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Human Anti–Human IL-18 Antibody Recognizing the IL-18–Binding Site 3 with IL-18 Signaling Blocking Activity

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IL-18 is an important regulator in both innate and acquired immune responses. The aberrant expression of IL-18 is associated with severe inflammatory conditions, such as autoimmune diseases and allergies. Thus, human antibodies with inhibitory activity on IL-18 signaling may be useful for therapeutic applications. We report here the first establishment of an antagonistic anti–IL-18 complete human antibody, h18-108, employing a human single chain antibody (scFv)–displaying phage library. The h18-108 scFv inhibited the IFN-γ production of a human myelomonocytic cell line, KG-1. Flow cytometry analysis showed that h18-108 blocked the binding of IL-18 to KG-1 cells. Epitope mapping analysis using two kinds of random peptide–displaying phage libraries and an IL-18 alanine mutant (D98A) demonstrated that the h18-108 scFv binds to the site 3 of IL-18, which is suggested to be an association site with the IL-18 receptor β. The complete human Fab and IgG forms of h18-108 have been successfully constructed to attain increases in both binding affinity and inhibitory activity.

Key words: allergy, epitope mapping, interleukin-18, human antibody, phage display.

Abbreviations: AP, alkaline-phosphatase; CDR, complementarity determining region; FR, framework; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; IC_{50}, 50% inhibitory concentration; IGIF, IFN-gamma inducing factor; IgG, immunoglobulin G; IgM, immunoglobulin M; IL-18, interleukin-18; IL-18Rα, interleukin-18 receptor α; IL-18Rβ, interleukin-18 receptor β; IL-1RAcPL, interleukin-1 receptor accessory protein–like; IL-1Rrp, interleukin-1 receptor related protein; LPS, lipopolysaccharide; mAb, monoclonal Ab; RU, response unit; scFv, single-chain variable fragment; TU, transformation unit.

IL-18 was initially designated as an IFN-γ–inducing factor (IGIF) belonging to the IL-1 cytokine family (*1*). A subsequent series of studies demonstrated the intriguing features of IL-18 in the pathogenesis of allergies, atopy, acute air inflammation, and parasite immunity (*2–5*). Thus, IL-18 is a potential therapeutic target for these inflammation disorders, and a neutralizing antibody should be a promising therapeutic reagent.

Pro-inflammatory cytokine IL-18 is constitutively and ubiquitously expressed as a precursor molecule (immature IL-18) and cleaved by both the caspase-1–dependent and caspase-1–independent pathways to form a biologically active protein. However, the limited IL-18 receptor expression in tissues restricts the ligand-acting sites and contributes to a specific response for IL-18. IL-18 has two receptor components, a ligand-binding α-chain (IL-18 receptor α: IL-18Rα or IL-1R–related protein: IL-1Rrp) and a signal-transducing β-chain (IL-18 receptor β: IL-18Rβ or IL-1R accessory protein–like: IL-1RAcPL). With regard to the involvement of IL-18Rα in IL-18 signaling, Azam *et al*., using various truncated recombinant IL-18Rα cDNAs, showed that the biologically active IL-18 complex requires the membrane-proximal third Ig-like domain in IL-18Rα for the formation of the IL-18R ternary complex, as well as for signal transduction (*6*). On the other hand, with respect to the importance of IL-18Rβ for IL-18 signaling, Born *et al*. showed that a truncated form of IL-18Rβ lacking the cytoplasmic domain inhibited IL-18 signaling (*7*). Debets *et al*. showed that cotransfectants with both IL-18Rα and IL-18Rβ provide low- and high-affinity binding to IL-18. They produced the rat anti–mouse IL-18Rβ antibody using IL-1RAcPL–transfecting cells. This antibody, TC30-28E3, strongly inhibited the IL-18–induced production of IFN-γ by Th1 cells (*8*). Recently, Kato *et al*. performed an NMR study that yielded advanced information about the functional receptor complex (*9*). By means of surface plasmon resonance measurements, it was shown that IL-18Rβ is not able to bind on its own to either IL-18 or IL-18Rα. On the other hand, IL-18Rβ associates with the IL-18/IL-18Rα complex to form a ternary complex, which is essential for the initiation of IL-18 receptor signaling. They also reported that receptor binding and cellular analyses using IL-18 mutants had revealed the presence of three

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sites: two of these are important for IL-18Rα binding, and the third site is involved in the cellular response but not in IL-18Rα binding.

Regarding the development of therapeutic antibodies, the mouse anti–human IL-18 antibody, 125-2H, was first established using cell-fusion technology since the discovery of IL-18 in 1997 (*10*). However, from the point of view of therapeutic agents, antibodies should be human, although a few humanized antibodies have recently been provided as antibody drugs. This is because mouse antibodies are conveniently prepared by the usual hybridoma technology.

Recently, we constructed a large scale of the human scFv–displaying phage library. This library was constructed using only complete human V genes amplified from IgM and IgG cDNA clones of human peripheral blood. It is a salient feature that these products have been tolerated by the human immune system and are immunologically the safest for humans.

We report here the successful establishment of an anti–human IL-18 scFv antibody, h18-108, which inhibits IL-18 signaling. The biochemical characterization of this antibody using two kinds of random peptide–displaying phage libraries showed that the binding epitope of the h18-108 scFv is directed to binding site 3 of IL-18, which is a crucial site for the association with IL-18R β. We directly confirmed this finding by demonstrating that h18-108 binds to native IL-18 but loses its binding activity to the recombinant IL-18 mutant, D98A. We successfully constructed the complete human Fab and IgG forms of h18-108, resulting in an approximately 14-fold increase of affinity at Kd in the case of Fab and a 78-fold increase for the IgG form. As these antibodies are the first complete human antibodies to human IL-18 and inhibit IL-18 signaling in accordance with their binding affinities, they may be useful not only as epitope-defined antibodies to investigate the mechanism of IL-18 signaling, but also as therapeutic reagents for human disorders involving IL-18.

MATERIALS AND METHODS

Phage Library—The human scFv–displaying M13 phage library constructed using the pCANTAB 5E (4.5 kb) phagemid vector was employed in this study as described (*11*).

Two kinds of peptide-displaying phage libraries (Ph.D-12, -C7) were purchased from New England Biolabs (Beverly, MA). The Ph.D-12 library contains linear peptides composed of 12 random amino acids. The Ph.D-C7 library contains circular random peptides of 7 amino acids constrained by a disulphide bond between two cysteines.

Biopanning—Human scFv library: Biopanning was performed as described previously (*12*, *13*). Briefly, an Immunotube (Nunc, Denmark) was coated with human IL-18 (MBL, Nagoya) at 5 μg/ml (0.1 M $NaHCO_3$, pH 8.6) for the first panning and 3 μg/ml for the second panning at 4°C overnight. The IL-18–coated tube was blocked with 0.5% gelatin for first-round selection and 5% skim milk for second-round selection. The phage library (3×10^{11} transforming units [TU]) was incubated in the IL-18–coated tube at room temperature for 1 h. The tube was washed with PBS containing 0.1% Tween 20. The phages were eluted with 1 ml of 0.1 M glycine-HCl (pH 2.2), immediately neutralized with 1 M Tris-HCl (pH 9.1) and amplified by infecting into *E. coli* TG1 cells, as described previously (*11*).

