Published OnlineFirst February 8, 2012; DOI:10.1158/0008-5472.CAN-11-3072

Microenvironment and Immunology

Cancer Research

Intracellular Tumor-Associated Antigens Represent Effective Targets for Passive Immunotherapy

Takuro Noguchi[1,4,8], Takuma Kato[2], Linan Wang[1,3], Yuka Maeda[1,5], Hiroaki Ikeda[3], Eiichi Sato[6], Alexander Knuth[7], Sacha Gnjatic[5,8], Gerd Ritter[8], Shimon Sakaguchi[5], Lloyd J. Old[†], Hiroshi Shiku[1,3], and Hiroyoshi Nishikawa[1,5]

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

Authors' Affiliations: Departments of [1]Cancer Vaccine, [2]Cellular and Molecular Immunology, and [3]Immuno-Gene Therapy, Mie University Graduate School of Medicine, Mie; [4]Department of Surgical Oncology, Hokkaido University Graduate School of Medicine, Hokkaido; [5]Experimental Immunology, Immunology Frontier Research Center, Osaka University, Osaka; [6]Department of Anatomic Pathology, Tokyo Medical University, Tokyo, Japan; [7]Department of Oncology, University Hospital Zurich, Zurich, Switzerland; and [8]Ludwig Institute for Cancer Research, New York Branch, Memorial Sloan-Kettering Cancer Center, New York

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

† Deceased.

Corresponding Authors: Hiroyoshi Nishikawa, Experimental Immunology, Immunology Frontier Research Center, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-4963; Fax: 81-6-6879-4464; E-mail: nisihiro@ifrec.osaka-u.ac.jp; and Hiroshi Shiku, Departments of Cancer Vaccine and Immuno-Gene Therapy, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. Phone: 81-59-231-5062; Fax: 81-59-231-5276; E-mail: shiku@clin.medic.mie-u.ac.jp

doi: 10.1158/0008-5472.CAN-11-3072

Published OnlineFirst February 8, 2012; DOI:10.1158/0008-5472.CAN-11-3072

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-1_{71-90}, ES121 (mouse IgG1) recognizing NY-ESO-1_{91-110}, 219–510 (mouse IgG1) recognizing NY-ESO-1_{21-40} (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-1_{81-88}-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-$1_{138-147}$ peptide SPSYVHQF (28), and NY-ESO-1_{81-88} 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 ×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

Published OnlineFirst February 8, 2012; DOI:10.1158/0008-5472.CAN-11-3072

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 mm^2). 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-1_{81-88} (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-1_{81-88}–specific $CD8^+$ T cells, the effector/memory status was analyzed. The

Published OnlineFirst February 8, 2012; DOI:10.1158/0008-5472.CAN-11-3072

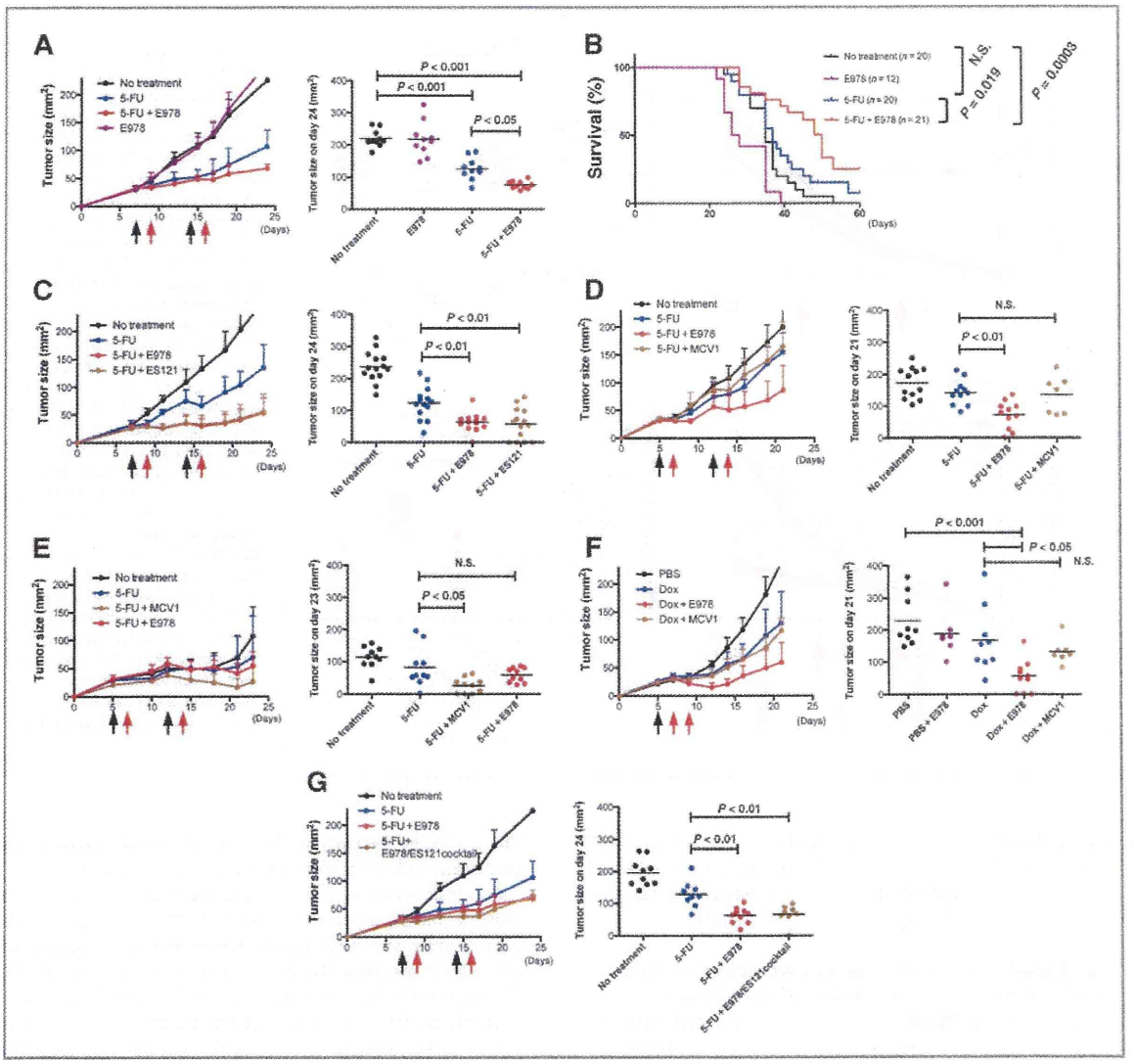

Figure 1. Combination of mAb and an anticancer drug exhibits augmented tumor growth inhibition. A–D and G, BALB/c mice were inoculated with CT26-NY-ESO-1 and treatment was started when tumors were palpable (around 25 mm^2, days 5–7). Mice received 5-FU intraperitoneally (black arrow) and/or anti-NY-ESO-1 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 μL, 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 μg) or cocktail of 2 anti-NY-ESO-1 mAbs (E978 50 μg and ES121 50 μg) 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 ± 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

Published OnlineFirst February 8, 2012; DOI:10.1158/0008-5472.CAN-11-3072

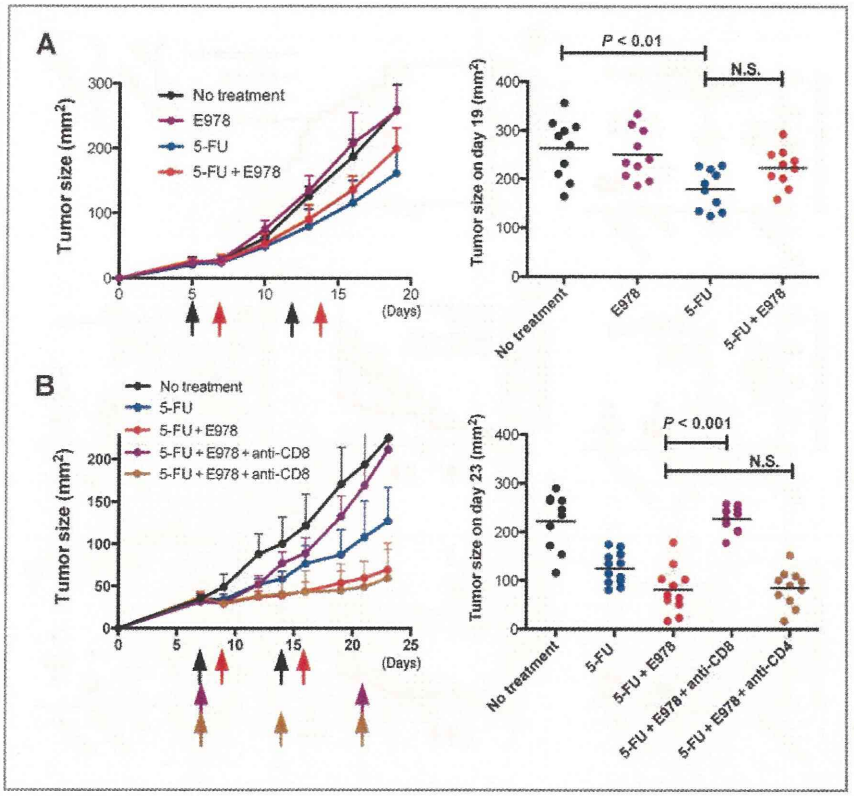

Figure 2. Augmented tumor growth inhibition by the combination treatment depends on CD8$^+$ T cells. A, BALB/c$^{nu/nu}$ mice were inoculated with CT26-NY-ESO-1 and treatment with 5-FU (days 5 and 12; black arrow) and anti-NY-ESO-1 mAb (E978, days 7 and 14; red arrow) was started as in Fig. 1A. Left, 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-1$_{81-88}$–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 (Fcer1g$^{-/-}$ 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

