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CD8⁺ T Cell Clones Specific for the 5T4 Antigen Target Renal Cell Carcinoma Tumor-Initiating Cells in a Murine Xenograft Model

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Abstract

The tumor antigen 5T4 is frequently expressed at high levels on renal cell carcinoma (RCC) and other epithelial carcinomas. Surveys of normal tissues demonstrate abundant 5T4 expression on placental trophoblast cells with limited expression elsewhere. 5T4 is the target for a therapeutic cancer vaccine (MVA-5T4) that elicits 5T4-specific serological, proliferative, and CTL responses. However, the anti-tumor activity of 5T4-specific CTL has not been extensively characterized. CD8⁺ T cells from HLA-A2⁺ healthy donors (n=4) or RCC patients (n=2) were stimulated *in vitro* with the HLA-A2-binding nonamer peptides 5T4_{17–25} or 5T4_{97–105} and screened by flow cytometry with specific tetramers (TET). CD8⁺/TET⁺ T cell clones specific for 5T4_{17–25} or 5T4_{97–105} peptide were isolated from 4/6 and 1/4 donors respectively. A subset of clones specific for 5T4_{17–25} was cytolytic for MVA-5T4 infected HLA-A2⁺ LCL target cells and for constitutively HLA-A2- and 5T4- expressing RCC tumor cell lines (including A498 RCC). In a xenoengraftment assay, the co-inoculation of a representative 5T4_{17–25}-specific CTL clone with A498 RCC tumors cells into immune deficient mice completely prevented growth of A498 tumors. Taken together, these data demonstrate high avidity CD8⁺ CTL able to recognize the naturally-processed 5T4_{17–25} epitope on RCC tumor cells including putative tumor-initiating cells are present in peripheral blood of both healthy donors and RCC patients. CD8⁺ T cell immunity targeting 5T4_{17–25} is therefore of substantial interest both as a potential target for further development of vaccination or adoptive cellular immunotherapy and for immune monitoring studies in association with nonspecific immunotherapies.

Keywords

cytotoxic T-lymphocyte; 5T4; renal cell carcinoma; tumor-initiating cells; xenograft

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Introduction

Renal cell carcinoma (RCC) is the most common malignant kidney tumor in adults representing 4% of new cancer diagnoses in the United States.¹ A majority of RCC patients either present with metastatic disease or relapse following surgical resection of the primary tumor.² Conventional systemic treatments with cytotoxic chemotherapy or hormonal therapy have minimal activity against metastatic RCC.³ Since 2005, seven new drugs targeting deregulated angiogenic and mitogenic signaling pathways in clear cell RCC tumors have been approved by the FDA for the treatment of advanced RCC. These include tyrosine kinase inhibitors (sorafenib, sunitinib, pazopanib, axitinib) that disrupt signaling mediated by the vascular endothelial growth factor (VEGF)-receptor; the VEGF-specific monoclonal antibody bevacizumab, and inhibitors of mammalian target of rapamycin (mTOR) (temsirolimus and everolimus). Although these targeted therapies have been rapidly adopted as first- and second-line treatments for metastatic clear cell RCC, the development of tumor resistance and disease progression has been uniformly observed in treated patients.^{4–10} Metastatic RCC is also sensitive to immune-based therapies. For example, high-dose interleukin-2 (IL-2) remains a first line treatment option for select RCC patients and has been associated with durable complete remissions (CR) in 5 to 7% of RCC patients.¹¹ The anti-tumor effect of IL-2 is thought to be indirect and mediated by recipient cellular immunity specific for RCC tumor. However, the antigenic targets for effector cells that mediate the anti-tumor effects of IL-2 remain undefined.

The 5T4 antigen is a cell surface glycoprotein expressed on many common epithelial tumors including 95% of primary RCC's (from both clear cell and papillary histologies) and is highly expressed on placental trophoblast cells, but is not detected on most other normal tissues.^{12–14} Transfection of *5T4* into epithelial cells has been associated with disruption of cell-cell contacts and increased cell motility; features associated with tumor cell metastasis.¹⁵ A higher frequency of 5T4 expression has been associated with more advanced disease in patients with cervical, colorectal (CRC), ovarian, gastric, and non-small cell lung cancers (NSCLC).^{16, 17} 5T4 expression on NSCLC tumor-initiating cells has also been recently described.¹⁷ 5T4 therefore represents a compelling target for cancer immunotherapy.