Peptide phage library: Biopanning was performed as described (*14*). Briefly, an Immunotube (Nunc, Denmark) was coated with h18-108 scFv at 5 μg/ml in 0.1 M $NaHCO_3$ (pH 8.6). The isolated phage clones were amplified by infecting into *E. coli* ER2738 cells.

Soluble scFv—The soluble scFv was prepared by infecting phage clones into *E. coli* HB2151. The scFv fragments were purified with an RPAS purification module from the supernatant or the periplasm fraction according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ). The elution was carried out with either 0.1 M glycine-HCl (pH 3.0) or 3 M KSCN. The purified scFv was analyzed by HPLC using HiLoad 16/60 Superdex (Amersham Biosciences).

The endotoxin contaminated in these preparations was measured using an Endospecy ES-50M Set (Associates of CAPE COD, Inc., Mainstreet Falmouth, MA). The h18-108 scFv contained 599 ng/ml endotoxin.

Flow Cytometry Analysis—IL-18 (100 μl at 50 μg/ml) was biotinylated by the addition of 5 μl of a 10 mM Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) solution on ice for 2 h. The anti–IL-18 mAb (mouse anti–human IL-18 antibody: 125-2H, MBL, Nagoya) recognized this preparation at a level equal to that of non-treated IL-18 by ELISA. KG-1 cells (human myelomonocytic cell line: 1×10^6 cells) were stained with biotinylated IL-18 (8 μg/ml) followed by the addition of phycoerythrin (PE)-conjugated streptavidin and analyzed with EPICS (Beckman-Coulter). To examine the inhibitory activity of scFv, biotinylated IL-18 was preincubated with scFvs at 4°C for 1 h before the flow cytometry analysis.

IL-18 Neutralizing Assay—The cells were cultured as described (*14*). The IL-18 (at a final concentration of 4 ng/ml) was pre-incubated with varying concentrations of scFv, Fab, or IgG at 25°C for 1 h, and then added to the KG-1 cell (3×10^6 cells/ml) culture. Alternatively, KG-1 cells (3×10^6 cells/ml) were stimulated with 4 ng/ml IL-18 in the absence or presence of varying concentrations of scFv, Fab, or IgG. The culture supernatants were collected 24 h later for the measurement of IFN-γ. It was confirmed that this assay was not influenced by the presence of up to 10 μg/ml of LPS contaminated with recombinant antibody preparations (data not shown).

ELISA—ELISA was performed as described (*11*, *12*). Gelatin, human AB serum, human serum albumin (Sigma, St. Louis, MO), bovine serum albumin (Sigma, St. Louis, MO), or skim milk (Becton, Dickinson, and Company, Sparks, MD) was used as a blocking agent or control protein.

Screening of IL-18–specific phage clones or scFv: An ELISA plate (Nunc, Denmark) was coated with various proteins (80 ng/40 μl/well) for 12 h at 4°C. The plates were then washed, and phage clones (8×10^{10} virions/40 μl/well) or soluble scFv (100 ng/40 μl/well) were added to each well. Phage clones were detected by a biotinylated anti-M13 monoclonal antibody (mAb, 1:1,000, Pharmacia) in combination with an alkaline-phosphatase (AP)–conjugated streptavidin (1:1,000, Vector Lab., Inc., Burlingame). Soluble scFv was detected by an anti–E-tag mAb (1:1,000, Pharmacia) in combination with an AP-conjugated anti–mouse IgG (Jackson ImmunoResearch, West Grove, PA). Absorbance was measured at 405 nm in incubation with

50 μl of a *p*-nitrophenyl phosphate/10% diethanol amine solution by use of a microplate reader (NJ-2300; Tokyo, Japan).

Cytokine ELISA: IFN-γ was measured by ELISA (Biosource, Camarillo, CA).

h18-108 epitope ELISA: This assay was performed to determine the h18-108 epitope by selecting h18-108–specific peptide phage clones. Briefly, the h18-108 scFv (100 ng/80 μl/well) was incubated in wells of anti–E tag mAb (80 ng/40 μl/well)-coated ELISA plates for 1 h. Peptide phage clones (8×10^{10} virions/40 μl/well) were added to each well for 1 h. The phage clones were detected by a biotinylated anti-M13 mAb in combination with AP-conjugated streptavidin.

Immunoblotting Analysis—The supernatant or periplasm fraction of an HB2151 culture was subjected to SDS-PAGE (12.5% gel) and the gel was blotted onto the PVDF membrane (Applied Biosystems) using a semi-dry electroblotter (Sartorius, Tokyo). After blocking with 5% skim milk/PBS, scFvs were detected by a horseradish peroxidase (HRP)–conjugated anti-Etag mAb (Pharmacia) using ECL reagents (Pharmacia) on an image analyzer LAS-1000 (Fuji Film, Tokyo). The NEB pre-stained protein marker (New England Biolabs, Beverly, MA) was used as a protein marker.

Sequencing—The DNA sequence of phages was determined by the Dye Terminator method using primer 1 (5′-CAACGTGAAAAAATTATTATTCGC-3′ for the scFv gene) or primer 2 (5′-CCCTCATAGTTAGCGTAACG-3′ for the peptide insert) and analyzed on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The nucleotide sequences were analyzed with the Genetyx-SV/R. The amino acid sequences deduced from the nucleotide data were aligned and are shown in Kabat format. The scFv nucleotide sequences were analyzed by searching the IMGT/V-QUEST database using DNAPLOT software. The complementary-determining regions (CDR1–CDR3) and flanking regions (FR1–FR4) were deduced according to Kabat *et al*.

Preparation of h18-108 Fab—The expression plasmid for the secretion of Fab was based on pTrc99A. Heavy and light chains are arranged as a dicistronic operon controlled under one trc promoter. Two pelB signal sequences were used for the secretion of two chains to the periplasm. Vh and Vl were precisely fused on their 3′ side to human IgG1 CH1 constant and kappa1 CL domains, respectively. The cysteine residues involved in disulfide bonding between the H and L chains were both mutated to serine by site-directed mutagenesis.

Vh and Vl of the h18-108 scFv gene were amplified by PCR and inserted into the Fab expression vector. The expression plasmid was transformed into *E. coli* JM83. Fab was induced with 1 mM IPTG at 30°C and purified with a HiTrap Protein G HP (Pharmacia Biotech) from the periplasm fractions. Fab construction was determined by SDS-PAGE. The binding specificity was confirmed by ELISA and BIAcore analyses.

This Fab preparation contained 433 ng/ml endotoxin.

