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Limited expression of cancer-testis antigens in renal cell carcinoma patients

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Abstract. The aim of this study was to evaluate the frequency of expression of the cancer-testis antigens (CTAs) NY-ESO-1, MAGE-A4 and SAGE, in renal cell carcinoma (RCC) patients compared to that in head and neck cancer (HNC) patients, which represent a positive control with a high incidence of CTA expression, to identify novel target antigens for immunotherapy. We prospectively examined frozen tissue samples collected from surgery or biopsy from 35 RCC and 40 HNC patients. Total RNA was extracted, and real-time reverse transcription-polymerase chain reaction (RT)-PCR was performed to determine the expression of MAGE-A4, NY-ESO-1 and SAGE. MAGE-A4 was not detected in any of the RCC samples, although a low incidence of NY-ESO-1 (5.7%; 2/35) and SAGE (2.9%; 1/35) expression was observed. No samples demonstrated co-expression of the three CTAs. By contrast, a comparatively high incidence of CTA expression was detected in squamous cell carcinoma (SCC) specimens of HNC patients. The actual incidence was 42.5% (17/40) for MAGE-A4, 20% (8/40) for NY-ESO-1 and 15% (6/40) for SAGE. The incidence of co-expression was 7.5% (3/40) for MAGE-A4 and NY-ESO-1, 7.5% (3/40) for MAGE-A4 and SAGE, 7.5% (3/40) for NY-ESO-1 and SAGE, and 2.5% (1/40) for the CTAs. The number of HNC samples positive for MAGE-A4 was significantly higher compared to that of RCC samples. The remaining two antigens, NY-ESO-1 and SAGE, were expressed at high levels in HNC compared to RCC samples. Limited frequency of CTA (NY-ESO-1, MAGE-A4 and SAGE) expression was demonstrated in RCC compared to HNC samples.

Introduction

Renal cell carcinoma (RCC) accounts for 2% of all types of cancer, and the reported incidence is on the increase due to advances in imaging technology (1). Distant metastases have been reported to develop in 24-30% of the patients who undergo radical nephrectomy (2,3). RCC patients with metastases who are not surgical candidates receive immunotherapy, chemotherapy or molecular target-based therapy as palliative treatment. However, the survival of these patients is limited. The median survival times reported are 7-8.5 months following immunotherapy, 6-10 months following systemic chemotherapy (4) and <19.3 months following molecular target-based therapy (5,6). Therefore, additional therapeutic options for RCC patients with unresectable metastases are needed.

Several novel regimens that use targeted immunotherapeutic strategies, such as cancer vaccines against MN/CA9 (7) or HIFPH3 (8), have been evaluated in RCC cases. Further investigation of these strategies is required for the precise evaluation of their therapeutic efficacy. It is crucial to define appropriate target antigens that are frequently and specifically expressed in RCC to establish effective RCC immunotherapy.

Cancer-testis antigens (CTAs) are particularly attractive targets for immunotherapy, due to their unique expression profiles. While these antigens are highly expressed in adult male germ cells or placenta, they are completely absent from other normal adult tissues and demonstrate aberrant expression in a variety of malignant neoplasms (9,10). As adult male germ cells do not express major histocompatibility complex (MHC) class I, CD8⁺ effector cells theoretically ignore these cells (11). MAGE, NY-ESO-1 and SAGE genes exhibit a similar expression pattern, and their immunogenicity as targets for cancer immunotherapy has been well-studied (12-16). In a recent study, we assessed the efficacy of immunotherapy for several types of cancer-targeting CTAs (17,18). As a first step towards the development of effective immunotherapy for RCC patients, it is crucial to evaluate the expression level of CTA in the targeted cancer. However, a limited number of studies has estimated the incidence of CTA expression in RCC cases, with controversial results (19-22). The aim of this study was to precisely evaluate the frequency of CTA expression in RCC with specific and quantitative methodology.

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We performed quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) for MAGE-A4, NY-ESO-1 and SAGE with specific primers in RCC compared to head and neck cancer (HNC) patients, which represent a positive control with a high incidence of CTA expression, to assess the future potential of CTA-targeted immunotherapy.

Materials and methods

Cases. Decisions regarding sample analysis were made following discussion with patients and obtaining their informed consent. Informed consent was obtained in accordance with the requirements of our Institutional Review Board (IRB) at the Mie University School of Medicine (Tsu, Japan).

Between September, 2009 and June, 2011, 35 RCC samples were obtained by surgery or biopsy. The 35 patients (29 males and 6 females) had a median age of 61.9 years (range, 41-87). The average tumor size was 5.0 cm (range, 1.8-12.0). Thirty-one patients had undergone radical nephrectomy, while one patient had initially undergone partial nephrectomy. In the remaining 3 cases computed tomography (CT)-guided biopsy was performed to obtain a tumor specimen prior to radiofrequency ablation.

Histological examination demonstrated that the 35 cases were clear cell RCC. Of these, 29 cases were grade ≤ 2 , 4 were grade ≥ 3 and in 2 cases the grade classification was unknown (Table I).

Samples from 40 HNC cases were collected by surgery or biopsy for the positive control with a high incidence of CTA expression between December, 2008 and August, 2011. The 40 patients (28 males and 12 females) had a median age of 63.9 years (range, 34-87). The average tumor size was 5.9 cm (range, 1.3-29.0). Regarding cancer location, there were 23 cases of oral cancer (14 cases of tongue, 5 of buccal mucosal and 4 of gingival cancers), and 10 cases of pharyngeal cancer. There were also 5 cases of laryngeal and 2 of maxillary cancer (Table II). In this group, 31 patients had undergone radical resection, while punch core biopsy was performed in 9 patients to obtain a tumor specimen.

RNA extraction and quantitative RT-PCR. Total RNA was extracted from frozen RCC and HNC tissue samples. Complementary DNA (cDNA) was prepared from 1 μ g of total RNA using the QuantiTect[®] Reverse Transcription kit (Qiagen, Hilden, Germany). The quantitative PCR analysis for the expression of MAGE-A4, NY-ESO-1 and SAGE was performed in buffer composed of 1X QuantiTect Multiplex PCR NoROX Master mix (Qiagen), 1X Pre-Developed TaqMan[®] Assay Reagents Control kits (Applied Biosystems, Carlsbad, CA, USA), and 0.4 mM of cognate primers and 0.2 mM of cognate probe. Following enzyme activation for 15 min at 95°C, 50 two-step cycles were performed (1 min at 94°C and 1 min at 60°C), using the Mx3000P system (Agilent Technologies, Santa Clara, CA, USA). Sequences of the primers and probes used in the present study were: MAGE-A4 F: 5'-GCAGTAATCCTGCGCGCTAT-3' and R: 5'-CATTGACCCTGACCACATGCT-3'; probe: 5'-FAM-CTCTGGCTGAAACCA-MGB-3'. NY-ESO-1 F: 5'-GGCTGAATGGA TGCTGCAGA-3' and R: 5'-CTGGAGACAGGAGCTGATGGA-3'; probe: 5'-FAM-TGTGTCCGGCAACATACTGAC

Table I. Baseline characteristics of RCC patients.

Patient demography	Value
Total no. of patients	35
Age (years), median (range)	61.9 (41-87)
<60	14
≥ 60	21
Gender	
Male	29
Female	6
Laterality	
Right	14
Left	21
Mean maximum diameter (cm)	
Median (range)	5.0 (1.8-12.0)
Treatment procedure to obtain specimens	
Radical nephrectomy	31
Partial nephrectomy	1
Biopsy before RFA	3
Histological cell type	
Clear cell	35
Tumor grade	
≤ 2	29
≥ 3	4
Unknown	2

RCC, renal cell cancer; RFA, radiofrequency ablation.

TATCCGA-TAMRA-3'. SAGE F: 5'-TGTCATTCACGATATCCAGGAG G-3' and R: 5'-GGTGGCATAACAATGTCCTGTCAT-3'; probe: 5'-FAM-TGTGTCCGGCAACATACTGACTATCCGA-TAMRA-3'. The GAPDH copy number was measured using Pre-Developed TaqMan Assay Reagents Control kits (Applied Biosystems). To transform the cycle threshold (Ct) values into absolute mRNA copy numbers, we used a dilution series of linearized plasmid containing each gene insert and constructed a calibration curve. Gene expression was evaluated as positive when the value exceeded 12.2 copies/GAPDH 10^4 copies for MAGE-A4, 5.96 copies/GAPDH 10^4 copies for NY-ESO-1 and 2.81 copies/GAPDH 10^4 copies for SAGE. These cut-off values were determined as the mean \pm 2 standard deviation (SD) of the expression levels in the cognate normal samples.

Statistical analysis. Differences between groups were assessed with the Chi-square test or direct Fisher's exact test. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed with the SPSS software version 20 (SPSS Japan, Tokyo, Japan).

Results

CTA expression levels were evaluated using real-time RT-PCR in a total of 35 RCC and 40 HNC cases (a positive control

Table II. Baseline characteristics of HNC patients.

Patient demography	Value
Total no. of patients	40
Age (years), median (range)	63.9 (34-87)
<60	11
≥60	29
Gender	
Male	28
Female	12
Mean maximum diameter (cm)	
Median (range)	5.9 (1.3-29.0)
Location of tumor	
Oral cancers	23
Tongues	14
Buccal mucosal	5
Gingival	4
Pharyngeal cancer	10
Laryngeal cancer	5
Maxillary cancer	2
Procedure to obtain specimens	
Resection	31
Biopsy	9
Histological cell type	
Squamous cell carcinoma	40

HNC, head and neck cancer.

with a high incidence of CTA expression). MAGE-A4 was not detected in any of the RCC cases, although a low incidence of NY-ESO-1 (5.7%; 2/35) and SAGE (2.9%; 1/35) expression was observed. No case demonstrated co-expression of the three CTAs investigated.

By contrast, a comparatively high incidence of expression was detected in squamous cell carcinoma (SCC) specimens of HNC patients. The actual incidence was 42.5% (17/40) for MAGE-A4, 20% (8/40) for NY-ESO-1 and 15% (6/40) for SAGE. The incidence of co-expression was 7.5% (3/40) for MAGE-A4 and NY-ESO-1, 7.5% (3/40) for MAGE-A4 and SAGE, 7.5% (3/40) for NY-ESO-1 and SAGE, as well as 2.5% (1/40) for the CTAs.

The number of HNC samples that were positive for MAGE-A4 was significantly higher compared to that of RCC samples ($P<0.001$). The remaining two antigens, NY-ESO-1 and SAGE, demonstrated higher expression levels in NSC compared to RCC samples (Table III).

Discussion

Since CTAs are expressed in tumors and not in normal tissue (except for testicular tissue), CTAs are optimal antigens for cancer-specific vaccines (23). As the first step in the strategy of designing a cancer vaccine, knowledge of the frequency of expression of each CTA in the targeted cancer is crucial.

Table III. Frequency of CTA expression in each type of cancer.

Antigen	RCC (n=35) n (%)	HNC (n=40) n (%)	P-value
MAGE-A4			
Positive	0 (0)	17 (42.5)	<0.001
Negative	35 (100)	23 (57.5)	
NY-ESO-1			
Positive	2 (5.7)	8 (20)	0.09
Negative	33 (94.3)	32 (80.0)	
SAGE			
Positive	1 (2.9)	6 (15.0)	0.11
Negative	34 (97.1)	34 (85.0)	

CTAs, cancer-testis antigens. RCC, renal cell carcinoma; HNC, head and neck cancer.

CTA expression is detected using several methods, including immunohistochemistry, RT-PCR and real-time RT-PCR. Among these methods, real-time RT-PCR is able to quantify the RNA expression of the targeted protein, contributing to a highly sensitive outcome.

