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# TNF receptor II fusion protein with tandemly repeated Fc domains

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**The extracellular domain of tumour necrosis factor (TNF) receptor II fused with the human IgG1 Fc region (TNFRII-Fc), as well as antibodies against TNF, has been used to treat rheumatoid arthritis. However, TNFRII-Fc is less effective than these antibodies in terms of antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against cells bearing TNF on the cell surface. We hypothesized that these activities could be increased by fusing TNFRII with tandemly repeated Fc (TNFRII-Fc-Fc). The affinities of TNFRII-Fc-Fc for soluble TNF-α and transmembrane TNF-α and the TNF-α cytotoxicity-inhibitory activity were as potent as those of TNFRII-Fc. TNFRII-Fc-Fc showed much higher binding avidity for Fcγ receptors than TNFRII-Fc and was more potent in terms of both ADCC and CDC against cells expressing transmembrane TNF-α. TNFRII-Fc-Fc of 80 kDa, as well as TNFRII-Fc-Fc of 200 kDa, was detected. TNFRII-Fc-Fc (80 kDa) was as potent as TNFRII-Fc in terms of both ADCC and CDC. These results suggest that Fc multimerization of receptor-Fc fusion proteins can augment effector functions such as ADCC and CDC, and thereby have the potential to provide a superior therapeutic effect. This may be the case not only for TNFRII-Fc but also for other receptor-Fc fusion proteins.**

*Keywords*: ADCC/CDC/Fc fusion protein/TNF/TNF receptor.

*Abbreviations*: ADCC, antibody-dependent cellular cytotoxicity; $(G_4S)_3$, a triple tandemly repeated peptide -Gly-Gly-Gly-Gly-Ser-; CDC, complement-dependent cytotoxicity; mAb, monoclonal antibody; RA, rheumatoid arthritis; TNF, tumour necrosis factor; sTNF-α, soluble TNF-α; tmTNF-α, transmembrane TNF-α.

More than 20 monoclonal antibodies (mAbs) are currently used as therapeutics in various parts of the world. mAbs have many advantages for therapeutics, including high selectivity for target antigens, multiple effector functions, low toxicity and long half-life. The effector functions of mAbs include neutralizing and blocking effects against ligands or receptors (*1*, *2*), antibody-dependent cellular cytotoxicity (ADCC) (*3*), complement-dependent cytotoxicity (CDC) (*4*) and induction of apoptosis (*5*). ADCC is a cytotoxic attack by immunocompetent cells such as natural killer (NK) cells, macrophages and neutrophils, and clinical studies have shown that ADCC is one of the most important mechanisms contributing to clinical efficacy (*6*–*9*). ADCC occurs via interaction between the Fc region of IgGs bound to the antigens on the surface of target cells and Fcγ receptors on the surface of effector cells (*10*). There are various Fcγ receptors, including FcγRIA (CD64A), FcγRIIA (CD32A), FcγRIIB (CD32B) and FcγRIIIA (CD16A). Of these Fcγ receptors, FcγRIIIA is considered to be the predominant contributor to ADCC induction of NK cells (*11*). The FcγRIIIA gene shows allelic polymorphism, encoding either valine or phenylalanine at position 158 (V158, F158), and this variation results in different affinities for human IgG1 antibodies (*12*). In addition to the fact that human IgG1 antibodies can bind to FcγRIIIA (V158) with higher affinities than to FcγRIIIA (F158), FcγRIIIA (V158) activates NK cells more effectively than does FcγRIIIA (F158) (*13*, *14*). It should be noted that several clinical studies have demonstrated the significance of ADCC in determining clinical outcomes (*6*, *7*).

Various modifications have been examined to increase the efficacy of mAbs. Amino acid mutations of IgG1 were reported to augment binding activities for Fcγ receptors and to enhance ADCC (*15*). Recently, three amino acid mutations, identified by computational design, achieved a 100-fold increased ADCC (*16*). In addition, amino acid mutations also increased the affinity for C1q, augmenting CDC (*17*). Modifications of the oligosaccharide linked to N297 in the CH2 domain of IgG1 also influence effector functions. The lack of fucose (*18*, *19*) and the existence of bisecting GlcNAc (*20*) in the oligosaccharide of IgG were shown to enhance binding activity to FcγRIIIA and ADCC, and the content of galactose in the oligosaccharide was reported to affect CDC (*21*). We previously reported that chimeric anti-CD20 mAbs with tandemly repeated Fc domains bound to Fcγ receptors, FcγRIA, FcγRIIA, FcγRIIB and FcγRIIIA, with higher avidities than the parental mAb, resulting in enhanced ADCC (*22*). However, many therapeutic

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mAbs directed against many kinds of antigens or ligands are currently in use, so further studies are needed to clarify whether Fc multimerization is generally effective.

An Fc fusion protein is an antibody-like molecule that is composed of the ligand-binding region of a receptor or an adhesion molecule and the Fc region of an immunoglobulin (*23*). Several kinds of Fc fusion proteins, including etanercept, abatacept, alefacept and rilonacept, have been approved as therapeutics. The Fc regions in these fusion proteins consist of the hinge region, CH2 domain and CH3 domain of IgG1. These fusion proteins share the characteristics of the Fc domain of IgG and have several advantages. First, they have greatly extended half-lives (*24*). Second, the dimeric ligand-binding regions that are linked to the amino terminals of the two Fc peptide chains bind to ligands with higher avidities than natural monovalent molecules. Third, they are capable of ADCC and CDC against target cells (*25*). Fourth, they can be purified using protein A column chromatography.

Etanercept is a type of Fc fusion protein consisting of two extracellular domains of the p75 tumour necrosis factor (TNF) receptor (TNFRII) linked to the Fc region of IgG1 (*26*). Etanercept is able to bind to soluble TNF-α (sTNF-α) and transmembrane TNF-α (tmTNF-α), and exerts a potent clinical effect on rheumatoid arthritis (RA) (*27*). The reason why etanercept has potent efficacy in RA is that the divalent molecule inhibits TNF-α more effectively than the monovalent molecule; the Fc fusion protein has a much longer half-life (68 h) than natural TNFRII (*28*).

Enhancement of binding avidities for Fcγ receptors, especially for FcγRIIIA, by Fc multimerization was shown to reinforce ADCC activity in an anti-CD20 antibody (*22*). In this study, we linked the extracellular domain of human TNFRII to the tandemly repeated Fc domain of human IgG1 (TNFRII-Fc-Fc) and investigated the effect of Fc multimerization on the effector functions of the Fc fusion protein. The binding activity to sTNF-α, the inhibitory efficacy against sTNF-α, the binding activity to tmTNF-α and the binding activities to individual Fcγ receptors, as well as ADCC and CDC, were examined, and TNFRII-Fc-Fc was confirmed to be more potent than TNFRII-Fc for all these effector functions.

