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BRIEF REPORT

TRAIL-receptor 1 IgM antibodies strongly induce apoptosis in human cancer cells *in vitro* and *in vivo*

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ABSTRACT

Agonistic tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-receptor-specific antibodies are attractive antitumor therapeutics. Recently, our group has generated several human monoclonal antibodies (mAbs) to TRAIL-receptor-1 (TRAIL-R1) (TR1-IgGs) using ISAAC technology. However, these TR1-IgGs did not demonstrate ideal apoptosis-inducing capacity in the absence of additional antibodies. To overcome this limitation, we class-switched the TR1-IgGs to TRAIL-R1 IgM antibodies (TR1-IgMs); TR1-IgMs might possess high valency and facilitate the crosslinking of the cell surface receptors. We showed that the TR1-IgMs bound TRAIL-R1, activated the caspase signal, and induced strong apoptosis (100-fold higher compared with the IgG form in one case) in human tumor cell lines without any additional crosslinking *in vitro*. We further demonstrated that these TR1-IgMs dramatically inhibited tumor growth in a xenograft model through the caspase activation cascade. These data suggest that TR1-IgMs may become potential immunotherapeutic agents for cancer therapy.

Abbreviations: DISC, death-inducing signaling complex; EC_{50}, 50% effective concentration; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; IgM, immunoglobulin M; mAbs, monoclonal antibodies; PE, phycoerythrin; SCID, severe combined immunodeficient; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptors; TR1-IgG, TRAIL-R1-specific IgG; TR1-IgM, TRAIL-R1-specific IgM

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Introduction

Apoptosis is a cellular process in which the organism regulates the number of cells in normal tissue compartments and eliminates unnecessary or damaged cells.[1] There are two major signaling pathways that trigger apoptosis in mammalian cells: the intrinsic and extrinsic pathways.[2] The TRAIL, a member of the TNF superfamily of cytokines, is a trimeric protein that is capable of activating both the extrinsic and intrinsic pathways for cellular apoptosis.[3,4] By binding to TRAIL-R1 and -R2, TRAIL induces the formation of the death-inducing signaling complex (DISC), which consists of a Fas-associated death domain and procaspase-8 and -10.[5,6] The activated caspase-8 then triggers the apoptotic pathway by activating caspase-3, -6, and -7, which leads to cell death.[6-8] In addition to TRAIL-R1 and -R2, there are other receptors, such as TRAIL-R3, -R4, and osteoprotegerin (OPG), although these receptors cannot induce cellular apoptosis because they lack functional death domains.[9-12]

It is worth noting that TRAIL-induced apoptosis only occurs in human cancer cell lines, whereas it rarely occurs in normal cells.[13-17] Accordingly, TRAIL-R1 and -R2 have been widely studied as potential therapeutic targets of cancer in the past, and TRAIL variants and mAbs of TRAIL-R1 and -R2 have been developed as possible reagents for cancer treatment. However, the hepatotoxicity and short half-life of TRAIL variants have hampered their application.[18] Therefore, TRAIL-R1/2 antibodies are expected to be more attractive for cancer therapy.

TRAIL-R1/2 can independently self-assemble into trimers without any other ligands.[19] Their further assembly with ligands is a prerequisite for activating apoptosis.[20] Previous studies using xenograft tumor-bearing mice that were immunedeficient and lacked endogenous IgGs demonstrated that the anti-TRAIL-R1 or -R2 antibodies showed ideal antitumor effects by crosslinking the Fcγ receptors (FcγRs) on immune cells.[21-25] However, a series of clinical trials based on these effective antitumor antibodies have failed[26,27] because the crosslinking with FcγR might be impaired in the presence of competitive endogenous IgGs.[28]

Regarding other crosslinking, the use of secondary antibodies is not suitable for clinical treatments in patients because these antibodies are usually prepared from animals and may induce side effects due to their antigenicity.[29] Therefore, agonistic TRAIL-R1/2-specific mAbs that induce cancer cell apoptosis in the absence of crosslinking reagents are desired for

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cancer therapy. In this context, Pukac et al. developed a fully human agonistic TRAIL-R1 mAb, HGS-ETR1, which achieved significant apoptosis of tumor cells *in vitro* and *in vivo*, without additional crosslinking.[22] However, HGS-ETR1 did not show antitumor effects in clinical trials.[30-32]

We have also developed a series of human TRAIL-R1-specific IgGs (TR1-IgGs) that induce apoptosis using the ISAAC technology[33] and human immunoglobulin γ and κ loci chimeric TransChromo (TC) mice.[34] However, these mAbs could not induce apoptosis in the absence of crosslinking antibodies.[35] Antibodies of the IgM class play an important role in primary immune responses.[36,37] The predominant structure of IgM in serum is a pentameric form.[38] Recently, Kunert's group constructed a class-switched HIV-specific IgM by combining the variable region of HIV-IgG with the constant region of IgM.[39,40] Considering this finding, we hypothesized that the conversion of TR1-IgG to pentameric IgM might facilitate the cell surface receptor crosslinking due to the high valency of IgM. In this study, we generated TRAIL-R1-IgM antibodies (TR1-IgMs) by combining the variable region of TR1-IgGs[35] with the constant region of IgM. The resulting TR1-IgMs induced strong apoptosis in not only various human cancer cell lines but also xenograft tumor-bearing mice. These TR1-IgMs antibodies may become an expectable immunotherapeutic agent for cancer therapy.

