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Molecular Characterization and Functional Activity of an IL-15 Antagonist MutIL-15/Fc Human Fusion Protein

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Abstract

Fc fusion proteins are a new emerging class of molecules for immune-targeted delivery of therapeutic proteins. Biophysical and bioanalytical characterization is critical for clinical development and delivery of therapeutic proteins. Here we report molecular and functional characterization of a recombinant human fusion protein Mutant IL-15/Fc. MutIL-15/Fc has a molecular weight of ~95kDa as determined by multi-angle laser light scattering with online size exclusion chromatography and migrated at a faster rate (lower retention time) in gel filtration column. The kinetics of binding of MutIL-15/Fc to Fc γ receptor is best fitted in a bivalent modal with K_{D1} 5 μ M and K_{D2} 9 μ M determined by surface plasmon resonance (BIAcore®). N-glycoprofiling analysis revealed extensive glycosylation of MutIL-15/Fc. The Fc and IL-15 components in the MutIL-15/Fc are detected using the dual mode ELISA. The HT-2 cell proliferation inhibition assay is qualified as a quantitative *in vitro* marker functional assay. Molecular state changes associated with forced stress analyzed by SEC-MALS resulted in changes in bioactivity and Fc:Fc γ receptor interaction affinity. These data provide a systematic approach to molecular and functional characterization of the MutIL-15/Fc to establish product consistency and stability monitoring during storage and under drug delivery conditions.

Keywords

fusion protein; bioactivity; SEC-MALS; N-glycoprofiling; Fc receptor binding; surface plasmon resonance

INTRODUCTION

Therapeutic fusion proteins are constructed by splicing two or more proteins or protein domains to obtain new non-natural polypeptides with the combined functionalities of the parent proteins. Therapeutic fusion proteins may exhibit advantages such as connecting

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desired but previously non-coupled pathways, increasing half-life, and increasing biological activity.^{1,2} MutIL-15/Fc is such a therapeutic cytokine/IgG fusion protein, in which a mutated form of interleukin (IL)-15 (MutIL-15) is fused with the Fc moiety of human IgG1. Protein stability and methods to characterize and predict stability are of major interest for pharmaceutical development, formulation and drug delivery studies of therapeutic fusion proteins.³

The Fc-based cytokines have been used as biotherapeutic agents to modulate inflammatory and immune responses.⁴ IL-15, as an important pro-inflammatory cytokine, stimulates the activation, proliferation, survival, and effector functions of a variety of immune cells.^{5,6} It is notable that increased IL-15 gene expression is evident during allograft rejection and in a variety of autoimmune disorders. In rheumatoid arthritis, an IL-15 blockade or neutralization is a very attractive strategy for treatment. A mutant IL-15 created through subtle changes at the C-terminus was shown to function as a high affinity IL-15R α -specific antagonist.⁷⁻¹¹ MutIL-15/Fc also showed Fc-related cytotoxic potential against IL-15R α expressing cells upon allograft survival.^{8,12} MutIL-15/Fc (γ 2a) fusion protein was also suggested as a promising tool capable of blocking transplant rejection.⁹ The MutIL-15/Fc fusion protein exhibited antagonism of IL-15 stimulation of cytotoxic T cells. It has been demonstrated that the “power mix” regimen of combined administration of rapamycin and agonist IL-2 and antagonist IL-15/Fc-related cytotoxic fusion proteins provides long-term engraftment/tolerance in exceptionally stringent mouse allogeneic models.^{11,12} This regimen is unique in its ability to enable long-term engraftment of allogeneic islets into autoimmune and overtly diabetic non-obese diabetic mice.^{11,12} However, the molecular characterization of this newly designed lytic IL-15 antagonist and its relationship to bioactivity and Fc: Fc γ receptor interaction affinities are not clear.

