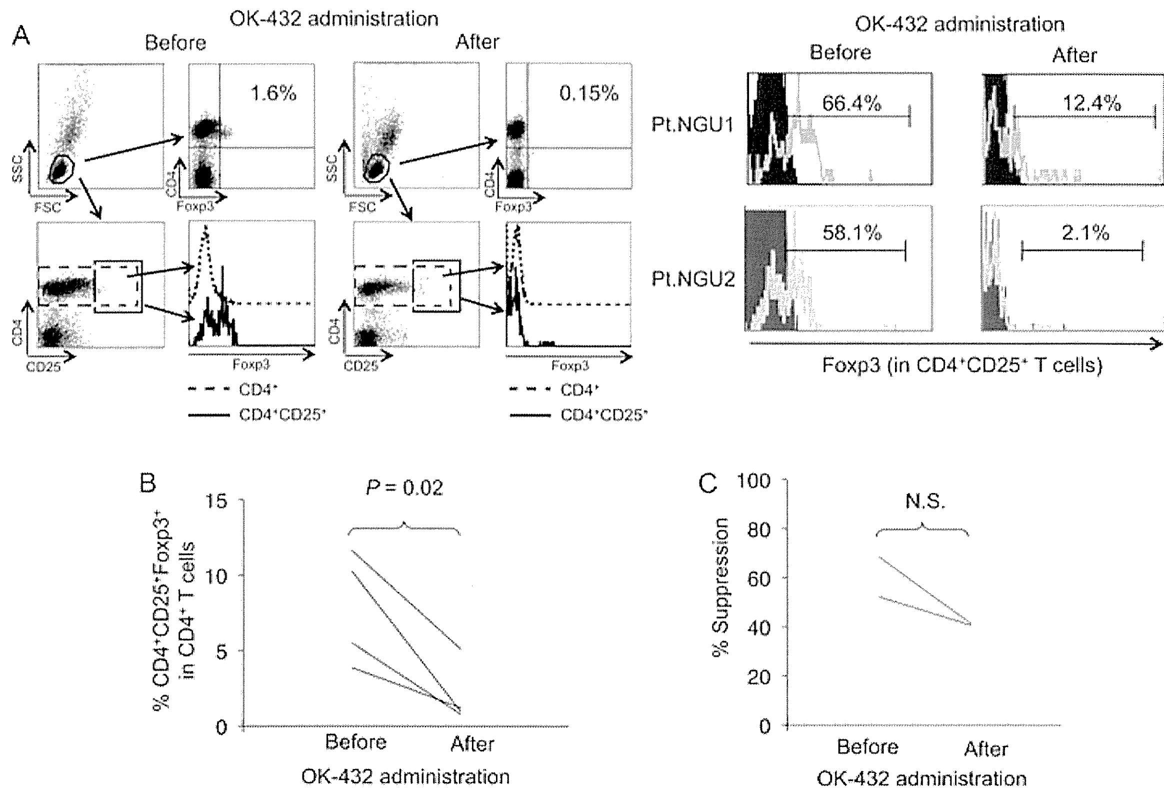

Figure 4. OK-432 administration into tumor-associated exudate fluids elicits reduction of local Treg-cell accumulation and function. Cells were isolated from tumor-associated exudate fluids (two pleural effusions or two ascites) and peripheral blood before and 2 days after OK-432 administration. (A) Cells were stained with anti-CD4, anti-CD25, and anti-Foxp3 Abs and were analyzed with flow cytometry. The staining pattern and gating method of a representative patient is shown (left); solid line histogram, Foxp3 expression in $CD4^+$ $CD25^+$ T cells; dotted line histogram, Foxp3 expression in $CD4^+$ T cells. Foxp3 expression in $CD4^+CD25^+$ T cells of two representative patients is shown (right); filled histogram, control staining; gray line histogram, Foxp3. (B) The percentage of $CD4^+CD25^+Foxp3^+$ cells in $CD4^+$ T cells is shown for $n = 4$ patients' samples. Statistical significance determined by Student's t-test. (C) 1×10^5 $CD4^+CD25^-$ T cells were isolated from PBMCs before OK-432 administration and cultured with irradiated autologous CD4-depleted PBMCs and anti-CD3 Ab in the presence or absence of 1×10^5 $CD4^+CD25^{high}$ T cells isolated from tumor-associated exudate fluids before and after OK-432 administration ($n = 2$). Data shown are from one experiment representative of two independent experiments.

induced NY-ESO-1_{91-110}–specific $CD4^+$ T cells were detected in the $CD4^+CD25^-CD45RO^+$ (effector/memory) T-cell population before and after vaccination. Following vaccination with NY-ESO-1 protein in the presence of OK-432, $CD4^+$ T-cell immune responses against NY-ESO-$1_{111-130}$ were newly elicited (Fig. 5A). These vaccine-induced NY-ESO-$1_{111-130}$–specific $CD4^+$ T cells were detected in the $CD4^+CD25^-CD45RO^+$ (effector/memory) T-cell population only after vaccination (Fig. 5A). In Pt #2, while specific $CD4^+$ T cells were not observed before vaccination, NY-ESO-$1_{119-141}$–specific $CD4^+$ T cells were elicited after vaccination. The vaccine-induced NY-ESO-$1_{119-141}$–specific $CD4^+$ T cells were also detected in the $CD4^+CD25^-CD45RO^+$ (effector/memory) T-cell population, as observed in Pt #1 (Fig. 5B).

NY-ESO-1 vaccination with OK-432 activates high-avidity preexisting NY-ESO-1–specific $CD4^+$ T-cells

We then asked whether vaccine-induced T cells had a high-affinity TCR that recognized naturally processed antigens [21, 28]. We established NY-ESO-1–specific $CD4^+$ T-cell clones. Four clones and a single clone that recognized different epitopes were generated from Pt #1 and Pt #2, respectively. Four minimal epitopes (NY-ESO-$1_{83-96,\ 94-109,\ 119-130, 121-134}$) were defined from $CD4^+$ T-cell clones derived from Pt #1 (Fig. 6A and data not shown). Both spontaneously induced (#2–11) and vaccine-induced (#3–1) $CD4^+$ T-cell clones recognized naturally processed NY-ESO-1 protein and as little as 0.1 nM of peptide (Fig. 6A). One minimal epitope defined from Pt #2 was NY-ESO-$1_{122-133}$ and the vaccine-induced $CD4^+$ T-cell clone (#1–1) again recognized both the naturally processed NY-ESO-1 protein and as little as 0.1 nM of peptide (Fig. 6B), indicating that these T-cell clones had high-affinity TCRs against NY-ESO-1. Together, OK-432 as an adjuvant could overcome Treg-cell suppression and activate high-affinity preexisting NY-ESO-1–specific $CD4^+$ T-cell precursors.

Discussion

While a subset of patients treated with immunotherapy has been shown to experience objective and durable clinical responses, it is becoming increasingly clear that several mechanisms

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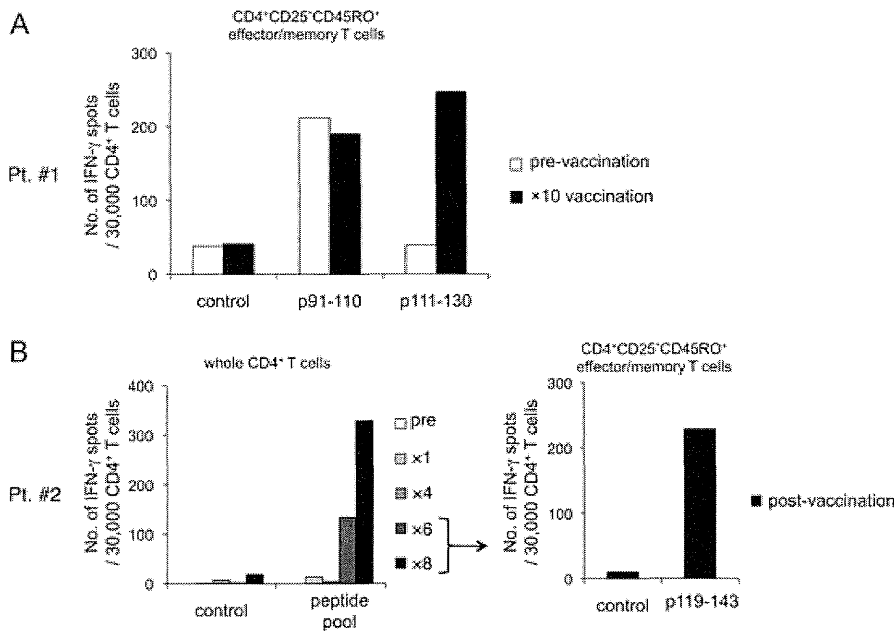

Figure 5. Vaccination with OK-432 elicits NY-ESO-1-specific effector/memory $CD4^+$ T cells $CD4^+CD25^-CD45RO^+$ T cells or unfractionated $CD4^+$ T cells were prepared from PBMCs of (A) Pt #1 (HLA-DR 4, 12 -DQ 4, 8) and (B) Pt #2 (HLA-DR 9, 15 -DQ 6, 9) before and after vaccination as described in the *Materials and Methods*; 3–5 × 10^5 $CD4^+CD25^-CD45RO^+$ T cells or unfractionated $CD4^+$ T cells were cultured with 3–5 × 10^5 CD4-depleted PBMCs pulsed with 10 μM pooled peptides covering the entire sequence of NY-ESO-1 for 3 weeks. Induction of NY-ESO-1–specific IFN-γ production was analyzed by ELISPOT. Data shown are from one experiment representative of at least two experiments performed.

downregulate antitumor immunity during the course of the immune response and play a major role in limiting the effectiveness of cancer immunity [6, 35, 36]. A plethora of cell types, cell surface molecules, and soluble factors mediate this suppressive activity [3, 6, 35, 36]. Among them, $CD4^+CD25^+Foxp3^+$ Treg cells play a crucial role by suppressing a wide variety of immune responses, and finding ways to control Treg-cell suppression is a major priority in this field [6, 7]. In this study, we showed the potential of OK-432 (a penicillin-inactivated and lyophilized preparation of *Streptococcus pyrogenes*) which stimulates TLR signals [30, 33, 34] to control Treg-cell suppression, supporting the idea that OK-432 may be a promising adjuvant for cancer vaccines by inhibiting Treg-cell suppression and by augmenting induction of tumor-specific T cells against coadministered protein antigens.