Preparation of h18-108 IgG—A heavy-chain variable gene or light-chain variable gene of h18-108 was cloned into mammalian cell expression vector pCAG-H with a human IgG_1 constant region derived from HCMV-$V_H$lys (Ref.15: pCAG-h18-108H) or pCAG-L with human Ck derived from HCMV-$V_L$lys (Ref.15: pCAG-h18-108L). In each vector, the promoter region was replaced with the chicken β-actin promoter (*16*), and the vector was equipped with a leader sequence (EMBL J00240) and Kozak sequence (*17*). CHO cells were co-transfected with an equimolar mixture of pCAG-h18-108H and pCAG-h18-108L by lipofectamine (Invitrogen) according to the manufacturer's instructions. After 48 h of culture in a selective medium, the supernatant was removed from the cells by centrifugation and filtered through a 0.22 mm membrane. The expressed h18-108-IgG was purified from the culture supernatants immediately after harvesting by protein A affinity chromatography. The cell culture supernatant (300 ml) was passed over a 2 ml column of rProsepA (Millipore), washed with 10-times column volumes of sterilized PBS (GIBCO), and eluted with 0.1 M acetate buffer, pH 3.5. The eluate was immediately neutralized with 1 M Tris-HCl, pH 8.0, and dialyzed against sterilized PBS (GIBCO). Purified h18-108-IgG was analyzed by SDS-PAGE, and endotoxin contamination was below detectable levels, as determined using the Endospecy ES-50M Set.

Surface Plasmon Resonance (SPR) Analysis—The binding kinetics of anti–human IL-18 antibodies to human IL-18 was measured by SPR using a BIAcore 2000 instrument (Biacore, Inc., Uppsala, Sweden). Recombinant human IL-18 in 10 mM sodium acetate (pH 4.0) was immobilized on a CM5 sensor chip at a flow rate of 1 μl/min using standard EDC-NHS coupling chemistry. Applying IL-18 (5 μg/100 μl) resulted in the immobilization of approximately 800 resonance units. Unreacted sites were masked with 1 M ethanolamine-HCl (pH 8.5). All experiments involving kinetic measurements of antibodies were conducted in HBS buffer (10 mM HEPES, pH 7.4 containing 0.15 M NaCl, 3 mM EDTA, and 0.005% Tween 20) at a flow rate of 30 μl/min. At the end of the cycle, the sensor chip surface was regenerated with 0.1 M glycine-HCl buffer (pH 2.5). The binding ability of the IL-18–immobilized chip was confirmed by estimating the RU value of an authentic murine anti–IL-18 antibody (125-2H) by BIAcore analysis. The association (k_{on}) and dissociation (k_{off}) rate constants were calculated by nonlinear fitting of the primary sensorgram data using BIAevaluation ver. 3.2 software (BIAcore, Inc., Uppsala, Sweden). The affinity constant, K_d, was calculated from the dissociation rate (k_{off})/association rate (k_{on}) ratio.

Computer Simulation—Modeling of the IL-18 and IL-18Rα complexes was based on both the PDB entry 1J0S (IL-18) and the PDB entry 1ITB [crystal structure of the IL-1β/IL-1RI complex (*18*)]. The Homology and Discover modules of Insight II were used for model construction and structure verification.

RESULTS

Human Single-Chain Fv Clones Specific to Human IL18—Human IL-18–specific scFv-phage clones were selected from the scFv-phage libraries. As we intended to select phage clones with varying affinities, two rounds of biopanning were carried out. The 108 clones were tested by ELISA. We selected three clones that showed fine specificity to IL-18 (Fig. 1). These phage clones bound to IL-18 but showed little binding to gelatin, human AB serum, HSA, BSA, or skim milk.

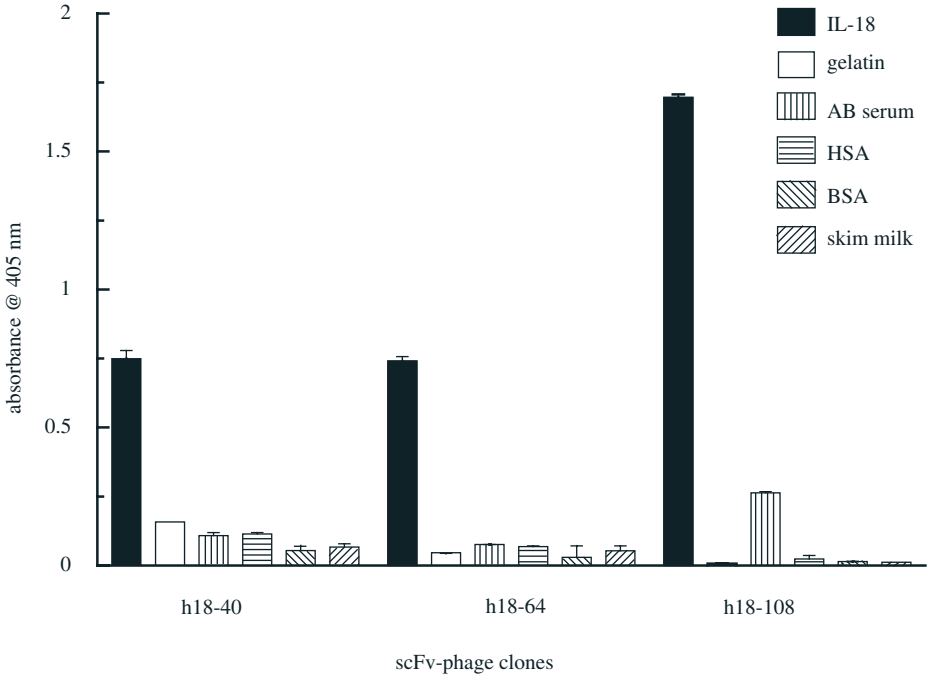


Fig. 1. **IL-18 binding activity of scFv-phage clones.** ELISA was performed using plates coated with IL-18, gelatin, AB serum, HSA, BSA, or skim milk (80 ng/40 μl/well) in the presence of each phage clone (8×10^{10} virions/40 μl/well) as described in "MATERIALS AND METHODS."