## Materials and Methods

### *Cell lines*

CHO-DG44 cells were purchased from Invitrogen (Carlsbad, CA, USA) and cultured in IMDM containing 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Murine L929 cells and human KHYG-1 cells were kindly provided by the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). L929 cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and KYHG-1 cells were cultured in RPMI1640 containing 10% FBS, 10 ng/ml IL-2 (Wako Pure Chemical Industries, Osaka, Japan), 100 U/ml penicillin and 100 μg/ml streptomycin. Human KHYG-1 cells stably expressing human FcγRIIIA (KHYG-1/ FcγRIIIA) were developed by culturing of the cells with the culture supernatants of PLAT-A cells that were transfected with retroviral vector pMXs-puro containing the FcγRIIIA complementary DNA (cDNA). Cells highly expressing FcγRIIIA were sorted by flow cytometry (data not shown; Kobayashi et al. 2009) and were cultured in a similar manner to the parental KHYG-1 cells.

### *Construction of cDNAs, production and purification of TNFRII-Fc and TNFRII-Fc-Fc*

cDNAs encoding TNFRII-Fc and TNFRII-Fc-Fc were constructed using PCR and restriction enzymes. cDNA encoding the extracellular domain of TNFRII was assembled with the cDNA of the human IgG1 Fc region (from hinge to CH3 domain) to construct cDNA of TNFRII-Fc in pcDNA3.1/Zeo (Invitrogen) as shown in Fig. 1A. To generate TNFRII-Fc-Fc, cDNA of the tandemly repeated Fc was connected downstream of cDNA encoding the extracellular domain of TNFRII. The gene encoding the flexible peptide linker [(a triple tandemly repeated peptide -Gly-Gly-Gly-Gly-Ser-$(G_4S)_3$]) was inserted between the two Fc cDNAs. The linearized expression vectors were transfected into CHO-DG44 cells using TransFast Transfection Reagent (Promega, Madison, WI, USA) and the transfected cells were cultured at 37°C in IMDM containing 2% FBS and 100 μg/ml zeocin (Invitrogen) in flat-bottomed BD Falcon 96-well microplates (BD Biosciences, San Jose, CA, USA) for 1 week. The cells were cloned by limiting dilution, and the clones were cultured in CD CHO medium (Invitrogen). TNFRII-Fc and TNFRII-Fc-Fc were purified from the culture supernatants by means of protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) column chromatography followed by gel-filtration high-performance liquid chromatography (HPLC) on TSK-GEL G3000SWXL (Tosoh, Tokyo, Japan) as previously described (*22*). The purified Fc fusion proteins were concentrated by ultrafiltration using an Amicon Ultra-4 50,000 MWCO (Millipore, Billerica, MA, USA) in 20 mM phosphate buffer (pH 7.4) containing 0.14 M NaCl (PBS).

### *Biochemical analyses of Fc fusion proteins*

The biochemical properties of the Fc fusion proteins were analysed by gel-filtration HPLC and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) as described previously (*22*). Briefly, the fusion proteins were analysed by gel-filtration HPLC on a Protein Pak G3000SWXL column (Tosoh) at a flow rate of 1 ml/min in 0.1 M sodium phosphate (pH 6.8). In addition, the fusion proteins were analysed by SDS–PAGE under reducing and non-reducing conditions on 10 and 6% polyacrylamide gels, respectively.

The number of free sulphhydryl residues in the individual fusion proteins was measured using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) according to the manufacturer's instructions. The protein concentrations were determined by measurement of the absorbance at 280 nm. The protein solutions were allowed to react with 4 mM DTNB in 0.1 M phosphate buffer (pH 8.0) containing 1 mM EDTA at 37°C for 15 min. The number of free sulphhydryl residues was determined by measurement of the absorbance at 412 nm.

### *ELISA assay of sTNF-α binding*

Binding activities of the Fc fusion proteins to sTNF-α were measured by ELISA. Flat-bottomed BD Falcon 96-well flexible PVC microplates (BD Biosciences) were coated with 50 μl of 1 μg/ml TNF-α (Wako Pure Chemical Industries) in PBS at 4°C for 18 h. The solution was discarded, and the plates were blocked with 150 μl of PBS containing 0.1% bovine serum albumin (BSA) at room temperature for 2 h. The blocking solution was discarded, and then 50 μl of serially diluted Fc fusion proteins was added and the plates were incubated at room temperature for 2 h and washed three times with 150 μl of PBS containing 0.05% Tween-20 (PBS-T). Then 50 μl of 0.2 μg/ml horseradish peroxidase (HRP)-conjugated goat anti-human IgG gamma chain (Tago, Burlingame, CA, USA) in PBS containing 0.1% BSA was added and incubation was continued. The plates were again washed three times with PBS-T, and 50 μl of ELISA substrate buffer (1 mM 3,3′,5,5′-tetramethylbenzidine, 1.3 mM *N*,*N*′-dimethylformamide, 1 mM benzenesulphonic acid sodium salt, 20 mM acetic acid, 0.03% hydrogen peroxide, pH 5.0) was added to each individual well, and the plates were allowed to stand at room temperature for 10 min with protection from light. The reaction was stopped by the addition of 50 μl of 0.5 M $H_2SO_4$. Absorbance at 450 nm was measured using a microplate reader, Model 550 (BIO-RAD, Hercules, CA, USA). Samples



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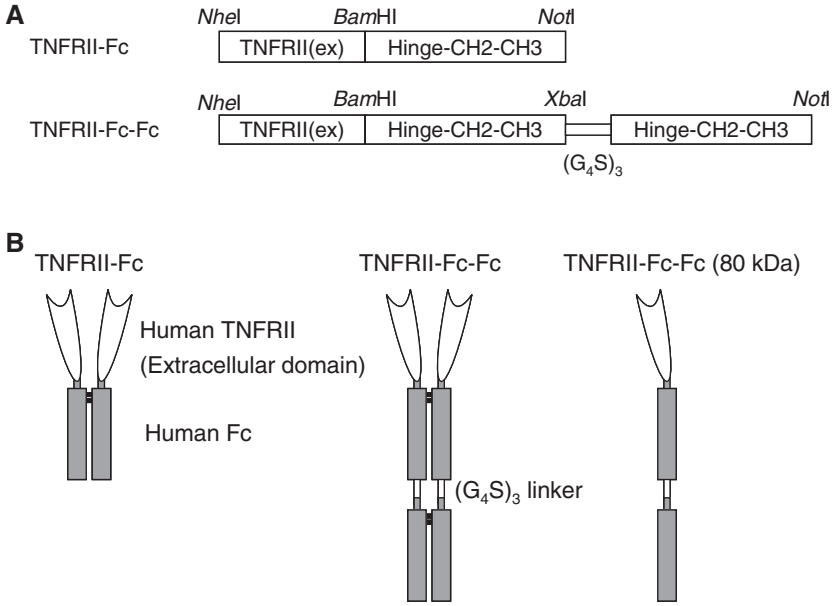