In the present study, the antigens MAGE-A4, NY-ESO-1 and SAGE demonstrated a low incidence of expression in RCC, but a comparably high incidence of expression in HNC, when assessed with the real-time RT-PCR method.

In previous studies on CTA expression in RCC cases, MAGE-A4 (previously denoted as MAGE4), a member of the MAGEA gene family, was expressed in 30% (15/50) of the RCC cases (19), while MAGE1 or 2 demonstrated a comparably low incidence of expression based on RT-PCR assay (19,20). However, additional studies showed no expression of MAGE-A4 in 8 RCC cells using RT-PCR (22). MAGE-A4 expression was detected in these cells when they were treated with 5-Aza-CdR. Immunohistochemistry using the monoclonal antibody 57B that reacts with MAGE-A4 and other members of the MAGE family, including MAGE-A1, 3, 6 and 12, was reported with 0% (0/9) staining with RCC tissues (21). In this study, none of the 35 RCC cases was found to express MAGE-A4 following utilization of specific and quantitative assessment with real-time RT-PCR.

In previous studies, NY-ESO-1 mRNA expression was not detected by RT-PCR in 37 (20), 6 (24) and 10 (12) RCC cases. Our results also suggested a significantly low incidence of NY-ESO-1 expression (5.7%, 2/35).

To the best of our knowledge, no previous study has evaluated the expression of SAGE in RCC. Our results showed a low incidence of SAGE expression in RCC samples.

Concerning HNC, the reported incidence of MAGE-A4 expression was 60.2-70% using immunofluorescence (25,26) and 60% using RT-PCR (27). A similar incidence of 42.5% (17/40) was found in this study.

NY-ESO-1 was expressed in 9.5-33% of HNC samples using immunofluorescence (25,26) and 6-6.7% using RT-PCR (26,28). Our results indicated that 20% of the 40 HNC cases expressed NY-ESO-1. SAGE was expressed in 27% of the

cases using RT-PCR (28), and in 15% of the HNC cases using real-time RT-PCR

A discrepancy related to CTA expression existed, depending on the method of evaluation (23). In general, immunohistochemical analysis suggested relatively high CTA expression levels compared to RT-PCR (27). This discrepancy may have occurred due to uncoupling of transcription with RT-PCR on the different tissue sections (23). By contrast, the possibility of non-specific binding in immunohistochemical studies cannot be excluded.

To select the tumor types that are optimal candidates for a cancer vaccine based on actual CTA expression, real-time RT-PCR in several sections of each specimen and several evaluation methods, including immunohistochemistry, should be used on the same samples. The immunological response to a cancer vaccine should also be evaluated, depending on the results of CTA expression obtained following the use of various methods.

The meaning of the CTA expression in these tumors, aside from its use in the strategy of designing a cancer vaccine, needs to be further investigated. The correlation between CTA expression and survival or staging has been previously evaluated. Recent studies have indicated that solitary CTA expression was associated with a higher risk of poor outcomes in HNC patients (27). Findings of another study demonstrated NY-ESO-1-positive cases to have a good prognosis, as opposed to MAGE-A-positive cases (26). According to these studies, the correlation between CTA expression and prognosis is controversial, since it depends on the specific CTA assessed. The co-expression of CTA is believed to be based on the order of tumorigenesis (23) or the amount of demethylation, related to the cancer type (29). A high incidence of simultaneous expression of CTA was observed in HNC, and simultaneous expression of CTA tended to correlate with advanced tumor stage, but not survival (25). However, it was difficult to determine a correlation between solitary CTA expression or co-expression with stage or survival, due to the low incidence of CTA expression itself in RCC cases. In the future, the correlation of solitary expression or co-expression of CTA associated with prognosis should be evaluated for various CTAs.

CTA expression was also evaluated as a diagnostic tool, focusing on RCC and differential diagnosis between chromophobe RCC and oncocytoma. The incidence of MAGE-A3/4 expression was 88.2% (15/17) for oncocytoma and 38.9% (7/18) for chromophobe RCC. In addition, the incidence of NY-ESO-1 expression was 88.2% (15/17) for oncocytoma and 33.3% (6/18) for chromophobe RCC (30). In this study, although a small number of cases was evaluated, NY-ESO-1 expression was demonstrated in 33.3% (1/3) of the chromophobe cases (data not shown), a fact which was in agreement with previous studies (30). We evaluated CTA expression in other types of RCC to determine its value for predicting poor prognosis or malignancy, including distinguishing between chromophobe RCC and oncocytoma.

Since quantitative real-time RT-PCR provided stable results compared to the methods used previously, our screening method should be useful as the starting point of designing immunotherapy strategies. The results of this study strongly suggest the need to explore additional antigens that are

expressed in the same frequency and tumor-specific manner in RCC, for the development of effective immunotherapy of this type of malignancy.

In conclusion, the results of this study have shown that with quantitative real-time PCR, NY-ESO-1, MAGE-A4 and SAGE are expressed at a significantly low frequency in RCC patients. Screening to detect additional CTAs should be continued in the future to develop targeted immunotherapeutic strategies for cancer vaccines in RCC.

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T-cell receptor gene therapy targeting melanoma-associated antigen-A4 inhibits human tumor growth in non-obese diabetic/SCID/ γ c^{null} mice

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Adoptive cell therapy with lymphocytes that have been genetically engineered to express tumor-reactive T-cell receptors (TCR) is a promising approach for cancer immunotherapy. We have been exploring the development of TCR gene therapy targeting cancer/testis antigens, including melanoma-associated antigen (MAGE) family antigens, that are ideal targets for adoptive T-cell therapy. The efficacy of TCR gene therapy targeting MAGE family antigens, however, has not yet been evaluated *in vivo*. Here, we demonstrate the *in vivo* antitumor activity in immunodeficient non-obese diabetic/SCID/ γ c^{null} (NOG) mice of human lymphocytes genetically engineered to express TCR specific for the MAGE-A4 antigen. Polyclonal T cells derived from human peripheral blood mononuclear cells were transduced with the $\alpha\beta$ TCR genes specific for MAGE-A4, then adoptively transferred into NOG mice inoculated with MAGE-A4 expressing human tumor cell lines. The transferred T cells maintained their effector function *in vivo*, infiltrated into tumors, and inhibited tumor growth in an antigen-specific manner. The combination of adoptive cell therapy with antigen peptide vaccination enhanced antitumor activity, with improved multifunctionality of the transferred cells. These data suggest that TCR gene therapy with MAGE-A4-specific TCR is a promising strategy to treat patients with MAGE-A4-expressing tumors; in addition, the acquisition of multifunctionality *in vivo* is an important factor to predict the quality of the T-cell response during adoptive therapy with human lymphocytes. (*Cancer Sci* 2012; 103: 17–25)

T-cell receptor (TCR) gene transfer using retroviral vectors has been shown to be an attractive strategy to redirect the antigen specificity of polyclonal T cells to create tumor- or pathogen-specific lymphocytes.^(1–6) This approach is a promising method for the treatment of patients with malignancies that might overcome the limitations of current adoptive T-cell therapies that have been hampered by difficulties in the isolation and expansion of pre-existing, antigen-specific lymphocytes in patients.^(7–10) For the treatment of metastatic melanoma, clinical trials using autologous lymphocytes that have been retrovirally transduced with melanoma/melanocyte antigen-specific TCR have reported objective cancer regression.^(11,12) These reports suggest that adoptive cell therapy using TCR gene-modified lymphocytes is a promising approach to immunotherapy in cancer patients; such reports have encouraged the development of novel TCR gene therapy-based approaches.

On-target adverse events, however, have been reported for TCR gene therapies targeting melanocyte differentiation antigens, such as melanoma antigen recognized by T-cells (MART)-1 or gp100. Normal tissues in which melanocytic cells exist, such as the skin, eyes, and inner ears, exhibited severe histological destruction, especially when high-avidity TCR were used.⁽¹²⁾ Gene-modified T cells targeting carcinoembryonic antigen also

induced a severe transient inflammatory colitis that served as a dose-limiting toxicity for all three patients enrolled.⁽¹³⁾ Case reports exploring the severe adverse events seen in patients receiving T cells transduced with chimeric antigen receptors bearing the variable regions of human epidermal growth factor receptor type 2 (HER2)/neu- or CD19-reactive antibodies have suggested that these adverse events might be related to the release of cytokines from transferred cells.^(14,15) These observations highlight the potential risk in the usage of receptor genes that render T cells reactive to both tumor cells and a subset of normal cells.

Cancer/testis antigens are particularly attractive targets for immunotherapy, because of their unique expression profiles. While these antigens are highly expressed on adult male germ cells or placenta, they are typically completely absent from other normal adult tissues, and demonstrate aberrant expression in a variety of malignant neoplasms.^(16,17) As adult male germ cells do not express MHC class I, CD8⁺ effector cells theoretically ignore these cells.⁽¹⁸⁾ MAGE-A, -B, and -C genes exhibit such an expression pattern, and their immunogenicity as targets for cancer immunotherapy has been well studied.^(19–21) MAGE-A4 expression was reported in 56.6% of serous carcinoma of the ovary, 61.4% of melanoma, 28.4% of non-small cell lung carcinoma, 20% of hepatocellular carcinoma, 22.3% of colorectal carcinoma, 90.2% of esophageal squamous cell carcinoma, and 6.7% of esophageal adenocarcinoma.^(22–28) These results suggest that TCR gene therapy targeting the MAGE family of antigens, including MAGE-A4, represents a promising treatment for malignancies that minimizes the risk of severe on-target toxicity. The feasibility of TCR gene therapy targeting MAGE family antigens *in vivo*, however, has not previously been evaluated.

In the present study, we isolated rearranged *TCR $\alpha\beta$* genes from a human CD8⁺ T-cell clone that recognizes a MAGE-A4-derived peptide, MAGE-A4_{143–151}, in the context of HLA-A*2402.⁽²⁹⁾ Polyclonal human lymphocytes that were retrovirally transduced with these TCR genes demonstrated stable transgene expression and specific cytotoxicity against MAGE-A4-expressing tumor cells *in vitro*.^(30,31) These results prompted us to confirm the efficacy of the TCR gene-modified T cells *in vivo* prior to clinical evaluation.

In this study, we investigated if human lymphocytes genetically engineered to express this MAGE-A4-specific TCR could inhibit the growth of MAGE-A4-expressing tumors when adoptively transferred into immunodeficient non-obese diabetic/SCID/ γ c^{null} (NOG) mice. We evaluated the *in vivo* function of the transferred cells, as well as their migration to the tumor

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site, and the resultant antitumor effect. We addressed if the combination of adoptive cell therapy and vaccination with peptide antigen could influence the antitumor activity of transferred cells.

Materials and Methods

Peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors who provided informed consent. Peripheral blood mononuclear cells were cultured in GT-T503 media (Takara Bio, Otsu, Japan) supplemented with 1% autologous plasma, 0.2% human serum albumin (HSA; Sigma-Aldrich, St. Louis, MO, USA), 2.5 mg/mL fungizone (Bristol-Myers Squibb, New York, NY, USA), and 600 IU/mL interleukin-2. This study was approved by the ethics review committees of Mie University Graduate School of Medicine (Tsu, Japan) and Takara Bio.

Mice. Studies were conducted using 8-week-old female NOG mice (Central Institute for Experimental Animals, Kawasaki, Japan) that had been established as described previously.⁽³²⁾ Mice were maintained at the Animal Center of Mie University Graduate School of Medicine. All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation (of Mie University Graduate School of Medicine).