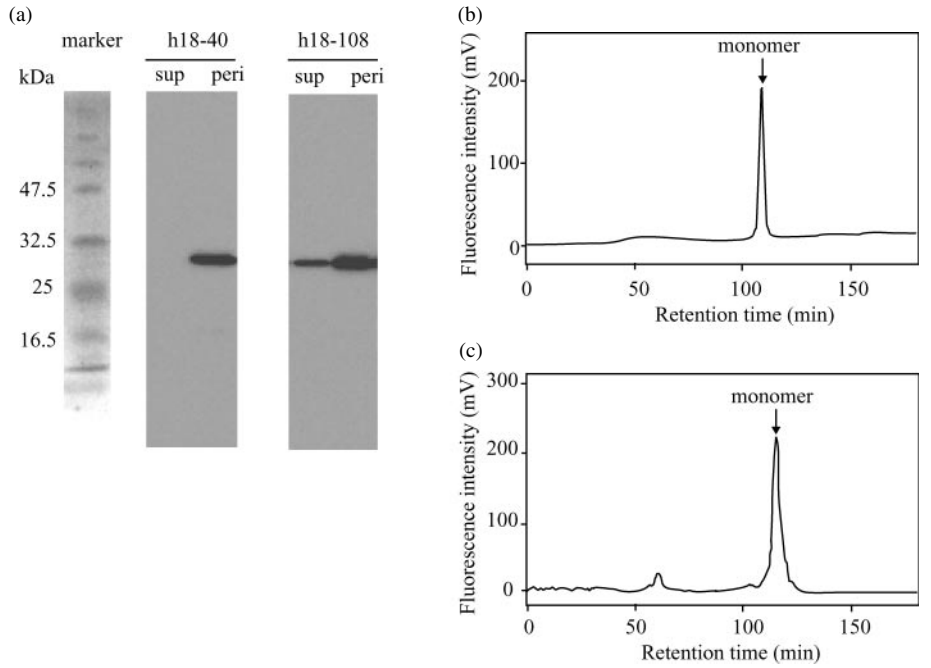


Fig. 2. **Characterization of soluble scFvs.** a: SDS-PAGE: The supernatant (sup) and periplasm (peri) fraction of an HB2151 cell culture infected with the indicated phage clone were subjected to 12.5% gel SDS-PAGE under reducing conditions. After electroblotting, the scFvs were detected with anti–E tag mAb. b and c: gel-permeation chromatography (HiLoad16/60) of h18-108 and h18-40, respectively.

The soluble scFvs were induced by IPTG after each phage clone was infected into *E. coli* HB2151. The supernatant or periplasmic fraction of each clone was electrophoresed in an SDS–polyacrylamide gel (12.5%) under reducing conditions and immunoblotted with an HRP-conjugated anti-E tag mAb. This analysis showed that the h18-40 scFv was expressed in the periplasmic fraction, while the h18-108 scFv was expressed in the supernatant and periplasmic fractions. For unknown reasons, the h18-64 scFv was not detected in any fraction. Both h18-40 and h18-108 were resolved at approximately 27–28 kDa (Fig. 2a). The h18-108 scFv was purified using an RPAS purification module with an elution buffer of 0.1 M glycine-HCl (pH 3.0), while the h18-40 scFv was recovered by elution with 3 M KSCN. In agreement with these results, gel-permeation chromatography showed that the h18-108 scFv was resolved in a monomer form, whereas the h18-40 scFv appeared to be a mixture of the monomer and aggregate forms (Fig. 2, b and c). Both purified scFvs showed fine binding specificity to IL-18 (Fig. 3). The scFv gene sequences of the selected phage clones were examined (Fig. 4, a and b). The germ-line Vh and Vl genes were assigned based on homology to a database (IMGT) of germ-line V genes compiled by Lefranc *et al.* (*19*). The h18-40 scFv and the h18-64 scFv shared an

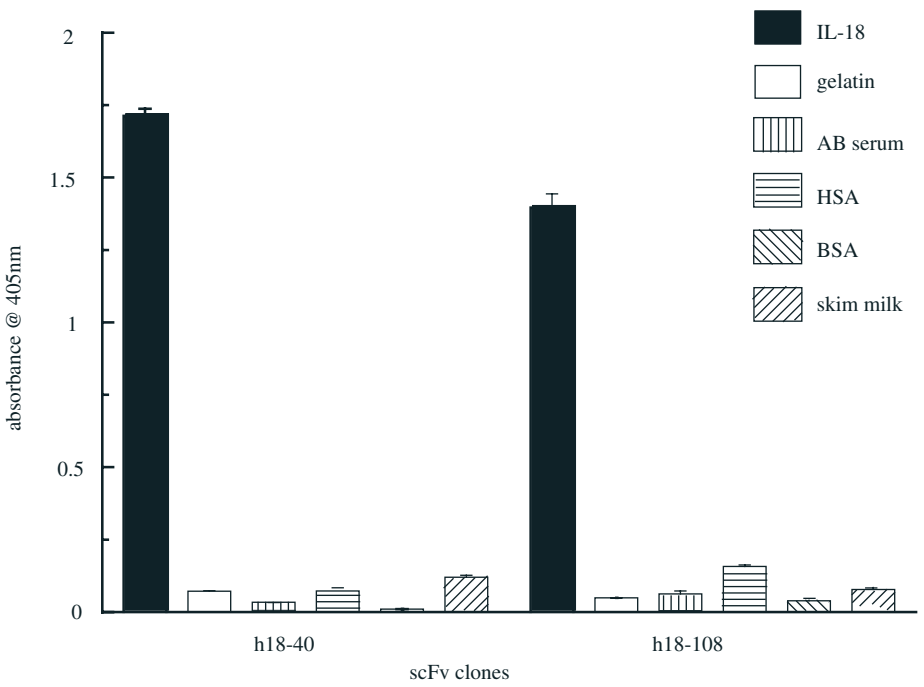


Fig. 3. **IL-18–binding activity of soluble scFvs.** ELISA was performed using plates coated with IL-18, gelatin, AB serum, HSA, BSA, or skim milk (80 ng/40 μl/well) in the presence of purified h18-108 or h18-40 scFv (100 ng/40 μl/well) as described in "MATERIALS AND METHODS."

(a) Amino acid sequences of the VH domains

	FR1	CDR1	FR2	CDR2
h18-40	QVQLQQSGPGLVTPSQTLSLTCAISGDSVS	**SNRVTWN**	FIRQSPSRGLEWLG	**RTYYRSKWYNDF**
h18-64	QVQLQQSGPGLVNPSQTLSLTCAISGDTVF	**TSSATWN**	WIRQSPSRGLEWLG	**RTYYRSKWYNDY**
h18-108	QVQLVQSGAEVRRPGASVRVSCKASGYTFT	**SHYIH--**	WVRQAPGQGLEWVA	**IINP-SDGRTDY**

	FR3	CDR3	FR4
h18-40	**AVSVKS** RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	**DDSISWPPAPFDY**	WGQRTLVTVS
h18-64	**ALSVKS** RITINPDTSKNQFSLHPNSVTPEDTAVYYCAR	**DLASAGYF---DY**	WGQGTLVTLS
h18-108	**AQKFQG** RVTVTRDTSASSVYMGISSLRSEDTAMYYCAR	**TARGFSYA---TD**	WGQGTLVTVS

(b) Amino acid sequences of the VL domains

	FR1	CDR1	FR2	CDR2	
h18-40	ETTLTQSPSSVSASVGDRVTITC	**RASQ-DISNSLA**	WYHQEPGKAPKLLIY	**SVSTLES**	GVPSR
h18-64	EIVLTQSPDTLSLSPGERATLSC	**RASQTLTSNSVA**	WFQQKRGQPPSLLMY	**GTSSRAT**	GIPDR
h18-108	SYELTQPPS-VSVSPGQTARITC	**SGDA-LPKKYAY**	WYQQKPGQAPVLVIY	**KDSERPS**	GIPER

