(OKT3, eBioscience) in round-bottom 96-well plates. $CD4^+CD25^{high}$ Treg cells (highest 3% of $CD4^+CD25^+$ cells) were purified with FACSAria (BD Biosciences), and graded numbers of them added in the culture as indicated in figure legends. Proliferation was evaluated by ^{3}H-thymidine with 1 μCi/well for the last 18 h of 6-day culture. ^{3}H-thymidine incorporation was measured by a scintillation counter.

ELISPOT (enzyme-linked immunospot) assay

The number of IFN-γ secreting antigen-specific $CD4^+$ T cells was assessed by ELISPOT assays as described [20, 21]. Briefly, flat-bottomed, 96-well nitrocellulose-coated microtiter plates (Millipore, Bedford, MA, USA) were coated with anti-IFN-γ Ab (1-D1K; MABTECH, Stockholm, Sweden). The presensitized T cells and phytohaemagglutinin (PHA HA15; Murex Diagnostics, Dartford, UK) activated $CD4^+$ T cells, EBV-transformed human B lymphocytes or DCs pulsed with 10 μM of peptides or 25 μg/mL protein overnight were added to each well and incubated for 24 h. Spots were developed using biotinylated anti-IFN-γ Ab (7-B6–1-biotin; MABTECH), alkaline phosphatase conjugated streptavidin (Roche, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma) and counted with C.T.L. Immunospot analyzer and software (Cellular Technologies, Cleveland, OH, USA).

Preparation of monocyte-derived DCs

Monocyte-derived DCs were generated from PBMCs as previously described with some modifications [51]. Briefly, $CD14^+$ monocytes were enriched by positive selection using CD14 Microbeads (Miltenyi Biotec). Monocytes were cultured in the presence of 20 ng/mL GM-CSF (Immunex, Seattle, WA, USA) and 20 ng/mL IL-4 (R&D systems) in RPMI1640 supplemented with 2.5% fetal calf serum. Medium was replaced by fresh medium containing cytokines 3 days later. On day 6, cells were harvested and used for subsequent experiments.

ELISA

The concentration of IL-12p70 and IL-10 was measured by ELISA Kit (eBioscicence) according to the instruction provided by the manufacturer.

Statistical analysis

Statistical significance was evaluated by Student's *t*-test; *p* values less than 0.05 are considered significant.

Acknowledgments: This article is dedicated to the memory of Lloyd J. Old, M.D. We thank Drs. T. Takahashi and J. B. Wing for critical reading of the manuscript, and L. Wang, C. Brooks, E. Krapavinsky, E. Ritter, and D. Santiago for technical support.
This study was supported by Grant-in-Aid for Scientific Research on Priority Areas (No. 17016031, H. Shiku, and No. 20015019, H. Nishikawa) and Grants-in-Aid for Scientific Research (B) (No. 23300354, 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), Takeda Science Foundation (H. Nishikawa), Kato Memorial Bioscience Foundation (H. Nishikawa), the Sagawa Foundation for Promotion of Cancer Research (H. Nishikawa), and Senri Life Science Foundation (H. Nishikawa). MH is a research fellow of the Japan Society for the Promotion of Science.

Conflicts of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviation: CHP: cholesteryl hydrophobized pullulan

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Received: 6/7/2012
Revised: 7/12/2012
Accepted: 28/1/2013
Accepted article online: 4/2/2013

Cancer Immunity (15 January 2013) Vol. 13, p. 3

Cancer Immunity
Article

A novel human-derived antibody against NY-ESO-1 improves the efficacy of chemotherapy

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We investigated whether antibodies against intracellular tumor-associated antigens support tumor-specific immunity when administered together with a treatment that destroys the tumor. We propose that released antigens form immune complexes with the antibodies, which are then efficiently taken up by dendritic cells. We cloned the first human monoclonal antibodies against the Cancer/Testis (CT) antigen, NY-ESO-1. We tested whether the monoclonal anti-NY-ESO-1 antibody (12D7) facilitates cross-presentation of a NY-ESO-1-derived epitope by dendritic cells to human CD8+ T cells, and whether this results in the maturation of dendritic cells *in vitro*. We investigated the efficacy of 12D7 in combination with chemotherapy using BALB/c mice bearing syngeneic CT26 tumors that express intracellular NY-ESO-1. Human dendritic cells that were incubated with NY-ESO-1:12D7 immune complexes efficiently stimulated NY-ESO-1$_{157\text{-}165}$/HLA-A2-specific human CD8+ T cells to produce interferon-γ, whereas NY-ESO-1 alone did not. Furthermore, the incubation of dendritic cells with NY-ESO-1:12D7 immune complexes resulted in the maturation of dendritic cells. Treatment of BALB/c mice that bear CT26/NY-ESO-1 tumors with 5-fluorouracil (5-FU) plus 12D7 was significantly more effective than chemotherapy alone. We propose systemic injection of monoclonal antibodies (mAbs) against tumor-associated antigens plus a treatment that promotes the local release of those antigens resulting in immune complex formation as a novel therapeutic modality for cancer.

Keywords: NY-ESO-1, antibody, chemotherapy

Introduction

Cancer/Testis (CT) antigens form an extended family of proteins that are frequently expressed in a large variety of malignancies but are absent from healthy tissue, except for the testis and placenta. Cancer patients often develop spontaneous immune responses toward CT antigens, which illustrate their immunogenicity (1-3). Their apparent immunogenicity and unique expression pattern make CT antigens attractive targets for immunotherapy, and a number of clinical trials in which cancer patients were immunized with CT antigens in different forms have been completed, some of which show objective clinical responses (4-12).

Dendritic cell (DC) maturation is a key prerequisite for the activation of T cells, and moreover, antigen presentation by steady-state DCs results in peripheral tolerance induction, a process that is considered crucial for the protection against autoimmunity (13, 14). DC maturation usually is induced by infection or inflammation—or by adjuvants for that matter—and can be a local event. Insufficient maturation of tumor-associated DCs may be one of multiple reasons for the compromised response of tumor-infiltrating T cells compared to peripheral T cells (15, 16). Cross-presentation of sufficient amounts of tumor-derived antigens may be another limiting factor, especially because the number of tumor-associated DCs often is low and cross-presentation is inefficient (17, 18). Therefore, we developed a novel immunotherapeutic approach that combines enhanced cross-presentation of epitopes derived from intracellular proteins with concomitant DC maturation. We hypothesized that administration of monoclonal antibodies (mAbs) against CT antigens together with a therapy that releases these usually intracellular antigens may support the local formation of immune complexes, which are efficiently taken up by DCs (19, 20) resulting in increased presentation of CT antigen-derived epitopes to CD8+ T cells. Because there is evidence that the uptake of immune complexes by DCs through the activating receptor for IgG (FcγRIIA) results in DC maturation (21), the use of mAbs against CT antigens may serve both purposes: DC activation and enhanced cross-presentation.

The fact that NY-ESO-1 is one of the best-characterized and most immunogenic CT antigens known to date (22, 23) and is frequently expressed by tumors of different origin (6, 24) prompted us to clone human-derived mAbs against NY-ESO-1 from patients who had high serum levels of NY-ESO-1-specific IgG and, thus, presumably a high frequency of NY-ESO-1-specific B cells. The obvious advantage of cloning a therapeutic antibody from humans is that adverse side effects of such an antibody are very unlikely and that it therefore can relatively be quickly tested in clinical trials. We report here the generation of the first human-derived IgG1 mAbs against NY-ESO-1 and the selection of a lead development candidate (12D7). We show that 12D7 facilitates cross-presentation of a NY-ESO-1-derived epitope to CD8+ T cells, that 12D7:NY-ESO-1 immune

complexes induce the maturation of human monocyte-derived DCs *in vitro*, and that 12D7 significantly enhances the therapeutic efficacy of chemotherapy using a preclinical syngeneic mouse model.