The interaction between the Fc region of Ig molecules and Fc receptors (FcR) is one of the major signaling pathways in adaptive immunity. Three major classes of human Fc γ Rs have been identified and studied intensively.^{13,14} The high-affinity receptor, Fc γ RI, and low-affinity receptors, Fc γ RII and Fc γ RIII, bind to IgG with dissociation constants in ranges of 0.1–10 nM and 0.1–10 μ M, respectively.¹³⁻¹⁶ Since pre-clinical studies have shown that besides the antagonism for IL-15 mediated cellular effect, the Fc portion of MutIL-15/Fc fusion protein may also contribute to the overall efficacy of the molecule *in vivo*, as well as in the immunomodulatory effect,¹⁰ it is interesting to investigate how MutIL-15/Fc interacts with the Fc receptor.

Recombinant proteins are often unstable, aggregate, and/or do not reach the fully native conformation compatible with proper biological activity.¹⁷ Aggregates within therapeutic protein formulations are a safety concern because of their potential for immunogenicity and altered efficacy in patients. Aggregation of therapeutic proteins is costly, requiring additional recovery and purification steps, reducing production yields and shortening shelf life. The phenomenon of protein aggregation is a common issue that compromises the quality, safety, and efficacy of antibodies and can happen at different steps of the manufacturing process, including fermentation, purification, final formulation, and storage. Aggregate levels in drug substance and final drug product are a key factor when assessing quality attributes of the molecule, since aggregation might impact biological activity of the biopharmaceutical.¹⁸ Therefore, the insight into the relationship between molecular characteristics, aggregation and bioactivity is necessary for evaluating stability and functions of MutIL-15/Fc.

Glycosylation can influence a variety of physiological processes at both the cellular (e.g., intracellular targeting) and protein levels¹⁹ (e.g., protein–protein binding, protein molecular stability). Recombinant MutIL-15/Fc expressed in CHO cells is known to be glycosylated at

the Fc and the cytokine domains. Glycosylation of Fc engineered proteins plays a significant role in the binding of the Fc domain to the receptor Fc γ RIIIa that subsequently results in the Fc-mediated antibody-dependent cell cytotoxicity.¹⁹ Human IL-15 is also known to be highly glycosylated and has a large degree of heterogeneity. It is also known that glycosylation affects IL-15 clearance rates in mice. Consequently, glycosylation plays a key role in the biological activity of both Human IL-2/Fc and MutIL-15/Fc. Therefore, understanding and controlling the glycoforms produced and purified are critical for successful delivery of IL-2/Fc and MutIL-15/Fc therapeutic proteins.

Here we report the characterization of MutIL-15/Fc fusion protein by SEC-MALS, Fc γ RIIIa interaction by surface plasmon resonance, N-glycosylation analysis, and describe the development of an HT-2 cell proliferation inhibition assay to analyze the *in vitro* biological activity of MutIL-15/Fc. The HT-2 cell proliferation assay is optimized and qualified to meet the requirement for a product release and stability monitoring assay for early-phase clinical investigations. Stress studies are conducted to force molecular aggregation or other changes, and the stressed samples are analyzed using SEC-MALS, BIAcore, and bioactivity assays.

MATERIALS AND METHODS

Materials

Human MutIL-15/Fc and IL-2/Fc fusion proteins were produced by the Biopharmaceutical Development Program (BDP) of the Biological Resources Branch, Frederick National Laboratory for Cancer Research. Mutant IL-15/Fc and IL-2/Fc expression plasmids were obtained from Dr. Terry Strom and Dr. Xin Xiao Zheng at the Beth Israel Deaconess Medical Center. Both plasmids were slightly modified to replace the original selection marker beta-lactamase gene with a chloramphenicol acetyltransferase gene. The fusion proteins were expressed in a CHO cell line transfected with either the modified MutIL-15/Fc or IL-2/Fc plasmid and purified by protein A and ion-exchange chromatographic separations (Manuscript under preparation). For stress studies, MutIL-15/Fc (in a solution of 10 mM sodium citrate, 150 mM NaCl, pH 7.0) was adjusted to pH 10.0 (high pH) for 12 h or heated at 55 °C for 8–12 h, or at 70 °C for 3 h, and at 70 °C for 6 h, respectively.

