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Cell Surface Antibody Retention Influences *In Vivo* Antitumor Activity Mediated by Antibody-dependent Cellular Cytotoxicity

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Abstract. *Background: Multiple factors affect the in vivo antitumor activity of antibody-based therapeutics; however, the influence of cell surface retention on antibody-dependent cellular cytotoxicity (ADCC) is not fully understood. Here we evaluated the importance of cell surface antibody retention in antitumor activity mediated by ADCC in vivo. Materials and Methods: Two mAbs against tumor-associated calcium signal transducer 2 (TACSTD2/TROP2), AR47A6.4.2 and Pr1E11, were used. Antitumor activities against BxPC3 and Colo205 cells were investigated through in vitro and in vivo assays. Results: Pr1E11 showed better cell surface retention than AR47A6.4.2 in vitro although Pr1E11 and AR47A6.4.2 showed equivalent ADCC activity. Complement-dependent cytotoxicity and antiproliferative activity were not observed for either antibody. Pr1E11 exhibited higher antitumor activity than AR47A6.4.2 in vivo. Conclusion: Our results suggest that high cell surface retention can result in potent ADCC activity in vivo. This observation could provide novel insight into how effectively screen for antibodies with strong in vivo antitumor activity.*

Monoclonal antibodies (mAb) represent an attractive format for cancer therapeutics due to their specificity and biological activity. For tumor antigen-targeting mAbs, naked human IgG1 (specifically, an IgG1 antibody that is not conjugated to an effector) has been widely used because this isotype can elicit effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (1). Evidence from several studies suggests the importance of these effector functions in mAb therapeutics. In particular, ADCC activity is believed to be important for antitumor action in the clinical setting because functional polymorphisms in FcγRIIIA (CD16), a major receptor that triggers ADCC, are associated with the therapeutic efficacy of mAbs (2). It has been reported that multiple factors affect ADCC activity, including antibody density on the cell surface, binding affinity, binding valency (monovalent or bivalent), and the binding epitope (3, 4).

In order to screen for mAbs with strong ADCC, *in vitro* assays are generally performed to select for candidate clones because, in most cases, *in vitro* ADCC activity generally correlates with *in vivo* ADCC activity. However, for other antibodies, especially those specific for highly internalized antigens, *in vitro* ADCC assays do not reflect *in vivo* ADCC activity.

Tumor-associated calcium signal transducer 2 (TACSTD2/TROP2), a type I glycoprotein that has high homology with epithelial cell adhesion molecule (EPCAM/TROP1), is a suitable target antigen for studying this issue (5). Increasing evidence suggests that TROP2 is an attractive target for mAb-based therapeutics because it is abundantly expressed in malignant tumors and contributes to tumor aggressiveness (6-8). RS7 is a well-known mAb to TROP2 that was established by Stein *et al.* (9, 10). This clone was found to have significant ADCC activity against TROP2-positive cancer cells *in vitro* but did not elicit *in vivo* antitumor activity in its naked format (11). Since RS7 is rapidly internalized after target antigen binding, these observations led us to speculate that antibody retention on the cell surface (after target antigen binding) is important for *in vivo* ADCC activity.

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In this study, we compared the *in vitro* and *in vivo* antitumor activity of two mAbs to TROP2, AR47A6.4.2 and Pr1E11, in order to determine the importance of cell surface antibody retention on antitumor activity. To date, AR47A6.4.2 is the only TROP2 mAb clone that has been found to display significant antitumor activity *in vivo* (12). Pr1E11 was established by our group from a modified adenovirus-based screening system. Pr1E11 was shown to bind to TROP2 with higher affinity than AR47A6.4.2, and recognized a unique epitope. In addition, cell surface retention of Pr1E11 was higher than that of AR47A6.4.2 *in vitro* (Table I) (13).

Materials and Methods

Cell lines. The human pancreatic cancer cell line BxPC3 was purchased from the American Type Culture Collection (Manassas, VA, USA). The human colon cancer cell line Colo205 was purchased from DS Pharma Biomedical (Osaka, Japan). These cells were maintained in RPMI-1640 medium (Nakalai tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 50 μg/ml gentamicin (Nakalai tesque). The Chinese hamster ovary cell line CHO/DG44 was a kind gift from Dr. Lawrence Chasin (Columbia University, New York, NY, USA) and maintained in iscove's modified dulbecco's medium (Nakalai tesque) supplemented with 10% dialyzed FBS (Gibco), HT supplement (Gibco) and 50 μg/ml gentamicin. CHO-K1 was purchased from RIKEN (Tsukuba, Japan) and maintained in EX-CELL325PF CHO serum-free medium (Sigma, St. Louis, MO, USA) supplemented with 6 mM L-glutamine (Nakalai tesque) and 50 μg/ml gentamicin. An α-1,6-fucosyltransferase (*FUT8*)-knockout CHO cell line, *FUT8*$^{-/-}$ CHO, for defucosylated antibody production was developed at Kyowa Hakko Kirin Co., Ltd., as previously described (14).