**Fig. 1** (A) cDNA constructs of TNFRII-Fc and TNFRII-Fc-Fc. Each cDNA was assembled in the pcDNA3.1/Zeo expression vector using restriction enzymes. TNFRII-Fc-Fc has a flexible $(G_4S)_3$ linker between the Fc domains. (B) Schematic illustrations of TNFRII-Fc, TNFRII-Fc-Fc and TNFRII-Fc-Fc (80 kDa).

were analysed in triplicate, and the values were expressed as the mean and standard deviation (SD).

### *TNF-α neutralization assay*

Neutralizing activities of the Fc fusion proteins against TNF-α-dependent cytotoxicity were measured using murine fibrosarcoma L929 cells as described previously (*29*). Briefly, 50-μl aliquots of $2 \times 10^5$ cells/ml L929 cells ($1 \times 10^4$ cells/well) in RPMI1640 were dispensed into flat-bottomed BD Falcon 96-well microplates and the plates were incubated at 37°C for 24 h. Then, 50 μl of 40 ng/ml TNF-α, 50 μl of serially diluted solutions of the Fc fusion proteins or human IgG Fc fragments as a control and 50 μl of 4 μg/ml actinomycin D (Wako Pure Chemical Industries Ltd) were added to individual wells. The plates were incubated at 37°C for 24 h and then centrifuged at 300*g* for 15 min, and 50-μl aliquots of the supernatants were transferred into flat-bottomed BD Falcon 96-well flexible PVC microplates. Lactate dehydrogenase activities in the individual supernatants were assessed using a Cytotoxicity Detection kit (Roche Diagnostics Corporation, Mannheim, Germany) as described in the manufacturer's instructions. The absorbance at 490 nm was measured using a microplate reader Model 550 (BIO-RAD). The neutralizing activities (%) were calculated according to the following formula: neutralizing activity = 100 × [1 − (sample absorbance − background absorbance)/(1% Triton-X100 absorbance − background absorbance)]. All samples were analysed in triplicate and the values were expressed as the mean and SD.

### *Flow cytometric assay of tmTNF-α binding*

CHO-DG44 stably expressing tmTNF-α was established by expression of tmTNF-α with two mutations (R77T and S78T) that enable it to resist TACE-mediated cleavage (*30*). The cDNA encoding the mutated tmTNF-α was cloned into pcDNA3.1/Zeo expression vector and transfected into CHO-DG44 with TransFast Transfection Reagent (Promega). CHO-DG44 stably expressing tmTNF-α (CHO-DG44/tmTNF-α) was cloned and established by the method described above.

CHO-DG44/tmTNF-α was used to evaluate the binding activities of Fc fusion proteins to tmTNF-α on the cell surface. CHO-DG44/tmTNF-α cells ($1.33 \times 10^5$ cells/ml) were blocked in cytometry buffer (PBS, 0.4% BSA, 3 mM EDTA) at 4°C for 30 min. After centrifugation at 600*g* at 4°C for 5 min, the supernatants were discarded and the cells were allowed to react with 1 μg/ml Fc fusion proteins in 100 μl of cytometry buffer at 4°C for 30 min. The cells were washed twice with 500 μl of cytometry buffer and then allowed to react with 7.8 μg/ml fluorescein isothiocyanate-conjugated goat anti-human IgG $F(ab')_2$ (Tago) in 100 μl of cytometry buffer at 4°C for 30 min. The cells were again washed twice and suspended in 500 μl of cytometry buffer. The fluorescence intensities of individual samples were measured using a Gemini XPS (Molecular Devices, Sunnyvale, CA, USA).

### *ELISA assay of Fcγ receptor binding*

Binding activities of Fc fusion proteins to individual Fcγ receptors were measured by ELISA as previously described (*22*). Briefly, FcγRIA, FcγRIIA, FcγRIIB, FcγRIIIA (V158) and FcγRIIIA (F158) in PBS were coated on 96-well microplates in the forms of their extracellular domains fused with glutathione S-transferase, and HRP-conjugated goat anti-human IgG gamma chain was used as the secondary antibody. The enzyme activities were measured using 3,3′,5,5′-tetramethylbenzidine.

### *ADCC assay*

CHO-DG44/tmTNF-α cells were used as the target cells of ADCC. The cells were suspended in Hanks Balanced Salt Solution (HBSS-F containing 10 mM HEPES and 5% FBS), and the cell suspension was centrifuged at 600*g* at 4°C for 5 min. The supernatant was discarded, and the cells were resuspended in 500 μl of HBSS-F. Then, 12.5 μl of 1 mM Calcein-AM (Dojindo Molecular Technologies Inc., Kumamoto, Japan) was added to the cell suspension, and the cells were incubated at 37°C for 30 min, washed twice with ADCC Buffer (RPMI1640, 2 mM L-glutamine, 10 mM HEPES at pH 7.2, 100 U/ml penicillin and 100 μg/ml streptomycin) and suspended in ADCC Buffer at $2 \times 10^5$ cells/ml. Aliquots of 50 μl of a suspension of CHO-DG44/tmTNF-α stained with Calcein-AM ($0.1 \times 10^5$ cells/well) were mixed with 50 μl of serially diluted Fc fusion proteins or human IgG Fc in the wells of round-bottomed BD Falcon 96-well microplates (BD Biosciences) and the plates were incubated at 37°C for 30 min. Then, effector cells or KHYG-1/ FcγRIIIA cells were added to the target cell suspensions. An aliquot of 50 μl of $50 \times 10^5$, $25 \times 10^5$ or $12.5 \times 10^5$ cells/ml KHYG-1/FcγRIIIA ($2.5 \times 10^5$, $1.25 \times 10^5$ or $0.625 \times 10^5$ cells/well) was added to each well. The ratios of effector cells to target cells (E/T) were 25/1, 12.5/1 and 6.25/1. The plates were incubated at 37°C for 4 h and then centrifuged at 300*g* for 10 min. The supernatants (50 μl) were transferred into individual wells of uClear fluorescence black plates with a transparent bottom (Greiner Bio-one, Frickenhausen, Germany). The fluorescence intensity of Calcein-AM released from damaged CHO-DG44/tmTNF-α was measured using an ARVO SX 1420 multilabel counter (Perkin Elmer, Waltham, MA, USA) with excitation

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at 485 nm and emission at 538 nm. ADCC cytotoxicity (%) was calculated according to the following formula. The fluorescence intensity of the well treated with 1% Triton-X 100 was used as the maximum cytotoxicity. ADCC cytotoxicity (%) = 100 × (sample fluorescence − background fluorescence)/(1% Triton-X100 fluorescence − background fluorescence). ADCC activities were measured in triplicate, and the results were expressed as the mean and SD.