A recombinant modified vaccinia Ankara (MVA) virus engineered to express 5T4 (MVA-5T4; TroVax®) has been tested as a therapeutic cancer vaccine in patients with RCC, CRC, or prostate cancer.¹⁶ Immune monitoring studies conducted as part of early phase testing of MVA-5T4 have confirmed that 5T4-specific antibody, proliferative, and/or CTL responses were elicited in subsets of vaccinated patients.^{18, 19} In a randomized, placebo-controlled, phase III trial for advanced clear cell RCC, MVA-5T4 vaccination was administered in a front-line setting paired with low-dose IL-2, IFN- α or sunitinib. No survival difference was observed in the overall study population, however, retrospective analyses identified patient subgroups with superior survival after MVA-5T4 vaccination, including good prognosis patients who received MVA-5T4 plus IL-2.²⁰

Further development of MVA-5T4 or other clinical reagents designed to target 5T4 would benefit from greater insight into immune mechanisms of 5T4-directed anti-tumor effects. The anti-tumor activity of cellular-immune responses specific for 5T4 has not been well established in preclinical models.¹⁶ Recently, two candidate CTL epitopes from the 5T4 antigen presented by HLA-A2 (residues 17–25 and 97–105) have been identified.^{21, 22} We now report the isolation of CTL as clones specific for peptides 5T4_{17–25} or 5T4_{97–105} in association with HLA-A2. The anti-tumor activity of these CTL for constitutively 5T4 expressing RCC tumor cells measured by *in vitro* assays and in a murine xenograft model is described.

Materials and Methods

Reagents

Synthetic peptides corresponding to 5T4 sequences 17–25 (RLARLALVL; “p17”) and 97–105 (FLTGNQLAV; “p97”) (GenScript Corporation, Piscataway, NJ) were dissolved at 10 mg/ml in 100% DMSO (Invitrogen, Carlsbad, CA) and stored at –20°C. APC-labeled HLA tetramers (TET) composed of HLA-A2 with 5T4 peptides p17 or p97 were generated by the Immune Monitoring Core Laboratory at our center.

Culture of 5T4-Peptide-Specific T Cell Lines and Clones

Peripheral blood leukapheresis products were obtained from four healthy donors and two patients with metastatic clear cell RCC. All donors gave written informed consent to participate in the research study that was approved by the Institutional Review Board at our center. Donor genotype was confirmed *HLA-A*0201* positive by PCR-based typing kits (Invitrogen) using genomic DNA template and the manufacturer’s protocol. RCC patient CRF had undergone a nephrectomy and then received systemic treatment with IL-2 without response followed by interleukin-21 (IL-21) plus sorafenib on a clinical trial. Leukapheresis was obtained after 20 months of therapy with IL-21 plus sorafenib at the time the patient had a very good partial remission. RCC patient DLG had undergone a nephrectomy and then received systemic treatment with IL-2 resulting in a partial response followed by sunitinib. The patient then underwent a metastectomy surgery. Leukapheresis was obtained 8 weeks after surgery at a time point the patient had no evidence of disease.

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation. Dendritic cells (DC’s) to serve as antigen presenting cells (APC’s) for peptide were generated from peripheral blood monocytes by two-day cultures as described.²³ CD8⁺ T cells were enriched from PBMC by negative selection using magnetic bead separation per the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). CD8⁺ T cell lines were established in T25 culture flasks that contained approximately 10 million CD8⁺ T cells and 5 million DC in CTL media²⁴ with 5T4 peptides at concentrations between 10 and 0.01 µg/ml at 10-fold increments or with no added peptide. After 24 hours, IL-7 (10 ng/ml) and IL-12 (10 ng/ml) (both from R&D Systems, Minneapolis, MN) were added to the cultures. Responder T cells were re-stimulated with peptide at 10–12 day intervals using monocyte APCs enriched from PBMC by plastic adherence in AIM-V media (Invitrogen)/ 1% human serum for one hour at 37°C. One day after the second and third peptide stimulation, IL-2 (Prometheus, San Diego, CA) was added at 25 IU/ml. T cell lines were evaluated by flow cytometry 10–12 days after the second or third peptide stimulation.

Flow-sorted CD8⁺/TET⁺ cells were cloned by limiting dilution in 96-well microtiter cultures. Wells contained 1×10^5 irradiated (35Gy) PBMC and 1×10^4 irradiated (70 Gy) EBV-LCL feeder cells in 200 µL CTL culture media containing 30 ng/mL OKT3 (Centocor Ortho Biotech Inc., Raritan, NJ) and 50 IU/mL IL-2. Responder T cells were added at 200 to 300 per 96-well plate. Positive wells were identified by microscopy after 10 to 12 days and aliquots of T cells then screened for lysis of T2 target cells with and without loading of specific peptide. Selected clones were then expanded and maintained in culture as described.²⁵

Cell Lines

Maintenance of the T2 cell line, EBV-transformed B LCL lines, RCC tumor lines A498, LB1828, DOBSKI, BB65, and breast tumor line MDA-231 have been previously described.²⁶ The RCC tumor line SST548 was isolated from a primary clear cell tumor. Dermal fibroblast derived cell lines were isolated and maintained as described.²⁷ Proximal

tubule endothelial cell (PTEC) cultures were isolated from normal renal cortex explants and maintained as described.²⁸ The 293 human embryonic kidney cell line and breast tumor lines BT-20 and BT-474 were obtained from ATCC (Manassas, VA).

