduced T cell products were highly cytotoxic to HLA-A11$^+$ and CT-RCC HERV-E-expressing ccRCC cells, but not HLA-A11$^-$ and/or CT-RCC HERV-E$^-$ cells (figure 7B).

DISCUSSION

Here, we characterize an HLA-A11-restricted TCR that recognizes a 10-mer peptide derived from the CT-RCC HERV-E that is selectively expressed in ccRCC and show for the first time that T cells transduced with this TCR acquire the ability to kill ccRCC tumors in vitro and in vivo. We also describe a GMP-compliant method for their large-scale ex vivo generation for clinical use.

Because the HERV-E TCR used in our experiments was found to have CD8-dependent recognition of its target antigen, we used a manufacturing strategy that incorporated CD4 depletion to obtain a population of predominantly CD8$^+$ T cells prior to their transduction. In vitro, HERV-E T cells demonstrated effector T cell functions that were HERV-E peptide-specific, including cytotoxic granule release and cytokine production. We show that TCR transduced T cells acquire the ability to selectively kill multiple ccRCC cell lines expressing both CT-RCC HERV-E and the restricting HLA-A11 allele for the

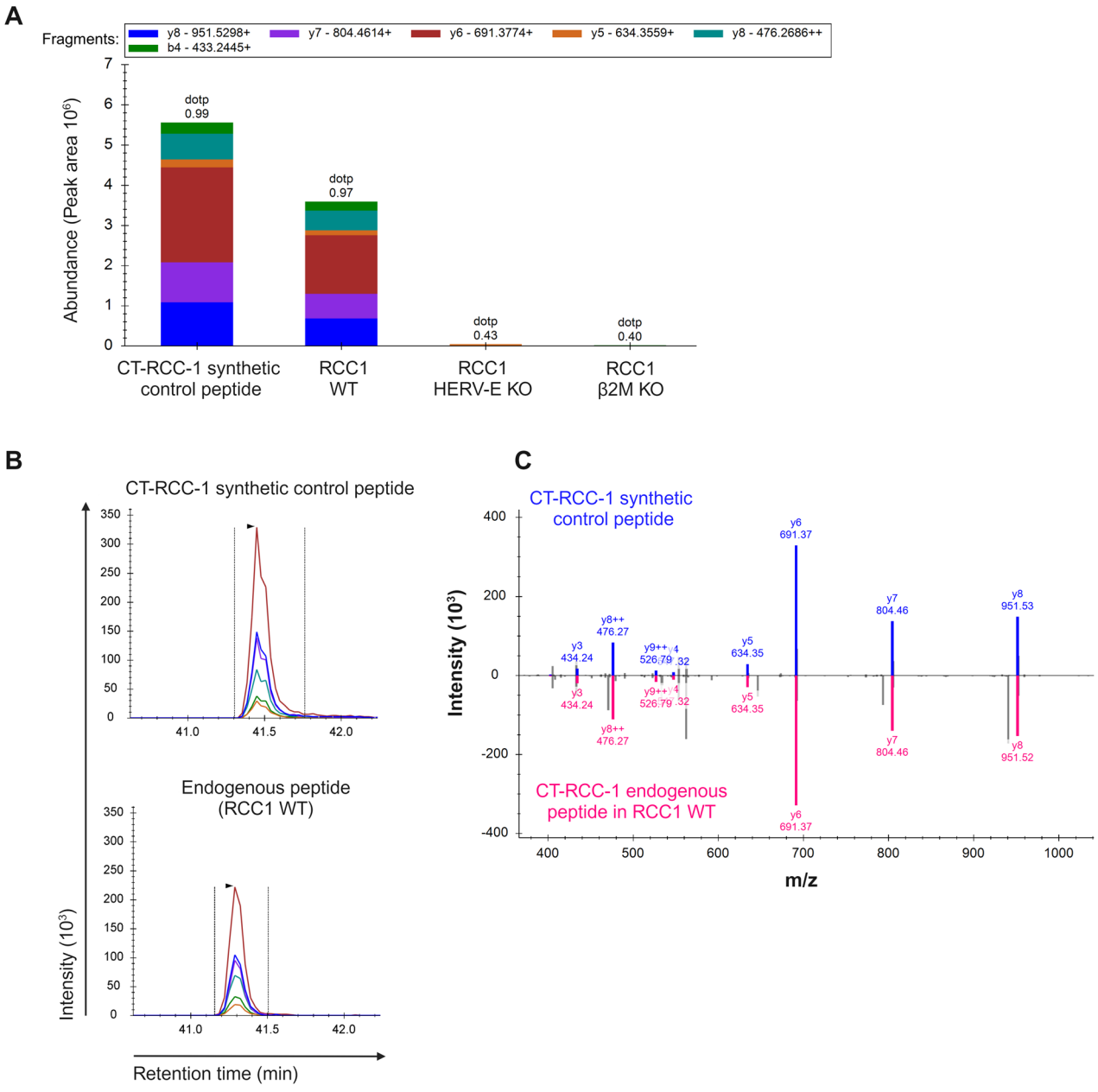

Figure 5 CT-RCC-1 peptide is exclusively derived from the CT-RCC HERV-E region. (A) Targeted PRM MS analysis for the presence of the CT-RCC-1 peptide among peptides presented by HLA class I molecules showed a distinct CT-RCC-1 peptide fingerprint to be present in