Cell lines. The KE4 (MAGE-A4⁺HLA-A*2402⁺ human esophageal carcinoma), QG56 (MAGE-A4⁺HLA-A*2402⁻ human lung carcinoma), and T2-A*2402 (human T, B hybridoma transfected with HLA-A*2402 cDNA)⁽²⁹⁾ cell lines were maintained in RPMI-1640 media (Sigma-Aldrich) supplemented with 10% FCS, penicillin (100 U/mL), and streptomycin (100 mg/mL).

Retroviral transduction. A retroviral vector encoding MAGE-A4-specific *TCRα* (*TRAV8-1*) and *TCRβ* (*TRBV7-9*) genes (MS-bPa retroviral vector) was described previously.⁽³⁰⁾ Peripheral blood mononuclear cells were stimulated with 30 ng/mL OKT-3 (Janssen Pharmaceutical, Titusville, NJ, USA) and 600 IU/mL interleukin-2 prior to transduction with MS-bPa particles. Briefly, retroviral solutions were preloaded onto RetroNectin-coated plates and centrifuged at 2000g for 2 h, then rinsed with PBS, according to the RetroNectin (Takara Bio)-bound virus infection method. Cells were then applied onto preloaded plates; PBMC transduced with the MS-bPa retroviral vector were designated as gene-modified cells. Control PBMC were treated similarly, except that MS-bPa was omitted from the cultures; these specimens were designated as unmodified cells.

Tumor challenge. KE4 tumor cells (2.5×10^6 in 0.2 mL PBS) were subcutaneously inoculated into the right flanks of mice. In the indicated experiments, QG56 tumor cells (2.5×10^6 in 0.2 mL PBS) were subcutaneously inoculated in a similar manner. Tumor size was determined by the product of perpendicular diameters measured with calipers. The mice were killed before the mean diameter of the tumor reached 20 mm, according to institutional guidelines. The statistical significance of the difference between groups in tumor growth was evaluated at the last time point.

Adoptive cell transfer. After two washes in saline containing 1% human serum albumin (HSA), gene-modified or unmodified cells (1×10^8) were suspended in 0.3 mL saline and intravenously injected into a lateral tail vein of the NOG mice. Prior to injection, gene-modified cells were analyzed for staining with MAGE-A4₁₄₃₋₁₅₁/HLA-A*2402 tetramer and antihuman CD8 mAb to calculate the proportion of tetramer⁺CD8⁺ T cells infused. When indicated, HLA-A*2402-positive PBMC were pulsed with 1 μM MAGE-A4₁₄₁₋₁₅₃ peptide and co-administered intravenously as a peptide vaccination.

In vitro stimulation and staining of cells. Cells were incubated for 2 h at 37°C with irradiated (45 Gy) stimulator T2-A*2402 cells, which had been pulsed with 1 μM MAGE-A4₁₄₁₋₁₅₃ or HER2₆₃₋₇₁ (an irrelevant peptide with HLA-A*2402 binding

activity) peptide, at an effector/stimulator ratio of four in the presence of 0.1 mg/mL phycoerythrin (PE)-conjugated anti-CD107a (BD Bioscience, San Diego, CA, USA). We then incubated samples for an additional 6 h in 1 mL GolgiStop (BD Bioscience). The cells were then stained with FITC-conjugated anti-CD8 (BD Bioscience) mAb. After permeabilization and fixation using a Cytotfix/Cytoperm kit (BD Bioscience) according to the manufacturer's instructions, the cells were stained intracellularly with allophycocyanin (APC)-conjugated anti-γ-interferon (IFN-γ) (BD Bioscience) and PE-Cy7-conjugated antitumor necrosis factor (TNF) (BD Bioscience) mAb.

Flow cytometric analysis. PE-conjugated MAGE-A4₁₄₃₋₁₅₁/HLA-A*2402 tetramer (provided by the Ludwig Institute for Cancer Research, New York, NY, USA) and FITC-conjugated antihuman CD4 (BD Bioscience), human CD8 (BD Bioscience), and PerCP-Cy5.5-conjugated antihuman CD3 (BD Bioscience) mAb were used to detect transduced TCR in specific cell populations. Polychromatic analyses were performed as previously described.⁽³³⁾ Cell staining data were acquired using a FACS CantoII flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and analyzed using FACSDiva (Becton Dickinson) and FlowJ (Tree Star, Ashland, OR, USA) software.

Immunohistochemical analysis. Formalin-fixed and paraffin-embedded specimens were used. After deparaffinization, tissue sections were pretreated with antigen retrieval solution (DAKO high pH solution, DAKO, Glostrup, Denmark) at 95°C for 20 min. As a primary antibody, antihuman CD8 (clone C8/144B; DAKO) was used. Dextran polymer method with EnVision plus (DAKO) was adopted for secondary detection. 3,3'-Diaminobenzidine was used as chromogen, and hematoxylin counterstain was performed. Infiltrated CD8-positive tumor-infiltrating lymphocytes (TIL) were counted in the selected 10 independent areas with most abundant TIL infiltration. Tumor-infiltrated, CD8-positive cells per high power field (0.0625 mm²) were counted using an ocular grid at ×400 magnification. Three independent counts were performed by a board-certified pathologist (E.S) with no knowledge of the earlier results. The average TIL counts of 10 fields was used for the statistical analyses.

Statistical analyses. Data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student's *t*-test. A *P*-value less than 0.01 denoted a statistically-significant difference.

Results

Adoptive transfer of MAGE-A4-specific, TCR-transduced lymphocytes inhibits tumor progression in a dose-dependent and antigen-specific manner. We previously reported the successful retroviral transduction of *TCRαβ* genes recognizing the MAGE-A4₁₄₃₋₁₅₁ peptide in an HLA-A*2402-restricted manner into polyclonally-activated human CD8⁺ T cells. The *TCRαβ*-transduced CD8⁺ T cells exhibited IFN-γ production and cytotoxic activity against both peptide-loaded T2-A*2402 cells and human tumor cell lines, such as KE4, that express both MAGE-A4 and HLA-A*2402.⁽³⁰⁾ To confirm the efficacy of these gene-modified T cells *in vivo* prior to clinical evaluation, we examined the antitumor efficacy of adoptive cell therapy with MAGE-A4-specific *TCR* gene-modified lymphocytes into NOG mice. We anticipated that a clinical trial to evaluate this therapy would involve the transduction of polyclonally-activated PBMC with *TCR* genes, followed by the transfer of these cells into patients without purification of the CD8⁺ T-cell subset. To mimic these conditions, the NOG mice received *TCR* gene-modified lymphocytes without further purification. The *TCR* gene-modified and unmodified cells used for the transfer experiments were stained with anti-CD8 mAb and a MAGE-A4₁₄₃₋₁₅₁/HLA-A*2402 tetramer that specifically detected the transduced TCR (Fig. 1A). As we reported previously, this TCR bound the tetramer in a

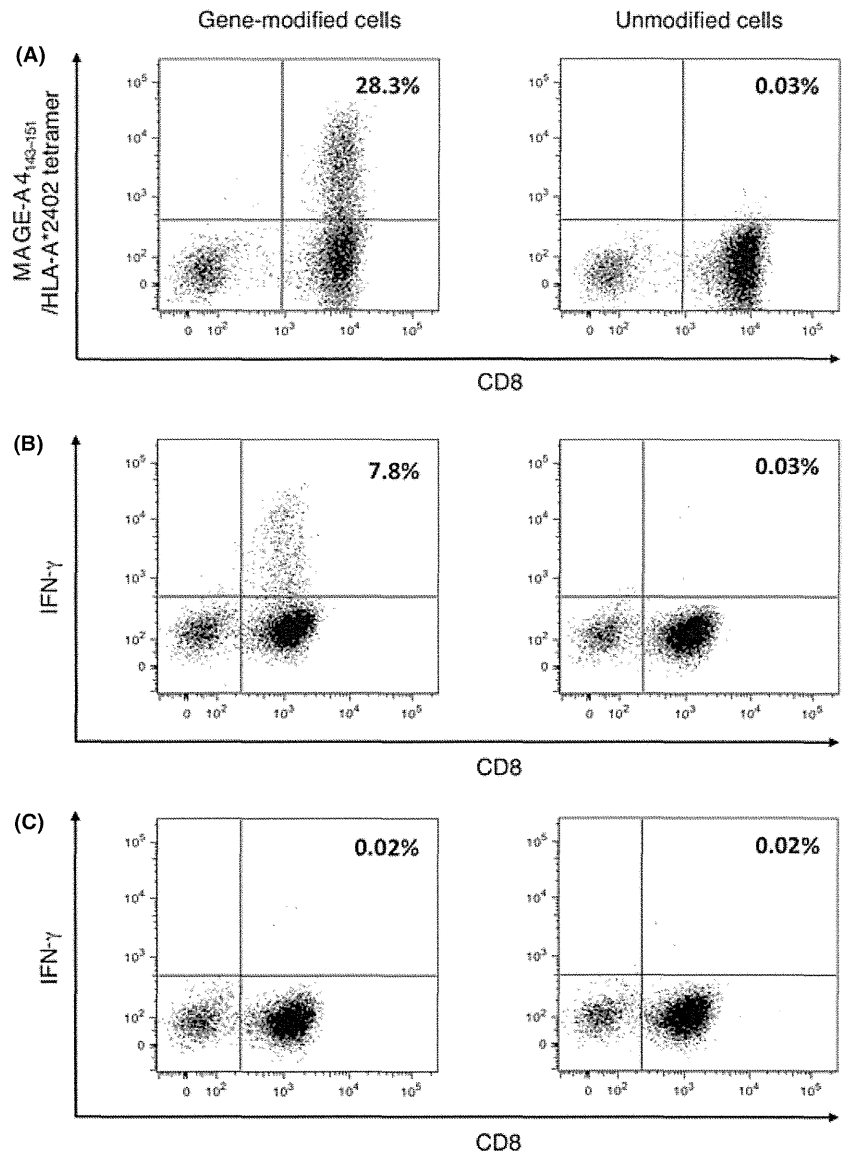


Fig. 1. Transduction of melanoma-associated antigen (MAGE)-A4-specific T-cell receptor (TCR) in human lymphocytes. Peripheral blood mononuclear cells from healthy donors were stimulated with anti-CD3 mAb and interleukin-2. Cells were cultured with or without retroviral vector encoding MAGE-A4-specific TCR, designated gene-modified or unmodified cells, respectively. (A) Representative staining for gene-modified and unmodified cells with MAGE-A4₁₄₃₋₁₅₁/HLA-A*2402 tetramer and antihuman CD8 mAb are shown. (B,C) Gene-modified and unmodified cells were stimulated with T2-A*2402 cells pulsed with the MAGE-A4₁₄₃₋₁₅₁ peptide (B) or HLA-A*2402-binding irrelevant control peptide (C). Representative specific intracellular interferon (IFN)- γ staining is displayed. Numerical value indicates the percentage of the tetramer⁺ cells or IFN- γ ⁺ cells among CD8⁺ cells.

CD8 molecule-dependent manner.⁽³⁴⁾ These T cells were tested for specific reactivity against antigen peptide presented on HLA-A*2402 (Fig. 1B,C).