Table 1

Binding of human monoclonal anti-NY-ESO-1 antibodies to NY-ESO-1. Comparison of EC_{50} and equilibrium affinity constants for the binding between NY-ESO-1 and different anti-NY-ESO-1 antibodies.

Antibody	EC_{50} [pM] (prok. NY-ESO-1)	K_D [M] (prok. NY-ESO-1)	K_D [M] (euk. NY-ESO-1)
12D7	1.14	$2.08x10^{-10}$	$1.56x10^{-10}$
1D4	2.23	$1.62x10^{-9}$	$2.24x10^{-10}$
30D6	1.09	$4.35x10^{-9}$	$2.65x10^{-9}$
31E4	9.52	$1.9x10^{-8}$	$2.23x10^{-8}$
15B12	72.6	---	---
E978 control	6.66	$2.56x10^{-8}$	$1.56x10^{-11}$

Results

Cloning of human-derived monoclonal antibodies from cancer patients

We cloned eight different NY-ESO-1-specific human-derived monoclonal antibodies (HD mAbs) from a melanoma patient, of which the following five were selected for further analysis based on their affinity to the target: 1D4, 12D7, 15B12, 30D6, and 31E4. All HD mAbs were of IgG1 isotype.

In vitro characterization of HD mAbs

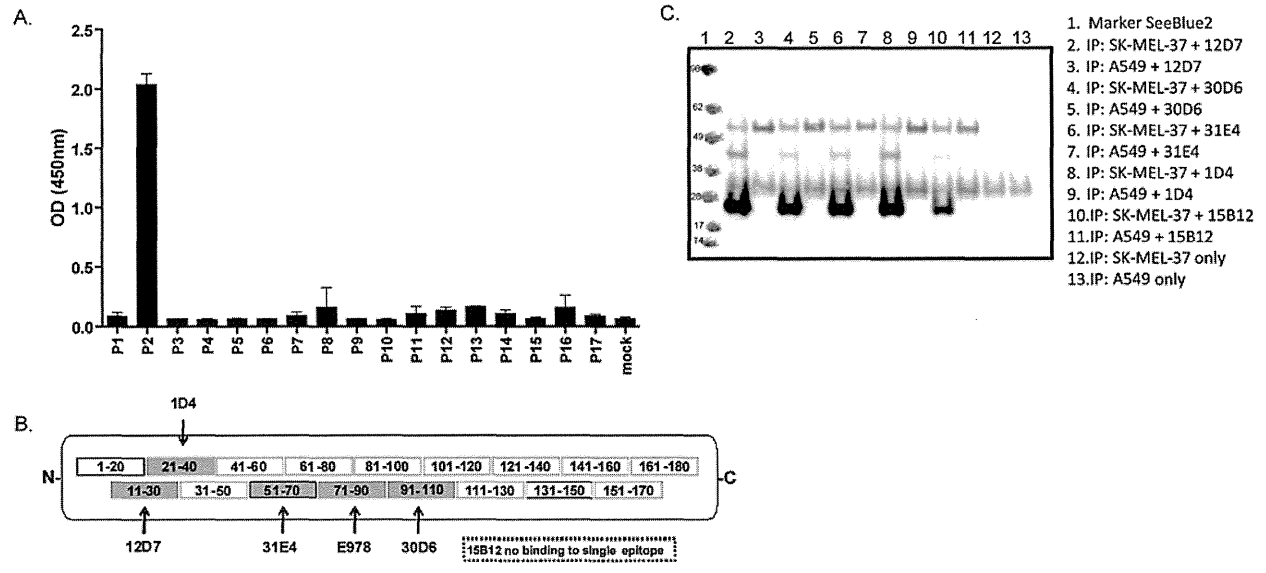
To compare the binding properties of five different anti-NY-ESO-1 HD mAbs to recombinant NY-ESO-1 protein, we determined the half-maximal effective concentration (EC_{50}) using a protein ELISA. All antibodies bound recombinant NY-ESO-1 produced in bacteria in the low pM range. Actual binding constants to recombinant NY-ESO-1 produced in bacteria and in eukaryotic cells were determined by surface plasmon resonance (Biacore Systems) (Table 1).

To determine the epitopes recognized by the different mAbs, we used a set of overlapping peptides spanning the complete NY-ESO-1 protein as coating antigen in ELISA. As shown in Figure 1A, 12D7 binds to a peptide representing the amino acids 11 to 30 from the NY-ESO-1 protein, but not to the two adjacent peptides that span amino acids 1-20 or 21-40. This suggests that the epitope recognized by 12D7 lies at the junction of these two peptides around amino acid 20 of NY-ESO-1. Figure 1B summarizes the epitope-specificity of all five anti-NY-ESO-1 antibodies. In addition, all antibodies were tested for binding to endogenous NY-ESO-1 from the human melanoma cell line SK-MEL-37 by immunoprecipitation. All antibodies precipitate NY-ESO-1 from a cell lysate of an NY-ESO-1+ cell line (SK-MEL-37) (Figure 1C). Because 12D7 had the highest affinity for eukaryotic NY-ESO-1, we performed further experiments with this mAb.

12D7 facilitates cross-presentation of NY-ESO-1 by DCs and induces concomitant DC maturation

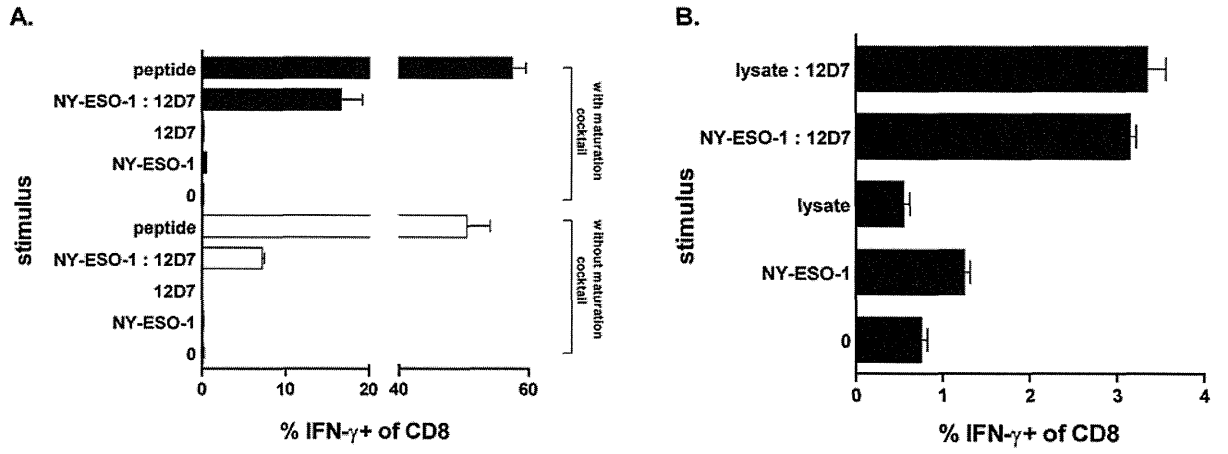
To test whether 12D7 facilitates the cross-presentation of NY-ESO-1-derived epitopes *in vitro*, we generated monocyte-derived, HLA-A*0201+ DCs and fed them with 12D7:NY-ESO-1 immune complexes, NY-ESO-1, or 12D7. DCs were subsequently incubated with cloned NY-ESO-$1_{157-165}$/HLA-A*0201-specific CD8+ T cells, and the percentage of T cells that produced IFN-γ was used as readout for antigen recognition. Mature DCs fed with NY-ESO-1 protein induced IFN-γ production in a low but discernible percentage of T cells (Figure 2A, black bars), which did not occur when DCs were not matured (Figure 2A, white bars). DCs fed with 12D7:NY-ESO-1 immune complexes induced the production of IFN-γ in a much

Figure 1



Epitope mapping of anti-NY-ESO-1 human monoclonal antibodies. (A) Representative peptide ELISA for antibody 12D7, where P1-P17 represent overlapping NY-ESO-1 peptides. (B) Overview of the specificities of different NY-ESO-1 specific human-derived mAbs. (C) Immunoprecipitation of NY-ESO-1 from a cell lysate of a NY-ESO-1+ cell line SK-MEL-37 or a NY-ESO-1- cell line A549 by human anti-NY-ESO-1 mAbs.