Published OnlineFirst February 8, 2012; DOI:10.1158/0008-5472.CAN-11-3072

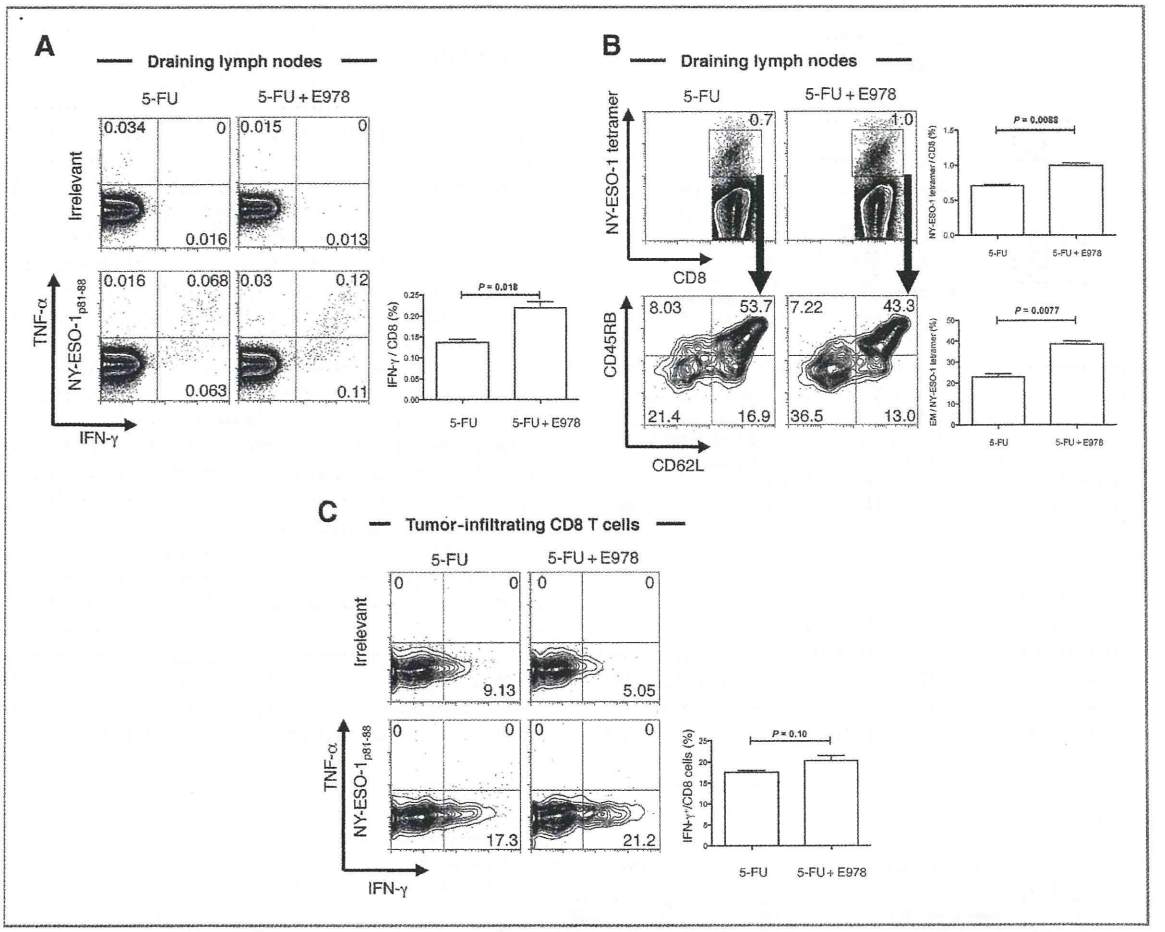

Figure 3. Combination treatment enhances NY-ESO-1–specific CD8$^+$ T-cell induction. A–C, BALB/c mice ($n = 3$) were inoculated with CT26-NY-ESO-1 and injected with 5-FU and anti-NY-ESO-1 mAb (E978). A, on day 14, dLNs were removed and incubated with NY-ESO-$1_{81–88}$ or control peptide. IFN-γ and TNF-α secretion by CD8$^+$ T cells was analyzed. B, dLN cells were isolated on day 16, and CD45RB and CD62L expression on NY-ESO-$1_{81–88}$–specific CD8$^+$ T cells identified as CD8$^+$ NY-ESO-$1_{81–88}$/D^d tetramer$^+$ T cells was analyzed. C, tumor-infiltrating lymphocytes were collected on day 16 and incubated with NY-ESO-$1_{81–88}$ or control peptide. IFN-γ and TNF-α secretion by CD8$^+$ T cells was analyzed. These experiments were repeated twice with similar results. Data are presented as mean ± SD. EM, effector/memory T cells.

(19–21), we examined the accumulation of the anti-NY-ESO-1 mAb to tumor sites for assessing the *in vivo* formation of antigen–antibody IC. For this purpose, we used a human anti-NY-ESO-1 mAb to detect and visualize the accumulation of anti-NY-ESO-1 mAb at the tumor sites. BALB/c mice bearing CT26-NY-ESO-1 received 5-FU and human anti-NY-ESO-1 mAb 2 days later. Tumors were removed several time points after the mAb injection. Anti-NY-ESO-1 mAb accumulated in CT26-NY-ESO-1 tumors after 24 hours and maintained thereafter when given in combination with 5-FU (Fig. 6A and B). In contrast, the accumulation of anti-NY-ESO-1 mAb in the tumors was lower without 5-FU treatment (Fig. 6A and B). We next tested whether the released NY-ESO-1 protein localized around the area of 5-FU–induced cell death. Anti-NY-ESO-1 mAb accumulated around the apoptotic area detected by cleaved caspase-3 staining (Fig. 6B), suggesting that 5-FU accentuated the natural release of intracellular NY-ESO-1 from dying tumor cells subsequently resulting in an increased accumulation of anti-NY-ESO-1 mAb in tumors and the formation of antigen–antibody IC.

Formation of antigen–antibody IC *in vivo* by the combination treatment induces sufficient maturation of dendritic cells for tumor eradication

We next analyzed the role of dendritic cells (DC) for this augmentation of antitumor effects. The activation status (CD80, CD86, MHC class II, and CD40) of CD11c$^+$ DCs at dLN after treatment was examined. The expression level of CD80, CD86, MHC class II, and CD40 in DCs was significantly enhanced in mice that received the combination treatment with anti-NY-ESO-1 mAb and 5-FU compared with mice treated with 5-FU alone (Fig. 6C).

Published OnlineFirst February 8, 2012; DOI:10.1158/0008-5472.CAN-11-3072

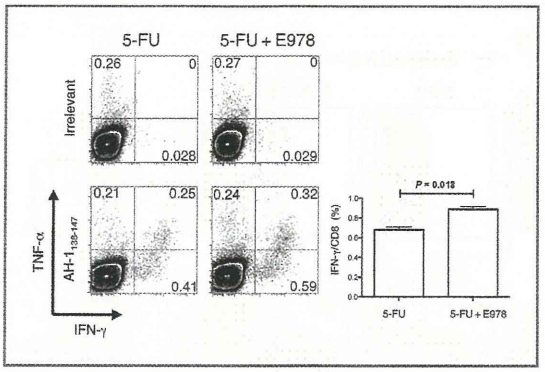

Figure 4. Antigen spreading is observed in mice that received the combination treatment. BALB/c mice ($n = 3$) were inoculated with CT26-NY-ESO-1 and injected with 5-FU and anti-NY-ESO-1 mAb (E978). On day 24, dLNs were removed and incubated with AH-$1_{138–147}$ or control peptide. IFN-γ and TNF-α secretion by $CD8^+$ T cells was analyzed. These experiments were repeated twice with similar results. Data are presented as mean ± SD.

