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Enhanced Fc-Dependent Cellular Cytotoxicity of Fc Fusion Proteins Derived from TNF Receptor II and LFA-3 by Fucose Removal from Asn-Linked Oligosaccharides

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Fucose removal from complex-type oligosaccharide of human IgGs results in a major enhancement of Fc-dependent cellular cytotoxicity. The aim of this study was to determine the effect of fucose removal on the effector function of another class of clinically important molecules that can effect cellular cytotoxicity, Fc fusion proteins. The receptors chosen for study were TNF receptor II and LFA-3, both of which have therapeutic significance. The fucosylated versions of these fusion proteins were produced in unmodified CHO cells, whereas the nonfucosylated counterparts were produced in CHO cells with α-1,6-fucosyltransferase, an enzyme required for fucosylation, knocked-out. Whilst binding activity of TNFRII-Fc and LFA-3-Fc were unchanged by fucose-removal, nonfucosylated Fc fusion proteins exhibited significantly higher Fc receptor γIIIa–binding and increased Fc-mediated cytotoxicity on target cells compared to fucosylated counterparts. Notably, in case of TNFRII-Fc, only the nonfucosylated protein exhibited potent Fc dependent cytotoxicity to transmembrane TNF-α expressing cells. These results prove that enhancement of Fc dependent cellular cytotoxicity by fucose-removal is effective in not only whole IgG but also Fc fusion proteins, and thus widens the potential of Fc-fusion proteins as therapeutic candidates.

Key words: cytotoxicity, defucosylation, Fc fusion, FcγRIIIa, oligosaccharide.

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; HPAC, high-performance anion exchange chromatography; Ig, immunoglobulin; LDH, lactate dehydrogenase; LFA-3, leukocyte function antigen-3; MFI, means of fluorescence intensity; NK, natural killer; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; TNFRII, tumor necrosis factor receptor II.

Antibodies of the human immunoglobulin (Ig) G1 isotype are commonly used for therapeutic applications as they can mediate multiple effector functions including antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and induce apoptosis (*1*–*3*). ADCC, a lytic attack on antibody-targeted cells, is triggered following binding of leukocyte receptors (FcγR) to the Fc region of antibody bound to a target. Several mouse and clinical studies indicate that ADCC is an important therapeutic mechanism of clinically effective antibodies (*4*–*7*). FcγRIIIa is the predominant FcγR of natural killer (NK) cells responsible for ADCC activation. The FcγRIIIa gene (*FCGR3A*) displays an allelic polymorphism that generates receptors containning either a phenylalanine (F) or a valine (V) at a position critical in mediating ADCC, amino acid position 158. This variation results in human IgG1 antibodies binding with higher affinity to the NK cells of homozygous *FCGR3A*-158V donors than those of homozygous *FCGR3A*-158F donors, and seems to result in more effective NK cell activation (*8*, *9*). Importantly, several reports have recently shown that *FCGR3A* genotype influences the clinical efficacy of human IgG1-type anti-CD20 antibody rituximab (*3*, *10*, *11*) with the clinical response of patients bearing FcγRIIIa-158F being significantly inferior to the patients with the FcγRIIIa-158V receptors (*5*–*7*). These reports underscore the importance of ADCC in clinical outcomes.

ADCC activity is influenced by the structure of complex-type oligosaccharides linked to CH2 domain of the IgG Fc region. The content of galactose (*12*, *13*), bisecting *N*-acetylglucosamine (*14*, *15*), and fucose (*16*, *17*) in the antibody oligosaccharide have each been reported to effect ADCC. In previous studies, we have shown that fucose is the most critical IgG oligosaccharide component and that the removal of fucose from IgG1 oligosaccharides results in a very significant enhancement of both ADCC *in vitro* (~100 fold) and antitumor activity *in vivo* (*17*, *18*). However, many therapeutic antibodies currently approved or under clinical development are produced using Chinese hamster ovary (CHO) cells that express high level of α1,6-fucosyltransferase and consequently produce low amounts of antibody lacking fucose (*17*). Therefore, we generated a Chinese hamster ovary cell line that can stably produce nonfucosylated antibodies with enhanced

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ADCC, by removing the fucosyltransferase gene (*19*), that behaves in other respects indistinguishable from the parental line.

