Novel Anti-Tn Single-Chain Fv-Fc Fusion Proteins Derived from Immunized Phage Library and Antibody Fc Domain

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Abstract. *Tn[GalNAc(α1-3)-Ser/Thr] antigen, a tumor-associated carbohydrate antigen, is highly expressed in various tumors and an attractive candidate for cancer immunotherapy. The generation of an anti-Tn antibody is a first step toward the construction of new anticancer molecules. However, because of the simple and small conformation of the Tn molecule, it is difficult to generate an anti-Tn antibody for therapeutic use by conventional hybridoma technology. The purpose of this study was to isolate anti-Tn single-chain antibody fragments (scFv) by phage display technology from a novel immunised library, to attach an antibody constant region (Fc) and to convert them to scFv-Fc fusion proteins. The scFv-Fcs obtained here showed strict specificity against the Tn antigen and also showed antibody-dependent cellular cytotoxicity. These results suggest a potential use of this antibody generating method by phage display and indicate the potential of Fc-fusion proteins as therapeutic candidates.*

Over the past decade, antibody-based cancer immunotherapy has demonstrated safety and efficacy in clinical trials and constitutes a major class of novel therapeutic agents for a broad range of indications (1-3). Therapeutic antibodies possess multiple mechanisms of action. Antibody-dependent cellular cytotoxicity (ADCC), a lytic attack on antibody-targeted cells that is triggered by the binding of FcγRIIIa on NK cells to the antibody constant region (Fc), is an established mechanism of antibody activity that is believed to be active in the clinic (4). The clinical importance of ADCC has been demonstrated in rituximab and trastuzumab therapies, in which FcγRIIIa functional polymorphisms of patients were significantly associated with clinical responses (5, 6).

Meanwhile, the strong antigenic similarities between malignant cells and their normal counterparts have prevented the use of tumor-associated antigens as targets for antibody-based immunotherapy (1). Nevertheless, interest still remains on tumor-associated antigens, whose expression patterns allow the isolation of cancer cells from normal cells.

Tn[GalNAc(α1-3)-Ser/Thr] antigen, a tumor-associated carbohydrate antigen, is highly expressed on various tumors (gastric, colorectal, ovarian, breast, and pancreatic carcinomas) while its expression is very limited in benign and normal tissues (7). Tn antigen expression has been shown to be an independent marker for poor prognosis in carcinomas (8-11), suggesting that the Tn antigen is an attractive candidate for cancer immunotherapy. However, carbohydrate structures mostly induce T-cell-independent humoral responses of the IgM isotype (12). Therapeutic use of IgM antibodies is limited by their short half life, lack of ADCC function and poor production in industrial processes (2). Therefore, the generation of an anti-Tn therapeutic antibody of IgG class would be of value for the novel treatment of solid tumors, since the conventional methodology to generate monoclonal antibodies from hybridomas is unsuitable.

This study reports the construction of an immunized single chain Fv (scFv) library and the selection of a specific binder against Tn antigens using phage display technology. The selected clones were converted to scFv-Fc fusion protein (scFv-Fc) which retained their binding characteristics and ADCC effecter function.

Materials and Methods

Construction of a single chain Fv library. BALB/c mice (6- to 8-week-old-females) were immunised by intraperitoneal injection with Tn-antigen-positive Jurkat cells (1.5×10^7 cells/mouse) with Bordetella pertussis as adjuvant (1×10^9/mouse). Weekly intraperitoneal injections were administered for six more weeks and the spleens were then removed.

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Key Words: Tn antigen, GalNAc(α1-3)-Ser/Thr, single chain antibody, phage-display, Fc fusion, antibody-dependent cellular cytotoxicity (ADCC).