Results

TR1-IgMs bind specifically to human TRAIL-R1

To produce the TR1-IgMs, we constructed the TR1-IgM cDNA by substituting the constant region of five clones (401, 404, 419, 422, and 438) of TR1-IgG with that of IgM.[35] We then co-transfected the cells with the heavy- and light-chain cDNAs together with or without the joining (J) chain cDNA. An anti-influenza IgM was also produced as a control using the same method. The purified TR1-IgMs and the control-IgM were confirmed by western blotting (Fig. 1A). Previous reports stated that the J chain existed only in pentameric IgMs, whereas the hexameric and pentameric forms of IgM were produced without the J chain.[41] Consistently, western blot analysis revealed that without J chain, the TR1-IgMs (TR1-IgM(J-)s) and control-IgM(J-) were present in both the pentameric and hexameric forms, whereas the TR1-IgM(J+)s and control-IgM(J+) with the J chain only formed pentamers (Fig. 1A). The TR1-IgM(J+)s and control-IgM(J+) were adopted in the subsequent studies because the pentameric form of IgM is predominant in human serum.[38]

To clarify whether the TR1-IgM(J+)s bind to recombinant human TRAIL receptor (TRAIL-R), we performed an enzyme-linked immunosorbent assay (ELISA) using chimeric recombinant human TRAIL-R-Fc proteins. All five TR1-IgM(J+)s speci-fically bound to TRAIL-R1-Fc, but not to TRAIL-R2-Fc, TRAIL-R3-Fc, TRAIL-R4-Fc, or OPG (Fig. 1B). The control-IgM did not bind to any of the proteins. The binding of TR1-IgM(J+) 401 to rhTRAIL was significantly weak. This weak binding may be due to its previously demonstrated low affinity.[35] In agreement with a previous study,[35] flow cytometry analysis also revealed that the TR1-IgM(J+)s bound to the surfaces of tumor cells that expressed TRAIL-R1 (Fig. 1C and Fig. S1). Together, these results indicated that our five clones of TR1-IgMs specifically bound to TRAIL-R1.

TR1-IgMs induce cell death by activating caspase signaling in human tumor cell lines

Next, we investigated whether the TR1-IgM(J+)s could induce cell death in cancer cell lines without additional crosslinking. Treatment of the TRAIL-sensitive Colo205 cell line[42] with various concentrations of TR1-IgM(J+)s resulted in a significant and dose-dependent reduction of cell viability at 24 h, whereas the groups treated with control-IgM(J+), control-IgG, or TR1-IgGs did not show cytotoxic activity, except that the TR1-IgG 438-treated group exhibited a weak induction of cell death (Fig. 2A). The 50% effective concentration (EC_{50}) of TR1-IgM (J+)s for the Colo205 cell line ranged from 0.5 to 100 ng/mL (Table 1 and Fig. S2). We compared the cytotoxic activity of TR1-IgG in the presence of the secondary crosslinking antibody with that of TR1-IgM (Fig. S3). TR1-IgG 422 with the secondary crosslinking antibody induced apoptosis in Colo205 cell line with EC_{50} of approximately 1,000 ng/mL, whereas that of TR-IgM 422 was 0.5 ng/mL, showing the remarkably higher cytotoxic activity of TR1-IgM compared to TR-1 IgG even in the presence of crosslinking antibody. TR1-IgM(J+) effectively induced apoptosis in the TRAIL-sensitive DU145 cell line[43] but not in the TRAIL-resistant A549[44] and MCF7[45] cell lines (Fig. S4). The current results demonstrated that the TR1-IgM(J+)s effectively induced apoptosis in the TRAIL-sensitive cell lines.

