Published in final edited form as:
Clin Cancer Res. 2009 January 1; 15(1): 169–180. doi:10.1158/1078-0432.CCR-08-1638.

Characterization of Genetically Modified T-Cell Receptors that Recognize the CEA:691-699 Peptide in the Context of HLA-A2.1 on Human Colorectal Cancer Cells

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Abstract

Purpose—Carcinoembryonic antigen (CEA) is a tumor-associated protein expressed on a variety of adenocarcinomas. To develop an immunotherapy for patients with cancers that overexpress CEA, we isolated and genetically modified a T-cell receptors (TCRs) that specifically bound a CEA peptide on human cancer cells.

Experimental Design—HLA-A2.1 transgenic mice were immunized with CEA:691-699. A CEA-reactive TCR was isolated from splenocytes of these mice and was genetically introduced into human peripheral blood lymphocytes via RNA electroporation or retroviral transduction. Amino acid substitutions were introduced throughout the complementarity determining regions (CDR1, CDR2, and CDR3) of both TCR α and β chains to improve recognition of CEA.

Results—Murine lymphocytes bearing the CEA-reactive TCR specifically recognized peptide-loaded T2 cells and HLA-A2.1$^+$ CEA$^+$ human colon cancer cells. Both CD8$^+$ and CD4$^+$ human lymphocytes expressing the murine TCR specifically recognized peptide-loaded T2 cells. However, only gene-modified CD8$^+$ lymphocytes specifically recognized HLA-A2.1$^+$ CEA$^+$ colon cancer cell lines, and tumor cell recognition was weak and variable. We identified two substitutions in the CDR3 of the α chain that significantly influenced tumor cell recognition by human peripheral blood lymphocytes. One substitution, T for S at position 112 (S112T), enhanced tumor cell recognition by CD8$^+$ lymphocytes, and a second dually substituted receptor (S112T L110F) enhanced tumor cell recognition by CD4$^+$ T cells.

Conclusions—The modified CEA-reactive TCRs are good candidates for future gene therapy clinical trials and show the power of selected amino acid substitutions in the antigen-binding regions of the TCR to enhance desired reactivities.

The adoptive transfer of melanoma-reactive tumor-infiltrating T lymphocytes can mediate cancer regression in ~50% of patients with metastatic melanoma (1, 2). Recent studies have shown that the adoptive transfer of normal peripheral lymphocytes genetically modified by the insertion of melanoma-reactive T-cell receptors (TCRs) can also mediate *in vivo* tumor

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Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

regression (3). However, the application of this immunotherapy approach to cancers other than melanoma is limited by the difficulty in identifying human TCRs reactive with antigens expressed on common epithelial cancers. This difficulty is likely due to the expression of these antigens at low levels on some normal tissues, thus resulting in central tolerance due to the negative selection of high-affinity TCRs during thymic development (4–6).

Carcinoembryonic antigen (CEA; CEACAM5; CD66e) is a 180-kDa glycoprotein often highly overexpressed in colorectal cancers and selected other epithelial cancers and is thus an attractive target for cell transfer immunotherapy (7–10). CEA is a normal, nonmutated self-protein expressed during fetal development as well as in some normal adult tissues and in thymic medullary epithelial cells (11). Thus, the development of T-cell reactivity to CEA is subject to normal mechanisms of immunologic tolerance. In the current study, to identify a CEA-reactive TCR, we employed a strategy previously used successfully to isolate TCRs specifically reactive with peptides derived from the tumor-associated antigens p53 and MDM2 (12–14). In particular, we isolated TCRs reactive with CEA by immunizing HLA-A2.1 transgenic mice with CEA-derived peptides containing amino acid sequences different from those present in any murine CEA-related protein. In addition, we evaluated amino acid substitutions in the complementarity determining regions (CDR) of the α and β chains from the CEA-reactive TCR. The combination of these two strategies enabled the generation of high-affinity TCRs capable of conferring CEA reactivity to both $CD4^+$ and $CD8^+$ human T cells.

Materials and Methods

Cell lines, primary human cell cultures, and peptides

The following cell lines were routinely cultured in DMEM supplemented with 25 mmol/L HEPES, 2 mmol/L L-glutamine, and 10% heat-inactivated fetal bovine serum (Invitrogen): colon cancer cell lines (SW1116, SW620, SW480, SW403, SW1463, and H508), COS-7 cells, 293 cells, and the SN renal cell carcinoma cell line (SN RCC). The following cell lines were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin (Invitrogen): melanoma cell lines (A375, 1383mel, 1861mel, Sk23mel, 624.38mel, 1300mel, and 2207mel), T2 cells, non-small cell lung cancer cell line H2087, and osteosarcoma cell line Saos2. COS-7 cells stably expressing HLA-A2.1 and either full-length CEA or NY-ESO-1 were generated previously by retroviral transduction with vectors encoding these proteins. 293 cells transiently expressing HLA-A2.1 and either full-length CEA or p53 were generated by transfection with pCDNA3.1 vectors encoding these proteins using Lipofectamine 2000 (Invitrogen). Murine lymphocytes were cultured in RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, MEM nonessential amino acids, 55 μmol/L 2-mercaptoethanol, 50 units/mL penicillin, and 50 μg/mL streptomycin (Invitrogen). Human lymphocytes, dendritic cells, and CD40L stimulated B cells were cultured in complete medium consisting of a 1:1 mixture of AIMV (Invitrogen) and RPMI 1640 containing 10% heat-inactivated human AB serum (Gemini Bio-Products; Valley Biomedical), 2 mmol/L L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin (Invitrogen).

Human dendritic cells were generated from adherent peripheral blood mononuclear cells (PBMC) cultured for 5 to 7 days in the presence of 1,000 units/mL GM-CSF and 1,000 units/mL interleukin (IL)-4 as described previously (15). Human B-cell cultures were generated by culturing human PBMCs with irradiated (18,000 rad) NIH 3T3 fibroblasts stably expressing CD40L and 200 units/mL IL-4.

All peptides were synthesized using a solid-phase method based on Fmoc chemistry using one of two multiple peptide synthesizers (model AMS 422; Gilson, or Pioneer, Applied Biosystems), and molecular weights were verified by laser desorption mass spectrometry (Bio-Synthesis; Tufts University Core Facility).

Immunization of HLA-A2.1 transgenic mice and in vitro *generation of murine CTL lines*

Transgenic mice expressing the full-length HLA-A2.1 gene were purchased from The Jackson Laboratory [C57Bl/6-Tg(HLAA2.1)1Enge/J] and were subsequently bred in the animal housing facility at the NIH. All mouse studies were approved by the National Cancer Institute Animal Care and Use Committee. HLA-A2.1 transgenic mice were injected subcutaneously at the base of the tail with 100 μg of one CEA peptide [CEA:691-699 (IMIGVLVGV), CEA:605-613 (YLSGANLNL), or CEA:694-702 (GVLVGVALI)] and 120 μg I-A^b binding HBVc:128-140 helper peptide (TPPAYRPPNAPIL) emulsified in 100 μL incomplete Freund's adjuvant (Montanide ISA 51). Two immunizations were given, separated by 1 week. Seven days after the second immunization, splenocytes were harvested and stimulated *in vitro* with irradiated T2 cells (18,000 rad) prepulsed with 1 μg/mL of the immunizing peptide. Splenocytes (1×10^6-3×10^6) were cocultured with 2×10^5 to 4×10^5 peptide-pulsed T2 cells in 24-well plates. Seven days later, bulk T-cell cultures were evaluated for specific peptide reactivity based on IFN-γ secretion assays. Peptide-reactive bulk cultures were then cloned at 10 cells per well in round-bottomed 96-well plates with 5×10^4 peptide-pulsed irradiated T2 cells and 5×10^4 irradiated (3,000 rad) C57BL/6 splenocytes as additional feeder cells per well in medium containing 60 IU/mL recombinant human IL-2 (rhIL-2; Chiron). Fourteen to 20 days later, growth-positive wells were selected, and the T lymphocytes from those wells were restimulated in individual 48-well plates with 2×10^5 peptide-pulsed irradiated T2 cells and 1×10^6 irradiated C57BL/6 splenocyte feeder cells per well in medium containing 60 IU/mL rhIL-2. One to 2 weeks later, these T-cell cultures were evaluated for specific recognition of peptide, tumor cells, and transfectants expressing full-length CEA and HLA-A2.1 by means of specific IFN-γ secretion. Some of the T cells from each of the tumor-reactive populations were frozen for subsequent RNA isolation for TCR cloning. Other cells were maintained in culture by restimulating once more in 24-well plates with 4×10^5 peptide-pulsed irradiated T2 cells and 1×10^6 irradiated C57BL/6 splenocyte feeder cells per well in medium containing 60 IU/mL rhIL-2 (Chiron).