Flow Cytometry

CD8⁺ T cell lines were stained with FITC-labeled anti-CD8⁺ monoclonal antibody (mAb) (1:20 dilution) (BD Biosciences, San Jose, CA), APC-labeled MHC-tetramers (10 µg/ml), and propidium-iodide (0.5 µg/ml) (PI). PI-excluding CD8⁺/TET⁺ cells were collected on a BD FACSAria cell sorter utilizing BD FACSDiva software (BD Biosciences). Flow cytometry based TCR Vβ-typing was performed on CD8⁺ T cell clones using a Beta Mark kit (Beckman Coulter, Miami, FL) following the manufacturer's protocol. Cell lines were stained with a 5T4-specific mAb clone B8 (1 µg/ml)¹⁴ or isotype-matched IgG1 control mAb (BD Biosciences) in PBS/2% FBS buffer. Cells were washed and stained with 10 µg/ml of FITC-labeled goat anti-mouse IgG1 secondary Ab (Southern Biotech, Birmingham, AL). All analytical flow studies were performed on a BD FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

Generation and Analysis of 5T4 Minigene Plasmid Vectors

Minigenes were designed with a Kozak consensus sequence followed by an initiating methionine and the coding sequence corresponding to the 5T4 p17 or p97 nonamer sequence by annealing complementary oligonucleotides designed with 3' overhanging deoxyadenosines. The minigenes were then cloned into the pcDNA3.1 expression plasmid using the pcDNA3.1/V5-His-TOPO TA Expression Kit (Invitrogen), according to the manufacturer's instructions. Oligonucleotide sequences for the p17 construct were (forward) 5'-GCCACCATGCGGCTGGCGCGACTAGCGCTGGTACTCTGA-3' and (reverse) 5'-ACGGTGGTACGCCGACCGCGCTGATCGCGACCATGAGAC-3' and for the p97 construct were (forward) 5'-GCCACCATGTTCCTTACCGGCAACCAGCTGGCCGTGTGA-3' and (reverse) 5'-ACGGTGGTACAAGGAATGGCCGTTGGTCGACCGGCACAC-3'. Minigene plasmid DNA (160 ng) was cotransfected into 293 cells along with a second plasmid encoding for an HLA gene (HLA-A*0201 or HLA-B*0702 as a control) using Lipofectamine (Invitrogen) and following the manufacturer's protocol. After 24 hrs, 2×10^4 T cell clones were added to wells in culture media supplemented with 25 IU/ml IL-2. After an additional 24 hrs. culture, supernatants were harvested and assayed for interferon (IFN)-γ by enzyme-linked immunosorbent assay (ELISA), (Endogen, Rockford, IL).

Cytotoxicity Assays

For some experiments, LCL were infected with wild-type MVA or MVA-5T4²⁹ at a 10:1 multiplicity of infection in serum-free media for 1 hour at 37°C. Culture media was then adjusted to 10% FCS. Chromium-release cytotoxicity assays were performed as previously described.³⁰

Real-Time Quantitative PCR Analyses for 5T4 Expression

Total RNA was isolated from 0.5 to 3.0×10^6 *in vitro* cultured RCC tumor cells or LCL using an RNeasy Plus Mini Kit following the manufacturer's instructions (Qiagen, Valencia, CA). First-strand cDNA was synthesized from 1 µg of total RNA using Oligo-dT and random hexamer priming with a Transcriptor First Strand cDNA Synthesis Kit following the manufacturer's instructions (Roche, Indianapolis, IN). Additional normal human tissue cDNA samples were obtained (human Multiple Tissue cDNA panels I,II, and Digestive System, Clontech Laboratories Inc, Mountain View, CA). 5T4 expression was normalized to *RPL13A* run in the same tray. Primer sequences for 5T4 amplification were (forward) 5'-

CTCAACAGTGCTGACCTGGA-3' and (reverse) 5'-TAGCGCCTATCAGGGCTAAA-3' and for *RPL13A* amplification were (forward) 5'-CCTGGAGGAGAAGAGGAAAGAGA-3' and (reverse) 5'-TTGAGGACCTCTGTGTATTTGTCAA-3' (Real Time Primers, Elkins Park, PA). Reactions were run in triplicate with FastStart Universal SYBR Green Master (Rox) (Roche) corresponding to reaction parameters and analysis methods previously described.²⁶

RCC Xenograftment into Immune-Deficient Mice

A firefly luciferase-expressing A498 RCC line (A498-L) was generated by retroviral transfection. Briefly, AmphiPhoenix retroviral packaging cells (National Gene Vector Biorepository, Indianapolis, IN) were transfected with 2 µg of MSCV luciferase-PGK Hygro plasmid DNA (plasmid 18782, Addgene, Inc., Cambridge, MA; Principal Investigator, Scott Lowe) using Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Retroviral supernatants were prepared as described.³¹ A498 monolayers were inoculated with the luciferase-encoding retrovirus in culture medium with 5 µg/mL polybrene (Millipore, Billerica, MA). After 24 hours, cells were placed in selective media containing 100 µg/mL hygromycin B (Fisher Scientific, Waltham, MA). Luciferase activity was assayed by the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's protocol.