Fc fusion proteins are an antibody-like molecule in which the ligand-binding region such as extracellular domain of a receptor or an adhesion molecule linked to the Fc portion of human IgG (*20*). The most common structure used for the Fc component contains the hinge region, CH2 domain, and CH3 domain, but not the CH1 domain of IgG. Fc fusion proteins and IgGs share two important properties that are significant to their potential as therapeutic agents (*20*). First, because of structural homology, Fc fusion proteins exhibit a pharmacokinetic half-life *in vivo* that is comparable to that of humanized IgGs of similar isotype. Second, Fc fusion proteins can be used in a similar manner to some IgGs to modulate biochemical interactions that play key roles in pathological processes. Given these similarities, Fc fusion proteins constitute a complementary therapeutic strategy to IgGs. Hence, Fc fusions designed as antagonists to inhibit deleterious interactions, as well as Fc fusions designed as agonists to enhance beneficial functions, hold promise as human therapeutics. The first two marketed Fc fusion proteins are etanercept [a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75 kDa (p75) tumor necrosis factor receptor II (TNFRII) linked to the Fc portion] (*21–25*) and alefacept [a dimeric fusion protein consisting of the extracellular CD2-binding portion of the human leukocyte function antigen-3 (LFA-3) linked to the Fc portion] (*26–29*). Etanercept, a TNFRII-Fc, is successfully demonstrated in the effectiveness of soluble TNFR-based agents in the treatment and management of rheumatoid arthritis to neutralize TNF-α activity (*22–24*). Because the TNFRII-Fc is dimeric by Fc portion, it binds to TNF-α with greater affinity than natural receptor, which is monomeric. Moreover, the linkage of soluble TNFR to Fc portion prolongs its terminal half-life, which ranges between 70 and 110 h (*25*). Alefacept, a LFA-3-Fc, has reduced disease expression in patients with chronic plaque psoriasis. It binds to CD2 on memory-effector T cells, inhibiting their activation and also reducing the number of these cells by Fc-dependent cytotoxicity (*27–31*).

Improvement of both binding to FcγRIIIa and Fc-dependent effector function by defucosylation has been demonstrated for whole IgG molecules (*17*, *32*) and single-gene–encoded antibody constructs (*33*). In the present study, we generated the nonfucosylated Fc fusion proteins, TNFRII-Fc (f–) and LFA-3-Fc (f–), using α-1,6-fucosyltransferase knock-out CHO cells, and conventional fucosylated TNFRII-Fc (f+) and LFA-3-Fc (f+), using CHO cell line. Then we investigated the effect of defucosylation on the effector function of the Fc fusion proteins.

MATERIALS AND METHODS

Blood Donors—Blood donors were randomly selected from healthy volunteers registered in BioFrontier Laboratory, Kyowa Hakko Kogyo Co. Ltd. All donors gave written informed consent prior to analyses.

Cell Lines—CHO cell line DG44, for the production of fucosylated Fc fusion protein, was kindly provided by Dr Lawrence Chasin (Columbia University). Establishment of FUT8 knockout CHO clone Ms705 used for production of nonfucosylated Fc fusion protein has been described previously (*19*). Mouse T-cell lymphoma cell line EL4 (ATCC TIB-39), tumorigenic murine fibroblast cell line L929 (ATCC CCL-1), human acute T-lymphoblastic leukemia cell line CCRF-CEM (ATCC CCL-119) and human acute T-cell leukemia cell line Jurkat (ATCC TIB-152) were purchased from American Type Culture Collection (Rockville, MD). These cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum at 37°C.

Establishment of Cells Expressing Nonsecretable Cell Surface Mutant of TNF-α—To evaluate the Fc-dependent cellular cytotoxicity of TNFRII-Fc a transformant expressing the nonsecretable cell surface mutant of TNF-α, mTNF-α/EL4, was generated. To generate uncleavable mutant of TNF-α we constructed a mutant of the wild-type TNFgene-α (Δ1-12TNF-α) in which the codons encoding the amino acid residues +1 to +12 including the TNF-α cleavage site was deleted using a PCR-based method (*34*). The cDNA fragment encoding Δ1-12TNF-α was inserted into a mammalian expression vector, pKANTEX93 (*35*). The resultant mutant TNF-α expression vector was transfected into EL4 cells and transfected cells were identified after selection with G418 (0.5 mg/ml). Single cell clones were isolated and subsequently analyzed for the expression of TNF-α on the cell surface by EPICS XL-MCL flowcytometer (Beckman Coulter, Tokyo, Japan).