Table I. *Primers sequence for construction of a single chain Fv library.*

Primer sequences		
(A) First-strand cDNA synthesis		
First-MGH$_1$	5'-AGGGGCCAGTGGATAGACAGATGGGGGTGT-3'	IgG$_1$
First-MGH$_{2a}$	5'-AGGGGCCAGTGGATAGACCGATGGGGCTGT-3'	IgG$_{2a}$
First-MGH$_{2b}$	5'-AGGGGCCAGTGGATAGACTGATGGGGGTGT-3'	IgG$_{2b}$
First-MGH$_3$	5'-AGGGACCAAGGGATAGACAGATGGGGCTGT-3'	IgG$_3$
First-κ	5'-GGATGGTGGGAAGATGGATACAGTTGGTGCAGC-3'	Cκ
First-λ	5'-AGGTGGAAACACGGTGAGAGTGGGAGTGGACTT-3'	Cλ
(B) Amplification of V_H and V_L domains		
$V_κ$-front and $V_λ$-front		
κ-front 1	5'-*CCATGG*CCGACATTGTGMTGWCACAGTC-3'	
κ-front 2	5'-*CCATG*GCCGATRTTKTGATGACCCARAC-3'	
κ-front 3	5'-*CCATGG*CCRAMATTGTGMTGACCCAATC-3'	
κ-front 4	5'-*CCATGG*CCSAAAWTGTKCTSACCCAGTC-3'	
κ-front 5	5'-*CCATGG*CCGAYATYCAGATGACMCAGWC-3'	
λ-back 1	5'-*CCATGG*CCCARSYTGTKSTSACTCAGKMATCT-3'	
Vκ-back and $V_λ$-back		
κ-back 1	5'-TCCAGAACCGCCACCGCCGCTACCGCCGCCACCTTTCAGYTCCARYTT-3'	
κ-back 2	5'-TCCAGAACCGCCACCGCCGCTACCGCCGCCACCTTTKATYTCCARYTT^3'	
κ-back 3	5'-TCCAGAACCGCCACCGCCGCTACCGCCGCCACCTTTBAKYTCTATCTTTGT-3'	
κ-back 1	5'-TCCAGAACCGCCACCGCCGCTACCGCCGCCACCTAGAGCAGTSASYTTGGT-3'	
VH-front		
VH-front 1	5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCT GAKGTRCAGCTTCAGGAGTCRGGA-3'	
VH-front 2	5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCT CAGGTGCAGCTGAAGSAGTCWGGM-3'	
VH-front 3	5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCT SAGGTYCAGCTGCARCAGTCWGGD-3'	
VH-front 4	5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCT SAGGTCCARCTGCAGSARYCTGGR-3'	
VH-front 5	5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCT GAGGTTCAGCTGCAGCAGTCTGGG-3'	
VH-front 6	5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCT GARGTGAAGCTGGTGGARTCTGGR-3'	
VH-front 7	5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCT GAGGTGAAGCTTCTCGAGTCTGGA-3'	
VH-front 8	5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCT GARGTGAAGCTKGAKGAGWCTGR-3'	
VH-front 9	5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCT GAVGTGMWGCTKGTGGAGTCTGGK-3'	
VH-front 10	5'-AGCGGCGGTGGCGGTTCTGGAGGCGGCGGTTCT SAGGTYCAGCTKCAGCAGTCTGGA-3'	
V_H-back		
VH-back 1	5'-*CCGCGG*TGMRGAGACDGTGASMGTRGTC-3'	
VH-back 2	5'-*CCGCGG*TGMRGAGACDGTGASMGTRGTG-3'	
VH-back 3	5'-*CCGCGG*TGMRGAGACDGTGASCAGRGTC-3'	
VH-back 4	5'-*CCGCGG*TGMRGAGACDGTGASTGARGTT-3'	

The primer sets $V_κ$-front and $V_λ$-front contain the restriction sites *Nco* I (in italics) and VH-back contain the restriction sites *Sac* II (in italics). Both the primer sets $V_κ$, $V_λ$-back and VH-front contain the coding sequence of the [(Gly4Ser)$_3$] linker (underline). B=C/G/T, D=A/G/T, K=G/T, M=A/C, R=A/G, S=C/G, W=A/T, Y=C/T

A scFv library was constructed as described previously (13). Total RNA was isolated from the spleen cells using RNeasy (Qiagen, Tokyo, Japan). First-strand cDNA was synthesized in two separate reactions using the First-Strand cDNA synthesis kit (Amersham Biosciences, Tokyo, Japan) and the mix primers (Table IA), binding to the C_H1 region of IgG$_1$, IgG$_{2a}$, IgG$_{2b}$ and IgG$_3$ heavy chains or C_L region of κ and λ light chains, respectively.

PCR amplification of the variable regions was performed using the following specific sets of primers binding to murine immunoglobulin variable regions of heavy (V_H) and light chains ($V_κ$ and $V_λ$) (Table IB).

V_H variable regions were amplified from the cDNA using PCR in combination with each of the V_H-front primers and an equimolar mixture of V_H-back primers. For V_L variable regions, a similar procedure was performed using each $V_κ$-front and a mixture of $V_κ$-back for κ chains and each $V_λ$-front with a mixture of $V_λ$-back for λ chains. A GeneAmp PCR System 9700 (Applied Biosystems, Foster city, CA) was used for PCR. DNA were then attached DNA segments encoding half of the peptide linker [(Gly4-Ser)$_3$] and *Not* I or *Sac* II restriction sites. Conditions for the amplifications were: 4 min denaturation at 94˚C, followed by 30 cycles at 94˚C for 30 seconds, 50˚C for 30 seconds, 68˚C for 1 min, with a final extension cycle at 68˚C for 4 min. PCR products of heavy chain and light chain gene segments were separately pooled and gel-purified using the QIAEX (Qiagen) DNA extraction kit and equimolar mixture of V_H and VL segments were overlapped by PCR. The following

program was used: Seven cycles of 5 min at 94˚C, 2 min at 55˚C, 10 min 72˚C without primers. Subsequently, external primers were added, followed by 30 cycles of 30 seconds at 94˚C, 30 seconds at 50˚C, 1 min at 68˚C and final extension at 68˚C for 4 min. PCR products were gel-purified and cut with restriction enzymes *Nco* I and *Sac* II. The resulting DNA fragments were gel-purified and ligated into the phagemid pTZ (kindly provided by Dr. Kumagai, Tohoku University, Japan) previously cut with the same restriction enzymes. Ligated DNA was electroporated into *E. coli* strain JM109. The sequences of the individual twenty clones were analyzed in the ABI PRISM3700 DNA Analyzer (Applied Biosystems).

