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Isolation and characterization of an HLA-DPB1*04:01-restricted MAGE-A3 T cell receptor for cancer immunotherapy

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Summary

Long-term tumor regressions have been observed in patients following the adoptive transfer of autologous tumor infiltrating lymphocytes (TILs) or genetically-modified T cells expressing MHC class I-restricted T cell receptors (TCRs), but clinical trials have not evaluated responses to genetically-modified T cells expressing anti-tumor MHC class II-restricted TCRs. Since studies carried out in a murine tumor model system have demonstrated that the adoptive transfer of CD4⁺ T cells could lead to the regression of established tumors, we plan to test the hypothesis that CD4⁺ T cells can also induce tumor regressions in cancer patients. In this study, two MAGE-A3-specific TCRs were isolated from a regulatory T cell clone (6F9) and an effector clone (R12C9), generated from the peripheral blood of two melanoma patients after MAGE-A3 vaccination. The results indicated that T cells transduced with 6F9 TCR mediated stronger effector functions than R12C9 TCR. The 6F9 TCR specifically recognized MAGE-A3 and the closely related MAGE-A6 gene product, but not other members of the MAGE-A family in the context of HLA-DPB1*04:01. To test the feasibility of a potential clinical trial using this TCR, a clinical-scale procedure was developed to obtain a large number of purified CD4⁺ T cells transduced with 6F9 TCR. Because HLA-DPB1*04:01 is present in ~60% of the Caucasian population and MAGE-A3 is frequently expressed in a variety of cancer types, this TCR immunotherapy could potentially be applicable for a significant portion of cancer patients.

Introduction

Adoptive immunotherapy using genetically modified T cells has become an important strategy for cancer therapy, and recent clinical trials have provided encouraging results.¹ In clinical trials involving TCR targeting HLA-A*0201-restricted NY-ESO-1, objective responses were observed in 61%, 55% and 80% of patients with synovial cell sarcoma,

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melanoma and myeloma, respectively.²⁻⁴ In addition, clinical response rates exceeding 50% have been observed in patients with acute lymphocytic leukemia, chronic lymphocytic leukemia or lymphoma who received treatment with autologous T cells transduced with a chimeric antigen receptor (CAR) targeting CD19.⁵⁻¹³ However, severe toxicities, including deaths, have been observed in several adoptive cell therapy trials targeting solid cancers, due to the recognition of normal tissues by TCRs or CARs.¹⁴⁻¹⁸ As a result, identifying ideal new targets has become one of the biggest challenges in recent years for T cell-based immunotherapies.

A class of tumor-associated antigens was identified, named cancer-germline antigens that frequently showed high levels of expression in a variety of common malignancies and only limited normal tissue expression, except in germline tissues, such as testes.^{19, 20} The first cancer-germline antigen MAGE-A1 (melanoma-associated antigen-A1) was identified and reported in 1991.²¹ In the subsequent studies, totally 12 related genes, including 1 pseudogene, were identified in the MAGE-A family.²² Among the MAGE-A family members, MAGE-A3 and MAGE-A6 are nearly indistinguishable, with 95.9% identical amino acid residues. Additionally, MAGE-A3 is frequently expressed in a variety of cancer types, such as melanoma, hepatocellular carcinoma and non-small cell lung cancer, whereas other members of the MAGE-A family are generally expressed at lower frequencies in cancers.²³⁻³³ As a result, MAGE-A3 is one of the best targets for cancer immunotherapy.

An affinity-enhanced TCR was generated to target HLA-A*01-restricted MAGE-A3 antigen, and this TCR gene therapy led to two deaths from cardiac toxicity, likely due to off-target recognition of a muscle protein Titin by this affinity enhanced TCR.^{18, 34} Two deaths were seen in nine patients treated in a TCR gene therapy trial targeting HLA-A*0201-restricted MAGE-A3/A9/A12.¹⁷ The most likely explanation was that the recognition of MAGE-A12 by TCR-transduced T cells induced neuronal cell destruction in these patients. MAGE-A12 was expressed at low levels in brain tissue specimens, but transferring a large number of T cells might lead to the recognition of MAGE-A12 in brain. Alternatively, this TCR was made in mice, with an amino acid substitution in the TCR α CDR3 region to enhance the affinity. As a result, it bypassed the natural negative selection in human thymus, increasing the likelihood of normal tissue recognition.²⁰

Because of the safety concerns raised by previous trials, we attempted to identify a TCR that specifically recognized MAGE-A3 and the closely related MAGE-A6 gene products, neither of which was expressed in human brain or any other normal tissues, as determined by quantitative PCR, NanoString and RNAseq analyses.¹⁷ In this study, we isolated TCRs from two T cell clones (6F9 and R12C9) obtained from the peripheral blood of melanoma patients after MAGE-A3 vaccination³⁵. Comparison of the specificity and affinity of these two TCRs led to the selection of the 6F9 TCR for a new TCR gene therapy targeting MHC class II-restricted MAGE-A3/A6.