Construction and Production of TNFRII-Fc and LFA-3-Fc—The cDNA encoding for the TNFRII-Fc was constructed by the PCR-based method. The NH_2-terminal 257 amino acids of TNFRII (GenBank accession no. BT019927) with same amino acid sequences as etanercept which has 467 amino acids in monomeric molecule, preceded by the signal sequence was fused to Fc portion of human IgG1 (*21*). The cDNA fragment encoding TNFRII-Fc, contained in a *Eco*RI–*Bam*HI fragment, was cloned into previously described pKANTEX93 vector (*34*). The TNFRII-Fc expression vector was introduced into CHO/DG44 cells and Ms705 cells as described previously (*19*) *via* electroporation, and transfected cells were selected in media G418-containing and lacking hypoxanthine and thymidine. TNFRII-Fc was purified on a protein A column (Amersham Biosciences, Piscataway, NJ) from serum-free cell culture supernatant of G418 resistant transfectant cells. Purified proteins were dialyzed in to 10mM phosphate buffer (pH 6.5, 34 mM NaCl). The concentration of the TNFRII-Fc was determined by measuring the absorbance at 280 nm. They were analyzed by SDS-PAGE on pre-cast 5–20% polyacrylamide tris-glycine gels (ATTO, Tokyo, Japan) with or without 2-mercaptoethanol.

LFA-3-Fc expression vector was prepared using the NH_2-terminal 92 amino acids of mature human LFA-3 sequence (GenBank accession no. BC005930), preceded by the LFA-3 signal sequence, was fused to Fc portion of human IgG1 as the same with that of alefacept (*26*) which has 10 amino acids of the hinge region of IgG1 and the CH2 and CH3 constant domains. Production and purification of LFA-3-Fc were performed using the same methods as for the TNFRII-Fc described above. The purity of LFA-3-Fc was confirmed by SDS-PAGE analysis.

Monosaccharide Composition of Fc Fusion Proteins—In order to measure the monosaccharide composition of *N*-linked oligosaccharide on CH2 domain of Fc portion,

Fc fragments were purified from Fc fusion protein using endoproteinase Lys-C (*14*). A 500 μg aliquot of protein was diluted to a concentration of 100 μg/ml with 50 mM Tris-HCl (pH 8.5). Samples were digested with Lys-C (Merck Biosciences, Darmstadt, Germany) for 1 h at 37°C using an enzyme-to-substrate ratio of 1:100. Lys-C digested samples containing Fc fragment were purified using MabSelect column (Amersham). The purity of Fc fragment was confirmed by SDS-PAGE (approximately 50 kDa). The monosaccharide composition of purified Fc fragment was analyzed by modified high-performance anion exchange chromatography (HPAEC) as described previously (*17*).

Inhibition of TNF-α Induced Cytotoxicity—TNF-α induced cytotoxicity was analyzed using L929 as target according to the method described previously (*36*). Briefly, L929 cells were plated in 96 well plates (Sumitomo Bakelite, Tokyo, Japan) at a density of 30,000 cells/well, in 100 μl of MEM (Invitrogen) containing 10% fetal bovine serum. The cells were incubated at 37°C for 24 h, supernatant was aspirated and then treated with mouse TNF-α (0.01 ng/ml) (R&D systems, Minneapolis, MN) and actinomycin D (0.5 μg/ml) (MBL, Nagoya, Japan) in the absence or presence of TNFRII-Fc. After 24 h incubation at 37°C, the cell survival was determined using crystal violet dye (Kanto Kagaku, Tokyo, Japan). The optical density of culture was assessed at 590 nm in an Emax plate reader (Molecular Devices, Menlo Park, CA) and percentage inhibition of cytotoxicity was determined as previously described (*37*).

Flow Cytometer Analysis—LFA-3-Fc was tested for the ability to bind CCRF-CEM cells expressing CD2. Cells were stained with serial dilutions of LFA-3-Fc (0.048 to 50 μg/ml). Phycoerythrin-conjugated anti–human IgG(γ) $F(ab')_2$ fragment (Beckman Coulter) was used as the secondary antibody. The stained cells were analyzed using a flowcytometer.