Peripheral blood mononuclear cells (PBMCs). Peripheral blood was collected from healthy volunteers registered at Kyowa Hakko Kirin Co., Ltd. All donors gave written informed consent before blood collection, in accordance with the process approved by the Institutional Ethical Committee of Kyowa Hakko Kirin (approval number #2009_024_00). PBMCs were prepared from heparinized blood using Lymphoprep (Axis Shield, Dundee, UK).

Mice. C.B-17/lcr-[severe-combined immunodeficient (SCID)] Jcl mice were purchased from CLEA Japan (Shizuoka, Japan) and maintained under specific pathogen-free conditions. All animal studies were performed in accordance with the Standards for Proper Conduct of Animal Experiments at Kyowa Hakko Kirin Co., Ltd., under the approval of the company's Institutional Animal Care and Use Committee (approval number #10-015).

Anti-TROP2 antibodies. Recombinant fucosylated [Fuc(+)] and defucosylated [Fuc(−)] chimeric AR47A6.4.2 (cAR47A6.4.2) and Pr1E11 (cPr1E11) antibodies, with a human IgG1 constant region, were produced by CHO/DG44 or *FUT8*$^{-/-}$ CHO cells as previously described (8). Recombinant mouse IgG1 AR47A6.4.2 (mAR47A6.4.2) and Pr1E11 (mPr1E11) antibodies were generated as follows. Polymerase chain reaction amplicons of each antibody variable region and mouse IgG1 constant region were inserted into pKANTEX93 plasmid vectors, and transfected into CHO/DG44 and CHO-K1 cells. Antibodies were purified from culture supernatants using Hitrap protein G HP (GE Healthcare, Tokyo, Japan).

ADCC assay. An ADCC assay was performed as previously described (10). Briefly, target cells (1×10^4 cells/well) and PBMCs (2.5×10^5 cells/well, as effector cells), were seeded in a 96-well U-bottom plate and incubated with different concentrations (0.1-1,000 ng/ml) of antibodies for 4 h at 37˚C. Supernatants were collected for a lactose dehydrogenase (LDH) release assay using an LDH-Cytotoxic test (Wako, Osaka, Japan) in order to evaluate cytotoxicity. Assay measurement was performed according to the manufacturer's protocol. The percentage of cytotoxicity was calculated according to the following formula:

$$\text{Cytotoxicity (\%)} = 100 \times (E - SE - ST)/(M - SE),$$

where E was the experimental release (supernatant activity from target cells incubated with antibody and effector cells), SE was the spontaneous release in the presence of effector cells (supernatant activity from target cells incubated with effector cells), ST was the spontaneous release of target cells (supernatant activity from target cells incubated with medium alone), and M was the maximum release of target cells (activity released from target cells lysed with 9% Triton X-100).

Complement-dependent cytotoxicity (CDC) assay. A CDC assay was performed as previously described (10). Briefly, target cells were seeded at 1×10^4 cells/well in a 96-well flat-bottom plate with different concentrations (1-10,000 ng/ml) of antibodies and incubated with a human complement serum (Sigma) at a 1/6 dilution in medium. After a 2-h incubation at 37˚C, WST-1 reagent (Roche Applied Science, Mannheim, Germany) was added and the plate was further incubated for 4 h to detect formazan dye production by living cells. The absorbance of each well was measured in accordance with the manufacturer's protocol. The percentage of cytotoxicity was calculated according to the following formula:

$$\text{Cytotoxicity (\%)} = 100 \times (C - E)/(C - B),$$

where C was the absorbance of target cells cultured in medium with complement alone, E was that in the presence of TROP2 mAb, and B was the absorbance of a blank well that contained medium and complement without target cells.

Cell proliferation assay. Cells were seeded at 500 cells/well in a 96-well flat-bottom plate with different concentrations (31.6-10,000 ng/ml) of antibodies. After 5-day incubation at 37˚C, WST-1 regent was added and further incubation was performed for 4 h. The independent effect of secondary antibody (ThermoFisher Scientific, Waltham, MA, USA), used for crosslinking TROP-2, was also evaluated. The effect on cell proliferation was calculated according to the following formula:

$$\text{Cytotoxicity (\%)} = 100 \times (C - E)/(C - B),$$

where C was the absorbance of target cells cultured in medium alone, E was that in the presence of TROP2 mAb, and B was the absorbance of a blank well containing medium without target cells.