Analytical size exclusion chromatographic columns, G3000SW_{XL} column and a TSKgel SW_{XL} guard column, were from Tosoh Bioscience LLC. Carboxymethyl dextran chip (GE Healthcare); Amine coupling reagents NHS and EDC (GE Healthcare); ethanolamine (GE Healthcare); HBS-EP buffer (0.01 M HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 [GE Healthcare]); Fc γ RIIIa receptor (R&D Systems); 10 M sodium acetate, pH 5.5 (immobilization buffer from GE Healthcare); 0.5% SDS (from Lonza); N-glyco profiling reagents (fetuin, 2-amino benzoic acid, di-sialylated, core-fucosylated bi-antennary complex type N-glycan [A2F], mono-sialylated bi-antennary complex-type N-glycan [A1], and asialo-bi-antennary complex-type N-glycan [NA2]) were obtained from QA-Bio, LLC. Analytical HPLC column Asahipak-NH2P-50 2D column and Asahipak 5S NH2P-50 0A guard column were obtained from Phenomenex. For chromatographic separation, a G3000SW_{XL} column and TSKgel SW_{XL} guard column (Tosoh Bioscience LLC, Japan) were used. The mobile phase for the isocratic SEC runs was 5.1 mM potassium phosphate, 15 mM sodium phosphate, 450 mM sodium chloride, pH 7.4. For system suitability checks, Gel Filtration Standards (Bio-Rad, CA, USA) and an Albumin Standard (Thermo-Scientific, IL, USA) were used. SEC-column calibration markers for MW estimation from column retention time were obtained from Biorad (Cat#151-1901). The calibration kit contained Thyroglobulin (670 kDa), Gamma Globulin (158 kDa), Ovalbumin (44 kDa), Myoglobin (17 kDa) and Vitamin B₁₂ (1.35 kDa). CellTiter96® Aqueous One Solution was obtained from Promega, WI, USA.

Cell and cell culture

HT-2 cells (IL-2/IL-15 dependent) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 200 U/mL IL-2 (Hoffmann-La Roche, NJ, USA).

Cell proliferation inhibition assay

The cells were harvested in their logarithmic phase and washed two times with the initial volume of Hank's buffered salt solution (1000 rpm, 5 min) and incubated them for 4 h in assay medium (RPMI-1640 supplement with 10% FBS without IL-2) in the CO₂ incubator. During this period, a 96-well tissue culture plate was set up. Reference lot and test samples were diluted to an initial concentration of 2,000 ng/mL, followed by serial twofold dilutions, and added to the wells in 100 µL of the assay medium containing 0.6 ng/mL rHuIL-15 (rHuIL-15 produced in E.coli was provided by BDP) in triplicate, as indicated in the template. After completion of 4 h incubation, the cell suspension was transferred to a sterile reservoir and seeded immediately in the wells of the above 96-well plate (containing 100 µL of MutIL-15/Fc at different concentrations) in 100 µL of the assay medium (final cell density: $[2.5 \sim 5 \times 10^4]$ cells/well; final rHuIL-15 concentration: 0.3 ng/mL; final MutIL-15/Fc concentration range: 3.9 to 1000 ng/mL) and incubated at 37 °C, 5% CO₂ for 48 h. After the 48 h incubation period, CellTiter96® AQueous One Solution was added (20 µL/well) and incubated for another 4 h at 37 °C and 5% CO₂, then 25 µL/well of 10% sodium dodecyl sulfate was added. The plate was then read at 490 nm. The background readings in the wells with medium were subtracted from the sample well read-outs. The data were analyzed using a 4-parameter curve fit (SoftMax Pro from Molecular Device). The ED₅₀ value is defined for the concentration of rhIL-15 required to induce the half-maximal stimulation and corresponds to the C parameter estimate of the 4-parameter logistic curve fit for the IL-15-induced dose-dependent response. The IC₅₀ value is used to denote the MutIL-15/Fc concentration corresponding to half-maximal cell proliferation inhibition.

For evaluation of bioassay variations, a reference lot (an earlier preparation of purified and vialled MutIL-15/Fc) and test lot (a subsequent purified and vialled lots of MutIL-15/Fc) were used. Variations in IC₅₀ values for both reference lot and the test lot was less than 10% for the intra-day assays, and less than 30% for the inter-day assays. Because of the considerable variation in IC₅₀ from day to day, the inter-assay variability was also evaluated by comparing the relative activity of a test lot to a reference lot. The activity of the test lot was $90 \pm 3\%$, relative to the reference lot. Excellent intra-day and inter-day assay consistency (less than 5%) was observed for the relative activity of the test lot, compared to a reference standard material, indicating that the HT-2 cell-based assay is qualified to serve as an index for determining functional activity of MutIL-15/Fc, comparing lot-lot consistency and monitoring the stability of MutIL-15/Fc.