Binding to Soluble Recombinant FcγRIIIa—Preparation of soluble recombinant FcγRIIIa and masurement of binding to FcγRIIIa has been described previously (*38*, *39*). Briefly, ELISA plates (Greiner, Frickenhausen, Germany) were coated with anti–Tetra His antibodies (QIAGEN, Hilden, Germany) in phosphate buffered saline (PBS). After blocking with PBS containing 1% BSA (Proliant, Ankeny, IA) (1%BSA-PBS), purified FcγRIIIa was incubated on the plates at room temperature for 2 h. Serial dilutions of Fc fusion proteins in 1%BSA-PBS were incubated for 2 h. Bound Fc fusion protein was detected using peroxidase-labeled goat anti–human IgG (H+L) antibodies (American Qualex, San Clemente, CA), with 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) as the substrate. The reaction was stopped with 5% SDS and the absorbance at 415 nm was measured on an Emax plate reader.

Fc-Dependent Cellular Cytotoxicity Assay—Cytotoxicity assays were performed by a lactate dehydrogenase (LDH) release assay using human peripheral blood mononuclear cells (PBMCs) as effector cells prepared from healthy donor as previously described (*17*). Target cells were either mTNF-α/EL4 for TNFRII-Fc or Jurkat for LFA-3-Fc. The target cells were plated with test Fc fusion protein and effector cells at an effector:target ratio was 25:1. After a 4 h incubation at 37°C the LDH activity in supernatant were measured using a cytotoxixity assay kit (Promega, Madison, WI). The percentage of specific cytolysis was calculated from the absorbance value of samples according to the formula,

$$\%\text{Cytotoxicity} = 100 \times (E\text{–}ES\text{–}TS)/(M\text{–}TS)$$

where E represents the experimental release (activity in the supernatant from target cells incubated with Fc fusion protein and effector cells), TS is the spontaneous release from target cells incubated with medium alone, ES is the spontaneous release in the presence of effector cells and M is the maximum release of target cells (activity released from target cells by lysed with 9% Triton-X.

RESULTS

Production and Characterization of Fc Fusion Proteins—We have reported previously that depletion of fucose from human IgG1 oligosaccharide improves its affinity for FcγRIIIa, *in vitro* ADCC (*17*, *39*) and *in vivo* anti-tumor activity (*18*). In this study the effect of defucosylation of Fc fusion proteins on the Fc-dependent cellular cytotoxicity was determined. In order to compare fusion protein with and without fucose expression vectors for TNFRII-Fc and LFA-3-Fc were transfected into CHO/DG44 cells and FUT8-knockout CHO cells Ms705 respectively. Fc fusion proteins produced by CHO/DG44 cells were designated TNFRII-Fc (f+) and LFA-3-Fc (f+), and those produced by Ms705 were designated TNFRII-Fc (f–) and LFA-3-Fc (f–). TNFRII-Fcs and LFA-3-Fcs were purified from culture media of cloned cell lines using protein A. SDS-PAGE analysis showed that TNFRII-Fc proteins and LFA-3-Fc proteins migrate under reducing conditions with the expected molecular size of approximately 70 kDa and 60 kDa, respectively, and migrate as disulfide-linked dimers of approximately 140 kDa and 115 kDa under non-reducing conditions, respectively (Fig. 1). Both TNFRII and LFA-3 are highly glycosylated molecules. TNFRII includes two potential sites for *N*-linked glycosylation, and a proline-, serine-, and threonine-rich region for potential *O*-linked glycosylation (*40*). The CD2 binding site of LFA-3 has three potential sites for *N*-linked glycosylation with 30% of its mass being carbohydrate (*41*). Therefore the Fc fusion proteins were digested with endoproteinase Lys-C to analyze the Asn-linked monosaccharide composition of Fc portion in these proteins. As shown in