It has been reported that TRAIL-induced apoptosis in human cancer cells is primarily triggered by the activation of caspase-8 and -3, the major caspases that are responsible for apoptosis.[6] To verify whether TR1-IgMs induce cell death via the caspase activation pathway, we incubated Colo205 cells with TR1-IgM(J+)s, and the caspase activation was investigated. As shown in Fig. 2B, caspase-8 and capase-3/7 were clearly activated in a dose-dependent fashion after 3 and 6 h of culture, respectively. The control-IgM(J+) did not induce caspase-8 or caspase-3 activity in Colo205 cells. Furthermore, the presence of a pan-caspase inhibitor, z-VAD-fmk, which has been reported to block caspase-mediated apoptosis,[46] prevented the TR1-IgM(J+)s-induced reduction of cell viability corresponding to TRAIL-induced apoptosis (Fig. 2C). These results showed that TR1-IgM(J+)s induced cell death in TRAIL-R1-expressing cell lines in a caspase-dependent fashion.

In vivo antitumor effect of the TR1-IgM antibodies without a crosslinking reagent

To assess the antitumor activity of TR1-IgM(J+)s *in vivo*, we evaluated the capacity of the most effective TR1-IgM(J+) 422 antibody (Table 1) in xenograft models using Colo205 tumor-transplanted SCID mice. The tumors were pre-established to a volume of approximately 100 mm^3, and different doses of TR1-IgM(J+) 422 (1, 3.3, and 10 mg/kg body weight) and control-IgM(J+) (10 mg/kg body weight) were then intravenously (i.v.) administered to the mice three times per week. As shown in Fig. 3A and 3B, treatment with TR1-IgM(J+) 422 resulted in an initial reduction in the tumor size and the retardation of tumor