### *CDC assay*

CDC assay was performed using CHO-DG44/tmTNF-α and low-expressing CHO-DG44/tmTNF-α as target cells. CHO-DG44/tmTNF-α cells were centrifuged at 300*g* at room temperature for 5 min and then suspended at $10 \times 10^5$ cells/ml in CDC Buffer (RPMI1640 containing 0.1% BSA, 20 mM HEPES (pH 7.2), 100 U/ml penicillin and 100 μg/ml streptomycin). Aliquots of 50 μl of cell suspension were dispensed into individual wells of uClear fluorescence black plates with a transparent bottom, and aliquots of 50 μl of serially diluted Fc fusion protein or human IgG Fc were added to the cell suspensions. Next, 50 μl of 5-fold-diluted fresh baby rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) was added to individual wells, and the plates were incubated at 37°C for 2 h. Then, 50 μl of Alamar Blue (TREK Diagnostic Systems, Cleveland, OH, USA) was dispensed into the wells, and the plates were further incubated at 37°C for 16 h to measure living cells. The fluorescence of individual wells was measured using an ARVO SX 1420 multilabel counter with excitation at 530 nm and emission at 590 nm. CDC cytotoxicity (%) was calculated according to the following formula: CDC cytotoxicity (%) = 100 × (background fluorescence − sample fluorescence)/background fluorescence. The cytotoxic activities were measured in triplicate, and the results were expressed as the mean and SD.

## Results

### *Biochemical properties of TNFRII-Fc and TNFRII-Fc-Fc*

TNFRII-Fc and TNFRII-Fc-Fc were purified from the culture supernatants of the respective transformed cells by means of protein A affinity chromatography followed by gel-filtration HPLC. Gel-filtration HPLC revealed two TNFRII-Fc-Fc proteins of different molecular weights (Fig. 2A): one larger and the other smaller than TNFRII-Fc. Those two TNFRII-Fc-Fc proteins were analysed by SDS–PAGE. Under reducing conditions, both proteins showed a single band of 100 kDa, which is equal to the calculated value of a single chain of TNFRII-Fc-Fc (Fig. 2B). Under non-reducing conditions, the large and small TNFRII-Fc-Fc molecules showed a molecular weight of ∼200 and 80 kDa, respectively (Fig. 2C). These results suggest that the smaller molecule is a half molecule of TNFRII-Fc-Fc without disulphide bonds between the two H chains at the hinge region. The structure of the smaller molecule is illustrated schematically in Fig. 1B. This protein product was designated as TNFRII-Fc-Fc (80 kDa). The free sulphhydryl residues of these molecules were measured using DTNB, and TNFRIIF-Fc, TNFR-Fc-Fc and TNFR-Fc-Fc (80 kDa) were found to have 0.64, 0.96 and 0.46 free sulphhydryl residues per molecule, respectively. The results suggest that cysteine residues of TNFRII-Fc-Fc (80 kDa) would form intrachain disulphide bonds instead of interchain disulphide bonds.

### *sTNF-α binding activity and neutralizing activity*

Binding activities of the Fc fusion proteins to sTNF-α were determined by ELISA using sTNF-α-coated plates (Fig. 3A). The dose–response curve of TNFRII-Fc-Fc was very close to that of TNFRII-Fc, implying that multimerization of the Fc domain does not affect the TNF-α binding activity. However, TNFRII-Fc-Fc (80 kDa) showed an absorbance of 0.6 at a concentration that was 10–20 times higher than that of TNFRII-Fc-Fc, suggesting that it has low affinity for sTNF-α.

Activity of neutralization of the cytotoxicity of sTNF-α was determined in the L929 assay system (*29*) to examine whether the neutralizing activity is influenced by Fc multimerization (Fig. 3B). The dose–response curve of TNFRII-Fc-Fc was close to that of TNFRII-Fc. On the other hand, TNFRII-Fc-Fc (80 kDa) showed 50% neutralizing activity at a concentration that was 10–20 times higher than that of TNFRII-Fc-Fc, suggesting that it has a low neutralizing activity for sTNF-α. Therefore, these results indicate that the binding and neutralizing activities of TNFRII for sTNF-α are not influenced by Fc multimerization.

### *tmTNF-α binding activity*

The binding activities of Fc fusion proteins towards tmTNF-α were determined by flow cytometry with CHO-DG44 cells that had been transfected with a TACE-resistant form of human tmTNF-α (Fig. 4). TNFRII-Fc and TNFRII-Fc-Fc bound to tmTNF-α at the same intensities in the cell lines with high and low expressions of tmTNF-α. However, the binding activity of TNFRII-Fc-Fc (80 kDa) to tmTNF-α was lower than that of TNFRII-Fc-Fc (200 kDa). These results suggest that Fc multimerization does not affect the binding activity of TNFRII to tmTNF-α on the surface of cells, and that the formation of the disulphide-linked dimers is important for binding to tmTNF-α.

### *Fcγ receptor binding activity*

The binding activities of Fc fusion proteins to Fcγ receptors were determined by ELISA to examine whether Fc multimerization augments the binding activity (Fig. 5). The binding activity of TNFRII-Fc-Fc to FcγRIIIA (V158) was about 20 times higher than that of TNFRII-Fc, and the binding activities of TNFRII-Fc-Fc to FcγRIA, FcγRIIA, FcγRIIB and FcγRIIIA (F158) were about 10 times higher than those of TNFRII-Fc. On the other hand, TNFRII-Fc-Fc (80 kDa) was weaker than TNFRII-Fc-Fc and as potent as TNFRII-Fc in terms of binding activities to all Fcγ receptors. Considering that FcγRIIIA has two genotypes at amino acid 158 and that FcγRIIIA (V158) binds to human IgG more strongly than FcγRIIIA (F158) (*12*), the enhancement of binding activity to FcγRIIIA by Fc multimerization appears to be unrelated to the genotype. Taken together, these results suggest that Fc multimerization augments the binding activities of an Fc fusion protein to all Fcγ receptors.