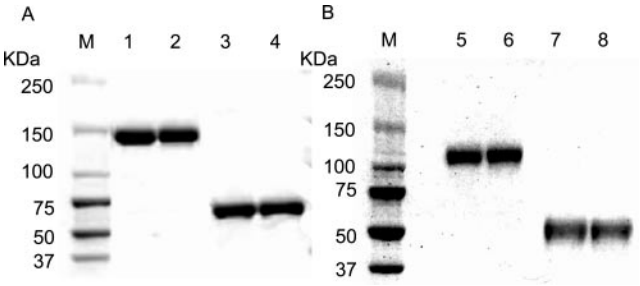

Fig. 1. **SDS-PAGE analysis of Fc fusion proteins.** Purified TNFRII-Fc (A) and LFA-3-Fc (B) were analyzed by SDS-PAGE under nonreducing (lanes 1, 2, 5 and 6) and reducing (lanes 3, 4, 7 and 8) conditions. Lanes: M, markers (sizes in kilodaltons at left); 1 and 3, TNFRII-Fc (f+); 2 and 4, TNFRII-Fc (f–); 5 and 7, LFA-3-Fc (f+); 6 and 8, LFA-3-Fc (f–).

Table 1. **Monosaccharide composition of *N*-linked oligosaccharide in CH2 domain of Fc-fusion protein.**[a]

Clone name	Host	Relative composition of monosaccharides			
		Fuc	Gal	Man	GlcNAc[a]
TNFRII-Fc (f+)	CHO	0.93	0.82	2.89	4
TNFRII-Fc (f–)	CHO/FUT8$^{-/-}$	n.d.[b]	0.79	2.9	4
LFA-3-Fc (f+)	CHO	0.93	1.16	2.74	4
LFA-3-Fc (f–)	CHO/FUT8$^{-/-}$	n.d.	0.99	2.84	4

[a]Molar ratio calculated *versus* 4 GlcNAc. [b]Not detected.

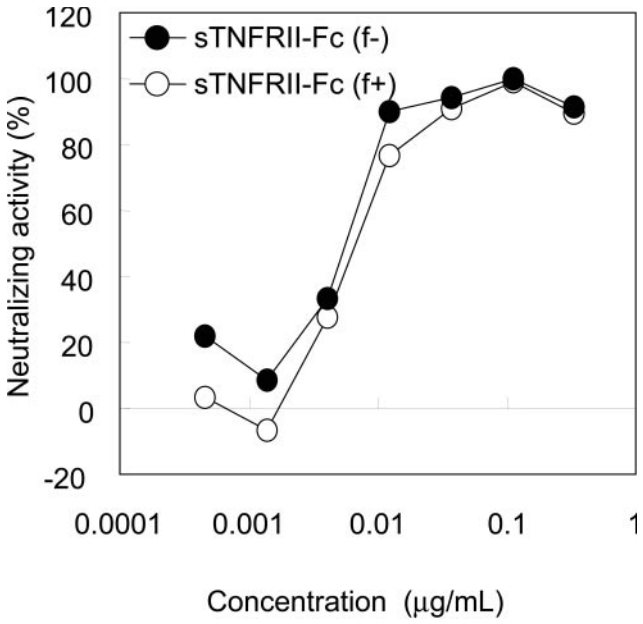

Fig. 2. **Neutralizing activity of TNFRII-Fc fusion proteins of L929 cell in response to TNF-α (10 pg/ml).** Serial diluted concentrations of the Fc fusion protein were incubated with TNF-α and Actinomysin D (0.5 μg/ml). The OD of L929 cells in the absence of TNF-α was indicated as 100% and in the presence of TNF-α was indicated as 0% neutralizing activity. The mean values of duplicate are shown.