Preparation of scFv phage library. The transformed JM109 cells were cultured at 37˚C in 40 ml of Luria-Bertani (LB) medium containing 100 μg/ml ampicillin. The cells were grown to an optical density $(OD)_{600}$ of 0.5 and then infected with 1×10^{10} colony forming units (cfu) of M13KO7 helper phage (Invitrogen, Tokyo, Japan). The culture was incubated for 1 h at 37˚C. Subsequently, the medium was exchanged with 40 ml of LB medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin, and culture was shaken for 12 h at 37˚C. The bacteria were removed by centrifugation (3000 ×g, 15 min, 4˚C), phage were precipitated from the supernatant (1/5 volume) with 20% polyethylene glycol 6000/2.5 M NaCl solution for at least 1 h on ice, collected by centrifugation (13000 ×g, 20 min, 4˚C), resuspended in 500 μl of phosphate-buffered saline (PBS), and subjected to selection.

Isolation of anti-Tn scFv by panning of phage-antibody repertories. Streptavidin MagneSphere Paramagnetic particles (Promega, Madison, WI, USA) were used to screen the phage antibody library (14, 15). Prior to panning, the magnetic beads were washed three times with 1 ml of PBS and then incubated for 1 h with Super Block blocking buffer (PIERCE, Rockford, IL, USA). The phage antibodies were incubated with 0.1-10 μg of UK blood group A trisaccharide-BSA-Biotin (Dextra laboratories, Reading, UK) for 1 h at room temperature as negative selection. After 1 h, a magnet was used to pull out the unbound phages. Unbound phages were incubated with 0.1-10 μg of synthetic Tn antigen-biotin (Dextra laboratories) for 1 h at room temperature as positive selection. After 1 h, bound phage was pulled out in the same way. The beads were washed extensively (3-10 times) with 1 ml of PBS containing 0.5% Tween 20, and 1 ml of PBS. Bound phages were eluted with 1-30 μg non-biotinylated soluble Tn antigen (non-labeled) for 10 min at room temperature and used to infect 40 ml culture (LB medium containing 100 μg/ml ampicillin) of early log phase *E.coli* JM109 cells. After incubation for 1 h at 37˚C, the cells were infected with 1×10^{10} colony forming units (cfu) of M13KO7 helper phage (Invitrogen). The culture was incubated for 1 h at 37˚C. Subsequently, the medium was exchanged with 40 ml of LB medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin, and culture was shaken for 12 h at 37˚C. After incubation, phage antibodies were prepared as described above and used for the next round of selection.

Phage ELISA. After each round of selection and amplification, the suspension was spread on LB plates containing 100 μg/ml ampicillin and cultured overnight at 37˚C. The next day, individual colonies were selected and phage antibodies prepared, as described above, for ELISA. Microtiter plates were coated overnight at 4˚C with 50 μl/well of Tn antigens or blood group A antigen (10 μg/ml in PBS) and then blocked for 1 h at 37˚C with SuperBlock blocking buffer. The plates were washed three times with PBS and incubated with individual 5×10^6 cfu scFv phages for 1 h at 37˚C. The plates were washed five times with PBS containing 0.05% Tween 20 (PBST) and incubated for 1 h at 37˚C with 1:3000 horseradish peroxidase-conjugated anti-M13 monoclonal antibody (Amersham Biosciences). After washing the plates five times with PBST, ABTS [2, 2'-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid)] was added as a substrate. The absorbance at 415 nm (contrast at 490 nm) was measured using a microtiter plate reader (Molecular Devices, Sunnyvale, CA).

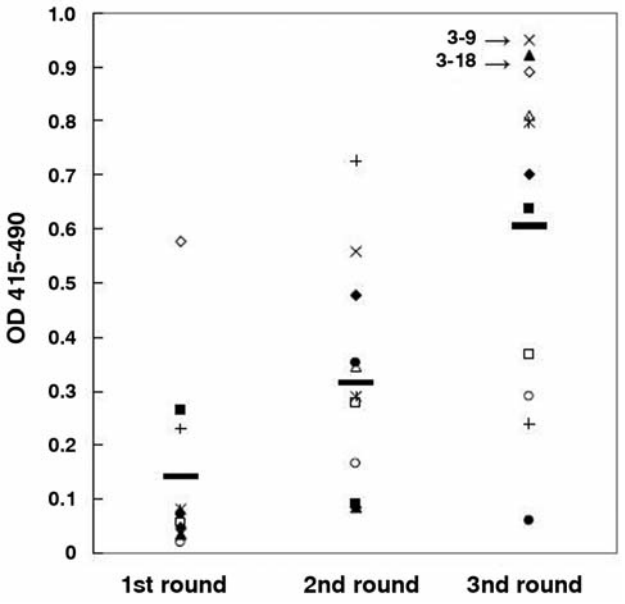

Figure 1. *Tn-binding of selected scFv-phage clones from each round of panning. Tn-binding of selected clones was analyzed by phage-ELISA. Each clones were prepared as 5×10^6 phages. scFv-phage binding to the immobilized Tn-antigen was detected by a perioxidase-labeled anti-M13 antibody.*