Before transfer, we stained the cells with the MAGE-A4₁₄₃₋₁₅₁/HLA-A*2402 tetramer to calculate the number of tetramer⁺CD8⁺ cells. The growth of implanted MAGE-A4⁺HLA-A*2402⁺ KE4 tumor cells was significantly inhibited when 9×10^6 of tetramer⁺CD8⁺ cells were intravenously injected into NOG mice on day 0 (Fig. 2A). The inhibition of KE4 growth required specific recognition of the MAGE-A4₁₄₁₋₁₅₃/HLA-A*2402 complex by the TCR, because unmodified cells derived from the same donor did not alter KE4 growth. In this experiment, 1×10^8 gene-modified or unmodified lymphocytes derived from the same donor were administered to mice. Although the CD4/CD8 ratio of the *in vitro* expanded lymphocytes depends on the donor, gene-modified and unmodified cells derived from the same donor demonstrated similar phenotypes, determined by the expression of cell surface markers, including CD3, CD4, CD8, CD45RA, CD45RO, CD62L, CCR7, CD152, CD25, CD27, and CD28 (data not shown). The growth of the QG56 tumors, which expressed MAGE-A4, but lacked HLA-A*2402, was indistinguishable in mice receiving

either gene-modified or unmodified cells (Fig. 2D). Only a modest inhibition of KE4 growth was seen when mice received only 3×10^6 of tetramer⁺CD8⁺ cells (Fig. 2B), while no effect was seen upon administration of 1×10^6 of tetramer⁺CD8⁺ cells (Fig. 2C).

We addressed the effect of the adoptive transfer of the gene-modified cells into the mice with established tumors. We adoptively transferred TCR-engineered T cells into NOG mice that were inoculated with KE4 tumor cells 3 days earlier. On the day of adoptive T-cell transfer, we observed the establishment of a KE4 tumor mass in the mice. As shown in Figure 2(E), the administration of gene-modified cells significantly inhibited the growth of KE4 tumors, although the effect was limited and appeared later compared to the treatment on day 0. Taken together, the adoptive transfer of MAGE-A4-specific TCR gene-modified lymphocytes inhibited human tumor growth in NOG mice in a dose-dependent and TCR-specific manner.

Adoptively-transferred human lymphocytes persist in NOG mice. We monitored the persistence of transferred human lymphocytes in peripheral blood by staining Ficoll-purified PBMC from NOG mice with mAb specific for human CD8 and CD4.

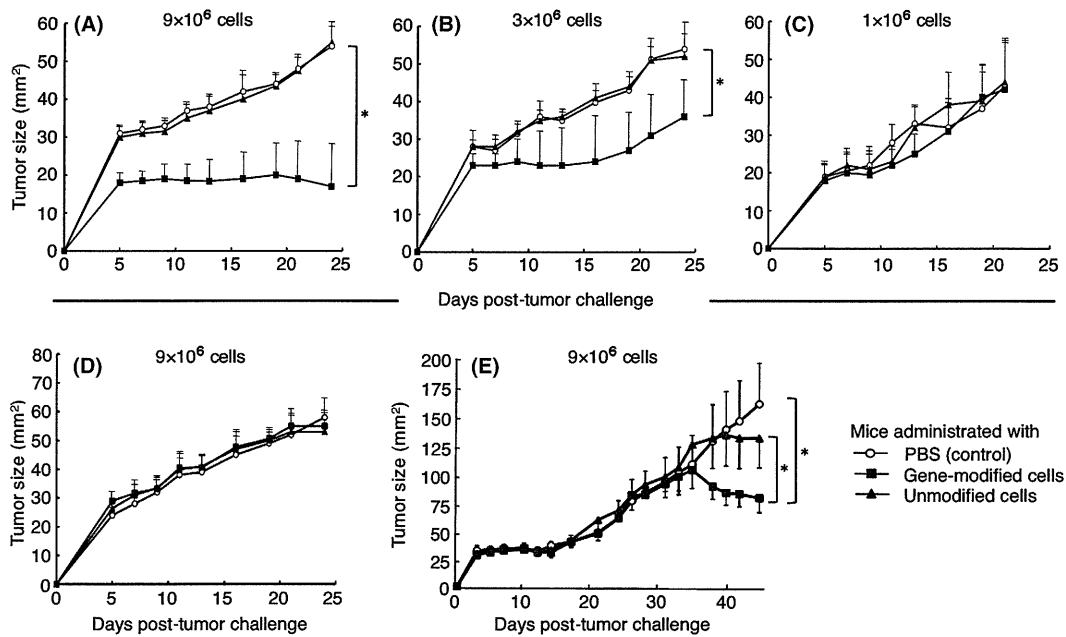


Fig. 2. Adoptive transfer of lymphocytes genetically engineered to express MAGE-A4-specific T-cell receptor inhibits human tumor progression in non-obese diabetic/SCID/ γ c^{null} mice. Non-obese diabetic/SCID/ γ c^{null} mice ($n = 4$ per group) were subcutaneously inoculated with 2.5×10^6 KE4 (A–C) or QG56 (D) tumor cells, and intravenously administered $\sim 1 \times 10^8$ gene-modified (■) or unmodified (▲) cells or PBS alone (control, ○) on day 0. Total of 9×10^6 (A,D), 3×10^6 (B), or 1×10^6 (C) tetramer⁺CD8⁺ cells were confirmed to be adoptively transferred; we subsequently monitored tumor growth over time. (E) Non-obese, diabetic/SCID/ γ c^{null} mice ($n = 4$ per group) received the treatment 3 days after the subcutaneous inoculation of 2.5×10^6 KE4. Total of 9×10^6 tetramer⁺CD8⁺ cells were transferred. Mean tumor size for each group is represented as the average + SD of four mice. Results are representative of three independent experiments. Differences between groups were examined for statistical significance using the Student's *t*-test. * $P < 0.01$. Numerical value indicates the number of tetramer⁺CD8⁺ cells administrated.

Human CD8⁺ T cells persisted in NOG mice for more than 40 days after transfer (Fig. 3A). The transferred human CD8⁺ cells comprised between 10% and 30% of the total peripheral mononuclear cells in NOG mice at almost all time points following transfer of 1×10^8 human lymphocytes. In these experiments, approximately 9×10^6 of the transferred 1×10^8 gene-modified cells were tetramer⁺CD8⁺. The percentage of specifically staining cells in the total peripheral mononuclear cell population was significantly less when mice received 5×10^7 human lymphocytes (Fig. 3B). There was no significant difference in transferred cell survival or percentages between mice receiving gene-modified and unmodified cells (Fig. 3A,B). Human CD4⁺ cells comprised less than 10% of all lymphocytes for the first 2 weeks following transfer, but a rapid increase in this population was evident after day 21 (Fig. 3C,D). This observation was consistent with reports suggesting that CD4⁺ T cells play a dominant role in the induction of graft-versus-host (GVH) reactions in hosts receiving transfusions.^(35,36) The NOG mice receiving human lymphocyte transfers demonstrated significant weight loss after day 21, a sign of GVH reactions (Fig. 3E).

Transferred TCR gene-modified T cells retain their ability to recognize specific antigens in NOG mice. Lymphocytes harvested from the peripheral blood of NOG mice administered TCR gene-modified lymphocytes were tested for their antigen-specific reactivity by intracellular cytokine staining with anti-IFN- γ mAb after incubation with peptide-loaded T2-A*2402 cells. Antigen-specific IFN- γ secretion was detectable by peripheral blood CD8⁺ cells isolated from mice throughout the 40-day period after adoptive transfer with either 1×10^8 (Fig. 4A) or 5×10^7 (Fig. 4B) gene-modified cells. No reactivity of these lymphocytes was seen against T2-A*2402 cells without loaded peptide (data not shown). Cells from mice that received unmodified lymphocytes did not demonstrate a specific response (Fig. 4A,B). These results indicate that

transferred TCR gene-modified cells remained functional *in vivo*, recognizing the MAGE-A4_{141–153} peptide in the context of HLA-A*2402. When 5×10^7 cells were transferred, these cells expanded more rapidly in the early phase compared to the group with 1×10^8 cells transferred. We speculate that the adoptive transfer of a lower number of antigen-specific T cells might induce these cells to expand more rapidly *in vivo* in the early expansion phase. At the later time points, more antigen-specific cells persisted in mice receiving 1×10^8 cells.

Intratumor infiltration of transferred human CD8⁺ T cells. To confirm the infiltration of transferred cells into tumor tissue, we examined implanted KE4 and QG56 tumors by immunohistochemical analysis. As antibodies specifically recognizing the transferred TCR (TCR α V8-1 or TCR β V7-9) are not available, we stained tumor specimens with a mAb against human CD8. Significant infiltration of human CD8⁺ cells was detectable in KE4 tumors harvested from mice as early as 2 weeks after the transfer of gene-modified cells (Fig. 5A,B). CD8⁺ cell infiltration in KE4 tumor specimens in the mice that received gene-modified cells was slightly better than in the mice that received unmodified lymphocytes. However, the difference was not statistically significant (Fig. 5A,B). A similar degree of infiltration was also observed in QG56 tumors. These data were consistent with previous reports analyzing the migration of tumor-specific T cells by two-photon laser microscopy that indicated tumor-specific T cells accumulate in both antigen-positive and -negative tumor tissues to comparable extents, but at different migratory velocities, according to tumor antigen expression.⁽³⁷⁾ The KE4 tumors in mice that did not receive human lymphocytes lacked any positive staining (Fig. 5B).

Combination of TCR gene therapy and peptide vaccine enhances antitumor efficacy. In animal models of adoptive cell therapy examining the effects against murine tumors with tumor-specific CD8⁺ T cells, *in vivo* vaccinations using agents, such as antigen-peptide or antigen-encoding viruses,

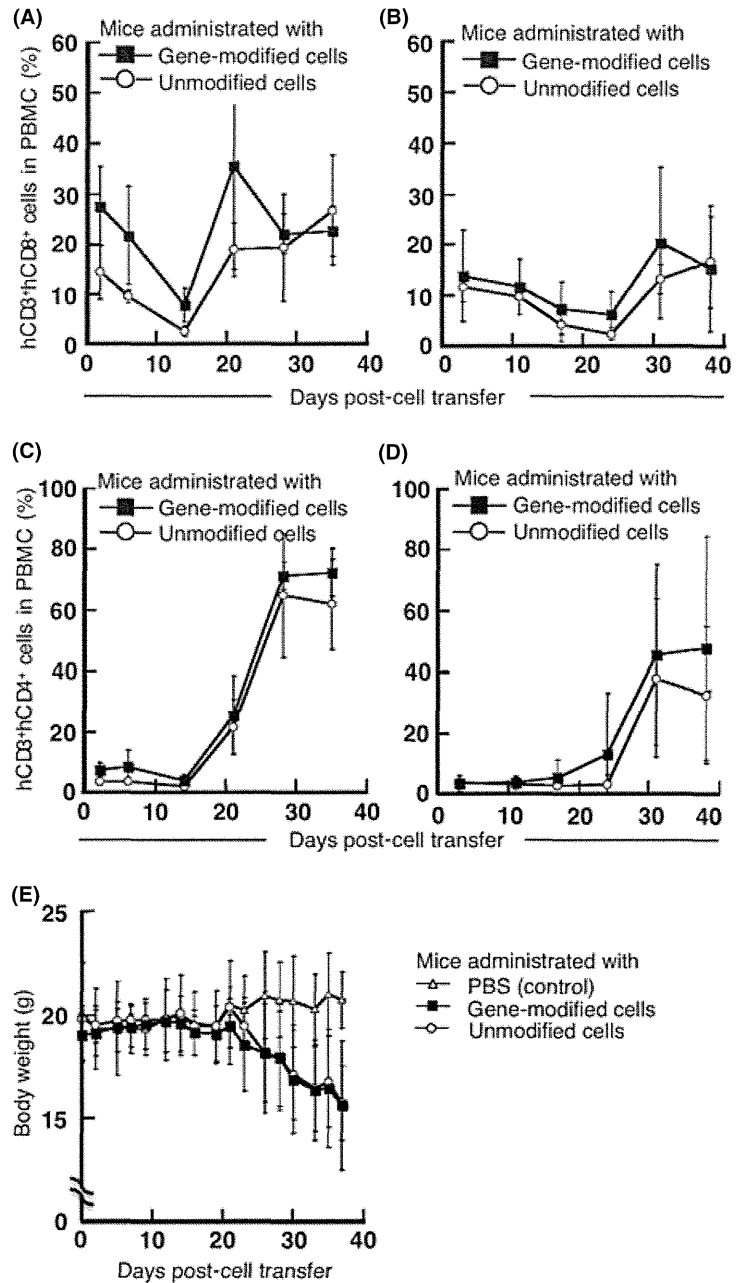


Fig. 3. Persistence of adoptively transferred human lymphocytes in non-obese, diabetic/SCID/ γc^{null} (NOG) mice. Non-obese, diabetic/SCID/ γc^{null} mice ($n = 4$ per group) were subcutaneously inoculated with 2.5×10^6 KE4 tumor cells, then intravenously administered 1×10^8 (A,C) or 5×10^7 (B,D) gene-modified (■) or unmodified (○) cells on day 0. Mononuclear cells were purified from peripheral blood collected from mice on the indicated days. We evaluated the proportion of human CD3⁺CD8⁺ (A,B) or CD3⁺CD4⁺ (C,D) cells among the mononuclear cell population. (E) We also monitored the body weight of NOG mice administered 1×10^8 gene-modified (■) or unmodified (○) cells or PBS (control, △) over time. Results are representative of three independent experiments. PBMC, peripheral blood mononuclear cells.

