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Efficacy of Long-Term Treatment with Low-Dose Thalidomide for Patients with Relapsed/Refractory Multiple Myeloma

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ABSTRACT

Introduction: *We report the results of a prospective study of long-tern treatment with single-agent thalidomide in patients who had responded in a preceding trial of the use of thalidomide for relapsed/refractory myeloma.* ***Patients and Methods***: *Nineteen patients were enrolled*: 11 *patients* (57.9%) *treated at a dosage of* 100 *mg/day*; 2 *patients* (10.5%) *at a dosage of* 200 *mg/day*; 2 *patients* (10.5%) *at a dosage of* 300 *mg/day*; *and* 4 *patients* (21.1%) *at a dosage of* 400 *mg/day. The median follow-up from the start of the preceding study was* 3.0 *years. At the time of entry to this study*, 5 *patients* (26.3%) *had partial response* (*PR*), *another* 5 *patients* (26.3%) *had a minimal response* (*MR*), *and the remaining* 9 *patients* (47.4%) *had shown no change* (*NC*). ***Results***: *The cumulative MR rate was* 78.9% (*at the* 32nd *week*) *and the cumulative PR rate was* 47.4% (*at the* 112th *week*). *The median progression-free survival was* 104 *weeks and the median time to next treatment was* 144 *weeks. No patients experienced grade* 4 *or greater hematologic toxicity or grade* 3 *or greater non-hematologic toxicity.* ***Conclusion***: *Long-term thalidomide maintenance therapy induced an increase in response rate, suppressed the progression to active myeloma without severe adverse events, and contributed to long survival with good activities of daily living.*

Keywords: *Maintenance Therapy, Progression-Free Survival, Time to Next Treatment, Efficacy, Safety*

1. Introduction

Whereas the positive role of thalidomide as a consolidation treatment after high-dose therapy with autologous stem cell transplantation (HDT-ASCT), in the context of newly diagnosed myeloma, has been clarified in the patients without high-risk cytogenetics who have obtained less than a very good partial response [1,2], its role as a maintenance therapy remains controversial because of its toxicity and the concern of potential induction of resistance to subsequent treatment [3-5]. In addition, we currently have much less information on the role of thalidomide as maintenance therapy, in the context of cases of relapsed/refractory multiple myeloma, after successful salvage treatment. Based on the results of phase II trials in which the cumulative dose of thalidomide did not have an impact on the efficacy of maintenance therapy and toxicity increased above a dosage of 200 mg/d [6,7], treatment with low-dose thalidomide is now the preferred option.

We have conducted a prospective study to evaluate the efficacy and safety of thalidomide given as a single-agent maintenance therapy to patients with relapsed and/or re-

fractory multiple myeloma who had been enrolled in a previous phase II study [8] and who achieved at least no change (NC) with thalidomide treatment as per the study protocol.

2. Patients and Methods

2.1. Eligibility

Patients were deemed eligible for enrollment in this study if they had responded and maintained at least NC assessed at the 16th week of the phase II study period with single-agent thalidomide treatment given for at least 4 weeks [8]. According to the phase II study protocol, patients who achieved at least a minimal response (MR) continued on thalidomide treatment at the dosage with which the response had been obtained until the cutoff of the study. Otherwise, the dose of thalidomide was escalated by 100 mg every 4 weeks until the cutoff of the study (16th week) to a maximum of 400 mg/d. In total, 19 patients had achieved and maintained a response of at least NC according to the European Group for Blood and Marrow Transplantation response criteria [9] by the cutoff of the phase II study and were studied.

All patients gave written informed consent and agreed to abide by strict contraception. The study and the written informed consent form were approved by the institutional review board of each participating hospital. The study was conducted in accordance with the Good Clinical Practice for Trials of Drugs and the Declaration of Helsinki.

2.2. Treatment Schedule

Single-agent thalidomide treatment was continued in the 19 patients at their individual final doses of the phase II study until disease progression or intolerance occurred, for a maximum of 3 years. Patients were evaluated every 4 weeks for response and drug toxicity. Thalidomide was supplied by the Fujimoto Pharmaceutical Corporation (Osaka, Japan) and was given orally before sleep. No antithrombotic prophylaxis was instituted because no patients experienced thromboembolic events during the phase II study.

2.3. Response, Progression-Free Survival, Time to Next Ttreatment, and Toxicity Criteria

Responses were assessed by the decrease in the monoclonal protein measured at the time of entry into the phase II study using the European Group for Blood and Marrow Transplantation response criteria [9]. Progressionfree survival (PFS) was measured from the date of initiation of thalidomide treatment in the phase II study until death or disease progression, whichever was earlier. Time to next treatment (TTNT) was measured from the date of initiation of thalidomide treatment until death or the date of initiation of the next treatment. The PFS and TTNT curves were constructed according to the Kaplan-Meier method. Toxicities were graded using the National Cancer Institute Common Toxicity criteria (version 3).

3. Results

3.1. Patient Characteristics

A total of 19 patients were enrolled between December 2005 and April 2006. Patients were followed until March 2009 and the median follow-up from the start of the phase II study was 3 years (156 weeks; range, 28 - 180 weeks). The characteristics of the 19 patients are shown in **Table 1**. The mean age was 60 years (range, 42 - 81 years). More than half of the patients had relapsed after HDT-ASCT.

Eleven patients (57.9%) were treated with thalidomide at a dosage of 100 mg/day, 2 patients (10.5%) were treated at a dosage of 200 mg/day, 2 patients (10.5%) were treated at a dosage of 300 mg/day, and 4 patients (21.1%) were treated at a dosage of 400 mg/day.

3.2. Response

At the time of entry to this study, 5 patients (26.3%) had partial response (PR), another 5 patients (26.3%) had MR,

Table 1. Patients characteristics.

Variables	Total
Number of cases	19
Mean age (yr)	60.0
Range (yr)	42 - 81
Time sinceDx(yr)	5.03
Range (yr)	0.17 - 17
Sex (male/female)	8/11
M protein type	
IgG	12
IgA	6
Light chain	1
PS (0/1/2)	15/3/1
ISS stage (I/II/III)	11/3/5
Prior therapy	
Chemotherapy	8
Lines (median, range)	1.5, 1 - 3
ASCT	11
β 2M (mg/L) median, range	2.50, 1.0 - 10.24
LDH (IU/L) median, range	162.5, 105 - 346

Dx; diagnosis, PS; performance status, β2M; β2 microgobulin.

and the remaining 9 patients (47.4%) had shown NC to the latest thalidomide therapy.

The cumulative response rate is shown in **Figure 1**. The reduction of M-protein was continued at the 112th week. MR was obtained in 73.7% at the 24th week and in 78.9% at the 32nd week. PR was obtained 31.6% at the 24th week, 31.6% at the 32nd week, 42.1% at the 48th week, and 47.4% at the 112th week.

3.3. Progression-Free Survival

Figure 2 shows the PFS curve of the 19 patients studied; the median PFS was 104 weeks (1.99 years). The PFS rates were 73.0% at 1 year, 55.1% at 2 years, and 28.0% at 3 years. Only 1 patient has died on the 31st week.