TCR cloning

For each tumor-reactive murine T-cell population, total RNA was isolated using RNeasy mini kits (Qiagen). TCR α and β chains from each tumor-reactive T-cell population were then identified using 5′-rapid amplification of cDNA ends (RACE)-PCR. 5′-RACE-ready first-strand cDNA was prepared from total RNA using a SMART-RACE cDNA amplification kit (Clontech). Full-length or 3′-truncated TCR α and β chain fragments were then amplified by PCR (Advantage 2 PCR kit; Clontech) using several different primers within or at the 3′-end of the α and β chain constant regions. Gel-purified PCR products were then either sequenced directly or cloned into a pcDNA3.1 vector via TA cloning (pcDNA3.1/V5-His TOPO TA Expression Kit; Invitrogen), and plasmid DNA from individual bacterial colonies was then sequenced (Macrogen). DNA sequences were then analyzed for the presence of specific murine TCR α and β chain sequences using the International Immunogenetics Information Systems Web site.[3] Once the 5′-variable ends of each α and β chains present have been identified as well as the specific constant regions of each β chain, full-length TCR α and β chains were then amplified by PCR from 5′-RACE-ready first-strand cDNAs using specific 5′-V region and 3′-C region primers using either

[3] http://imgt.cines.fr/IMGT_vquest/vquest?livret=0&Option=mouseTcR

Advantage HF polymerase mix (Clontech) or Phusion high-fidelity DNA polymerase (New England Biolabs). cDNAs encoding codon-optimized versions of some TCR α and β chains were designed and produced by GENEART.

Generation of DNA constructs and in vitro *transcription of RNA*

cDNAs encoding wild-type TCR α and β chains were generated as described above. Single and dual amino acid substitutions were introduced into the CDRs of these TCR α and β chains via site-directed mutagenesis using an overlapping PCR technique as described previously (16, 17). Briefly, for each desired amino acid substitution, forward and reverse primers overlapping the mutation site were designed encompassing a new codon for the appropriate amino acid. These primers were then used to generate two overlapping PCR products, which were then stitched together via another round of PCR to create a full-length α or β chain encoding the desired amino acid. PCR products encoding full-length TCR α and β chains were used as template for a second round of PCR in which a T7 promoter sequence and Kozak consensus sequence (CACC) were added to the 5′-end of each gene and a 66 poly(A) tail was added to the 3′-end of each gene. These PCR products were then gel-purified and used as template for *in vitro* mRNA transcription reactions (mMESSAGE mMACHINE T7 Kit; Ambion). The *in vitro* transcribed (IVT) RNA was purified using RNeasy mini kits (Qiagen) and was eluted in RNase-free water at 0.5 to 1 mg/mL.

Genetic modification of T lymphocytes via RNA electroporation

Transient transfection of T lymphocytes with murine TCR α and β chains was done by electroporation with IVT RNA as described previously (18). Briefly, whole PBMCs were stimulated with 30 ng/mL OKT3 and 300 IU/mL rhIL-2. For some experiments, $CD8^+$ and/or $CD4^+$ T cells were purified 2 to 4 days later using anti-CD8 and/or anti-CD4 coated magnetic beads (Miltenyi Biotec). Five to 7 days after stimulation, T cells were washed and gently resuspended in Opti-MEM (Invitrogen) at 2×10^7/mL. Subsequently, 0.05 to 0.2 mL cells were mixed with IVT RNA (2 μg each of α and β chain encoding RNA per 1×10^6 T cells) and electroporated with a single pulse in a 2 mm cuvette (Harvard Apparatus BTX) using an ECM 830 Electro Square Porator at 500 V and 500 μs (Harvard Apparatus BTX). Immediately after electroporation, the cells were transferred to fresh complete medium and incubated at 37°C. In some experiments, these cells were used in cytokine release assays 2 to 4 h after the electroporation. Alternatively, they were evaluated for TCR expression by fluorescence-activated cell sorting (FACS) and/or were used in ^{51}Cr release cytotoxicity assays 16 to 24 h after electroporation.

Construction of retroviral vectors encoding TCR α and β chains

Retroviral vectors encoding both α and β chains of wild-type CEA-reactive murine TCR and additional TCRs containing amino acid substitutions in the CDR3 of the α chain were constructed using a MSGV1 backbone. Each vector encoded both an α chain and a β chain separated by a cleavable picorovirus peptide sequence in which the furin cleavage site was separated from the P2A sequence by a spacer encoding the amino acids, SGSG (19, 20). Retroviral supernatants were generated by cotransfecting 293 cells that stably expressed MMLV gag and pol proteins with each MSGV1 TCR vector and a vector encoding the RD114 feline endogenous virus retroviral envelope protein using Lipofectamine 2000 (Invitrogen) as described previously (17). Supernatants were collected 2 and 3 days post-transfection and were diluted 1:1 with fresh DMEM containing 10% FCS.

Genetic modification of T lymphocytes via retroviral transduction

MSGV1 retroviral particles encoding TCR α and β chains produced as described above were used to transduce PBMCs twice on days 2 and 3 after stimulation with 30 ng/mL

OKT3 and 300 IU/mL rhIL-2. Nontissue culture-treated plates were coated overnight with Retronectin (Takara Bio) at 4°C (10 μg/mL; 2 mL/6-well plate or 0.5 mL/24-well plate). Retronectin-coated plates were subsequently blocked with HBSS containing 2% bovine serum albumin for 30 min at room temperature and were then washed with HBSS containing 25 mmol/L HEPES. After aspirating the wash buffer, the diluted retroviral supernatants described above were added to the Retronectin-coated plates (4 mL/6-well plate or 1-2 mL/24-well plate). The plates were then centrifuged at 2,000 × *g* for 2 h at 32°C. During the centrifugation, 2-day stimulated PBMCs were harvested and resuspended at ~0.5 × 10^6/mL in medium containing 300 IU/mL rhIL-2. After spin-loading the plates, approximately half of the retroviral supernatant was aspirated, and PBMCs were added to the wells (4 mL or 2 × 10^6 cells/6-well plate or 1 mL or 0.5 × 10^6 cells/24-well plate). The cells were then centrifuged at 1,000 × *g* for 10 min and then incubated overnight at 37°C. Transductions were repeated on day 3 post-OKT3 stimulation using the same protocol. Four to 24 hours after the second transduction, T cells were harvested and resuspended in fresh medium containing 300 IU/mL rhIL-2 and allowed to expand *in vitro*. For some experiments, $CD8^+$ and/or $CD4^+$ T cells were purified 2 to 4 days after the second transduction using anti-CD8 and/or anti-CD4 coated magnetic beads (Miltenyi Biotec). One to 3 weeks after transduction, TCR expression was evaluated by FACS, and T-cell function was evaluated by measuring IFN-γ secretion in response to target cells.