### *ADCC activity*

ADCC activities of Fc fusion proteins were determined using KHYG-1 cells transfected with FcγRIIIA (KHYG-1/FcγRIIIA) and tmTNF-α-expressing CHO-DG44 cells (CHO-DG44/tmTNF-α). Figure 6A



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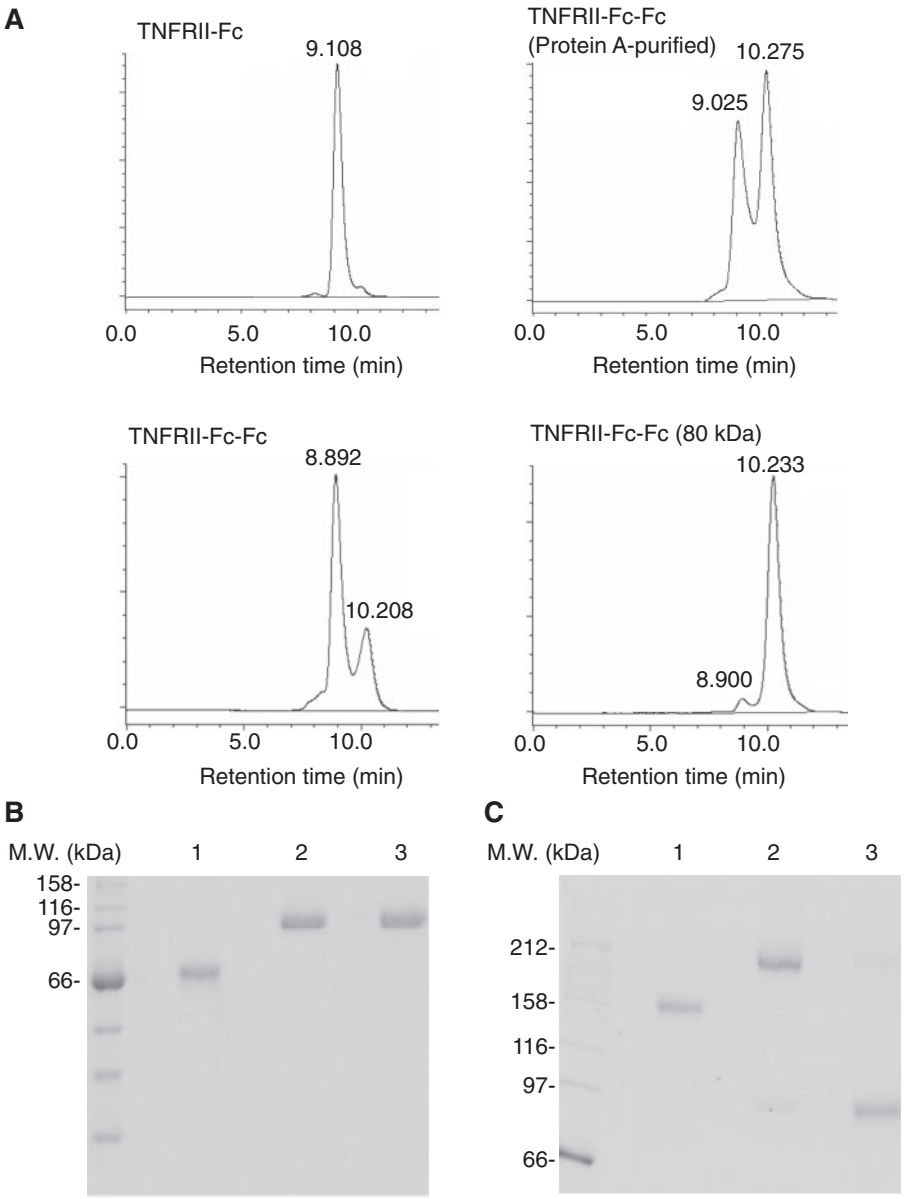


**Fig. 2 Gel-filtration HPLC and SDS–PAGE analyses of Fc fusion proteins.** (A) Gel-filtration HPLC. TNFRII-Fc-Fc (Protein A-purified) proteins are purified by protein A-agarose and TNFRII-Fc-Fc and TNFRII-Fc-Fc (80 kDa) proteins are separated by gel-filtration HPLC. These proteins were electrophoresed under reducing (B) and non-reducing (C) conditions. Lane 1: TNFRII-Fc, lane 2: TNFRII-Fc-Fc and lane 3: TNFRII-Fc-Fc (80 kDa).

shows the ADCC activities of three Fc fusion proteins against tmTNF-α high-expressing CHO-DG44 with KHYG-1/FcγRIIIA at an E/T ratio of 25/1. Human IgG Fc alone did not bind to target cells and showed no significant cytotoxicity. TNFRII-Fc-Fc was found to be cytotoxic at concentrations of 0.74 nM and above, and TNFRII-Fc at concentrations of 6.67 and 20 nM were as cytotoxic as TNFRII-Fc-Fc at 0.74 nM. TNFRII-Fc-Fc (80 kDa) was as potent as TNFR-Fc in terms of ADCC. Also, at lower E/T ratios (12.5/1 and 6.25/1), the ADCC activity of TNFRII-Fc-Fc was more potent than that of TNFRII-Fc at every concentration examined (Fig. 6B). CHO-DG44 with lower tmTNF-α expression also showed higher susceptibility to ADCC with TNFRII-Fc-Fc than with TNFRII-Fc (Fig. 6C). Therefore, TNFRII-Fc-Fc exerts more potent ADCC activity than TNFRII-Fc against tmTNF-α-expressing target cells.

### *CDC activity*

CDC activities of Fc fusion proteins were determined using baby rabbit complement and tmTNF-α-expressing CHO-DG44 cells (Fig. 7). TNFRII-Fc-Fc mediated CDC against CHO-DG44/tmTNF-α cells at concentrations equal to or above 2.2 nM, while human IgG Fc alone did not (Fig. 7A). The cytotoxicity of TNFRII-Fc-Fc at 2.2 nM was equivalent to that of TNFRII-Fc at 6.6 nM, implying that TNFRII-Fc-Fc is three times as potent as TNFRII-Fc in terms of CDC. TNFRII-Fc-Fc (80 kDa) was less active than TNFRII-Fc-Fc and similar to TNFRII-Fc in terms of CDC. Similar results were found using CHO-DG44