3.4. Time to Next Treatment

Figure 3 shows the TTNT curve of the 19 patients. The median TTNT was 144 weeks (2.76 years). The TTNT rate was 73.7% at 1 year, 63.2% at 2 years, and 47.4% at 3 years. Thalidomide/high-dose dexamethasone treatment was conducted in 6 patients and melphalan/prednisolone/-thalidomide treatment was conducted in 3 patients after the discontinuance of single-agent thalidomide maintenance therapy. Eight patients continued single-dose thalidomide treatment over 40 months after progressive disease. Thalidomide was discontinued in only 2 patients.

3.5. Toxicities

All 19 patients experienced at least Grade 1 toxicity; however, no patient experienced Grade 4 or greater hematologic toxicity or Grade 3 or greater non-hematologic toxicity. The toxicity profile observed in at least 10 patients is shown in **Table 2**. The most common Grade 3 hematologic toxicities were neutropenia in 9 patients (47.4%), lymphopenia in 5 (26.3%), and leucopenia in 4 (21.1%). The most common Grade 2 non-hematologic toxicities were peripheral neuropathy in 3 patients (15.8%), constipation in 2 (10.5%), and skin rash in 2 (10.5%). One patient receiving 200 mg/d thalidomide but no thromboprophylaxis experienced deep vein thrombosis on the 71st week.

Toxicity or intolerance that resulted in the discontinuation or dose reduction of thalidomide occurred in 4 of

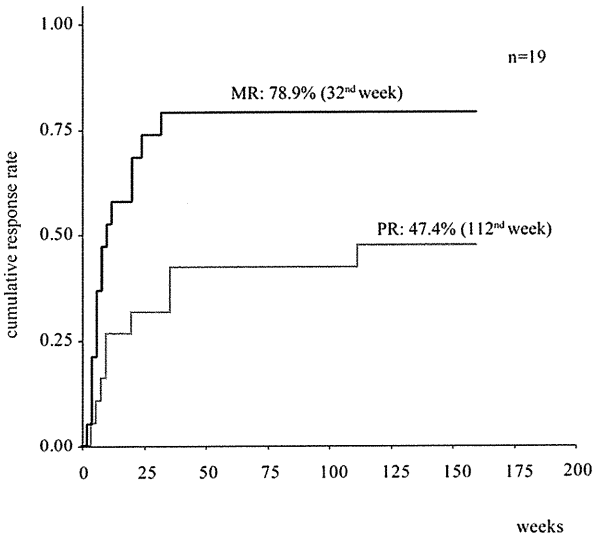

Figure 1. The cumulative response rate in patients treated with long-term thalidomide maintenance. The black line shows minimal response rate and the gray line shows partial response rate.

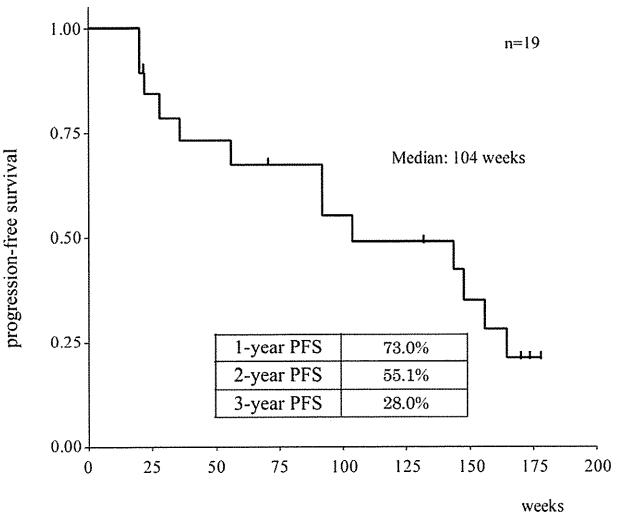

Figure 2. Progression-free survival in patients treated with long-term thalidomide maintenance. The curve was constructed according to the Kaplan-Meier method.

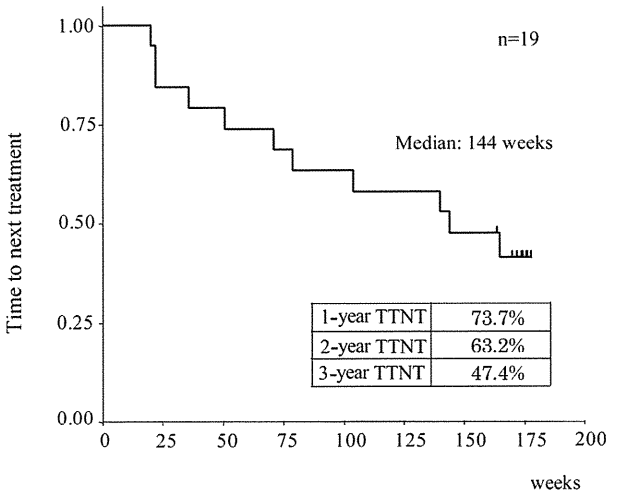

Figure 3. Time to next treatment in patients treated with long-term thalidomide maintenance. The curve was constructed according to the Kaplan-Meier method.

Table 2. Toxicity profile.

Toxicity	n (%)			
	Total	Grade 1	Grade 2	Grade 3
Hematological				
Neutropeni	14 (73.7)	1 (5.3)	4 (21.1)	9 (47.4)
Lymphopenia	10 (52.6)	1 (5.3)	4 (21.1)	5 (26.3)
Leucopenia	10 (52.6)	2 (10.5)	4 (21.1)	4 (21.1)
Basophilia	11 (57.9)	11 (57.9)	0	0
Non-hematological				
Constipation	16 (84.2)	14 (73.7)	2 (10.5)	0
Peripheral neuropathy	15 (78.9)	12 (63.2)	3 (15.8)	0
Somnolence	14 (73.7)	13 (68.4)	1 (5.3)	0
Dry mouth	11 (57.9)	11 (57.9)	0	0
Edema	10 (52.6)	10 (52.6)	0	0
Tremor	10 (52.6)	10 (52.6	0	0
Skin rash	10 (52.6)	8 (42.1)	2 (10.5)	0

the 19 patients (21.1%). These 4 patients were treated with thalidomide at a dosage of over 200 mg/day. Thalidomide treatment was discontinued in 2 patients: deep vein thrombosis occurred in 1 patient treated with 200 mg/d on the 71st week, and nephrotic syndrome occurred in 1 patient on the 132nd week. Thalidomide dosage was reduced in another 2 patients because of peripheral neuropathy and neutropenia. No patients treated with 100 mg/d thalidomide discontinued treatment due to toxicity.

4. Discussion

This study was conducted prospectively to evaluate the efficacy and safety of long-term treatment with single-agent thalidomide in patients who had been enrolled and obtained at least NC in a phase II trial of the use of thalidomide in patients with relapsed/refractory multiple myeloma [8]. The dosage of thalidomide in this continuous treatment study was determined by the one with which each patient had responded by achieving at least NC in the preceding phase II trial.

Nineteen patients were enrolled. Eleven patients (57.9%) were treated with continuous thalidomide treatment at a dosage of 100 mg/day, 2 patients (10.5%) were treated at a dosage of 200 mg/day, 2 patients (10.5%) were treated at a dosage of 300 mg/day, and 4 patients (21.1%) were treated at a dosage of 400 mg/day. The discontinuation or dose reduction of thalidomide occurred in 4 patients (21.1%). These 4 patients were treated with thalidomide at a dosage of over 200 mg/day. In contrast, no patients treated with 100 mg/d discontinued thalidomide due to toxicity. According to these findings, low-dose thalidomide might be adequate for the maintenance treatment for multiple myeloma.