Conversion of selected scFv to scFv-Fc fusion proteins. Anti-Tn scFv-Fc fusion protein expression vector was generated by inserting the cDNA, coding the selected scFv derived from phage display selection as previously described (16-18). The cDNA was amplified by PCR and cut with restriction enzyme, then inserted into the previously described pKNATEX93 vector (19). The anti-Tn scFv-Fc fusion proteins were produced using CHO/FUT8$^{-/-}$ cells. CHO/FUT8$^{-/-}$, a FUT8 knockout cell line for fucose-negative scFv-Fc fusion proteins production, has been described previously (20). pKANTEX93/scFv-Fcs was introduced into CHO/FUT8$^{-/-}$ cells *via* electroporation, and transfected cells were grown in Iscove's modified Dulbecco's medium (IMDM) containing 0.5 mg/ml G418 sulfate to obtain G418-resistant clones. Then G418-resistant clones were selected for gene amplification in methotrexate-containing medium. A high producing cell clone, as determined by ELISA, was grown in serum-free EX-CELL301 medium (JRH Bioscience, Lenexa, KS, USA). The culture supernatant was collected, centrifuged to remove cellular debris, and affinity-purified on a Prosep A column (Millipore, Tokyo, Japan) as described by the manufacture. The eluted scFv-Fc fusion proteins were dialyzed in to 10 mM citrate buffer (pH 6.5, 150 mM NaCl), sterile-filtered

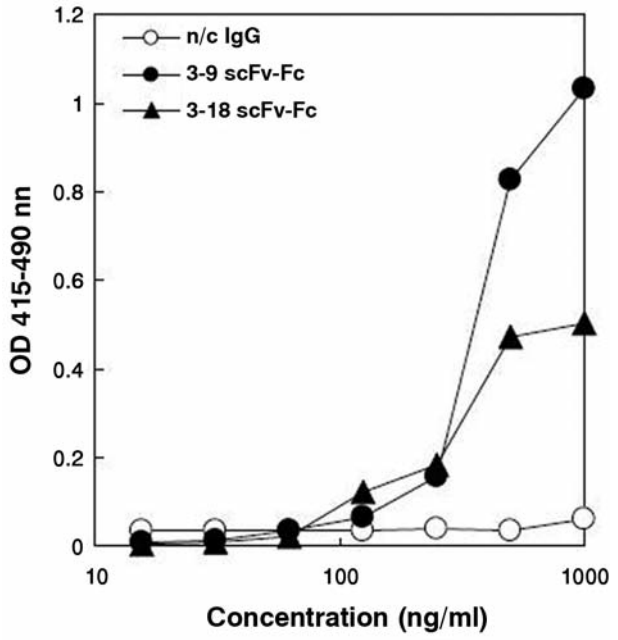

Figure 2. *Tn-binding of scFv-Fcs was analyzed by ELISA. Negativi contorol IgG (○) or 3-9 scFv-Fc (●) or 3-18 scFv-Fc (▲) binding to [{Ser(GalNAc)-Thr(GalNAc)-Thr(GalNAc)}-Gly6-Lys] immobilized plates.*

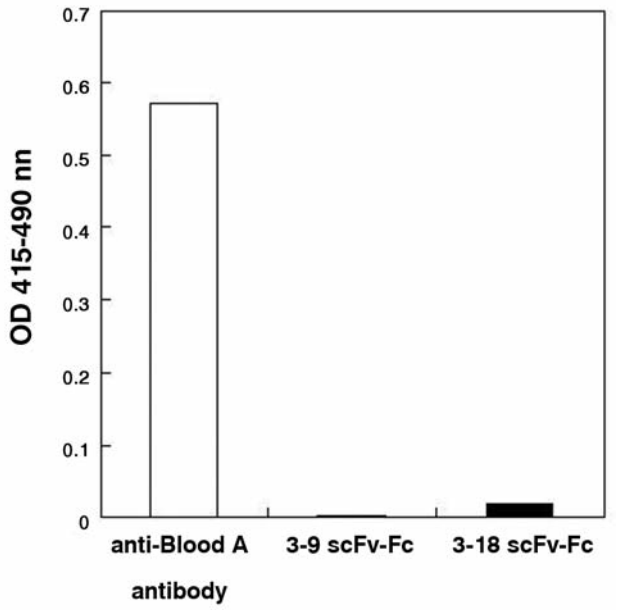

Figure 3. *Cross-reactivity of scFv-Fcs against blood group A antigen. Binding to the immobilized blood group A was analyzed by ELISA. anti-blood A antigen antibody was used as positive control.*

(0.22 μm), and stored at 4˚C. The concentration of scFv-Fc fusion proteins were determined by measuring the absorbance at 280 nm.

Characterization of anti-Tn scFv-Fc fusion proteins binding properties by ELISA. Anti-Tn scFv-Fc fusion proteins biding activities were analysed by ELISA with [{Ser(GalNAc)-Thr(GalNAc)-Thr(GalNAc)}-Gly6-Lys] (Tn3-G6K) as Tn-positive antigen, synthesised by classical methods (21). The specificity of each scFv-Fc fusion protein was also evaluated with blood group A antigen as negative control.

Microtiter plates were coated overnight at 4 C with 50 μl/well of each antigen (1 μg/ml in PBS) and then blocked for 1 h at 37 C with PBS containing 1 % BSA. The plates were washed three times with PBS and incubated with scFv-Fc fusion protein in various concentrations for 1 h at 37˚C. The plates were washed five times with PBST and incubated for 1 h at 37˚C with 1:3000 horseradish peroxidase-conjugated goat anti-human IgG monoclonal antibody (American Qualex, San Clemente, CA, USA). After washing the plates five times with PBST, ABTS was added as a substrate. The absorbance at 415 nm (contrast at 490 nm) was measured using a microtiter plate reader (Molecular Devices).