Appropriate adjuvant combinations, such as those that are MyD88-dependent or MyD88-independent, or those that are TRIF-coupled and include endosomal signals, are known to synergistically activate DCs with regard to the production of inflammatory cytokines [37, 38]. As OK-432 is derived from bacterial components, its capacity to bind a combination of various TLRs makes it attractive. It has been shown that OK-432 exhibits antitumor effects through TLR-2, TLR-4, and TLR-9 using knockout mice for each TLR [30, 33, 34]. Alternatively, OK-432 reportedly stimulates DCs through the β_2-integrin system rather than via TLR signals [29]. In the presence of OK-432, Treg cells slightly proliferated with TCR stimulation. TLR2 triggering results in a temporary loss of the anergic status of Treg cells and is associated with loss of Treg-cell suppressive function [24, 25]. The perturbation of Treg-cell anergy by OK-432 through TLR2 stimulation may play a role, at least in part, in the inhibition of Treg-cell suppressive function.

In accordance with previous reports [29, 34], we showed that APCs, including $CD11c^+$ and $CD14^+$ cells (monocytes, macrophage, and DCs), stimulated with OK-432 exhibited significantly higher production of IL-12 as compared with that of LPS- or TNF-α–matured APCs, and that OK-432–induced IL-12 from these APCs was a critical component for abrogating Treg-cell activity. Additionally, we found that monocyte-derived DCs stimulated with OK-432 produced significantly higher amounts of IL-12 compared with DCs stimulated with LPS or TNF-α (Supporting Information Fig. 2). It has been reported that IL-12 receptor expressed on effector T cells, but not on Treg cells has a critical role for abrogating Treg-cell suppression by IL-12 in mice [39, 40]. In accordance with this, downregulation of IL-12 receptors by siRNA on effector cells partially abrogated the OK-432–induced inhibition of Treg-cell suppressive activity (Supporting Information Fig. 3). IL-12 receptor was induced in both effector T cells and Treg cells after activation (Supporting Information Fig. 3). We attempted to downregulate the IL-12 receptor on Treg cells with siRNA to explore the exact target(s) of IL-12, however, the limitation in the availability of human materials hampered these analyses. Thus, IL-12 produced by APCs on the OK-432 stimulation could have two (or more) mutually compatible activities, (i) rendering effector cells resistant to Treg-cell suppression and (ii) inhibiting Treg-cell suppressive function directly, though the in vivo data argue against direct inhibition of Treg-cell suppression [39, 40].

Local administration of OK-432 reduced the number of $CD4^+CD25^+Foxp3^+$ Treg cells in tumor-associated exudate fluids. After administration of OK-432, local chemokine gradient may be changed and infiltration of Treg cells may be blocked [6, 13]. Alternatively, the inflammatory environment after OK-432 administration may be suitable for effector T-cell activation and IL-2, that is critical for Treg-cell survival and function [41], may not be adequately provided, as observed during severe *Toxoplasma gondii* infection [42]. In addition, suppressive function of $CD4^+CD25^{high}$ T cells in tumor-associated

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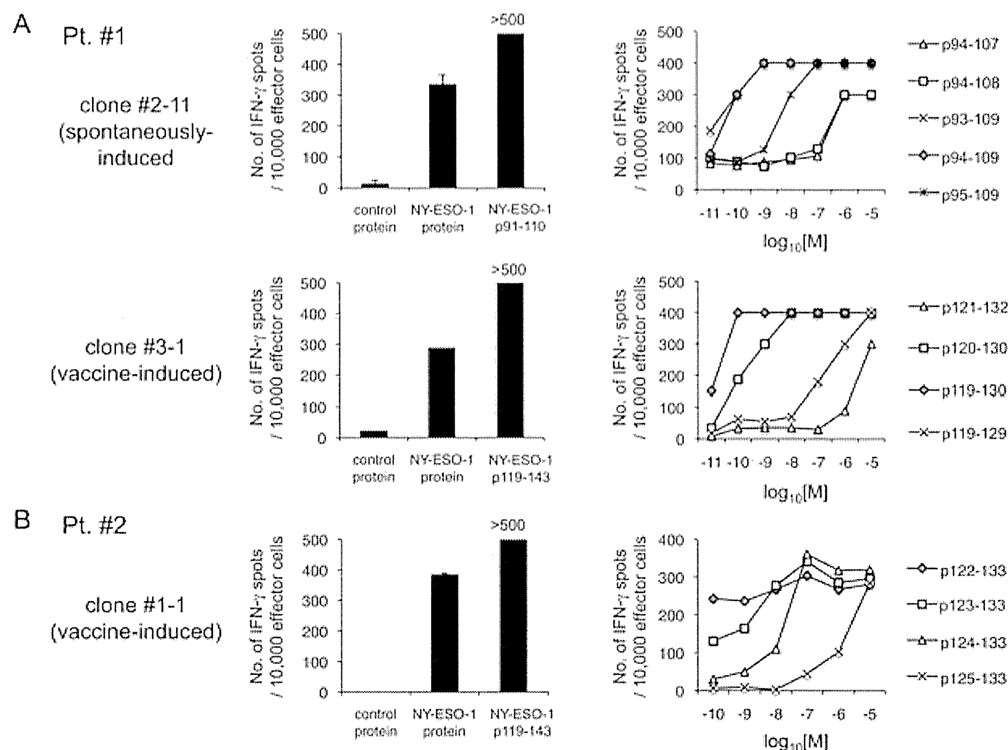

Figure 6. Vaccination with OK-432 activates high-avidity NY-ESO-1–specific CD4$^+$ T cells. NY-ESO-1–specific CD4$^+$ T-cell clones were generated from PBMCs of patients harboring NY-ESO-1–specific CD4$^+$ T cells. CD4$^+$ T-cell clones from (A) Pt. #1 and (B) Pt. #2 were stimulated and expanded by 30 ng/mL anti-CD3 Ab in the presence of 20 U/mL IL-2 and irradiated PBMCs and EBV-transformed human B lymphocytes as feeder cells. A couple of weeks later, these NY-ESO-1–specific CD4$^+$ T cell clones (1×10^4/well) were cultured with 1×10^4 NY-ESO-1 protein-pulsed DCs or 2×10^4 EBV-B cells pulsed with graded amounts of peptides and NY-ESO-1–specific IFN-γ production was analyzed by ELISPOT. Data are shown as mean ± SD of two replicates and are from one experiments representative of at least two experiments performed.

exudate fluids was reduced after OK-432 treatment in accordance with decreased expression of Foxp3 [43]. Considering the fact that IL-12, a main effector molecule induced by OK-432, renders effector cells resistant to Treg-cell suppression, direct administration of OK-432 may change the immunological balance in the local microenvironment from suppression by Treg cells to activation of helper T cells by augmenting helper T-cell activity. However, the sample size of patients analyzed in this study was relatively small and warrants cautious interpretation.

We have previously shown that while naive NY-ESO-1–specific CD4$^+$ T-cell precursors are present in wide range of healthy individuals and cancer patients, their activation is kept under stringent CD4$^+$CD25$^+$ Treg-cell control [20, 21, 28]. Using OK-432 as an adjuvant, we detected high-affinity NY-ESO-1–specific CD4$^+$ T cells in effector/memory population after vaccination in two esophageal cancer patients. In Pt #1, we found two responses; spontaneous and vaccine-induced NY-ESO-1–specific CD4$^+$ T cells. Both of them exhibited a similar efficiency to recognize titrated peptide, indicating that these NY-ESO-1–specific CD4$^+$ T cells had TCRs with similar affinity and were likely activated from naive high-affinity NY-ESO-1–specific CD4$^+$ T-cell precursors. Vaccination with minimal peptide in incomplete Freund's adjuvant fails to activate high-affinity NY-ESO-1–specific CD4$^+$ T-cell precursors, rather it dominantly expands low-avidity effector/memory CD4$^+$ T cells that cannot recognize naturally processed antigens [21]. In addition, following DNA vaccination covering the entire sequence of NY-ESO-1, high-avidity NY-ESO-1–specific CD4$^+$ T cells were not detected persistently because of rapid suppression by Treg cells [44]. While these data suggest a critical role for the inhibition of Treg-cell suppression by OK-432 in the activation of high-affinity NY-ESO-1–specific CD4$^+$ T-cell precursors, it is still difficult to obtain conclusive evidence without direct in vivo Treg-cell inhibition/depletion. To formally address this issue, clinical trials using Treg-cell depletion reagents and another clinical trial having two arms of patients receiving NY-ESO-1 with/without OK-432 would be required.

Certain types of immunization methods or DC stimulations elicit/augment CD4$^+$CD25$^+$ Treg cells in vivo [10–12, 45]. As many tumor-associated antigens recognized by autologous tumor-reactive lymphocytes are antigenically normal self-constituents [1–3], they also could be recognized with CD4$^+$CD25$^+$ Treg cells. Given that a proportion of cancer/testis antigens are targets of Treg cells [46], it is necessary to avoid unwanted activation of cancer/testis antigen-specific CD4$^+$CD25$^+$ Treg cells. Though the sample size of patients analyzed in this study was small and warrants cautious interpretation, including OK-432 in vaccine components as an adjuvant would be a promising strategy to establish favorable circumstances for stimulating effector T cells by inhibiting Treg-cell activation. Furthermore, since this agent has a long history and is widely applied as an anticancer drug, particularly

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in Japan, its clinical safety profile has been already established. Our data provide a critical cue for effective cancer vaccines and immunotherapy during antigen priming through modulation of $CD4^+CD25^+$ Treg-cell function.

Materials and methods

Blood samples

All healthy donors were subjects with no history of autoimmune disease. PBMCs, pleural effusions, or ascites from cancer patients were collected before and after local administration of OK-432 based on the protocol approved by the Human Ethics Committees of Mie University Graduate School of Medicine and Nagasaki University Graduate School of Medicine. PBMCs from esophageal cancer patients enrolled in a clinical trial of CHP-NY-ESO-1 and CHP-HER2 vaccination with OK-432 [47] (Supporting Information Fig. 1) were collected based on the protocol approved by the Human Ethics Committees of Mie University Graduate School of Medicine and Kitano Hospital. The clinical trial was conducted in full conformity with the current version of the Declaration of Helsinki and was registered as NCT00291473 of Clinical Trial.gov, and 000001081 of UMIN Clinical Trial Registry. All samples were collected after written informed consent.