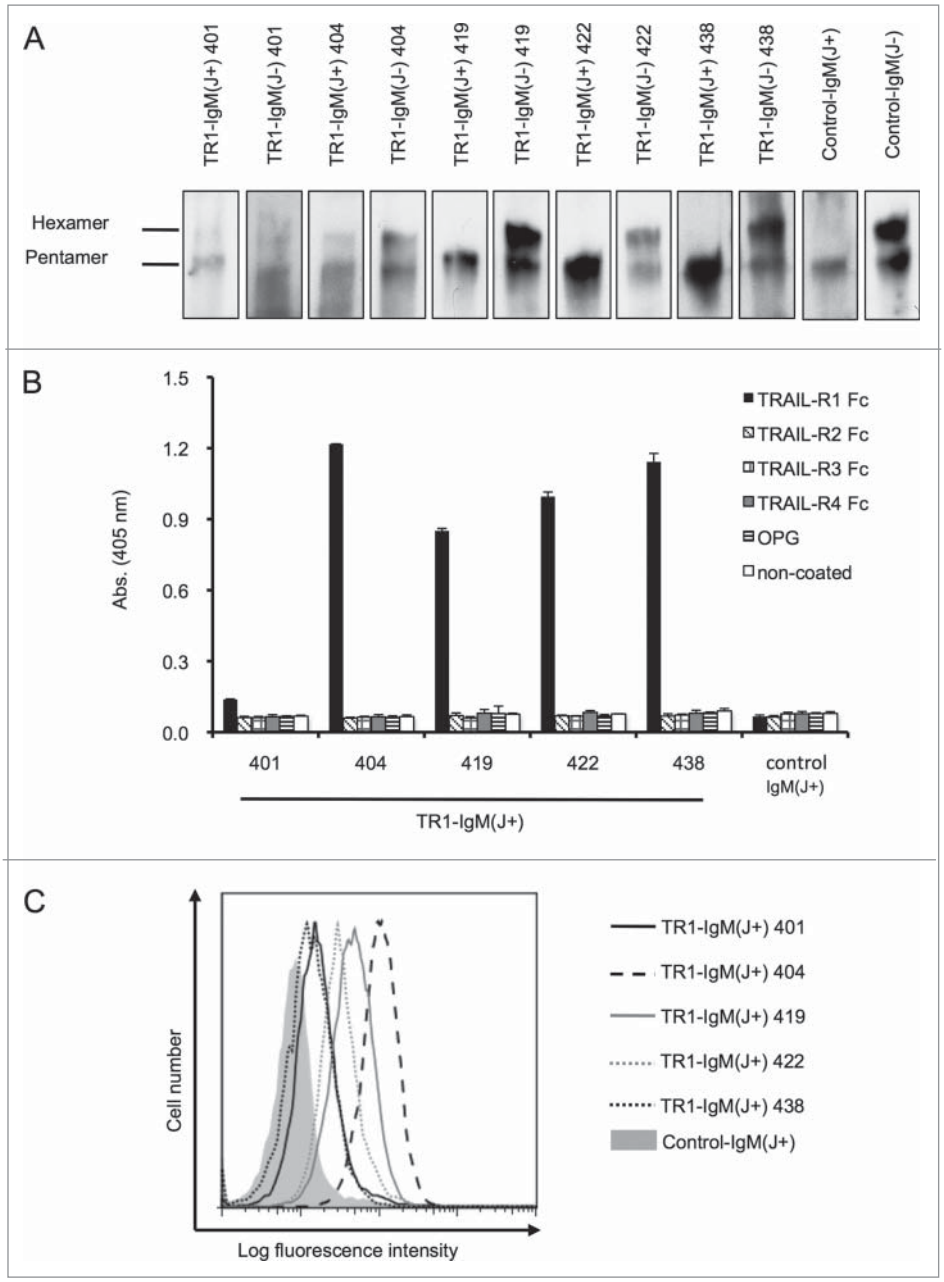

Figure 1. Construction and evaluation of the TR1-IgMs. (A) Western blot analysis of the recombinant purified TR1-IgMs. Ten nanograms of each of the biotinylated-TR1-IgM(+/−)s (TR1-IgM(J+/−)-401, 404, 419, 422, and 438) and control-IgM(+/−) was immunoblotted with horseradish peroxidase-conjugated streptavidin. (B) Reactivity of the TR1-IgM(J+)s with the recombinant human TRAIL-R family. The binding of the TR1-IgM(J+)s to human TRAIL-R1-Fc, TRAIL-R2-Fc, TRAIL-R3-Fc, TRAIL-R4-Fc, or OPG was examined by ELISA. Control-IgM(J+) was used as a control. The data are shown as the mean ± SD of triplicate cultures. (C) The binding of the TR1-IgM(J+)s to the Colo205 cell line was analyzed by flow cytometry. The cells were stained with 5 μg/mL of biotinylated-TR1-IgM(J+)s and control-IgM(J+) as indicated, followed by R-PE-conjugated streptavidin, and analyzed with flow cytometry. *X*-axis, fluorescence intensity of PE; *Y*-axis, relative cell number. Representative data of three independent experiments in which similar results were obtained are shown.

growth in a dose-dependent manner. The control-IgM(J+) treatment did not inhibit tumor growth. Histological analysis of the tumor sections demonstrated that TR1-IgM(J+) 422 induced cell death within 2 d after treatment (Fig. 3C). To clarify whether the cell death was due to the caspase-dependent apoptosis, we analyzed caspase-3 activation in the tumors that had been treated with the TR1-IgM(J+) 422 antibody. We found that antibody administration clearly induced caspase-3 activation in the pre-developed Colo205 tumor cells 4 h after administration (Fig. 3D). These data demonstrate that the TR1-IgM(J+) 422 antibody penetrated the tumor *in vivo* to induce immediate caspase-dependent apoptosis and cell death, exhibiting a potent antitumor effect.

Discussion

Previously, we used TC mice to develop fully human TR1-IgGs with tumoricidal activity,[35] but these mAbs required additional crosslinking antibodies to induce apoptosis in the target cells.[35] Additional antibodies may induce serious side effects due to their antigenicity[29] and the formation of immune complexes with blood IgGs. To overcome this limitation, we converted the