Flow cytometer analysis. Anti-Tn scFv-Fc fusion proteins binding to cell surface Tn antigens were analysed by flow cytometry. The CHO-Lec8 cells and Jurkat cells as Tn-antigen positive cell line and CHO-DG44 as Tn-antigen negative cell line were stained with 5 μg/ml of scFv-Fc fusion proteins. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG antibody was used as the secondary reagent. The stained cells were analyzed using an EPICS XL-MCL flow cytometer (Beckman Coulter, Brea, CA).

ADCC assay. Peripheral blood mononuclear cells (PBMC) were separated from the peripheral blood of a healthy donor using Lymphoprep (Fresenius Kabi, Norway) and used as the effector cells. The tumor cells, Jurkat and KPL4 as Tn-positive cell lines and Namalwa as Tn-negative cell lines, were prepared as 1×10^4 cells/well and effector cells as 2.5×10^5 cells/well (E/T ratio is 25/1) and put in 96-well microtiter plates and incubated with various concentrations of scFv-Fc fusion proteins for 4 h at 37˚C. After centrifugation, 50 μl of the each supernatant was transferred to new microtiter plates and cytolysis was measured using the CytoTox96 Non-Radioactive Cytotoxicity Assay kit according to the manufacture's instructions (Promega). Cytotoxicity was determined as a function of lactate dehydrogenase (LDH) enzymatic activity released from the cytosol of damaged cells into supernatant as measured by microtiter plate reader at 490 nm. Cytolysis percentage was calculated using the following equation: Cytotoxicity (%)=100×[{(effector and target cell mix–effector cell control)–spontaneous release}/(maximum release–spontaneous release)].

Results

Construction of the immunized mouse scFv phage library. Mice were immunized with Tn-positive Jurkat cells to induce anti-Tn antibody production. The scFv library was generated by RT-PCR from total RNA from immunized mouse spleen cells. To amplify cDNAs of IgG class antibody specifically, first-strand cDNAs were generated in two separate reverse transcriptase PCR reactions, using primers binding to constant Ig domains (Table IA). The amplified cDNAs were subsequently used for the amplification of variable (V) regions of either IgG_1, IgG_{2a}/b or IgG_3 heavy or κ or λ light chains using extended sets of immunoglobulin-specific

Table II. *Number of scFv-pahage clones during bio-panning*[a].

Rounds of panning	Input number (cfu)[b]	Output number (cfu)[b]	Ratio (output/input)
1	7.0×10^{13}	5.0×10^{6}	7.1×10^{-8}
2	9.1×10^{12}	4.2×10^{6}	4.6×10^{-7}
3	3.7×10^{13}	2.2×10^{9}	5.4×10^{-5}

[a] Experimental procedures are described in Material and methods. [b] The procedure for estimating colony forming unit (cfu) is that of Tsumoto *et al*., 1998.

primers (Table IB). *Via* this PCR reaction, the $[(Gly_4Ser)_3]$-coding linker sequence were inserted between the 5' and 3' site of variable heavy and light chain regions, respectively. The complementary $[(Gly_4Ser)_3]$-coding sequence overlap of 30 bp enabled random assembly of scFv-encoding fragments by splice-overlap PCR. These assembled scFv-encoding fragments were reamplified to incorporate flanking *Not* I- and *Sac* II-cloning sites at the 5' and 3' end. The assembled products were purified, digested with *Not* I and *Sac* II, and ligated to the pIII gene of the pTZ phagemid. The diversity of the scFv library was estimated by DNA sequence analysis on twenty independent scFv clones, which resulted in approximately 6.0×10^{5} independent clones for the size of the library constructed.

Isolation and characterization of specific scFvs against Tn antigen. To optimize the capture of Tn-antigen-specific phage and minimize the binding of irrelevant phage antibodies, a simultaneous positive and negative selection strategy was employed (22-24), in which the clones reactive to the blood group A antigen which has the same terminal α anomeric GalNAc residue were removed as negative-selection. After three rounds of negative- and positive-selection, phage antibodies showed binding activity evaluated by phage ELISA using 96-well plates coated with Tn-antigen (Figure 1). The result revealed that clones with increased affinity for the target antigen were enriched through the selection (Figure 1 and Table II). The DNA sequence of the selected clones that underwent three cycles of screening was determined. Interestingly, all selected clones had different sequences (data not shown), but complementarily determining region (CDR) sequences showed close homology (Table III).

Production and characterization of anti-Tn scFv-Fc fusion proteins. Antibody-dependent cellular cytotoxicity (ADCC) is a lytic attack on antibody-targeted cells that is triggered by the binding of lymphocyte receptors (FcγRs) to the antibody constant region (Fc). ADCC is considered to be one of the major effector functions of therapeutic antibodies.