METHODS

Isolation of TCRs from CD4⁺ T cell clones

The generation of CD4⁺ T cell clones was described previously.³⁵ Briefly, Patient EB97 was vaccinated with 300 µg MAGE-A3 protein mixed with an immunological adjuvant AS-15 (GlaxoSmithKline), and later a set of MAGE-A3 peptides at sites close to the protein/adjuvant injection site. Patient R12 received dendritic cells pulsed with a pool of MAGE-A3 peptides. Peripheral blood mononuclear cells (PBMC) from vaccinated patients were labeled with DP4/MAGE-A3 tetramers, and single CD4⁺CD8⁻ tetramer⁺ cells were sorted and stimulated with irradiated DP4⁺ feeder cells pulsed with MAGE-A3.DP4 peptide KKLLTQHFVQENYLEY. TCR sequences were obtained from DP4/MAGE-A3 tetramer⁺ clones by following the manufacturer's protocol for 5' RACE (rapid amplification of cDNA ends) (Clontech, CA), using a TCRα constant region reverse primer (5'-CAC TGT TGC TCT TGA AGT CC-3') and a TCRβ constant region reverse primer (5'-CAG GCA GTA TCT GGA GTC ATT GAG-3').

Retrovirus transduction for T cells

The detailed protocol has been described previously, with some minor modifications described here.³⁶ TCRα and TCRβ sequences linked by a furinSGSGP2A linker (RAKRSGSGATNFSLLKQAGDVEENPGP) were synthesized and cloned into the MSGV1 retroviral expression vector.³⁷ 1.5 µg of MSGV1-TCR plasmid and 0.75 µg of VSV-G (RD114) plasmid were co-transfected into 1×10⁶ 293GP cells in each 6-well using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific). After 48 hr, the supernatant was harvested and spun at 3000 rpm for 10 min to remove debris. The retrovirus supernatant was loaded on RetroNectin (Takara, Otsu, Japan) coated 6-well plates by centrifugation at 2000 g for 2 hr.

Separately, 1×10⁶/mL PBMCs from health donors were stimulated with 50 ng/mL anti-CD3 mAb OKT3 and 300 IU/mL IL-2 in AIM V medium containing 5% human serum. After 2 days, stimulated cells were harvested and resuspended in the same medium without OKT3. Stimulated PBMCs were added to each retrovirus-loaded well at 2×10⁶ cells/well and spun at 1000 g for 10 min. Plates were incubated overnight at 37°C, and the next day the PBMCs were transferred to new retrovirus-loaded wells and the transduction procedure was repeated. TCR-transduced T cells were continuously cultured in AIM V medium with 300 IU/mL IL-2 and 5% human serum for 5 more days before experiments.

Co-culture assays and flow cytometric analyses

1×10⁵ T cells were co-cultured with 1×10⁵ target cells overnight in 96-well U-bottom plates. The supernatant was harvested and the secretion of IFN-γ from T cells was determined by ELISA (Thermo Fisher Scientific). As indicated in the figure legends, 10 µM peptides were pulsed for 2 hr on 293-CIITA cells or HLA-DPB1*0401(+) EBV-transformed B cells, generated from Patient 2556, and then co-culture with T cells. For siRNA knockdown assays, cell lines were transfected with ON-TARGETplus siRNAs (Dharmacon) and Lipofectamine RNAiMAX (Life Technologies) for 48 hr, according to the manufacturer's instruction. For intracellular IFN-γ staining, T cells were stimulated for 4 hr in the presence

of GolgiPlug (BD Biosciences). These T cells were then fixed, permeabilized and stained according to the manufacturer's instruction (eBiosciences). All the antibodies for flow cytometric analyses were purchased from BD Biosciences, except human anti-TCR V β antibodies from Beckman Coulter.