Figure 2



Human monoclonal anti-NY-ESO-1 antibody (12D7) facilitates cross-presentation of a NY-ESO-1-derived, HLA-A2-restriced epitope (NY-ESO-$1_{157-165}$). (A) HLA-A2+, monocyte-derived DCs were incubated with 20 μg NY-ESO-1 protein, 200 μg human monoclonal anti-NY-ESO-1 antibody (12D7), with immune complexes (12D7:NY-ESO-1) or with media for 3 h, were washed and cultured for 36 h with (black bars) or without (white bars) 25 ng/mL TNF-α + 1 μg/mL sCD40L (maturation cocktail). 6 x 10^4 cloned, NY-ESO-$1_{157-165}$ /HLA-A2-specific CD8+ T cells were added to 10^5 DCs in the presence of 10 μg/mL Brefeldin A, followed by a 5 h incubation and subsequent surface staining for CD8 and intracellular staining for IFN-γ. 10^{-6} M peptide was added to DCs as positive control. All cultures were performed in triplicate. (B) HLA-A2+, monocyte-derived DCs were incubated with 20 μg NY-ESO-1 protein, 200 μg human monoclonal anti-NY-ESO-1 antibody (12D7), with lysate of 10^7 NY-ESO-1+ SK-MEL-37 cells (lysate), with immune complexes (NY-ESO-1:12D7 or lysate:12D7), or with media (0) for 3 h, were washed and cultured for 36 h with 25 ng/mL TNF-α + 1 μg/mL sCD40L (maturation cocktail). 6 x 10^4 cloned, NY-ESO-$1_{157-165}$ /HLA-A2-specific CD8+ T cells were added to 10^5 DCs in the presence of 10 μg/mL Brefeldin A, followed by a 5 h incubation and subsequent surface staining for CD8 and intracellular staining for IFN-γ. All cultures were performed at least in duplicate.

higher percentage of T cells and, importantly, also did so when DCs that were not deliberately matured were used (Figure 2A, compare black and white bars). None of the negative controls—DCs fed with 12D7, mock immune complexes, or medium—induced IFN-γ production (Figure 2A and data not shown). To exclude that our observations are a peculiarity of recombinant NY-ESO-1, we incubated 12D7 with a cell lysate of SK-MEL-37 cells, which naturally express NY-ESO-1, and subsequently fed this mixture to DCs. DCs fed with the 12D7:lysate or with 12D7:NY-ESO-1 presented NY-ESO-1-derived epitopes approximately equally well (Figure 2B).

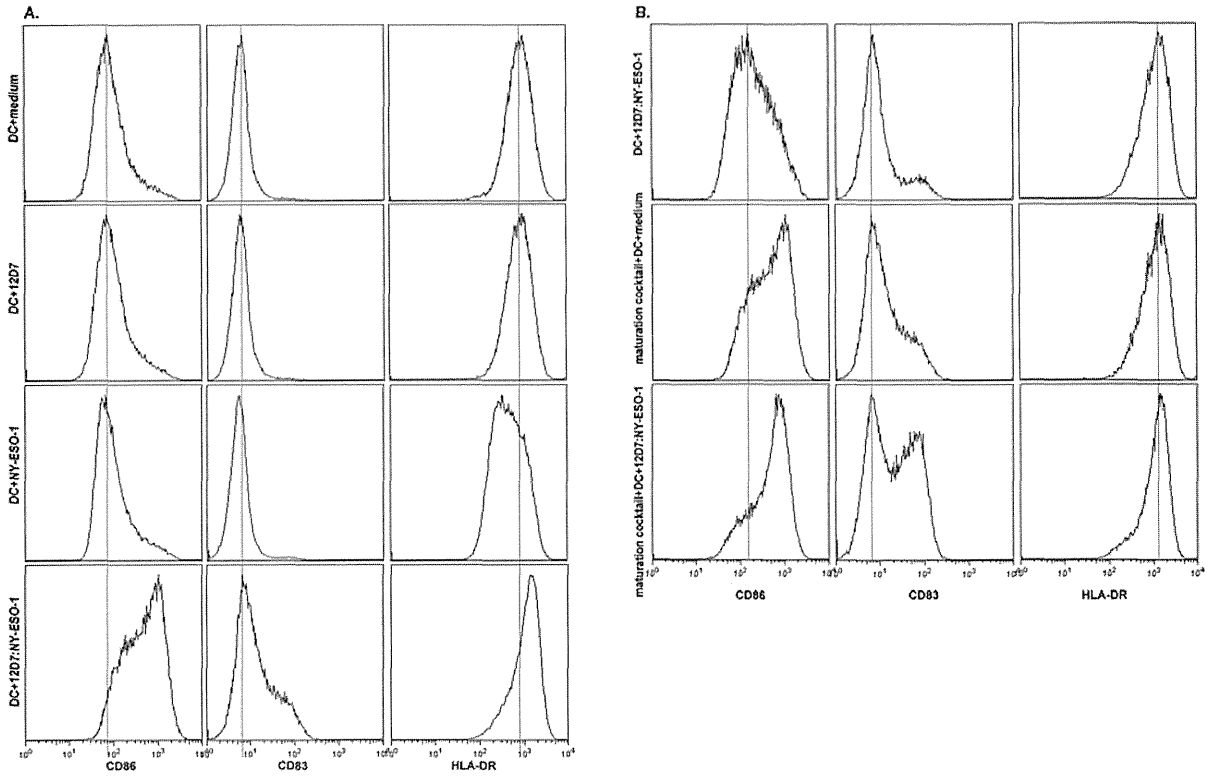
Because presentation of 12D7:NY-ESO-1 immune complexes seemed not to require deliberate DC maturation, we addressed whether the uptake of immune complexes, but not the uptake of uncomplexed protein, induced DC maturation *in vitro*. We therefore compared the expression of three surface molecules that are upregulated on mature DCs (CD83, CD86, and MHC class II) after incubation with media, 12D7, NY-ESO-1, or with 12D7:NY-ESO-1 immune complexes in the absence of maturation cocktail. We found that only immune complexes induced an upregulation of CD86, CD83, and MHC II (Figure 3A; left, middle, and right panels, respectively). We then compared the expression of CD83, CD86, and MHC II on DCs that were incubated with the maturation cocktail, with immune complexes, or with both, in order to determine the relative potency of immune complexes with respect to DC maturation. We found that immune complexes were almost as potent in inducing DC maturation as the classical maturation cocktail (sCD40L plus TNF-α) (Figure 3B). A combination of immune complexes plus maturation cocktail resulted in the most pronounced upregulation of CD86 and CD83 (Figure 3B; left and middle panels, respectively), whereas MHC II was not further upregulated compared to any of the two treatments alone (Figure 3B, right panels).

12D7 increases the therapeutic efficacy of chemotherapy in mice with NY-ESO-1+ tumors

To test the therapeutic efficacy of 12D7 *in vivo*, we injected 10^6 syngeneic, NY-ESO-1-transfected CT26 tumor cells s.c. in BALB/c mice. To induce release of intracellular NY-ESO-1, mice were treated with 75 mg/kg 5-FU when tumors reached a size of approximately 25 mm^2, which was typically around 2 weeks after injection of tumor cells. The treatment with 5-FU was repeated one week later and, in some groups, was combined with 100 μg 12D7 given systemically 2 d after each 5-FU injection. As can be seen from the growth curves, 5-FU has the expected therapeutic effect. Importantly, this was enhanced by 12D7 (Figure 4A). Treatment with 12D7 alone had no effect, presumably because the amount of spontaneously released antigen is not sufficient in this particular model. A compilation of end-point tumor sizes from 4 independent experiments shows a highly significant difference between mice treated with 5-FU plus 12D7, and mice treated with 5-FU alone (Figure 4B).