Discussion

In view of the recent clinical successes of targeted mAbs to tumor antigens expressed on the surface of tumors for cancer therapy (3–5), we explored the feasibility to extend this approach of targeted mAb therapy to intracellular molecules as the majority of tumor antigens identified to date, are exclusively expressed and located inside the cell (14–16). Appropriate maneuvers that facilitate access of mAbs to these intracellular antigenic targets are critical requirement for this approach. Nucleoside analogues, such as 5-FU, predominantly induce apoptosis in target cells (31), but we found that NY-ESO-1 protein was released from tumor cells after 5-FU treatment in similar amounts as released by necrosis. The injected mAb accumulated into CT26-NY-ESO-1 tumors, suggesting the *in vivo* formation of antigen–antibody ICs. Furthermore, DCs in dLN that captured these ICs exhibited a mature phenotype and were associated with the induction of higher numbers of NY-ESO-1–specific $CD8^+$ T cells. This augmented antitumor immunity by combination treatment with anti-NY-ESO-1 mAb, and 5-FU was abrogated in nude mice and wild-type mice depleted of $CD8^+$ cells, arguing that a major involvement of ADCC or complement is less likely. Furthermore, this augmented antitumor effect by intracellular antigen-specific mAb combined with chemotherapy was observed in another tumor system using doxorubicin, indicating the broader application of this combination treatment.

A combination of anti-Her2 mAb and HER2/neu-expressing granulocyte macrophage colony-stimulating factor (GM-CSF)-secreting tumor vaccine augmented the antitumor effect compared with either treatment alone, and the improved therapeutic efficacy was dependent on Fc-mediated activation of APCs (11). TA-99 (recognizing Trp1) mAb enhanced DNA vaccination–induced antitumor effects (12). More recently, Park and colleagues showed that the therapeutic effect of an

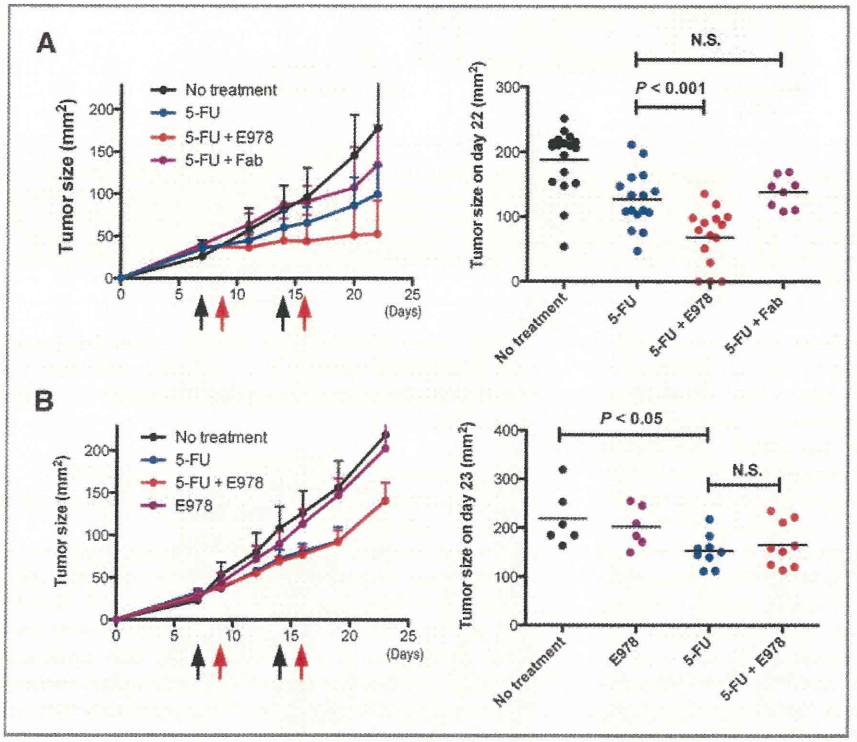

Figure 5. The Fc receptor signals are required for augmented antitumor effects by the combination treatment. A, BALB/c mice were inoculated with CT26-NY-ESO-1 and injected with 5-FU (days 7 and 14) and intact or Fc-depleted F(ab) anti-NY-ESO-1 mAb (E978, days 9 and 16). B, Fcγ receptor knockout mice were inoculated with CT26-NY-ESO-1 and injected with 5-FU (days 7 and 14) and anti-NY-ESO-1 mAb (E978, days 9 and 16). Tumor size was monitored 3 times a week. Each group consisted of 3 to 10 mice. Left, tumor growth curves representative of 2 independent experiments; right, summary of tumor size of 2 independent experiments on day 22 (A) and day 23 (B). Data are presented as mean ± SD. N.S., not significant.

Published OnlineFirst February 8, 2012; DOI:10.1158/0008-5472.CAN-11-3072

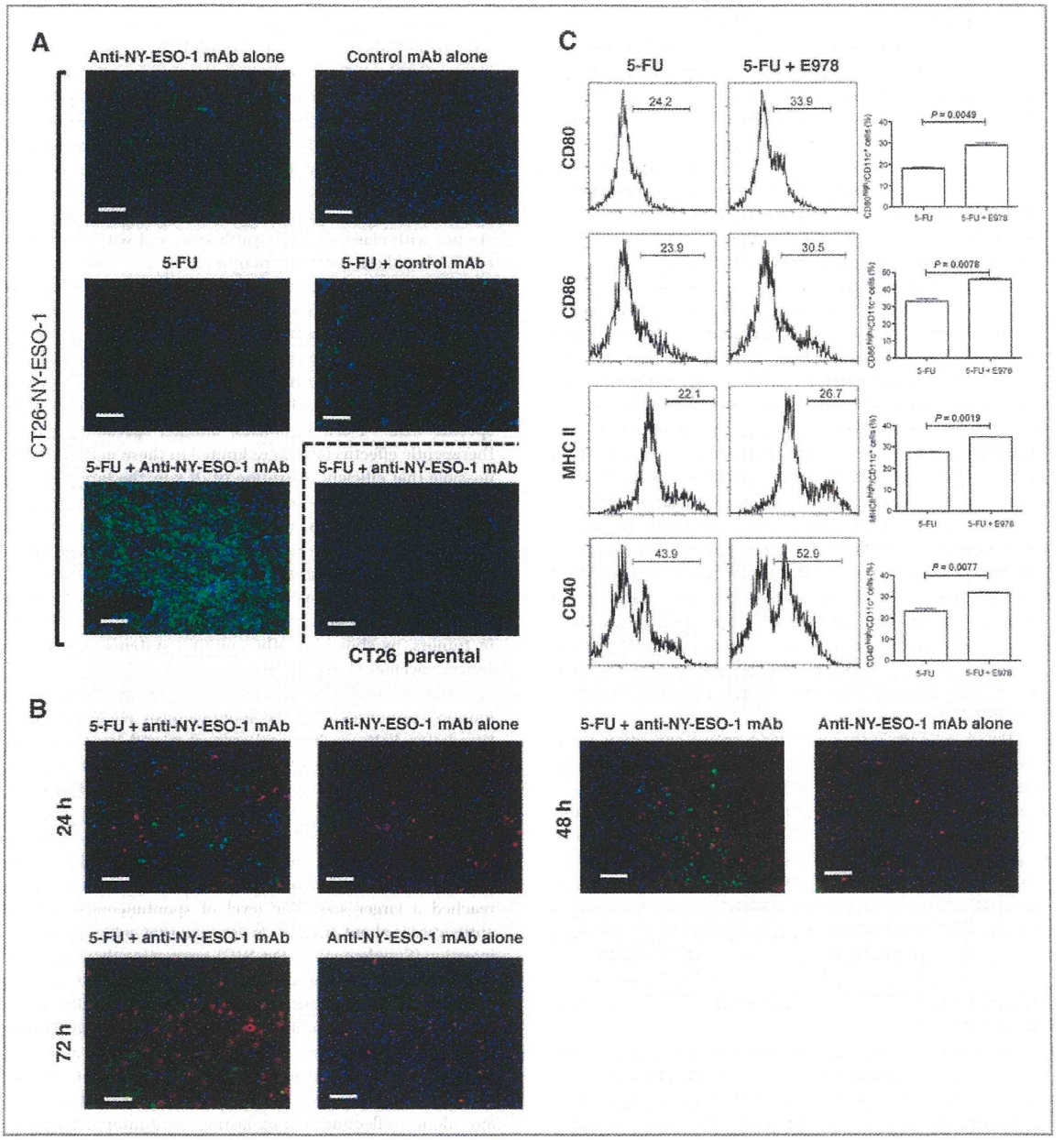

Figure 6. The combination treatment results in accumulation of injected antibody at the tumor site and induces maturation of DCs. A, BALB/c mice were inoculated with CT26-NY-ESO-1 and injected with 5-FU (day 5) and human anti-NY-ESO-1 mAb (12D7, day 7) or human anti-A33 mAb as a control (day 7). Twenty-four hours after mAb injection, tumors were removed and the accumulation of human anti-NY-ESO-1 mAb into tumors was examined by immunohistochemistry. Bar, 50 mm). B, CT26-NY-ESO-1 tumors as in A were removed at the indicated time points after mAb injection and costained with antihuman IgG mAb (green) and anti-cleaved caspase-3 mAb (red). Bar, 50 μm. C, two days after the last 5-FU injection. dLNs were harvested. CD80, CD86, CD40, and MHC class II expression on $CD11c^{high}$ DCs was analyzed. These experiments were repeated twice with similar results.

anti-HER2/neu mAb was associated with adaptive cellular immune responses, such as $CD8^+$ T cells (13). While these data clearly implicated a critical role for Fc-mediated APC activation and cross-priming correlated with enhancement of antigen-specific $CD8^+$ T-cell induction, other or additional mechanisms may include direct signal blocking and other Fc-mediated antitumor effects as the target antigens were expressed on the cell surface. These data, therefore, do not

Published OnlineFirst February 8, 2012; DOI:10.1158/0008-5472.CAN-11-3072

unambiguously suggest a possible application of mAb therapy to intracellular molecules. Here, we show that Fc-mediated antigen-specific $CD8^+$ T-cell induction was an important element of mAb therapy using mAbs against tumor antigens that are exclusively expressed in the intracellular compartment and we suggest the potential application of targeted mAb therapy also to intracellular tumor antigens. As a result, it is of interest to readdress the correlation between antitumor effect of $CD8^+$ T-cell response and clinical response by trastuzumab (anti-Her2/neu) treatment, as trastuzumab is able to enhance cross-presentation *in vitro* (32).