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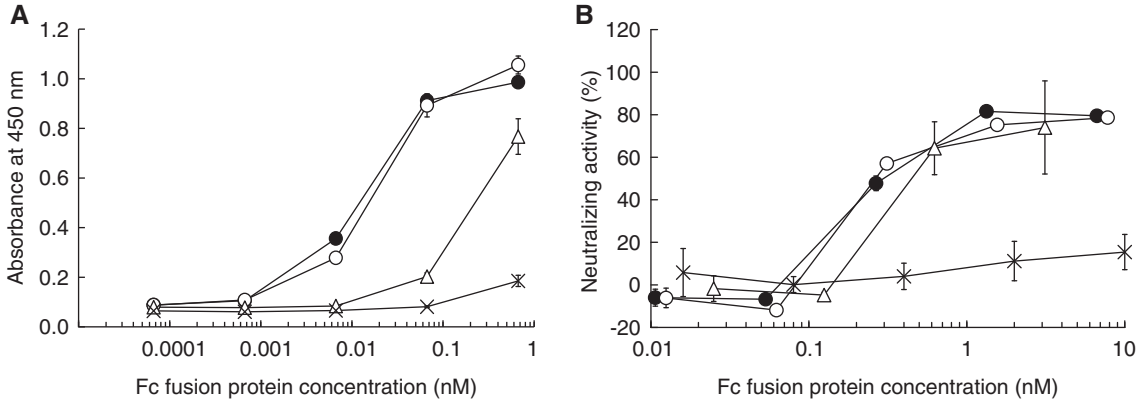


**Fig. 3** (A) Binding activities of Fc fusion proteins to sTNF-α. Serially diluted Fc fusion proteins were allowed to react with sTNF-α coated on ELISA plates. The bound Fc fusion proteins were measured using HRP-conjugated goat anti-human IgG gamma chain. (B) Neutralizing activities of Fc fusion proteins against sTNF-α cytotoxicity. L929 cells were incubated with serially diluted Fc fusion proteins and sTNF-α. The absorbance at 450 nm in the absence of sTNF-α was used as background absorbance and that in the absence of Fc fusion protein was used as maximum absorbance. Samples were analysed in triplicate, and the mean values ± SD are shown. TNFRII-Fc (closed circles), TNFRII-Fc-Fc (open circles), TNFRII-Fc-Fc (80 kDa) (open triangles) and human Fc (crosses).

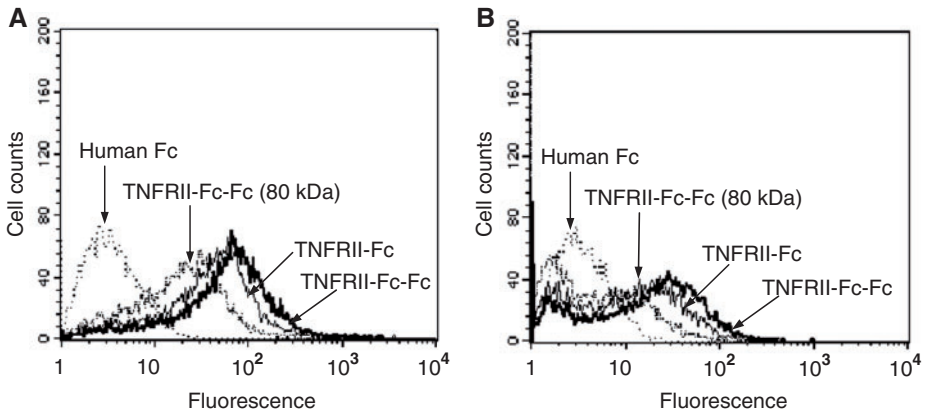


**Fig. 4** Binding activities of Fc fusion proteins to tmTNF-α on (A) high-expressing and (B) low-expressing CHO-DG44. Fc fusion proteins were allowed to react with CHO-DG44 cells expressing high or low levels of tmTNF-α at 4°C for 30 min. The bound Fc fusion proteins were measured using fluorescein isothiocyanate-conjugated goat anti-human IgG $F(ab')_2$. Fluorescence intensities were analysed by means of flow cytometry.

with lower tmTNF-α expression (Fig. 7B). These results suggest that Fc multimerization enhances the CDC activity of Fc fusion proteins.

## Discussion

Currently, five TNF-α antagonists, infliximab, adalimumab, etanercept, certolizumab pegol and golimumab, are in clinical use for therapy of immune-mediated inflammatory diseases. However, their efficacy profiles are different. Etanercept differs from other antagonists in its lack of efficacy in Crohn's disease (*31*) and is less efficacious in psoriasis (*32*). These differences may result from their biological characteristics. Etanercept can bind to TNF-α in 1 : 1 molar ratio, whereas three molecules of the other mAb agents bind to one molecule of TNF-α, and the dissociation rate is faster for etanercept than for the other antagonists (*33*). Regarding cytotoxicity, several studies have shown that infliximab and adalimumab induce ADCC and CDC in tmTNF-α-transfected cells, whereas etanercept induces little or no cytotoxicity (*34*, *35*).

We previously showed that tandemly repeated Fc domains linked to an anti-CD20 antibody result in augmented binding avidities for Fcγ receptors, leading to enhanced ADCC activity (*22*). However, because many kinds of antibody therapeutics, such as whole antibody molecules, a variety of antibody fragments and Fc fusion proteins, are increasingly being used for various diseases, further studies are required to determine whether Fc multimerization is applicable to other types of antibody therapeutics. Here, we describe an Fc fusion protein composed of tandemly repeated Fc domains following the extracellular domain of p75 TNF receptor (TNFRII) with a flexible linker of $(G_4S)_3$. In this study, TNFRII-Fc-Fc, which has two Fc domains, was developed and its binding activities to sTNF-α, tmTNF-α and Fcγ receptors, activity of neutralizing the cytotoxicity of sTNF-α, and ADCC and CDC activities were evaluated. TNFRII-Fc-Fc was as potent as TNFRII-Fc not only in binding activities to sTNF-α and tmTNF-α but also in sTNF-α neutralizing activity (Figs 3 and 4).