can increase the antitumor efficacy of adoptive cell therapy.^(9,38) Therefore, we explored if a peptide vaccination in conjunction with TCR gene-modified cell transfer could increase the inhibition of tumor growth seen in this model. As the administration of 1×10^6 tetramer⁺CD8⁺ cells alone was incapable of inducing tumor growth inhibition in this model (Fig. 2C), we examined if the combination of an *in vivo* peptide vaccination with cell transfer under these conditions could enhance tumor inhibition. As NOG mice do not possess endogenous antigen-presenting cells capable of presenting peptide in an HLA-A*2402-restricted manner, we used HLA-A*2402-positive human PBMC pulsed with the MAGE-A4₁₄₃₋₁₅₁ peptide. Tumor-inoculated NOG mice receiving gene-modified cells were also administered peptide-loaded HLA-A*2402-positive PBMC derived from the same donor on days 2 and 8 of the tumor challenge. KE4 tumor growth was significantly inhibited in the mice receiving a

combination of cell therapy and peptide vaccination in comparison to mice treated by cell therapy alone (Fig. 6A). The peptide vaccination did not alter KE4 growth when combined with the transfer of unmodified cells. The growth of the HLA-A*2402-negative QG56 tumor was identical in both groups (Fig. 6B).

Increased multifunctionality in adoptively-transferred cells when inoculated with peptide vaccine. We previously reported that the multifunctionality of effector cytotoxic T cells (CTL) is a critical determinant of the quality of the T-cell response and the resultant immunological control of tumor.^(33,39) We therefore compared the multifunctionality of transferred cells from NOG mice treated with TCR gene-modified cells and peptide vaccination with that from mice treated by TCR gene cell therapy alone. We assessed IFN- γ and TNF- α production and CD107a mobilization by CD8⁺ T cells at the single-cell level in specimens harvested from mice on days 2, 7, and 14 after transfer. We

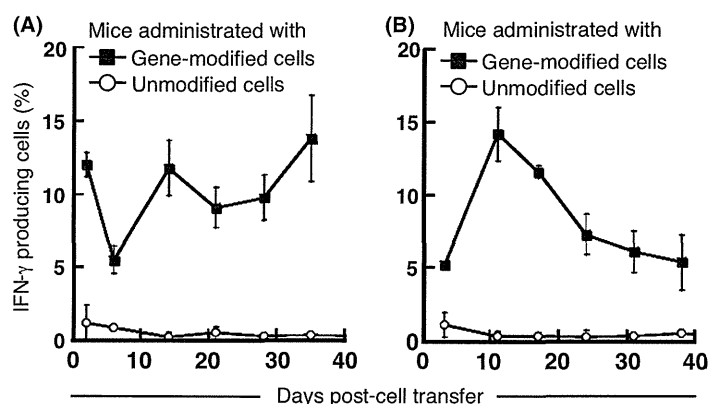


Fig. 4. Lymphocytes genetically engineered to express MAGE-A4-specific T-cell receptor-maintained specific reactivity after *in vivo* passage. Non-obese, diabetic/SCID/ γc^{null} mice ($n = 4$ per group) were subcutaneously inoculated with 2.5×10^6 KE4 tumor cells, then intravenously administered 1×10^8 (A) or 5×10^7 (B) gene-modified (■) or unmodified (○) cells on day 0. Mononuclear cells were purified from peripheral blood collected from mice on the indicated days. Intracellular γ -interferon (IFN- γ) production by these cells was assessed after being stimulated with $1 \mu M$ MAGE-A4₁₄₁₋₁₅₃ peptide for 6 h. Data are shown as the percentage of IFN- γ -producing cells within the total human CD8⁺ cell population. Results are representative of three independent experiments.

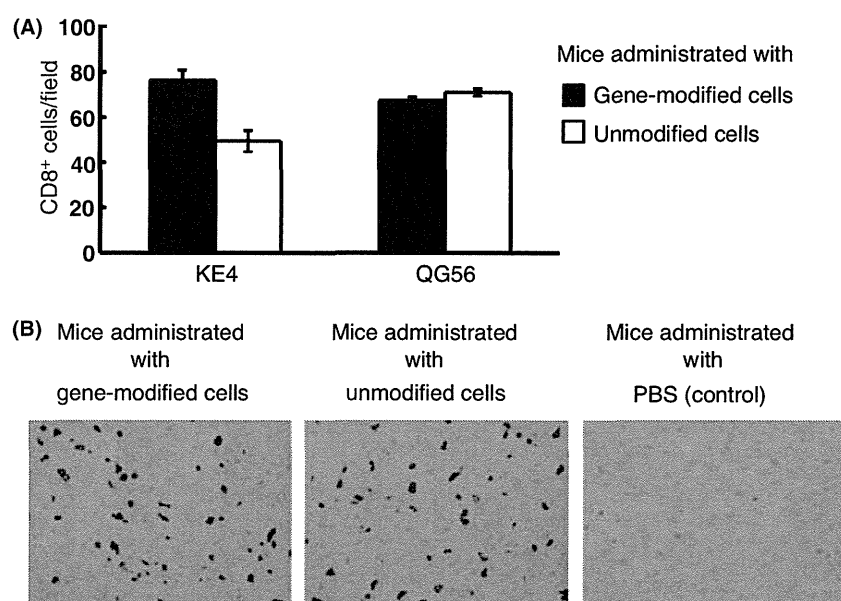


Fig. 5. Adoptively-transferred human CD8⁺ T cells infiltrate into tumor tissues. Tumor specimens were harvested from non-obese, diabetic/SCID/ γc^{null} mice 14 days after subcutaneous inoculation with 2.5×10^6 KE4 or QG56 tumor cells, and intravenous administration of 1×10^8 gene-modified or unmodified cells or PBS (control). We stained formalin-embedded tumor specimens with an antihuman CD8 monoclonal antibody, clone C8/144B. Average CD8⁺ TIL counts \pm SD in KE4 or QG56 (A) and the representative images from KE4 tissue sections (B) are shown.

selected these functional measures because multifunctionality assessed by these factors defines a sensitive correlate of the immunological control of tumors.^(33,39)

The mice received human lymphocytes with or without peptide vaccination; isolated peripheral blood specimens were tested for their antigen-specific reactivity of component CD8⁺ T cells at the indicated time points. On day 2 or 7 after adoptive transfer, we were barely able to detect cells with two or three functions in mice receiving gene-modified cells without peptide vaccination (Fig. 7); cells with three functions comprised 3.7% of all CD8⁺ T cells, while bifunctional cells comprised 2.4% on day 14. In contrast, mice receiving combination therapy with gene-modified cells and peptide vaccination exhibited a population of cells with three and two functions of 1.4% and 2%

of the total CD8⁺ cells, respectively, as early as day 2. Therefore, multifunctional effector CD8⁺ T cells appear earlier in mice receiving combination therapy in comparison to those receiving cell therapy alone. On day 7, trifunctional and bifunctional cells in mice receiving combination therapy comprised 1.7% and 4.8% of all cells, respectively. The cells with three or two functions were retained as part of the peripheral mononuclear cell population in these animals on day 14.

Discussion

Successful clinical responses using adoptive cell therapy with tumor-reactive T cells in patients with advanced melanoma have encouraged the development of genetic engineering approaches

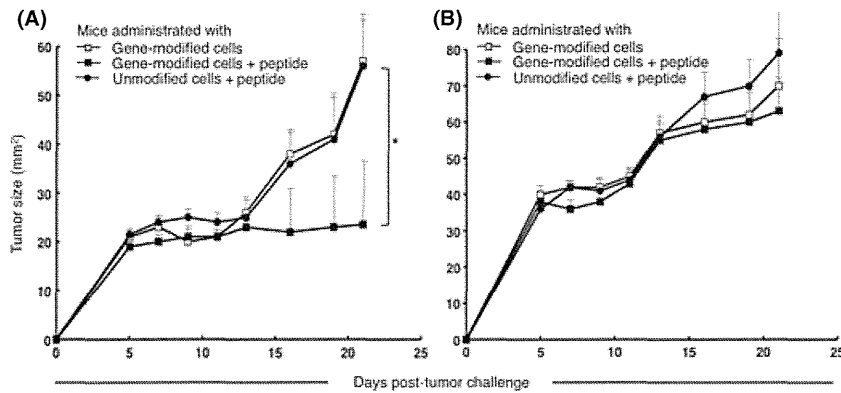


Fig. 6. Peptide vaccination enhanced the antitumor efficacy of adoptive therapy using T-cell receptor, gene-modified cells. Non-obese, diabetic/SCID/ γc^{null} mice ($n = 4$ per group) were subcutaneously inoculated with 2.5×10^6 KE4 (A) or QG56 (B) tumor cells, and intravenously administered 1×10^8 gene-modified (\square) or unmodified (\bullet) cells on day 0. Gene-modified population included 1×10^6 tetramer $^+$ CD8 $^+$ cells. We pulsed 4×10^7 peripheral blood mononuclear cells derived from the same donor (HLA-A*2402 positive) with $1 \mu M$ MAGE-A4₁₄₁₋₁₅₃ peptide, and intravenously administered these cells into the animals on days 1 and 8 (\blacksquare and \bullet). Results are representative of three independent experiments.