In vivo antitumor activity of TROP2 antibody in mouse xenograft models. BxPC3 or Colo205 cells (5×10^6 cells) were inoculated *s.c.* into 5- to 6-week-old male SCID mice. After 5 to 6 days, a tumor

Table I. *Summary of biochemical characteristics of Pr1E11 and AR47A6.4.2 monoclonal antibodies to tumor-associated calcium signal transducer 2 (TACSTD2/TROP2) (8).*

Antibody	Binding kinetics[a]			Binding epitope[b]	Antibody retention on cell surface[c]
	k_a (1/Ms)	k_d (1/s)	K_D (nM)		
Pr1E11	4.16×10^5	5.80×10^{-5}	0.143	Cysteine-rich domain (*N*-terminal domain)	50%
AR47A6.4.2	2.21×10^5	2.18×10^{-4}	1.01	Cysteine-poor domain (membrane proximal domain)	33%

[a]Kinetic rate constants and equilibrium constants of each chimeric antibody against TROP2/Fc/6×His were determined by surface plasmon resonance analysis. [b]Determined by western blotting using TROP2 domain deletion mutants. [c]Evaluated by flow cytometric analysis of the percentage of antibody remaining bound to the cell surface after 180 min.

volume of approximately 100 mm^3 was achieved; the mice were randomly assigned to groups of five to eight animals each, based on tumor volume, and treated with antibody solution or phosphate-buffered saline (PBS)(−) by *i.p.* administration twice per week. The tumor volume was calculated according to the following formula:

Tumor volume (mm^3)=0.5 × major diameter (mm) × [minor diameter (mm)]^2

Statistical analysis. All *in vitro* experiments were performed in triplicate and results are reported as the mean with standard error (SEM). Statistical analysis of *in vivo* experiments was performed with SAS software (release 9.2; SAS Institute Inc., Cary, NC, USA). Statistical significance between the PBS-treated group and the mAb-treated groups was determined by the Kruskal–Wallis and Steel tests.

Results

AR47A6.4.2 and Pr1E11 showed equivalent ADCC activity in vitro. The ADCC activity of chimeric TROP2 mAb was evaluated using BxPC3 (Figure 1A) and Colo205 (Figure 1B) cells, as TROP2 is highly expressed in these cell lines (8). PBMCs were prepared from four independent donors and used as effector cells. When fucosylated chimeric mAbs were used, weak ADCC activity was observed for cAR47A6.4.2/Fuc(+) and cPr1E11/Fuc(+) against the two cell lines. A high antibody concentration (greater than 100 ng/ml) was needed to induce apparent cytotoxicity. In contrast, when defucosylated chimeric mAbs were used, cAR47A6.4.2/Fuc(−) and cPr1E11/Fuc(−) exhibited ADCC activity from 1 ng/ml. The activity reached a maximum at 100 ng/ml and was higher than those of fucosylated mAbs. Maximum ADCC activity for the two mAbs was comparable.

AR47A6.4.2 and Pr1E11 did not elicit CDC and antiproliferative activity in vitro. CDC assays were performed using defucosylated TROP2 mAbs and human complement. When BxPC3 and Colo205 cell lines were used as target cells, neither cAR47A6.4.2/Fuc(−) nor cPr1E11/Fuc(−) had any cytotoxic activity (Figure 2).

In order to assess *in vitro* antiproliferative activity, BxPC3 and Colo205 cells were cultured with chimeric TROP2 mAbs in the presence or absence of anti-human IgG polyclonal antibody, as cross-linker. As shown in Figure 3, neither cAR47A6.4.2/Fuc(−) nor cPr1E11/Fuc(−) affected proliferation in the two cell lines.

In vivo antitumor effect of TROP2 antibody using mouse xenograft models. The *in vivo* antitumor effects of defucosylated chimeric mAbs were examined in mouse xenograft models. In the BxPC3 xenograft model (Figure 4), cPr1E11/Fuc(−) showed stronger anti-tumor activity than cAR47A6.4.2/Fuc(−). The minimum tumor growth inhibition ratio (T/C_{min}) at day 36 for cPr1E11/Fuc(−) and cAR47A6.4.2/Fuc(−) was 0.36 and 0.47, respectively. Comparable to this result, cPr1E11/Fuc(−) had superior antitumor activity in the Colo205 xenograft model compared with cAR47A6.4.2/Fuc(−) and showed significant antitumor activity. The T/C_{min} at day 44 for cPr1E11/Fuc(−) and cAR47A6.4.2/Fuc(−) was 0.29 and 0.48, respectively.

In order to elucidate the *in vivo* mechanism of action for Pr1E11 and AR47A6.4.2, we compared the antitumor activity of defucosylated human IgG1 mAbs and mouse IgG1 mAbs. Human IgG1-type mAbs were expected to exert *in vivo* activity through both Fc-dependent and independent functions. In contrast, the effector function of mouse IgG1 is known to be weak (15, 16); therefore, *in vivo* activity mostly depends on its Fc-independent function. Both mAR47A6.4.2 and mPr1E11 retained their binding activity to TROP2-expressing cell lines (data not shown). However, as shown in Figure 5, mouse IgG1 mAbs were completely devoid of antitumor activity.