Abs and reagents

Synthetic peptides of NY-ESO-1_{1-20} (MQAEGRGTGGSTGDADGPGG), NY-ESO-1_{11-30} (STGDADGPGGPGIPDGPGGN), NY-ESO-1_{21-40} (PGIPDGPGGNAGGPGEAGAT), NY-ESO-1_{31-50} (AGGPGEAGATGGRGPRGAGA), NY-ESO-1_{41-60} (GGRGPRGAGAARASGPGGGA), NY-ESO-1_{51-70} (ARASGPGGGAPRGPHGGAAS), NY-ESO-1_{61-80} (PRGPHGGAASGLNGCCRCGA), NY-ESO-1_{71-90} (GLNGCCRCGARGPESRLLEF), NY-ESO-1_{81-100} (RGPESRLLEFYLAMPFATPM), NY-ESO-1_{91-110} (YLAMPFATPMEAELARRSLA), NY-ESO-$1_{101-120}$ (EAELARRSLAQDAPPLPVPG), NY-ESO-$1_{111-130}$ (QDAPPLPVPGVLLKEFTVSG), NY-ESO-$1_{119-143}$ (PGVLLKEFTVSGNILTIRLTAADHR), NY-ESO-$1_{131-150}$ (NILTIRLTAADHRQLQLSIS), NY-ESO-$1_{139-160}$ (AADHRQLQLSISSCLQQLSLLM), NY-ESO-$1_{151-170}$ (SCLQQLSLLMWITQCFLPVF), NY-ESO-$1_{161-180}$ (WITQCFLPVFLAQPPSGQRR), and HIV $P17_{37-51}$ (ASRELERFAVNPGLL) [48] were obtained from Invitrogen (Carlsbad, CA, USA). Recombinant NY-ESO-1 protein was prepared using similar procedures as described previously [49]. OK-432 was purchased from Chugai Pharmaceutical (Tokyo, Japan). LPS (*Escherichia coli* 055:B5) was obtained from Sigma (St. Louis, MO, USA). Purified and FITC-conjugated anti-IL-12 (C8.6; mouse IgG1), purified anti-IL-6 (MQ2–13A5; rat IgG1), purified anti-IFN-γ (NIB42; mouse IgG1), purified anti-IL-23 (HNU2319; mouse IgG1), PE-conjugated anti-CD20 (2H7; mouse IgG2b) and PE-conjugated anti-CD56 (MEM188; mouse IgG2a) Abs were purchased from eBioscience (San Diego, CA, USA). Purified anti-IL-1β Ab (8516; mouse IgG1) was purchased from R&D Systems (Minneapolis, MN, USA). PE-conjugated anti-CD14 (MφP9; mouse IgG2b), PE-conjugated anti-CD45RA (HI100; mouse IgG2b), PerCP-conjugated anti-CD4 (RPA-T4; mouse IgG1), and FITC-conjugated anti-CD4 (RPA-T4; mouse IgG1), Foxp3 (259D; mouse IgG1), and CD45RO (UCHL1; mouse IgG2a) Abs were purchased from BD Biosciences (Franklin Lakes, NJ, USA). PerCP-Cy5.5-conjugated anti-CD11c Ab (3.9; mouse IgG1) was obtained from Biolegend (San Diego CA, USA). PE-conjugated anti-CD25 Ab (4E3; mouse IgG2b) was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Recombinant IL-6, IL-12, and TNF-α were purchased from PeproTech (Rocky Hill, NJ, USA).

Intracellular cytokine staining

PBMCs were cultured with/without OK-432 and GolgiStop reagent (BD Biosciences) for 20 h. Cells were stained for cell surface markers and then for intracellular cytokine (IL-12) after permeabilization. Results were analyzed by flow cytometry (FACSCanto; BD Biosciences).

Generation of NY-ESO-1–specific $CD4^+$ T cells

NY-ESO-1–specific $CD4^+$ T cells were elicited as described previously [20]. Briefly, $CD4^+$ T cells and $CD4^+CD25^-$ T cells were isolated from PBMCs using a $CD4^+CD25^+$ Treg Isolation Kit (Miltenyi Biotec). $CD4^+CD25^-$ T cells were further separated into $CD45RO^+$ T cells or $CD45RA^+$ T cells by FACSAria (BD Bioscience) after staining with anti-CD45RO and CD45RA Abs. $CD4^-$ PBMCs pulsed with 10 μM of peptide overnight were used as APCs. After irradiation, 5×10^5 APCs were added to round-bottom 96-well plates (Nunc, Roskilde, Denmark) containing 1–5×10^5 unfractionated $CD4^+$ or $CD4^+CD25^-CD45RO^+$ T cells and were fed with 10 U/mL IL-2 (Kindly provided by Takeda Pharmaceutical, Osaka, Japan) and 20 ng/mL IL-7 (R&D Systems). Subsequently, one-half of medium was replaced by fresh medium containing IL-2 (20 U/ml) and IL-7 (40 ng/mL) twice per week.

Generation of NY-ESO-1–specific $CD4^+$ T-cell clones

Cloning was performed by limited dilution as described previously [50]. Briefly, NY-ESO-1–specific $CD4^+$ T cell lines (0.3 cells/well) were stimulated and expanded in the presence of irradiated 5×10^4 cells/well PBMCs and 1×10^4 cells/well irradiated EBV-transformed human B lymphocytes with 10% AB serum, 20 U/ml IL-2, and 30 ng/mL anti-CD3 Ab (OKT3; eBioscience) in 96-well round-bottom plates.

Proliferation assay

$CD4^+CD25^-$ T cells were cultured with 1×10^5 irradiated CD4-depleted PBMCs and stimulated with 0.5 μg/mL anti-CD3 Ab

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(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.

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Abbreviation: CHP: cholesteryl hydrophobized pullulan

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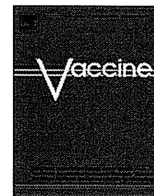

Establishment of animal models to analyze the kinetics and distribution of human tumor antigen-specific CD8$^+$ T cells

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ABSTRACT

Many patients develop tumor antigen-specific T cell responses detectable in peripheral blood mononuclear cells (PBMCs) following cancer vaccine. However, measurable tumor regression is observed in a limited number of patients receiving cancer vaccines. There is a need to re-evaluate systemically the immune responses induced by cancer vaccines. Here, we established animal models targeting two human cancer/testis antigens, NY-ESO-1 and MAGE-A4. Cytotoxic T lymphocyte (CTL) epitopes of these antigens were investigated by immunizing BALB/c mice with plasmids encoding the entire sequences of NY-ESO-1 or MAGE-A4. CD8$^+$ T cells specific for NY-ESO-1 or MAGE-A4 were able to be detected by ELISPOT assays using antigen presenting cells pulsed with overlapping peptides covering the whole protein, indicating the high immunogenicity of these antigens in mice. Truncation of these peptides revealed that NY-ESO-1-specific CD8$^+$ T cells recognized D^d-restricted 8mer peptides, NY-ESO-1$_{81-88}$. MAGE-A4-specific CD8$^+$ T cells recognized D^d-restricted 9mer peptides, MAGE-A4$_{265-273}$. MHC/peptide tetramers allowed us to analyze the kinetics and distribution of the antigen-specific immune responses, and we found that stronger antigen-specific CD8$^+$ T cell responses were required for more effective anti-tumor activity. Taken together, these animal models are valuable for evaluation of immune responses and optimization of the efficacy of cancer vaccines.

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1. Introduction

A number of cancer vaccine strategies targeting tumor antigens recognized by the human immune system have been tested [1–3]. While many of these cancer vaccines elicited measurable immune responses detectable in peripheral blood mononuclear cells (PBMCs), only a subset of treated patients experienced clinical benefits, such as tumor regression [4,5]. Because of the weak clinical effectiveness of currently available cancer vaccines, not only new immunogenic antigens, effective adjuvant formulations, vectors or vaccination methods but also new methodologies to evaluate efficacy of cancer vaccines are required.

NY-ESO-1, a germ line cell protein detected by SEREX (serological identification of antigens by recombinant expression cloning) using the serum of an esophageal cancer patient, is often expressed by cancer cells, but not by normal somatic cells [3,6]. This ideal expression pattern facilitated the study of this antigen; including immuno-monitoring of cancer patients with NY-ESO-1-expressing tumors and clinical trials that focused on NY-ESO-1 [3]. While these studies have revealed that a number of different cancer vaccines, including short and overlapping peptides, protein, viral vectors and DNA, resulted in development of measurable immune responses,

Abbreviations: APC, antigen presenting cells; CTL, cytotoxic T lymphocyte; dLN, draining lymph node; ELISPOT assay, enzyme-linked immunospot assay; IFN, interferon; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells.

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correlations between immunological and clinical responses were often week or difficult to observe [3].

MAGE-A4, another cancer/testis (CT) antigen, elicits MAGE-A4-specific $CD4^+$ and $CD8^+$ T cell responses in some patients with MAGE-A4-expressing cancers, indicating that MAGE-A4 is also an immunogenic protein [7–9]. We have recently reported a novel MAGE-A4 epitope presented by human leukocyte antigen (HLA)-A*2402 using a $CD8^+$ T cell clone 2-28 [9]. As this clone effectively killed tumor cell lines that expressed both MAGE-A4 and HLA-A*2402, this antigen could be a candidate for a cancer vaccine.

Given the poor correlation between the immune responses detected in PBMCs and clinical responses [2,3,5], it is necessary to re-evaluate existing cancer immunotherapy strategies in detail using animal models, namely reverse translational research. To this end, we developed animal models involving human tumor antigens, such as NY-ESO-1 or MAGE-A4 in this study.

2. Materials and methods

2.1. Mice

Female BALB/c mice were purchased from SLC Japan (Shizuoka, Japan) and used at 7–10 weeks of age. They were maintained at the Animal Center of Mie University Graduate School of Medicine (Mie, Japan). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Mie University Graduate School of Medicine.