Immune-deficient NOD/SCID/IL2R $\gamma^{-/-}$ mice (NSG)³² received low-dose irradiation (275 cGy) and were inoculated in contralateral flanks with 1×10^5 wild-type A498 RCC cells mixed with 1×10^6 of the 5T4₁₇₋₂₅ specific CTL clone ALS-17 or a control clone KSN-7A7 specific for an antigen target not expressed on A498.³³ Tumor-cells and CTL were mixed together in culture media, kept on ice, and 100 µl of the cell suspension injected within 1 hour into mice. Alternatively, A498-L tumor cells and CTL were mixed together in culture media plus Matrigel (BD Biosciences) at a 1:1 (v/v) ratio in a final volume of 50 µl and implanted in the sub kidney capsule space of NSG mice. All mice received 20,000 IU of IL-2 by intraperitoneal (ip) injection daily for 5 days following cell transfers. Bioluminescent imaging was performed weekly for the A498-L tumors following ip injection of 150 mg/kg D-luciferin (Caliper Life Sciences, Hopkinton, MA) with an IVIS Spectrum imager and Living Image software (Caliper Life Sciences).

Immunohistochemistry

Serial sections of formalin-fixed, paraffin-embedded (FFPE), clear cell RCC tumors representing primary (CRF) or metastatic (DLG) tumor were used. Sections underwent de-waxing and heat induced epitope retrieval. Sections were stained using a Leica Bond III autostainer (Leica Microsystems, Buffalo Grove, IL). Immunostaining for carbonic anhydrase IX was performed with mAb M75 (a generous gift of Egbert Oosterwijk, Nijmegen, The Netherlands) as described.³⁴ Immunostaining for 5T4 was performed with mAb clone MAB4975 (R&D Systems) at 10 µg/ml.¹⁷ A498 xenograft tumors were bisected and tumors frozen in OCT media in parallel with FFPE processing. Frozen A498 tumor in OCT was sectioned and stained for 5T4 with primary mAb clone B8 as described.¹⁴ FFPE A498 tumor was stained for HLA expression with pan-HLA reactive mAb EMR8-5 according to the vendor's instructions (Abcam, Cambridge, MA). Sections were counter stained with hematoxylin. Digital images were obtained with a Magnafire digital imaging system (Olympus America, Melville, NY).

Results

In Vitro Isolation of CD8⁺ CTL Clones Specific for HLA-A2-Binding 5T4 Peptides

To isolate CD8⁺ T cell clones specific for the nonamer peptides 5T4_{17–25} or 5T4_{97–105}, peptide-stimulated *in vitro* primary CD8⁺ T cell lines were initiated from HLA-A2-positive leukapheresis products collected from 4 healthy donors and 2 patients with metastatic RCC. Following 2 or 3 rounds of peptide stimulation, CD8⁺ T cell lines were then screened by flow cytometry with corresponding HLA-A2/5T4_{17–25} or HLA-A2/5T4_{97–105} specific tetramers. These analyses demonstrated expansion of CD8⁺/TET⁺ cells in 4/6 CD8⁺ T cell lines representing 1.0% to 3.7% of CD8⁺ cells stimulated with the 5T4_{17–25} peptide including lines established from 3 of 4 healthy donors and 1 of 2 RCC patients (Figure 1). Expansion of CD8⁺/TET⁺ cells was also observed in 1/4 CD8⁺ T cell lines and represented 17.3% of CD8⁺ T cells stimulated with the 5T4_{97–105} peptide (Figure 1). These data indicated that both 5T4 peptide sequences were immunogenic in the stimulation conditions tested, but suggest CD8⁺ T cell responses against the 5T4_{17–25} peptide may be more frequently observed.

CD8⁺/TET⁺ cells were then flow-sorted from peptide-stimulated T cell lines and cloned by limiting dilution. CD8⁺ T cell clones were successfully isolated from each of the four CD8⁺ T cell lines that were positive by HLA-A2/5T4_{17–25} tetramer screening and were cytolytic for T2 target cells pulsed with 5T4_{17–25} peptide (Figure 2A). Half-maximal lysis of peptide-loaded T2 targets cells was observed at peptide concentrations between 10 and 100 pM by CTL clones isolated from both healthy donors and a patient with mRCC (Figure 2A). CD8⁺ T cell clones were also isolated from the one CD8⁺ T cell line positive for CD8⁺/TET⁺ cells by HLA-A2/5T4_{97–105} tetramer screening. These clones were similarly cytolytic for T2 target cells pulsed with 5T4_{97–105} peptide with half-maximal target lysis also at 10 to 100 pM (Figure 2B).