Clinical scale production of TCR-transduced CD4⁺ T cells

Peripheral blood leukocytes (PBLs) from cancer patients were isolated by leukapheresis. Lymphocytes were separated by centrifugation on a Lymphocyte Separation Medium (LSM) cushion, and then washed in HBSS. By following Miltenyi Biotech's standard operating procedure, CD8⁺ lymphocytes were depleted by clinical-grade CD8-antibody coated magnetic particles and CliniMACS clinical-scale cell separation apparatus. The CD8⁺ T cell-depleted PBMCs were then resuspended for stimulation in AIM V medium, supplemented with 300 IU/ml IL-2, and 5% human serum. 3×10^8 CD8⁺ T cell-depleted PBMCs were cultured for 2 days in the presence of 50 ng/ml OKT3 antibody. Stimulated cells were harvested, spun to pellet, and resuspended in AIM V medium supplemented with 300 IU/ml IL-2 and 5% human serum. The stimulated cells were transduced with a γ -retroviral vector encoding the MAGE-A3 TCR (6F9mC) as described previously.³⁶ Clinical-grade retrovirus supernatant was produced from a master cell bank derived from a selected high-titer PG13 cell-based producer cell line (PG13 MAGE-A3 DP4 6F9mC TCR clone B8) under good manufacturing practice conditions (Surgery Branch Vector Production Facility, National Cancer Institute, Bethesda, MD). The biosafety of master cell bank and vector supernatant was tested and passed all currently required US Food and Drug Administration (FDA) guidelines for recombinant γ -retrovirus production for clinical application.

Results

Evaluation of two TCRs isolated from an anti-MAGE-A3 CD4⁺ effector T cell clone and a regulatory T cell clone

For this study, we selected two MAGE-A3-specific CD4⁺ T cell clones generated from the peripheral blood of vaccinated patients. One was a CD4⁺CD25⁺FOXP3⁺ regulatory T cell (Treg) clone 6F9, and the other was a CD4⁺CD25⁻ effector T cell clone R12C9. The TCR α and β chains expressed by the two clones were then sequenced in order to generate recombinant retroviruses encoding the appropriate TCRs. Experiments were carried out to analyze the reactivity of 6F9 TCR- and R12C9 TCR-transduced T cells against target cells, generated by the stable transduction of CIITA (class II, major histocompatibility complex, transactivator), which led to the increased expression of MHC class II molecules on transduced tumor cells. The results indicated that T cells transduced with either the 6F9 or R12C9 TCR strongly recognized HLA-DPB1*04:01 (DP4)(+) 293-CIITA cells pulsed with the MAGE-A3:243-258 peptide (Fig. 1A). Titration of the MAGE-A3:243-258 peptide indicated that T cells transduced with the 6F9 and R12C9 TCRs released comparable levels of IFN- γ in response to targets pulsed with a minimum of 0.1 μ g/ml of the target peptide. In contrast to the results using peptide-pulsed target cells, 6F9 TCR-transduced T cells produced significantly higher levels of IFN- γ in response to DP4(+) EBV-B cells pulsed with MAGE-A3 protein than the R12C9 TCR-transduced T cells (Fig. 1B). Further comparisons indicated that 6F9 TCR-transduced T cells but not R12C9 TCR-transduced T

cells recognized 293-CIITA cells transfected with full-length MAGE-A3 cDNA or MAGE-A6 cDNA. However, both 6F9 TCR- and R12C9 TCR-transduced T cells failed to recognize 293-CIITA cells transfected with full length MAGE-A1 cDNA, MAGE-A12 cDNA or NY-ESO-1 cDNA (Fig. 1C). Comparing the amino acid sequences in the corresponding regions of the MAGE family members, MAGE-A6 differs from MAGE-A3 in one residue (249), whereas MAGE-A12 differs in two residues (249 and 250) and MAGE-A1 differs in three residues (249, 250 and 254) (Fig. 1D). These findings indicated that 6F9 TCR might have a higher functional avidity than R12C9 TCR, because 6F9 TCR strongly recognized the processed MAGE-A3 protein as well as 293-CIITA cells overexpressing MAGE-A3 or MAGE-A6.

To test the tumor reactivity of both TCRs, a panel of tumor lines were transduced with CIITA to increase the expression of MHC class II molecules (Fig. 2A and Fig. S1B). Cells transduced with the 6F9 TCR recognized the MAGE-A3(+)/DP4(+) cell lines Mel 526-CIITA and H1299-CIITA, but failed to recognize the MAGE-A3(+)/DP4(-) Mel 624-CIITA, whereas R12C9 TCR-transduced T cells failed to recognize either of the tested cell lines (Fig. 2B). In addition, 6F9 TCR-transduced T cells recognized DP4(+) dendritic cells pulsed with MAGE-A3(+) tumor cell lysates (Fig. 2C), indicating that these T cells could react to APCs that have processed and presented MAGE-A3 antigen expressed by dead and dying tumor cells in the tumor microenvironment.