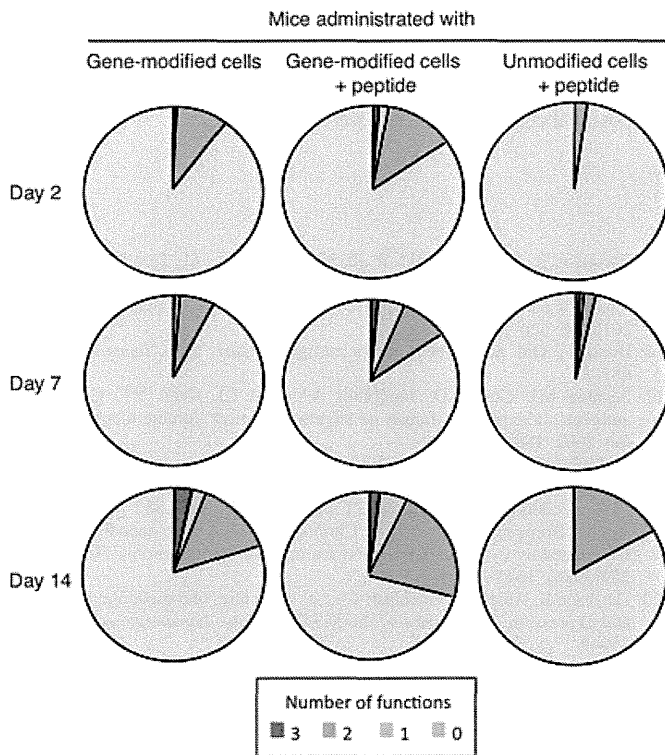


Fig. 7. Peptide vaccination increased the multifunctionality of transferred gene-modified cells. Mice were subcutaneously inoculated with 2.5×10^6 KE4 tumor cells, then intravenously administered 1×10^8 gene-modified or unmodified cells with or without peptide vaccination. Two, 7, and 14 days after transfer, we collected peripheral blood from mice. After purifying the mononuclear cells in these samples, we evaluated their multifunctionality by measuring γ -interferon (IFN- γ) and tumor necrosis factor- α (TNF- α) production and CD107a mobilization. Data are summarized in the pie chart, where each wedge represents the frequency of human CD8 $^+$ cells expressing all three functions (3), any two functions (2), a single function (1), or no function (0). Results are representative of three independent experiments.

using patient lymphocytes; these studies aim to extend the range of tumor types that can be treated with this technique and to improve the quality of the lymphocytes employed.⁽⁴⁰⁻⁴²⁾ In a

recent clinical trial for metastatic synovial cell sarcoma and melanoma, patients were administered autologous lymphocytes genetically engineered to express a high-avidity TCR against NY-ESO-1; objective clinical responses were observed in four (60%) of six patients with synovial cell sarcoma, and five (45%) of 11 patients with melanoma.⁽⁴³⁾ In this trial, the transferred TCR contained two amino-acid substitutions in the third complementary determining region of the native TCR α chain that conferred CD8 $^+$ T cells with an enhanced avidity. No on-target toxicities were seen in this trial, in contrast to previous observations of vigorous on-target toxicity in patients receiving lymphocytes engineered to express melanocyte differentiation antigen-specific TCR. Genetic engineering also offers the means to endow T cells with enhanced function, as well as resistance to tumor-mediated immunosuppression through the addition of genes encoding homeostatic or pro-inflammatory cytokines,^(44,45) chemokine receptors,⁽⁴⁶⁾ anti-apoptotic molecules,⁽⁴⁷⁾ and costimulatory molecules,^(48,49) as well as the silencing of co-inhibitory molecules,⁽⁵⁰⁾ although these modifications await clinical evaluation. As increased effector function and/or *in vivo* persistence of cells bearing these modifications might increase on-target toxicity during therapy, the selection of appropriate target antigens is critical to induce favorable anti-tumor effects and avoid severe adverse events.

The establishment of an animal model suitable for evaluating the *in vivo* efficacy and safety of human adoptive cell therapy is an important challenge to facilitate the development of these therapies and prevent toxicity. Non-obese diabetic/SCID/ γc^{null} -immunodeficient mice that lack T, B, and natural killer cells, and demonstrate impaired dendritic cell activity, are a helpful animal model to evaluate the *in vivo* activity of human hematopoietic cells.⁽³²⁾ The NOG mouse model, however, still has limitations, including a homeostatic expansion effect on infused T cells, an allo-reactive response between infused effector cells and transplanted target cells, and potential GVH reactions. In this study, mice receiving human lymphocytes exhibited severe weight loss, consistent with GVH reaction, which worsened after day 21. Therefore, antitumor efficacy in this model is best evaluated before day 21. Future studies will need to evaluate if the homeostatic proliferation of infused cells and/or a suboptimal allo-reactivity influenced the treatment effect seen in this model. The lack of an effect by unmodified cells (Fig. 2) and the increased efficacy upon co-administration of an antigen-peptide vaccine (Fig. 6), however, strongly suggest that the observed antitumor effect was achieved in a MAGE-A4-specific, TCR-mediated manner. The future devel-

opment of improved humanized mice will help to better evaluate the optimization of human immunotherapy.

Multifunctionality is the ability of T cells to exhibit multiple functions, including the simultaneous secretion of multiple cytokines, chemokines, or cytotoxic granules at the single-cell level.⁽⁵¹⁾ The importance of T-cell multifunctionality has been reported in multiple animal infection models^(52,53) and in humans infected with HIV, cytomegalovirus, hepatitis B virus, or tuberculosis.^(53–60) We reported the importance of effector T-cell multifunctionality in antitumor immune response. Specifically, the appearance of multifunctional CD8⁺ effector cytotoxic T cells *in vivo* is a critical determinant of effective immunological control of tumors. Regulatory T cells were found to play a role in the inhibition of transferred tumor antigen-specific T-cell multifunctionality.^(33,39) In the present study, effector T-cell multifunctionality appeared to correlate with the quality of T-cell responses in adoptive T-cell therapy utilizing genetically-engineered human lymphocytes (Figs 6,7). The peptide vaccination did not significantly change the percentage of human CD3⁺CD8⁺ cells in the PBMC of NOG mice (data not shown). The TCR-transduction efficiency in this study was not very high in general. We found that the combination of vaccination with the adoptive transfer of antigen-specific T cells increased effector T-cell multifunctionality and made the antitumor effect visible, even with a low number of specific TCR-transduced T cells transferred. The unmodified cells with background reactivity were the IFN- γ single producers. We speculate that these cells are positive for IFN- γ because of their non-specific activation due to GVH reaction.

To our knowledge, this study represents the first demonstration *in vivo* of an antitumor effect following the adoptive transfer of human lymphocytes genetically engineered to express a TCR specific for MAGE family antigen. The retroviral vector used in this report is currently under evaluation in a phase I clinical trial designed to treat patients with MAGE-A4-expressing esophageal cancer.

In summary, our data suggest that adoptive cell therapy with human lymphocytes engineered to express MAGE-A4-specific TCR through retroviral transduction is a promising strategy to treat patients with MAGE-A4-expressing tumors. Combination therapy with gene-modified cell-adoptive transfer and *in vivo* vaccination might improve antitumor efficacy, even with low numbers of transferred tumor-reactive T cells. These data support the rationale to explore clinical trials utilizing gene-modified lymphocytes prepared using the vector described in this report.

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Disclosure Statement

No potential conflicts of interest were disclosed.

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Intracellular Tumor-Associated Antigens Represent Effective Targets for Passive Immunotherapy

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Abstract

Monoclonal antibody (mAb) therapy against tumor antigens expressed on the tumor surface is associated with clinical benefit. However, many tumor antigens are intracellular molecules that generally would not be considered suitable targets for mAb therapy. In this study, we provide evidence challenging this view through an investigation of the efficacy of mAb directed against NY-ESO-1, a widely expressed immunogen in human tumors that is expressed intracellularly rather than on the surface of cells. On their own, NY-ESO-1 mAb could neither augment antigen-specific CD8⁺ T-cell induction nor cause tumor eradication. To facilitate mAb access to intracellular target molecules, we combined anti-NY-ESO-1 mAb with anticancer drugs to accentuate the release of intracellular NY-ESO-1 from dying tumor cells. Strikingly, combination therapy induced a strong antitumor effect that was accompanied by the development of NY-ESO-1-specific effector/memory CD8⁺ T cells that were not elicited by single treatments alone. The combinatorial effect was also associated with upregulation of maturation markers on dendritic cells, consistent with the organization of an effective antitumor T-cell response. Administration of Fc-depleted F(ab) mAb or combination treatment in Fcγ receptor-deficient host mice abolished the therapeutic effect. Together, our findings show that intracellular tumor antigens can be captured by mAbs and engaged in an efficient induction of CD8⁺ T-cell responses, greatly expanding the possible use of mAb for passive cancer immunotherapy. *Cancer Res*; 72(7): 1672–82. ©2012 AACR.

Introduction

With the molecular identification of tumor antigens recognized by the human immune system, antigen-specific immunotherapy for cancers has been developed and is explored in the clinic (1–3). Particularly, monoclonal antibodies (mAb) that recognize surface antigens, such as trastuzumab (anti-Her2/neu) and rituximab (anti-CD20), as a single agent or in combination with chemotherapy, are used in the clinic for

frontline or salvage therapy and have resulted in objective and durable clinical responses (3–5). One of the major therapeutic mechanisms of mAb is considered to be the selective interruption of vital signaling pathways in which the targeted antigens are critically involved (3, 5). In addition, there is accumulating evidence that mAb therapy also works through antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells or through the activation of complement, both of which depend on the Fc portion of the mAbs (6–9). Furthermore, Fc receptor-mediated uptake of immune complexes results in activation of antigen-presenting cells (APC) and facilitates cross-presentation of those antigens to tumor-specific CD8⁺ T cells and inhibition of tumor growth, as was shown recently in HER2/*neu* and melanoma differentiation antigen tyrosinase-related protein-1 (Trp1; gp75) models (10–13).

However, many well-characterized tumor-associated antigens, including cancer/testis (CT) antigens, are intracellular antigens and thus not accessible for antibodies (14–16). An exception is mAb TA99, which targets gp75 and was shown to induce NK and CD4⁺ T-cell-dependent antitumor responses *in vivo* (17). However, the fact that gp75 is expressed both on the cell surface and intracellularly makes it difficult to define the precise targets for the antitumor responses induced by mAb TA99 (12, 17).

NY-ESO-1, a CT antigen discovered by SEREX (serologic identification of antigens by recombinant expression cloning) using the serum of a patient with esophageal cancer, is frequently expressed in cancer cells of various tissue origins

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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but not in normal somatic cells except for germ cells in the testis (2, 18). Spontaneous cellular and humoral immune responses against NY-ESO-1 are found in patients with cancer, which underscores its immunogenicity (2, 18). It has an intracellular location and lacks cell surface expression (2, 18), thus curtails it from being a candidate of mAb therapy. Interestingly, NY-ESO-1 protein/IgG antibody complexes (immune complexes, IC) are efficiently cross-presented to the MHC class I pathway (19, 20) and there is a close correlation between antibody and CD8⁺ T-cell responses (2, 21), suggesting that NY-ESO-1-specific CD8⁺ T-cell induction by cross-priming *in vivo* is associated with the induction of specific antibodies. These data prompted us to analyze the possibility whether mAb therapy could be applied to an intracellular molecule NY-ESO-1 and inhibit tumor growth by enhancing CD8⁺ T-cell induction.

We have established syngeneic tumor models in BALB/c mice using CT26 colon carcinoma cells and CMS5a sarcoma cells that are stably transfected with NY-ESO-1 (22, 23). Using these models, we addressed whether NY-ESO-1 mAb combined with chemotherapy augmented NY-ESO-1-specific CD8⁺ T-cell induction and inhibited tumor growth.

Materials and Methods

Mice

Female BALB/c mice and BALB/c^{nu/nu} mice were obtained from SLC Japan or Jackson laboratory and used at 7 to 10 weeks of age. BALB/c mice deficient in the γ -chain subunit of Fc receptors were obtained from Taconic and used at 7 to 10 weeks of age. Mice were maintained in accordance with the NIH and American Association of Laboratory Animal Care Regulations. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Mie University Graduate School of Medicine (Mie, Japan) and by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee (New York, NY).

Tumors

CT26 is a colon epithelial tumor derived by intrarectal injections of *N*-nitroso-*N*-methylurethane in BALB/c mice (24). CT26 expressing NY-ESO-1 (CT26-NY-ESO-1) was established as described previously (23). CMS5a is a subcloned cell line obtained from CMS5 (25). CMS5a-NY-ESO-1 was established as described previously (22).