The beneficial effect of long-term treatment with thalidomide was observed at least until week 112 (2.15 years). The PR rate was 26.3% at the time of initiation of maintenance treatment (16th week) and continuously increased up to 47.4% at the 112th week. The MR rate also increased from 26.3% at the initiation of maintenance treatment to 78.9% at the 32nd week. Singhal *et al.* reported that the response of thalidomide treatment was obtained within 4 months in patients with refractory myeloma [10]; however, our study revealed the response of long-term thalidomide maintenance treatment gradually increased over 4 months after initiation of thalidomide.

The PFS was fairly long in this study. It is difficult to compare this result with those in the published reports on single-agent thalidomide treatment in cases of relapsed/refractory multiple myeloma, because most trials used a starting thalidomide dose of 200 mg/d and utilized a dose escalation up to 800 mg/d, and also because the follow-up period is not as long as that in our study [6,11]. The case series studied may be mostly composed of low-risk patients in terms of age, International Staging System stages,

β2-microglobulin levels, and lactate dehydrogenase levels. Recent studies have shown the importance of obtaining complete response (CR) not only in patients with newly diagnosed multiple myeloma but also relapsed/-refractory multiple myeloma [12,13]. However, in patients in the low-risk category, survival is not significantly different between patients with CR and those with PR [14]. Another recent study has disclosed that maintaining CR is more important than obtaining CR in terms of longer survival duration [15]. It is also noted that patients with low-risk disease can survive longer with PR status [14]. In our patients, long-term maintenance treatment with thalidomide upgraded the initial response status and sustained the upgraded response status, which resulted in prolonged PFS.

The TTNT was extremely long compared with the PFS. Eight patients continued single-dose thalidomide treatment after progressive disease because of the absence of progression to active myeloma, namely clinical relapse [16]. According to this finding, one of the reasons for the long TTNT might depend on the slow progression to active myeloma during thalidomide maintenance treatment. Our results are in agreement with the comment of Stewart [17] that, in a slower-tempo relapse, sequencing of drugs may offer superior overall survival results.

With regard to a long-term treatment with thalidomide, there has been a concern of the late development of neuropathy if given sufficient length of time with low-dose thalidomide [18]. However, long-term treatment with low-dose thalidomide for as long as 3 years in the present study did not result in delayed development of adverse events. Furthermore, because of the lower toxicity of low-dose thalidomide, the patients could stay on the treatment and enjoyed a long-term survival with good activities of daily living.

5. Acknowledgements

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Cancer Research

Intracellular Tumor-Associated Antigens Represent Effective Targets for Passive Immunotherapy

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Abstract

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

Introduction

With the molecular identification of tumor antigens recognized by the human immune system, antigen-specific immunotherapy for cancers has been developed and is explored in the clinic (1–3). Particularly, monoclonal antibodies (mAb) that recognize surface antigens, such as trastuzumab (anti-Her2/neu) and rituximab (anti-CD20), as a single agent or in combination with chemotherapy, are used in the clinic for frontline or salvage therapy and have resulted in objective and durable clinical responses (3–5). One of the major therapeutic mechanisms of mAb is considered to be the selective interruption of vital signaling pathways in which the targeted antigens are critically involved (3, 5). In addition, there is accumulating evidence that mAb therapy also works through antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells or through the activation of complement, both of which depend on the Fc portion of the mAbs (6–9). Furthermore, Fc receptor–mediated uptake of immune complexes results in activation of antigen-presenting cells (APC) and facilitates cross-presentation of those antigens to tumor-specific $CD8^+$ T cells and inhibition of tumor growth, as was shown recently in HER2/*neu* and melanoma differentiation antigen tyrosinase–related protein-1 (Trp1; gp75) models (10–13).

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

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

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

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

Materials and Methods

Mice

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

Tumors

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

Antibodies and reagents

Anti-NY-ESO-1 mAbs [E978 (mouse IgG1) recognizing NY-ESO-1_{71-90}, ES121 (mouse IgG1) recognizing NY-ESO-1_{91-110}, 219–510 (mouse IgG1) recognizing NY-ESO-1_{21-40} (Supplementary Fig. S3; ref. 26)]; anti-CD4 (GK1.5, rat IgG2b), anti-CD8 (19/178, mouse IgG2a), and anti-MAGE-A4 (MCV1, mouse IgG1) were purified from hybridoma supernatant by protein G affinity chromatography. The F(ab) fragment of E978 was generated using the ImmunoPure Fab Preparation Kit (Thermo Fisher Scientific). Anti-CD8 (53–6.7), anti-CD45RB (16A), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-CD40 (3/23), anti-IFN-γ (XMG1.2), anti-CD62L (MEL-14), anti-CD11c (HL3), anti-TNF-α (MP6-XT22), and antimouse IgG1 (A85-1) mAbs were purchased from BD Biosciences, Biolegend, or eBioscience. Phycoerythrin (PE)-labeled NY-ESO-1_{81-88}-D^d tetramers were provided by Drs. P. Guillaume and I. Luescher (Ludwig Institute Core Facility, Lausanne, Switzerland). An anti-NY-ESO-1 human IgG1 mAb (12D7) was obtained from CT Atlantic. p63 (T) peptide TYLPTNASL (27), AH-$1_{138-147}$ peptide SPSYVHQF (28), and NY-ESO-1_{81-88} peptide RGPESRLL (23) were purchased from Operon Biotechnologies and BioSynthesis and Sigma.

Chemotherapeutic agents

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

Tumor challenge

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

Staining and flow cytometry

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

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

Fluorescent immunohistochemistry

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

Statistical analysis

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

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

Results

Establishment of CT26-NY-ESO-1

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

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

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

BALB/c mice were inoculated with CT26-NY-ESO-1 and were injected with 5-FU (75 mg/kg) and anti-NY-ESO-1 mAb (clone; E978, 100 μg, 2 days after 5-FU injection) when the tumor was palpable (around 25 mm^2). Treatment was repeated after 1 week. The combination treatment with anti-NY-ESO-1 mAb and 5-FU exhibited a significantly augmented antitumor effect and longer survival compared with control mice or mice that had received either 5-FU or anti-NY-ESO-1 mAb alone (Fig. 1A and B). This augmented antitumor effect was also observed when another anti-NY-ESO-1 mAb (clone; ES121, 100 μg) was used, but not with a control mAb, against another immunogenic CT antigen MAGE-A4, which is not expressed in the CT26-NY-ESO-1 cells (Fig. 1C and D). In contrast, combination treatment with anti-MAGE-A4 mAb (clone; MCV1, 100 μg), but not control antibody and 5-FU, exhibited an augmented antitumor effect against CT26-MAGE-A4 (Fig. 1E). To show that the effect of this combination treatment is not limited to the CT26, we examined the antitumor effect using CMS5a fibrosarcoma cells. BALB/c mice were inoculated with CMS5a-NY-ESO-1 and were injected with doxorubicin (50 μL intratumoral injection, 0.25 mmol/L) and anti-NY-ESO-1 mAb. As systemic administration of doxorubicin did not induce effective killing of CMS5a-NY-ESO-1, we used an intratumoral injection method. This combination treatment with anti-NY-ESO-1 mAb (but not an isotype control antibody) and doxorubicin exhibited a significantly augmented antitumor effect as well (Fig. 1F). These data suggest that the augmented antitumor effect is an antigen-specific phenomenon and that this combination treatment could be applicable to a broader range of intracellular antigens and tumors.