Discussion

ADCC activity is mediated by multiple factors such as binding affinity, epitope, and antigen expression level; therefore, we often observe that different clones exhibit different biochemical characteristics but have equivalent

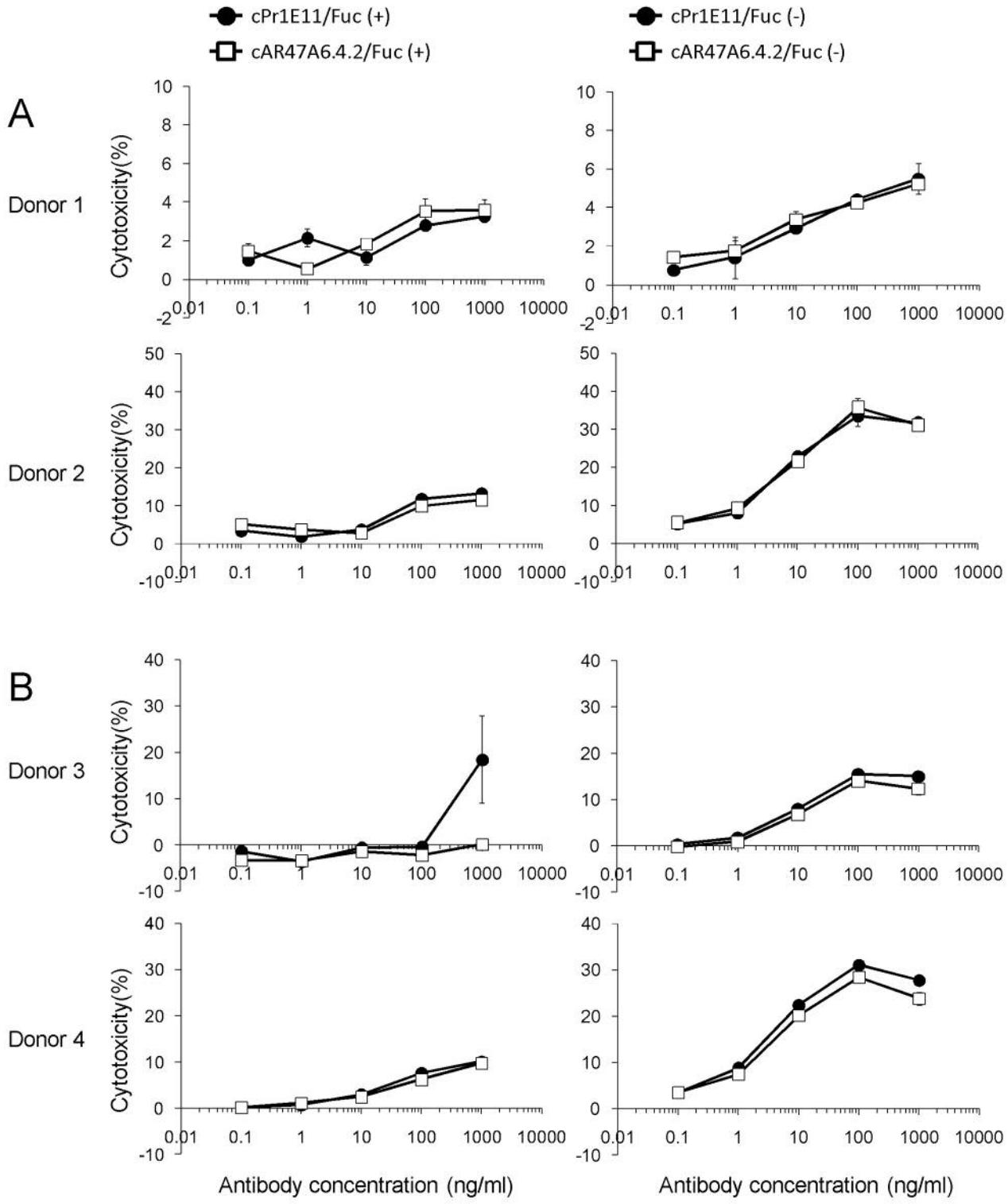

Figure 1. *Antibody-dependent cellular cytotoxicity activity of monoclonal antibodies to tumor-associated calcium signal transducer 2 (TACSTD2/TROP2) against epithelial cancer cell lines BxPC3 (A) and Colo205 (B). Target cells were incubated with human peripheral blood mononuclear cells as effector cells (effector/target=25/1) and different concentrations of recombinant fucosylated [Fuc(+)] and defucosylated [Fuc(−)] chimeric AR47A6.4.2 (cAR47A6.4.2) and Pr1E11 (cPr1E11) monoclonal antibodies to TROP2, with a human IgG1 constant region. After a 4-h incubation, the activity of released lactose dehydrogenase in the supernatants was measured and cytotoxicity (%) was calculated as described in the Material and Methods. All data are reported as the mean±SEM (n=3).*