To investigate whether treatment with 5-FU plus 12D7 supported tumor-specific immunity, we injected mice with Brefeldin A 4 h before euthanasia, followed by staining for CD45.2, CD8, and intracellular IFN-γ. This way of analysis shows which cells actually are making IFN-γ *in vivo* and not which cells potentially can do this upon *in vitro* restimulation with peptide. This method obviously does not allow discrimination between single peptide specificities, but it is of higher biological relevance (25) particularly because we envisaged that DC activation, which we have shown to occur upon cross-presentation (Figure 3), may also support the presentation of other epitopes besides those derived from NY-ESO-1. Treatment with 5-FU plus 12D7 supported CD8+ and

Figure 3



Immune complexes induce maturation of monocyte-derived DCs *in vitro*. (A) CD14-derived DCs were incubated with media, 200 μg NY-ESO-1, 20 μg 12D7 or preformed immune complexes of 20 μg 12D7 + 200 μg NY-ESO-1, and were analyzed 36 h later for surface expression of CD86, CD83, or MHC II. (B) CD14-derived DCs were incubated with preformed immune complexes of 20 μg 12D7 + 200 μg NY-ESO-1, maturation cocktail (sCD40L + TNF-α) or with preformed immune complexes plus maturation cocktail, and were analyzed 36 h later for surface expression of CD86, CD83, or MHC II.

effector function in the tumor (Figure 4C). Treatment with 5-FU (Figure 4C) or 12D7 (data not shown) did not have this effect.

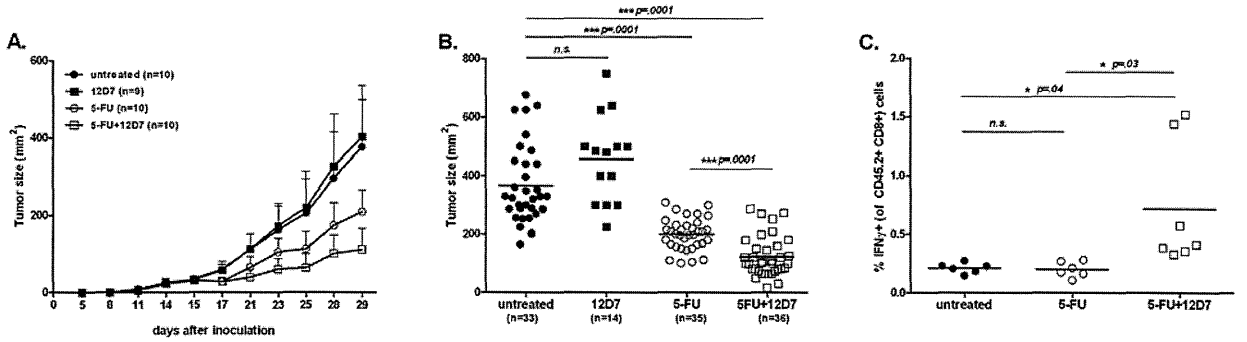
Discussion

We hypothesized that antibodies against intracellular, tumor-associated antigens support tumor-specific immunity when used in combination with a therapy that induces cell death such as chemo- or radiotherapy. We envisaged that such antibodies form immune complexes with the released tumor antigens. These immune complexes are subsequently taken up with higher efficiency compared to protein (fragments) by DCs (26), which then cross-present relevant epitopes to local CD8+, tumor-specific T cells. This presumed sequence of events may be of particular interest as evidence is accumulating that both chemo- and radiotherapy support tumor-specific immunity (27), and we therefore reasoned that additional stimulation of tumor-specific immunity could further improve the efficacy of these standard therapies.

For this purpose, we have cloned the first fully human mAbs to NY-ESO-1 using Epstein-Barr virus (EBV)-transformed B cells from a melanoma patient and subjected those to preclinical experiments to obtain proof of principle. We found that 12D7, a fully human IgG1 mAb specific for the immunogenic CT antigen NY-ESO-1, supported cross-presentation of NY-ESO-1 *in vitro* resulting in an approximate 15-fold increase of the number of responding CD8+ T cells. Of the other four NY-ESO-1-specific mAbs we generated here, 1D4 and 30D6 improved cross-presentation of NY-ESO-1 (data not shown), whereas 15B12 and 31E4 seemed not effective (data not shown). This difference may be explained by the difference in affinity, as 15B12 did not show binding to NY-ESO-1 by Biacore—although it did bind weakly to NY-ESO-1 in ELISA—and 31E4 had at least a 1-log lower affinity than 12D7, 1D4, and 30D6. At present, we have no reason to think that the epitope recognized by the mAb impacts on its ability to support cross-presentation. Our observation that 12D7:NY-ESO-1 immune complexes are considerably less efficient than peptide-loaded DCs in stimulating IFN-γ production illustrates that cross-presentation is a rather inefficient process, but underscores the therapeutic potential of antibodies against tumor-associated antigens.

It is well accepted now that activation of T cells *in vivo* crucially depends on antigen presentation by mature or activated DCs (14, 28). Many cues, including inflammation and infection but also endogenous signals, can induce DC maturation (29), and the lack of such signals in the tumor environment may be one reason why tumor-infiltrating T cells often have compromised functions (16, 30). Because the uptake of immune complexes was shown to result in DC maturation (19), we specifically addressed this issue here. We found that the *in vitro* uptake of immune complexes resulted in DC maturation that was comparable to sCD40L plus TNF-α, which is a classical maturation cocktail. Therefore, the use of mAbs against CT antigens may serve both purposes: DC activation and enhanced

Figure 4



A human, monoclonal anti-NY-ESO-1 antibody (12D7) increases the therapeutic efficacy of 5-FU chemotherapy in mice bearing NY-ESO-1+ syngeneic tumors. Female BALB/c mice were injected s.c. with 10^6 CT26/NY-ESO-1+ cells and treatment was started when tumors reached a surface of approximately 25 mm^2 (~ d13-15). (A) Mice received 75 mg/kg 5-FU i.p. at days 15 and 22 and/or 100 μg 12D7 i.p. on days 17 and 24. The results are shown as mean±SD. A representative experiment of 4 experiments is shown. (B) Compilation of 4 independent experiments, each symbol represents the tumor surface of an individual mouse at the end of the experiment (d 29). (C) Mice were injected i.p. 1 week after the last injection with 12D7 (d 29) with 250 μg Brefeldin A and were euthanized 4 h later. Processing of tumors and staining with antibodies for CD45.2, CD8 (surface), and IFN-γ (intracellular) was performed in the presence of 10 μg/mL Brefeldin A. Each symbol represents values from individual mice at the end of the experiment.

cross-presentation at the relevant anatomic location. This is not trivial, as systemic activation of DCs may not be without risk as systemic side effects such as the release of cytokines or autoimmunity may ensue (31, 32).

We found that 12D7 improved the efficacy of chemotherapy in a preclinical mouse model of transplanted, syngeneic NY-ESO-1-expressing tumors, thus supporting our concept. Further support comes from the fact that more CD8+ T cells infiltrate the tumor and that those cells have increased effector function. By itself, however, 12D7 had no therapeutic effect, suggesting that the amount of released tumor antigen is limiting without deliberate destruction of the tumor. Our *in vivo* experiments require the binding of human IgG to mouse Fcγ receptors (FcγR), which was previously described (33, 34). Improved efficacy of chemotherapy by the use of tumor-associated antigen-specific antibodies will presumably work for chemotherapies especially, which are not immunosuppressive or—even more important—promote immunogenic cell death (35).