Further studies were carried out to analyze the specificity of 6F9 TCR. The response was significantly blocked by a MAGE-A3 siRNA by not a MART-1 control siRNA when 6F9 TCR-transduced T cells co-cultured with siRNA-treated Mel 526-CIITA cells (Fig. 3A). In addition, transfection of an HLA-DP-specific siRNA but not an HLA-DR-specific siRNA decreased the reactivity of 6F9 TCR-transduced T cells against both Mel 526-CIITA and H1299-CIITA cell lines (Fig. 3B). Additional experiments were conducted to compare the reactivity of this MHC class II TCR expressed in purified CD4⁺ and CD8⁺ T cells. The results indicated that 6F9 TCR-transduced CD4⁺ T cells strongly recognized cells transfected with constructs encoding MAGE-A3 and MAGE-A6 cDNA, but failed to recognize cells transfected with constructs encoding MAGE-A12 or other MAGE-A family members (Fig. 3C). The 6F9 TCR-transduced CD8⁺ T cells produced low but significant levels of IFN- γ in response to MAGE-A3 and MAGE-A6 transfectants, but not other MAGE-A family members. Furthermore, 6F9 TCR-transduced CD4⁺ T cells recognized MHC class II-negative 293 cells that were transfected with the combination of MAGE-A3, HLA-DPA1*0103 and HLA-DPB1*04:01 constructs, but not HLA-DPB1*02:01, 03:01, 04:02 or 06:01 construct (Fig. 3D). These results further demonstrated that the recognition of the MAGE-A3 epitope was mediated by the 6F9 TCR was restricted to the HLA-DPB1*04:01.

Substitutions of murine TCR α and β constant regions enhanced the reactivity of 6F9 TCR transduced T cells

Previous studies have demonstrated that replacing human TCR α / β constant region sequences with mouse counterparts could enhance the reactivity of TCRs.³⁸ Here we evaluated the effects of replacing the 6F9 TCR α / β constant regions with murine sequences by constructing

a new TCR, designated 6F9mC, into a retroviral vector. Surface expression levels of the modified 6F9mC TCR-transduced T cells were higher than T cells transduced with the native 6F9 TCR, as demonstrated by staining with a V β 6.7 antibody and a mouse TCR β (constant region) antibody, as well as a MAGE-A3/HLA-DPB1*04:01 tetramer (Fig. S2A).

Further analyses revealed that 6F9mC TCR-transduced T cells released two to five-fold higher levels of IFN- γ than those transduced with the native 6F9 TCR in response to a panel of six MAGE-A3(+)/DP4(+) tumor cell lines (Fig. 4A). Substitution of the murine constant regions also significantly enhanced the responses of purified CD4 $^{+}$ and CD8 $^{+}$ T cells against MAGE-A3(+)/DP4(+) tumor cell lines (Fig. 4B and 4C).

Evaluation of the cytokine responses in CD4 $^{+}$ T cells transduced with the 6F9mC TCR revealed that these cells produced IFN- γ , TNF- α , and IL-2 in response to MAGE-A3(+)/DP4(+) tumor targets (Fig. 4D). Up-regulation of the T cell activation markers 4-1BB, CD25, and CD69, was also observed on 6F9mC TCR-transduced CD4 $^{+}$ T cells following an overnight incubation with target tumor cell lines (Fig. S2B). In the standard 4 hr Chromium-51 release assay, we could not detect any significant killing activity in 6F9mC TCR-transduced CD4 $^{+}$ T cells (data not shown). However, 6F9mC-transduced CD4 $^{+}$ T cells up-regulated CD107a expression upon stimulation by MAGE-A3(+)/DP4(+) tumor targets and lysed the appropriate target cell lines after 18 hr co-culture (Fig. 4E and 4F). These results indicated that 6F9mC TCR-transduced CD4 $^{+}$ T cells were capable of killing tumor cells directly, with much slower kinetics comparing to CD8 $^{+}$ T cells.