A serological typing assay was used to identify the TCR Vβ gene segment family expressed by a subset of 5T4_{17–25} peptide-specific clones isolated from each of 4 donors. These analyses identified three different TCR Vβ gene segment families (Vβ13.2, Vβ14, and Vβ18, data not shown) expressed on the cell surface of individual CTL clones specific for the 5T4_{17–25} peptide. Taken together, these observations suggest a diverse repertoire of CD8⁺ T cells with high avidity for the HLA-A2-associated 5T4_{17–25} epitope that is available in the peripheral blood of both healthy donors as well as patients with mRCC.

Immunohistochemical staining was carried out on FFPE samples of tumor from the two patients with clear cell RCC included in peripheral blood T cell studies. Both tumors stained strongly positive for the clear cell RCC-associated marker carbonic anhydrase IX³⁴ and were also shown to have regional expression of 5T4 (Figure 3A–D). These data confirmed that 5T4-peptide reactive CTL clones were isolated from an RCC patient whose tumor clearly expressed the target antigen.

5T4_{17–25} is a Naturally-Processed T Cell Epitope Presented by HLA-A2

To determine if the HLA-A2-binding peptides 5T4_{17–25} and 5T4_{97–105} would associate with HLA-A2 and form cell surface complexes when expressed endogenously within cells, minigene plasmid vectors were designed to express an initiating methionine followed by the nonamer sequence corresponding to 5T4_{17–25} or 5T4_{97–105}. The 293 cell line was transfected with the 5T4 minigene plasmids with or without a second plasmid coding for HLA-A2 and co-cultured with 5T4-specific CD8⁺ T cell clones. HLA-A2-dependent stimulation of robust IFN-γ release from 5T4_{17–25} or 5T4_{97–105} specific CTL clones was observed and suggested efficient presentation of 5T4 peptide/HLA-A2 complexes on the cell surface of minigene and HLA-A2 co-transfected 293 target cells (Figure 4A,C).

Processing and presentation of the 5T4₁₇₋₂₅ and 5T4₉₇₋₁₀₅ peptide epitopes from the native 5T4 protein was then tested by infecting HLA-A2-expressing, EBV-transformed LCL target cells with a recombinant vaccinia virus vector encoding a full length 5T4 cDNA (MVA-5T4).²⁹ Representative 5T4₁₇₋₂₅-specific CTL clones isolated from both healthy donors and from an RCC patient efficiently recognized HLA-A2 positive LCL targets infected with MVA-5T4 but not control LCL infected with a wild type vaccinia virus or mock infected targets (Figure 4B). In contrast, a 5T4₉₇₋₁₀₅-specific CTL clone showed no lysis of MVA-5T4 infected LCL targets (Figure 4D), despite high levels of cell surface 5T4 expressed on MVA-5T4 infected LCL measured by flow cytometry with a 5T4 specific mAb (data not shown). Taken together, these data support the conclusion that 5T4₁₇₋₂₅ represents a naturally-processed T cell epitope.

***In Vitro* Cultured Tumor Cell Lines Express 5T4 and are Targets for 5T4₁₇₋₂₅-Specific CTL Clones**

A panel of RCC tumor cell lines was screened for cell surface 5T4 expression by flow cytometry as previously described.¹⁴ Four RCC tumor cell lines known to express cell surface HLA-A2 in addition to the HLA-A2-negative SST548 RCC line all had significantly brighter immunofluorescent staining for cell surface 5T4 than LCL, fibroblast, or PTEC cells obtained from *in vitro* culture and analyzed in parallel (Figure 5A). To determine if *in vitro* cultured tumor cells representing histologies other than RCC also expressed cell surface 5T4, a panel of colorectal, ovarian, and breast carcinoma cell lines was screened for 5T4 by flow cytometry. Bright cell surface 5T4 expression was seen on the HLA-A2-positive breast tumor line MDA-231 and the HLA-A2-negative lines BT-20 and BT-474 (Figure 5A).