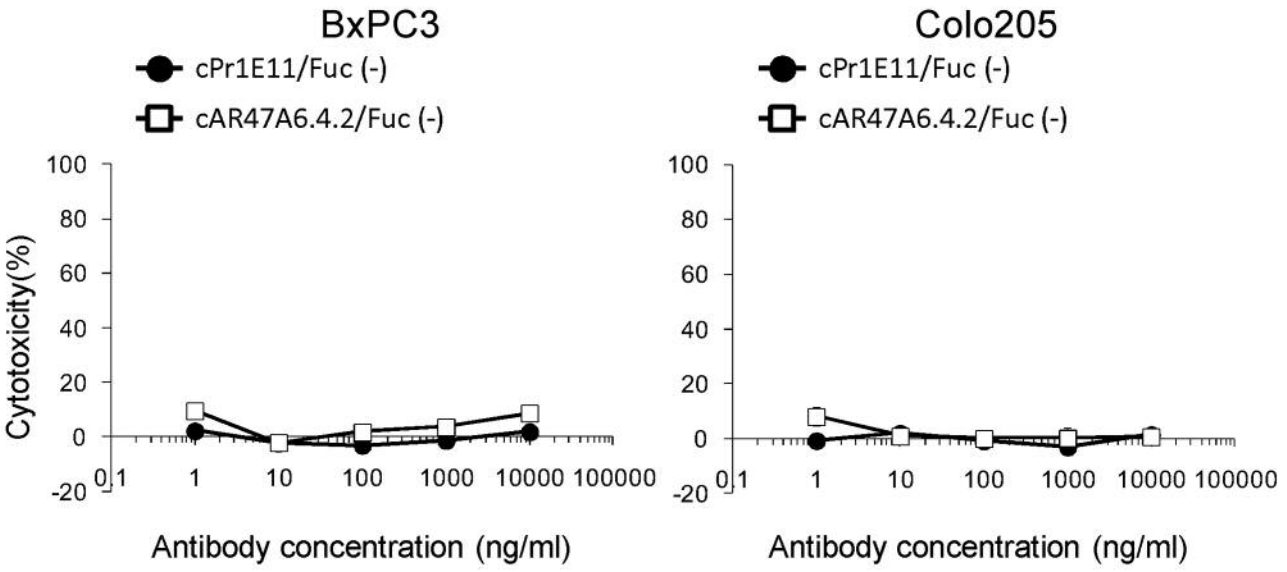

Figure 2. *Complement-dependent cytotoxicity activity of monoclonal antibodies to tumor-associated calcium signal transducer 2 (TACSTD2/TROP2) against epithelial cancer cell lines. Two epithelial cancer cell lines, BxPC3 and Colo205, were incubated with human complement and recombinant defucosylated chimeric AR47A6.4.2 [cAR47A6.4.2/Fuc(−)] and Pr1E11 [cPr1E11/Fuc(−)]monoclonal antibodies to TROP2. After a 2-h incubation, WST-1 reagent was added to quantify cytotoxicity (%). All data are reported as the mean±SEM (n=3).*

ADCC activities *in vitro*. In addition, *in vitro* ADCC activity sometimes does not reflect *in vivo* ADCC activity. Thus, these problems make the process of how to select strong ADCC-inducing clones unclear. In this study, we evaluated the *in vitro* and *in vivo* antitumor activities of two anti-TROP-2 mAbs, AR47A6.4.2 and Pr1E11, in order to identify the key factor important for *in vivo* ADCC activity. To date, AR47A6.4.2 is the only antibody clone that has shown *in vivo* antitumor activity in its naked format. Pr1E11 was established by our group, and has different biochemical properties (high affinity, different epitope, and high cell surface retention) compared to AR47A6.4.2.