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

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

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

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

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

Considering a critical role of $CD8^+$ T cells, we examined NY-ESO-1–specific T cells in dLNs. BALB/c mice were inoculated with CT26-NY-ESO-1 and received the combination treatment. dLNs and tumors were harvested on days 14 to 16, and cells were incubated with NY-ESO-1_{81-88} (23) or control peptide, and cytokine secretion was analyzed. Combination treatment with anti-NY-ESO-1 mAb and 5-FU elicited significantly higher numbers of NY-ESO-1–specific $CD8^+$ T cells producing IFN-γ and/or TNF-α than 5-FU alone (Fig. 3A). Furthermore, there was a trend of higher numbers of NY-ESO-1–specific $CD8^+$ T cells in tumors treated with the combination treatment than those treated with 5-FU alone (Fig. 3C).

To explore further differences in NY-ESO-1_{81-88}–specific $CD8^+$ T cells, the effector/memory status was analyzed. The

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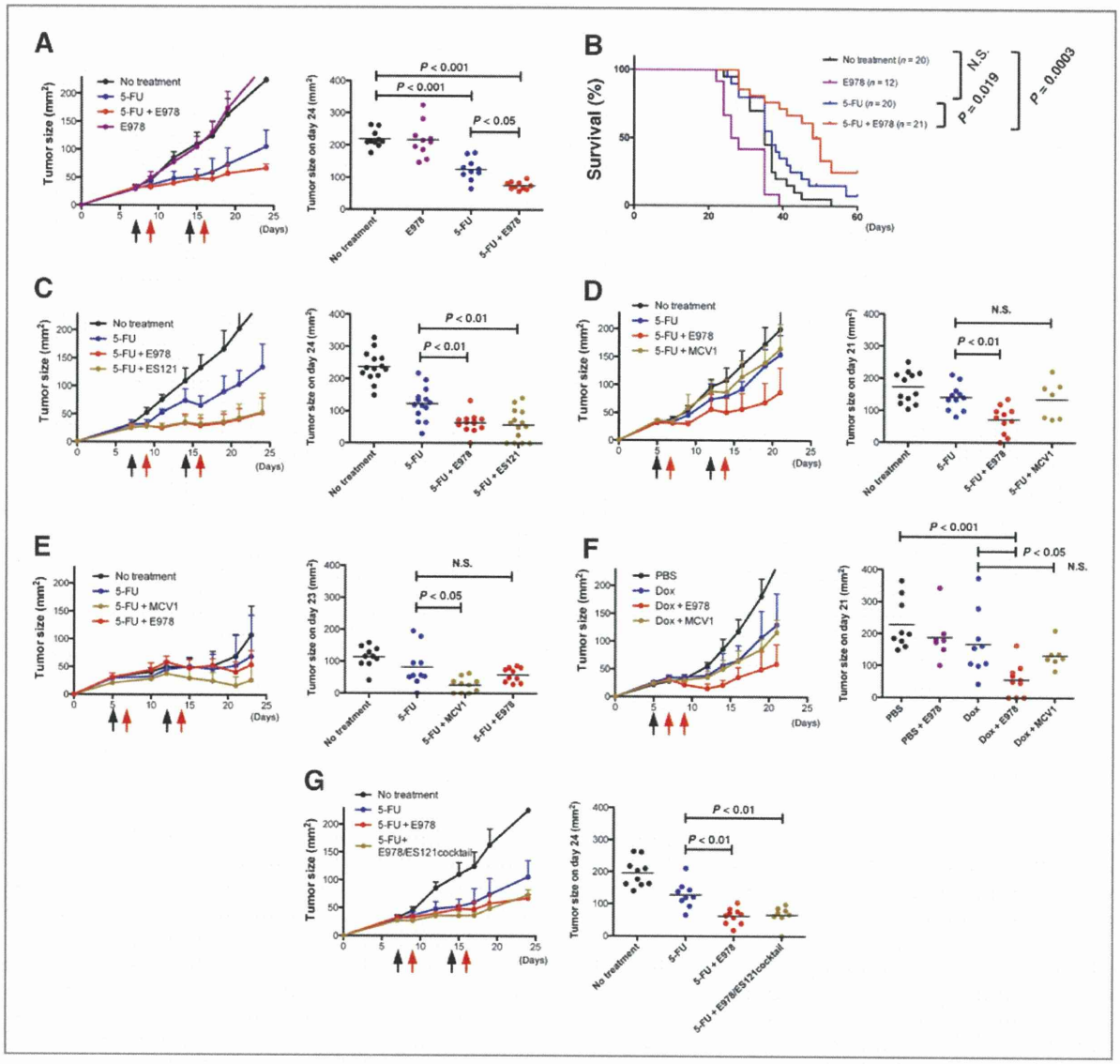

Figure 1. Combination of mAb and an anticancer drug exhibits augmented tumor growth inhibition. A–D and G, BALB/c mice were inoculated with CT26-NY-ESO-1 and treatment was started when tumors were palpable (around 25 mm^2, days 5–7). Mice received 5-FU intraperitoneally (black arrow) and/or anti-NY-ESO-1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

frequency of NY-ESO-1–specific $CD8^+$ T cells as measured by $CD8^+$NY-ESO-1/D^d $tetramer^+$ T cells was higher in mice treated with the combination therapy than in mice treated with 5-FU alone, confirming the data from the intracellular cytokine assays. The frequency of effector/memory ($CD62L^{low}CD45RB^{low}$) T cells was higher in mice treated with

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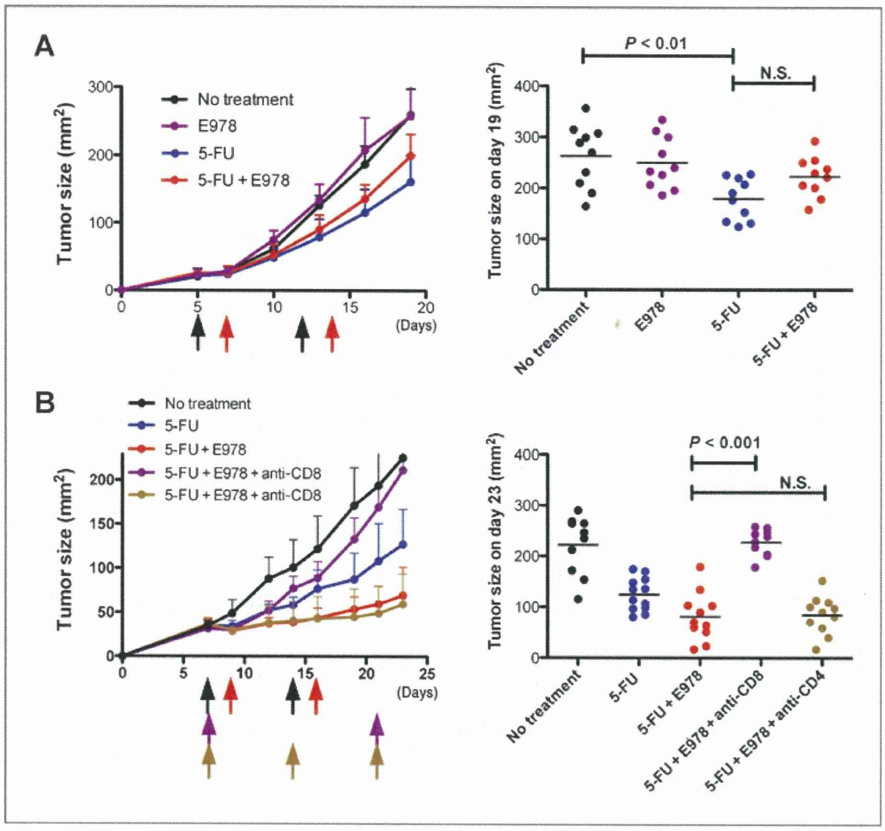