In contrast to the binding activity to TNF-α, TNFRII-Fc-Fc bound to Fcγ receptors with 10–20



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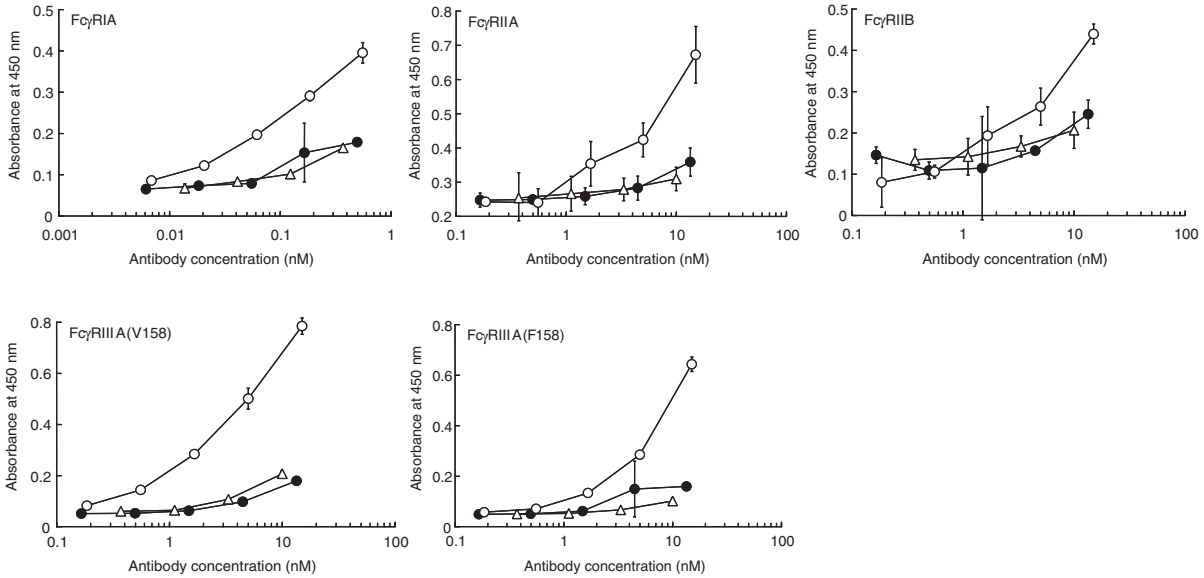


**Fig. 5 Binding activities of Fc fusion proteins to Fcγ receptors.** Serially diluted Fc fusion proteins were allowed to react with FcγIA, FcγIIA, FcγIIB, FcγIIIA(V158) and FcγIIIA(F158) receptors coated on ELISA plates. The bound Fc fusion proteins were measured using HRP-conjugated goat anti-human IgG gamma chain. Samples were analysed in triplicate and the absorbances at 450 nm are shown as the mean ± SD. TNFRII-Fc (closed circles), TNFRII-Fc-Fc (open circles) and TNFRII-Fc-Fc (80 kDa) (open triangles).

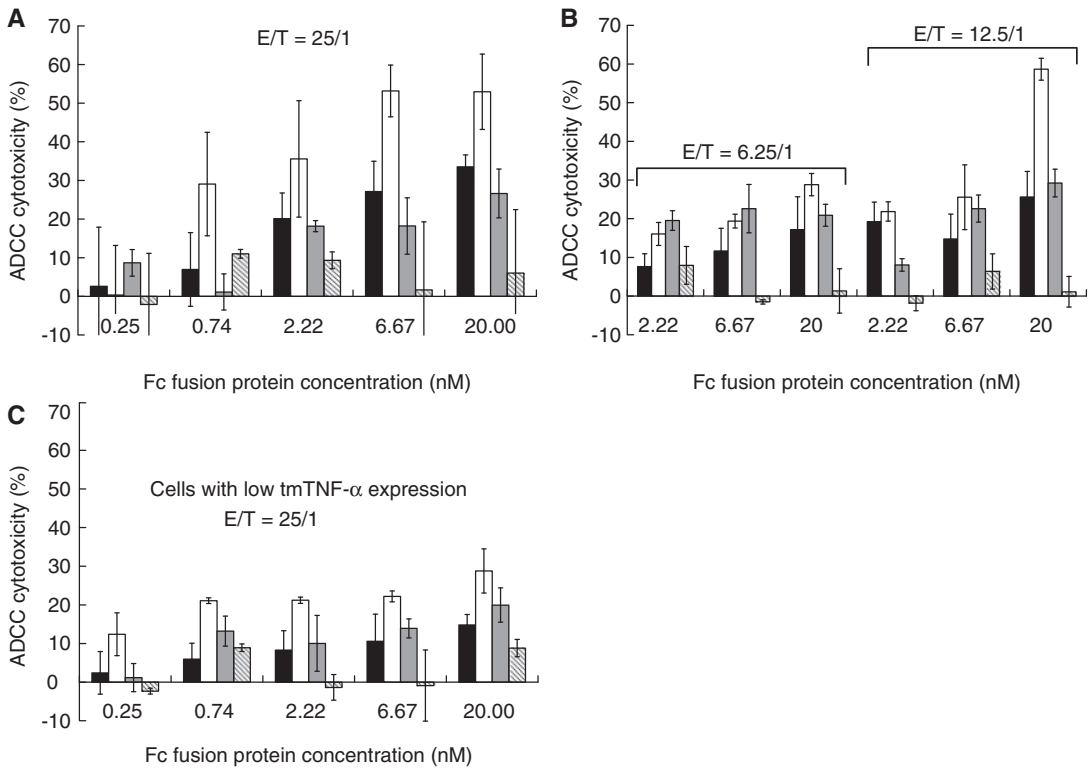


**Fig. 6 ADCC activities of Fc fusion proteins.** (A) Calcein-labelled CHO-DG44 cells highly expressing tmTNF-α were incubated with serially diluted Fc fusion proteins at 37°C for 30 min. KHYG-1 cells transfected with FcγIIIA(V158) were added at an E/T ratio of 25/1 and the cell suspensions were further incubated at 37°C for 4 h. The fluorescence intensity of released calcein was measured with excitation at 485 nm and emission at 538 nm. (B) Cells highly expressing tmTNF-α were used as target cells at E/T ratios of 12.5/1 or 6.25/1. (C) Cells weakly expressing tmTNF-α were used as target cells at an E/T ratio of 25/1. The samples were analysed in triplicate and the mean values ± SD are shown. TNFRII-Fc (black), TNFRII-Fc-Fc (white), TNFRII-Fc-Fc (80 kDa) (grey) and human Fc (slashed).

times higher avidities (Fig. 5) and induced ADCC at 10–20 times lower concentration than TNFRII-Fc (Fig. 6A). Enhancement of ADCC by Fc multimerization was also observed when E/T ratios, which were between 25/1 and 6.25/1, were analysed (Fig. 6A and 6B), and when cells with a low density of tmTNF-α on the cell surface were used (Fig. 6C). Fcγ receptors play a pivotal role in linking antibody-mediated immune