Anti-Fc/anti-IL-15 dual probes ELISA

A 96-well Nunc plate was coated with 100 µL per well of goat anti-human IgG (5 µg/mL). The coated plate was covered with sealing film and incubated at 2–8 °C overnight. The plate was washed with PBST, using a plate washer. The plate was incubated with 200 µL/well of blocking buffer [PBST-1% Bovine Serum Albumin (BSA)] at 37 °C for 1 h and washed with PBST. Human MutIL-15/Fc (100 µL/well) diluted in serial dilutions (0.17 ng/mL to 10 µg/mL in blocking buffer) was added to the plate (blocking buffer as blank) and incubated at 37 °C for 1 h. The plate was washed with PBST. The biotinylated goat anti-human IL-15 (100 µL/well, 1:2000 diluted in blocking buffer) was added to the plate and incubated at 37 °C for 1 h. The plate was washed with PBST, and Streptavidin-HRP in blocking buffer (100 µL/well, 1:200 dilution) was added to the plate and incubated at 37 °C for 1 h. The plate was washed with PBST and TMB (100 µL/well) was added to the plate and incubated at room

temperature for 10 min. The reaction was arrested by adding 100 μL /well of 2N H_2SO_4 . The plate was read at 450 nm.

Online size-exclusion chromatography (SEC) coupled with multi-angle light scattering (MALS)

Online size-exclusion chromatography (SEC) coupled with multi-angle light scattering (MALS) with embedded QELS (quasi-elastic-light scattering), refractive index (RI) and UV-Vis detectors were used in the determination of molar mass, particle size, and detection of aggregates in the product. Chromatographic separation was achieved under isocratic conditions using a G3000SW_{XL} column and a TSKgel SW_{XL} guard column from Tosoh Bioscience. The method used 3X phosphate buffered saline, pH 7.4 (prepared from 10X PBS) as a mobile phase at a flow rate of 0.75 ml/min and a sample injection volume of 100 μL . Duration of each run was 30 min. UV detection was at A₂₁₀ and A₂₈₀. The column was connected to an Agilent 1100 HPLC system equipped sequentially with a diode array detector (DAD, Agilent 1100), a MALS detector (Wyatt Technology Corporation, Santa Barbara, CA), and a refractive index (RI) detector (Wyatt Technology Corporation, Santa Barbara, CA). The MALS detector employed a laser light at 658 nm and 18 detectors at angles evenly positioned between 22.5° and 147°. The output signals from DAD, MALS and RI were imported into Astra V® software for data processing. HPLC (UV/VIS) data analysis was performed, using HP Chemstation software, and Astra V 5.3.4 was used for analyzing the MALS data. System suitability was assessed by analyzing the standard protein BSA (Mw 65kDa). A blank run, using sample buffer injection, was performed and corrected from sample runs. Percentage aggregates were calculated from the integrated UV-VIS peak areas of the component peaks.

Kinetic analysis of the interaction of Mut-IL15/Fc with Fc γ RIIIa receptor

Approximately 25 ng of Fc γ RIIIa receptor (R & D Systems, MN, USA) was immobilized on a BIAcore CM5 sensor chip by amine coupling. The running buffer was 0.01 M HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 (HBS-EP buffer supplied by GE Healthcare Biosciences). MutIL-15/Fc was initially diluted to 2 μM with HBS-EP buffer. Two fold serial dilutions were then prepared up to 15.6 nM, using HBS-EP buffer. These samples were applied onto the CM5 chip at a flow rate of 20 μL /min. Each sample, 20 μL , was injected onto the prepared sensor chip surface and then it was allowed to dissociate for 5 minutes. After the dissociation, the binding surface was regenerated with 15 μL of 0.05% SDS. All data analyses were performed using the instrument, manufacturer-supplied software, BiaEvaluation.²⁰ A bivalent binding model was used to determine the equilibrium dissociation constants for the binding of MutIL-15/Fc to the Fc γ RIIIa receptor.