	FR3	CDR3	FR4
h18-40	FSGSGSGADFALTISNLQPEDFGTYYC	**QQAKSFP--LT**	FGQGTKLEIK---
h18-64	FSGSGSGTDFTLTISRLEPEDFAVYYC	**QQYGYSPG-LT**	FGGGTKVDIK---
h18-108	FSGSSSGTTVTLTISGVQAEDEADYYC	**QSADSSGTYVV**	FGGGTQLTVL---

(c) Variable gene usage

	Heavy chain			Light chain	
clone #	V	D	J	V	J
h18-40	IGHV6-1*01	IGHD6-13*01	IGHJ4*02	IGKV1-12*01	IGKJ2*01
h18-64	IGHV6-1*01	IGHD6-13*01	IGHJ4*03	IGKV3-20*01	IGKJ4*01
h18-108	IGHV1-46*03	IGHD5-5*01	IGHJ4*02	IGLV3-25*03	IGLJ3*01

Fig. 4. **Amino acid sequences and gene usage of anti–human IL-18 scFvs.** Sequencing was performed by the Dye Terminator method. The amino acid sequences deduced from the nucleotide data were aligned and are shown in the Kabat format. The scFv nucleotide sequences were analyzed by searching the IMGT/V-QUEST database using DNAPLOT software. The regions of CDR1-CDR3 and FR1-FR4 were deduced according to Kabat *et al*.

identical V-D segment. The h18-108 scFv was unique in Vh and Vl usage.

h18-108 Inhibits the IFN-γ Production of KG-1 Cells Stimulated with Human IL-18—KG-1 cells produce IFN-γ by the stimulation with IL-18 (*10*). To evaluate the inhibitory activity of these scFvs for IL-18 signaling, KG-1 cells were stimulated with IL-18 preincubated with varying concentrations of scFvs. The amount of IFN-γ in the culture supernatant was determined by ELISA 24 h later. As shown in Fig. 5, h18-108 showed 50% inhibition of IFN-γ production at 10 μg/ml and complete inhibition at 43 μg/ml. On the other hand, the h18-40 scFv showed very weak inhibition at the concentrations examined. The unrelated scFv M36 showed no inhibition. These results suggested that the h18-108 scFv might inhibit the binding of IL-18 to IL-18 receptors expressing on KG-1 cells.

Flow Cytometry Analysis: h18-108 Inhibits the Binding of IL-18 to KG-1 Cells—KG-1 cells were incubated with

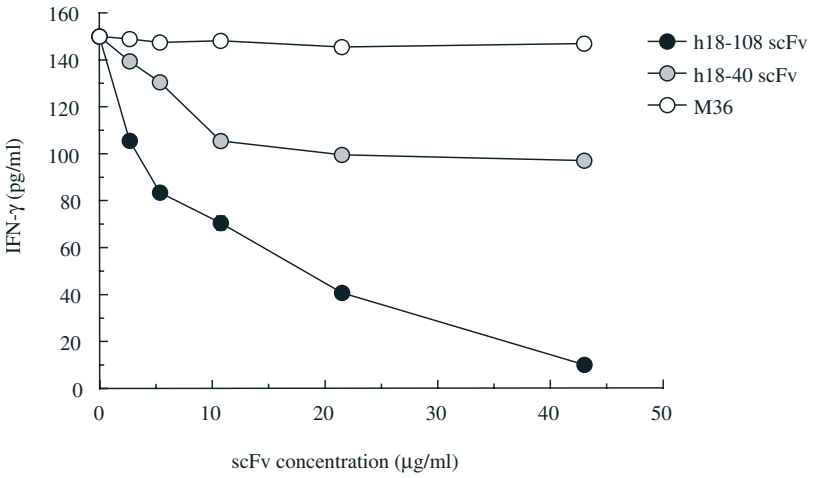


Fig. 5. **The h18-108 scFv inhibits IFN-γ production by KG-1 cells stimulated with IL-18.** IL-18 (at a final concentration of 4 ng/ml) was pre-incubated with varying concentrations of scFvs at 25°C for 1 h and then added to a KG-1 cell (3×10^6 cells/ml) culture. Twenty-four hours later, IFN-γ in the culture supernatant was measured by ELISA. M36: An irrelevant scFv. It was confirmed that this assay was not influenced by the presence of up to 10 μg/ml of LPS (data not shown). The E-tag column-purified scFv was used in this assay.

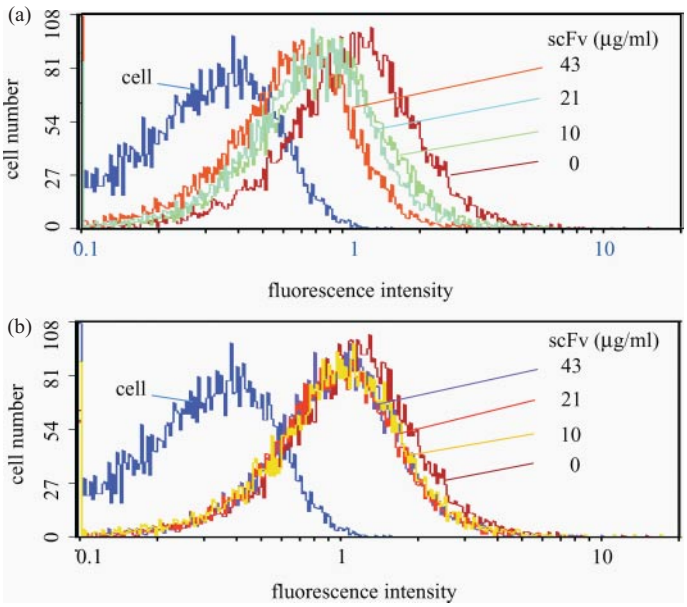


Fig. 6. **The h18-108 scFv inhibits IL-18 binding to KG-1 cells.** KG-1 cells (1×10^6 cells/50 μl) were incubated with biotinylated IL-18 (8 μg/ml), which was pre-incubated with varying concentrations of scFvs for 30 min. at 4°C. IL-18–binding KG-1 cells were stained with PE-conjugated streptavidin and analyzed by flow cytometry. a: h18-108; b: M36: An irrelevant scFv.

biotinylated IL-18 in the presence of varying concentrations of scFvs. IL-18 binding was monitored with PE-conjugated streptavidin by an EPICS flow cytometer. As shown in Fig. 6, h18-108 inhibited the binding of IL-18 to KG-1 cells in a dose-dependent manner, and showed partial inhibition at 43 μg/ml of scFv. The unrelated M36 scFv had no influence on the binding of IL-18 to KG-1 cells.