5T4 transcription in RCC and breast tumor cell lines was compared to LCL using a real-time (RT)-PCR assay. 5T4 transcript levels were > 8.7-fold higher for all tumor cell lines compared to LCL (Figure 5B). 5T4 transcription in a representative RCC tumor line (BB65 RCC) and LCL (BB65 LCL derived from the same patient) was then compared to 12 normal human tissues. These analyses showed the highest expression of 5T4 transcripts in RCC tumor and placenta (4.7-fold) versus all other normal tissue samples (Figure 5C) consistent with prior immunohistochemical analyses that demonstrated abundant 5T4 protein in placenta and tumor tissues.^{12, 13} A low level of 5T4 protein expression has been previously observed in specialized epithelia in a subset of normal tissues including esophagus, stomach and large intestine. Therefore, 5T4 transcripts within the GI tract were analyzed in more detail. 5T4 transcription in representative RCC tumor and LCL was then compared to a cDNA array representing 11 sub-segments of GI tract tissues. This analysis demonstrated > 13-fold higher expression of 5T4 transcripts in RCC tumor versus any individual GI tract region (Figure 5D). Thus, the RT-PCR analyses of normal tissues closely correlated with previous immunohistochemical studies that documented high levels of 5T4 protein expression in RCC tumors and placenta versus normal tissues.^{13, 14}

HLA-A2- and 5T4-expressing RCC tumor cell lines were then tested as targets for 5T4₁₇₋₂₅ specific CTL clones isolated from healthy donors or an RCC patient in cytotoxicity assays. These studies demonstrated HLA-A2 dependent specific recognition of RCC tumor by 5T4₁₇₋₂₅ specific CTL clones (Figure 6A). No recognition (< 2% specific lysis) of HLA-A2⁺ LCL, fibroblast, or PTEC target cells by these clones was observed (Figure 6B). To determine if tumor cells representing histologies other than RCC could also be killed by 5T4₁₇₋₂₅ specific CTL clones, 5T4-expressing breast tumor lines were also tested as CTL targets in cytotoxicity assays (Figure 6C). These data demonstrated HLA-A2 dependent specific recognition of the breast tumor cell line MDA-231 comparable to RCC tumor (Figure 6C).

A 5T4₁₇₋₂₅-Specific CTL Clone Prevents RCC Xenoengraftment into Immune-Deficient Mice

The 5T4- and HLA-A2-expressing RCC tumor cell line A498 can be engrafted into immune deficient NSG mice growing as either a sub kidney-capsule or flank tumor (data not shown). A luciferase-expressing A498 subline (A498-L) was generated, allowing for serial monitoring of A498 tumor growth in NSG mice via bioluminescence imaging. A phenotypic description of RCC tumor cell subpopulations with tumor-initiation function has not been definitively established.³⁵ Therefore, to functionally assess the expression of the 5T4₁₇₋₂₅ epitope on putative tumor-initiating A498 cells, tumor inoculations were performed into NSG mice with A498 or A498-L mixed at the time of implantation with the 5T4₁₇₋₂₅ specific CTL clone ALS-17 or an irrelevant CTL (clone KSN 7A7 recognizing an HLA-A3-restricted minor H antigen³³ not present on A498 cells; data not shown). A498 was then administered as a flank injection or A498-L implanted under the kidney capsule of NSG mice. The ALS-17 CTL clone completely prevented engraftment and tumor development of A498 (4/4) at flank injection sites and of A498-L (4/4) after sub-capsule inoculations. In contrast, all NSG mice implanted with A498 or A498-L mixed with control KSN 7A7 T cells developed tumors with growth kinetics similar to A498 or A498-L inoculations performed without the addition of CTL (Figure 7A–C and data not shown). Immunohistochemical analyses of A498 and A498-L tumor xenografts harvested from NSG mice demonstrated homogenous expression of both 5T4 and HLA antigens confirming the maintenance of target antigen expression for 5T4₁₇₋₂₅ specific CTL on A498-derived tumors grown as xenografts versus *in vitro* culture (Figure 7D, E, and data not shown).

Discussion

Several lines of evidence suggest the possibility for spontaneous cellular immunity to RCC including spontaneous remissions of advanced RCC³⁶, and the isolation of CTL lines and clones from tumor-infiltrating lymphocytes or peripheral blood mononuclear cells (PBMC) of some RCC patients capable of recognizing autologous tumor.^{37, 38} Immunotherapies intended to augment T cell mediated anti-tumor effects such as IL-2, IL-21, or blocking antibodies targeting the inhibitory receptors CTLA-4 or PD-1 on activated T lymphocytes have also been associated with objective responses of metastatic RCC.^{39–41} However, the antigen targets of spontaneous or induced RCC-reactive T cell responses remain poorly characterized.