Antibodies and reagents

Anti-NY-ESO-1 mAbs [E978 (mouse IgG1) recognizing NY-ESO-1₇₁₋₉₀, ES121 (mouse IgG1) recognizing NY-ESO-1₉₁₋₁₁₀, 219-510 (mouse IgG1) recognizing NY-ESO-1₂₁₋₄₀ (Supplementary Fig. S3; ref. 26)]; anti-CD4 (GK1.5, rat IgG2b), anti-CD8 (19/178, mouse IgG2a), and anti-MAGE-A4 (MCV1, mouse IgG1) were purified from hybridoma supernatant by protein G affinity chromatography. The F(ab) fragment of E978 was generated using the ImmunoPure Fab Preparation Kit (Thermo Fisher Scientific). Anti-CD8 (53-6.7), anti-CD45RB (16A), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-CD40 (3/23), anti-IFN- γ (XMG1.2), anti-CD62L (MEL-14), anti-CD11c (HL3), anti-TNF- α (MP6-XT22), and antimouse IgG1 (A85-1) mAbs were purchased from BD Biosciences, Biolegend, or

eBioscience. Phycoerythrin (PE)-labeled NY-ESO-1₈₁₋₈₈-D^d tetramers were provided by Drs. P. Guillaume and I. Luescher (Ludwig Institute Core Facility, Lausanne, Switzerland). An anti-NY-ESO-1 human IgG1 mAb (12D7) was obtained from CT Atlantic. p63 (T) peptide TYLPTNASL (27), AH-1₁₃₈₋₁₄₇ peptide SPSYVHQF (28), and NY-ESO-1₈₁₋₈₈ peptide RGPESRLL (23) were purchased from Operon Biotechnologies and BioSynthesis and Sigma.

Chemotherapeutic agents

5-Fluorouracil (5-FU; Kyowa Hakko Kirin.), doxorubicin (Kyowa Hakko Kirin), CPT-11 (Yakult), and paclitaxel (Bristol-Myers Squibb) were injected intraperitoneally as indicated.

Tumor challenge

Mice were inoculated with 0.5×10^6 to 1×10^6 CT26-NY-ESO-1 cells, 1×10^6 CMS5a-NY-ESO-1, or 1×10^6 CT26-MAGE-A4 cells in the right hind flank subcutaneously. Mice were monitored 3 times a week and were sacrificed when tumors reached greater than 20 mm.

Staining and flow cytometry

To collect tumor-infiltrating T cells, tumors were minced and treated with 1 mg/mL of collagenase IA (Sigma) in Hanks' balanced salt solution (HBSS) for 90 minutes at room temperature.

Cells harvested from draining lymph node (dLN) and tumors were stained for surface markers in PBS with 0.5% FBS for 15 minutes at 4°C. For intracellular cytokine staining, 1×10^6 to 3×10^6 cells from tumors or dLNs were cultured with peptide for 5 hours at 37°C, and GolgiPlug was added for the last 4 hours of culture. These cells were stained for surface markers and intracellularly with allophycocyanin-conjugated anti-IFN- γ and PE-conjugated anti-TNF- α mAbs after permeabilization and fixation using Cytofix/Cytoperm Kit (BD Bioscience). Dead cells were excluded by LIVE/DEAD Fixable Dead Cell Stain Kits (Invitrogen). Cells were analyzed on FACSCanto or FACSCalibur (BD Bioscience) and FlowJo software (Tree Star).

Fluorescent immunohistochemistry

Three micrometers of tissue sections prepared from fresh-frozen tumor specimens were fixed with ice-chilled acetone for 15 minutes. Alexa 488-labeled antihuman IgG antibody (Invitrogen) was applied and incubated at room temperature for 2 hours. For double immunolabeling, sections were fixed with 3% paraformaldehyde for 15 minutes, incubated with anti-cleaved caspase-3 (Cell Signaling Technology) at room temperature for 2 hours and then incubated with Alexa 488-labeled anti-human IgG antibody and Alexa 568-labeled antirabbit IgG Ab (Invitrogen) at room temperature for 2 hours. Sections were rinsed with PBS, counterstained with 4',6-diamidino-2-phenylindole (DAPI), and mounted. Images were captured using $\times 40$ magnification objective by Zeiss Axiocam system (Carl Zeiss).

Statistical analysis

Tumor curves were assessed by one-way ANOVA with a Bonferroni multiple comparisons posttest. Single measurement

comparison between 2 groups was evaluated by 2-sided Student *t* test. *P* values <0.05 were considered statistically significant.

Results

Establishment of CT26-NY-ESO-1

We established a syngeneic colon carcinoma model (CT26-NY-ESO-1) with stable NY-ESO-1 expression (2, 22, 23). NY-ESO-1 expression in CT26-NY-ESO-1 cells was exclusively intracellular, and no NY-ESO-1 protein was detected on the cell surface (Supplementary Fig. S1A), consistent with the expression of NY-ESO-1 protein in human cancer cells (2). These CT26-NY-ESO-1 cells maintained the same tumor growth capacity as their parental CT26 cells in both wild-type Balb/c and C.B-17 SCID (severe combined immunodeficient) mice, indicating that there was no alteration of tumorigenicity caused by the NY-ESO-1 transfection (Supplementary Fig. S1B). When BALB/c mice were inoculated with CT26-NY-ESO-1 cells, spontaneous antibody and CD8⁺ T-cell responses were detected after 7 days and increased thereafter (Supplementary Fig. S1C and S1D). These spontaneous immune responses closely paralleled spontaneous NY-ESO-1-specific immune responses found in humans (2).

We used this tumor model to explore the antitumor effects of mAbs against NY-ESO-1 alone and in combination with an anticancer drug. To select anticancer drugs suitable for this model, we examined the antitumor capacity of several anticancer drugs (5-FU, CPT-11, paclitaxel, and doxorubicin) against CT26-NY-ESO-1. Of the 4 drugs, 5-FU exhibited a significant antitumor effect (Supplementary Fig. S2A). When CT26-NY-ESO-1 cells were cultured with 5-FU, NY-ESO-1 protein was released from CT26-NY-ESO-1 cells into the culture supernatant but not from parental CT26 cells (Supplementary Fig. S2B). On the basis of these data, we chose 5-FU for our further experiments.

Combination treatment with anti-NY-ESO-1 mAb and 5-FU results in augmented tumor growth inhibition

BALB/c mice were inoculated with CT26-NY-ESO-1 and were injected with 5-FU (75 mg/kg) and anti-NY-ESO-1 mAb (clone; E978, 100 µg, 2 days after 5-FU injection) when the tumor was palpable (around 25 mm²). Treatment was repeated after 1 week. The combination treatment with anti-NY-ESO-1 mAb and 5-FU exhibited a significantly augmented antitumor effect and longer survival compared with control mice or mice that had received either 5-FU or anti-NY-ESO-1 mAb alone (Fig. 1A and B). This augmented antitumor effect was also observed when another anti-NY-ESO-1 mAb (clone; ES121, 100 µg) was used, but not with a control mAb, against another immunogenic CT antigen MAGE-A4, which is not expressed in the CT26-NY-ESO-1 cells (Fig. 1C and D). In contrast, combination treatment with anti-MAGE-A4 mAb (clone; MCV1, 100 µg), but not control antibody and 5-FU, exhibited an augmented antitumor effect against CT26-MAGE-A4 (Fig. 1E). To show that the effect of this combination treatment is not limited to the CT26, we examined the antitumor effect using CMS5a fibrosarcoma cells. BALB/c mice were inoculated with CMS5a-NY-ESO-1 and were injected with doxorubicin (50 µL intratumoral

injection, 0.25 mmol/L) and anti-NY-ESO-1 mAb. As systemic administration of doxorubicin did not induce effective killing of CMS5a-NY-ESO-1, we used an intratumoral injection method. This combination treatment with anti-NY-ESO-1 mAb (but not an isotype control antibody) and doxorubicin exhibited a significantly augmented antitumor effect as well (Fig. 1F). These data suggest that the augmented antitumor effect is an antigen-specific phenomenon and that this combination treatment could be applicable to a broader range of intracellular antigens and tumors.

We next investigated whether a cocktail of 2 different anti-NY-ESO-1 mAbs (E978 50 µg and ES121 50 µg) that recognize 2 different nonoverlapping epitopes on the NY-ESO-1 protein (Supplementary Fig. S3) further augmented antitumor effects. We observed no additive antitumor effects when mice were treated with the combination of 2 different anti-NY-ESO-1 mAbs and 5-FU compared with mice treated with a single anti-NY-ESO-1 mAb and 5-FU (Fig. 1G).

Augmented tumor growth inhibition by combination treatment with anti-NY-ESO-1 mAb and 5-FU is dependent on CD8⁺ T cells

To gain insight into the cellular components involved in the augmented antitumor effects by the combination treatment, we initially examined the role of T cells using BALB/c^{nu/nu} mice. BALB/c^{nu/nu} mice were inoculated with CT26-NY-ESO-1 and combination treatment with 5-FU and anti-NY-ESO-1 mAb was initiated when the tumor was palpable. The augmented antitumor effect by the combination treatment in wild-type BALB/c mice was abrogated in BALB/c^{nu/nu} mice (Fig. 2A).

Given the critical role of T cells in this augmentation of antitumor effects, we next explored the outcome of CD4⁺/CD8⁺ T-cell depletion on the augmented antitumor effect. BALB/c mice bearing CT26-NY-ESO-1 tumors were injected with 5-FU and anti-NY-ESO-1 mAb and received anti-CD4 (days 7, 14, and 21) or anti-CD8 mAb (days 7 and 21). The depletion of CD8⁺ T cells totally abolished the augmented antitumor effects (Fig. 2B). In contrast, CD4⁺ T-cell depletion did not affect the augmented antitumor effects (Fig. 2B).

Combination treatment with anti-NY-ESO-1 mAb and 5-FU enhances NY-ESO-1-specific CD8⁺ T-cell induction

Considering a critical role of CD8⁺ T cells, we examined NY-ESO-1-specific T cells in dLNs. BALB/c mice were inoculated with CT26-NY-ESO-1 and received the combination treatment. dLNs and tumors were harvested on days 14 to 16, and cells were incubated with NY-ESO-1₈₁₋₈₈ (23) or control peptide, and cytokine secretion was analyzed. Combination treatment with anti-NY-ESO-1 mAb and 5-FU elicited significantly higher numbers of NY-ESO-1-specific CD8⁺ T cells producing IFN-γ and/or TNF-α than 5-FU alone (Fig. 3A). Furthermore, there was a trend of higher numbers of NY-ESO-1-specific CD8⁺ T cells in tumors treated with the combination treatment than those treated with 5-FU alone (Fig. 3C).