Another unique point in our study is that our mAb treatment targeting an intracellular antigen does not require *in vitro* formation of IC or a combination with antigen immunization, such as protein or DNA vaccines for the formation of antigen–antibody IC (10–12, 33). When the mAb was injected alone, an augmented antitumor effect was not observed in our model, suggesting the essential role of chemotherapy for releasing sufficient amounts of antigen to form antigen–antibody IC. Other modalities for facilitating antigen release from tumors, such as radiation therapy, cryoablation, or other agents, that may result in partial destruction of tumor cells could be applicable to this combination therapy. These results are particularly important for considering the clinical application of targeted mAb therapy because combination of chemotherapy and mAbs have already been widely used in the clinic (3–5). Furthermore, combining a mAb therapy with protein or DNA cancer vaccines is very expensive and enormous effort is required to translate into the clinic.

$CD4^+$ T cell help is necessary for a proper activation and a long-lasting memory formation of $CD8^+$ T cells (34, 35). While combination treatment with anti-NY-ESO-1 mAb and chemotherapy provided an augmented antitumor efficacy and induced higher numbers of NY-ESO-1–specific $CD8^+$ T cells with effector/memory type, these effects were dependent on $CD8^+$ T cells but not $CD4^+$ T cells. One can envisage that as a major role of $CD4^+$ T cells is to stimulate APCs, such as DCs, to activate $CD8^+$ T cells (licensing; refs. 34, 35), signals provided through Fc receptors may compensate the $CD4^+$ T-cell help for stimulating/activating APCs. Alternatively, inflammation induced by anticancer drugs further supports the stimulating/activating of APCs.

One intriguing question is why the combination of mAb and 5-FU exhibited a strong antitumor effect, despite a possible inhibitory signal through a subclass of IgG, namely, IgG1 used in this study (8). Because we used anti-NY-ESO-1 mAbs (mouse IgG1) for this combination therapy, IgG1 may show inhibitory function by activating inhibitory Fc receptor (7–9). Some protocols of anticancer chemotherapy induce the stimulation of immune responses by Toll-like receptor ligands released from tumor cells (36). The possibility that 5-FU–induced tumor destruction stimulates inflammation signals, such as Toll-like receptor signals, and these inflammation signals may change the ratio of stimulatory/inhibitory Fc receptor expression to a more stimulatory condition (8) is less likely because our preliminary data show that the balance between activating Fcγ receptor III and inhibitory Fcγ receptor IIB expression on $CD11c^+$ cells was not influenced by 5-FU treatment. This raises several possibilities as follows: (i) the balance between those receptors changes on other hematopoietic cells, (ii) signaling pathways through those Fcγ receptors are altered by chemotherapy-induced inflammation, and (iii) antibody specificity is not good enough to address this point and proper knockout animals are required. In addition, it will be crucial to compare the effect of immunologic responses by other IgG subclasses, and studies with class-switched antibodies and with Fcγ receptor IIB knockout mice are planned.

We observed that mAb and 5-FU combination treatment resulted in the development of an immune response against tumor antigens that have not been directly targeted by the antibody but that are expressed in tumor cells, a phenomenon known as antigen spreading (22, 29, 30). While mice treated with 5-FU alone or without treatment elicited NY-ESO-1–specific $CD8^+$ T-cell responses, antigen spreading and its therapeutic effectiveness were limited in these mice. It is also possible that efficient activation of DCs by the targeted mAb and 5-FU combination treatment provides the opportunity to stimulate subsequently additional $CD8^+$ T cells specific for other antigens derived from the tumor cells. Therefore, effective antitumor responses, such as tumor eradication, may require $CD8^+$ T cells specific for the single antigen used for immunization but also multiple antigens that were contained in tumors, as shown in other murine systems and human cancer vaccines (1, 22, 37, 38).

In our model as well as in patients with cancer, NY-ESO-1 humoral responses could be spontaneously elicited. While a correlation between humoral responses and longer survival was not reported, NY-ESO-1–specific $CD8^+$ T-cell induction by cross-priming *in vivo* is associated with the induction of specific antibodies (2, 39). Spontaneous NY-ESO-1 humoral responses are correlated with progression of tumor stage in humans (2, 39). In our mouse system, spontaneously induced anti-NY-ESO-1 antibodies were observed when tumors reached a larger size. The level of spontaneously induced antibodies is about 10 times lower than that achieved by mAb injection (Supplementary Fig. S1C), suggesting that spontaneously induced humoral responses may potentially have some antitumor effects, but the amount of antibodies may be too low to exhibit effective antitumor activity, such as facilitating tumor regression. Our data revealed that mAb and 5-FU combination treatment induced higher numbers of effector/memory NY-ESO-1–specific $CD8^+$ T cells than by chemotherapy alone, reflecting a long-lasting antitumor capacity as shown by improved survival. In conclusion, combination treatment with targeted mAbs and chemotherapy opens a new era of antibody cancer immunotherapy for tumor antigens with intracellular expression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

Authors' Contributions

Conception and design: T. Noguchi, T. Kato, A. Knuth, S. Gnjatic, G. Ritter, L.J. Old, H. Shiku, H. Nishikawa

Published OnlineFirst February 8, 2012; DOI:10.1158/0008-5472.CAN-11-3072

Development of methodology: T. Noguchi, H. Ikeda, E. Sato, A. Knuth, G. Ritter, L.J. Old
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Noguchi, G. Ritter
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Noguchi, T. Kato, Y. Maeda, S. Gnjatic, G. Ritter, L.J. Old, H. Shiku, H. Nishikawa
Writing, review, and/or revision of the manuscript: T. Noguchi, T. Kato, H. Ikeda, A. Knuth, S. Gnjatic, G. Ritter, S. Sakaguchi, L.J. Old, H. Shiku, H. Nishikawa
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Noguchi, L. Wang, H. Ikeda, G. Ritter, S. Sakaguchi
Study supervision: T. Kato, G. Ritter, L.J. Old, H. Shiku

In Memoriam

This article is dedicated to the memory of L.J. Old.

Acknowledgments

The authors thank the contribution of the late Dr. S. Kondo for discussion. The authors also thank Drs. T. Takahashi, M. van den Broek, and J. Wing for critical reading of the manuscript and M. Yamane for technical assistance.

Grant Support

This study was supported by Grants-in-Aid for Scientific Research (B; no. 23300354 to H. Nishikawa), the Cancer Research Institute Investigator Award (H. Nishikawa) and Cancer Vaccine Collaborative Grant for Immunological Monitoring (S. Gnjatic, G. Ritter, and L.J. Old), Cancer Research Grant from Foundation of Cancer Research Promotion (H. Nishikawa), and Atlantic Philanthropies (G. Ritter, L.J. Old).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 15, 2011; revised December 27, 2011; accepted January 18, 2012; published OnlineFirst February 8, 2012.