In order to evaluate the fine specificity of antigen recognition mediated by cells transduced with the 6F9 or 6F9mC TCR, DP4(+) 293 CIITA cells were pulsed with truncations of MAGE-A3:243-258 peptide or related peptides from the MAGE family members. The results indicated that 11-mer peptide QHFVQENYLEY, corresponding to amino acids 248-258 of the MAGE-A3 protein, represented the minimal peptide that elicited a response comparable to MAGE-A3:243-258 peptide (Table 1). This 11-mer peptide was nearly identical to the 12-mer minimal epitope previously described but lacked the N-terminal threonine residue.³⁹ Consistent with the previous findings, both 6F9 TCR and 6F9mC TCR-transduced T cells recognized MAGE-A6:248-258 peptide containing a single substitution of tyrosine for histidine at position 249. Minimal reactivity was observed against additional members of the MAGE family containing 2~5 amino acid substitutions from the MAGE-A3:248-258 peptide (Table 1). T cells transduced with 6F9 or 6F9mC TCR also failed to recognize target cells pulsed with a Necdin (NDN) that containing 5 amino acid substitutions from the MAGE-A3 peptide (Table 1). A BLAST search of the NCBI database indicated that no additional peptides shared more than five residues with the core MAGE-A3:248-258 sequence. These findings indicated that the 6F9 and 6F9mC TCRs had a high degree of specificity for the MAGE-A3:248-258 epitope, with little risk of cross-reactivity with peptides derived from other proteins produced in human.

Clinical production of 6F9mC TCR-transduced CD4 $^{+}$ T cells

For the clinical production, the 6F9mC TCR was selected over the native 6F9 TCR because 6F9mC TCR conferred higher activity than the 6F9 TCR in the majority of the assays. In addition, mouse constant region modification could prevent the risk of mispairing, which

could potentially induce off-target toxicity in immunotherapy.^{38, 40} Three test runs were carried out for the clinical-scale production of TCR-transduced CD4⁺ T cells, which has not been published in the literature before. A CliniMACS clinical-scale cell separation apparatus was used to deplete CD8⁺ lymphocytes from PBLs (Fig. 5A). Almost all CD8⁺ T cells were depleted after the CliniMACS separation, and 68% of the cells after separation were CD4⁺ T cells (Fig. 5B). The experimental results from three donors were summarized, and in average 59% of the cells were CD4⁺ after the CliniMACS separation (Fig. 5C). CD8-depleted PBL were stimulated by OKT-3 for 2 days and then transduced twice with retrovirus containing 6F9mC TCR (Fig. 5A). Flow cytometric analysis of transduced cells showed that 96% of transduced cells were CD4⁺ T cells, with less than 0.1% CD8⁺ T cells. In addition, 88% of TCR-transduced cells were CD4⁺ and mouse TCRβ⁺, detected by an anti-mouse TCRβ constant region antibody (Fig. 5D). The experimental results from three donors were summarized in Figure 5E. TCR-engineered CD4⁺ T cells were co-cultured with tumor cell lines or MAGE-A3 peptide-pulsed cells to assess the specific recognition of tumor cells. High levels of IFN-γ were observed from the co-culture wells with specific target cells but not negative controls (Fig. 5F). To obtain a sufficient cell number for patient treatment, TCR-engineered CD4⁺ T cells were expanded following the rapid expand protocol (REP). A mean of 973-fold expansion was achieved in 14 days (Fig. 6A). Similar experimental results were obtained after the cell expansion, and 99% of the cells were CD4⁺ and 92% of the cells were TCR-transduced (CD4⁺/mTCRβ⁺) (Fig. 6B). In Figure 6C, the results from three donors were summarized, and in average 98% of the cells were CD4⁺ and 92% of the cells were CD4⁺/mTCRβ⁺. Specific release of IFN-γ from TCR-engineered CD4⁺ T cells was also observed after REP (Fig. 6D). Taken together, this clinical-scale process could produce a large number of highly purified CD4⁺ TCR-transduced T cells for patient treatment.

Discussion

The functions of MAGE-A3 and other family members have been largely unknown. Limited evidence suggested that MAGE-A3 could modulate cancer progression through accelerating cell cycle and enhancing cell migration/invasion.⁴¹ Other studies showed that the suppression of MAGE-A family proteins could induce apoptosis through p53-mediated pathways.^{42–44} We have proposed that targeting essential gene products for carcinogenesis is likely more effective than targeting non-essential gene products.⁴⁵ As a result, cancer immunotherapy targeting MAGE-A3 might be more effective than targeting tissue specific differentiation antigens, such as MART-1 and gp100.^{14, 36} Consistent with our hypothesis, 5 out of 9 patients experienced clinical tumor regressions after the treatments with T cells targeting HLA-A*0201-restricted MAGE-A3 epitope KVAELVHFL, but the unexpected neurological toxicity that likely came from the recognition of a similar epitope KMAELVHFL from MAGE-A12 expressed at a low level in the brain, resulted in the closure of this clinical trial.^{17, 46} The sequence for another potential A*0201 epitope FLWGPRALV is identical in both MAGE-A3 and MAGE-A12. As a result, it's difficult to generate a TCR that can recognize MAGE-A3 but not MAGE-A12 in an HLA-A*0201-restricted manner.