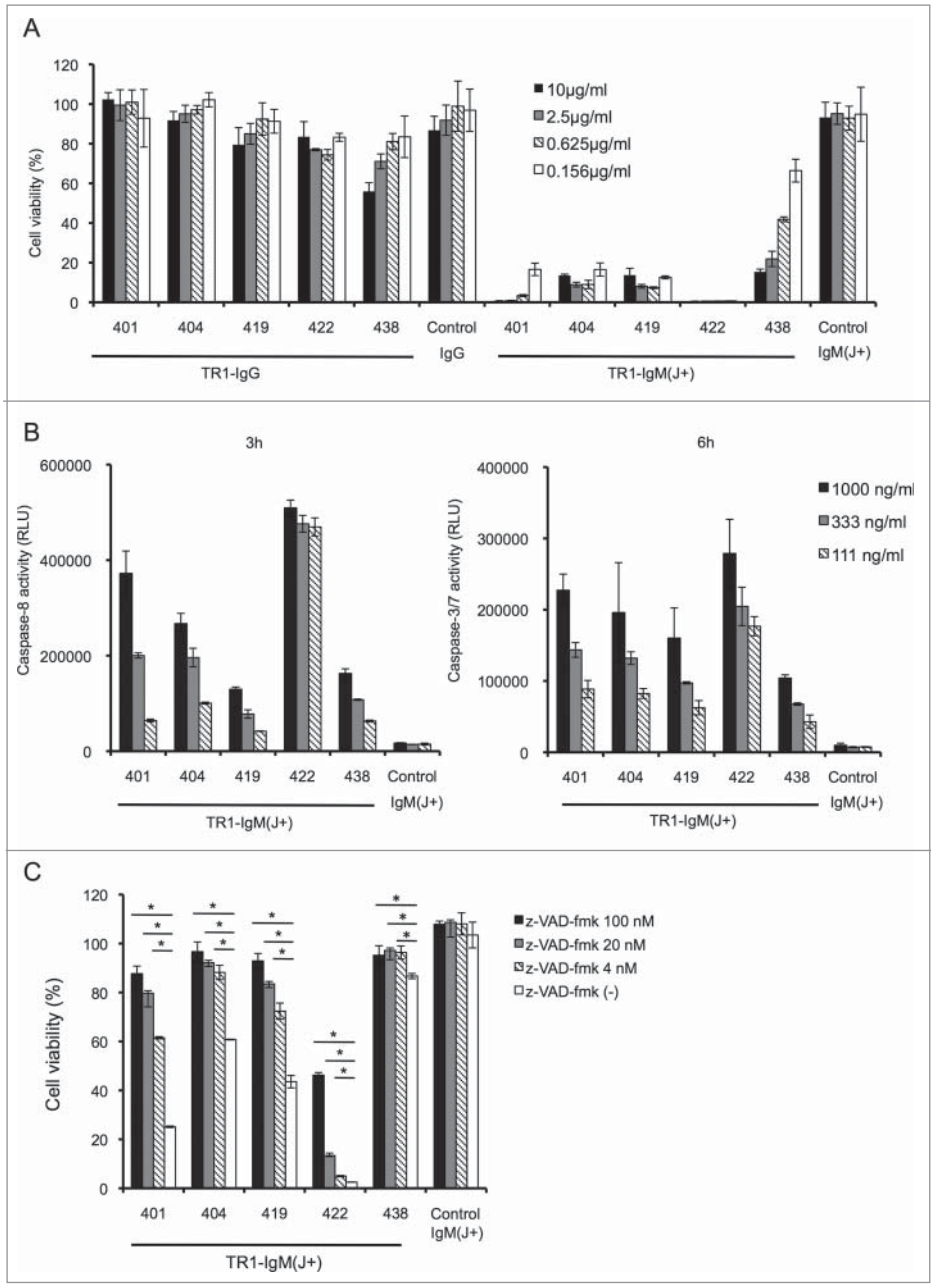

Figure 2. TR1-IgMs induce tumor cell killing by apoptotic signaling. (A) Cell viability assay. Colo205 cells were incubated with the indicated antibodies for 24 h, and cell viability was measured. (B) Measurement of activated caspases. The cells were incubated with the indicated concentrations of TR1-IgM(J+)s or control-IgM(J+). Activated caspase-8, and -3/7 were measured after 3 and 6 h of culture, respectively. (C) Caspase-dependent cell death in Colo205 cells. The cells were incubated with or without the indicated concentrations of z-VAD-fmk for 4 h. Then, the cells were cultured with TR1-IgMs or control-IgM for 24 h. After cell culture, the cell viability was determined. The data are shown as the mean ± SD of triplicate cultures. $^{*}p < 0.05$ by Student's t-test.

TR1-IgGs to TR1-IgMs because secreted IgMs have 10 antigen-binding sites and have a superior capacity to crosslink molecules compared with IgGs.[47] The resulting TR1-IgMs are expected to induce apoptosis *in vitro* and *in vivo* in the absence of additional crosslinking and, thus, would be more suitable for cancer therapy.

Table 1. EC_{50} values of the TR1-IgM(J+)s

IgM	EC_{50} (ng/mL)
TR1-IgM(J+) 401	70
TR1-IgM(J+) 404	9
TR1-IgM(J+) 419	12
TR1-IgM(J+) 422	0.5
TR1-IgM(J+) 438	100

Note: EC_{50} values were determined from the data in Fig. S2.

High functionality is one of the critical parameters for antibodies to be used as potent and efficacious therapeutics.[48] In the present study, we demonstrated that one of the TR1-IgMs, TR1-IgM(J+) 422, showed high antitumor activity on the Colo205 cell line *in vitro*, with an EC_{50} of approximately 0.5 ng/mL. This result was approximately 200-fold higher than that of HGS-ETR1, an agonistic anti-TR1 antibody used in clinical trials.[22] Therefore, TR1-IgM(J+) 422 could be an efficient antitumor agent in cancer therapy.

One of drawbacks of IgM-form antibodies as therapeutic antibodies is that they are difficult to produce compared with