References

1. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P. Human T cell responses against melanoma. Annu Rev Immunol 2006;24:175–208.
2. Gnjatic S, Nishikawa H, Jungbluth AA, Gure AO, Ritter G, Jager E, et al. NY-ESO-1: review of an immunogenic tumor antigen. Adv Cancer Res 2006;95:1–30.
3. Dougan M, Dranoff G. Immune therapy for cancer. Annu Rev Immunol 2009;27:83–117.
4. Coiffier B, Lepage E, Briere J, Herbrecht R, Tilly H, Bouabdallah R, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med 2002;346:235–42.
5. Hudis CA. Trastuzumab–mechanism of action and use in clinical practice. N Engl J Med 2007;357:39–51.
6. Regnault A, Lankar D, Lacabanne V, Rodriguez A, Thery C, Rescigno M, et al. Fcγ receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. J Exp Med 1999;189:371–80.
7. Nimmerjahn F, Ravetch JV. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. Science 2005;310:1510–2.
8. Nimmerjahn F, Ravetch JV. Fcγ receptors as regulators of immune responses. Nat Rev Immunol 2008;8:34–47.
9. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate *in vivo* cytotoxicity against tumor targets. Nat Med 2000;6:443–6.
10. Wolpoe ME, Lutz ER, Ercolini AM, Murata S, Ivie SE, Garrett ES, et al. HER-2/neu-specific monoclonal antibodies collaborate with HER-2/neu-targeted granulocyte macrophage colony-stimulating factor secreting whole cell vaccination to augment $CD8^+$ T cell effector function and tumor-free survival in Her-2/neu-transgenic mice. J Immunol 2003;171:2161–9.
11. Kim PS, Armstrong TD, Song H, Wolpoe ME, Weiss V, Manning EA, et al. Antibody association with HER-2/neu-targeted vaccine enhances $CD8^+$ T cell responses in mice through Fc-mediated activation of DCs. J Clin Invest 2008;118:1700–11.
12. Saenger YM, Li Y, Chiou KC, Chan B, Rizzuto G, Terzulli SL, et al. Improved tumor immunity using anti-tyrosinase related protein-1 monoclonal antibody combined with DNA vaccines in murine melanoma. Cancer Res 2008;68:9884–91.
13. Park S, Jiang ZJ, Mortenson ED, Deng LF, Radkevich-Brown O, Yang XM, et al. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. Cancer Cell 2010;18:160–70.
14. Kawakami Y, Rosenberg SA. Human tumor antigens recognized by T-cells. Immunol Res 1997;16:313–39.
15. Old LJ, Chen Y-T. New paths in human cancer serology. J Exp Med 1998;187:1163–7.
16. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer 2005;5:615–25.
17. Hara I, Takechi Y, Houghton AN. Implicating a role for immune recognition of self in tumor rejection: passive immunization against the brown locus protein. J Exp Med 1995;182:1609–14.
18. Chen YT, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proc Natl Acad Sci U S A 1997;94:1914–8.
19. Dhodapkar KM, Krasovsky J, Williamson B, Dhodapkar MV. Antitumor monoclonal antibodies enhance cross-presentation of cellular antigens and the generation of myeloma-specific killer T cells by dendritic cells. J Exp Med 2002;195:125–33.
20. Nagata Y, Ono S, Matsuo M, Gnjatic S, Valmori D, Ritter G, et al. Differential presentation of a soluble exogenous tumor antigen, NY-ESO-1, by distinct human dendritic cell populations. Proc Natl Acad Sci U S A 2002;99:10629–34.
21. Valmori D, Souleimanian NE, Tosello V, Bhardwaj N, Adams S, O'Neill D, et al. Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. Proc Natl Acad Sci U S A 2007;104:8947–52.
22. Nishikawa H, Sato E, Briones G, Chen LM, Matsuo M, Nagata Y, et al. *In vivo* antigen delivery by a *Salmonella typhimurium* type III secretion system for therapeutic cancer vaccines. J Clin Invest 2006;116:1946–54.
23. Muraoka D, Kato T, Wang LA, Maeda Y, Noguchi T, Harada N, et al. Peptide vaccine induces enhanced tumor growth associated with apoptosis induction in $CD8^+$ T cells. J Immunol 2010;185:3768–76.
24. Griswold DP, Corbett TH. A colon tumor model for anticancer agent evaluation. Cancer 1975;36:2441–4.
25. Ikeda H, Ohta N, Furukawa K, Miyazaki H, Wang L, Kuribayashi K, et al. Mutated mitogen-activated protein kinase: a tumor rejection antigen of mouse sarcoma. Proc Natl Acad Sci U S A 1997;94:6375–9.
26. Stockert E, Jager E, Chen Y-T, Scanlan MJ, Gout I, Karbach J, et al. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. J Exp Med 1998;187:1349–54.
27. Nagata Y, Furugen R, Hiasa A, Ikeda H, Ohta N, Furukawa K, et al. Peptides derived from a wild-type murine proto-oncogene c-*erb*B-2/HER2/*neu* can induce CTL and tumor suppression in syngeneic hosts. J Immunol 1997;159:1336–43.
28. Huang AY, Gulden PH, Woods AS, Thomas MC, Tong CD, Wang W, et al. The immunodominant major histocompatibility complex class I-restricted antigen of a murine colon tumor derives from an endogenous retroviral gene product. Proc Natl Acad Sci U S A 1996;93:9730–5.
29. Lehmann PV, Forsthuber T, Miller A, Sercarz EE. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. Nature 1992;358:155–7.
30. Vanderlugt CL, Miller SD. Epitope spreading in immune-mediated diseases: implications for immunotherapy. Nat Rev Immunol 2002;2:85–95.
31. Sampath D, Rao VA, Plunkett W. Mechanisms of apoptosis induction by nucleoside analogs. Oncogene 2003;22:9063–74.
32. Kono K, Sato E, Naganuma H, Takahashi A, Mimura K, Nukui H, et al. Trastuzumab (Herceptin) enhances class I-restricted antigen

Published OnlineFirst February 8, 2012; DOI:10.1158/0008-5472.CAN-11-3072

presentation recognized by HER-2/neu-specific T cytotoxic lymphocytes. Clin Cancer Res 2004;10:2538–44.
33. Rafiq K, Bergtold A, Clynes R. Immune complex-mediated antigen presentation induces tumor immunity. J Clin Invest 2002;110:71–9.
34. Kennedy R, Celis E. Multiple roles for $CD4^+$ T cells in anti-tumor immune responses. Immunol Rev 2008;222:129–44.
35. Arens R, Schoenberger SP. Plasticity in programming of effector and memory $CD8^+$ T-cell formation. Immunol Rev 2010;235:190–205.
36. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med 2007;13:1050–9.
37. Germeau C, Ma WB, Schiavetti F, Lurquin C, Henry E, Vigneron N, et al. High frequency of antitumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens. J Exp Med 2005;201:241–8.
38. Lurquin C, Lethe B, De Plaen E, Corbiere V, Theate I, van Baren N, et al. Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. J Exp Med 2005;201:249–57.
39. Gure AO, Chua R, Williamson B, Gonen M, Ferrera CA, Gnjatic S, et al. Cancer-testis genes are coordinately expressed and are markers of poor outcome in non-small cell lung cancer. Clin Cancer Res 2005;11:8055–62.

Cancer Science
The official journal of the Japanese Cancer Association JCA

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

Yoshitaka Shirakura,[1,2,7] Yukari Mizuno,[1,2,7] Linan Wang,[2] Naoko Imai,[2] Chisaki Amaike,[2] Eiichi Sato,[3] Mamoru Ito,[4] Ikuei Nukaya,[5] Junichi Mineno,[5] Kazutoh Takesako,[5] Hiroaki Ikeda[2,6] and Hiroshi Shiku[1,2,6]

Departments of [1]Cancer Vaccine, [2]Immuno-Gene Therapy, Mie University Graduate School of Medicine, Tsu; [3]Department of Pathology, Tokyo Medical University, Tokyo; [4]Central Institute for Experimental Animals, Kawasaki; [5]Center for Cell and Gene Therapy, Takara Bio Inc., Otsu, Japan

(Received June 20, 2011/Revised September 14, 2011/Accepted September 17, 2011/Accepted manuscript online September 23, 2011/Article first published online November 9, 2011)

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/γc^{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/γc^{null} (NOG) mice. We evaluated the *in vivo* function of the transferred cells, as well as their migration to the tumor

[6]To whom correspondence should be addressed.
E-mail: shiku@clin.medic.mie-u.ac.jp; hikeda@clin.medic.mie-u.ac.jp
[7]These authors contributed equally to this work.