2.2. Antibodies and reagents

Anti-H2-K^d (KD40, mouse IgG2a), anti-H2-D^d (DD98, mouse IgG2a), and anti-H2-L^d (30-5-7, mouse IgG2a) were produced and purified from each hybridoma. FITC-conjugated anti-CD8 mAb (53-6.7, rat IgG2a) and APC-conjugated anti-CD4 mAb (GK1.5, rat IgG2b) were purchased from BD Biosciences (Franklin Lakes, NJ). PE-conjugated anti-Foxp3 mAb (Fjk16s, rat IgG2a) was purchased from eBiosciences (San Diego, CA). Synthetic NY-ESO-1 and MAGE-A4 peptides (summarized in Supplementary Table 1) were obtained from Sigma Genosys (Hokkaido, Japan).

2.3. Immunization using a gene gun

Naive BALB/c mice were immunized twice at two-week intervals. Gold particles coated with 1 μg of each plasmid DNA were prepared and delivered into the shaved skin of the abdominal wall of BALB/c mice using a Helios Gene Gun System (BioRad, Hercules, CA) at a helium discharge pressure of 350–400 psi, as described previously [10,11].

2.4. Cell isolation

Spleen cell suspensions were mixed with CD8 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and separated into $CD8^+$ T cells by positive selection on a MACS column. The isolated $CD8^+$ T cell populations were confirmed to contain >95% $CD8^+$ T cells.

2.5. Enzyme-linked immunospot (ELISPOT) assay

The number of IFN-γ secreting antigen-specific $CD8^+$ T cells was assessed by ELISPOT assay as described previously [10,11]. Briefly, purified $CD8^+$ T cells were cultured for 24 hours with 5×10^5 irradiated CD90-depleted splenocytes pulsed with the indicated peptides in 96-well nitrocellulose-coated microtiter plates (Millipore, Bedford, MA) coated with rat anti-mouse IFN-γ mAb (R4-6A2, BD Biosciences). Spots were developed using biotinylated anti-mouse IFN-γmAb (XMG1.2, BD Biosciences), alkaline phosphatase conjugated streptavidin (MABTECH, Sweden) and alkaline phosphatase substrate kit (BioRad), and subsequently counted.

2.6. ELISA

96-well flat-bottomed microliter plates (Immuno-NUNC) were coated with 20 ng/50 μml of NY-ESO-1 or MAGE-A4 protein, respectively, at 4 °C overnight. Wells were blocked with 1% BSA/PBS for 1 hour at room temperature and washed three times. Serum (1:100 dilution) was added and incubated at 4 °C overnight. After washing, goat anti-mouse IgG antibody conjugated with horseradish peroxidase (Promega, Madison, WI) was added (1:5000 dilution). Two hours later, color was developed with TMB substrate solution (Thermo scientific, IL) and stopped with H_2SO_4. The absorbance was measured at 450 nm and calculated after subtraction of the absorbance value of control wells without sera.

2.7. Flow cytometry and tetramer staining

Tetramer staining was performed as described previously [11]. Briefly, cells were stained with PE-labeled NY-ESO-1_{81-88}/D^d or MAGE-$A4_{265-273}$/D^d tetramers (prepared at the Ludwig Institute Core Facility, Lausanne, Switzerland) for 10 minutes at 37 °C before additional staining of surface markers for 15 minutes at 4 °C. After washing, the results were analyzed on FACSCanto (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

2.8. Tumors

CT26 is a colon epithelial tumor derived by intrarectal injections of N-nitroso-N-methylurethane in BALB/c mice [12]. CT26 expressing NY-ESO-1 or MAGE-A4, a human cancer/testis antigen were established as described previously [11,13].

2.9. Statistical analysis

Values were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student's *t*-test. *p* values <0.05 were considered statistically significant.

3. Results

3.1. NY-ESO-1-specific $CD8^+$ T cells recognize D^d-restricted NY-ESO-1_{81-88} peptide

We analyzed the minimal epitope recognized by NY-ESO-1-specific $CD8^+$ T cells after immunization with NY-ESO-1. To this end, we employed a Helios Gene Gun System as we have previously detected NY-ESO-1-specific $CD8^+$ T cell responses [10,11]. To identify minimal epitopes, naive BALB/c mice were immunized twice at two-week intervals with plasmids encoding the entire sequence of NY-ESO-1. $CD8^+$ T cells were obtained from spleens and specific T cell responses were analyzed by ELISPOT assay using peptide pools shown in Supplementary Table 1. A significant number of NY-ESO-1-specific $CD8^+$ T cells was detected against peptide pool #3 (Fig. 1A). To identify the NY-ESO-1 epitope, NY-ESO-1-specific $CD8^+$ T cells were stimulated with each of these peptides. IFN-γ secretion was observed when NY-ESO-1-specific $CD8^+$ T cells were stimulated with 71–90 and 81–100 NY-ESO-1 peptides, suggesting the presence of a minimal epitope within 81–90 residues (Fig. 1B). To determine the minimal epitope, the 81–90 peptide of NY-ESO-1 was further truncated. NY-ESO-1-specific $CD8^+$ T cells recognized the 80–88 and 81–88, but not 80–87 or 82–88 peptides, thus

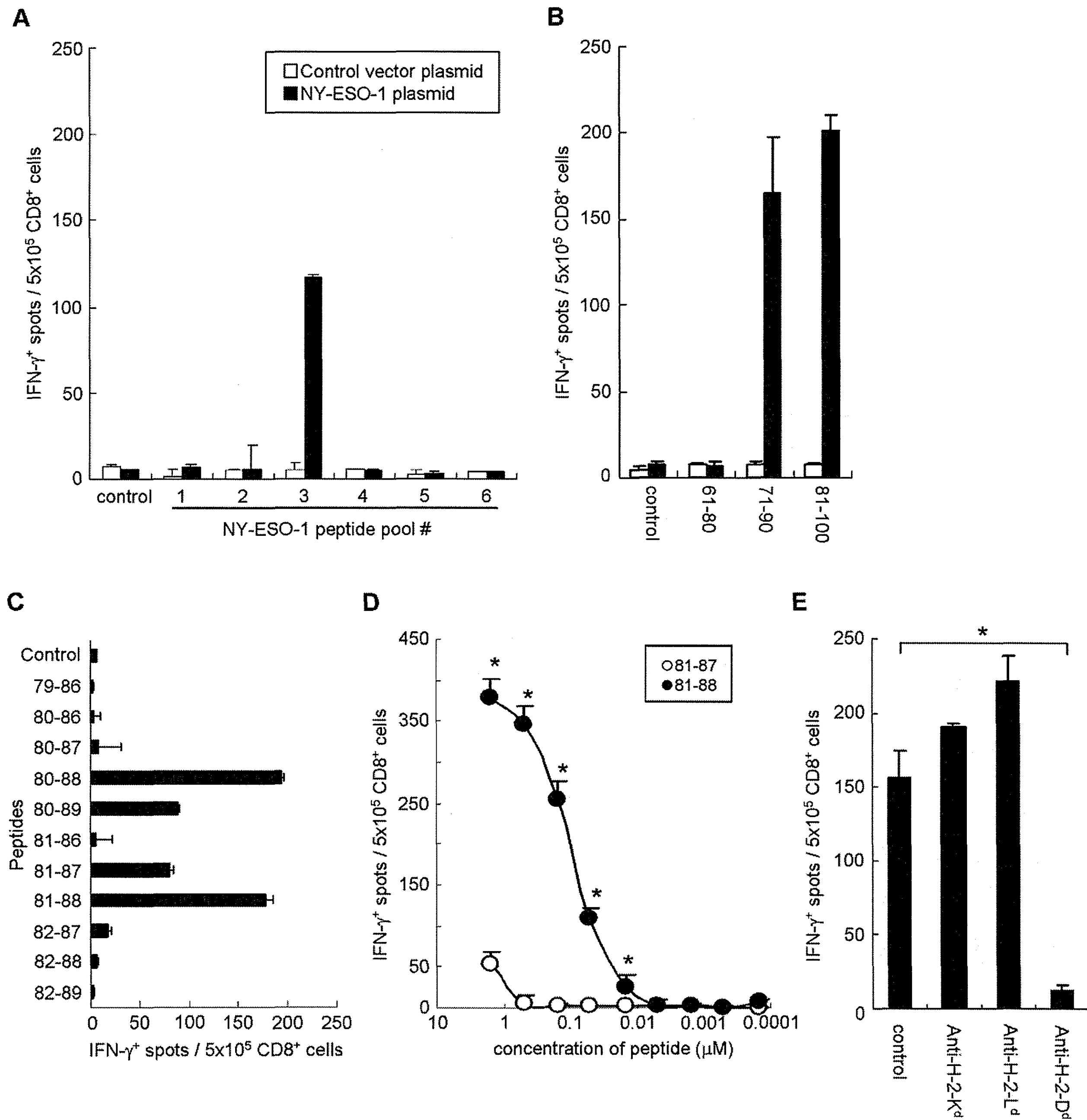

Fig. 1. NY-ESO-1-specific CD8+ T cells recognize D^d-restricted NY-ESO-1_{81-88} peptide. (A–C) BALB/c mice were immunized by gene gun twice at two-week intervals with plasmids encoding the entire sequence of NY-ESO-1. CD8+ T cells were obtained from spleens and specific T cells were analyzed with ELISPOT assay using APCs pulsed with the indicated peptides. (D) Avidity of induced NY-ESO-1-specific CD8+ T cells was analyzed with ELISPOT assay using APCs pulsed with graded doses of peptides ranging from 3 to 0.0001 μM. (E) MHC restriction of induced NY-ESO-1-specific CD8+ T cells was analyzed with ELISPOT assay by the addition of anti-H-2-K^d, anti-H-2-D^d or anti-H-2-L^d mAbs. These experiments were repeated two to four times with similar results. Data are mean ± SD.

the minimal epitope was identified to be NY-ESO-1_{81-88} peptide (Fig. 1C).

To confirm this, graded doses of the peptides were pulsed on antigen presenting cells (APCs) and specific IFN-γ secretion was analyzed by ELISPOT assay. NY-ESO-1-specific CD8+ T cells were high-avidity, and capable to recognize as little as 30 nM of peptide (Fig. 1D), confirming that NY-ESO-1_{81-88} peptide is the minimal epitope. Next, we assessed the restriction of this response using blocking antibodies. NY-ESO-1-specific CD8+ T cell responses were completely blocked by addition of anti-H-2-D^d mAb (Fig. 1E). Taken together, NY-ESO-1-specific CD8+ T cells recognize D^d-restricted NY-ESO-1_{81-88} peptide.