One potential alternative is targeting MHC class II-restricted epitopes. It has been long thought that CD8⁺ T cells play the direct role of killing tumor cells through cytotoxicity,

while CD4⁺ T cells merely provide help during the immune responses against tumor.^{47, 48} As a result, the majority of current immunotherapies utilize MHC class I-restricted TCR or non-MHC-restricted CAR technologies to genetically modify CD8⁺ T cells or bulk T cells for patient treatments. However, some evidence suggested that CD4⁺ T cells could be in the driver's seat to eliminate tumors. In a B16 melanoma mouse model, tumor-specific CD4⁺ T cells could eradicate established melanoma, and the anti-tumor activity could be further enhanced by the CTLA-4 blockade, OX40 stimulation or Th17-polarization of CD4⁺ T cells.^{49–51} The *in vivo* anti-tumor activity of CD4⁺ T cells was dependent on the IFN- γ secretion from CD4⁺ T cells and the MHC class II molecules expressed on B16 melanoma, up-regulated following *in vivo* IFN- γ stimulation.^{49, 51} It appeared that tumor-specific CD4⁺ T cells could play a direct role in eradicating tumors *in vivo*, since the tumor regression was not dependent on endogenous CD8⁺ T cells, B cells, NK cells or NKT cells.^{51, 52} Limited evidence also suggested that Eomes and granzyme B were essential for the cytotoxic activity of CD4⁺ T cells.^{50, 51} However, according to the published data, 12 ~ 24 hr co-culture was required to detect the killing activity of CD4⁺ T cells *in vitro*, similar to the observation in this study (Fig. 4F).^{50, 51} As a result, CD4⁺ T cells might be less potent than CD8⁺ T cells, and more CD4⁺ T cells might be required to induce significant tumor regressions *in vivo*. In spite of these findings, a patient with metastatic melanoma was treated with an autologous DP4-restricted NY-ESO-1-specific CD4⁺ T cell clone, and this patient experienced a long-term complete remission beyond 2 years.⁵³ In our recent clinical trial, a 95% pure population of mutated ERBB2IP-reactive CD4⁺ T cells was transferred to a patient with metastatic cholangiocarcinoma, and she experienced a partial response ongoing for two years.⁵⁴ These clinical studies indicated that CD4⁺ T cells could induce long-term tumor regression in human, similar to CD8⁺ T cells. Taken together, these findings suggest that CD4⁺ T cells are capable of inducing tumor regressions and can play a direct role in cancer immunotherapy. More studies are necessary to understand the detailed mechanisms on how CD4⁺ T cells eliminate tumors *in vivo*.

Because of the therapeutic potentials of MAGE-A3-reactive CD4⁺ T cells, we attempted to isolate TCRs recognizing MHC class II-restricted MAGE-A3 epitopes. Previous studies have identified MAGE-A3 epitopes that were recognized by CD4⁺ T cells in the context of either HLA-DRB1*01, *04, *07, *11 or *13.^{55–58} However, among the Caucasian population only 20%~30% of individuals have each individual DRB1 allele, according to the Allele Frequency Net Database (AFND).⁵⁹ In contrast, HLA-DPB1*0401 is the most conserved allele, and ~60% of Caucasians are DPB1*0401(+), calculated from 21 studies in AFND. Therefore, the use of HLA-DPB1*0401-restricted TCR may extend the reach of TCR gene therapy for cancer patients among the Caucasian population.