doi: 10.1111/j.1349-7006.2011.02111.x

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 2000*g* 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-A4$_{143-151}$/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-A4$_{141-153}$ 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-A4$_{141-153}$ or HER2$_{63-71}$ (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/mL GolgiStop (BD Bioscience). The cells were then stained with FITC-conjugated anti-CD8 (BD Bioscience) mAb. After permeabilization and fixation using a Cytofix/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-A4$_{143-151}$/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 CantoI 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, Glostrum, 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 mm^2) 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-A4$_{143-151}$ 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-A4$_{143-151}$/HLA-A*2402 tetramer that specifically detected the transduced TCR (Fig. 1A). As we reported previously, this TCR bound the tetramer in a

doi: 10.1111/j.1349-7006.2011.02111.x

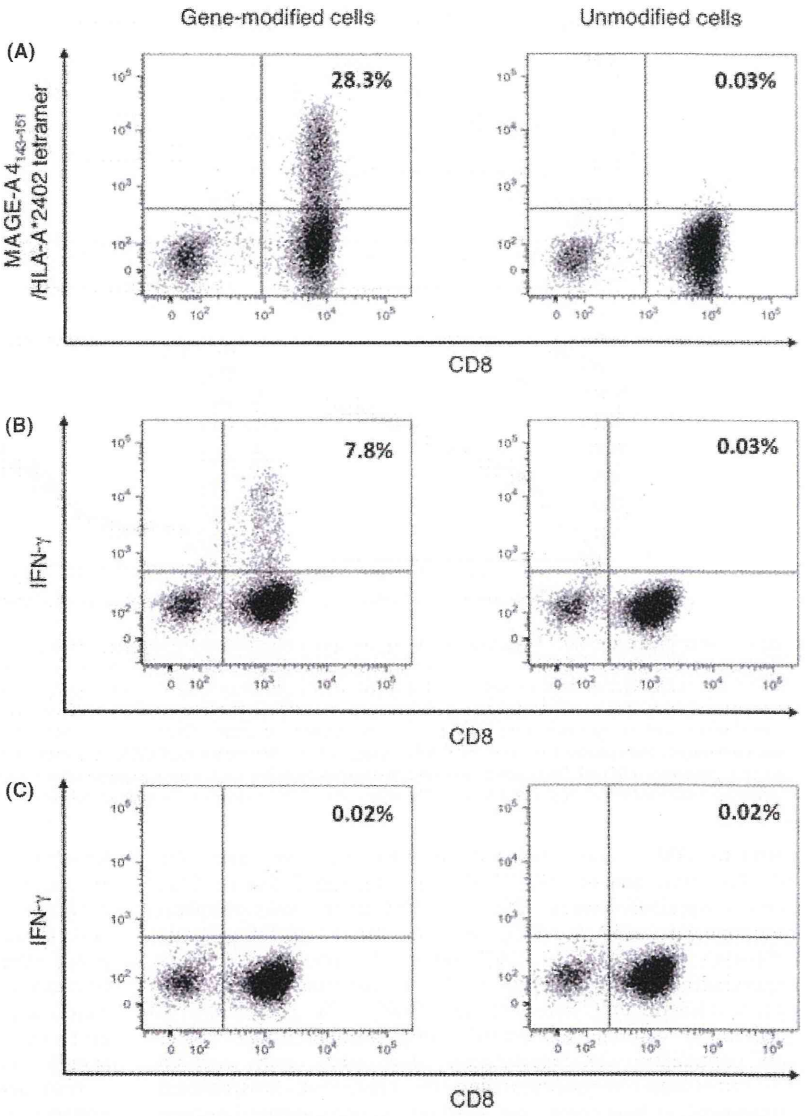

Fig. 1. Transduction of melanoma-associated antigen (MAGE)-A4-specific T-cell receptor (TCR) in human lymphocytes. Peripheral blood mononuclear cells from healthy donors were stimulated with anti-CD3 mAb and interleukin-2. Cells were cultured with or without retroviral vector encoding MAGE-A4-specific TCR, designated gene-modified or unmodified cells, respectively. (A) Representative staining for gene-modified and unmodified cells with MAGE-$A4_{143-151}$/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-$A4_{143-151}$ 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-g$^+$ 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-$A4_{143-151}$/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-$A4_{141-153}$/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.

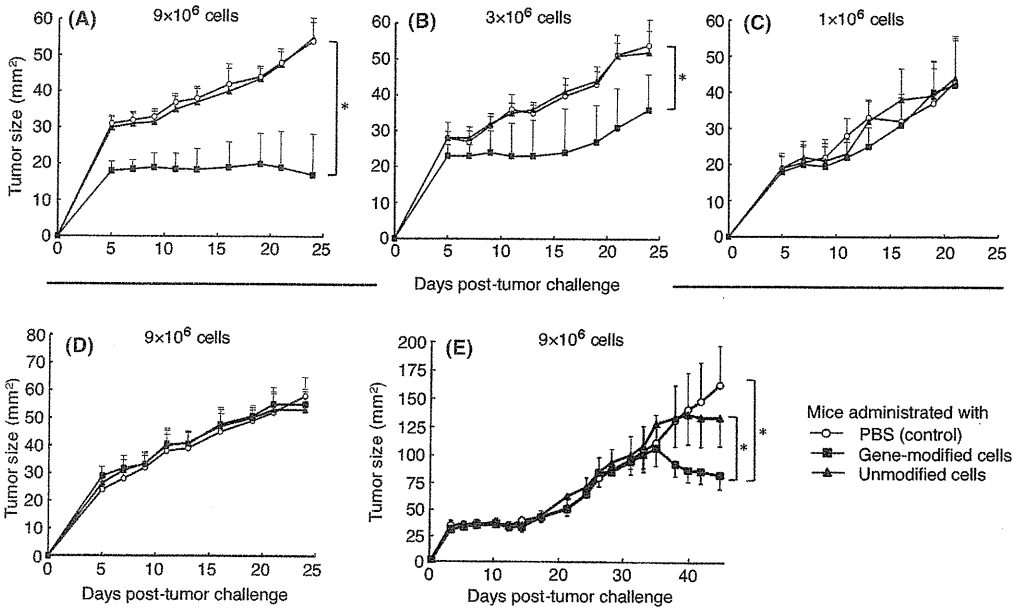

Fig. 2. Adoptive transfer of lymphocytes genetically engineered to express MAGE-A4-specific T-cell receptor inhibits human tumor progression in non-obese diabetic/SCID/γc^{null} mice. Non-obese diabetic/SCID/γc^{null} mice ($n = 4$ per group) were subcutaneously inoculated with 2.5×10^6 KE4 (A–C) or 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/γc^{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 (TCRα V8-1 or TCRβ 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,

doi: 10.1111/j.1349-7006.2011.02111.x

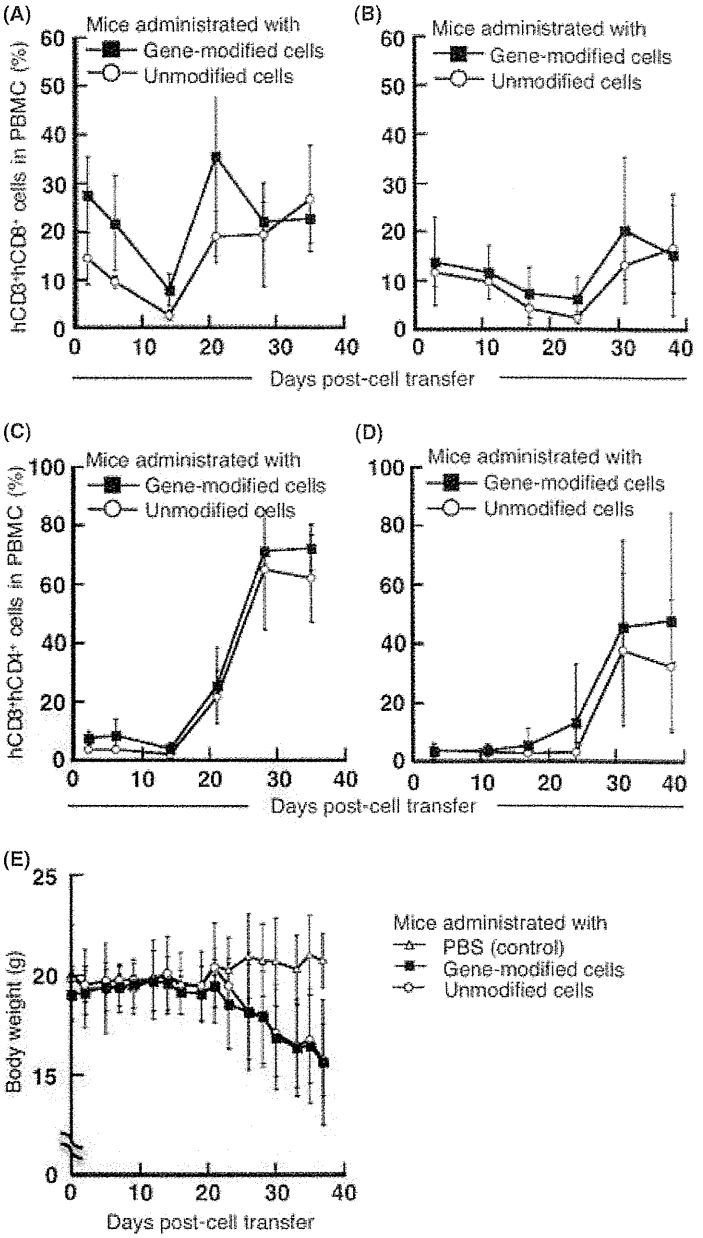

Fig. 3. Persistence of adoptively transferred human lymphocytes in non-obese, diabetic/SCID/γc^{null} (NOG) mice. Non-obese, diabetic/SCID/γc^{null} mice ($n = 4$ per group) were subcutaneously inoculated with 2.5×10^6 KE4 tumor cells, then intravenously administered 1×10^8 (A,C) or 5×10^7 (B,D) gene-modified (■) 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-$A4_{143-151}$ 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

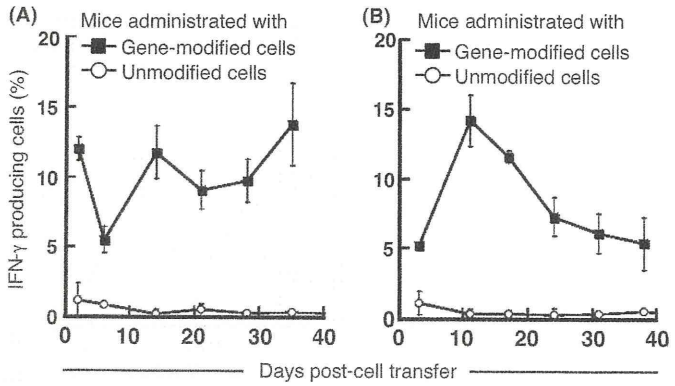

Fig. 4. Lymphocytes genetically engineered to express MAGE-A4-specific T-cell receptor-maintained specific reactivity after *in vivo* passage. Non-obese, diabetic/SCID/γc^{null} mice ($n = 4$ per group) were subcutaneously inoculated with 2.5×10^6 KE4 tumor cells, then intravenously administered 1×10^8 (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 μM MAGE-$A4_{141-153}$ 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.