We propose the concept of antibody-facilitated T cell induction in cancer (AFTIC) as a novel type of immunotherapy. AFTIC is based on the application of mAbs against tumor-associated antigens, including CT antigens, plus a treatment that promotes the local release of those antigens, such as chemo- or radiotherapy. The locally released antigens and the mAb form immune complexes, which facilitate the uptake and subsequent presentation of antigen-derived peptides by tumor-associated DCs. As the uptake of immune complexes induces concomitant maturation of DCs, AFTIC supports boosting as well as *de novo* activation of tumor-specific CD8+ T cells. Furthermore, administration of antibodies against a particular tumor-associated antigen may promote the presentation of the same antigen when administrated as a cancer vaccine, thereby improving the efficacy of immunotherapy. Alternatively, better antigen presentation of immune complexes and concomitant DC maturation may support the activity of adoptively transferred T cells provided they have the same antigen specificity as the therapeutic antibodies.

Abbreviations

CT, Cancer/Testis; DC, dendritic cell; mAb, monoclonal antibody

Acknowledgements

We thank Alexandre Ruffieux and Victor Escalante (Institute of Laboratory Animal Science, University of Zürich) for expert animal care. This work was supported by Atlantic Philanthropies (New York, USA), the Cancer Research Institute, the Science Foundation for Oncology (Zürich), the Ellinger Foundation Zürich, the Hartmann Müller Foundation Zürich, and the Hanne Liebermann Foundation (Zürich).

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Materials and methods

Patient material

Serum and peripheral blood was collected from cancer patients. All patients were admitted at the University Hospital Zürich and provided written informed consent in accordance with the Declaration of Helsinki. The local ethics committee approved the study.

Memory B cell culture

PBMC were incubated with anti-CD22 coupled to magnetic beads (Miltenyi Biotec), PE-conjugated anti-IgD, and APC-conjugated antibodies to IgM, CD3, CD8, and CD56 (Becton Dickinson). B cells were isolated by positive selection of CD22+ cells using a midi-MACS device and LS columns (Miltenyi Biotec), followed by sorting PE-APC- cells using a MoFlo cell sorter (Beckman Coulter). CD22+ IgD- IgM- memory B cells were incubated with 10% EBV-containing supernatant from B95-8 cells (from European Collection of Cell Cultures, ECACC) in the presence of 2.5 μg/mL CpG 2006 at 37°C for 4 h. Cells were seeded in 96-well U-bottom plates at 10 cells per well plus 3 x 10^4 irradiated allogeneic PBMCs in RPMI 1640 medium supplemented with 10% human serum, antibiotics, 10% supernatant from B95-8 cells, and 2.5 μg/mL CpG 2006. Supernatants were tested for NY-ESO-1-specific antibodies after 2 weeks by ELISA.

Single cell-RT-PCR

B cell cultures were harvested and single cells were deposited into a 96-well PCR plate (Applied Biosystems) using a MoFlo XDP cell sorter (Beckman Coulter). RT-PCR was performed using random hexamer primers for cDNA synthesis and specific primers to amplify the immunoglobulin variable and constant regions. Immunoglobulin heavy and light chain variable regions were amplified using a nested PCR approach as described (36). Primer-encoded amino acid sequences and J-C regions of the antibodies were corrected to represent the authentic amino acid sequence as it occurred in the patient in a subsequent step prior to antibody production.

Antibody production and purification

293-T human embryonic kidney cells were transfected with 25 kDa branched polyethylenimine (PEI, Polysciences, Warrington, PA) plus DNA plasmids (heavy and light chain in equal ratios) in a 1.3:1 ratio and were incubated for 15 min at room temperature. Following transfection, the cells were cultured in serum free Opti-MEM I + GlutaMAX-I (Invitrogen) supplemented with 10 U penicillin-streptomycin (Lonza, Switzerland). After 72 h supernatants were collected and IgG was purified on a protein A column (GE Healthcare, Sweden) using FPLC (GE Healthcare, Sweden).

Biacore analysis

Antibody binding kinetics with NY-ESO-1 proteins derived from *E. coli* (LICR New York Branch) and HEK293 cells (OriGene Technologies, Inc.) were determined by Biacore technology (model Biacore 2000; Biacore AB) using CM5 sensor

chips, EDC-NHS conjugation, and BIAevaluation software. Technical details have been described previously (37).

ELISA

- Protein or peptide ELISA

96-well half-area microtiter plates (Costar, USA) were coated with 30 µL/well of 1 µg/mL recombinant NY-ESO-1 protein, or 10 µg/mL 20-mer peptides spanning the entire NY-ESO-1 protein (Peptides & Elephants, Germany) diluted in PBS overnight at 4°C. After coating, plates were washed with PBS + 0.05% Tween-20 (PBS-T) and blocked for 1 h at room temperature with 2% BSA/PBS (Sigma). B cell-conditioned medium, patient serum, or recombinant antibody was incubated for 2 h at room temperature (RT) at indicated concentrations or dilutions in PBS. Plates were washed with PBS-T and incubated for 1 h at RT with HRPO-conjugated goat-anti-human Fcγ antibody (Jackson ImmunoResearch), diluted 1:4000 in 0.5% BSA/PBS, followed by measurement of the HRPO activity using a TMB substrate solution (Sigma, Buchs, Switzerland). The mouse IgG1 monoclonal anti-NY-ESO-1 antibody E978 (38) and HRPO-conjugated goat-anti-mouse Fcγ antibody (Jackson ImmunoResearch) at 1:4000 dilution in 0.5% BSA/PBS served as positive control.

- Cellular ELISA

4 x 10^4 SK-MEL-37 cells were seeded in a 96-well flat bottom plate and cultured under standard conditions overnight. Cells were fixed in ice-cold ethanol/acetone mix (1:1) for 15 min on ice. After two wash steps with PBS, cells were blocked and permeabilized with 100 µL of PBS + 0.5% BSA + 0.5% Triton X 100 for 2 h at 4°C. B cell-conditioned medium or recombinant antibody was incubated at indicated concentrations for 2 h at 4°C. Bound antibodies were detected after 1 h incubation at 4°C with HRPO-labeled goat anti-human Fc secondary antibody (Jackson ImmunoResearch).

Immunoprecipitation

SK-MEL-37 tumor cells were lysed with Triton X 100/Glycerol-based lysis buffer for 15 min at 4°C. Cell debris was separated by centrifugation at maximum speed in a table centrifuge and protein concentration of the supernatant analyzed by standard Bradford assay. 300 ng of antibody was used to precipitate NY-ESO-1 from 250 µg of SK-MEL-37 cell lysate in a 16 h incubation at 4°C. The immune complex was isolated by adding magnetic Protein G beads (New England Biolabs, Ipswich, MA) for 1 h at 4°C under constant agitation. Beads were washed, resuspended in NuPAGE LDS sample buffer (Invitrogen) and boiled prior to Gradient SDS Polyacrylamide Gel Electrophoresis (NuPAGE 4-12% Bis-Tris Gel, Invitrogen). NY-ESO-1 protein was detected by Western blot using murine antibody E978 (38).