FACS analyses

Tumor cell lines were characterized for expression of HLA-A2.1 and CEA (CEACAM5; CD66e) using a FITC-conjugated monoclonal antibody against HLA-A2 (One Lambda) and a PE-conjugated anti-CEA monoclonal antibody (Chemicon). T cells that have been electroporated with IVT RNAs or transduced with retroviral vectors encoding murine TCR α and β chains were characterized for expression of CD4, CD8, and TCR using a FITC-conjugated monoclonal antibody specifically reactive with the constant region of the murine β chain (eBiosciences) and either PE- or APC-conjugated antibodies against human CD4 or CD8 (BD Biosciences). FACS analyses were done on a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Cytokine release and cytotoxicity assays

Recognition of CEA by both murine and human T-cell populations was evaluated based on specific IFN-γ secretion in response to T2 cells preincubated with peptide, $HLAA2^+$ CEA^+ colon carcinoma cells, and, in some experiments, 293 or COS-7 cells expressing HLA-A*0201 and CEA or NY-ESO-1 as a negative control using overnight coculture assays. In experiments with murine T cells and preliminary experiments with human T cells electroporated with wild-type TCR α and β chains, tumor cells were treated with IFN-γ (20 ng/mL) and TNFα (3 ng/mL) overnight before the assays. In later experiments with codon-optimized and amino acid-substituted TCR α and β chains, tumors cells were not pretreated with any cytokines. Responder T cells (10^5) were coincubated with 0.5 × 10^5 to 1 × 10^5 stimulator cells (250 μL total) ~20 h at 37°C, and the concentration of murine or human IFN-γ in coculture supernatants was measured by ELISA using commercially available reagents (Pierce-Endogen). For cytokine secretion assays comparing wild-type and amino acid-substituted TCRs, statistical comparisons were made using a one-tailed *t* test comparing actual fold increases in IFN-γ secretion in response to relevant targets over negative controls to theoretical no-fold increases. In some experiments, 4 h ^{51}Cr release cytotoxicity assays were also done to evaluate the recognition of CEA by human T-cell populations as described previously (21).

Results

Generation of CEA-reactive T cells from HLA-A2.1 transgenic mice

HLA-A2.1 transgenic mice were immunized with one of three different CEA peptides: CEA:691-699 (IMIGVLVGV), CEA:605-613 (YLSGANLNL), and CEA:694-702 (GVLVGVALI). These peptides were selected based on three criteria: (*a*) relatively high binding affinities for HLA-A2.1 as predicted using published binding algorithms[4] or previously published binding affinity data (22–24), (*b*) previously published data suggesting that these peptides were naturally processed and presented on the surfaces of tumor cells in the context of HLA-A2.1 (22–25), and (*c*) the lack of any completely homologous peptide sequences from any known murine proteins as determined by BLAST searching.[5]

T-cell populations generated after *in vivo* and *in vitro* stimulation with peptide were evaluated in coculture assays for specific recognition of peptide and tumor cells and transfectants expressing full-length CEA and HLA-A2.1. For CEA:694-702 (GVLVGVALI), no peptide-reactive T-cell cultures were generated. Using CEA:605-613 (YLSGANLNL), two peptide-reactive T-cell populations were identified. However, these T cells did not recognize human colon cancer cell lines or COS-7 or 293 cells expressing HLA-A2.1 and full-length CEA (data not shown). In contrast, using CEA:691-699 (IMIGVLVGV), four T-cell populations were identified after limiting-dilution cloning that specifically secreted IFN-γ in response to peptide loaded T2 cells, COS-7 and 293 cells genetically engineered to express HLA-A2.1 and full-length CEA, and human colon cancer cell lines that expressed both HLA-A2.1 and CEA (Table 1). It is unclear why CEA:691-699 was the only peptide capable of stimulating a functional T-cell response, but two possible explanations seem plausible: (*a*) CEA:694-702 and CEA:605-613 may not be processed and presented on the surfaces of colon cancer cells in the context of HLA-A2.1 or (*b*) the methodologies we employed were not sufficient to stimulate highly avid T cells that recognize these peptides.

Identification, cloning, and function in human T cells of a murine CEA-reactive TCR

From the four CEA:691-699-reactive murine splenocyte cultures, the α and β chains of every TCR present were cloned, and these cDNAs were used as templates for the *in vitro* transcription of RNA encoding these proteins as described previously (26, 27). Based on DNA sequencing results of preliminary 5′-RACE reaction products, two α chains (TRAV7-D-3*01 TRAJ15*01 and TRAV8-1*01 TRAJ6*01) and two β chains (TRBV26*01 TRBD1*01 TRBJ2-7*01 TRBC2 and TRBV3*01 TRBD1*01 TRBJ1-5*01 TRBC1) were identified. Full-length TCR α and β chains were then amplified by PCR from 5′-RACE-ready first-strand cDNAs using specific 5′-V region and 3′-C region primers. DNA sequencing of the full-length TCR chains actually revealed the presence of three α chains and three β chains because two genetic variations were identified within the CDR3 of both the TRBV26 β chain and the TRAV8-1 α chain (Supplementary Fig. S1). The nucleotide variation in the TRBV26 β chain was such that an amino acid difference was encoded at position 117 (a T or L was encoded at this position), whereas the genetic variation in the TRAV8-1 α chain was silent, such that the encoded proteins were identical. RNAs encoding each of the TCR α and β chains were then used in all nine possible combinations to electroporate human $CD8^+$ T cells that had been stimulated to proliferate with anti-CD3 and IL-2. T-cell function was evaluated within 24 h after the electroporation by measuring specific IFN-γ secretion in response to appropriate target cells. One α/β TCR combination (TRAV8-1/TRBV26) induced previously nonreactive human $CD8^+$ T cells to secrete IFN-γ

[4] http://www.syfpeithi.de/ and http://www-bimas.cit.nih.gov/molbio/hla_bind/
[5] http://www.ncbi.nlm.nih.gov/blast/Blast.cgi

specifically in response to CEA:691-699 peptide-loaded T2 cells, COS cells genetically engineered to express HLA-A2.1 and full-length CEA, and human colon cancer cell lines that expressed both HLA-A2.1 and CEA (Table 2), although tumor cell recognition was weak and variable in different experiments. In addition, the TRBV26 β chain encoding T at amino acid position 117 induced higher amounts of IFN-γ secretion in response to CEA^+ target cells than did that encoding L at this position. Not surprisingly, both TRAV8-1 α chains, which encoded identical proteins, induced similar amounts of IFN-γ secretion.

Optimization of the murine CEA-reactive TCR

Codon optimization and removal of mRNA-destabilizing sequences have been shown previously to enhance the expression and function of genetically introduced TCRs in comparison with their wild-type counterparts (28). The company GENEART has developed GeneOptimizer software that optimizes every codon within a given gene for predicted maximal expression of the encoded protein in mammalian cells. Codon optimization of the CEA:691-699-reactive TCR by GENEART resulted in a slight enhancement in expression following RNA electroporation. However, no significant advantage was observed for the codon-optimized TCR α and β chains in terms of T-cell function as measured by specific IFN-γ secretion in response to appropriate target cells (Supplementary Figs. S2 and S3). A new set of experiments was then initiated aimed at enhancing TCR function by introducing amino acid substitutions in the CDRs of the TCR using previously described techniques (17). In particular, we introduced amino acid substitutions throughout the CDR1, CDR2, and CDR3 of both α and β chains of the TCR, IVT RNA using each of the mutated TCR chains as templates, and electroporated PBMCs to determine if any of the modified TCRs induced more specific cytokine secretion than the wild-type receptor. Initially, alanine substitutions were introduced at each position within the CDR1, CDR2, and CDR3 of both α and β chains predicted to form loops that would directly contact the MHC molecule and/or the MHC/peptide complex.[3] For any given position, if T-cell function remained unchanged, no additional amino acid substitutions were pursued. In contrast, if T-cell function was significantly enhanced or diminished by a particular alanine substitution, we made additional amino acid substitutions at that particular position. Using this semiempirical approach, no modifications were identified within any of the CDRs of the β chain or within the CDR1 or CDR2 of the α chain that significantly enhanced T-cell function (data not shown).

In preliminary screening experiments, we evaluated the function of TCRs containing alanine substitutions in the CDR3 of the α chain. Because tumor cell recognition by $CD4^+$ T cells was very weak or even nonexistent using the wild-type CEA-reactive TCR, mutated TCRs were initially screened by electroporating $CD4^+$ T cells with IVT RNAs encoding wild-type β chain and modified α chains. Results from one representative screen are presented in Table 3. Alanine substitutions at positions 111, 112, and 114 within the α chain significantly decreased the reactivities of $CD4^+$ T cells expressing these modified receptors in comparison with the wild-type receptor. An alanine for glycine substitution at position 113 appeared to enhance reactivity slightly against peptide-pulsed target cells, and the alanine for leucine substitution at position 110 enhanced reactivity more significantly against peptide-pulsed T2 cells as well as target cells endogenously expressing HLA-A2.1 and full-length CEA. At this point, no additional modifications were pursued at positions 113 and 114 because the alanine for glycine substitution was the most conservative possible; instead, we focused on making additional amino acid substitutions at positions 110, 111, and 112 in the α chain (see Supplementary Fig. S4 for a complete list of CDR3 α chain substitutions evaluated).