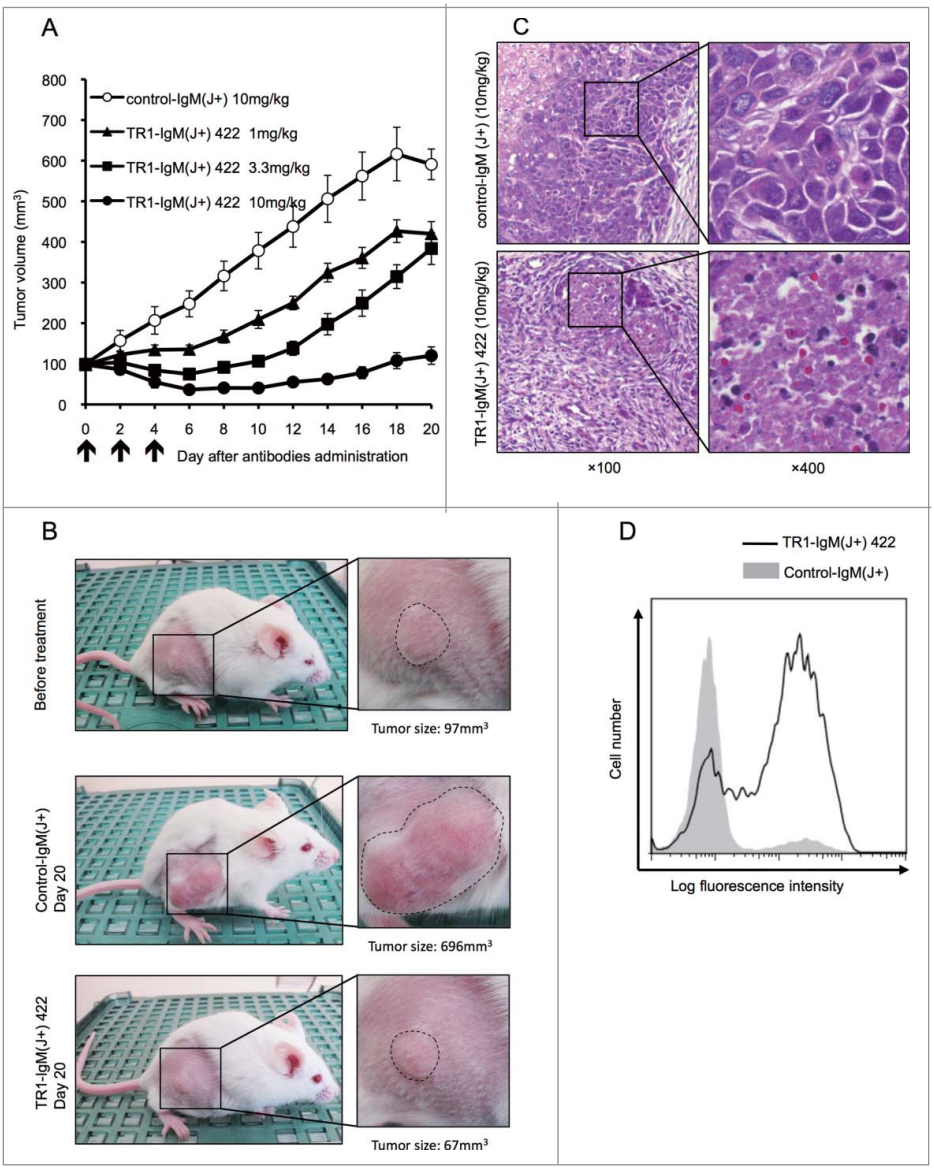

Figure 3. Antitumor efficacy of TR1-IgMs on Colo205 cell-derived mouse xenograft tumors *in vivo*. (A) The tumor growth curves of each group of tumor-bearing mice. SCID mice (10 per group) bearing Colo205 xenograft tumors were i.v. injected with TR1-IgM(J+) 422 or control-IgM(J+) on the indicated days (arrows). Each time point represents the mean value (±S .E.M.) of the tumor sizes within the treated group on the day of measurement. (B) Representative photographs of the Colo205 tumor-bearing mice in each group on day 0 or day 20 after treatment. The magnified photo shows that the tumors (within dotted lines) in the TR1-IgM(J+) 422 (10mg/kg)-treated mice were notably reduced compared with the tumors in the control-IgM(J+) (10mg/kg)-treated mice. (C) Representative photomicrographs of hematoxylin and eosin staining of the tumor tissues from each group of mice at 2 d post-treatment. Expanded views of the region marked with black boxes are shown (right). Original magnification, ×100 (left), ×400 (right). (D) Caspase-3 activation following the injection of TR1-IgM(J+) 422. SCID mice that had pre-established Colo205 tumor xenografts were injected with a single dose (10 mg/kg body weight) of TR1-IgM(J+) 422 (solid line) or control-IgM (shaded peak). The tumors were then excised 4 h after injection, and activated caspase-3 induction was analyzed by flow cytometry. *X*-axis; fluorescence intensity of fluorescein isothiocyanate (FITC), *Y*-axis; relative cell number.

IgG-form antibodies. In this context, Tchoudakova et al developed a PER.C6® cell line that could produce large amounts of IgMs, which promotes the scale-up of IgM production for clinical use.[49] The second drawback of IgM-form antibodies is that their half-life in the blood is shorter than that of IgG.[50] In fact, in the xenograft model, the administration of a high dose (10 mg/kg) of TR1-IgM(J+) 422 elicited significant tumor regression during the first 8 d in all of the mice. However, beginning day 9, the tumors slowly began to re-grow. With these drawbacks, the protocol for TR1-IgM production and administration should be further optimized.