Analysis of N-glycosylation profile

N-linked carbohydrates on the glycoprotein were cleaved using a glycosidase, Peptide-N4-(acetyl- β -glucosaminyl)-asparagine amidase (PNGase F) from QA-Bio, LLC (CA, USA). A procedure supplied with the PNGase F kit (contents: PNGase F, 5 x reaction buffer, denaturation solution and 15% Triton X-100) was used for this purpose. The cleaved carbohydrates were separated from the protein and lyophilized. The lyophilized carbohydrate samples were labeled using a fluorophore, 2-amino benzoic acid (2-AA). The labeling procedure was supplied with the 2-AA labeling kit. The labeled samples were dialyzed extensively and the samples were lyophilized again. The dried, labeled samples were dissolved in 500 μL water and were analyzed on a HPLC fitted with a fluorescent detector. A Phenomenex Asahipak-NH2P-50 2D column and Phenomenex Asahipak 5 μ NH2P-50 0A guard column were used for the separation. The runs were performed using a linear gradient of mobile phase A (5% acetic acid, 4% diethylamine in Direct-Q water) and mobile phase B (2% acetic acid, 1% diethylamine in acetonitrile) with a flow rate of 0.2 mL/

min. The column compartment was kept at 50 °C. The fluorescence detector was set at an excitation wave length of 320 nm and an emission wave length of 420 nm. Carbohydrates were released from fetuin; a glycoprotein, using the procedure described above, was used as a system suitability/assay control. Di-silaylated, core-fucosylated bi-antennary complex type N-glycan (A2F), mono-silaylated bi-antennary complex-type N-glycan (A1), and asialo-bi-antennary complex-type N-Glycan (NA2) were also used as controls for the HPLC runs.

Determination of carbohydrate composition

Sialic acid content of MutIL-15/Fc was determined by RP-HPLC, as described by Anumula²¹ with some modification. The sialic acids were released from the glycoproteins by mild acid hydrolysis, followed by derivatization with o-phenylenediamine (OPD) to yield a fluorescent quinoxaline derivative. The derivative was separated from excess reagent by a Glycosep RP-HPLC column (4.6 × 150 mm, 5 µm, Prozyme Cat#GKI-4727) for quantitation using fluorescence detection (Agilent 1200 with a quaternary pump and a fluorescence detector with excitation λ = 230 nm and emission λ = 425 nm). The column was equilibrated with solvent A (acetonitrile: methanol: H₂O 9:7:84% v/v) for 10 min. The separation was performed under isocratic conditions with the same buffer for 15 min, followed by the column regeneration with solvent B (0.1% butylamine, 0.25% phosphoric acid, 0.5% tetrahydrofuran, 50% acetonitrile in water [v/v]) for 5 min. The flow rate was at a constant 0.5 mL/min.

Monosaccharide analysis was also performed, following the procedure described by Anumula.²² An Agilent HPLC 1200 system with a quaternary pump and a fluorescence detector was used to perform the assay. To calculate the mole ratio of carbohydrate composition vs protein, the molecular weight of 78 kDa of the protein part of MutIL-15/Fc molecule derived from amino acid sequence, not apparent molecular weight determined by SDS-PAGE or MALS, was used. The protein concentration was determined by A280 and molar extinction coefficient was estimated from amino acid composition (both depends only on amino acid composition and not carbohydrate).