3.2. MAGE-A4-specific CD8+ T cells recognize D^d-restricted MAGE-$A4_{265-273}$ peptide

To establish a MAGE-A4 animal model, we determined the minimal epitope of MAGE-A4-specific CD8+ T cells after immunization with MAGE-A4. Naive BALB/c mice were immunized twice at two-week intervals with plasmids encoding the entire sequence of MAGE-A4. Splenic CD8+ T cells were prepared and specific T cell responses were analyzed by ELISPOT assay using peptide pools shown in Supplementary Table 1. MAGE-A4-specific CD8+ T cells were induced in mice immunized with plasmids encoding MAGE-A4 within peptide pool #5 (Fig. 2A). To elucidate the dominant

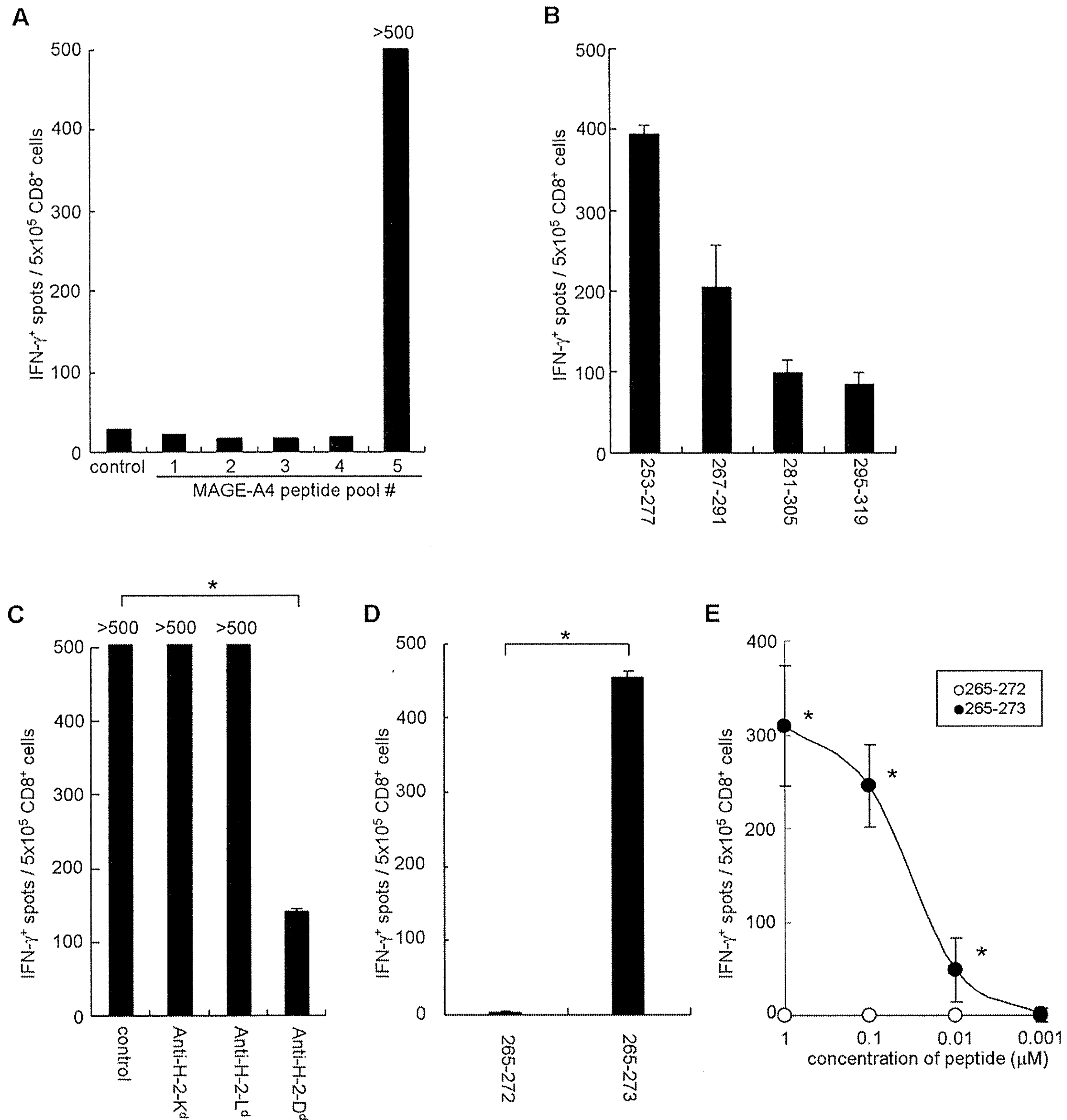

Fig. 2. MAGE-A4-specific CD8+ T cells recognize D^d-restricted MAGE-$A4_{265-273}$ peptide. (A, B) BALB/c mice were immunized by gene gun twice at two-week intervals with plasmids encoding the entire sequence of MAGE-A4. CD8+ T cells were obtained from spleens, and specific T cells were analyzed with ELISPOT assay using APCs pulsed with the indicated peptides. (C) MHC restriction of induced MAGE-A4-specific CD8+ T cells was analyzed with ELISPOT assay by the addition of the indicated mAb. (D) MAGE-$A4_{253-277}$ was subjected to BIMAS program and the highest score within MAGE-$A4_{253-277}$ for a D^d binding motif was predicted in 265–272 and 265–273 of MAGE-A4. These predicted peptides were analyzed with ELISPOT assay for identification of MAGE-A4 epitope peptide. (E) Avidity of MAGE-A4-specific CD8+ T cells was analyzed with ELISPOT assay using APCs pulsed with graded doses of peptides. These experiments were repeated two to four times with similar results. Data are mean ± SD.

MAGE-A4 epitope, MAGE-A4-specific CD8+ T cells were stimulated with each of the peptides from pool #5. The 253–277 peptide was most effective for stimulating MAGE-A4-specific CD8+ T cells (Fig. 2B). We next assessed the restriction of this response using blocking antibodies. MAGE-A4-specific CD8+ T cell responses were completely blocked by anti-H-2 D^d mAb (Fig. 2C). Given the H-2 D^d restriction of this CD8+ T cell response, we employed computer-based BIMAS program to predict optimized MHC class I epitope within the MAGE-$A4_{253-277}$ peptide. This program ranks all the possible MHC class I epitopes within a given polypeptide sequence. MAGE-$A4_{253-277}$ was subjected to this program and the highest score within MAGE-$A4_{253-277}$ for a D^d binding motif was predicted in 265–272 and 265–273 of MAGE-A4 (Supplementary Table 2). MAGE-A4-specific CD8+ T cells recognized the 265–273, but not 265–272 peptides, thus the minimal epitope was considered to be the MAGE-$A4_{265-273}$ peptide (Fig. 2D). To confirm this minimal epitope, graded doses of peptides were pulsed on APC and specific IFN-γ secretion was analyzed by ELISPOT assay. MAGE-A4-specific CD8+ T cells were high avidity, and could recognize as little as 10 nM of the peptide (Fig. 2E). We conclude that MAGE-A4-specific CD8+ T cells recognize D^d-restricted MAGE-$A4_{265-273}$ peptide.

3.3. *Kinetics and distribution of NY-ESO-1/MAGE-A4-specific CD8+ T cells after gene gun immunization*

Next, we generated MHC/peptide tetramers based on the data of minimal epitope and MHC restriction for NY-ESO-1/MAGE-A4-specific CD8+ T cells. BALB/c mice were immunized with plasmids

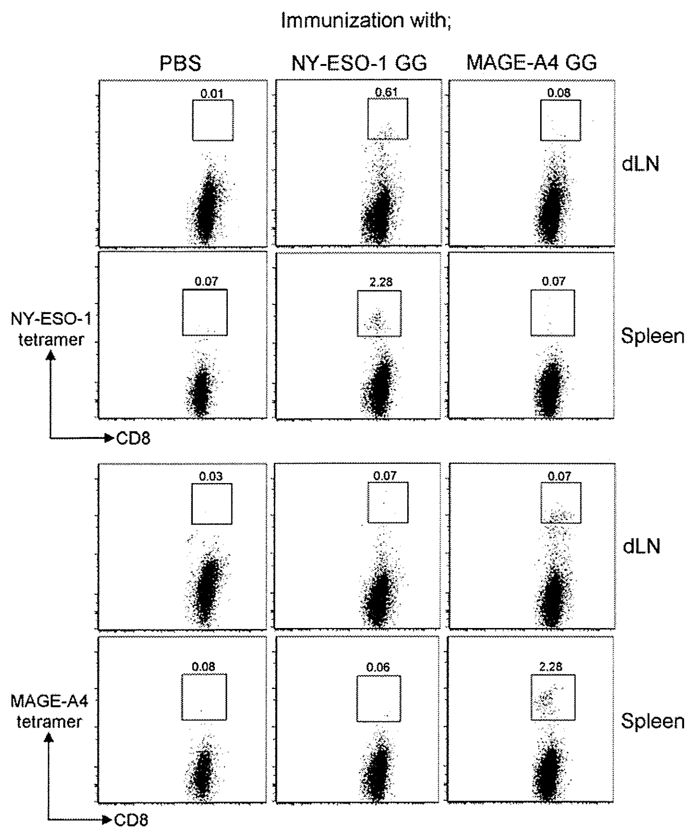

Fig. 3. NY-ESO-1/MAGE-A4-specific CD8$^+$ T cells are detected after immunization with a gene gun. BALB/c mice were immunized twice at two-week intervals with plasmids encoding the entire sequences of NY-ESO-1 or MAGE-A4 using a gene gun. Seven days after the second vaccination, CD8$^+$ T cells were obtained from the draining lymph nodes (dLNs) and spleens, and specific T cells were analyzed with MHC/peptide tetramer assay. These experiments were repeated two to four times with similar results. GG: gene gun.

encoding the whole sequences of NY-ESO-1 or MAGE-A4 by gene gun and the kinetics and distribution of NY-ESO-1/MAGE-A4-specific CD8$^+$ T cells were analyzed with MHC/peptide tetramers. NY-ESO-1-specific CD8$^+$ T cells were detected 7–10 days after the primary immunization both in draining lymph nodes and spleens in mice immunized with plasmids encoding NY-ESO-1, but not in mice immunized with MAGE-A4 or control mice (Fig. 3, 4A and 4B). NY-ESO-1-specific T cell responses were further enhanced by the secondary vaccination in both the draining lymph nodes and spleens (Fig. 4A and B). Similarly, MAGE-A4-specific CD8$^+$ T cells were detected 7–10 days after the primary immunization by gene gun in both the draining lymph nodes and spleens and were enhanced after the booster vaccination (Figs. 3, 4C and 4D), suggesting that these assays are useful tools for analyzing the kinetics and distribution of these antigen-specific CD8$^+$ T cells.