We reveal that cPr1E11 and cAR47A6.4.2 have equivalent ADCC activity *in vitro*; however, the *in vivo* antitumor activity of cPr1E11 was higher than that of cAR47A6.4.2. Truong *et al*. reported that AR47A6.4.2 exerts CDC-mediated cell killing of BxPC3 cells (12). In order to confirm their observations, we evaluated the CDC activity of cPr1E11 and cAR47A6.4.2 in two different cell lines, including BxPC3. However, under our experimental conditions, we did not observe CDC activity for either antibody. In Truong *et al*.'s study, rabbit serum was used for the CDC assay to maximize CDC activity. In contrast, we employed human complement. This difference in complement source might explain the discrepancy in CDC activity. It was also reported that AR47A6.4.2 partially suppressed mitogen-activated protein kinase phosphorylation in response to serum stimulation, possibly contributing to its antiproliferative activity. However, in our study, neither cPr1E11 nor cAR47A6.4.2 showed any antiproliferative effect. Antibody hyper-crosslinking, using secondary polyclonal antibodies, was shown to enhance antiproliferative activity for some antigens (17, 18). This reaction condition mimics FcγR-mediated antibody crosslinking *in vivo*. We evaluated the combined effect of TROP2 mAbs and a secondary antibody but antiproliferative activity was not observed. In order to confirm the *in vivo* mechanism of action, we prepared mouse IgG1-type mAbs and evaluated their *in vivo* activities. This was because mouse IgG1 is incapable of mediating Fc-dependent cytotoxicity. Using the Colo205 xenograft model, we found that both mPr1E11 and mAR47A6.4.2 IgG1-type mAbs were completely devoid of antitumor activity. These results indicate that the *in vivo* efficacies of cPr1E11 and cAR47A6.4.2 are mostly dependent on ADCC.

From these results, it is apparent that sustained exposure to antibody might affect the *in vivo* antitumor activity for antibodies that primarily function through ADCC. It has been reported that target antigen-mediated internalization reduces the *in vivo* half-life and local concentration of antibodies (19, 20). In addition, antibody-mediated antigen internalization reduces the antigen density on target cell surfaces, which restricts antibody-mediated effector functions. Therefore, a high level of antigen–antibody internalization potentially relucts ADCC activity *in vivo*, but not *in vitro*. In this study, we did not evaluate the antibody half-life and changes in antigen levels in antibody-treated tumors *in vivo*. Further studies are needed to evaluate this hypothesis.