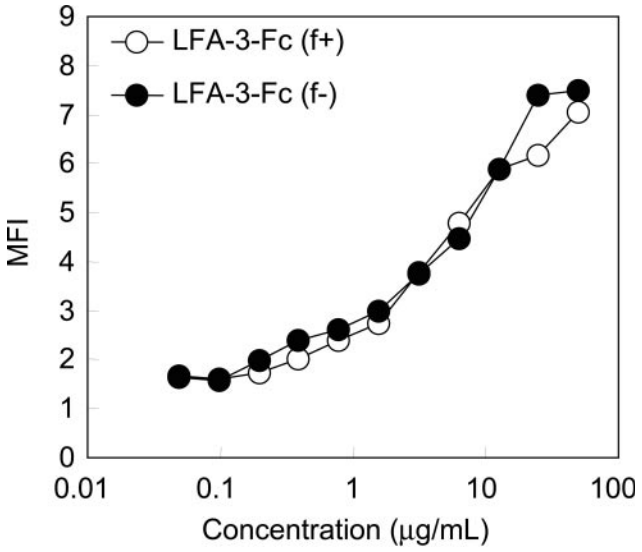

Fig. 3. **Binding of LFA-3-Fc fusion proteins to CD2 expressed cell, CCRF-CEM by flow cytometry.** Cells were incubated with serial diluted concentration of the Fc fusion proteins for 30 min on ice, washed, and incubated with R-PE conjugated goat anti–human Fc(γ) antibody. Mean fluorescence intensity (MFI) of R-PE is indicated on the *Y*-axis. The mean values of duplicate are shown.

Table 1, approximately 90% of the TNFRII-Fc (f+) and LFA-3-Fc (f+) oligosaccharides were fucosylated. In contrast the TNFRII-Fc (f–) and LFA-3-Fc (f–) had no detectable fucose.

Biological Activity of TNFRII-Fc and LFA-3-Fc—The TNF-α neutralizing activities of both TNFRII-Fc (f+) and TNFRII-Fc (f–) were almost equal as measured with a TNF-α dependent cell cytotoxicity assay (Fig. 2). As shown in Fig. 3, the CD2 binding activities of LFA-3-Fc (f+) and LFA-3-Fc (f–) measured by flowcytometry analysis was comparable. These results indicate that the functional activities measured for the TNFRII and LFA-3 are not influenced by fucose removal of Fc portion.

Binding of Fc Fusion Proteins to FcγRs—We investigated the binding profiles of Fc portion of TNFRII-Fcs and LFA-3-Fcs to FcγRIIIa, the predominant leukocyte receptor responsible for Fc-dependent cellular cytotoxicity. An ELISA method was employed using two immobilized recombinant FcγRIIIa polymorphic variants, low-affinity variant FcγRIIIa-158F and high-affinity FcγRIIIa-158V. As shown in Fig. 4, TNFRII-Fc (f–) bound better to both allotypes of FcγRIIIa than TNFRII-Fc (f+). Fucose removal of TNFRII-Fc resulted in a greater than 10-fold increase in binding to FcγRIIIa. Similarly, LFA-3-Fc (f–) bound with higher affinity to both FcγRIIIa allotypes than LFA-3-Fc (f+). These results indicate that nonfucosylated Fc fusion proteins have a greater affinity for FcγRIIIa than fucosylated Fc fusion proteins in common with intact IgGs (*17*, *32*).

Fc Dependent Cellular Cytotoxicity of Fc Fusion Proteins—The purified Fc fusion proteins were compared for their ability to induce Fc-dependent cellular cytotoxicity of a target cell line. To measure the cellular cytotoxicity of TNFRII-Fc, we constructed a transformant expressing an uncleavable cell surface mutant of TNF-α as target cells. Membrane-bound TNF-α was detected by flowcytometry with anti–TNF-α antibody on the surface of the transfectant cell line, mTNF-α/EL4 (data not shown). As shown in Fig. 5, TNFRII-Fc (f–) showed potent cytotoxicity to target cells. On the other hand, TNFRII-Fc (f+) lysis of target cells in this assay condition was very modest despite both Fc fusion proteins bound to TNF-α with equivalent affinities.

Fc-mediated cytotoxicity of LFA-3-Fc produced by CHO cell line had been reported against CD2 expressing cells. In this study, Fc-mediated cytotoxicity of LFA-3-Fc was measured using CD2+ Jurkat cells as target cells and human PBMCs as effector cells. As shown in Fig. 5, both LFA-3-Fc (f+) and LFA-3-Fc (f–) revealed cytotoxicity against CD2