3.4. *NY-ESO-1-specific CD8$^+$ T cell responses are primed spontaneously after tumor inoculation and these cells partially inhibit tumor growth*

It is important to establish tumor models to re-evaluate cancer immunotherapy strategies in detail. To this end, we employed CT26 (a murine colon carcinoma) tumor transplantation model with stable expression of NY-ESO-1 and examined NY-ESO-1-specific CD8$^+$ T cell and humoral responses spontaneously primed in tumor bearing animals. BALB/c mice were inoculated with CT26-NY-ESO-1 and specific CD8$^+$ T cell and Ab responses were analyzed with MHC/peptide tetramers and ELISA, respectively. NY-ESO-1-specific CD8$^+$ T cells were spontaneously primed 7 days after tumor inoculation in the draining lymph nodes, spleens and peripheral blood in mice inoculated with CT26-NY-ESO-1 and augmented thereafter (Fig. 5A). We then addressed whether these spontaneously-primed NY-ESO-1-specific CD8$^+$ T cells were involved in tumor growth inhibition. To deplete these CD8$^+$ T cells, tumor bearing mice were injected with anti-CD8 mAb and tumor growth was analyzed. Anti-CD8 mAb administration augmented tumor growth compared with the control group without any treatment (Fig. 5C), suggesting an anti-tumor role of spontaneously-primed NY-ESO-1-specific CD8$^+$ T cells. On the other hand, NY-ESO-1-specific Ab responses were not observed 7 days after tumor inoculation, but detected 21 days after tumor inoculation (Fig. 5B). This is compatible with immunological monitoring in humans showing that higher stage of melanoma patients frequently develop humoral immune responses against NY-ESO-1 [3,14].

We next immunized these mice with plasmids encoding the entire sequence of NY-ESO-1 and anti-tumor activity was examined. Tumor growth was significantly reduced by immunization with NY-ESO-1 as compared to the control group without treatment (Fig. 5C). Furthermore, CD8$^+$ T cell depletion totally abolished the anti-tumor effects induced by DNA vaccine (Fig. 5C). As CD4$^+$ regulatory T cells (Tregs) are reportedly associated with spontaneously-primed and treatment-induced anti-tumor immune responses [15], we also investigated tumor-infiltrating Tregs. While Tregs were present in tumors, their frequency was not associated with anti-tumor activity induced by immunization with plasmids encoding NY-ESO-1 (Fig. 5D). Together, CD8$^+$ T cell and Ab responses to NY-ESO-1 in this tumor model closely parallel NY-ESO-1 immune responses in humans. Spontaneous tumor antigen-specific immune responses restrained, albeit incomplete, tumor growth, but tumor growth were vigorous and overwhelmed the tumor growth inhibition, thus additional augmentation of these immune responses are required for effective control of tumor growth.

3.5. *MAGE-A4-specific CD8$^+$ T cell responses is primed spontaneously after tumor inoculation*

We established another tumor transplantation model with stable expression of MAGE-A4 and examined MAGE-A4-specific CD8$^+$ T cell and humoral responses spontaneously primed in tumor bearing mice. BALB/c mice were inoculated with CT26-MAGE-A4 and specific CD8$^+$ T cell and humoral responses were analyzed with MHC/peptide tetramers and ELISA. In these mice, MAGE-A4-specific CD8$^+$ T cells were spontaneously primed 7 days after tumor inoculation in the draining lymph nodes, spleen and PBMC, and augmented thereafter (Fig. 6A). MAGE-A4-specific Ab responses were not observed 7 days after tumor inoculation, but detected 21 days after tumor inoculation (Fig. 6B). Like as the result of NY-ESO-1 models, cellular and humoral immune responses to MAGE-A4 in this model closely parallel MAGE-A4 immune responses in humans.

4. Discussion

We have established mouse models that allowed studies on NY-ESO-1 and MAGE-A4 immunity. Using these models, we evaluated the kinetics and distribution of antigen-specific CD8$^+$ T cells after tumor growth and immunization. While it has been recently shown that CD4$^+$CD25$^+$ Tregs and the ratio of CD8$^+$ effector T cells to Tregs in tumors critically influenced the prognosis of cancer patients [16,17], limitation of samples usually makes it difficult to investigate the function of effector T cells and Tregs at tumor sites in humans. Given the importance of tumors as active sites of anti-tumor responses, it is important to examine not only the draining

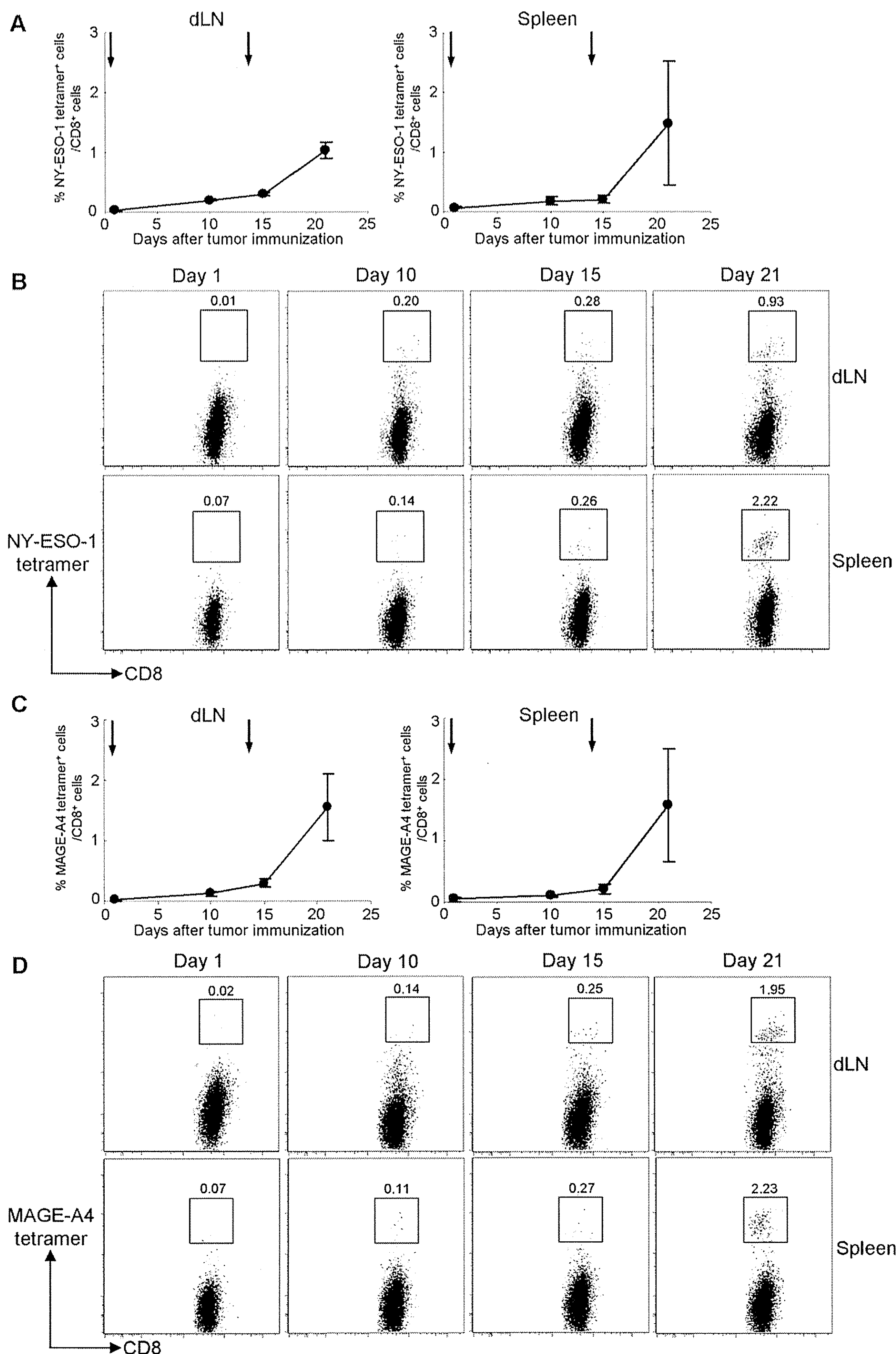

Fig. 4. Kinetics and distribution of NY-ESO-1/MAGE-A4-specific $CD8^+$ T cells after immunization with a gene gun. Kinetics and distribution of NY-ESO-1 (A and B)/MAGE-A4 (C and D)-specific $CD8^+$ T cells were analyzed by MHC/peptide tetramer assay. BALB/c mice were immunized twice at two-week intervals with plasmids encoding the entire sequence of either NY-ESO-1 or MAGE-A4 using a gene gun. $CD8^+$ T cells were obtained from the draining lymph nodes (dLNs) and spleens on the indicated days, and specific T cells was analyzed with MHC/peptide tetramer assay. These experiments were repeated two to four times with similar results. Data in (A) and (C) are mean ± SD. Arrows in (A) and (C) represent the first and second immunization.