Extensive analyses have revealed that RCC cells do not commonly express tumor antigens encoded by cancer-testis (CT) genes (for example, the *MAGE* gene family and *NY-ESO-1*) – that represent antigen targets for numerous clinical trials in other cancer diagnoses.^{42, 43} However, the expression profile of the 5T4 antigen shares common features with CT antigens. In addition to tumor and testicular expression, a number of CT antigens are also highly expressed in placenta. As seen with 5T4, higher frequency of CT antigen expression is often correlated with advanced disease and worse outcomes. Spontaneous serologic and cellular immune responses specific for CT antigens including MAGE-A, NY-ESO-1 and SSX have been observed in antigen-positive cancer patients. Similarly, immune monitoring assays performed in support of clinical studies with the MVA-5T4 vaccine have detected spontaneous humoral or cellular immune responses in the pretreatment blood samples of some patients.^{44–46}

Substantial evidence demonstrates the potential for CD8⁺ T cell recognition of the 5T4 antigen. Following vaccination with MVA-5T4, ELISPOT assays with peripheral blood samples demonstrate T cell reactivity to nonamer and decamer peptide libraries tiling the length of the 420 amino acid 5T4 protein.^{19, 22} Correlation of the immunogenicity of individual peptide sequences with HLA-binding data has identified many candidate CTL epitopes within the 5T4 sequence including HLA-A2-binding peptides 5T4₁₇₋₂₅ or

5T4₉₇₋₁₀₅.^{21, 22} Independent of MVA-5T4 vaccination, reports from other investigators have described candidate CTL epitopes in 5T4 presented by HLA-A2 or HLA-Cw7.^{47, 48} However, to our knowledge the functional assessment of the cytolytic potential of 5T4 specific CTL for RCC tumor cells constitutively expressing 5T4 and measured by conventional *in vitro* cytotoxicity assays or in more complex three-dimensional tumor models has never been reported.

Our data now demonstrate that CTL were present in the peripheral blood of 4 of 6 individuals tested including a patient with metastatic clear cell RCC, and capable of detecting T2 targets cells pulsed with the 5T4₁₇₋₂₅ peptide at concentrations as low as 10 pM. Such CTL when isolated as clones were also capable of detecting the target epitope on constitutively 5T4- and HLA-A2-expressing RCC target cells measured by conventional *in vitro* cytotoxicity assays. Expression of three unique TCR V β chains among the 5T4₁₇₋₂₅-specific CTL clones isolated from four donors suggests the potential for heterogeneous and polyclonal CTL reactivity to the 5T4₁₇₋₂₅ epitope for responding individuals. Our studies also demonstrate that peptide reactivity is not a sufficiently rigorous marker for anti-tumor T cell activity. CTL specific for 5T4₉₇₋₁₀₅, despite recognition of T2 pulsed with 10 pM peptide, showed no significant lytic activity for MVA-5T4 infected target cells or RCC tumor cells. Recognition of target cells expressing the pre-processed 5T4₉₇₋₁₀₅ as a minigene but not MVA-5T4 infected target cells by CTL specific for 5T4₉₇₋₁₀₅ suggests that inefficient processing of 5T4₉₇₋₁₀₅ from native 5T4 protein may limit CTL recognition of this epitope.

Our research group has previously used tumor engraftment into immune deficient mice as a functional assay to detect T cell recognition of a tumor-initiating cell population for acute leukemia.^{49, 50} While a functional hierarchy among tumor cells is more clearly established for human acute leukemias than for RCC, the capacity of a CTL clone specific for 5T4₁₇₋₂₅ to prevent engraftment of the A498 RCC tumor cell in NSG mice is consistent with 5T4-expression and thus, 5T4₁₇₋₂₅ expression, by a putative tumor-initiating cell population. We note with interest a recent report demonstrating that 5T4 expression in NSCLC cells is a marker associated with a tumor-initiating cell phenotype for this tumor.¹⁷ Further, the observation of sustained 5T4 expression by A498 tumor propagated as a xenograft in NSG mice represents a model system that will allow examination of the ability of 5T4₁₇₋₂₅ specific T cells to eliminate established A498 tumors. The anti-tumor activity of 5T4₁₇₋₂₅ specific T cells delivered by intra-tumor inoculation or by intravenous injection represents an area of ongoing investigation and reflects a treatment approach similar in kind to the treatment of established human tumor by adoptive T cell therapy.

In conclusion, the validation of a naturally-processed CTL epitope within the 5T4 antigen presented by HLA-A2 is of substantial interest due to the favorable expression pattern of 5T4, high frequency of 5T4 expression in RCC tumors, and high population prevalence of HLA-A2. CTL reactivity for 5T4₁₇₋₂₅ will represent a suitable target for developing clinical trials of adoptive T cell therapy for metastatic RCC. In addition, 5T4₁₇₋₂₅ represents a rational target for immune monitoring studies in association with nonspecific immune therapies applied to the treatment of advanced RCC.⁵¹

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Figure 1. Flow cytometry analyses of peptide-stimulated CD8⁺ T cell lines

Peptide-stimulated CD8⁺ T cells lines were cultured from HLA-A2-positive healthy donors or RCC patients. After two or three *in vitro* stimulations with 10 µg/ml of peptides 5T4₁₇₋₂₅ (p17) or 5T4₉₇₋₁₀₅ (p97), T cell lines were then stained with an anti-CD8 mAb and specific TET and analyzed by flow cytometry. The percentage of TET-binding CD8⁺ T cells is noted in each panel.