Figure 2. Augmented tumor growth inhibition by the combination treatment depends on CD8$^+$ T cells. A, BALB/c$^{nu/nu}$ mice were inoculated with CT26-NY-ESO-1 and treatment with 5-FU (days 5 and 12; black arrow) and anti-NY-ESO-1 mAb (E978, days 7 and 14; red arrow) was started as in Fig. 1A. Left,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

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

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

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

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

We next explored the mechanism(s) of the augmented antitumor effect and the differences of NY-ESO-1$_{81-88}$–specific CD8$^+$ T cells. The mAb therapy can exhibit immunostimulatory effects through the Fc portion of a mAb (7, 8). We investigated whether the augmented antitumor effect by combination treatment depended on the Fc portion of the mAb. BALB/c mice bearing CT26-NY-ESO-1 tumors were injected with 5-FU (days 7 and 14) and intact antibody or an Fc-depleted form of the anti-NY-ESO-1 mAb (days 9 and 16). The antitumor effect induced by the combination treatment with the intact anti-NY-ESO-1 mAb and 5-FU was totally abolished when F(ab) antibodies were administered (Fig. 5A). We further examined the critical role of the Fc portion for this augmented antitumor effect by the combination treatment using activating Fcγ receptor knockout mice (Fcer1g$^{-/-}$ mice). In these mice, we did not observe the augmented antitumor effect by the combination treatment compared with mice treated with 5-FU alone (Fig. 5B), confirming the critical role of the antibody–Fc portion for this augmented antitumor effect.

Accumulation of antibody to tumor sites by combination treatment

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

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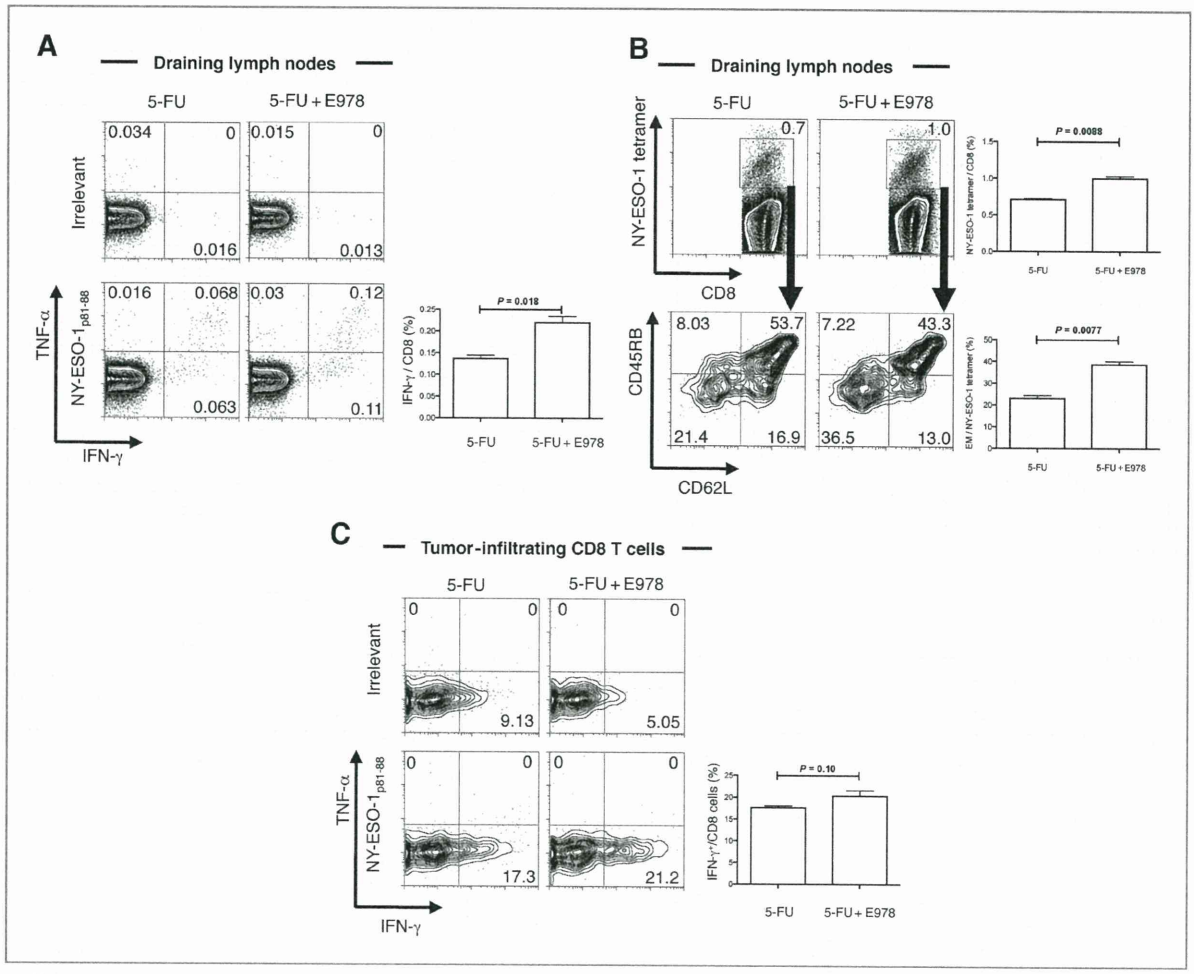

Figure 3. Combination treatment enhances NY-ESO-1–specific CD8$^+$ T-cell induction. A–C, BALB/c mice ($n = 3$) were inoculated with CT26-NY-ESO-1 and injected with 5-FU and anti-NY-ESO-1 mAb (E978). A, on day 14, dLNs were removed and incubated with NY-ESO-1_{81-88} or control peptide. IFN-γ and TNF-α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

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

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

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

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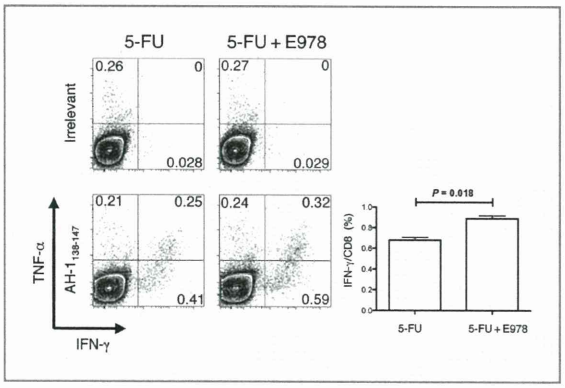