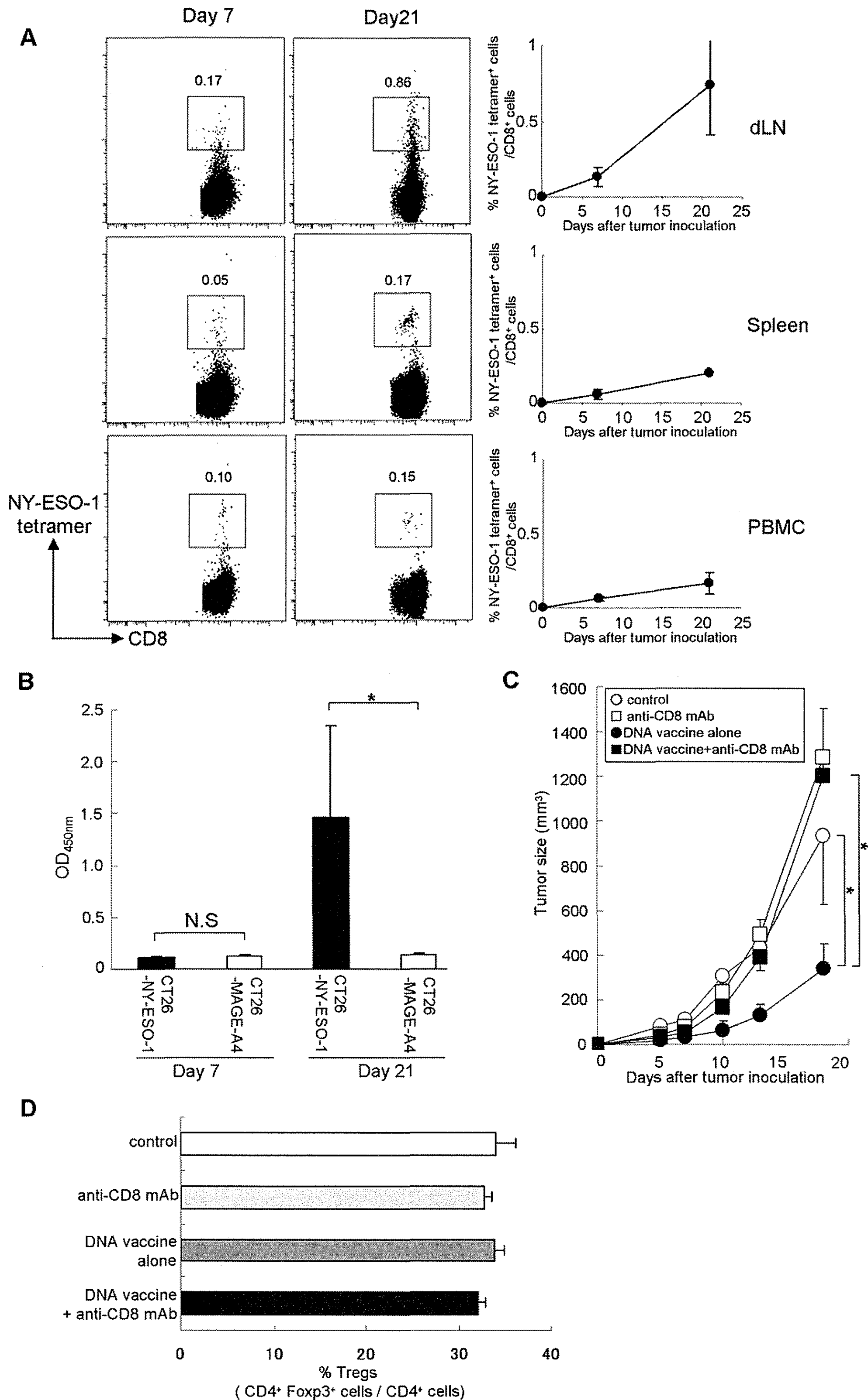

Fig. 5. NY-ESO-1-specific CD8+ T cells are primed spontaneously after tumor inoculation, and these cells partially inhibit tumor growth. BALB/c mice were inoculated with CT26-NY-ESO-1. (A) CD8+ T cells were isolated from the draining lymph nodes (dLNs), spleens and PBMCs on the indicated days, and NY-ESO-1-specific T cells were analyzed with MHC/peptide tetramer assay. (B) Sera were obtained on the indicated days and Ab responses against NY-ESO-1 were analyzed with ELISA. These experiments were repeated twice with similar results. Data are mean ± SD. $^{*}p < 0.05$ as compared to control. (C) BALB/c mice were immunized with plasmids encoding the entire sequence of NY-ESO-1, and CT26-NYESO-1 was inoculated on day 21 (one week after final immunization). Indicated groups of mice were administered with anti-CD8 mAb (clone 19/178, 100 μg/mouse, every 12 days). More than 95% of CD8+ T cells were depleted by this method (data not shown). Each group consisted of four mice. Mice were monitored thrice a week. These experiments were repeated two times with similar results. (D) Tumor infiltrating lymphocytes were collected 21 days after tumor inoculation and the frequency of Foxp3+ cells in CD4+ T cells was analyzed.

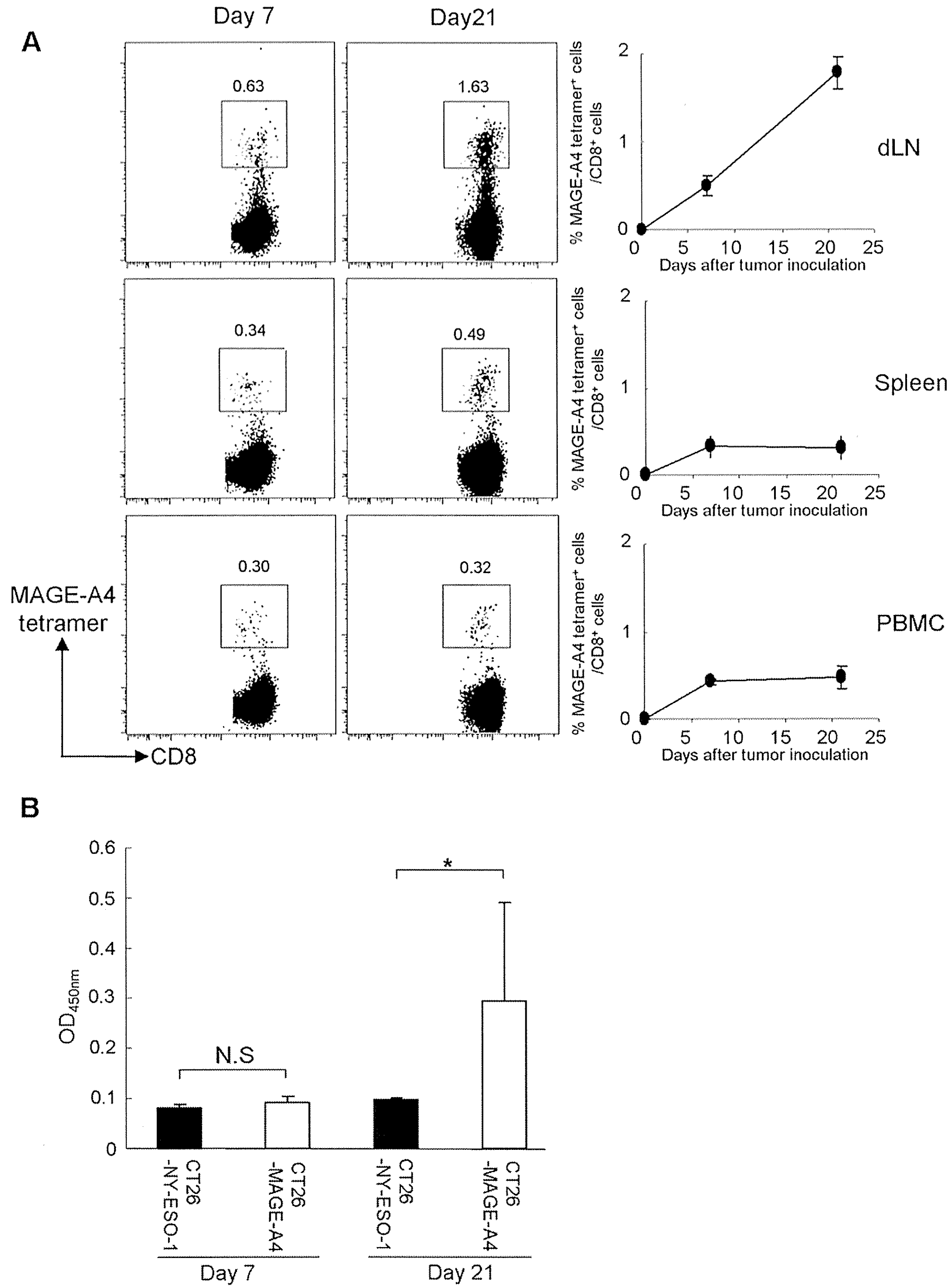

Fig. 6. Spontaneous MAGE-A4-specific CD8+ T cells are primed after tumor inoculation. BALB/c mice were inoculated with CT26-MAGE-A4. (A) CD8+ T cells were isolated from the draining lymph nodes (dLNs), spleens and PBMCs on the indicated days, and MAGE-A4-specific T cells was analyzed with MHC/peptide tetramer assay. (B) Sera were obtained on the indicated days, and Ab responses against MAGE-A4 were analyzed with ELISA. These experiments were repeated twice with similar results. Data are mean ± SD. $^{*}p < 0.05$ as compared to control.

lymph nodes, spleens and PBMCs, but also tumor local sites. Our models could be valuable tools for analyzing antigen-specific T cells in both novel cancer immunotherapy and cancer immunotherapy that have been already tested in humans.

In our models, we found that spontaneous CD8+ T cell and Ab responses were primed and increased along with tumor progression in both NY-ESO-1 and MAGE-A4 models [3,9]. Accumulating data show that induction/augmentation of anti-tumor immune responses are often detected in patients with larger tumors [3,14], suggesting that immune responses found in our NY-ESO-1 and MAGE-A4 tumor models closely parallel NY-ESO-1 and MAGE-A4 immune responses in humans. It has been a long debate whether spontaneous anti-tumor responses detected in cancer patients impact on tumor growth, as tumors continuously grow in patients harboring spontaneous anti-tumor immune responses. Our tumor model provides a clear answer for this conundrum. Although the immune responses spontaneously primed in tumor-bearing hosts partly inhibit a tumor growth, this immune response

is not strong enough to completely reject the tumor in the host. This means that further activation of immune responses by appropriate immunotherapy is essential for tumor rejection. Indeed, when these spontaneous immune responses were augmented by DNA vaccine, tumor growth was significantly inhibited. In contrast, we have reported that peptide vaccine using this NY-ESO-1 peptide enhanced tumor growth rather than inhibiting tumor growth unless it is vaccinated with proper adjuvants [13].