It has been reported that combination treatments with anti-TRAIL-R1 or R2 antibodies and different chemotherapeutics resulted in enhanced cell killing *in vitro* compared to antibodies or chemotherapeutics alone.[22-25,28,51,52] The enhanced cell death may be because the chemotherapeutics can upregulate TRAIL-R1/2[53,54] and proapoptotic protein[55] and decrease anti-apoptotic protein expression levels.[53] In addition, Tuthill et al. found that an anti-TRAIL-R2 antibody and recombinant TRAIL can synergize to kill cancer cells.[56] In our previous study, the combination of TR1-IgGs and recombinant TRAIL significantly induced cell death compared with TR1-IgG or recombinant TRAIL alone.[35] Therefore, the combination of TR1-IgMs with TRAIL or chemotherapeutics should be examined to determine whether they enhance the antitumor activity in non-sensitive cell lines.

Regarding the correlation between tumoricidal activity and TR1-IgM affinity, we prepared five clones of TR1-IgMs in this study (Table 1 and Fig. S2). Among them, TR1-IgM (J+) 422 showed the highest antitumor activity (EC_{50} = 0.5 ng/mL) and moderate affinity (1.4×10^{-8} M).[35] By contrast, TR1-IgM(J+) 404 showed approximately 10-fold higher affinity (1.5×10^{-9} M)[35] compared with TR1-IgM(J+) 422, but the EC_{50} of TR1-IgM(J+) 404 was approximately 20-fold lower (9 ng/mL) than that of TR1-IgM(J+) 422. These results suggest that the antitumor effect was not correlated with the IgM affinity. We previously analyzed the relative epitopes of the TR1-IgG antibodies[57] and showed that the TR1-404 antibody and TRAIL competitively bound to TR1, but the TR1-422 antibody did not. Thus, the antitumor activity may depend on the epitope that is recognized by the individual antibodies. Taken together, we believe that TR1-IgMs may greatly contribute to the promotion of apoptosis for the clinical treatment of cancer.

Materials and methods

Production and purification of the antibodies

We cloned the J chain cDNA (GenBank accession number: NM_144646) and inserted it into a pcDNA3.4 expression vector (Life Technologies, Carlsbad, CA). We also amplified the antibody cDNAs encoding the V_H of TR1-IgGs[35] or an anti-influenza IgG (control antibody)[33] and cloned them together with cDNA encoding the entire immunoglobulin constant region for IgM (GenBank accession number: X57086) into a pcDNA3.4 expression vector, respectively. We co-transfected Expi293F™ cells (Life Technologies, Carlsbad, CA) with the TR1-IgMs or control-IgM heavy chain and light chain vectors with or without the J chain expression vectors. We then collected the culture supernatants and purified the antibodies using HiTrap IgM Purification HP Columns (GE Healthcare, Marlborough, MA), according to the manufacturer's instructions. We then conjugated biotin to the IgMs using the biotin Labeling Kit-NH_2 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions.

SDS-PAGE and western blotting analysis of the recombinant IgMs

We resolved the biotinylated IgMs on polyacrylamide-agarose composite gels under non-reducing conditions, as previously described.[58] Briefly, the gel was prepared using 3% acrylamide-N,N''diallyltartardiamide (29/1), 0.5% agarose, 0.1 M phosphate buffer (pH 7.0), 0.1% SDS, and 15% glycerol. The sample buffer contained 0.02 M phosphate buffer (pH 7.0), 30% glycerol, 1% SDS, and 0.1% bromophenol blue. The electrophoresis buffer contained 0.1 M phosphate buffer (pH 7.0) and 0.1% SDS. After electrophoresis, we transferred the proteins from the gel onto an Immobilon-P nylon membrane (Millipore, Bedford, MA). We treated the membrane with 0.04% skim milk and probed it with the antibodies. Visualization was performed using an ECL detection system (GE Healthcare, Marlborough, MA).

ELISA

We coated 96-well MaxiSorp plates (Nunc, Neptune, NJ) with 50 μL per well of either 1 μg/mL recombinant TRAIL-R1-Fc, TRAIL-R2-Fc, TRAIL-R3-Fc, TRAIL-R4-Fc, or OPG (R&D Systems, Inc., Minneapolis, MN) in PBS and subsequently blocked the plates with 3% bovine serum albumin (BSA) in PBS. After washing, we added 1 μg/mL of the antibodies to the plates and incubated them at room temperature (r.t.) for 1 h. We detected the antibodies that bound to the antigens using IgM-specific antibodies conjugated to alkaline phosphatase (Sigma, St. Louis, MO) and p-nitrophenylphosphate (Sigma, St. Louis, MO). We measured the optical absorbance at 405 nm with an ELISA reader (BMG LABTECH, Offenburg, Germany), according to the manufacturer's instructions.