To explore further differences in NY-ESO-1₈₁₋₈₈-specific CD8⁺ T cells, the effector/memory status was analyzed. The

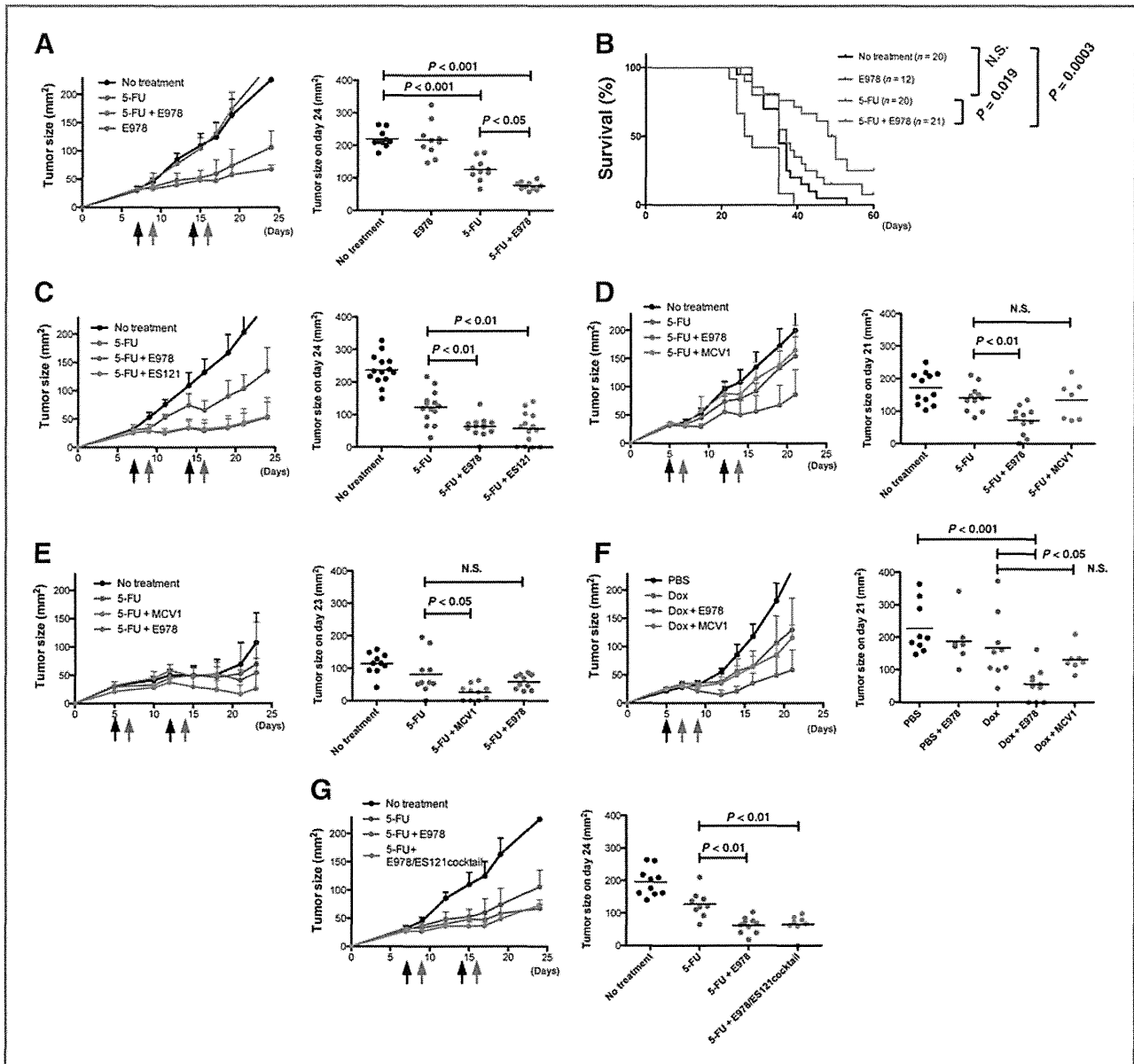


Figure 1. Combination of mAb and an anticancer drug exhibits augmented tumor growth inhibition. A–D and G, BALB/c mice were inoculated with CT26-NY-ESO-1 and treatment was started when tumors were palpable (around 25 mm², days 5–7). Mice received 5-FU intraperitoneally (black arrow) and/or anti-NY-ESO-1 mAb (clone; E978 or ES121) or anti-MAGE-A4 mAb (clone; MCV1) 2 days after 5-FU injection (red arrow). Treatment was repeated twice at 1-week intervals. A, left, tumor growth curves representative of 2 independent experiments; right, summary of tumor size on day 24 of 2 independent experiments. B, survival rate curves summarized from another 3 independent experiments (separate from tumor growth data) are shown. C and D, left, tumor growth curves representative of 2 independent experiments. Right, summary of tumor size of 2 independent experiments on days 24 and 21, respectively. E, BALB/c mice were inoculated with CT26-MAGE-A4 and treatment was started as in A. Mice received 5-FU (black arrow) and anti-MAGE-A4 mAb (clone; MCV1) or anti-NY-ESO-1 mAb (clone; E978) 2 days after 5-FU injection (red arrow). Left, tumor growth curves representative of 2 independent experiments; right, summary of tumor size on day 23 of 2 independent experiments. F, BALB/c mice were inoculated with CMS5a-NY-ESO-1 and treatment was started as in A. Mice received doxorubicin (Dox; 50 μ L, 0.25 mmol/L) intratumorally (black arrow) and/or anti-NY-ESO-1 mAb (clone; E978) 2 and 4 days after doxorubicin administration (red arrow). Left, tumor growth curves representative of 2 independent experiments; right, summary of tumor size on day 21 of 2 independent experiments. G, mice were injected with 5-FU (black arrow) and anti-NY-ESO-1 mAb (E978, 100 μ g) or cocktail of 2 anti-NY-ESO-1 mAbs (E978 50 μ g and ES121 50 μ g) 2 days after 5-FU injection (red arrow). Left, tumor growth curves representative of 2 independent experiments; right, summary of tumor size on day 24 of 2 independent experiments. Tumor size was monitored 3 times a week. Each group consisted of 3 to 8 mice. Data are presented as mean \pm SD. N.S., not significant.

frequency of NY-ESO-1-specific CD8⁺ T cells as measured by CD8⁺NY-ESO-1/D^d tetramer⁺ T cells was higher in mice treated with the combination therapy than in mice treated

with 5-FU alone, confirming the data from the intracellular cytokine assays. The frequency of effector/memory (CD62L^{low}CD45RB^{low}) T cells was higher in mice treated with

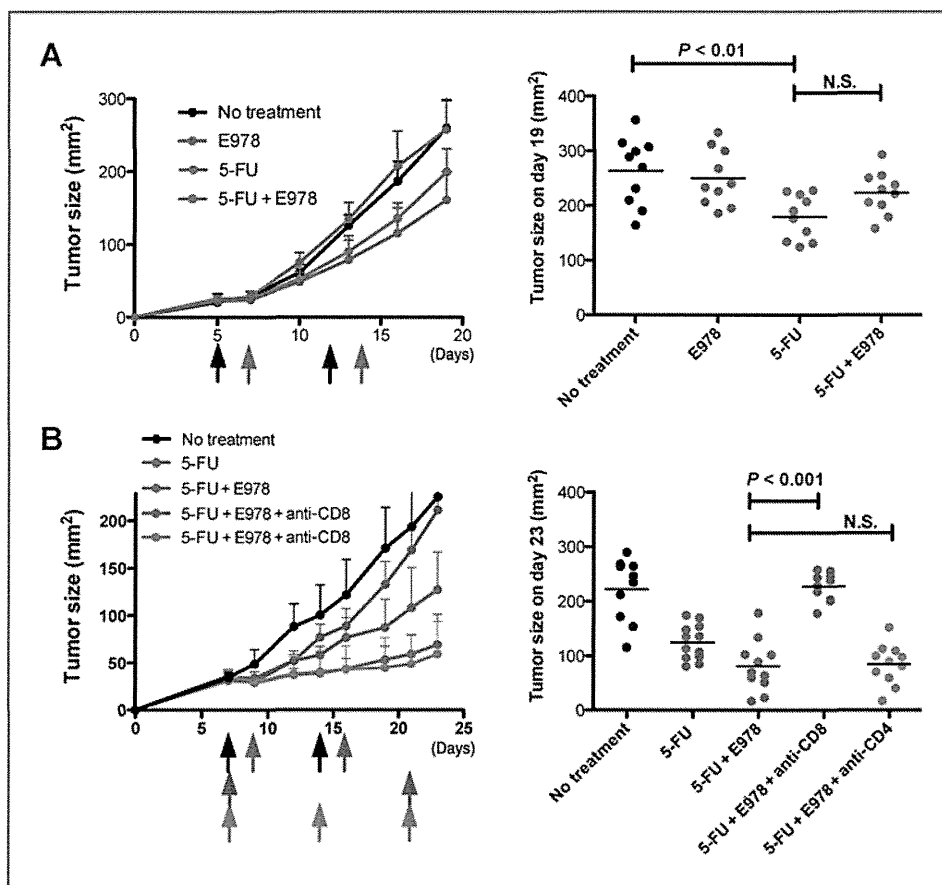


Figure 2. Augmented tumor growth inhibition by the combination treatment depends on CD8⁺ T cells. A, BALB/c^{nu/nu} mice were inoculated with CT26-NY-ESO-1 and treatment with 5-FU (days 5 and 12; black arrow) and anti-NY-ESO-1 mAb (E978, days 7 and 14; red arrow) was started as in Fig. 1A. Left, tumor growth curves representative of 2 independent experiments; right, summary of tumor size on day 19 of 2 independent experiments. B, BALB/c mice bearing CT26-NY-ESO-1 were injected with 5-FU (days 7 and 14; black arrow) and anti-NY-ESO-1 mAb (E978, days 9 and 16; red arrow) and received anti-CD4 (days 7, 14, and 21; brown arrow) or anti-CD8 mAb (days 7 and 21; purple arrow), resulting in more than 95% depletion of CD4/CD8 cell depletion. Left, tumor growth curves representative of 2 independent experiments; right, summary of tumor size on day 23 of 2 independent experiments. Tumor size was monitored 3 times a week. Each group consisted of 5 to 7 mice. Data are presented as mean ± SD. N.S., not significant.

the combination treatment (Fig. 3B). In contrast, frequency of naive (CD62L^{high}CD45RB^{high}) T cells was higher in mice treated with 5-FU alone, indicating that the combination treatment efficiently activated antigen-specific CD8⁺ T cells.

Therapeutically effective antigen spreading is observed in mice treated with the combination treatment

Certain immunization strategies result in the development of an immune response against tumor antigens that are not contained in the vaccine but are found in tumor cells, a phenomenon known as antigen spreading (22, 29, 30). Therefore, we explored whether the combination treatment resulted in the development of an immune response against other antigens expressed in tumor cells. As we used CT26 tumors, we examined CD8⁺ T cells recognizing AH-1 peptide, which is derived from the envelope protein (gp70) of an endogenous ecotropic murine leukemia provirus expressed by CT26 and previously shown to be a target of CD8⁺ T cells (28). Mice bearing CT26-NY-ESO-1 received treatment with anti-NY-ESO-1 mAb and 5-FU. Given that antigen spreading is observed after the antigen release from killed tumor cells, AH-1-specific CD8⁺ T-cell induction was analyzed at later time point (day 24). Significantly higher numbers of AH-1-specific CD8⁺ T cells was detected in mice treated with anti-NY-ESO-1 mAb and 5-FU than in mice treated with 5-FU alone (Fig. 4).

The antibody-Fc portion is required for the augmented antitumor effect by the combination treatment

We next explored the mechanism(s) of the augmented antitumor effect and the differences of NY-ESO-1₈₁₋₈₈-specific CD8⁺ T cells. The mAb therapy can exhibit immunostimulatory effects through the Fc portion of a mAb (7, 8). We investigated whether the augmented antitumor effect by combination treatment depended on the Fc portion of the mAb. BALB/c mice bearing CT26-NY-ESO-1 tumors were injected with 5-FU (days 7 and 14) and intact antibody or an Fc-depleted form of the anti-NY-ESO-1 mAb (days 9 and 16). The antitumor effect induced by the combination treatment with the intact anti-NY-ESO-1 mAb and 5-FU was totally abolished when F(ab) antibodies were administered (Fig. 5A). We further examined the critical role of the Fc portion for this augmented antitumor effect by the combination treatment using activating Fcγ receptor knockout mice (Fcγr1g^{-/-} mice). In these mice, we did not observe the augmented antitumor effect by the combination treatment compared with mice treated with 5-FU alone (Fig. 5B), confirming the critical role of the antibody-Fc portion for this augmented antitumor effect.

Accumulation of antibody to tumor sites by combination treatment

Given the importance of the Fc portion and the antigen-antibody IC formation for an enhancement of CD8⁺ T cells