Figure 2. Recognition of peptide-pulsed T2 targets by 5T4-peptide specific CD8⁺ T cell clones
 Representative CD8⁺ T cell clones isolated from 5T4₁₇₋₂₅-stimulated (panel A) or 5T4₉₇₋₁₀₅-stimulated (panel B) T cell lines were tested for lysis of T2 target cells pulsed with specific peptide in a standard 4-hour cytotoxicity assay. The effector:target (E:T) ratio was 10:1 for all clones.

Figure 3. Immunohistochemical staining of RCC tumors

Serial sections of FFPE clear cell RCC tumor from patients CRF (primary tumor - panels A, B) and from patient DLG (metastatic tumor - panels C, D) were immunostained for carbonic anhydrase IX (CAIX) (panels A, C)³⁴ or 5T4 (panels B, D)¹⁷ and counter stained with hematoxylin.

Figure 4. Endogenous HLA-A2-presentation of CTL epitopes 5T4₁₇₋₂₅ or 5T4₉₇₋₁₀₅ to CD8⁺ CTL clones

Representative CD8⁺ T cell clones specific for 5T4₁₇₋₂₅ (panel A, B) or 5T4₉₇₋₁₀₅ (panel C, D) were tested by IFN- γ ELISA for recognition of 293 target cells transfected with plasmid vectors encoding for 5T4₁₇₋₂₅ or 5T4₉₇₋₁₀₅ minigenes paired with or without a second plasmid encoding for HLA-A2 (panel A, C). CD8⁺ T cell clones were also tested for recognition of HLA-A2 positive LCL target cells loaded with specific peptide (1 μ g/ml) or infected with MVA-5T4 or a control wild-type vaccinia virus (MVA-wt) in 4-hour cytotoxicity assays with a 10:1 E:T (panel B, D).

A

Figure 5. 5T4 expression by *in vitro*-cultured cell lines and normal tissues

RCC (BB65, A498, DOBSKI, LB1828, and SST548), T2, LCL, PTEC, or breast carcinoma (MDA-231, BT-20 and BT-474) cells were stained with a 5T4-specific mAb (solid line) or IgG1 isotype control mAb (shaded) and analyzed by flow cytometry (panel A). Relative levels of 5T4 transcript were measured by quantitative RT-PCR from cDNA templates generated from five RCC, three breast carcinoma, and two LCL cell lines. 5T4 expression was normalized to *RPL13A* amplification run in the same tray and plotted relative to the BB65 RCC tumor cell line (BB65 RCC expression = 1, arbitrary units) (panel B). In similar analyses, relative levels of 5T4 transcript were then compared for a representative RCC

tumor line (BB65 RCC), and an array of 12 normal human tissues (panel C) or an array representing 11 GI-tract sub-segments (panel D).

Figure 6. 5T4- and HLA-A2 expressing RCC tumor cell lines are targets for 5T4₁₇₋₂₅-specific CTL clones

Representative CD8⁺ T cell clones specific for 5T4₁₇₋₂₅ were used in cytotoxicity assays with T2 (without or with the addition of 5T4₁₇₋₂₅ peptide at 1 µg/ml), RCC and LCL target cell lines (panel A), cultured human renal PTEC or fibroblast cell lines (panel B), or breast carcinoma tumor cell lines (panel D) in 4-hour cytotoxicity assays with a 10:1 E:T. All target cells for cytotoxicity assays were HLA-A2-positive except SST548 RCC (panel A), and BT-20 and BT-474 (panel C).

Figure 7. Xenoengraftment of A498 RCC tumor cells into immune-deficient NSG mice

A group of 4 NSG mice received contralateral flank inoculations with 1×10^5 A498 RCC tumor cells mixed with 1×10^6 of the 5T4₁₇₋₂₅-specific CD8⁺ CTL clone ALS-17 or the control CD8⁺ CTL clone KSN 7A7 (panel A). Mice were examined for tumor growth weekly. Two of 4 animals from the control group were sacrificed for large tumor burden after week 9 (noted by an arrow in the panel). Groups of 4 NSG mice received sub kidney capsule implantations of A498-L mixed with CTL clones as in panel A. Mice were evaluated weekly by bioluminescent imaging following i.p. injection of D-luciferin (panel B). Serial images of representative mice following D-luciferin injection are shown (panel C). Frozen-sectioned A498 xenograft tumor was immunostained for 5T4 expression as described (panel D).¹⁴ FFPE A498 xenograft tumor was immunostained for HLA expression with the pan-HLA reactive mAb EMR8-5 (panel E). Slides were counter stained with hematoxylin.