By screening modified TCRs in $CD4^+$ T cells, two modifications were identified that enhanced T-cell reactivities against peptide-pulsed T2 cells as well as $HLA\text{-}A2.1^+$ CEA^+

target cells [F for L at amino acid position 110 (L110F) and T for S at position 112 (S112T); preliminary screening data not shown]. These substitutions were further combined (L110F S112T), and the functions of these modified TCRs were evaluated in both $CD4^+$ and $CD8^+$ human T cells by specific IFN-γ secretion (Fig. 1; Table 4). In these experiments, all T cells electroporated with the wild-type receptor specifically recognized peptide-pulsed T2 cells, but neither $CD4^+$ nor $CD8^+$ T cells specifically recognized tumor cells or genetically modified COS cells expressing HLA-A2.1 and full-length CEA. In contrast, the doubly substituted TCR induced both specific peptide and tumor cell recognition by $CD4^+$ T cells. Transfected $CD8^+$ T cells that expressed the TCR containing both L110F and S112T substitutions, as well as the receptor containing the single L110F substitution, released significant levels of IFN-γ in response to tumor cells that expressed HLA-A2.1 and CEA as well as multiple targets that lacked expression of CEA. Both $CD4^+$ and $CD8^+$ T cells transfected with the S112T TCR showed enhanced peptide reactivity in comparison with the wild-type receptor, and $CD8^+$ T cells transfected with this TCR showed increased reactivity to COS-A2 cells expressing CEA as well as a low reactivity to tumor cell targets relative to cells transfected with the wild-type receptor. Similar patterns of reactivity were observed when lysis of target cells by TCR-electroporated T lymphocytes was evaluated (Supplementary Fig. S5). In all experiments, TCR expression was comparable as evaluated by FACS using a monoclonal antibody against the constant region of the murine TCR β chain (see Supplementary Fig. S6 for representative data).

Recognition of CEA:691-699 homologues from other CEA family member proteins

The CEA gene product that is the subject of this report and has been most extensively analyzed is one member of a family of 29 different genes and has been designated CEACAM5 (10). Sequence comparisons revealed that several additional members of this family of gene products possess amino acid sequences that are similar to that of CEACAM5:691-699. An attempt was then made to determine whether T cells that recognize the CEACAM5:691-699 epitope also recognize peptides derived from other members of the CEA gene family (Supplementary Table S1). $CD8^+$ T cells transfected with the L110F/S112T TCR recognized target cells pulsed with any of the four peptides derived from additional family members, although the levels of these peptides required for recognition were between 10- and 10,000-fold higher than those needed for recognition of the wild-type peptide (Supplementary Table S1). In addition, $CD8^+$ T cells transfected with the S112T TCR recognized targets pulsed with three of the four peptides evaluated, although, again, the levels of these peptides required for recognition were between 10- and 10,000-fold greater than those required for recognition of the wild-type peptide. It is possible that these cross-reactivities contributed, in part, to the IFN-γ secretion previously observed in response to many $HLAA2.1^+$ target cells regardless of CEA expression. However, this is clearly not the only factor involved because the IFN-γ secretion in response to T2 cells pulsed with the irrelevant control HBVc peptide was significantly higher from $CD8^+$ T cells expressing the L110F S112T receptor than the native TCR. This suggests that the doubly substituted TCR binds with greater affinity to the HLA-A2.1 molecule regardless of what peptide is in the MHC binding groove.

Activity of human T cells retrovirally transduced with vectors encoding murine CEA-reactive TCRs

Several previous clinical trials have been conducted in which human PBMCs were genetically modified to express tumor-reactive TCRs and were subsequently adoptively transferred to the autologous patient (3). In these trials, TCRs were introduced into the PBMCs using retroviral vectors, not RNA electroporation, to allow for longer-term expression and viability of the gene-modified cells. Therefore, to determine which CEA:691-699-reactive receptor might be most suitable for use in a clinical therapy protocol, we

constructed retroviral vectors encoding the wild-type TCR and two of the three modified TCRs described above (S112T and L110F S112T). Each vector was composed of a MSGV1 backbone and encoded both an α chain and a β chain separated by a "self-cleaving" picorovirus peptide sequence as described previously (19, 20). Retroviral supernatants were generated by cotransfecting 293 cells that stably expressed gag and pol proteins with each MSGV1 TCR vector and a vector encoding the RD114 envelope protein as described previously (17). These retroviral particles were then used to transduce HLA-A2.1$^+$ PBMCs that had been stimulated with anti-CD3 and IL-2 as described previously (17). One to 3 weeks after transduction, TCR expression was evaluated by FACS using a monoclonal antibody against the constant region of the murine β chain, and T-cell function was evaluated by measuring IFN-γ secretion in response to target cells (Table 5). The results of coculture assays with transduced T cells showed that CD8$^+$ T cells expressing the S112T TCR released significantly higher levels of IFN-γ in response to HLA-A2.1$^+$ CEA$^+$ tumor targets than CD8$^+$ T cells expressing the wild-type TCR (Table 5). In three independent experiments involving transductions of six different PBMCs, the fold enhancement in IFN-γ production with the S112T TCR compared with the wild-type TCR in CD8$^+$ T cells in response to each of the HLA-A2.1$^+$ CEA$^+$ target cells was as follows: (*a*) H508, 8.5 ± 4.2 (average ± SD), range 3.6 to 13.4 ($P < 0.01$); (*b*) SW1463, 5.6 ± 6.1, range 1.7 to 19.4 ($P = 0.05$); (*c*) SW403, 11.8 ± 14.0, range 2.4 to 41.8 ($P = 0.04$); and (*d*) COS-A2-CEA, 3.9 ± 2.2, range 2.0 to 7.6 ($P < 0.01$). No recognition of tumor targets was observed in CD8$^+$ T cells transduced with the L110F/S112T TCR; however, transduction efficiencies as evaluated by FACS were consistently lower in PBMCs and CD8$^+$ T cells transduced with this doubly substituted receptor, and these populations proliferated to a much lesser extent than those transduced with any of the other receptors (data not shown). Perhaps this was related to the nonspecific cytokine secretion and lysis previously observed in the short-term, transient expression experiments by T cells electroporated with RNA encoding this receptor. These results suggest that the L110F/S112T TCR may confer a significant level of autoreactivity, and it is possible that the nonspecificity of the T cells transduced with this receptor resulted in fratricide due to the expression of HLA-A2.1 alone. CD4$^+$ T cells transduced with the S112T TCR as well as the L110F/S112T TCR released significant levels of IFN-γ in response to HLA-A2.1$^+$ CEA$^+$ tumor targets in comparison with CD4$^+$ T cells expressing the wild-type TCR that failed to recognize tumor cells (Table 5). This was consistently observed in three independent experiments involving transductions of six different PBMCs. Further results indicated that the transduction of both CD8$^+$ and CD4$^+$ T cells derived from the peripheral blood of multiple HLA-A2.1$^+$ individuals with the S112T TCR lead to enhanced tumor recognition in comparison with the wild-type TCR without apparent loss of specificity, indicating this genetically modified receptor may represent a potential candidate for use in clinical trials for the treatment of patients with common epithelial cancers that overexpress CEA.

Discussion

The adoptive transfer of tumor-reactive tumor-infiltrating T lymphocytes following lymphodepleting chemotherapy administered in conjunction with IL-2 can mediate the regression of melanoma in ~50% of patients. However, this treatment is not readily available to patients with other cancers because it is not usually possible to expand large numbers of tumor-reactive T lymphocytes from resected tumors other than melanoma. In a recent clinical trial, and in numerous *in vitro* studies, it has been shown that this limitation can be overcome by genetically introducing tumor-reactive TCR α and β chains into previously nonreactive PBMCs (3, 13, 17, 29). Therefore, to extend current adoptive cell transfer immunotherapies to patients with common epithelial tumors, we initiated the current investigation to isolate a high-affinity CEA-reactive TCR.