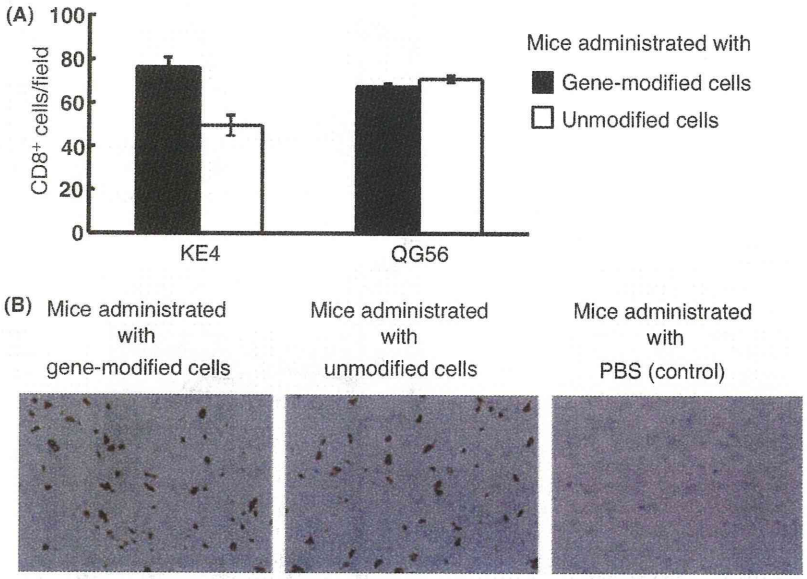

Fig. 5. Adoptively-transferred human $CD8^+$ T cells infiltrate into tumor tissues. Tumor specimens were harvested from non-obese, diabetic/SCID/γc^{null} mice 14 days after subcutaneous inoculation with 2.5×10^6 KE4 or QG56 tumor cells, and intravenous administration of 1×10^8 gene-modified or unmodified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

doi: 10.1111/j.1349-7006.2011.02111.x

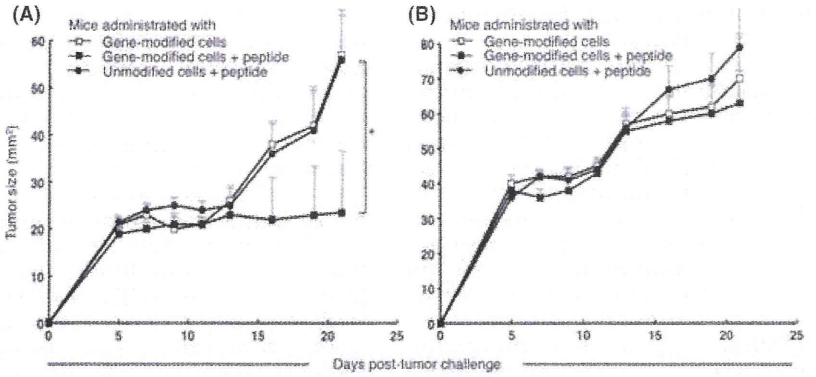

Fig. 6. Peptide vaccination enhanced the antitumor efficacy of adoptive therapy using T-cell receptor, gene-modified cells. Non-obese, diabetic/SCID/γc^{null} mice ($n = 4$ per group) were subcutaneously inoculated with 2.5×10^6 KE4 (A) or QG56 (B) tumor cells, and intravenously administered 1×10^8 gene-modified (□) or unmodified (●) 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 μM MAGE-A4$_{141-153}$ peptide, and intravenously administered these cells into the animals on days 1 and 8 (■ and ●). Results are representative of three independent experiments.

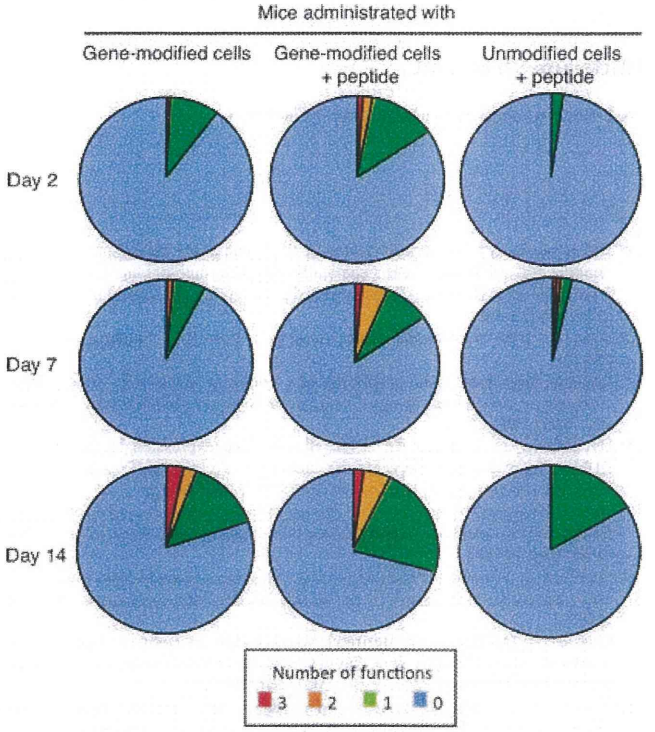

Fig. 7. Peptide vaccination increased the multifunctionality of transferred gene-modified cells. Mice were subcutaneously inoculated with 2.5×10^6 KE4 tumor cells, then intravenously administered 1×10^8 gene-modified or unmodified cells with or without peptide vaccination. Two, 7, and 14 days after transfer, 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 TCRα 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 antitumor 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.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Disclosure Statement

No potential conflicts of interest were disclosed.