***In vitro* cross-presentation assay**

Human, HLA-A*0201/NY-ESO-$1_{157\text{-}165}$ specific CD8+ T cells were cloned as previously described (39). To generate DCs, CD14+ cells were MACS-purified according to the manufacturer's instructions (Miltenyi Biotech) from PBMC from HLA-A*0201+ healthy donors and cultured at 10^6 cells/mL in serum-free CellGro DC media (CellGenix), supplemented with 800 U/mL GM-CSF and 500 U/mL IL-4 (R&D Systems) to generate DCs. Medium was exchanged the following day and DCs were harvested on d 4 of culture and resuspended at 10^6/mL in Opti-MEM (Gibco). Immune complexes were generated by incubating 20 µg recombinant NY-ESO-1 with 200 µg 12D7 in a total volume of 500 µL Opti-MEM (Gibco) for 30 min at 37°C. Human IgG1 (Sigma Aldrich), 12D7 alone, or NY-ESO-1 alone were used as controls. Alternatively, 200 µg 12D7 was incubated with a lysate of an equivalent of 10^7 NY-ESO-1+ SK-MEL-37 cells in 500 µL Opti-MEM. DCs (5x10^5 in 0.5 mL Opti-MEM) were added to the immune complexes and controls. The mixture was incubated at 37°C for 3 h. DCs were then centrifuged and resuspended in CellGro DC media at 10^6/mL. Hundred µL (10^5 DCs) were cultured in 96-well flatbottom plates at 37°C in the presence or absence of maturation cocktail (1 µg/mL soluble CD40L (sCD40L) trimer (PeproTech) plus TNF-α (25 ng/mL; R&D Systems)). After 36 h, approximately 6 x 10^5 HLA-A*0201/NY-ESO-$1_{157\text{-}165}$ specific CD8+ T cells in 100 µL RPMI + 10% human serum + antibiotics + 20 µg/mL Brefeldin A (Sigma Chemicals) were added to the different DC-cultures. After 4 h, cultures were harvested in FACS buffer (PBS + 2% FCS + 2 mM EDTA + 0.05% NaN_3) and surface stained with anti-CD8 followed by intracellular staining for IFN-γ as previously described (39). CD8+ T cells plus unloaded DCs served as negative control, and CD8+ T cells plus DCs with 10^{-6} M of NY-ESO-$1_{157\text{-}165}$ (SLLMWITQC, Thermo Fisher Scientific) served as positive control. All cultures were performed at least in duplicates.

Mice and cell lines

BALB/c mice were originally obtained from Jackson Laboratories and were bred and kept under specific pathogen-free conditions in the Institute of Laboratory Animal Sciences (University of Zürich). Age- and sex-matched mice of 9-12 weeks old were used for all experiments. Mice were housed under specific pathogen-free conditions at University Hospital Zürich. All experiments were performed in agreement with the federal and cantonal laws on animal protection.

The colon carcinoma cell line CT26 was transfected to stably express intracellular NY-ESO-1 (40) and was cultured in RPMI + 10% FCS + antibiotics + 10 µg/mL puromycin under standard tissue culture conditions. CT26/NY-ESO-1 and the human melanoma cell line SK-MEL-37 were cultured in RPMI + 10% FCS + antibiotics under standard tissue culture conditions. 293-T cells were cultured in DMEM (Lonza, Switzerland) supplemented with 10% FCS (Linaris) and 10 U penicillin-streptomycin (Lonza, Switzerland) under standard tissue culture conditions.

Treatment of mice

Mice were injected s.c. into the right flank with 10^6 CT26/NY-ESO-1+ cells in 100 µL RPMI. The tumor surface was measured at least twice a week with a calliper. Treatment was started (d 0) when tumors reached a size of approximately 25 mm^2. 5-Fluorouracil (5-FU, TEVA Pharma, Aesch, Switzerland) was diluted in saline and were given i.p. on d 0 and d 7 at 75 mg/kg, respectively. 12D7 (100 µg in 100 µL PBS) was given i.p. on d 2 and d 9. All animal experiments were performed in accordance with the Swiss federal and cantonal law on animal protection.

Flow cytometry

At the end of the experiment (1 week after the last injection of 12D7), mice were injected i.p. with 250 µg Brefeldin A and were euthanized 4 h later. Subsequent processing and staining was performed in the presence of 10 µg/mL Brefeldin A (25). Tumors were cut into small pieces and subsequently digested with 1.5 mg/mL collagenase + 100 µg/mL DNase for 1 h at 37°C followed by filtration through a 50 µm cell strainer. Single cell suspensions were surface stained in FACS buffer (FB, PBS + 2% FCS + 0.03% NaN_3 + 20 mM EDTA) with anti-CD45.2 pacific

blue and anti-CD8bPE. For intracellular staining to detect IFN-γ, cells were permeabilized with permeabilization buffer (PB, FB + 0.1% saponin) and stained intracellularly with anti-IFN-γ APC. All antibodies were obtained from BioLegend, San Diego, CA, USA. Samples were measured with a CyAn ADP9 (Beckman Coulter, Brea, CA, USA) and analyzed using FlowJo Analysis Software (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

Statistics were done using an unpaired Student two-tailed *t*-test. Error bars represent SD. *p* values less than 0.05 were considered significant.

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Cancer Immunity (1 May 2012) Vol. 12, p. 2

Cancer Immunity
Reflection

Cell surface antigens: invaluable landmarks reflecting the nature of cells

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Keywords: cell surface antigens, leukemia, Lyt antigen, TL antigen, Lloyd Old

Although Lloyd J. Old was involved in various studies of the interactions between cancer and the immune system, it seems to us that his ideas often originated from the studies of serological identification of mouse lymphocyte antigens. The findings from these studies allow us to distinguish cells of different lineages and differentiation stages, and also to distinguish leukemia cells from normal lymphocytes (1). It is amazing that essentially a single serological technique, i.e., the Trypan blue exclusion test (presumably introduced in Peter Gorer's laboratory by Edward A. Boyse), was used to define a series of these antigens. This technique was used in conjunction with absorption analysis to elucidate the specificity of antisera, based on a vast knowledge of mouse immunogenetics. In the laboratory, Elisabeth Stockert (who was a technical assistant at that time) was the great master of these serological techniques, carrying out her own projects of cell surface antigens, as will be described below, while managing the day-to-day business of the laboratory. Similarly, Elizabeth A. Carswell and Gayla Geering investigated tumor necrosis factor (TNF) and retrovirus-associated intracellular antigens, respectively, while working as technical assistants.

When Toshitada Takahashi left Nagoya, Japan, for New York in 1968, the TL and Lyt (Ly) series of antigens of thymocytes and peripheral T cells had already been identified by Drs. Old, Boyse, and Stockert (1-3), and congenic strains of mice for each antigen system had been established. Contemporaneously, Katsuaki Itakura was preparing to initiate genetic linkage studies of these differentiation antigens. In the late 1960s, the concept of T cells and B cells was becoming more and more widespread, and after the First International Immunology Congress in Washington, D.C., in 1971, this concept became widely accepted all over the world. The Thy-1 (Θ, CD90) and Lyt series of antigens are considered to be markers for thymocytes and peripheral T cells, whereas TL is a marker for thymocytes. Thus, Takahashi's project was focused on B cells. When he started his study on cell surface antigen analysis of plasma cell leukemias (PCL), Yoshiyuki Hashimoto kindly taught him how to carry out day-to-day experiments. At that time, Dr. Hashimoto was a visiting investigator from the Biochemistry Institute in Tokyo, Japan, working on cell-mediated immunity against TL-positive leukemias. It is noteworthy that, as the founder of the Japanese Association of Cancer Immunology, Dr. Hashimoto greatly contributed to the progress in tumor immunology in Japan. Dr. Takahashi defined a novel PC.1 alloantigen that is expressed on PCL cells and mature antibody-forming cells (IgG type hemolytic plaque-forming cell, IgG-PFC), but not on peripheral B cells (4). Furthermore, he demonstrated reciprocal expression of surface immunoglobulin, which is expressed on peripheral B cells, but on neither IgG-PFC nor PCL cells. We had to wait until 1973 to see the first report of a B cell alloantigen by Hidetoshi Sato (5), although it is named Lyb-2 (CD72). This protein was later shown to be a ligand for Lyt-1 (CD5) and appears to be present on all B lymphocytes except for plasma cells.