RESULTS

Dual probe ELISA shows MutIL-15/Fc binding to both anti-IgG and anti-IL-15

Human MutIL-15/Fc is designed as a human fusion protein consisting of a two points-mutated IL-15 and the hinge-CH₂-CH₃ human IgG1. We used a dual probe ELISA to verify the identity of both IL-15 and Fc portions of the protein. Using plates coated with goat anti-human IgG Fc to capture MutIL-15/Fc, the binding of MutIL-15/Fc to the anti-IgG Fc coated plate was determined by the reactivity of the IL-15 portion of the bound molecule with the biotinylated anti-human IL-15 antibody, followed by incubation with horseradish peroxidase conjugated streptavidin. The bound HRP-streptavidin was quantified using reactivity with an HRP substrate. As shown in Figure 1A, human MutIL-15/Fc showed a dose-dependent binding activity. In contrast, the human IgG isotype control and HuMik β 1, a humanized anti-IL-2/IL-15R β ²³ (data not shown), did not show any activity under similar conditions. Similarly, IL-2/Fc showed a dose-dependent reactivity determined by a capture ELISA on an IgG-Fc coated plate in combination with the biotinylated anti-IL-2 detection antibody (data not shown). These Fc-anti-IL-15 dual probes ELISA results support the identity of an intact immunocytokine fusion molecule of the human MutIL-15/Fc.

SEC-MALS analysis of MutIL-15/Fc

The molecular properties (molecular weight [Mw], polydispersity [Pd], hydrodynamic radius [R_H] and molecular aggregates), were analyzed using an online size-exclusion chromatography–high pressure liquid chromatography (SEC-HPLC) coupled with MALS,

RI, and UV/VIS detectors. BSA was run as the standard marker for MALS system calibration. The MALS estimated average Mw for BSA was 64 kDa. The SEC column calibration marker mixtures are routinely run to check the SEC column performance and suitability. Earlier we have reported that MALS estimated Mw for a variety of immunoglobulins, and standard proteins such as chymotrypsin, ribonuclease and lysozyme are in the anticipated range consistent with values reported in literature.²⁴ Evaluation of MALS estimated Mw for the calibration marker kit run with MutIL-15/Fc in three different experiments showed average Mw values of 709 kDa for thyroglobulin, 151 kDa for gamma globulin, 45.2 kDa for ovalbumin and 19.7 kDa for myoglobulin.

Figure 1B shows the UV280 nm-LS (90° angle)-RI overlay of MutIL-15/Fc. Compared to the UV/VIS signal, light scattering is more sensitive in detecting high molecular weight oligomers and molecular aggregates. By SEC-HPLC separation, MutIL-15/Fc showed a major peak eluting at a retention time (RT) of ~10.8 min. The main peak (UV) constituted ~98% of the total peak area, with <1.5% aggregate consisting of higher molecular weight components near the 7–8 minute region of the chromatogram. The SEC showed a shorter column retention time (RT ~10.845 min) for MutIL-15/Fc peak, compared to γ Globulin (RT ~11.447 min; theoretical M_W of ~150 kDa). The SEC estimated Mw for MutIL-15/Fc, based on column retention time and calibration standards, was ~187 kDa. The MALS estimated molecular weight (M_W) of the major component peak was ~95 kDa with a hydrodynamic radius (R_H) of ~4.8 nm and a polydispersity (Pd) of 1.001. A recombinant protein IL-2/Fc, expressed in the same CHO cell line, was also analyzed using SEC-MALS (data not shown). The column retention time for IL-2/Fc was 12.3 min, which was longer compared to 10.8 min for MutIL-15/Fc. The SEC estimated Mw for IL-2/Fc was 68 kDa. The MALS data analysis showed an absolute Mw of 88 kDa with a hydrodynamic radius of 4.3 nm. Online SEC with a quadruple detector system from Viscotek (now Malvern Instruments) was also used in earlier studies, following the procedure described earlier²⁴, and similar results were obtained. Batch mode dynamic light scattering using Dynapro also showed comparable results (data not shown).

Molecular weights estimated using SDS-PAGE on Novex Bis-tris (4–12%) gel showed molecular weights of MutIL-15/Fc in the range of 52–55kDa under reducing conditions and 93–101 kDa under non-reducing conditions in different runs (data not shown). These data indicated that the MALS estimated Mw is consistent with Mw determined by non-reducing SDS-PAGE.