Flow cytometry analysis

Colo205, MCF7, DU145, and A549 cell lines were incubated with 5 μg/mL of biotinylated-IgM(J+)s for 20 min at r.t. The cells were then washed and stained with R-phycoerythrin (PE)-conjugated streptavidin for 20 min at r.t. After washing, the cells were analyzed with a FACSCanto flow cytometer (Becton Dickinson Biosciences, San Jose, CA). FlowJo 8.4.7 software (Tree Star, Inc.., Ashland, OR) was used for data analysis.

Cell viability assays

Colo205, DU145, A549, and MCF7 cell lines were cultured in 96-well white plates (Nunc, Neptune, NJ) (1×10^4 cells per well) and incubated with the indicated concentrations of antibodies at 37°C and 5% CO_2 for 24 h. Cell viability was determined by CellTiter-Glo™ Luminescent Cell Viability Assay (Promega, San Luis Obispo, CA), according to the manufacturer's instructions.

Caspase activity assays

The Colo205 cell line was cultured in 96-well white plates (1×10^4 cells per well) and incubated with the indicated concentration of antibodies at 37°C and 5% CO_2 for 3 and 6 h to analyze the caspase-8 and caspase-3/7 activities, respectively. The caspase-8 and caspase-3/7 activities were measured using Caspase-Glo™ 8 Assays (Promega, San Luis Obispo, CA) and Caspase-Glo™ 3/7 Assays (Promega, San Luis Obispo, CA), respectively, according to the manufacturer's instructions. The potency of caspase activation was compared with the levels in non-treated cells.

For the caspase inhibition experiments, Colo205 cells were cultured in 96-well white plates and incubated with or without the indicated concentrations of the pan-caspase inhibitor z-VAD-fmk (R&D Systems, Inc.., Minneapolis, MN) at 37°C and 5% CO_2 for 4 h and then cultured with the indicated concentration of antibodies for 24 h in 5% CO_2. Cell viability was determined as described above.

In vivo tumor models

The experiments using mice were approved by the Committee on Animal Experiments at the University of Toyama. Seven-week-old female severe combined immunodeficient (SCID) mice (C.B-17/IcrHsd-*Prkdc*^{scid}; Sankyo Lab Service, Tokyo, Japan) were used. Colo205 cells (1×10^7) were subcutaneously (s.c.) inoculated in the lower right flank of the mouse. Treatment was initiated when the tumor volume reached ~100 mm^3, and 10 mice per group were used for each treatment group. TR1-IgM(J+) or control-IgM(J+) was administered to the animals i.v. via the tail vein in a dose/weight-matched fashion on the indicated days. The tumors were measured in the two largest dimensions, and the tumor volumes in mm^3 were calculated using the following formula: tumor volume = length × width^2 × 1/2. The experiments were concluded three weeks after the initial dosing.

Histological analysis of the xenograft tumors

The mice bearing the Colo205 cell-derived xenograft tumors were i.v.-treated with three doses of TR1-IgM(J+) 422 or control-IgM(J+). 2d after the antibody treatment, the mice were sacrificed, and the tumors were excised. The tumor tissues were placed into formalin for overnight fixation at r.t. The tissues were processed into paraffin blocks, and 4-μm-thick sections were stained with hematoxylin and eosin (Wako, Osaka, Japan).

Cleaved caspase-3 activity in xenograft tumors

The mice bearing the Colo205 cell-derived xenograft tumors were i.v. injected with a single dose of TR1-IgM(J+) or control-IgM(J+). 4h after treatment, the mice were sacrificed, and the tumors were excised. Single-cell suspensions were prepared using the Tumor Dissociation Kit, human (Miltenyi Biotec, Bergisch Gladbach, Germany), and the gentleMACS^{TM}Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the standard protocol provided with the kit. After fixation and permeabilization with IntraPrep^{TM} (Beckman Coulter, Miami, FL), the single-cell suspensions were incubated with a rabbit anti-cleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA) for 20 min at r.t. The cells were then washed and stained with R-PE-conjugated streptavidin for 20 min at r.t. After washing, the cells were analyzed using a FACSCanto flow cytometer (BD Biosciences, Bedford, MA).

Disclosure of potential conflicts of interest

The ISAAC method is covered by patents that have been exclusively licensed to Valneva (Nantes, France).

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Author contributions

X.P. and T.O. performed and analyzed the experiments. All authors designed the experiments. X.P., T.O., H.K., and A.M. wrote the manuscript.

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