Figure 4. Antigen spreading is observed in mice that received the combination treatment. BALB/c mice ($n = 3$) were inoculated with CT26-NY-ESO-1 and injected with 5-FU and anti-NY-ESO-1 mAb (E978). On day 24, dLNs were removed and incubated with AH-$1_{138-147}$ or control peptide. IFN-γ and TNF-α secretion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

Discussion

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

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

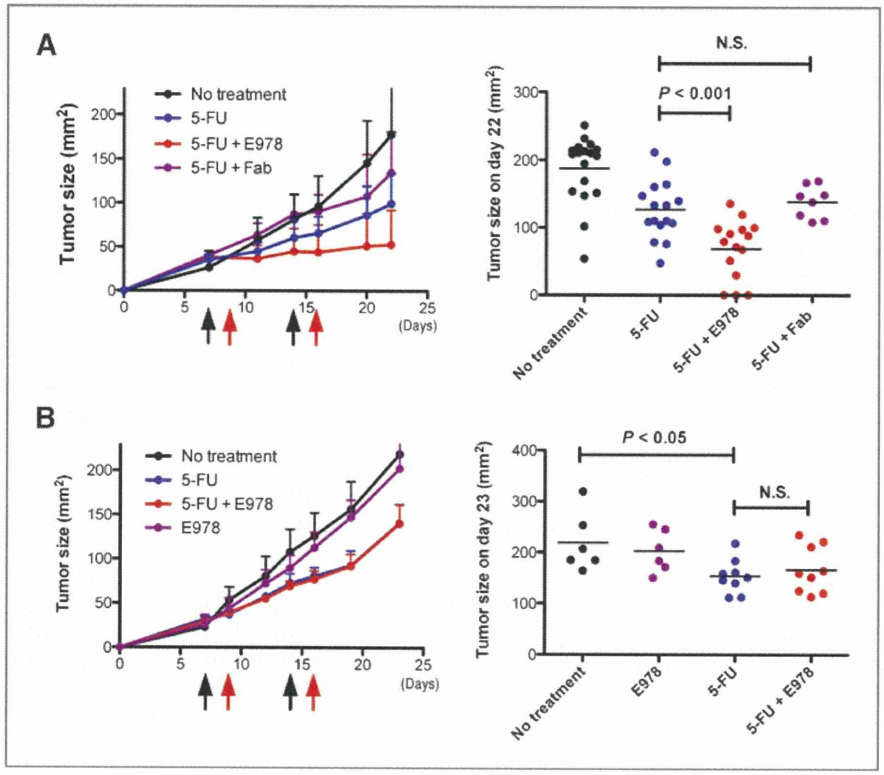

Figure 5. The Fc receptor signals are required for augmented antitumor effects by the combination treatment. A, BALB/c mice were inoculated with CT26-NY-ESO-1 and injected with 5-FU (days 7 and 14) and intact or Fc-depleted F(ab) anti-NY-ESO-1 mAb (E978, days 9 and 16). B, Fcγ receptor knockout mice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

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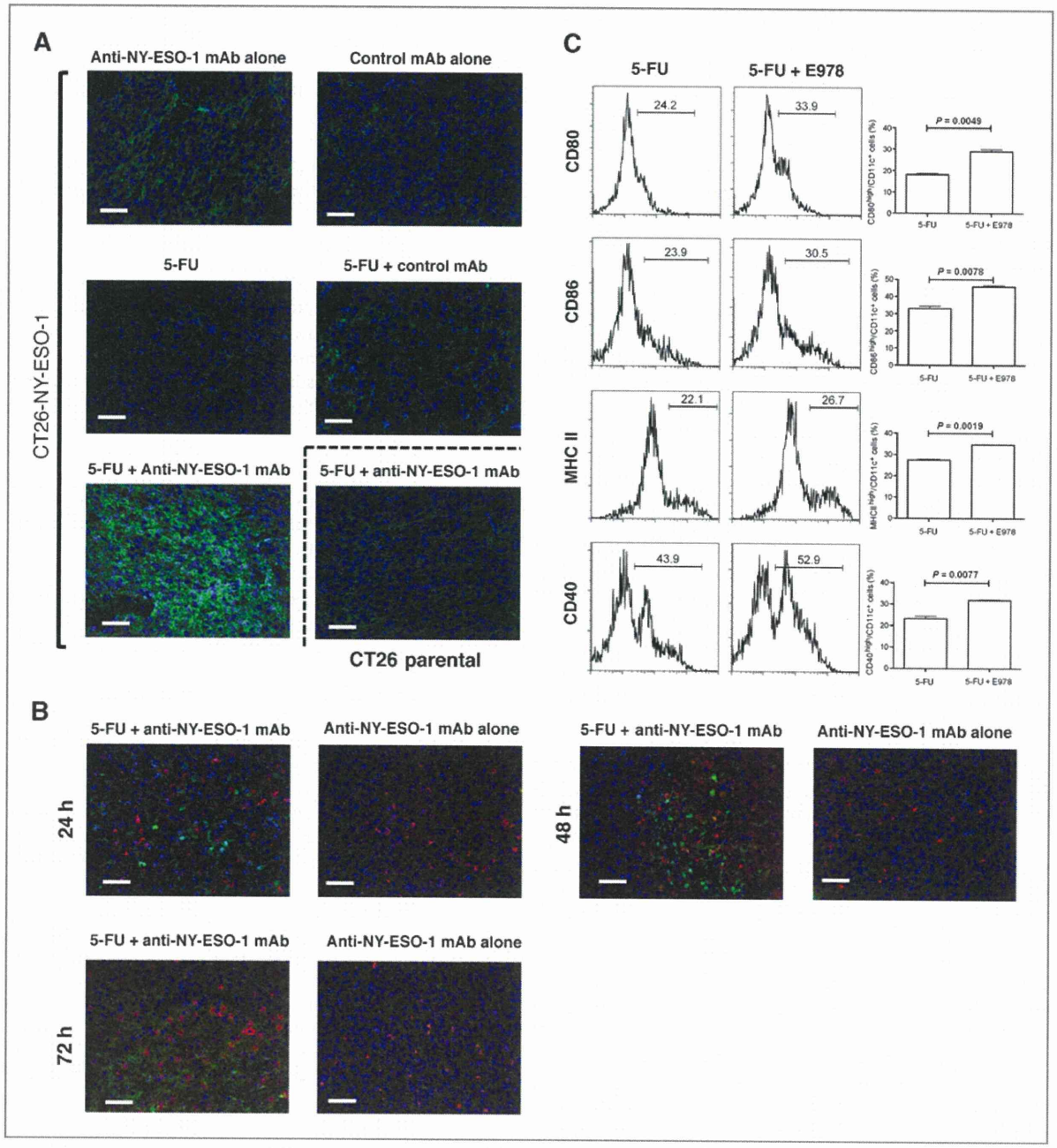

Figure 6. The combination treatment results in accumulation of injected antibody at the tumor site and induces maturation of DCs. A, BALB/c mice were inoculated with CT26-NY-ESO-1 and injected with 5-FU (day 5) and human anti-NY-ESO-1 mAb (12D7, day 7) or human anti-A33 mAb as a control (day 7). Twenty-four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