In 1973, Hiroshi Shiku, also from Nagoya, went to the Sloan-Kettering Institute for Cancer Research (SKI) and started working on cell-mediated immunity with Herbert F. Oettgen and Michael A. Bean. Although the chromium release assay had already been established by Cerottini *et al.* and was widely used to target suspension cells, this assay was not always suitable for monolayer cell cultures derived from solid tumors. In order to detect killing activity against attached target cells, Dr. Bean developed a new microassay using [3H]proline for labeling, instead of chromium 51; this assay was used by Dr. Shiku for his phenotype analysis of T cell subpopulations. A very exciting result was obtained in 1975, namely, that the killer T cell population is relatively rich in Lyt-2/3 (CD8) surface antigens, but relatively poor in Lyt-1 antigen, suggesting that T cells with different functions could be distinguished on the basis of their Lyt phenotypes (6). Subsequently, Eiichi Nakayama clearly demonstrated selective blocking of cytotoxic cells by Lyt-2 and Lyt-3 antisera *in vitro* in the absence of complement, suggesting that Lyt-2/3 determinants on the surface of cytotoxic T cells have a close spatial relationship to the T cell receptor (7, 8). Furthermore, it was shown later by Drs. Nakayama and Akiko Uenaka in Japan that *in vivo* administration of Lyt-2/3 antibodies is able to block the cytotoxicity of killer T cells responsible for tumor rejection (9). Thus, Lyt-2/3 was demonstrated to be not merely the marker of killer T cells, but a molecule that is important to their key function. As for the phenotype of effector cells (Th1) and regulatory T cells (Treg), Shimon Sakaguchi and his colleagues demonstrated in 1982 that both populations express Lyt-1, but not Lyt-2/3, i.e., the CD4 phenotype (10, 11); they accomplished this by analysis of an organ-localized autoimmune disease model that develops in mice thymectomized on day 3 after birth. It is now well known that CD8 and CD4 molecules are involved, respectively, in the interactions between T cell receptor and target antigen peptides presented on major histocompatibility complex (MHC) class I and class II molecules. This research laid the groundwork for identifying the cell surface markers that allow cells to be experimentally and diagnostically separated and distinguished,

revolutionizing immunology and medicine as it is practiced today.

Unlike the Lyt series of antigens, the TL antigen system has unique characteristics, e.g., TL expression is restricted to thymocytes in certain (TL^+) strains of the mouse, indicating a character of differentiation antigens, whereas TL appears in a proportion of T cell leukemias (lymphomas) developed even in TL^- strains that normally never express TL during fetal or adult life; these can be regarded as leukemia-specific antigens. Yuichi Obata, who worked with Dr. Old for 12 years, succeeded in 1985 in cloning genomic *TL* genes as members of *MHC* genes of chromosome 17 (12). These may be regarded as the first set of cloned genes encoding tumor antigen; the P815 mast cell tumor antigen gene, *P1A*, was reported by Boon's group in 1991. In 1985, Dr. Obata then returned to Japan to be a staff member at the Aichi Cancer Center Research Institute in Nagoya, where he continued his TL project. One of the interesting findings obtained thereafter is that when a chemical carcinogen (NBU) was administered to C57BL/6 and C3H strains, T cell leukemia development was slower than in T3B-TL gene-transduced counterpart strains expressing TL ubiquitously as self antigens, suggesting that anti-TL immunity may play a protective role in immune surveillance (13). Another important finding is that immunization with TL-positive skin from T3B-TL transgenic mice produced cytotoxic T cells exhibiting TL- (but not H-2-) restriction (14). This indicated that TL antigen is a transplantation antigen, although in the 1970s it was considered to be a serologically defined antigen.

In the late 1960s and early 1970s, cancer research focused on viral carcinogenesis, since the Epstein-Barr virus (EBV) had been discovered as a candidate for human tumor virus, while retroviruses such as murine leukemia virus (MuLV) and murine mammary tumor virus (MTV) had been shown to be associated with development of murine leukemia and mammary tumor, respectively. In Dr. Old's laboratory, cell surface and intracellular antigens of MuLV and MTV were extensively studied, and many antigenic systems were defined. Particularly, detailed analyses of the relationship between gp70 of MuLV classes and cell surface antigens were conducted by Drs. Stockert and Obata (15-18). Four antigens were defined as markers for each of the MuLV classes: GIX and G(RADAI) distinguished two types of ecotropic MuLV; G(ERLD) identified all xenotropic MuLV; and G(AKSL2) served as a marker for dual-tropic viruses in high-leukemia strains such as AKR mice. GIX antigen has characteristics of a differentiation antigen and is expressed on thymocytes in low-leukemia strains such as 129 mice; a linkage study conducted by Hisami Ikeda demonstrated that the antigen expression is controlled by linkage group 8 on chromosome 4 (19). During the course of these studies, Kouhei Kawashima obtained an interesting finding: thymocytes exhibit amplified expression of gp70-associated antigens during the late preleukemic period in the AKR strain, which is accompanied by the emergence of MuLVs with dual-tropic (but not ecotropic) properties (20, 21), leading to the belief that dual-tropic MuLVs are the proximal vectors of leukemogenesis in the AKR strain.

In addition to the studies of leukemia, an attempt was also made to detect individual antigens of chemically induced mouse sarcomas by use of the complement-dependent cytotoxicity microassay. The presence of such antigens was demonstrated in the 1960s by *in vivo* transplantation experiments, but the nature of the antigens was not well understood. In 1977, Albert B. DeLeo produced antisera that detected antigens with an exceedingly restricted distribution on two BALB/c methylcholanthrene-induced sarcomas, Meth A and CMS4 (22). During the course of these studies, he defined a transformation-related intracellular antigen of sarcomas and leukemias with a molecular weight of 53 kilodaltons (23); this antigen is now known to be the product of the *p53* tumor suppressor gene. Two genes encoding for individual transplantation antigens detected by cytotoxic T cells were subsequently isolated in Japan from different tumors: *RAMP* (exon extension mutation) from Meth A, by Dr. Nakayama in 2003 (24), and *ERK2* (point mutation) from another sarcoma, CMS5, by Hiroaki Ikeda *et al.* in 1997 (25). To date, the relationship between these gene products and the serologically defined antigens has not been elucidated.

Here, we have only introduced a part of the mouse cell surface antigen studies initiated at SKI, and briefly discussed their further development in Japan. It is noteworthy that not only genes encoding these antigens, but also fertilized eggs of the pertinent congenic and transgenic mouse strains are maintained even now at the BioResource Center of Riken Tsukuba Institute (director, Dr. Obata), and are available to researchers interested in this field.

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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

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(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

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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