References

1 Morgan RA, Dudley ME, Yu YY *et al.* High efficiency TCR gene transfer into primary human lymphocytes affords avid recognition of melanoma tumor antigen glycoprotein 100 and does not alter the recognition of autologous melanoma antigens. *J Immunol* 2003; **171**: 3287–95.
2 Rubinstein MP, Kadima AN, Salem ML *et al.* Transfer of TCR genes into mature T cells is accompanied by the maintenance of parental T cell avidity. *J Immunol* 2003; **170**: 1209–17.
3 Zhao Y, Zheng Z, Robbins PF, Khong HT, Rosenberg SA, Morgan RA. Primary human lymphocytes transduced with NY-ESO-1 antigen-specific TCR genes recognize and kill diverse human tumor cell lines. *J Immunol* 2005; **174**: 4415–23.
4 Hughes MS, Yu YY, Dudley ME *et al.* Transfer of a TCR gene derived from a patient with a marked antitumor response conveys highly active T-cell effector functions. *Hum Gene Ther* 2005; **16**: 457–72.
5 Coccoris M, Swart E, de Witte MA *et al.* Long-term functionality of TCR-transduced T cells *in vivo*. *J Immunol* 2008; **180**: 6536–43.
6 Abad JD, Wrzensinski C, Overwijk W *et al.* T-cell receptor gene therapy of established tumors in a murine melanoma model. *J Immunother* 2008; **31**: 1–6.
7 Sadelain M, Riviere I, Brentjens R. Targeting tumours with genetically enhanced T lymphocytes. *Nat Rev Cancer* 2003; **3**: 35–45.
8 Murphy A, Westwood JA, Teng MW, Moeller M, Darcy PK, Kershaw MH. Gene modification strategies to induce tumor immunity. *Immunity* 2005; **22**: 403–14.
9 June CH. Adoptive T cell therapy for cancer in the clinic. *J Clin Invest* 2007; **117**: 1466–76.
10 Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer* 2008; **8**: 299–308.
11 Morgan RA, Dudley ME, Wunderlich JR *et al.* Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 2006; **314**(5796): 126–9.
12 Johnson LA, Morgan RA, Dudley ME *et al.* Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 2009; **114**: 535–46.
13 Parkhurst MR, Yang JC, Langan RC *et al.* T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Mol Ther* 2011; **19**: 620–6.
14 Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* 2010; **18**: 843–51.
15 Brentjens R, Yeh R, Bernal Y, Riviere I, Sadelain M. Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. *Mol Ther* 2010; **18**: 666–8.
16 Boon T, Old LJ. Cancer tumor antigens. *Curr Opin Immunol* 1997; **9**: 681–3.
17 Scanlan MJ, Gure AO, Jungbluth AA, Old LJ, Chen YT. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. *Immunol Rev* 2002; **188**: 22–32.
18 Uyttenhove C, Godfraind C, Lethe B *et al.* The expression of mouse gene P1A in testis does not prevent safe induction of cytolytic T cells against a P1A-encoded tumor antigen. *Int J Cancer* 1997; **70**: 349–56.
19 van der Bruggen P, Traversari C, Chomez P *et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991; **254**(5038): 1643–7.
20 De Plaen E, Arden K, Traversari C *et al.* Structure, chromosomal localization, and expression of 12 genes of the MAGE family. *Immunogenetics* 1994; **40**: 360–9.
21 Chomez P, De Backer O, Bertrand M, De Plaen E, Boon T, Lucas S. An overview of the MAGE gene family with the identification of all human members of the family. *Cancer Res* 2001; **61**: 5544–51.
22 Yakirevich E, Sabo E, Lavie O, Mazareb S, Spagnoli GC, Resnick MB. Expression of the MAGE-A4 and NY-ESO-1 cancer-testis antigens in serous ovarian neoplasms. *Clin Cancer Res* 2003; **9**: 6453–60.
23 Peng JR, Chen HS, Mou DC *et al.* Expression of cancer/testis (CT) antigens in Chinese hepatocellular carcinoma and its correlation with clinical parameters. *Cancer Lett* 2005; **219**: 223–32.
24 Li M, Yuan YH, Han Y *et al.* Expression profile of cancer-testis genes in 121 human colorectal cancer tissue and adjacent normal tissue. *Clin Cancer Res* 2005; **11**: 1809–14.
25 Lin J, Lin L, Thomas DG *et al.* Melanoma-associated antigens in esophageal adenocarcinoma: identification of novel MAGE-A10 splice variants. *Clin Cancer Res* 2004; **10**: 5708–16.
26 Tajima K, Obata Y, Tamaki H *et al.* Expression of cancer/testis (CT) antigens in lung cancer. *Lung Cancer* 2003; **42**: 23–33.
27 Yoshida N, Abe H, Ohkuri T *et al.* Expression of the MAGE-A4 and NY-ESO-1 cancer-testis antigens and T cell infiltration in non-small cell lung carcinoma and their prognostic significance. *Int J Oncol* 2006; **28**: 1089–98.
28 Forghanifard MM, Gholamin M, Farshchian M *et al.* Cancer-testis gene expression profiling in esophageal squamous cell carcinoma: identification of specific tumor marker and potential targets for immunotherapy. *Cancer Biol Ther* 2011; **12**: 191–7.

doi: 10.1111/j.1349-7006.2011.02111.x

29 Miyahara Y, Naota H, Wang L *et al.* Determination of cellularly processed HLA-A2402-restricted novel CTL epitopes derived from two cancer germ line genes, MAGE-A4 and SAGE. *Clin Cancer Res* 2005; **11**: 5581–9.
30 Hiasa A, Hirayama M, Nishikawa H *et al.* Long-term phenotypic, functional and genetic stability of cancer-specific T-cell receptor (TCR) alphabeta genes transduced to CD8+ T cells. *Gene Ther* 2008; **15**: 695–9.
31 Okamoto S, Mineno J, Ikeda H *et al.* Improved expression and reactivity of transduced tumor-specific TCRs in human lymphocytes by specific silencing of endogenous TCR. *Cancer Res* 2009; **69**: 9003–11.
32 Ito M, Hiramatsu H, Kobayashi K *et al.* NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. *Blood* 2002; **100**: 3175–82.
33 Imai N, Ikeda H, Tawara I, Shiku H. Tumor progression inhibits the induction of multifunctionality in adoptively transferred tumor-specific CD8+ T cells. *Eur J Immunol* 2009; **39**: 241–53.
34 Hiasa A, Nishikawa H, Hirayama M *et al.* Rapid alphabeta TCR-mediated responses in gammadelta T cells transduced with cancer-specific TCR genes. *Gene Ther* 2009; **16**: 620–8.
35 Bendle GM, Linnemann C, Hooijkaas AI *et al.* Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. *Nat Med* 2010; **16**: 565–70. 1p following 70.
36 Schroeder ML. Transfusion-associated graft-versus-host disease. *Br J Haematol* 2002; **117**: 275–87.
37 Mrass P, Takano H, Ng LG *et al.* Random migration precedes stable target cell interactions of tumor-infiltrating T cells. *J Exp Med* 2006; **203**: 2749–61.
38 Overwijk WW, Theoret MR, Finkelstein SE *et al.* Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *J Exp Med* 2003; **198**: 569–80.
39 Imai N, Ikeda H, Tawara I *et al.* Glucocorticoid-induced tumor necrosis factor receptor stimulation enhances the multifunctionality of adoptively transferred tumor antigen-specific CD8+ T cells with tumor regression. *Cancer Sci* 2009; **100**: 1317–25.
40 Dudley ME, Wunderlich JR, Robbins PF *et al.* Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002; **298**: 850–4.
41 Dudley ME, Wunderlich JR, Yang JC *et al.* Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol* 2005; **23**: 2346–57.
42 Dudley ME, Yang JC, Sherry R *et al.* Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* 2008; **26**: 5233–9.
43 Robbins PF, Morgan RA, Feldman SA *et al.* Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* 2011; **29**: 917–24.
44 Hsu C, Hughes MS, Zheng Z, Bray RB, Rosenberg SA, Morgan RA. Primary human T lymphocytes engineered with a codon-optimized IL-15 gene resist cytokine withdrawal-induced apoptosis and persist long-term in the absence of exogenous cytokine. *J Immunol* 2005; **175**: 7226–34.
45 Liu K, Rosenberg SA. Interleukin-2-independent proliferation of human melanoma-reactive T lymphocytes transduced with an exogenous IL-2 gene is stimulation dependent. *J Immunother* 2003; **26**: 190–201.
46 Kershaw MH, Wang G, Westwood JA *et al.* Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. *Hum Gene Ther* 2002; **13**: 1971–80.
47 Charo J, Finkelstein SE, Grewal N, Restifo NP, Robbins PF, Rosenberg SA. Bcl-2 overexpression enhances tumor-specific T-cell survival. *Cancer Res* 2005; **65**: 2001–8.
48 Topp MS, Riddell SR, Akatsuka Y, Jensen MC, Blattman JN, Greenberg PD. Restoration of CD28 expression in CD28- CD8+ memory effector T cells reconstitutes antigen-induced IL-2 production. *J Exp Med* 2003; **198**: 947–55.
49 Stephan MT, Ponomarev V, Brentjens RJ *et al.* T cell-encoded CD80 and 4-1BBL induce auto- and transcostimulation, resulting in potent tumor rejection. *Nat Med* 2007; **13**: 1440–9.
50 Borkner L, Kaiser A, van de Kasteele W *et al.* RNA interference targeting programmed death receptor-1 improves immune functions of tumor-specific T cells. *Cancer Immunol Immunother* 2010; **59**: 1173–83.
51 Perfetto SP, Chattopadhyay PK, Roederer M. Seventeen-colour flow cytometry: unravelling the immune system. *Nat Rev Immunol* 2004; **4**: 648–55.
52 Chan KS, Kaur A. Flow cytometric detection of degranulation reveals phenotypic heterogeneity of degranulating CMV-specific CD8+ T lymphocytes in rhesus macaques. *J Immunol Methods* 2007; **325**: 20–34.
53 Darrah PA, Patel DT, De Luca PM *et al.* Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat Med* 2007; **13**: 843–50.
54 De Rosa SC, Lu FX, Yu J *et al.* Vaccination in humans generates broad T cell cytokine responses. *J Immunol* 2004; **173**: 5372–80.
55 Casazza JP, Betts MR, Price DA *et al.* Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. *J Exp Med* 2006; **203**: 2865–77.
56 Betts MR, Nason MC, West SM *et al.* HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 2006; **107**: 4781–9.
57 Precopio ML, Betts MR, Parrino J *et al.* Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J Exp Med* 2007; **204**: 1405–16.
58 Beveridge NE, Price DA, Casazza JP *et al.* Immunisation with BCG and recombinant MVA85A induces long-lasting, polyfunctional mycobacterium tuberculosis-specific CD4+ memory T lymphocyte populations. *Eur J Immunol* 2007; **37**: 3089–100.
59 Almeida JR, Price DA, Papagno L *et al.* Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J Exp Med* 2007; **204**: 2473–85.
60 Duvall MG, Precopio ML, Ambrozak DA *et al.* Polyfunctional T cell responses are a hallmark of HIV-2 infection. *Eur J Immunol* 2008; **38**: 350–63.