Epitope Mapping of h18-108 scFv—In order to investigate the inhibitory mechanism of h18-108 scFv on IL-18 signaling, the binding epitope of h18-108 scFv was determined using two kinds of random peptide-displaying phage libraries, Ph.D-12 and Ph.D-C7. We isolated four clones from the Ph.D-12 library and three clones from the Ph.D-C7 library. These clones specifically bound to the h18-108 scFv (Fig. 7). We determined the DNA sequences of these clones for the elucidation of the deduced amino acid sequences, and searched their homologous regions with IL-18 using CLUSTAL W ver. 3.1 (Fig. 8a). All sequences, including four clones from the Ph.D-12 library and three clones from the Ph.D-C7 library, consistently showed homology in the sequence region from # 95–99 of the mature form (# 131 to # 135 of the immature form). Particularly, it is of note that these sequences share K S/T D at # 96–98 (# 132–134 of the immature form) of IL-18. This epitope is mapped in the region of binding site 3 of IL-18, which has been suggested by Kato *et al*. to be an important region for the association with IL-18Rβ. These results suggest the importance of Asp98 of IL-18 for the recognition of h18-108.

To demonstrate directly the involvement of Asp98 in the binding epitope of h18-108, we tested the binding activity of h18-108 to the IL-18 mutant, D98A. As shown in Fig. 9, h18-108 completely lost binding activity to D98A, whereas it retained binding activity to wild-type IL-18. The murine anti–IL-18 mAb, 125-2H, which recognizes the conformation of IL-18 (*20*), bound to both wild-type IL-18 and D98A, indicating that D98A was refolded into correct conformation. These results demonstrated that the h18-108 binds

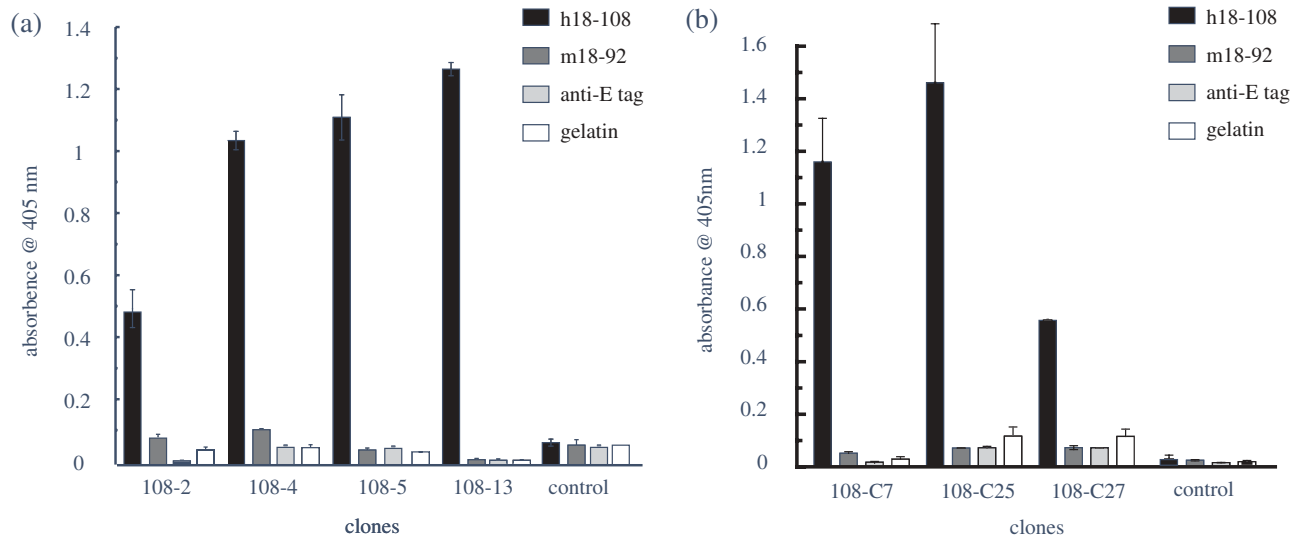


Fig. 7. **Random peptide–displaying phage clones specifically recognized by the h18-108 scFv.** a: Ph.D-12 clones. b: Ph.D-C7 clones. The ELISA plate was coated with or without anti–E tag mAb (80 ng/40 μl/well) to capture the h18-108 scFv (100 ng/80 μl/well). Gelatin was used as a blocking agent. The phage clones (8×10^{10} virions/40 μl/well) were added to these wells. The binding phage clones were detected by biotinylated anti-M13 mAb in combination with AP-conjugated streptavidin. Control: m18-92 as an irrelevant clone.

(a)

```
          1                                                          60
hIL-18    YFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDSDCRDNAPRTIFIISMYKDSQPRGM
108-2     ------------------------------------------------------------
108-4     ------------------------------------------------------------
108-5     ------------------------------------------------------------
108-13    ------------------------------------------------------------
108-C7    ------------------------------------------------------------
108-C25   ------------------------------------------------------------
108-C27   ------------------------------------------------------------
125-2H    ------------------------------------------------------------

          61                                                        120
hIL-18    AVTISVKCEKISTLSCENKIISFKEMNPPDNIKDTKSDIIFFQRSVPGHDNKMQFESSSY
108-2     ---------------------------------NKTDFLPCARFA--------------
108-4     -------------------------------YKSTNKTDYLFS----------------
108-5     ---------------------------------MKSDLASGSFLS--------------
108-13    ---------------------------------NTKTDINWNYVS--------------
108-C7    ---------------------------------CKSDYRTTC-----------------
108-C25   ---------------------------------CKSDLWSSC-----------------
108-C27   ---------------------------------CKSDCGRSC-----------------
125-2H    ------------------------------------------------------------

          121                                157
hIL-18    EGYFLACEKERDLFKLILKKEDELGDRSIMFTVQNED
108-2     -------------------------------------
108-4     -------------------------------------
108-5     -------------------------------------
108-13    -------------------------------------
108-C7    -------------------------------------
108-C25   -------------------------------------
108-C27   -------------------------------------
125-2H    -------------------**********----------
```

(b)

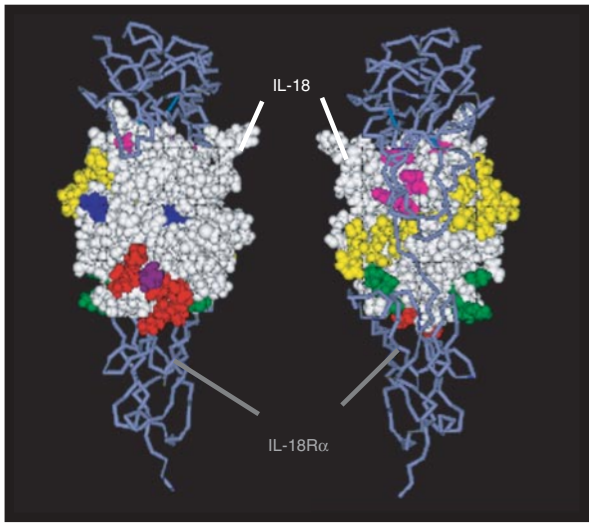


Fig. 8. **Epitope mapping.** a: Amino acid sequences of the human IL-18 and h18-108 scFv epitopes. The h18-108 scFv epitopes were determined by isolation of random-peptide phage clones. Clones 108-2, 108-4, 108-5, and 108-13 are derived from the Ph.D-12 library. Clones 108-C7, 108-C25, and 108-C27 are derived from the Ph.D-C7 library. The highly homologous region between the IL-18 and scFv epitopes is marked by a red box according to CLUSTAL W ver. 3.1. The partial epitope of 125-2H is indicated by asterisks (*20*). b: The computer simulation of the IL-18/IL-18Rα complex was performed on the basis of the crystal structure of the IL-1β/IL-1RI complex (PDB # 1ITB) using FAMS and Discovery. The right figure is a view from the opposite side of the left figure. The h18-108 epitope is shown in red. Asp98 is shown in purple. The binding regions of IL-18 to its receptors are indicated in pink (Site 1), green (Site 2), and blue (Site 3). The epitope regions of 125-2H mAb are shown in yellow.