RCC1 WT (HLA-A11$^+$/CT-RCC HERV-E$^+$) ccRCC tumors but not in its subclones (RCC1 HERV-E KO and RCC1 β2m KO) that had CT-RCC HERV-E or β2m knocked out using CRISPR. (B) Elution profiles of synthetic and endogenous CT-RCC-1 peptide. (C) The MS/MS fragmentation pattern further confirms the presence of the endogenous peptide. HERV-E, human endogenous retrovirus type E; KO, knockout; MS, mass spectrometry; PRM, parallel reaction monitoring; ccRCC, clear cell renal cell carcinoma.

CT-RCC-1 peptide antigen. Specificity for recognition of the HERV-E encoded antigen by TCR-transduced T cells was established by the observation that tumor recognition and killing by HERV-E T cells was completely lost when HERV-E was KO'ed using CRISPR. Notably, the specificity of this TCR for the HLA-A11/CT-RCC-1 peptide complex was further confirmed by showing tumor recognition and killing of an HLA-A11 negative/HERV-E$^+$ ccRCC was observed only when the tumor was transduced with HLA-A11. Finally, HERV-E T cells demonstrated the ability to induce tumor regression in mice with ccRCC tumors, significantly prolonging their survival compared with controls that either received non-transduced T cells or no T cells. These data suggest HERV-E T cells are able to home, target, and kill ccRCC tumors even after they have become well-established in the host. We note that in our animal model, a single infusion of HERV-E T cells was not curative, even when the tumor burden was