Kinetic analysis of the interaction of Mut-IL15/Fc with Fc γ RIIIa receptor

Fc γ RIIIa is responsible for triggering antigen-dependent cellular cytotoxicity that is primarily mediated by natural killer cells.²⁵ The Fc portion of MutIL-15/Fc fusion protein has been demonstrated to contribute to the overall efficacy of the molecule *in vivo*.¹⁰ We therefore looked at the interaction between MutIL-15/Fc and the Fc receptor by BIAcore®. The BIAcore® technology platform is a label-free detection system used to study the interaction between molecules using the surface plasmon resonance (SPR) phenomenon. This platform technology can be used to determine the specificity of an interaction, strength of an interaction, and speed of an interaction between two molecules (protein–protein, protein–DNA, etc.). Using amine coupling reagents, Fc γ RIIIa is immobilized onto a carboxymethyl dextran (CM5) sensor chip. When MutIL-15/Fc binds to this immobilized Fc γ RIIIa, a mass change occurs near the sensor chip surface with subsequent change in refractive Index (RI). This change in RI results in a change in the angle of the light (SPR angle) that is responsible for causing surface plasmon resonance. A sensorgram depicts this change in SPR angle in real time and is measured in response units (RU). The kinetics properties of MutIL-15/Fc binding to Fc γ RIIIa were analyzed. The assay was performed with an initial concentration of 2000 nM and a 1:2 serial dilution thereafter, up to 15.6 nM.

The data obtained from the kinetic runs are analyzed using the BIA evaluation program. The data showed a better fit to a bivalent model for the MutIL-15/Fc binding. In a bivalent model, there is an initial fast binding followed by a slow binding. Subsequently there is a fast dissociation followed by a slow dissociation. This kinetic analysis yielded two equilibrium dissociation constants, K_{D1} and K_{D2} , for the binding of MutIL-15 to the Fc γ RIIIa receptor. The MutIL-15/Fc showed a concentration-dependent binding to Fc γ RIIIa receptors with a K_{D1} of 5.04 μ M and a K_{D2} of 9.12 μ M, respectively (Figure 1C). The equilibrium dissociation constants (K_D) are in the micromolar range consistent with the observed range for IgGs (0.1–10 μ M).^{13–16}

N-linked glycosylation profile of MutIL-15/Fc

N-Linked glycosylation is an important determinant of protein structure and function^{26–28}. MutIL-15/Fc used in this study is produced in CHO cells and as such is anticipated to be glycosylated. We analyzed the N-glycosylation profile of MutIL-15/Fc using the procedure described in Materials and Methods. As shown in Figure 2A, MutIL-15/Fc showed an N-glycan profile with a cluster of five peaks, indicating that there is extensive N-glycosylation of MutIL-15/Fc. By contrast, the N-glycan derivatives from human IL-2/Fc are eluted very early in the chromatography (Figure 2B). Since only O-linked glycosylation has been reported in IL-2, the N-glycosylation in IL-2/Fc may be attributed to Fc glycosylation. Since the Fc construct in both MutIL-15/Fc and IL-2/Fc are the same and the expression cell systems are identical, the N-glycosyl moieties arising from the Fc portion are expected to be identical. Thus, the clusters of peaks that are not present in IL-2/Fc but are present in MutIL-15/Fc are attributable to N-glycosyl moieties from the IL-15 portion.

Measurement of the carbohydrate composition of MutIL-15/Fc

Four preparations of MutIL-15/Fc were analyzed for sialic acid content, following the procedure described in Materials and Methods. An average sialic acid content of MutIL-15/Fc was 12.0 ± 2.67 mol/mol protein. Under the experimental conditions, we obtained a sialic acid content of 12.3 mol/mol protein for the fetuin standard. The MutIL-15/Fc showed a carbohydrate content of 26.9 ± 1.0 (GluN), 2.6 ± 0.13 (GalN), 14.6 ± 0.83 (Gal), 13.43 ± 1.0 (Man), and 5.08 ± 0.96 (Fucose) mol/mol protein. There was no detectable Glu. The fetuin standard showed a carbohydrate composition of 6.3 (GluN), 1.4 (GalN), 4.8 (Gal), and 3.3 (Man) mol/mol protein, but no detectable fucose or glucose.