In summary, the MAGE-A3 TCR characterized in this study possesses several unique properties suitable for TCR therapy. (1) MAGE-A3 can be frequently expressed in a variety of common malignancies, and is not expressed in any normal tissue, except testes. (2) HLA-DPB1*0401 is the most frequent HLA allele among the Caucasian population. (3) The 6F9mC TCR is highly specific and has no cross-reactivity with other MAGE family member, except its homolog MAGE-A6. To study the safety and efficacy of adoptive T cell therapy using this TCR, we have recently started a CD4⁺ T cell immunotherapy trial based on the data obtained this study (ClinicalTrials.gov Identifier: NCT02111850). This cancer

immunotherapy trial will further test the hypothesis that adoptively transferring tumor-specific CD4⁺ T cells can induce tumor regressions in cancer patients. To our knowledge, this clinical trial represents the first genetically-modified CD4⁺ T cell immunotherapy against metastatic cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Reactive TCRs were isolated from MAGE-A3 specific CD4⁺ T cell clones. (A) PBMCs were transduced with either R12C9 TCR or 6F9 TCR, and then co-cultured with 293-CIITA cells pulsed with MAGE-A3:243-258 peptide at indicated concentration overnight. UT: untransduced control. (B) HLA-DPB1*04:01 (DP4)(+) EBV transformed B cells were incubated with MAGE-A3 full-length protein at indicated concentrations for 18 hr, and then co-cultured with T cells transduced with either R12C9 TCR or 6F9 TCR overnight. (C) 293-CIITA cells were transfected with full-length cDNA constructs as shown, and these transfectants were co-cultured with R12C9 TCR-transduced T cells or 6F9 TCR-transduced T cells overnight. The secretion of IFN- γ from T cells was determined by ELISA. Representative data from one of four different donors are shown. (D) Sequence comparison of the corresponding regions of MAGE-A1, MAGE-A3, MAGE-A6 and MAGE-A12.

Figure 2. 6F9 TCR-transduced T cells recognized tumor cell lines. (A) The list of tumor cell lines stably transfected with CIITA. (B) T cells transduced with either R12C9 TCR or 6F9 TCR were co-cultured with three tumor lines overnight. MART-1 TCR-transduced T cells were served as the positive control. (C) DP4(+) dendritic cells were incubated with MAGE-A3 protein, as well as cell lysates from MAGE-A3(+) or (-) tumor cells for 18hr, and then co-cultured with 6F9 TCR-transduced T cells overnight. The concentration of IFN- γ in the supernatant was determined by ELISA. Representative data from one of three different donors are shown.

Figure 3. 6F9 TCR was MAGE-A3/A6 specific and HLA-DPB1*04:01-restricted. (A) Mel 526-CIITA cells were transfected with siRNA specific for MAGE-A3 or MART-1. After 48 hr, tumor cells were then co-cultured with T cells transduced with MART-1 TCR or 6F9 TCR overnight. (B) Mel 526-CIITA and H1299-CIITA cells were transfected with siRNA specific for HLA-DR or HLA-DP. After 48 hr, tumor cells were then co-cultured with T cells transduced with 6F9 TCR overnight. (C) 293-CIITA cells were transfected with a panel of cDNA constructs isolated from MAGE-A family members, and then these transfectants were co-cultured with 6F9 TCR-transduced CD4⁺ or CD8⁺ T cells. (D) 293 cells were transfected with different HLA and MAGE-A3 cDNA constructs, and then co-cultured with CD4⁺ or CD8⁺ T cells transduced with 6F9 TCR. The secretion of IFN- γ from T cells was determined by ELISA. Representative data from one of three different donors are shown.

Figure 4.

The replacements of TCR constant regions enhanced the tumor reactivity of 6F9 TCR. (A) The human constant regions of 6F9 TCR was replaced by murine constant regions, designated 6F9mC. T cells transduced with either 6F9 or 6F9mC TCRs were co-cultured with 9 tumor lines overnight. The expression data of HLA-DPB1*04:01 and MAGE-A3 have been shown in Figure 2A. (B and C) Enriched CD4⁺ or CD8⁺ T cells transduced with either 6F9 or 6F9mC TCRs were co-cultured with 9 tumor lines overnight. The concentration of IFN-γ in the supernatant was measured by ELISA. (D) 6F9mC TCR-transduced CD4⁺ T cells were co-cultured with tumor targets for 18 hr. The production of IFN-γ, TNF-α, and IL-2 were determined by flow cytometric analysis. (E) 6F9mC TCR-transduced CD4⁺ T cells were co-cultured with tumor targets for 18 hr. The expression of CD107a was determined by flow cytometric analysis. (F) 6F9mC TCR-transduced CD4⁺ T cells were co-cultured with tumor targets for 18 hr. Cell viability was determined by Propidium iodide (PI) staining and flow cytometry, and PI (–) cells were counted as the viable tumor cells. Representative data from one of three different donors are shown.