Previously, it has been reported that ADCC is greatly enhanced (by ~100-fold) by fucose depletion from oligosaccharides in the Fc region (25). Therefore, anovel structure of therapeutic proteins composed of anti-Tn scFvs and the Fc with nonfucosylated oligosaccharides was desigend, to confer potent ADCC function to the anti-Tn scFvs obtained above. For the production of completely nonfucosylated proteins, a Chinese hamster ovary cell line was previously established, in which FUT8 gene encodingα-1,6-fucosyltransferase was knocked out (CHO/FUT8$^{-/-}$) (20). In this study, two scFv clones were selected, 3-9 and 3-18, the two best Tn binders (Figure 1), and their Fc-fusion proteins (scFv-Fc) were produced using CHO/FUT8$^{-/-}$ as host cells.

To test their binding activities and specificities, purified scFv-Fcs were analysed to immobilized synthetic Tn-positive antigen, [{Ser(GalNAc)-Thr(GalNAc)-Thr(GalNAc)}-Gly6-Lys] and blood group A antigen, which share the same terminal α anomeric GalNAc structure with Tn antigen as negative control. As shown in Figure 2 and 3, both scFv-Fcs showed specific binding to Tn-positive antigen (Figure 2) and showed no detectable cross-reactivity to blood group A antigen (Figure 3).

In addition, to confirm its binding activity against native Tn antigen, flow cytometric analysis with Tn-positive Jurkat cells and CHO-Lec8 cells and also Tn-negative CHO-DG44 cells were carried out. As shown in Figure 4, both scFv-Fcs bound equivalently to Tn-positive Jurkat cells and CHO-Lec8 cells, but they showed no specific binding to Tn-negative CHO-DG44 cells. These results indicate that the selected scFvs specifically recognised Tn-antigen expressed on the cell surface. Taken together, by the use of negative- and positive-panning, these data indicate that selected scFvs have high selectivity against Tn-antigen.

ADCC activities of anti-Tn scFv-Fc fusion proteins. The human PBMC-mediated ADCC of scFv-Fcs was measured against Jurkat cells, Tn-positive tumor cell line and Namalwa cells, Tn-negative tumor cell line. As shown in Figure 5, both scFv-Fcs exhibited antigen-dependent ADCC. These results indicate that Fc-fusion molecules, which consist of target binding domains and the Fc portion of human IgG antibody, have ADCC activity.

Discussion

In the present study, a scFv phage library was constructed from immunised mice, the specific binders from the library were screened, and then scFv-Fc fusion protein was generated based on the selected scFv fragment. ADCC function against Tn, tumor-associated carbohydrate, antigens was achieved successfully.

Tn-antigen is a potential target for anticancer therapy (7, 26), however it isdifficult to generate an anti-Tn antibody for therapeutic use with mouse hybridoma technology, since Tn-antigen is monosaccharide carbohydrate and less immunogenic. Furthermore there are various cognate antigens which share the