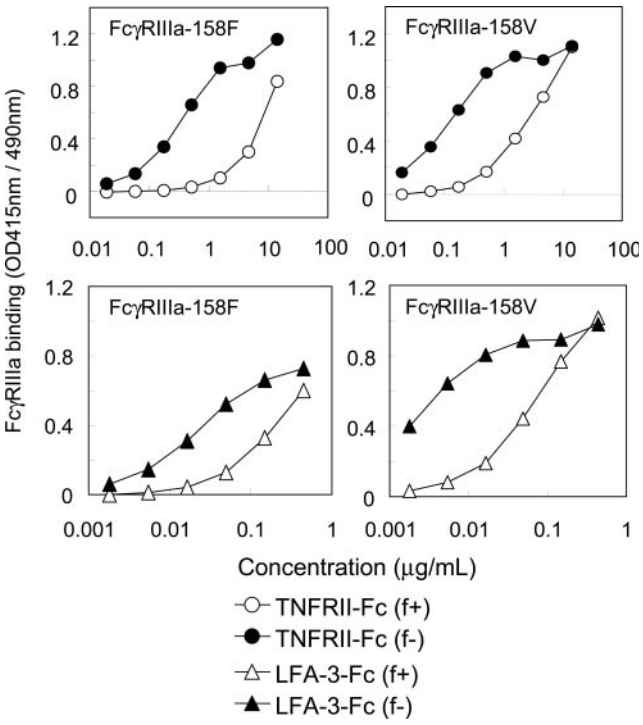

Fig. 4. **Binding assays of Fc fusion proteins to recombinant soluble FcγRIIIa.** TNFRII-Fc binding to FcγRIIIa-^{158}F (A), FcγRIIIa-158V) (B), LFA-3-Fc binding to FcγRIIIa-158F (C) and FcγRIIIa-^{158}V (D). Serial dilutions of Fc fusion protein were added to the plates coated with 6× His-tagged FcγRIIIa-^{158}F or FcγRIIIa-^{158}V. After washing, the Fc fusion proteins bound were detected with HRP labeled anti-hIgG polyclonal antibody by ELISA. The mean values of duplicate are shown.

positive target cells. However the extent of cellular cytotoxicity of LFA-3-Fc (f–) was approximately 100-fold higher than that of LFA-3-Fc (f+). These results indicate that the removal of fucose from Fc fusion proteins enhances both FcγRIIIa binding and Fc-mediated cytotoxicity of both fusion proteins.

DISCUSSION

Fc fusion proteins have many biological characters in common with IgG, including pharmacokinetic properties and target-recognition capacity. Therefore, they are often considered as an alternative to IgGs, both in research and in therapeutics. Fc fusion proteins may be preferred in certain contexts, for example, when high affinity IgGs capable of neutralizing or agonistic activity to a given antigen are difficult to obtain but the natural receptor that binds to the antigen and shows desired function is readily available (*20*). In addition, some Fc fusion proteins show broad cross reactivity to multiple ligand species while most therapeutic IgGs are highly specific for the target antigen. For example, TNFRII-Fc can bind to both TNF and lymphotoxin α (*42*, *43*), and this feature might contribute to its clinical benefits. To date, several Fc fusion proteins are marketed including TNFRII-Fc, etanercept and LFA-3-Fc, alefacept as therapeutic biologic agents for indication of RA and psoriasis, respectively (*21–29*).

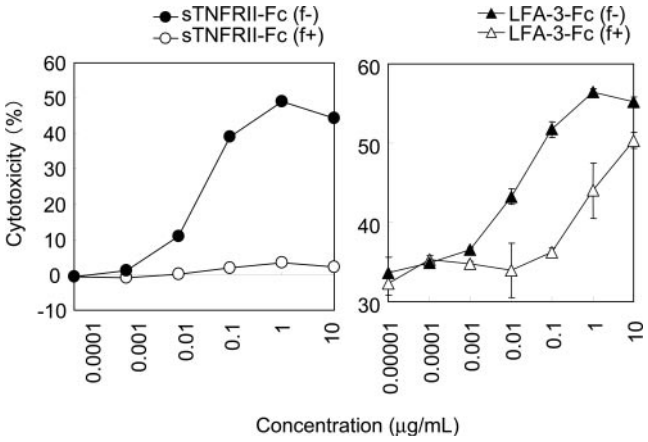

Fig. 5. **Fc-dependent cell cytotoxicity assays of Fc fusion proteins.** Assays were carried out using mTNF-α/EL4 for TNFRII-Fc, and Jurkat cells for LFA-3-Fc as targets, and PBMC as effector cells (effector/target ratio = 25:1). Serially diluted Fc fusion protein, target cells, and effector cells were added to 96-well U-bottomed plates. After 4 h incubation at 37°C, the lactate dehydrogenase activity in the supernatant was measured. Each concentration was tested in triplicate and the results are expressed as means ± SD.