CEA was originally described in 1965 by Gold and Freeman as an oncofetal antigen expressed during fetal development and in cancers of the human digestive system but not in normal adult tissues (7–10). Since that time, CEA has been extensively investigated as a tumor-specific marker for the detection, diagnosis, prognosis, treatment monitoring, localization, and therapy of a variety of different cancers. Expression of CEA protein by tumor cells has been extensively studied using immunohistochemistry with many different polyclonal and monoclonal antibodies. Immunohistochemically, CEA has been found in cancers of the colorectum, breast, lung, cervix, gallbladder, stomach, pancreas, liver, prostate, urinary bladder, ovaries, uterus, and head and neck and in neuroendocrine tumors from the larynx, lung, and thyroid (10). CEA expression in normal adult tissues is considerably more limited but is present in columnar epithelial cells and goblet cells in the colon, in mucous cells in the neck and stomach, in squamous epithelial cells of the tongue, esophagus, and cervix, in secretory epithelial and duct cells of sweat glands, and in epithelial cells of the prostate (30, 31).

At least two investigations have compared amounts of CEA protein or mRNA transcripts in colon cancer samples and surrounding autologous histologically normal colon tissue (32, 33). In one study using a sensitive radioimmunoassay, when samples were normalized for total protein, CEA levels were significantly elevated in tumor lesions in comparison with normal colonic mucosa. Similar results were found for CEA mRNA transcripts in samples normalized for total RNA. However, on a per cell basis, it has been shown that normal highly differentiated colonic epithelial cells produce comparable amounts of CEA to colon cancer cells (34, 35). Nonetheless, several studies have shown that radiolabeled anti-CEA antibodies effectively localize to gastrointestinal cancers (36, 37). Furthermore, many clinical trials have been conducted in which CEA was the target antigen in attempting to treat a variety of cancers with a variety of different immunotherapeutic strategies (38). An anti-idiotypic antibody vaccine targeting CEA has been extensively evaluated in multiple phase II and III clinical trials (39). Recombinant CEA protein has been used as an immunogen in phase I clinical trials to evoke cellular and humoral immune responses to CEA in patients with Dukes' A to C colon cancer (40). Recombinant poxviruses encoding CEA have also been tested in multiple clinical trials to immunize patients with a variety of different cancers that express CEA protein (41–44). Overall objective clinical response rates in previously published immunotherapy-based clinical trials targeting CEA have been low. Although many published reports have shown the existence of human T lymphocytes that can specifically recognize peptide epitopes derived from CEA (22–24, 45, 46), it appears that these T cells are not sufficiently avid to mediate the regression of tumors expressing CEA.

In many early attempts to isolate CEA-reactive T lymphocytes, we stimulated human PBMCs *in vitro* with previously published HLA-A2.1-restricted CEA-derived peptides. Although we often generated peptide-reactive CTL, these T cells never showed specific recognition of HLA-A2.1$^+$ tumor cells that endogenously expressed CEA. The predominant hypothesis was that high-avidity CEA-reactive CTLs were likely to be eliminated during thymic selection or anergized due to peripheral tolerance mechanisms because CEA is a normal, nonmutated self-protein expressed during fetal development, in some normal adult tissues, and in thymic medullary epithelial cells (11). Therefore, we employed an alternate strategy to isolate CEA-reactive T cells from HLA-A2.1 transgenic mice as described previously for p53 and MDM2 (12–14). In those investigations, transgenic mice expressing fully human HLA-A2.1 were used to try to identify high-affinity, CD8-independent TCRs because murine CD8 does not bind efficiently to the a3 domain of the HLA-A2.1 molecule. Therefore, we also used these mice in the studies described here. The most homologous murine protein to human CEA (CEACAM5) is murine CEACAM1 with a sequence identity of only 60%. In addition, by BLAST searching, no completely identical murine peptide

sequences were found for the three previously published HLA-A2.1-restricted CEA epitopes. Therefore, it seemed possible that we could isolate high-avidity CEA-reactive CTL by immunizing HLA-A2.1 transgenic mice with these peptides. Using CEA:691-699 (IMIGVLVGV), murine T cells were isolated that specifically secreted IFN-γ in response to peptide-loaded T2 cells, COS-7 and 293 cells genetically engineered to express HLA-A2.1 and full-length CEA, and human colon cancer cell lines that expressed both HLAA2.1 and CEA. However, when the α and β chains of the CEA-reactive TCR were cloned and genetically introduced into human peripheral blood lymphocytes, specific peptide recognition was observed in both $CD8^+$ and $CD4^+$ T cells. However, only gene-modified $CD8^+$ T cells specifically recognized $HLAA2.1^+$ CEA^+ colon cancer cell lines, and this tumor cell recognition was weak and variable, sometimes nonexistent.

We first attempted to improve the function of this TCR by codon optimization and removal of mRNA-destabilizing sequences (GENEART), which have been shown previously to enhance the expression and function of genetically introduced TCRs in comparison with their wild-type counterparts (28). Although increased TCR expression on the surfaces of genetically modified cells was observed, T-cell function was not significantly enhanced. Therefore, a different avenue was pursued to improve TCR function by introducing amino acid substitutions in CDRs of the TCR as described previously (17). In particular, amino acid substitutions were introduced throughout the CDR1, CDR2, and CDR3 of both α and β chains of the TCR, and two potentially improved modified TCRs were identified with amino acid substitutions in the CDR3 of the α chain. The singly substituted TCR (S112T) enhanced T-cell function, most notably in whole PBMCs and $CD8^+$ T cells with less reactivity in $CD4^+$ T cells. The doubly mutated TCR (L110F S112T) significantly enhanced highly specific tumor cell recognition by $CD4^+$ T cells. However, this modified receptor also caused a significant increase in nonspecific IFN-γ secretion and target cell lysis by $CD8^+$ T cells regardless of CEA expression. This is similar to previously published observations with a modified high-affinity NY-ESO-1-reactive TCR (47).

The identification of these two modified TCRs provides significant flexibility for use in clinical trials of TCR gene-modified lymphocytes. Highly specific anti-CEA $CD8^+$ T cells could be generated using the S112T receptor. The role of $CD4^+$ T cells in antitumor immunity is currently the subject of much debate. Evidence from murine models strongly suggests that $CD4^+$ T-cell help can significantly enhance the function and persistence of tumor-reactive $CD8^+$ T lymphocytes and is associated with enhanced tumor treatment. However, $CD4^+$ regulatory T cells may significantly impair the efficacy of adoptive cell transfer immunotherapy (48). If desired, highly specific CEA-reactive $CD4^+$ T helper cells could be generated using the doubly substituted TCR (L110F S112T) and adoptively transferred to patients along with $CD8^+$ T cells expressing the singly substituted TCR (S112T).

One potential concern related to the use of these murine TCRs in human gene therapy trials is the potential development of anti-transgene immune responses by human lymphocytes against murine TCR proteins. We are currently evaluating this possibility in two ongoing clinical trials in the Surgery Branch of the National Cancer Institute. In particular, we have genetically introduced murine TCRs against peptides derived from p53 and gp100 into human PBMCs by retroviral transduction. These cells were then expanded to large numbers and adoptively transferred back to the autologous patient after lymphodepleting but nonmyeloablative chemotherapy as described previously (3). Thus far, we have treated 29 patients between these two protocols and have observed clear evidence of persistence of the gene-modified T cells in the peripheral circulation out to several months. In addition, we have not found evidence of anti-murine TCR antibodies in the serum of these patients. However, these studies are continuing, and the potential development of anti-murine TCR

immune responses needs to be more fully evaluated before any definitive conclusions can be drawn.

