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.
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/cnu/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\(_{11-90}\), ES121 (mouse IgG1) recognizing NY-ESO-1\(_{101-116}\), 219–510 (mouse IgG1) recognizing NY-ESO-1\(_{211-40}\) (Supplementary Fig. S3; ref. 26); anti-CD4 (GK1.5, rat IgG2b), anti-CD8 (19/178, mouse IgG2a), and anti-MAGE-A4 (MCVI, 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 (XM612), anti-CD62L (MEI-14), anti-CD11c (HL3), anti-TNF-α (MP6-XT22), and antimouse IgG1 (A85-1) mAbs were purchased from BD Biosciences, Biologend, or eBioscience. Phycoerythrin (PE)-labeled NY-ESO-1\(_{141-148}\)-D\(^3\) 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 TLYPTNASL (27), AH-1\(_{128-147}\) peptide SPSTYHQF (28), and NY-ESO-1\(_{131-148}\) peptide RGPSERLL (23) were purchased from Operon Biotecnologies 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 GolgiPlugs was added for the last 4 hours of culture. These cells were stained for surface markers and intracellularly with allopurinol-conjugated anti-IFN-γ and PE-conjugated anti-TNF-α mAbs after permeabilization and fixation using Cytofix/Cytoperm Kit (BD Biosciences). Dead cells were excluded by LIVE/DEAD Fixable Dead Cell Stain Kits (Invitrogen). Cells were analyzed on FACSCan or FACSCalibur (BD Biosciences) 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 anti-human 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 anti-rabbit 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 Axioscan system (Carl Zeiss).

**Statistical analysis**

Tumor curves were assessed by one-way ANOVA with a Bonferroni multiple comparisons posttest. Single measurement
comparison between 2 groups was evaluated by 2-sided Student t test. P values <0.05 were considered statistically significant.

Results

Establishment of CT26-NY-ESO-1
We established a syngeneic colon carcinoma model (CT26-NY-ESO-1) with stable NY-ESO-1 expression (2, 22, 23). NY-ESO-1 expression in CT26-NY-ESO-1 cells was exclusively intracellular, and no NY-ESO-1 protein was detected on the cell surface (Supplementary Fig. S1A), consistent with the expression of NY-ESO-1 protein in human cancer cells (2). These CT26-NY-ESO-1 cells maintained the same tumor growth capacity as their parental CT26 cells in both wild-type Balb/c and C.B-17 SCID (severe combined immunodeficient) mice, indicating that there was no alteration of tumorigenicity caused by the NY-ESO-1 transfection (Supplementary Fig. S1B). When BALB/c mice were inoculated with CT26-NY-ESO-1 cells, spontaneous antibody and CDS+ 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 mm³). 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 CDS+ 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/cnu/nu mice. BALB/cnu/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/cnu/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 CDS+ 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-1-88 (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-1-88–specific CD8+ T cells, the effector/memory status was analyzed. The
frequency of NY-ESO-1–specific CD8⁺ T cells as measured by CD8⁺ NY-ESO-1/1D₃ 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 (CD62LlowCD45RBlow) T cells was higher in mice treated with

Figure 1. Combination of mAb and an anticancer drug exhibits augmented tumor growth inhibition. A–D, BALB/c mice were inoculated with CT26-NY-ESO-1 and treatment was started when tumors were palpable (around 25 mm², days 5–7). Mice received 5-FU intraperitoneally (black arrow) and/or anti-NY-ESO-1 mAb (clone; E978 or ES121) or anti-MAGE-A4 mAb (clone; MCV1) 2 days after 5-FU injection (red arrow). Treatment was repeated twice at 1-week intervals. A, left, tumor growth curves representative of 2 independent experiments; right, summary of tumor size on day 24 of 2 independent experiments. B, survival rate curves summarized from another 3 independent experiments (separate from tumor growth data) are shown. C and D, left, tumor growth curves representative of 2 independent experiments. Right, summary of tumor size of 2 independent experiments on days 24 and 21, respectively. E, BALB/c mice were inoculated with CT26-MAGE-A4 and treatment was started as in A. Mice received 5-FU (black arrow) and anti-MAGE-A4 mAb (clone; MCV1) or anti-NY-ESO-1 mAb (clone; E978) 2 days after 5-FU injection (red arrow). Left, tumor growth curves representative of 2 independent experiments; right, summary of tumor size on day 23 of 2 independent experiments. F, BALB/c mice were inoculated with CMSs-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.
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 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). 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 antibody–Fc portion is required for the augmented antitumor effect by the combination treatment**

We next explored the mechanism(s) of the augmented antitumor effect and the differences of NY-ESO-181–88–specific CD8$^+$ T cells. The mAb therapy can exhibit immunostimulatory effects through the Fc portion of a mAb (7, 8). We investigated whether the augmented antitumor effect by combination treatment depended on the Fc portion of the mAb. BALB/c mice bearing CT26-NY-ESO-1 tumors were injected with 5-FU (days 7 and 14) and anti-NY-ESO-1 mAb (E978, days 7 and 14; red arrow) or anti-CD8 mAb (days 7 and 14; brown arrow) or anti-CD8 mAb (days 7 and 14; 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. Each group consisted of 5 to 7 mice. Data are presented as mean ± SD. N.S., not significant.
(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).

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-181–88 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-181–88–specific CD8+ T cells identified as CD8+ NY-ESO-181–88/Dd tetramer+ T cells was analyzed. C, tumor-infiltrating lymphocytes were collected on day 16 and incubated with NY-ESO-181–88 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.
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 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-1138–147 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.

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.
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

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 anti-human 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^+ DCs was analyzed. These experiments were repeated twice with similar results.
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 readress 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, cryoaiblation, 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 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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In Memoriam
This article is dedicated to the memory of L.J. Old.

References


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