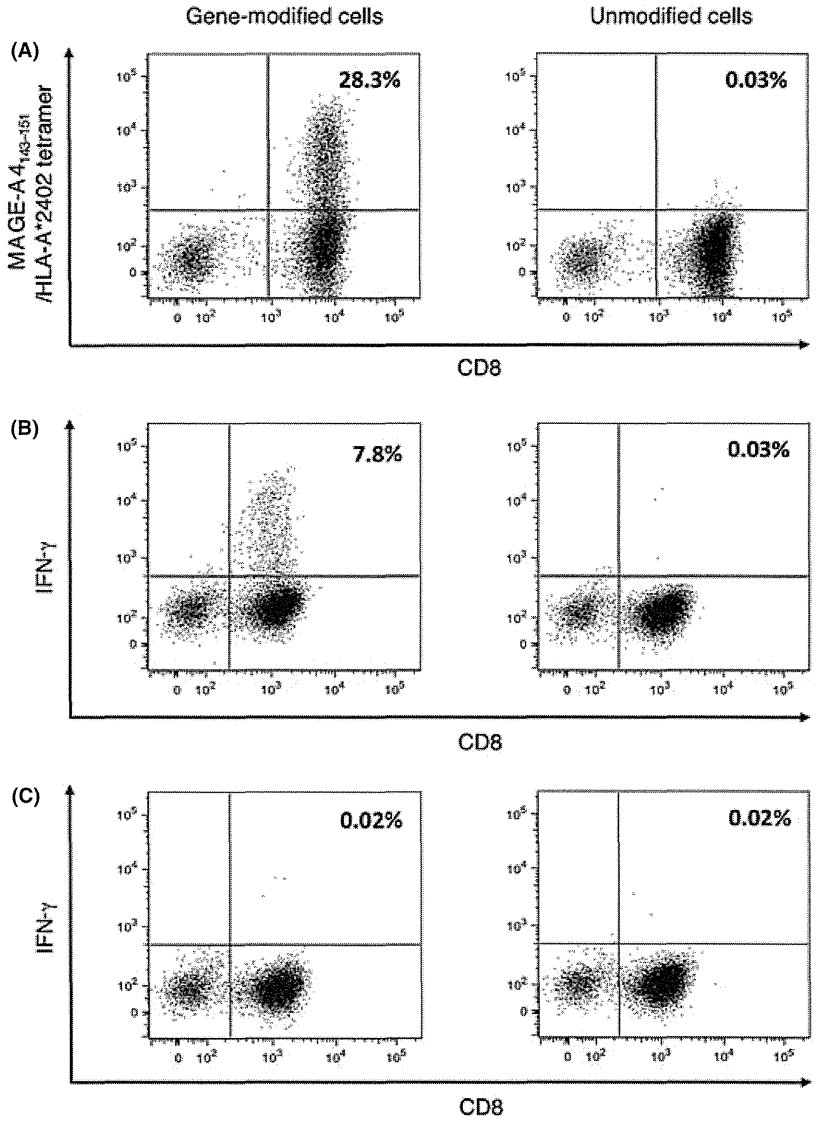

Fig. 1. Transduction of melanoma-associated antigen (MAGE)-A4-specific T-cell receptor (TCR) in human lymphocytes. Peripheral blood mononuclear cells from healthy donors were stimulated with anti-CD3 mAb and interleukin-2. Cells were cultured with or without retroviral vector encoding MAGE-A4-specific 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.

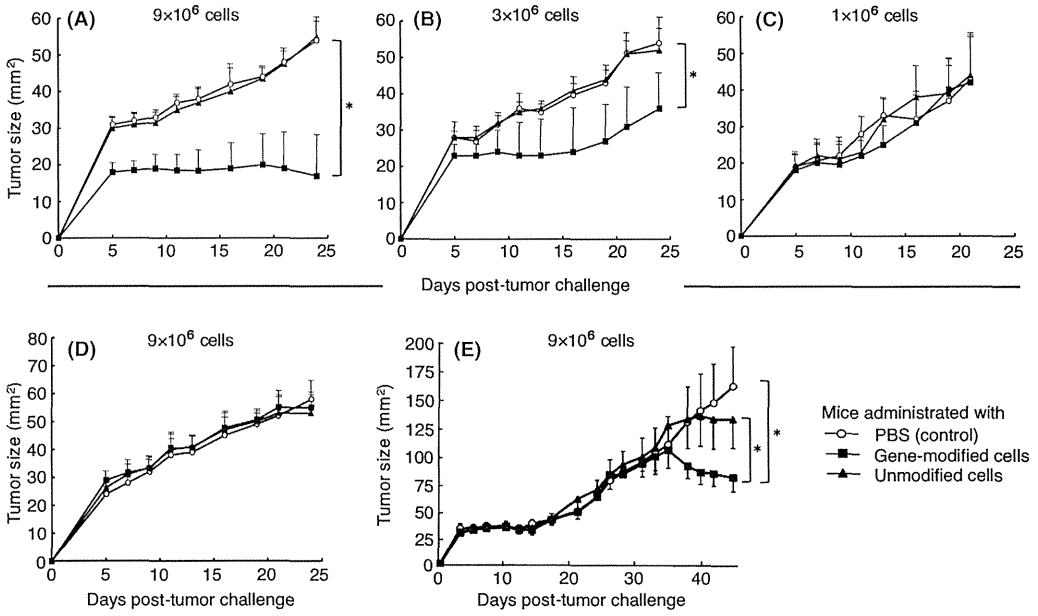

Fig. 2. Adoptive transfer of lymphocytes genetically engineered to express MAGE-A4-specific T-cell receptor inhibits human tumor progression in non-obese diabetic/SCID/γc^{null} mice. Non-obese diabetic/SCID/γc^{null} mice (n = 4 per group) were subcutaneously inoculated with 2.5×10^6 KE4 (A–C) or QG56 (D) tumor cells, and intravenously administered ~1×10^8 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

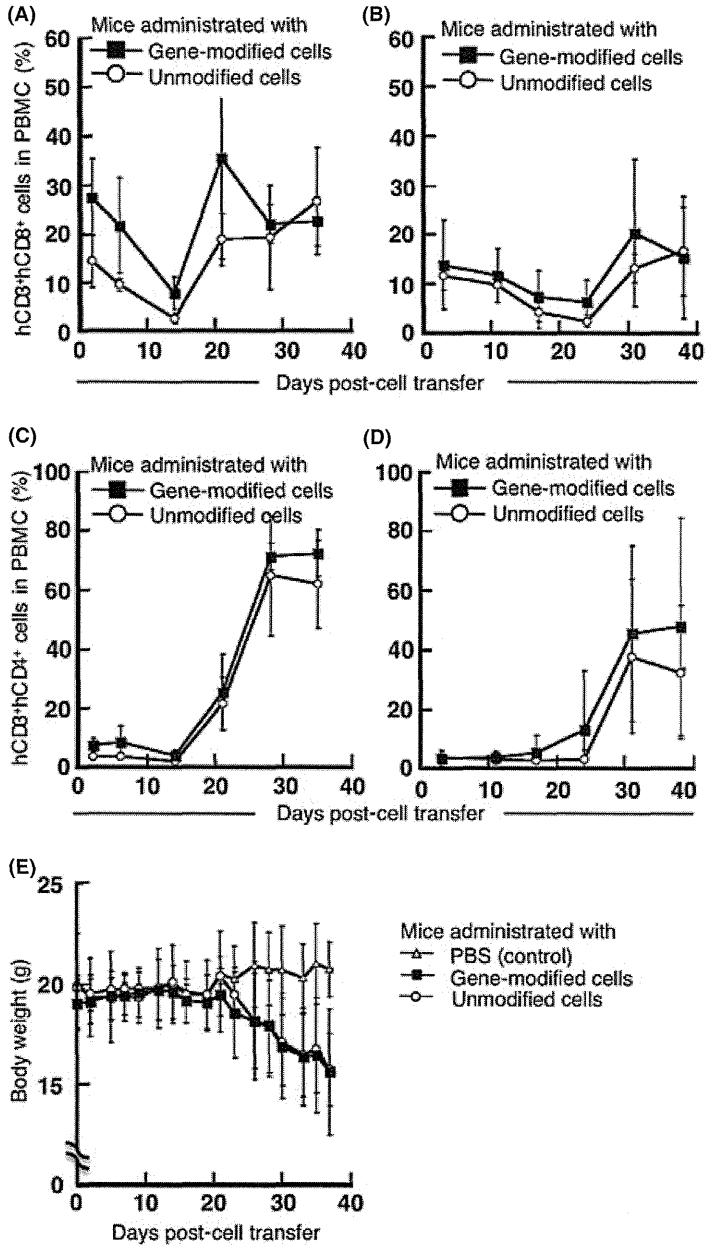

Fig. 3. Persistence of adoptively transferred human lymphocytes in non-obese, diabetic/SCID/γc^{null} (NOG) mice. Non-obese, diabetic/SCID/γc^{null} mice ($n = 4$ per group) were subcutaneously inoculated with 2.5×10^6 KE4 tumor cells, then intravenously administered 1×10^8 (A,C) or 5×10^7 (B,D) gene-modified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