CEA is clearly expressed on a variety of different cancers but is also expressed in some normal adult tissues, most notably colonic epithelial cells in the upper third of colonic crypts (10). Thus, there is significant concern about potential toxicities that might be induced by the adoptive transfer of large numbers of CEA-reactive T cells. In a previously published clinical trial, patients with melanoma were treated with adoptively transferred PBMCs genetically engineered to express a TCR reactive with the melanoma antigen MART-1, which is also expressed on normal melanocytes (3). In that study, significant melanocyte-directed autoimmune toxicities were not observed. However, in other clinical trials in which patients with melanoma received large numbers of tumor-reactive T cells, melanocyte destruction was observed in the forms of uveitis and vitiligo (2). Therefore, targeting CEA in clinical trials must proceed with caution. In summary, this article describes anti-CEA TCRs active in $CD8^+$ and $CD4^+$ T cells and shows the power of selected amino acid substitutions in the antigen-binding regions of the TCR to enhance desired reactivities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

This article describes the isolation and genetic modification of a CEA-reactive TCR from HLA-A2.1 transgenic mice. Introduction of the modified TCRs into human peripheral blood lymphocytes by either RNA electroporation or retroviral transduction enabled the previously nonreactive T cells to recognize peptide-pulsed target cells as well as HLA-A2.1^{+} CEA^{+} human colorectal cancer cells. The modified TCRs are good candidates for future gene therapy clinical trials for the treatment of patients with common epithelial cancers that overexpress CEA. Specifically, the modified TCRs could be genetically introduced into human peripheral blood lymphocytes to confer tumor reactivity, and these peripheral blood lymphocytes could then be adoptively transferred into patients with CEA-positive tumors. Similar modalities have been used previously for the treatment of patients with melanoma, and these new CEA-reactive TCRs would enable such adoptive cell transfer therapies to be extended to patients with common epithelial cancers.

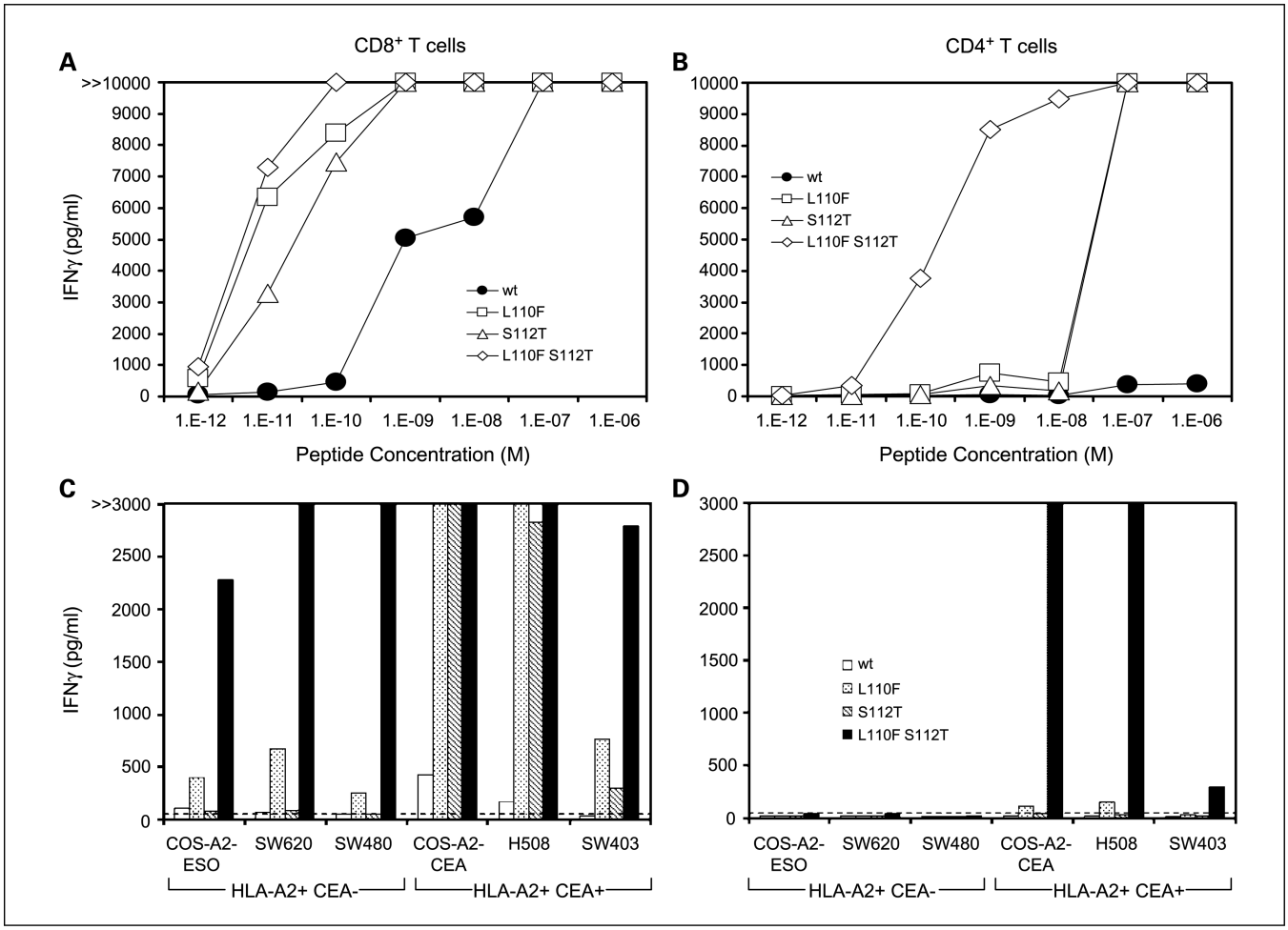

Fig. 1.

Recognition of peptide and target cells expressing HLA-A2.1and CEA by $CD8^+$ and $CD4^+$ Tcells transfected with RNAs encoding CEA:691-699-reactive TCR-modified α chains and wild-type β chains. RNAs encoding TCR amino acid-substituted α and wild-type β chains were used to electroporate human $CD8^+$ (*A* and *C*) and $CD4^+$ (*B* and *D*) T cells previously stimulated to proliferate with anti-CD3 and IL-2.Two to 4 h later,T cells were cocultured with various target cells. Approximately 20 h later, IFN-γ secretion was measured in response to CEA:691-699 peptide-pulsed T2 cells (*A* and *B*) and HLA-$A2.1^+$ CEA^+ tumor cells or genetically engineered COS cells (*C* and *D*).

Table 1

Recognition of peptide and target cells expressing HLA-A2.1 and CEA by CEA:691-699-reactive murine T-cell populations

	HLA-A2.1	CEA	T cell 1	T cell 2	T cell 3	T cell 4
Medium*	-	-	54	276	618	126
T2 + HBVc: 18-27	+	-	7	221	753	2,395
T2 + CEA: 691-699	+	+	**>140,000** †	**>140,000**	**>140,000**	**>140,000**
SW620	+	-	0	308	594	86
SW480	+	-	0	324	483	62
SW403	+	Weak	**269** ‡	348	626	118
H508	+	+	0	**5,363**	**1,800**	**2,022**
SW1463	+	+	**149**	**28,239**	**5,094**	**12,006**
COS-A2-CEA	+	-	15	538	649	173
COS-A2-CEA	+	+	**5,689**	**>140,000**	**18,478**	**102,361**
293-A2$^+$ p53	+	-	0	364	546	165
293-A2$^+$ CEA	+	+	**237**	**13,085**	**7,609**	**27,763**

NOTE: IFN-γ secretion (pg/mL) in 20 h coculture supernatants of target cells with murine T-cell cultures derived from HLA-A2.1 transgenic mice immunized with CEA:691-699 and subsequently stimulated with peptide *in vitro* under limiting-dilution conditions.

*IFN-γ release in the absence of stimulator cells.

†Bold values in this row indicate that IFN-γ release in response to T2 cells preincubated with 1 μg/mL CEA:691-699 was ≥100 pg/mL and at least twice background with medium and T2 cells preloaded with 1 μg/mL of the negative control peptide HBVc:18-27(23Y).

‡Bold values in this row and additional rows indicate that IFN-γ release in response to HLA-A2.1$^+$ CEA$^+$ target cells was ≥100 pg/mL and at least twice background with medium and HLA-A2.1$^+$ CEA$^-$ target cells and that specific peptide reactivity was observed in the same culture.