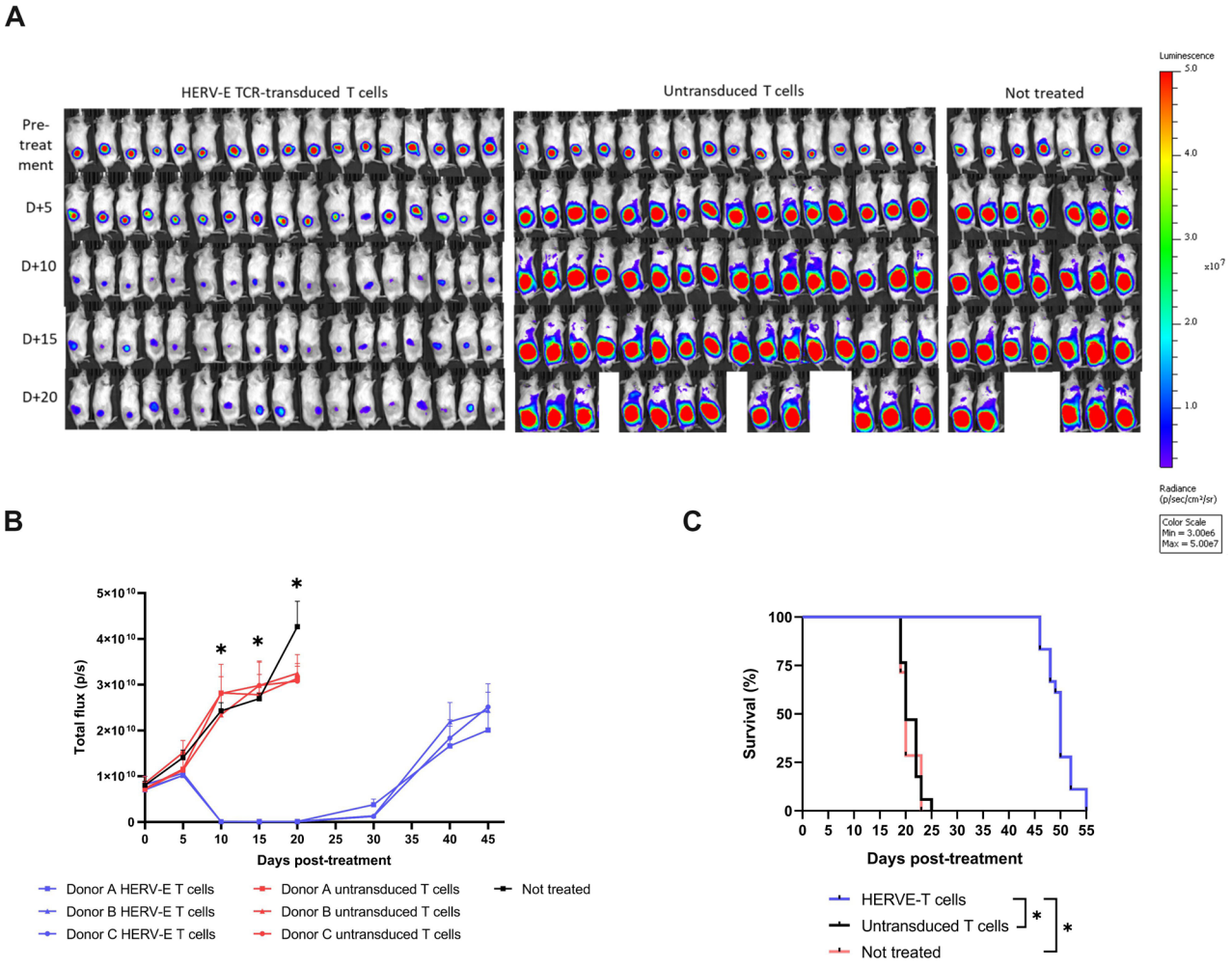

Figure 6 HERV-E T cells mediate regression of ccRCC tumors in vivo in a tumor-bearing mouse model. NSG mice with established (14 days) luciferase expressing subcutaneous RCC1 WT tumors were treated with a single intravenous injection of either HERV-E T cells, untransduced T cells, or did not receive T cells. Tumor burden was evaluated by serial BLI at the indicated time points. (A) Bioluminescence signal shows tumor burden in each treatment group at indicated time points after the i.v. T cell injection. (B) Bioluminescent quantification of ccRCC tumor burden in mice. Error bars represent the SEM (n=17 in the group treated with HERV-E T cells, n=16 in the group treated with untransduced T cells, and n=7 in the no T cell treated group (Not treated). *p<0.05 (C) Kaplan-Meyer survival curves show that mice treated with HERV-E T cells had significantly prolonged survival (median survival: 50 days, p<0.001) compared with mice that received untransduced T cells from the same donor (median survival: 20 days, p<0.001) or mice that did not receive T cells (median survival: 20 days, p<0.001). Mice that developed tumor ulceration were euthanized as per animal use committee standards. *p<0.001. ccRCC, clear cell renal cell carcinoma; HERV-E, human endogenous retrovirus type E.

reduced and HERV-E T cells were given sooner, i.e. closer to tumor inoculation time (online supplemental item 2). It is important to note that HERV-E T cells could not be detected in the circulation of animals bled on day 15 and that tumors that eventually grew in mice that received HERV-E T cells showed they had persistent HERV-E expression.

Taken altogether, these data suggest that strategies bolstering the number and/or in vivo survival and persistence of HERV-E TCR transduced T cells could amplify their antitumor effects in vivo. Such strategies include giving multiple T cell infusions, prolonging T cell exposure to IL-2 and/or transducing the HERV-E reactive TCR into T cells that are less terminally differentiated and possess a greater "stem cell-like" ability of self-renewal.