to IL-18–binding site 3, including Asp98, resulting in the inhibition of IL-18 signaling.

As the h18-108 scFv showed inhibitory activity on IL-18 signaling, we constructed the Fab and IgG form by recloning the Vh and Vl fragments by PCR as described in "MATERIALS AND METHODS." We confirmed their binding specificities to be identical to the original scFv by ELISA and BIAcore (Fig. 10).

SPR Analysis—To characterize the binding affinity of the h18-108 scFv, Fab, or IgG form, SPR analysis was performed by BIAcore. The human IL-18 was immobilized onto the sensor chip CM5, and varying concentrations of analytes were injected. All of the antibody forms, scFv, Fab, and IgG of h18-108, were purified in monomer forms. The responses were dose-dependent in each antibody (Fig. 10a). The binding parameters are summarized in comparison with the results of murine anti–IL-18 IgG, 125-2H (Fig. 10b). The association rate of h18-108 Fab (18×10^4 M^{-1} s^{-1}) was approximately 6.4-fold faster than that for the scFv (2.8×10^4 M^{-1} s^{-1}), resulting in an approximately 14-fold increase in K_d. It is of note that the dissociation rate of the h18-108 IgG form (16×10^{-5} s^{-1}) was approximately 4.3-fold slower than that of the Fab form (68×10^{-5} s^{-1}), resulting in an approximately 78-fold increase in K_d in comparison with scFv. In the case of the Fab form, the interaction with CH1 and Cκ stabilize the conformation, which leads to an increase in the affinity in comparison with scFv. Furthermore, the IgG form possesses a disulfide bond in the hinge region of the CH1 domain. These factors may give the marked influence on the stability and the binding ability of the whole IgG molecule, resulting in a relatively prominent decrease in the K_d value. Thus, the K_d value of h18-108 IgG was improved by approximately 2.8-fold over that of the 125-2H murine monoclonal IgG.

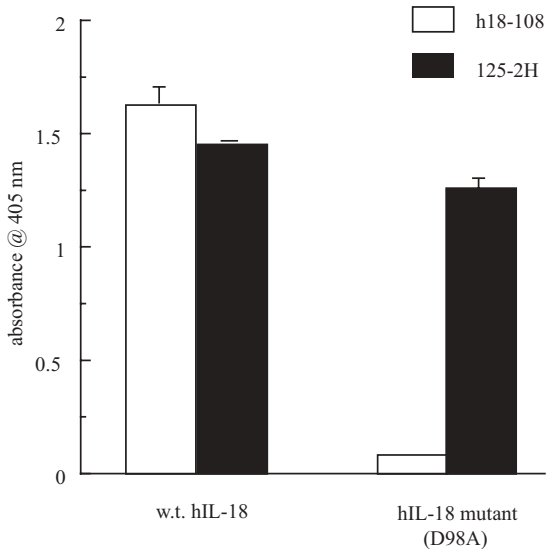
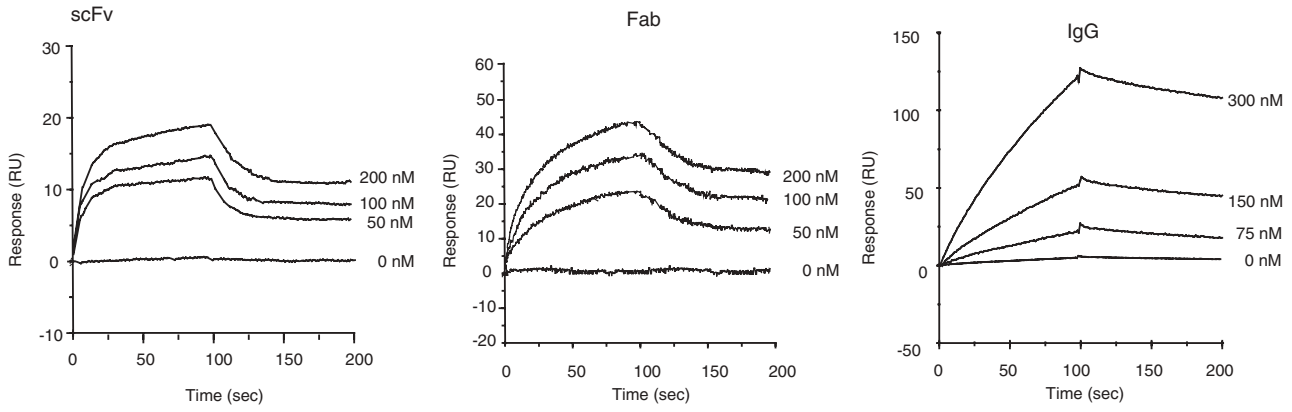


Fig. 9. **The h18-108 scFv recognizes the determinant of binding site 3 of IL-18 composed of Asp98.** The reactivity of wild-type IL-18 or an IL-18 mutant (D98A: 60 ng/40 μl/well) with either the h18-108 scFv or 125-2H mAb (80 ng/40 μl/well) was examined by ELISA as described in "MATERIALS AND METHODS." The 125-2H mAb [(*18*): MBL Co., Ltd., Nagoya] was used to confirm the correct conformation of IL-18 (D98A).

IC50 of the IL-18–Signaling–Inhibitory Activity of Various Antibody Forms of h18-108—To compare the IL-18–signal inhibitory activity of h18-108 Fab and IgG with scFv, the IFN-γ production of KG-1 cells was assayed in the presence of varying doses of these antibodies.