Clin Cancer Res. Author manuscript; available in PMC 2012 October 17.

Table 2

Recognition of peptide and target cells expressing HLA-A2.1 and CEA by $CD8^+$ T cells transfected with RNAs encoding murine TCR α and β chains derived fromCEA:691-699-reactive murine T-cell populations

α Chain	AV7-D-3	AV7-D-3	AV7-D-3	AV8-1 (GAT)	AV8-1 (GAT)	AV8-1 (GAT)	AV8-1 (GAC)	AV8-1 (GAC)	AV8-1 (GAC)	
β Chain	**BV26 (117T)**	**BV26 (117L)**	**BV3**	**BV26 (117T)**	**BV26 (117L)**	**BV3**	**BV26 (117T)**	**BV26 (117L)**	**BV3**	**Mock**
Medium	19	20	34	6	10	14	4	6	14	6
T2 + HBVc:18-27	115	182	108	105	221	151	324	101	43	51
T2 + 10^{-12} mol/L CEA:691-699	86	137	175	203	103	240	89	41	74	64
T2 + 10^{-11} mol/L CEA:691-699	83	166	113	**219***	48	79	82	51	47	106
T2 + 10^{-10} mol/L CEA:691-699	69	76	119	**516**	95	62	448	81	78	76
T2 + 10^{-9} mol/L CEA:691-699	197	119	366	**6,007**	**1,471**	55	**5,338**	**1,640**	47	65
T2 + 10^{-8} mol/L CEA:691-699	154	134	127	**11,359**	**5,057**	76	**13,331**	**6,166**	81	101
T2 + 10^{-7} mol/L CEA:691-699	84	76	318	**>20,000**	**17,060**	104	**>20,000**	**18,926**	48	58
T2 + 10^{-6} mol/L CEA:691-699	66	113	239	**>20,000**	**20,000**	52	**>20,000**	**15,716**	56	51
SW620 ($A2^+$ CEA^-)	39	58	78	34	32	30	26	28	29	25
SW480 ($A2^+$ CEA^-)	66	102	113	55	48	54	53	47	76	39
H508 ($A2^+$ CEA^+)	61	85	82	**292** †	**141**	38	**346**	**150**	50	26
SW1463 ($A2^+$ CEA^+)	59	72	86	**830**	**318**	38	**875**	**360**	38	34
COS-A2-ESO	54	78	84	77	65	57	69	58	56	69
COS-A2-CEA	68	106	130	**935**	**384**	58	**1,080**	**429**	74	52

NOTE: IFN-γ secretion (pg/mL) in 20 h coculture supernatants of target cells with electroporated human $CD8^+$ T cells.

*Bold values for peptide-pulsed targets indicate that IFN-γ release in response to T2 cells preincubated with the indicated concentration of CEA:691-699 was ≥50 pg/mL and at least twice background with medium and T2 cells preloaded with 1 μg/mL of the negative control peptide HBVc:18-27(23Y).

†Bold values for tumor cells and transduced COS-7 cells indicate that IFN-γ release in response to HLA-$A2.1^+$ CEA^+ target cells was ≥50 pg/mL and at least twice background with medium and HLA-$A2.1^+$ CEA^- target cells and that specific peptide reactivity was observed in the same culture.

Table 3

Recognition of peptide and target cells expressing HLA-A2.1 and CEA by $CD4^+$ T cells transfected with RNAs encoding alanine-substituted CDR3-modified α chains and wild-type β chains derived from CEA:691-699-reactive murine T cells

α Chain modification	L110A	T111A	S112A	G113A	G114A	Wild-type	Mock
Medium	9	3	15	8	10	13	9
T2 + HBVc	16	4	13	15	29	17	26
T2 + 10^{-12} mol/L CEA	25	14	11	11	11	14	19
T2 + 10^{-11} mol/L CEA	10	5	20	12	15	19	10
T2 + 10^{-10} mol/L CEA	27	9	8	14	10	12	10
T2 + 10^{-9} mol/L CEA	**265** *	9	16	**128**	7	**83**	8
T2 + 10^{-8} mol/L CEA	**399**	9	14	**273**	9	**84**	13
T2 + 10^{-7} mol/L CEA	**>2,000**	15	**94**	**1,054**	18	**714**	10
T2 + 10^{-6} mol/L CEA	**>2,000**	7	**172**	**1,920**	12	**1,181**	13
SW480 ($A2^+$ CEA^-)	11	8	15	19	13	13	19
SW620 ($A2^+$ CEA^-)	7	5	18	9	17	24	11
SW1463 ($A2^+$ CEA^+)	**63** †	13	32	39	28	37	25
H508 ($A2^+$ CEA^+)	**63**	13	24	30	16	35	28
COS-A2-ESO	11	12	10	17	14	13	23
COS-A2-CEA	**286**	10	8	49	18	32	9

NOTE: IFN-γ secretion (pg/mL) in 20 h coculture supernatants of target cells with electroporated human $CD4^+$ T cells.

*Bold values for peptide-pulsed targets indicate that IFN-γ release in response to T2 cells preincubated with the indicated concentration of CEA:691-699 was ≥50 pg/mL and at least twice background with medium and T2 cells preloaded with 1 μg/mL of the negative control peptide HBVc:18-27(23Y).

†Bold values for tumor cells and transduced COS-7 cells indicate that IFN-γ release in response to HLA-$A2.1^+$ CEA^+ target cells was ≥50 pg/mL and at least twice background with medium and HLA-$A2.1^+$ CEA^- target cells and that specific peptide reactivity was observed in the same culture.

Table 4

Recognition of peptide and target cells expressing HLA-A2.1 and CEA by $CD8^+$ and $CD4^+$ T cells transfected with RNAs encoding modified TCR α chains and wild-type β chains derived from CEA:691-699-reactive murine T cells

	HLA-A2* (MFI)	CEA (MFI)	$CD4^+$ T cells					$CD8^+$ T cells				
			None	Wild-type	L110F	S112T	L110FS112T	None	Wild-type	L110F	S112T	L110FS112T
Medium	-	-	0	0	0	0	0	0	0	0	0	0
T2 + HBVc	+	-	0	0	0	0	11	10	1	138	8	204
T2 + 10^{-12} mol/L CEA	+	-	0	0	2	0	35	0	0	178	27	194
T2 + 10^{-11} mol/L CEA	+	-	0	0	**63**	11	**159**	0	5	**346**	**150**	**692**
T2 + 10^{-10} mol/L CEA	+	-	0	0	**198**	**93**	**610**	0	**68**	**1,950**	**413**	**3,024**
T2 + 10^{-9} mol/L CEA	+	-	0	0	**286**	**145**	**2,271**	4	**108**	**3,422**	**816**	**5,882**
T2 + 10^{-8} mol/L CEA	+	-	0	**70**	**4,158**	**917**	**>10,000**	3	**729**	**>10,000**	**8,678**	**>10,000**
T2 + 10^{-7} mol/L CEA	+	-	0	**4,497**	**>10,000**	**>10,000**	**>10,000**	0	**>10,000**	**>10,000**	**>10,000**	**>10,000**
T2 + 10^{-6} mol/L CEA	+	-	0	**6,799** †	**>10,000**	**>10,000**	**>10,000**	0	**>10,000**	**>10,000**	**>10,000**	**>10,000**
COS-A2-ESO	+ (286)	- (3)	0	0	2	0	56	6	30	115	7	433
COS-A2-CEA	+ (284)	+ (142)	0	0	**127** ‡	**50**	**2,836**	6	74	**1,737**	**407**	**3,951**
SW1116	- (8)	+ (368)	0	0	0	0	0	0	0	0	0	0
A375mel	+ (63)	- (2)	30	11	12	8	58	0	0	87	0	501
SW620	+ (73)	- (2)	0	0	0	0	65	0	0	134	0	828
SW480	+ (79)	- (3)	0	0	0	0	30	0	0	75	0	386
H2087	+ (91)	- (3)	0	0	0	0	80	8	1	150	0	2,803
Saos2	+ (241)	- (9)	0	0	0	0	13	0	0	30	0	175
1383mel	+ (318)	- (3)	21	0	41	0	118	19	25	415	19	8,094
1861mel	+ (325)	- (2)	0	0	7	0	109	9	17	297	0	3,684
Sk23mel	+ (345)	- (4)	0	0	0	0	85	64	29	164	71	2,347
624.38mel	+ (388)	- (2)	0	0	99	0	607	0	41	4,197	32	>10,000
SN RCC	+ (517)	- (3)	0	0	0	0	0	0	0	0	0	98
1300mel	+ (533)	- (3)	11	0	83	0	325	53	33	1,914	27	8,603
2207mel	+ (853)	- (3)	24	23	105	9	197	32	42	4,652	171	9,564
SW403	+ (256)	Weak (19)	0	0	64	14	851	1	0	1,436	129	9,411