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

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

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

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

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

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

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

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Noguchi, G. Ritter
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Noguchi, T. Kato, Y. Maeda, S. Gnjatic, G. Ritter, L.J. Old, H. Shiku, H. Nishikawa
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Study supervision: T. Kato, G. Ritter, L.J. Old, H. Shiku

In Memoriam

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

Acknowledgments

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Heteroclitic serological response in esophageal and prostate cancer patients after NY-ESO-1 protein vaccination

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NY-ESO-1 is a prototypic cancer/testis antigen. In a recent phase I clinical trial, we vaccinated 13 patients bearing NY-ESO-1-expressing tumors with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein (CHP-NY-ESO-1) and showed efficient induction of NY-ESO-1 antibody, and CD4 and CD8 T cell responses using peripheral blood from the patients. In our study, we analyzed heteroclitic serological responses in those patients after vaccination. Serological response against 11 tumor antigens including MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SOX2, SSX2, XAGE1B and p53 was examined by enzyme-linked immunosorbent assay (ELISA) using sera from ten vaccinated patients. Expression of tumor antigens was determined by reverse transcription-polymerase chain reaction or immunohistochemistry. Eight of nine patients who showed antibody responses against NY-ESO-1 also showed an antibody response against at least 1 of these 11 tumor antigens after vaccination. In one patient, seven tumor antigens were recognized. Specificity analysis of the antibody response by ELISA using control recombinant proteins and synthetic peptides and by Western blot showed that the response was not against His6-tag and/or bacterial products included in a preparation of CHP-NY-ESO-1 used for vaccination. Thus, heteroclitic serological responses appear to be indicative of the overall immune response against the tumor, and their analysis could be useful for immune monitoring in cancer vaccine.

NY-ESO-1 antigen was originally identified in esophageal cancer by serological expression cloning (SEREX) using autologous patient serum[1,2] and has been shown to be strongly immunogenic. Patients bearing NY-ESO-1-expressing tumors often show antibody and CD4 and CD8 T cell responses spontaneously.[3–5] Clinical trials using NY-ESO-1 peptide,[6–9] protein[10,11] and viral constructs[12] as cancer vaccine have been conducted. Those studies have demonstrated efficient induction of antibody, and CD4 and CD8 T cell responses, and have also shown in some sporadic cases efficacy of immunotherapy.[2,6–12]

In a recent phase I clinical trial, we vaccinated 13 patients including eight Stage IV esophageal cancer patients, four

Key words: NY-ESO-1, cancer vaccine, cancer testis antigen, heteroclitic antibody response

Abbreviations: CHP: cholesterol-bearing hydrophobized pullulan; CMV: Cytomegalovirus; CT antigen: cancer/testis antigen; CTL: cytotoxic T lymphocytes; DHFR: dihydrofolate reductase; EBV: Epstein–Barr virus; OD: optical density; OLPs: overlapping peptides; RT-PCR: reverse transcription-polymerase chain reaction; SEREX: serological expression cloning

Additional Supporting Information may be found in the online version of this article

The authors declare that there is no conflict of interest

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Stage D3 prostate cancer patients and a Stage IV malignant melanoma patient with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein (CHP-NY-ESO-1).[13–16] We showed that the vaccine was well tolerated and had a potent capacity to induce NY-ESO-1 antibody and CD4 and CD8 T cell responses using peripheral blood from patients. Beneficial effects such as tumor regression or no tumor growth for a prolonged period were observed in six of seven disease-evaluable patients. However, all patients died eventually. Immunohistochemical analysis of the tumors that grew after vaccination suggested involvement of different mechanisms resulting in immune impairment.[15] NY-ESO-1-antigen loss was observed in a patient, disappearance of tumor-infiltrating CD4 and CD8 T cells was observed in two patients and an increase in the number of $CD68^+$ macrophages was observed in another patient. Further study will be necessary to elucidate cellular mechanisms including regulatory T cells causing immune suppression at the local tumor site.

It has been shown that cytotoxic T cell responses to tumor antigens other than the antigen used for immunization occur after vaccination.[17–26] This heteroclitic immune response following the initial response to a specific antigen was originally described in autoimmune disease and has been suggested to play a crucial role in clinical responses mediated by cancer vaccines.[27,28] Occurrence of CD8 T cell responses to unrelated tumor antigens was shown in studies of vaccination with MAGE-A1 and/or MAGE-A3,[19,20,25] HER-2/neu,[17,18,21,22] MART-1/Melan-A[23–25] and gp100.[26]

In our study, we analyzed the heteroclitic serological response against a panel of tumor antigens. Specificity analysis of antibody response was performed by enzyme-linked immunosorbent assay (ELISA) using control recombinant proteins and synthetic peptides, and by Western blot. The findings indicate frequent occurrence of heteroclitic serological responses in patients after CHP-NY-ESO-1 vaccination.

Material and Methods

Patients and sera

Eight advanced esophageal cancer patients (E-1, E-2, E-3, E-4, E-5, E-6, E-7 and E-8) and two prostate cancer patients (P-2 and P-3) were enrolled in the clinical trial (protocol LUD 2002-005 of the Ludwig Institute for Cancer Research, New York, NY) (http://clinicaltrials.gov/ct2/show/NCT00106158?term=CHP+NY-ESO-1&rank=2).[13–16] Peripheral blood was drawn from patients with written informed consent with the permission of the ethics committees of Osaka and Okayama Universities. Sera from MAGE-A4 seropositive esophageal cancer patients (EC-1, EC-2 and EC-3) and five healthy donors were drawn with written informed consent with the permission of the ethics committees of Osaka University. Sera were stored in −80°C freezer until use.

Preparation of a complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 protein (CHP-NY-ESO-1)

Preparation of recombinant NY-ESO-1 protein for vaccine was described elsewhere.[29] A complex of CHP and NY-ESO-1 protein (CHP-NY-ESO-1) and the schedule of vaccine were described previously.[14] Briefly, patients with advanced cancers expressing NY-ESO-1 were injected 2–31 times subcutaneously at biweekly intervals with 100 μg of NY-ESO-1 recombinant protein formulated with 2 mg of CHP.

Recombinant protein and overlapping peptides

N-His6-tagged recombinant proteins, NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, p53, SOX2, SSX2, XAGE1B, dihydrofolate reductase (DHFR) and Akt,[30–32] were produced by using pQE30 vector (QIAGEN, Hilden, Germany) and expressed in M15 *E. coli* cells. N-His6-tagged p53 produced in a *Baculovirus* system was purchased (Enzo Life Sciences, New York, NY). N-His6-tagged CCDC-62 protein was produced in a *Baculovirus* system at Okayama University.[33]

A series of 22 25-mer MAGE-A4 overlapping peptides (OLPs) were synthesized using standard solid-phase methods based on *N*-(9-fluorenyl)-methoxycarbonyl chemistry on an ABIMED Multiple Peptide Synthesizer (AMS422, ABIMED, Langenfeld, Germany) at Okayama University. MAGE-A4 OLPs: 1 (1–25), 2 (15–39), 3 (29–53), 4 (43–67), 5 (57–81), 6 (71–95), 7 (85–109), 8 (99–123), 9 (113–137), 10 (127–151), 11 (141–165), 12 (155–179), 13 (169–193), 14 (183–207), 15 (197–221), 16 (221–235), 17 (225–249), 18 (239–263), 19 (253–277), 20 (267–291), 21 (281–305), 22 (295–317).