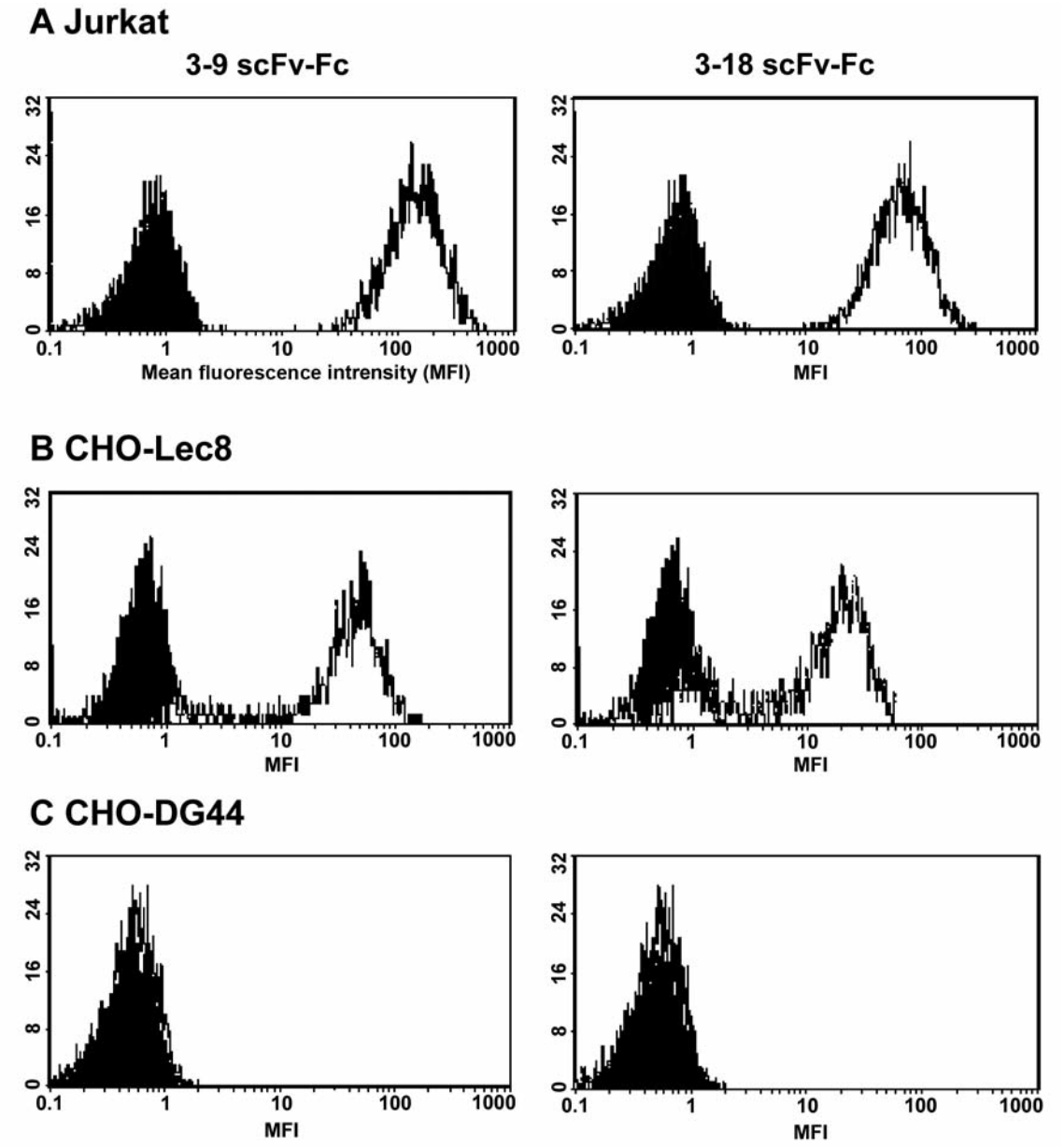

Figure 4. *Reactivity of 3-9 scFv-Fc and 3-18 scFv-Fc with A: Jurkat (Tn-positive), B: CHO-Lec8 (Tn-positive), C: CHO-DG44 (Tn-negative) cells. Cells were evaluated with the respective scFv-Fc (light trace) or negative control chimeric IgG (dark trace), and then analyzed by fluorescent flow cytometry as described in the Materials and Methods. Each scFv-Fc and chimeric IgG has same human IgG1 Fc region and are shown in the same panel.*

terminal α anomeric GalNAc residue, such as human blood group A or Forssman antigens (27, 28).

Phage display technology has proven to be an efficient and convenient technology for the generation of antigen-specific antibody fragments *in vitro*. Various antibody therapeutics obtained from phage display library are in clinical trial or on the market (29). One of the advantages of this technology is that the selection of antibodies can be achieved *in vitro*. Therefore, phage display technology is a highly effective method to generate antibodies, especially against antigens that have difficulty in immunization (30-32) (for example, toxic, and less immunogenic antigens).

On the other hand, the property of antibody fragments selected by phage display depends primarily on the quality and diversity of the library. However, the use of a 'naive antibody library' often falls into insufficient affinity, which requires further optimization such as *in vitro* affinity maturation method (33, 34). To generate antibodies for therapeutic use, a

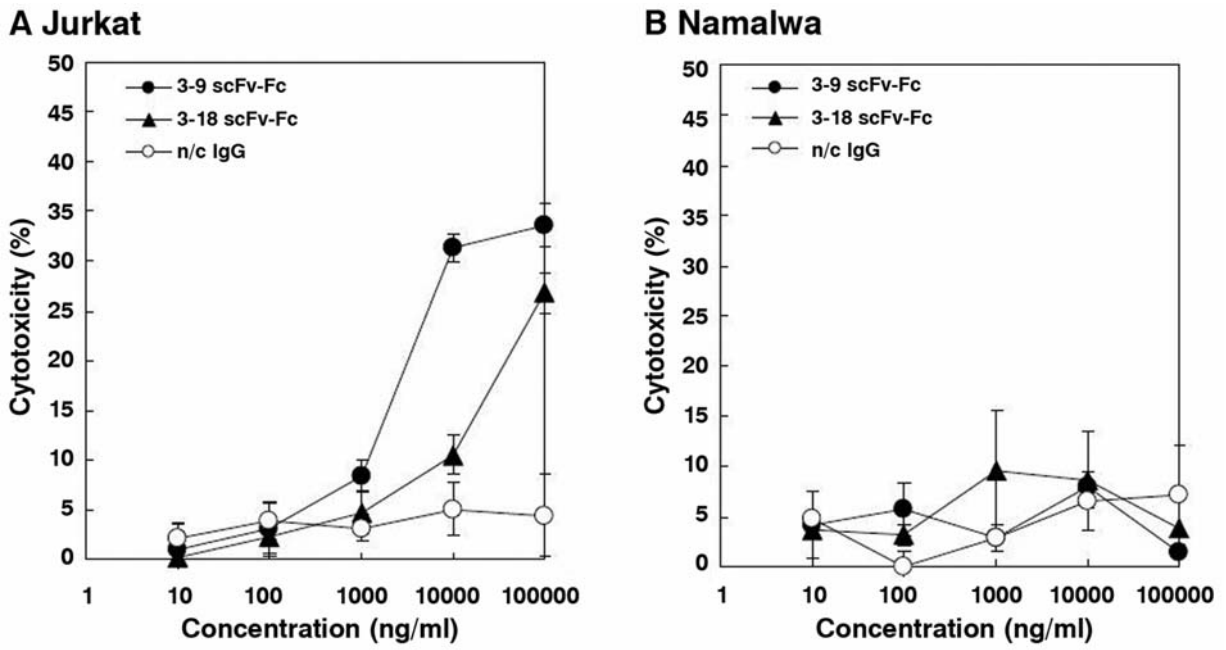

Figure 5. *Antibody-dependent cellular cytotoxicity (ADCC) of anti-Tn scFv-Fcs. Cytotoxicity was measured with lactate dehydrogenase (LDH) enzymatic activity in the presence of negative control IgG (○) or 3-9 scFv-Fc (●) or 3-18 scFv-Fc (▲) and human peripheral blood mononuclear cells as effector cells. Tn-positive Jurkat cells (A) and Tn-negative Namalwa cells (B) were analyzed as target tumor cells. E/T ratio was held constant at 25.*

novel scFv immunized library (6.0×10^5 variants) was constructed from spleens of mice immunized with Tn antigen.