In fact, antigen-specific antibody may not play an important role for tumor rejection in our models, because 1) most tumor antigens including NY-ESO-1 and MAGE-A4 focused on this study, was exclusively expressed intracellularly by the tumor cells, thus not accessible for antibody [3] and 2) Ab responses were detected on day 21, namely later than $CD8^+$ T cell responses. Nevertheless, we have reported that injection of NY-ESO-1 mAb with chemotherapy, that can accentuate the release of intracellular tumor antigens to facilitate mAb access to intracellular target molecules, augmented anti-tumor effect in the same model system, though Ab administration alone did not inhibit tumor growth [18]. In our mouse model, spontaneously-primed anti-NY-ESO-1 Ab was detected when tumors reached a larger size. The level of spontaneously-primed antibody was, however, about 10-times lower than that achieved by mAb injection [18], suggesting that spontaneously-primed Ab responses may potentially have some anti-tumor effects, but the amount of Abs is too low to exhibit effective anti-tumor activity.

Since no NY-ESO-1 homologue is present in mice, the detected immune responses against NY-ESO-1 are considered to reflect a foreign antigen, rather than a self-antigen [3]. Whereas cancer-testis antigens like NY-ESO-1 are only expressed by cancer cells and testis, but not by normal somatic cells, mimicking foreign antigens, some cancer-testis antigens are reportedly expressed in medullary thymic epithelial cells under control of AIRE (Autoimmune regulator) [3,19]. It is plausible that cancer-testis antigens like NY-ESO-1 could be considered self-antigens during thymic selection, resulting in a repertoire of NY-ESO-1-specific T cells that are either subject to central or peripheral tolerance [3,20–22]. Thus, studies using mice in which NY-ESO-1 is a self-antigen should allow resolving this issue.

A unique finding of our study is that NY-ESO-1-specific $CD8^+$ T cell epitopes were present in an immunogenic part defined in humans [3]. This finding implies that immunogenicity may be characterized with similar components between humans and mice, further supporting the usefulness of our models. Our animal models provide important tools for the development of effective cancer vaccines.

In conclusion, we established animal models involving human tumor antigens, such as NY-ESO-1 or MAGE-A4 protein. These models allowed us to study the kinetics and distribution of antigen-specific immune responses in detail, and hence providing tools to optimize the efficacy of current cancer immunotherapy.

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Contributions: Designed the experiments: DM, HN, TK, HS. Performed the experiments: DM, HN, TN, LW, ES. Analyzed the data: DM, HN, NH, TK, HS. Contributed reagents/materials/analysis tools: IL, EN. Wrote the paper: DM, HN, TK. *Conflicts of interest:* D.M. and N.H. are employees of ImmunoFrontier, Inc. The other authors have no potential conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.02.056.

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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$_{157-165}$/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

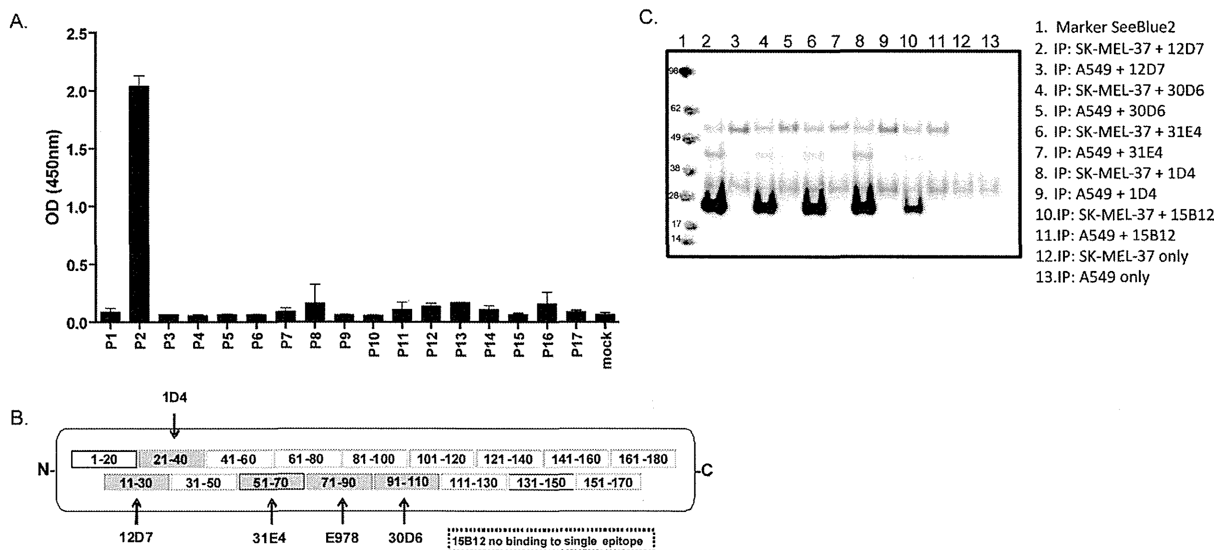
To compare the binding properties of five different anti-NY-ESO-1 HD mAbs to recombinant NY-ESO-1 protein, we determined the half-maximal effective concentration (EC_{50}) using a protein ELISA. All antibodies bound recombinant NY-ESO-1 produced in bacteria in the low pM range. Actual binding constants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

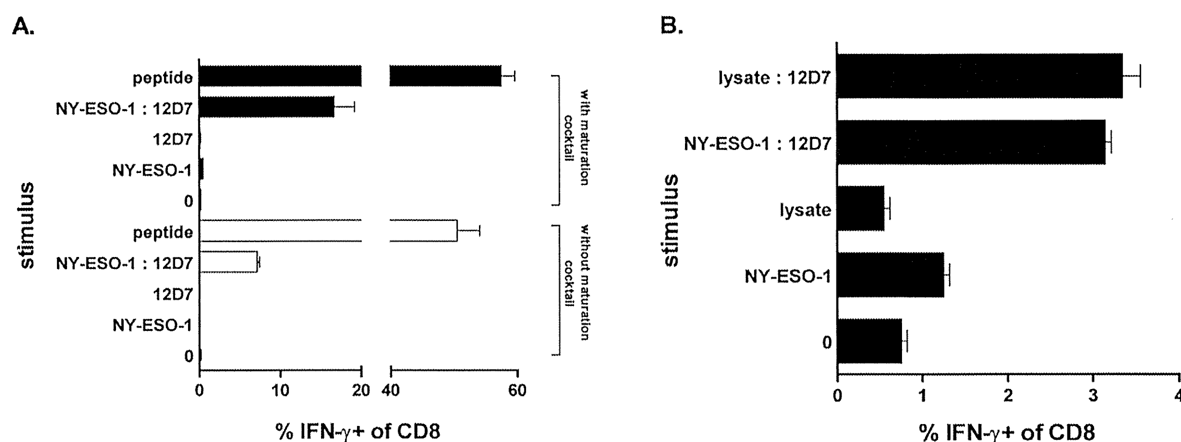
To test whether 12D7 facilitates the cross-presentation of NY-ESO-1-derived epitopes *in vitro*, we generated monocyte-derived, HLA-A*0201+ DCs and fed them with 12D7:NY-ESO-1 immune complexes, NY-ESO-1, or 12D7. DCs were subsequently incubated with cloned NY-ESO-$1_{157-165}$/HLA-A*0201-specific CD8+ 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-$1_{157\text{-}165}$). (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-$1_{157\text{-}165}$ /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-$1_{157\text{-}165}$ /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).

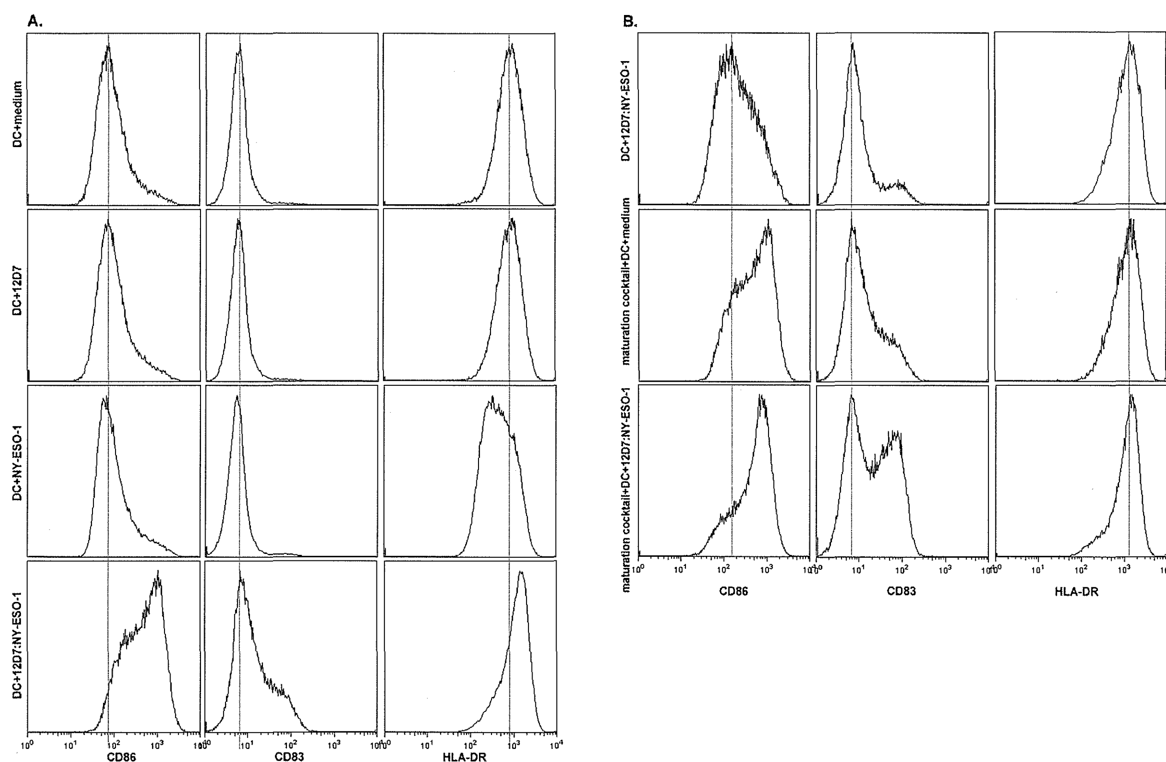
Because presentation of 12D7:NY-ESO-1 immune complexes seemed not to require deliberate DC maturation, we addressed whether the uptake of immune complexes, but not the uptake of uncomplexed protein, induced DC maturation *in vitro*. We therefore compared the expression of three surface molecules that 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