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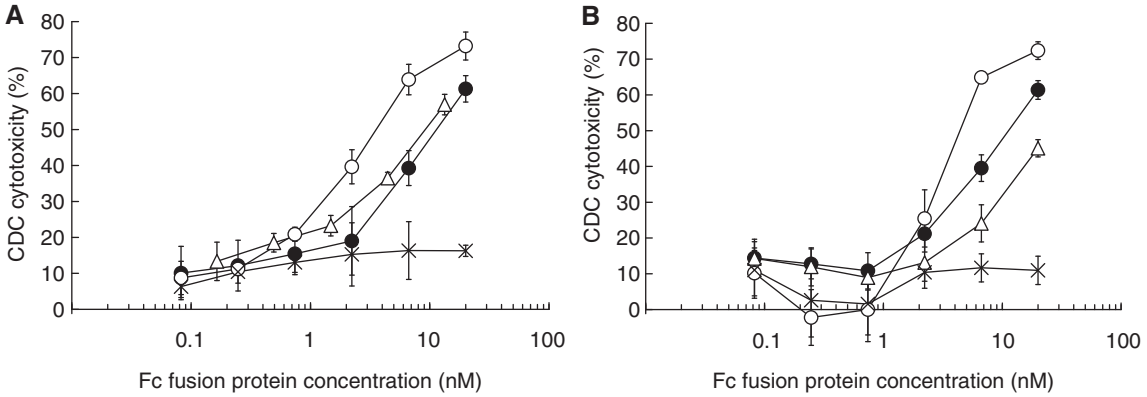


**Fig. 7 CDC activities of Fc fusion proteins.** (A) CHO-DG44 cells highly expressing tmTNF-α and (B) CHO-DG44 cells with low expression of tmTNF-α were incubated with serially diluted Fc fusion proteins and 5-fold-diluted baby rabbit complement at 37°C for 2 h. Alamar Blue was added, and the cell suspensions were further incubated at 37°C for 16 h. The fluorescence intensities of the wells were measured with excitation at 530 nm and emission at 590 nm. The samples were analysed in triplicate and the mean values ± SD are shown. TNFRII-Fc (closed circles), TNFRII-Fc-Fc (open circles), TNFRII-Fc-Fc (80 kDa) (open triangles) and human Fc (crosses).

responses to cellular effector functions (*10*, *11*), and FcγRIIIA is considered to be the predominant Fcγ receptor of NK cells for ADCC induction (*10*). Thus, it can be concluded that tandemly repeated Fc domains augment binding avidities for Fcγ receptors, resulting in enhanced ADCC activity.

TNFRII-Fc-Fc exerted stronger CDC activity than TNFRII-Fc irrespective of the expression level of tmTNF-α on the target cell surface (Fig. 7). In ELISA-based C1q binding assay, the binding activity of TNFRII-Fc-Fc to C1q was augmented as compared with that of TNFRII-Fc (data not shown). CDC is triggered by the binding of C1q to Fc regions of more than two IgG molecules bound on the cell surface. CDC has been reported to be enhanced by amino acid mutations (*19*) and insertion of an IgG3 sequence (*35*) at the hinge and Fc domains. Four amino acid residues, D270, K322, P329 and P331, are known as epicenters (*17*), and tandemly repeated TNFRII-Fc-Fc has two binding sites in a single molecule. Thus, the enhancement of CDC activity of TNFRII-Fc-Fc appears to be caused by the strong binding activity to C1q. Therefore, fusion proteins with tandemly repeated Fc domains have dual mechanisms: cytotoxicity through ADCC and CDC to the target cells and neutralization of biological activities of the target molecules.

Purification of TNFRII-Fc-Fc by gel-filtration HPLC yielded TNFRII-Fc-Fc (80 kDa) as well as TNFRII-Fc-Fc of 200 kDa (Fig. 2A). SDS–PAGE analysis suggested that TNFRII-Fc-Fc (80 kDa) is a single peptide chain, which is schematically drawn in Fig. 1B. TNFRII-Fc-Fc (80 kDa) had 0.46 free sulphhydryl residues per molecule, implying that the two cysteine residues would form a disulphide bond. The peptide sequence at the hinge region is -Cys-Pro-Pro-Cys-. Because these two cysteine residues are very close, they may form an intrachain disulphide bond, but not an interchain bond. The reason why the binding activities of TNFRII-Fc-Fc (80 kDa) to sTNF-α (Fig. 3A) and tmTNF-α (Fig. 4) are lower than those of the disulphide-linked dimer is presumably due to the difference in their binding valencies. It seems that TNFRII-Fc-Fc (80 kDa) binds monovalently to TNF-α, while TNFRII-Fc-Fc (200 kDa) binds divalently. Our results are consistent with a previous report regarding monovalent and divalent TNFRII molecules (*26*). The low binding activity of TNFRII-Fc-Fc (80 kDa) to sTNF-α resulted in slightly lower neutralizing activity as compared with TNFRII-Fc and TNFRII-Fc-Fc (Fig. 3B).

TNFRII-Fc-Fc (80 kDa) induced ADCC (Fig. 6) and CDC (Fig. 7), and its activities were as potent as those of TNFRII-Fc. Actually, the binding avidities of TNFRII-Fc-Fc (80 kDa) for Fcγ receptors were as strong as those of TNFRII-Fc (Fig. 5), and TNFRII-Fc-Fc (80 kDa) showed binding avidity for C1q as well (data not shown). Because the size of the entire IgG molecule is as large as ~150 kDa, attempts have been made to obtain smaller antibody derivatives with similar biological activities, aiming at better tissue penetration *in vivo* (*36*). Thus, TNFRII-Fc-Fc (80 kDa) should be compared with TNFRII-Fc-Fc (200 kDa) in an *in vivo* study.

The mechanisms of action of TNF antagonists have been studied (*33*, *34*), particularly for infliximab and etanercept, but many issues remain unresolved. Recently, *in vitro* studies using certorizumab suggested that induction of apoptosis, ADCC and CDC may not be required for clinical efficacy of a TNF-α antagonist in Crohn's disease (*25*). Pharmacokinetic effects such as administration route, dosage and half-life, as well as differences in the binding kinetics between Fc fusion proteins and mAb, may influence the clinical effects. Although our results in this study suggested that Fc multimerization would improve clinical efficacy and practicality, further studies will be necessary to clarify the mode of action in Crohn's disease.

In conclusion, we have shown that Fc multimerization of TNFRII-Fc augments its binding activities to Fcγ receptors and complement, leading to enhancement of ADCC and CDC, and does not affect its binding activity to TNF-α or its neutralizing activity. The simultaneous enhancement of multiple effector



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functions is expected to be highly beneficial for therapeutics. Furthermore, we unexpectedly generated TNFRII-Fc-Fc (80 kDa) and found it may also have therapeutic potential. In agreement with a previous study on anti-CD20 tandemly repeated antibodies, Fc multimerization was found to augment effector functions of Fc fusion protein. These results suggest that the usefulness of Fc multimerization is not limited to antibody therapeutics, but may extend to many varieties of protein therapeutics, including receptor-Fc fusions.

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**Conflict of interest**
None declared.

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