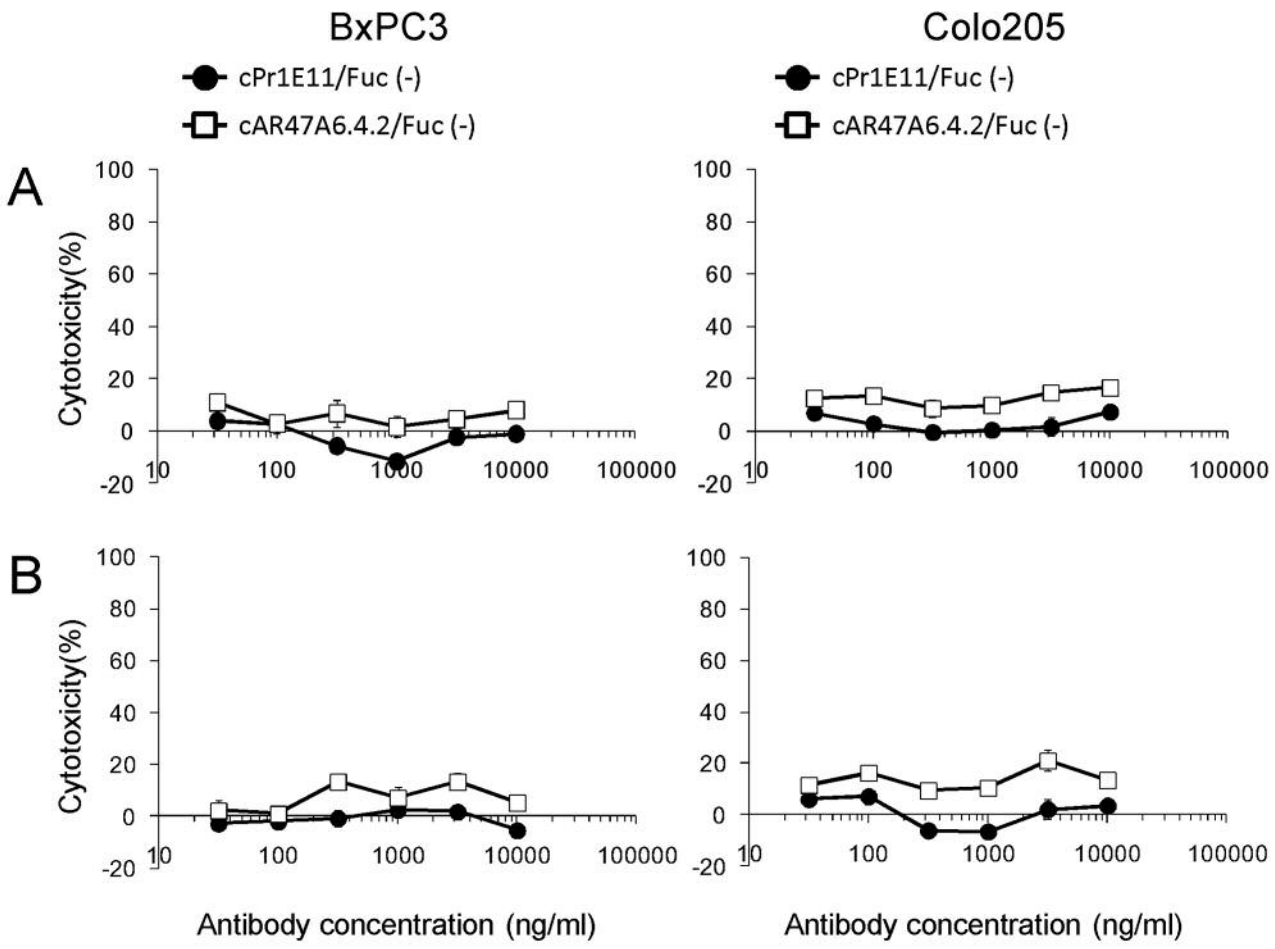

Figure 3. *In vitro antitumor activity of cPr1E11and cAR47A6.4.2. Effect of monoclonal antibodies to tumor-associated calcium signal transducer 2 (TACSTD2/TROP2) on cell proliferation were evaluated in the absence (A) and presence (B) of a secondary antibody as a cross-linker. Two epithelial cancer cell lines, BxPC3 and Colo205, were incubated with recombinant defucosylated chimeric AR47A6.4.2 [cAR47A6.4.2/Fuc(−)] and Pr1E11 [cPr1E11/Fuc(−)] monoclonal antibodies to TROP2. After a 5-day incubation, WST-1 reagent was used to determine antiproliferative activity (%). All data are reported as the mean±SEM (n=3).*

In conclusion, we demonstrated that Pr1E11 exerts higher ADCC *in vivo* than AR47A6.4.2, despite their *in vitro* ADCC activities being comparable. Our results led us to the hypothesis that cell surface retention of the antibody is an important factor for *in vivo* ADCC activity, and would affect antigen and antibody down-regulation *in vivo*. If this were true, the assessment of cell surface antibody retention activity *in vitro* would represent an easy approach to selecting clones capable of eliciting strong ADCC *in vivo*. Cell surface retention is the net result of dissociation and internalization of antibodies bound to cell surface antigens, and is easy to measure using appropriate methods (specifically, flow cytometry and cell-based enzyme-linked immunosorbent assay). We also demonstrated that TROP2 is a suitable target not only for antibody-drug conjugate but also for ADCC-dependent naked antibody-based therapeutics. To our knowledge, Pr1E11 is the best TROP2-targeting clone for this application to date. Further evaluation is needed to confirm its efficacy and safety for clinical use.

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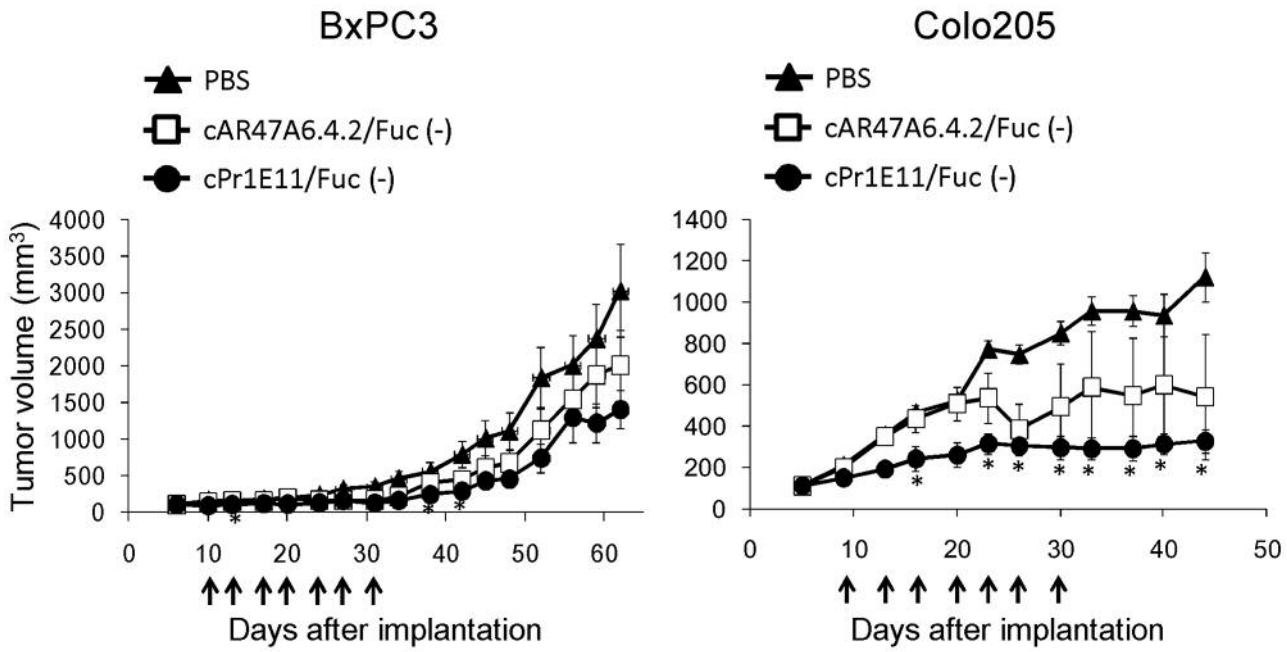