In this study, we selected LFA-3-Fc as one of the examples of Fc fusion proteins to clarify the effect of fucose removal on the effector function of Fc fusion proteins. Psoriasis is an immune-mediated disease, with memory T cells playing a key role in disease pathogenesis (*28*). The costimulatory molecule CD2 is highly expressed on activated memory T cells. This increased expression of CD2 provides a specific marker that can be targeted by therapeutic agents (*28*). The results of experiments *in vitro* and in rodents have shown that LFA-3-Fc, alefacept has a dual mechanism of action. First, since it binds to CD2 on T cells, thereby blocking their natural interaction with LFA-3 on antigen-presenting cells (*26*, *27*). Second, LFA-3-Fc binds to Fcγ receptors on effector cells (such as NK cells) and depletes T-cell *via* Fc-dependent cellular cytotoxicity (*30*, *31*). The clinical importance of Fc-mediated cellular cytotoxicity of memory-effector T cells by LFA-3-Fc is supported by the relationship between the magnitude of change in memory T-cell counts and probability of clinical response. Moreover, a greater degree of reduction in memory T-cell counts was generally associated with a more favorable clinical outcome (*44*, *45*). In this study, we confirmed that defucosylation of Fc portion of LFA-3-Fc enhances both FcγRIIIa binding and Fc-mediated cellular cytotoxicity of CD2-expressing target cells. Considering the importance Fc-mediated cellular cytotoxity of LFA-3-Fc, nonfucosylated LFA-3-Fc might be more effective than conventional LFA-3-Fc in lowering the number of CD45RO+ cells and hence improving clinical outcomes.

The second model of Fc fusion protein used in this study is TNFRII-Fc, etanercept (*21–25*). TNFRII-Fc inhibits TNF-α activity by competing for TNF-α with cell surface TNF receptors. In contrast to LFA-3-Fc, the major mechanism of action of TNFRII-Fc is inhibition of TNF-α activity, and Fc-dependent cellular cytolysis of TNF-α–expressing cells by TNFRII-Fc has not been reported yet. To measure

the Fc-mediated cellular cytotoxicity of TNFRII-Fc, we first established EL4 cells expressing a transmembrane variant of TNF that could not be cleaved as target cells. The data presented herein clearly shows that both fucose variants could bind to FcγRIIIa, suggesting TNFRII-Fc is inherently capable of triggering Fc-mediated cytotoxicity, although the activation of the effector cells by the molecule was not sufficient to provide measurable lysis of the target cells with the fucosylated glycoform of TNFRII-Fc [TNFRII-Fc (f+)]. Importantly, TNFRII-Fc exhibited measurable cytotoxicity only after the fucose was removed. Since nonfucosylated TNFRII-Fc and fucosylated counterpart show the same TNF-α blocking activity, only nonfucosylated TNFRII-Fc has dual mechanism action: blocking the function of TNF-α and depletion of TNF-α–expressing cells. Interestingly, anti–TNF-α chimeric antibody, infliximab, has been reported to bind to mTNF-α expressing cells such as activated lymphocytes (*46*) and monocytes (*47*), and to induce apoptosis to these cells. TNFRII-Fc and anti–TNF-α antibody have been shown to bind to soluble TNF-α and mTNF-α and to be equally effective in rheumatoid arthritis. However, in Crohn's disease, remission is induced only by anti–TNF-α antibody and not by TNFRII-Fc (*48*). This difference between anti–TNF-α antibody and TNFRII-Fc in a clinical setting might be due to the differential binding characteristics; infliximab has strong and multivalent binding via the two Fab arms each binds independently to one TNF molecule, whereas the binding mode of TNFRII-Fc is monovalent and has rapid dissociation (*49*). However, our results suggest that the addition of the Fc receptor–mediated cytotoxic effect by fucose removal might lead to the improvement of its clinical efficacy of TNFRII-Fc and broaden the clinical utility.

In conclusion, the current study demonstrated that fucose removal of *N*-linked oligosaccharide of Fc portion potently enhances Fc-mediated cellular cytotoxicity of Fc fusion proteins that are constructed using Fc components containing the hinge region, CH2 domain, and CH3 domain of IgG. This result indicates that the methodology used expands the therapeutic possibility of Fc-fusion proteins for which the depletion of the target cells is considered beneficial.

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