Figure 5.

Clinical-scale cell separation by CliniMACS and the subsequent retroviral transduction of 6F9mC TCR. (A) The schedule of clinical-scale cell production for patient treatment. Peripheral blood lymphocytes (PBL) were CD8⁺ T cells depleted on day 0 and transduced with 6F9mC TCR on day 2 and 3. (B) Flow cytometric analysis of PBL before and after CliniMACS separation on day 0. (C) The summary of results on day 0 from three donors. (D) Flow cytometric analysis of transduced T cells on day 9. (E) The summary of flow cytometric analyses on day 9 from three donors. The purity of CD4⁺ T cells and the transduction efficiency were determined by CD4, CD8 and mTCRβ antibody staining. (F) Recognition of a tumor cell line and MAGE-A3 peptide-pulsed cells on day 10 after CliniMACS separation. MAGE-A3 DP4 (6F9mC) TCR-transduced CD4⁺ T cells were co-cultured overnight with MAGE-A3:248-258 peptide-pulsed 293-CIITA cells or tumor cell lines [2630-CIITA: MAGE-A3(+), DP4(+); 624-CIITA: MAGE-A3(+), DP4(-); 1764 CIITA: MAGE-A3(-), DP4(+)]. The secretion of IFN-γ was determined by ELISA. Unseparated, un-transduced lymphocytes were functioned as the negative control. Representative data from one of the three different donors are shown.

Figure 6.

Rapid expansion of TCR-transduced CD4⁺ T cells for patient treatment. Transduced CD4⁺ T cells underwent rapid expansion protocol (REP) on day 10 after CliniMACS separation. (A) After REP, the expansion of T cells from three donors was evaluated. (B) Flow cytometric analysis of transduced CD4⁺ T cells on day 13 after REP. (C) The summary of results on day 13 from three donors. (D) Recognition of tumor cell lines and MAGE-A3:248-258 peptide-pulsed cells on day 13 after REP. 6F9mC TCR-transduced CD4⁺ T cells were co-cultured for 16 hr with tumor cell lines or MAGE-A3 peptide-pulsed 293 CIITA cells. The secretion of IFN-γ was determined by ELISA. Representative data from one of the three different donors are shown.

Table 1

The specificity of 6F9 TCR and 6F9mC TCR.

Protein name & amino acid position	Amino Acid Sequence	Un-transduced control IFN- γ (pg/mL)	6F9 TCR IFN- γ (pg/mL)	6F9mC TCR IFN- γ (pg/mL)
MAGE-A3:243-258	KKLLTQHFVQENYLEY	33	10,220	15,210
MAGE-A3:243-256	KKLLTQHFVQENYL	72	1,018	1,815
MAGE-A3:243-255	KKLLTQHFVQENY	29	76	137
MAGE-A3:243-254	KKLLTQHFVQEN	67	28	0
MAGE-A3:243-253	KKLLTQHFVQE	38	0	40
MAGE-A3:245-258	LLTQHFVQENYLEY	84	9,290	14,970
MAGE-A3:246-258	LTQHFVQENYLEY	56	7,140	12,700
MAGE-A3:247-258	TQHFVQENYLEY	30	6,710	10,600
MAGE-A3:248-258	QHFVQENYLEY	52	6,220	9,000
MAGE-A3:249-258	HFVQENYLEY	57	669	1,643
MAGE-A6:248-258	QYFVQENYLEY	54	6,440	11,800
MAGE-A2/A12:248-258	QDLVQENYLEY	49	33	66
MAGE-A4/A9:249-259	QDWVQENYLEY	32	0	23
MAGE-A8:251-261	QEWVQENYLEY	79	43	58
MAGE-A1/B4:241-251	QDLVQEKYLEY	55	129	126
MAGE-B2:250-260	KDLVQEKYLEY	43	0	0
MAGE-B10:250-260	KDLVKENYLEY	69	22	18
MAGE-B16:252-262	KDFVKEKYLEY	16	0	27
MAGE-C1:113-123	KVWVQEHYLEY	30	9	0
MAGE-D4:300-315	RKLITDDFVKQKYLEY	81	193	234
MAGE-D2:413-428	KKLITDEFVKQKYLDY	56	82	43
MAGE-L2:582-597	KKLITEFVVRQKYLEY	58	45	56
MAGE-G1:220-235	KKLITDEFVRQRYLEY	68	0	29
Necdin (NDN):237-247	EEFVQMNYLKY	59	0	22
No peptide		58	0	5