TCR cross-reactivity against healthy tissues has challenged the clinical development of otherwise promising TCR-based T cell therapies.[35 36] The likelihood of HERV-E T cells causing autoimmune damage seems relatively low, especially given that the HERV-E TCR used in our experiments did not undergo any genetic modification or affinity enhancement and was isolated from a transplant patient with no signs of allo/autoimmunity,[22] having previously been subject to human thymic selection. Despite establishing the antigen specificity of HERV-E TCR and the selective expression of CT-RCC HERV-E in ccRCC,[22 24 25] off-tumor and off-target toxicities of TCR transduced T cells can be challenging to predict and cross-reactivity to non-cognate peptide sequences was not directly assessed. However, in our phase I trial

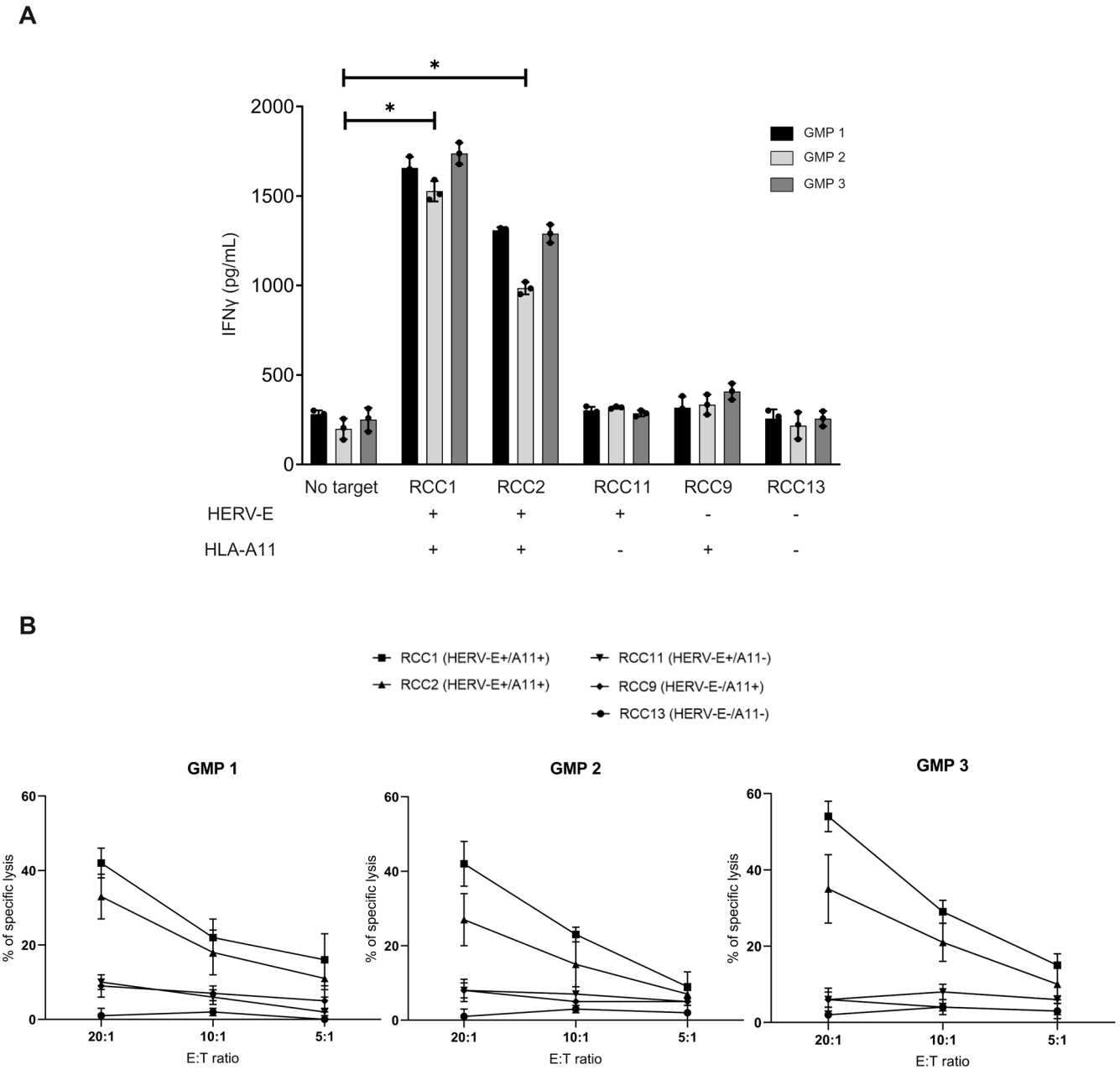

Figure 7 Antigen specificity of clinical-grade HERV-E TCR-transduced T cells manufactured from three healthy donors. (A) IFNγ secretion was determined by ELISA after coculturing HERV-E TCR-transduced T cells with different ccRCC targets. Compared with cultures without targets, IFNγ secretion levels were significantly higher only in cocultures with HLA-A11^{+}/CT-RCC HERV-E^{+} tumor cells ($p<0.05$) and not in cocultures with other cell lines. The assay was done in triplicates. (B) Dose-dependent cytotoxic activity of the three clinical-grade HERV-E TCR-transduced T cell products after coculturing with different ccRCC cell lines at 20:1, 10:1, and 5:1 effector to target (E:T) ratios, as determined by an LDH release cytotoxicity assay. The assay was done in triplicates. ccRCC, clear cell renal cell carcinoma; HERV-E, human endogenous retrovirus type E; LDH, lactate dehydrogenase.

(NCT03354390), no off-target toxicity of HERV-E T cells has been observed.[37]

Our preclinical data led us to pursue the development of a method to produce and expand HERV-E T cells for clinical use. The culture conditions used for the GMP-compliant production of HERV-E T cells resulted in robust T cell expansions achieving sufficient numbers of cells for use in a phase I clinical trial (up to at least 5×10^{7} transduced cells per kg body weight). Although clinical grade HERV-E T cells appeared to have slightly lower in vitro cytotoxicity compared with preclinical counterparts, different methods were used to quantitate their cytotoxicity, potentially accounting for the observed differences.

The data presented here should be considered within the limitations of using such a strategy in the clinic. First, the CT-RCC-1 antigen targeted by the HERV-E TCR