As shown in Fig. 11a and b, the IC_{50} values of scFv, Fab, and IgG were estimated to be 200 nM, 40 nM, and 5 nM, respectively. Thus, antibody engineering of scFv towards the Fab or IgG form was accomplished by increasing

(a)



(b)

	kon(x 10^4 $M^{-1}s^{-1}$)	koff (x $10^{-4}s^{-1}$)	Kd (nM)
scFv	2.8	14	50
Fab	18	6.8	3.7
IgG	25	1.6	0.64
125-2H	2.6	0.46	1.8

Fig. 10. **BIAcore analysis of h18-108.** IL-18 was immobilized onto the sensor chip CM5, and the kinetic parameters were evaluated by BIA evaluation 3.2 as described in "MATERIALS AND METHODS." a: Sensorgram of h18-108 scFv, Fab, and IgG forms. b: Kinetic parameters of anti–IL-18 antibodies, h18-108 scFv, Fab, IgG forms, and 125-2H mAb.

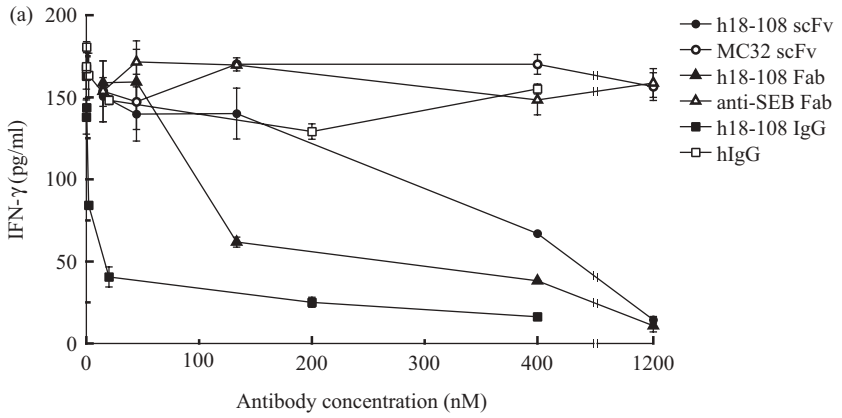


Fig. 11. **Inhibitory activity of the h18-108 Fab and IgG forms on IL-18 signaling.** KG-1 cells (3.6×10^5 cells/well) were stimulated with IL-18 (4 ng/ml) in the presence or absence of varying concentrations of h18-108 scFv, Fab, or IgG for 24 h. IFN-γ in the culture supernatant was measured by ELISA. a: MC32 scFv, anti–staphylococcus enterotoxin B (SEB) Fab, and IgG were used as negative controls. b: The IC50 values were derived from (a). As this assay was not influenced by the presence of up to 10 μg/ml of LPS, the E-tag column–purified scFv or Fab was used in this assay. The IgG form contained endotoxin below the detectable level of the endotoxin-measuring kit, as described in "MATERIALS AND METHODS."

(b)

	h18-108 scFv	h18-108 Fab	h18-108 IgG
IC50	250 nM	100 nM	5 nM

the IL-18–signaling–inhibitory activity according to the improvement in their binding affinities.

DISCUSSION

Until now, a rat antibody (TC30-28E3: anti–IL-18Rβ mAb) and a murine antibody (125-2H: anti–IL-18 mAb) have been reported as inhibitory reagents of IL-18 signaling (*8*, *10*). In this study, we established human scFv antibodies specific to human IL-18 using a naïve human antibody–displaying phage library. Among these antibodies, h18-108 showed a marked neutralizing activity in spite of its monovalency, inhibiting the production of IFN-γ by KG-1 cells stimulated with IL-18.

Recently, receptor binding and cellular response analyses using IL-18 mutants by Kato *et al*. revealed the presence of three sites. Two of these are important for IL-18Rα binding, and the third is involved in the cellular response but not in IL-18Rα binding (*9*). In their experiments using BIAcore analysis, they showed that IL-18Rβ was unable to bind on its own to either IL-18 or IL-18Rα. In contrast, in the presence of IL-18, IL-18Rβ was capable of binding to IL-18Rα, showing that IL-18Rβ associates with the IL-18/IL-18Rα complex to form a ternary complex. This region is postulated to be a binding site of site 3.

In the h18-108 epitope-mapping experiments, all selected sequences, including four clones from the Ph.D-12 library and three clones from the Ph.D-C7 library, showed homology with residues 96–98 (KSD) of human IL-18. This region belongs to binding site 3, according to the analysis by Kato. Employing an IL-18 mutant, D98A, it was confirmed that the h18-108 scFv recognizes the determinant of binding site 3 of IL-18 composed of Asp98 (Fig. 9).

The h18-108 scFv showed partial inhibition in the binding of IL-18 to the IL-18R complex of KG-1 cells at 43 μg/ml (1.7 μM: Fig. 6). On the other hand, the h18-108 at 43 μg/ml almost completely inhibited the production of IFN-γ by KG-1 cells (Fig. 5). These results suggested that the h18-108 scFv might allow the low-affinity interaction of IL-18 with IL-18Rα but block the association of IL-18 with IL-18Rβ, resulting in the inhibition of IL-18 signaling. Indeed, this might be due to the experimental conditions, *i.e*., flow cytometry analysis needed 8 μg/ml IL-18 to visualize the IL-18 binding to the IL-18 receptor complex, while IFN-γ production by KG-1 cells required 4 ng/ml of IL-18. Inevitably, higher concentrations of inhibitors (anti-IL18 antibodies) are required for flow cytometry analysis than for the KG-1 assay. According to the data in Fig. 11, the IC_{50} of the h18-108 scFv is estimated to be 250 nM (5 μg/ml). Considering the relatively strong inhibitory activity of h18-108 scFv in spite of its binding affinity with 10^{-8} order K_d, the association of IL-18Rβ with the IL18/IL-18Rα complex might be highly sensitive to binding inhibitors in transducing IL-18 signaling. In the case of the h18-108 Fab or IgG forms, their IC_{50} values appear to be greatly influenced by the stability of their molecular structures in addition to their K_d values.

When the h18-108 human IgG is compared with the 125-2H murine antibody, a contrasting feature is noted. The h18-108 IgG has a k_{on} value indicating 9.6-faster association than 125-2H and a k_{off} value indicating 3.4-faster dissociation than 125-2H, resulting in almost the same K_d values for both. However, when the inhibitory activity of these antibodies on IFN-γ production was compared, 125-2H showed strong activity with IC_{50} = 0.01 nM (data not shown), which is 500 times higher than that of h18-108 IgG, IC_{50} = 5 nM (Fig. 11). This marked difference can not be simply explained by the binding affinity. Previous attempts to map the 125-2H epitope using overlapping peptides were unsuccessful, suggesting that 125-2H may be against a conformational epitope of human IL-18 (*20*). The tertiary structure of IL-18 for 125-2H recognition might be critical for IL-18–signal transduction.

Although 125-2H is a murine antibody, the h18-108 IgG is a complete human antibody with an IC_{50} of 5 nM (Fig. 11) and a Kd of 0.64 nM (Fig. 10). The h18-108 epitope was also defined as specific to IL-18–binding site 3, as shown in Fig. 8. Taking these results together, the h18-108 antibody may be useful for both therapeutic applications for

IL-18–involving allergic or autoimmune disorders and basic research on the ligand–receptor interaction on IL-18 signal transduction.

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