Figure 4. *Therapeutic efficacy of monoclonal antibodies to tumor-associated calcium signal transducer 2 (TACSTD2/TROP2) in mouse xenograft models. SCID mice were inoculated s.c. with 5×10^6 cells (BxPC3 or Colo205) (day 0). When the tumor volume reached approximately 100 mm^3, mice were treated with PBS(−) (control), or 200 μg of defucosylated chimeric AR47A6.4.2 [cAR47A6.4.2/Fuc(−)] or Pr1E11 [cPr1E11/Fuc(−)] monoclonal antibodies to TROP2 twice a week for 4 weeks and tumor volumes were measured. Arrows indicate antibody treatment. *Statistically significantly different from the control group at p<0.05. All data are reported as the mean±SEM (n=5).*

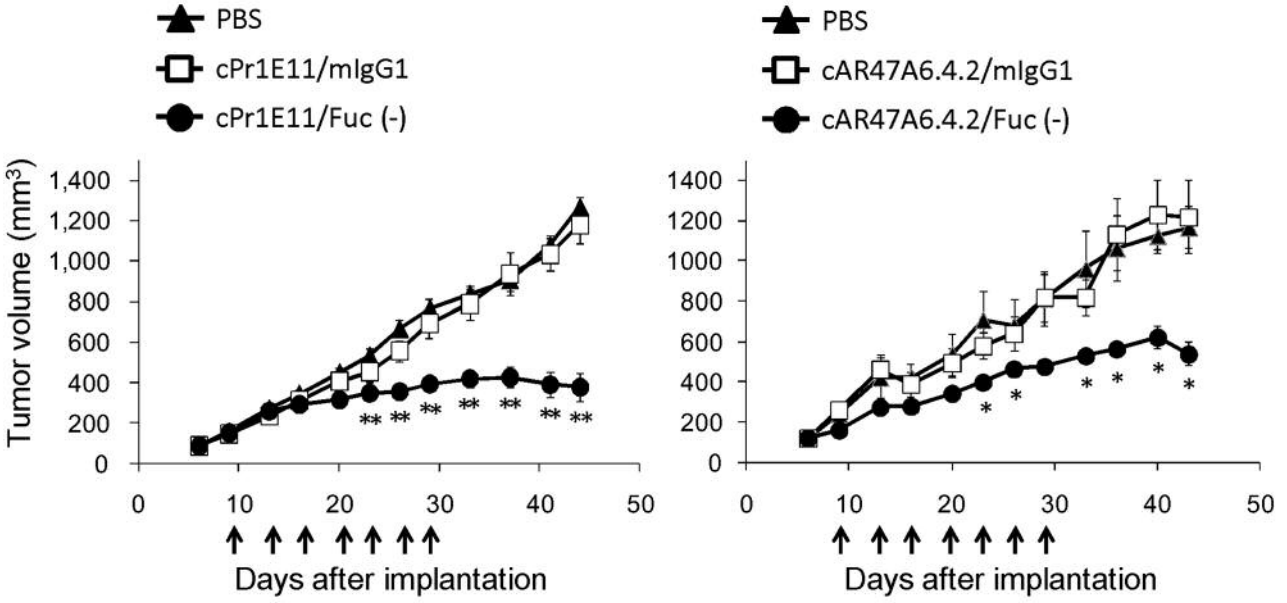

Figure 5. *In vivo mechanism of action for antitumor effect of monoclonal antibodies to tumor-associated calcium signal transducer 2 (TACSTD2/TROP2). SCID mice were inoculated s.c. with 5×10^6 Colo205 cells (day 0). When tumor volume reached approximately 100 mm^3, mice were treated with PBS(−) (control), 200 μg of mouse IgG1 or defucosylated [Fuc(−)] chimeric AR47A6.4.2 (cAR47A6.4.2) and Pr1E11 (cPr1E11) monoclonal antibodies to TROP2, twice a week for 4 weeks and tumor volumes were measured. Arrows indicate antibody treatment. Statistically significantly different from the control group at *p<0.05 and **p<0.01. All data are reported as the mean±SEM (n=5 or 8).*

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