MAGE-A4 transfectants

CMS5a, a murine fibrosarcoma cell line from a strain of BALB/c origin, was stably transfected with pcDNA3.1 MAGE-A4 plasmid as described previously.[34] The plasmid was provided by Dr. A. Kuroda and Dr. M. Miyamoto (Hokkaido University, Sapporo, Japan) and purified using plasmid Mini kit.

ELISA

Serially diluted sera were added to 96-well plates coated overnight at 4°C with 1 μg/ml recombinant protein or 5 μg/ml peptide solution and blocked for 2 hr at room temperature. After overnight incubation, plates were extensively washed with PBS containing 0.2% Tween 20. For determining reciprocal titer by ELISA, shown in Figure 1, serum IgG bound to antigens was detected by alkaline phosphatase-conjugated specific monoclonal antibody (Southern Biotech, Birmingham, AL). After addition of ATTOPHOS substrate (Fisher Scientific, Waltham, MA), absorbance was measured using a Cytofluor Series 4000 fluorescence reader (PerSeptive Biosystems, Framingham, MA). A reciprocal titer was calculated for each sample as the maximal dilution still significantly reacting to a specific antigen. Specificity was determined by

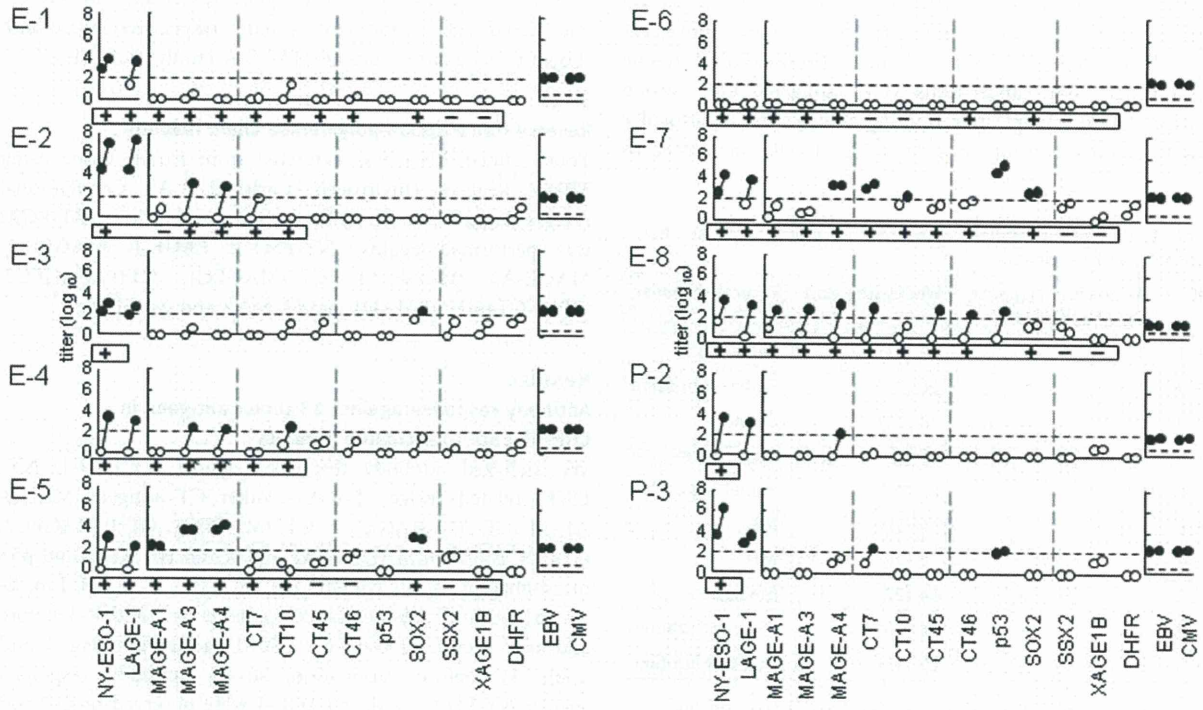

Figure 1. Reciprocal antibody titer against 13 tumor antigens in sera from patients vaccinated with CHP-NY-ESO-1 by ELISA. Serially diluted sera obtained before and after vaccination were assayed against N-His6-tagged recombinant proteins NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, p53, SOX2, SSX2, XAGE1B and DHFR. The reciprocal titer was the maximal dilution showing significant reaction (open and closed circles). Closed circles indicate reciprocal titers exceeding 100 (positive reaction). In each assay, antibody positive and negative sera were included as controls. Positive (+) and negative (–) expression of tumor antigens indicated in boxes under each panel was analyzed by RT-PCR and/or IHC when sample was available (see Supporting Information Table). Titer of EBV and CMV antibody in sera were measured by EBV and CMV kits, respectively. Values exceeding 4.0 were positive by manufacturer's indication. +*; 57B and M3H67 mAbs generated against MAGE-A3 and MAGE-A4 recombinant proteins, respectively, were both shown to recognize multiple MAGE-A family molecules.

comparing seroreactivity among the various antigens tested.[31] In each assay, positive and negative control sera were included. A positive result was defined as reciprocal titers >100. For conventional ELISA, peroxidase-conjugated goat anti-human IgG or IgM (Jackson Immuno Research Laboratory, West Grove, PA) was added to the wells for second antibody. After washing, signals were developed with *o*-phenylene diamine dihydrochloride, and absorbance at 490 nm was read using an ELISA reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA). Positivity was defined as sample optical density (OD) greater than three times that of the value for irrelevant control protein. Titers of Epstein–Barr virus (EBV) and Cytomegalovirus (CMV) antibody in sera were measured by EBV VCA kit (Denka Seiken, Tokyo, Japan) and CMV kit (Denka Seiken), respectively.

Western blot

Recombinant protein (20 ng) or cell lysate (20 μg) in sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS and 1 mM dithiothreitol) was boiled for 5 min and subjected to SDS-PAGE with 10–20% polyacrylamide BioRad Ready-Gels (Bio-Rad). After electrophoresis, the membrane (Hybond-P membrane, Amersham Pharmacia Biotech, Buckinghamshire, UK) was blocked with 5% FCS/PBS and then incubated with patients' sera diluted 1:1,000 for recombinant protein or 1:200 for cell lysate for 1 hr at room temperature. After washing, alkaline phosphatase-conjugated goat anti-human IgG (Jackson Immuno Research Laboratory) was added to the membrane. Signals were developed with a 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chromogenic substrate kit (Bio-Rad). Polyclonal rabbit anti-MAGE-A1 serum (Abcam, Cambridge, UK) and monoclonal anti-MAGE-A4 (clone 3D12; Abnova, Taipei, Taiwan), anti-p53 (clone PAb421; Enzo) and anti-His6-tag (clone OGHis; MBL, Nagoya, Japan) antibodies were used for positive controls at 1:1,000 dilution.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using formalin-fixed paraffin-embedded specimens. Monoclonal antibodies