To optimise the capture of antigen-specific phage and minimize the binding of irrelevant phage antibodies, a simultaneous positive and negative selection strategy against Tn-antigen and blood group A, which both have the same terminal α anomeric GalNAc residue, was applied.

After three rounds of panning, multiple clones were selected randomly and their binding to Tn-antigen was evaluated by ELISA. The results indicated that some of the clones selected showed a marked signal for the target antigen (Figure 1), and the output/input ratio was increased in correlation with the number of rounds (Table II). Interestingly, the selected 9 clones had different V region sequences, while their CDR sequences showed close homology (Table III). These results indicate that specific scFv fragments are enriched by the phage-display selection employed in this study. As a result, two anti-Tn clones (3-9 and 3-18), which showed highest affinity for Tn-antigen, were selected for further analyses.

A series of analyses on the cross-reactivity to various analogous antigens, such as nonglycosylated core peptide, blood group A antigen and Tn-negative cell lines, revealed that the obtained scFv-Fcs recognize Tn with highly strict specificity. This could be attributed to the dual selection steps developed in this study: (i) *in vivo* immunological deletion of antibodies reactive to murine cognate antigens during immunization, and (ii) the subsequent negative panning step using blood group A antigen that further warrants the fine specificity for GalNAc moiety in complex with the backbone core amino acid (Ser or Thr).

Furthermore, several groups report that ADCC enhancement can be achieved by amino acid (35) or glycoform (25, 36) modification in the Fc region. Elsewhere, it has been reported that removal of fucose from IgG Fc region enhances their ADCC>50-fold (25, 37) and establishes CHO/FUT8$^{-/-}$ cells (20), a FUT8 knockout cell line for fucose-negative IgG production.

To provide strong effector function of the nonfucosylated Fc, two scFv-Fc fusion proteins (3-9 scFv-Fc, 3-18 scFv-Fc) were developed using CHO/FUT8$^{-/-}$ cells as host cells.

Therapeutic potential of the obtained scFv-Fcs appears to be promising, since they bind to a Tn-positive tumor cell line Jurkat with high intensity and induce significant cytotoxic activity in ADCC experiments against Jurkat cells. In addition, scFv-Fc format has potential benefits for therapeutic development and use in comparison with wild-type IgG molecules. Firstly, because of their small molecular weight (110 kDa), scFv-Fc molecules are thought to have better tissue penetration than whole IgG molecules (150 kDa). Secondly, scFv-Fc is composed of a single polypeptide chain that does not require any heteromeric disulfide association. Consequently, expression of functional scFv-Fcs have been reported not only with mammalian cells but also using yeast (38).

Table III. *Sequence information of selected clones. Amino acid sequences of the VH and VL CDR regions of the anti-Tn scFvs are provided.*

Clone	CDR-L1	CDR-L2	CDR-L3	CDR-H1	CDR-H2	CDR-H3
3-3	TASSSVSSSYLY	STSNLAS	HQYHRSPLT	SYWMH	EINPSNGGTNYNEKFKR	RGNAMDY
3-4	SVSSSISSSNLH	GTSNLAS	QQWSSYPLT	NYWLG	RIYPGTGTIYYNERFKV	ELVPFDY
3-8	KSSQSLLNSGNQKNYLA	GASTRES	QNDHSYPLT	RDYWN	NIGYSGRTYYNPSLKS	GDRYNWYFDV
3-9	SASSSVSYMH	DTSKLAS	HQRSSYPLT	RDYWN	NIGYSGRTYYNPSLKS	GDRYNWYFDV
3-10	KSSQSLLNSGNQKNYLA	GASTRES	QNDHSYPLT	RDYWN	NIGYSGRTYYNPSLKS	GDRYNWYFDV
3-18	SASSSVSSSYLY	GASTRES	QNDHSYPLT	RDYWN	NIGYSGRTYYNPSLKS	GDRYNWYFDV
3-19	SASSSVSSSYLY	STSNLAS	HQQSSYPLT	DHAIH	YISPGNDDIKYNEKFKG	STDDTFDV
3-20	SASSSVSSSYLY	GASTRES	QNDHSYPLT	RDYWN	NIGYSGRTYYNPSLKS	GDRYNWYFDV
3-23	KSSQSLLNSGNQKNYLA	GASTRES	QNDHSYPLT	RDYWN	NIGYSGRTYYNPSLKS	GDRYNWYFDV

In conclusion, the current study demonstrates a useful strategy for construction of Tn-specific immunotherapeutic proteins which have potential therapeutic activities. Moreover, the methotology can also be expanded to the development of antibodies for therapeutic use, especially for antigens difficult to generate antibodies by conventional hybridoma technology.

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