	HLA-A2* (MFI)	CEA (MFI)	CD4+ T cells					CD8+ T cells				
			None	Wild-type	L110F	S112T	L110FS112T	None	Wild-type	L110F	S112T	L110FS112T
SW1463	+ (357)	+ (175)	0	0	90	29	**3,051** §	0	11	182	168	834
H508	+ (396)	+ (267)	0	0	98	25	**2,046**	0	13	436	215	1,613

NOTE: IFN-γ secretion (pg/mL) in 20 h coculture supernatants of target cells with electroporated human $CD8^+$ and $CD4^+$ T cells.

*Expression of HLA-A2.1 and CEA on target cells was evaluated by FACS, and data are presented as mean fluorescence intensity (MFI).

†Bold values for peptide-pulsed targets indicate that IFN-γ release in response to T2 cells pulsed with the indicated concentration of CEA:691-699 was ≥50 pg/mL and at least twice background with medium and T2 cells preloaded with 1 μg/mL of the negative control peptide HBVc:18-27(23Y).

‡Bold values for transduced COS-7 cells indicate that IFN-γ release in response COS-7 cells transduced with HLA-A2.1 and CEA was ≥50 pg/mL and at least twice background with medium and COS-7 cells transduced with HLA-A2.1 and NY-ESO-1 HLA and that specific peptide reactivity was observed in the same culture.

§Bold values for tumor cells indicate that IFN-γ release in response to HLA-$A2.1^+$ CEA^+ tumors was ≥50 pg/mL and at least twice background with medium, HLA-$A2.1^+$ CEA^- tumors, and HLA-A2.1-CEA^+ tumors and that specific peptide reactivity was observed in the same culture.

Table 5

Recognition of peptide and target cells expressing HLA-A2.1 and CEA by whole PBMCs and purified $CD8^+$ and $CD4^+$ T cells from a HLA-A2.1-positive donor transduced with retroviruses encoding modified TCR α chains and wild-type β chains derived fromCEA:691-699-reactive murine T cells

	A2[†]	CEA	PBMC				CD4+ T cells				CD8+ T cells			
			None	Wild-type	S112T	L110F S112T	None	Wild-type	S112T	L110F S112T	None	Wild-type	S112T	L110F S112T
	%CD8+ [*]		45.4	56.0	55.9	34.8	2.5	7.3	6.8	6.7	86.4	93.1	92.8	93.5
	%CD4+		49.8	38.4	39.5	59.6	97.1	92.2	92.1	88.8	2.6	0.4	0.5	0.2
	%CD8+ TCR+		0.0	43.5	41.8	8.6	0.0	5.9	5.3	2.8	0.0	71.8	71.7	21.5
	%CD4+ TCR+		0.0	24.9	24.3	19.0	0.0	48.1	36.2	16.2	0.0	0.3	0.4	0.1
Medium	-	-	11	31	12	53	7	13	0	33	5	0	0	0
T2 + HBVc	+	-	59	252	130	61	2	24	3	33	36	278	131	0
T2 + 10^{-12} mol/L CEA	+	-	48	221	134	65	0	25	5	40	29	234	92	0
T2 + 10^{-11} mol/L CEA	+	-	66	215	179	**150**	0	37	37	**98**	27	274	141	3
T2 + 10^{-10} mol/L CEA	+	-	63	**772** [≠]	**1,658**	**2,786**	0	**157**	**370**	**2,420**	30	**768**	**1,399**	**113**
T2 + 10^{-9} mol/L CEA	+	-	67	**8,028**	**>10,000**	**>10,000**	0	**2,259**	**3,597**	**>10,000**	24	**6,145**	**9,961**	**809**
T2 + 10^{-8} mol/L CEA	+	-	64	**>10,000**	**>10,000**	**>10,000**	0	**8,089**	**>10,000**	**>10,000**	32	**>10,000**	**>10,000**	**2,242**
T2 + 10^{-7} mol/L CEA	+	-	81	**>10,000**	**>10,000**	**>10,000**	0	**>10,000**	**>10,000**	**>10,000**	33	**>10,000**	**>10,000**	**2,100**
T2 + 10^{-6} mol/L CEA	+	-	51	**>10,000**	**>10,000**	**>10,000**	0	**>10,000**	**>10,000**	**>10,000**	38	**>10,000**	**>10,000**	**2,443**
COS-A2-ESO	+	-	97	738	173	459	0	67	2	367	58	595	152	22
COS-A2-CEA	+	+	93	**1,674** [§]	**2,838**	**3,393**	21	117	**252**	**3,421**	61	**1,668**	**3,296**	**75**
Allo. dendritic cells 1	-	-	60	23	20	142	56	11	9	107	0	0	10	0
Allo. B cells 1	-	-	51	70	49	203	49	72	41	131	26	33	24	32
Allo. dendritic cells 2	+	-	2	30	0	426	0	0	0	225	0	21	0	14
Allo. B cells 2	+	-	57	87	72	187	64	74	59	172	41	50	34	39
Auto. dendritic cells	+	-	0	0	0	105	0	0	0	65	0	0	0	0

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	A2[†]	CEA	PBMC				CD4[+] T cells				CD8[+] T cells			
			None	Wild-type	S112T	L110F S112T	None	Wild-type	S112T	L110F S112T	None	Wild-type	S112T	L110F S112T
Auto. B cells	+	-	16	49	33	99	23	30	27	84	29	38	24	54
Sk23	+	-	113	57	39	152	8	9	0	50	50	120	89	13
SW620	+	-	37	111	77	533	0	6	0	94	141	75	44	96
SW480	+	-	51	118	61	162	0	9	1	403	19	108	73	10
H508	+	+	1	**870** [//]	**6,092**	**8,352**	0	17	0	121	18	85	68	14
SW1463	+	+	5	**496**	**2,418**	**7,875**	0	54	**810**	**7,625**	0	**714**	**5,223**	47
SW403	+	Weak	4	67	**206**	**1,560**	15	64	**403**	**7,327**	0	**543**	**2,407**	45
							0	9	24	**1,542**	0	67	161	56

NOTE: IFN-γ secretion (pg/mL) in 20 h coculture supernatants of target cells with electroporated human $CD8^+$ and $CD4^+$ T cells.

[*] Percentages of $CD8^+$ and $CD4^+$ cells were determined by FACS as were the percentages of cells doubly expressing CD8 or CD4 and the murine TCR.

[†] Expression of HLA-A2.1 and CEA on target cells was evaluated by FACS.

[‡] Bold values for peptide-pulsed targets indicate that IFN-γ release in response to T2 cells pulsed with the indicated concentration of CEA:691-699 was ≥50 pg/mL and at least twice background with medium and T2 cells preloaded with 1 μg/mL of the negative control peptide HBVc:18-27(23Y).

[§] Bold values for transduced COS-7 cells indicate that IFN-γ release in response COS-7 cells transduced with HLA-A2.1 and CEA was ≥50 pg/mL and at least twice background with medium and COS-7 cells transduced with HLA-A2.1 and NY-ESO-1 HLA and that specific peptide reactivity was observed in the same culture.

[//] Bold values for tumor cells indicate that IFN-γ release in response to HLA-$A2.1^+$ CEA^+ tumors was ≥50 pg/mL and at least twice background with medium, HLA-$A2.1^+$ CEA^- tumors, and HLA-$A2.1^-$ CEA^+ tumors and that specific peptide reactivity was observed in the same culture.