used in our experiments is presented by HLA-A11. The allele frequency of HLA-A11:01 is highly variable: it is only present in around 15% of the world population, ranging from 0% to 63%,[38] which limits the therapeutic applicability of HERV-E T cells in a broad patient cohort. Second, the extent of cytotoxicity mediated by HERV-E T cells is dependent on both HLA-A11 surface density and CT-RCC HERV-E mRNA expression levels in tumor cells, which our experiments show vary among different ccRCC tumors. Recently published data confirm that transcription is the limiting step for the presentation of HLA-restricted peptides, especially those derived from noncanonical regions such as HERVs.[39] Although we and others have shown the majority of ccRCC express the CT-RCC HERV-E,[22–25] lower HERV-E expression levels in ccRCC tumors would hinder the ability of HERV-E T cells to recognize such tumors. A better understanding of factors regulating the expression of this HERV could lead to strategies that boost its expression in ccRCC cells, which would enhance recognition by TCR-modified T cells through augmented antigen presentation.

In conclusion, this is the first study to show that human tumor cells can be selectively killed by T cells that had their TCR genetically engineered to recognize an antigen derived from a HERV. These data provided the background for a first-in-human clinical trial exploring infusions of autologous HERV-E T cells in patients with metastatic ccRCC (NCT03354390).

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Contributors SB designed and conducted the experiments, analyzed the data, created figures and wrote the paper. EC conducted the experiments and wrote the paper. RN and RR gave advice and wrote the paper. EMB, AVD, GMS, TTS, SP, LC, MI, US, GZ, GP, DFS and SH designed and conducted the experiments. RWC and MIN initiated the hypothesis, designed the experiments, gave advice and wrote the paper. RWC is the guarantor that the above contributions are accurate.

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Competing interests The authors (EC, MIN and RWC) declare a filed patent WO2018006054A1, licensed by T-Cure BioScience, represented by GZ and GP.

Patient consent for publication Not applicable.

Ethics approval Human ccRCC cell lines (RCC1-RCC13) were established from surgically resected tumors procured at the NIH Clinical Center on IRB-approved protocols 04-H-0012, 97-C-0147, and 97-H-0196. All animal experiments described here were approved by the NIH Animal Care and Use Committee on the protocol H-0334.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. All data relevant to the study are included in the article or uploaded as online supplemental information.

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