MutIL-15/Fc inhibits IL-15-induced HT-2 cell proliferation in a dose-dependent manner

MutIL-15/Fc is a unique lytic antagonistic fusion protein that is known to have an antagonistic effect on IL-15 activity. HT-2 cells are IL-2/IL-15 dependent cells and they cannot survive without IL-15 (or IL-2) supply. The survival rate of HT-2 cells can be used as an indicator for IL-15 bioactivity.²⁹ Therefore, by using the HT-2 cell line, we analyzed functions of MutIL-15/Fc on IL-15-mediated cell proliferation. First, the half-maximal stimulation values (ED_{50}) of IL-15-induced HT-2 cell proliferation curves were measured when HT-2 cells were treated at different concentrations of MutIL-15/Fc. As shown in Table 1, the ED_{50} value of IL-15, increased with increasing MutIL-15/Fc concentration in the growth medium. The half-maximal inhibition concentration values (IC_{50}) for the MutIL-15/Fc were also examined when the concentration of IL-15 for HT-2 stimulation was changed. As shown in Figure 3, the IC_{50} values for MutIL-15/Fc inhibiting IL-15 responses increased as the concentration of IL-15 increased. These data suggested that ED_{50} values for IL-15-induced stimulation and the IC_{50} values for MutIL-15/Fc inhibitory effect were mutually dependent on the concentrations of each other.

Specificity of the inhibitory effect of MutIL-15/Fc on IL-15-induced dose-dependent responses

Since IL-15 is a member of the four α -helix bundle cytokine family with closest homology to IL-2,^{10,30} we verified the specificity of MutIL-15/Fc functions by comparing the effect on IL-15, IL-2, and IL-2-Fc-mediated stimulation of HT-2 cells. Like IL-15, both IL-2 and IL-2/Fc stimulated HT-2 cell proliferation in a dose-dependent manner. MutIL-15/Fc treatment showed significant inhibition on IL-15-induced cell proliferation (Figure 4A); the ED₅₀ of the IL-15 response curve was right-shifted from 0.064 ng/mL to 11.287 ng/mL after treatment of MutIL-15/Fc at 10 μ g/mL. However, MutIL-15/Fc did not affect IL-2/Fc (Figure 4B); or IL-2 (Figure 4C) triggered HT-2 cell proliferation. These findings demonstrated the specificity of the inhibitory effect of MutIL-15/Fc on IL-15-induced dose-dependent cell proliferative responses.

Stability under the storage condition and in infusion study

Stability of the MutIL-15/Fc under normal storage condition was routinely followed for nearly three years using SDS-PAGE and analytical size SEC-HPLC. The MutIL-15/Fc was stable during the storage period studied.

The stability of MutIL-15/Fc was also studied by using the biological activity assay. The data obtained from the experiments under two testing conditions performed over a three-year period are shown in Figure 5. In the first series of experiments (Figure 5A), the reference lot had been examined at a cell density of 5×10^4 /well. The average IC₅₀ value from 15 independent runs was 74.32 ± 20.90 ng/ml with a CV of 28%. In the second series of experiments (Figure 5B), the reference lot had been examined at a cell density of 2.5×10^4 /well. The experiments were performed on different days as indicated in the figure legends. The average IC₅₀ value from 8 independent runs was 32.27 ± 8.10 ng/ml, with a CV of 25%. These data suggested that the bioactivity of MutIL-15/Fc was also stable under the normal storage condition.

Apart from the storage stability of therapeutic candidates, the stability and bioactivity of the molecules during the clinical administration for the entire administration period are of critical importance. Mock studies mimicking the pre-clinical and clinical administration procedure are to be conducted and the bioactivity and other properties have to be evaluated to address the stability of the molecule. A mock infusion model study was performed to evaluate the stability of MutIL-15/Fc following a proposed pre-clinical evaluation mock study. The antagonistic activity of MutIL-15/Fc of samples collected at different intervals of the mock infusion study was assessed by following the blockade of IL-15-induced proliferation of the HT-2 cells. Results are shown in Table 2. Though there is some loss of protein at the low dosage infusion mock modeling, the relative or specific activity of the recovered material was comparable to that of the control, suggesting that the recovered material maintains functional activity.

Effect of stress on the molecular and functional characteristics of MutIL-15/Fc

MutIL-15/Fc was subjected to forced stress by high pH and elevated temperature exposure. Changes in molecular characteristics were determined using SEC-MALS (Figure 6A). The high pH-stressed MutIL-15/Fc sample was comparable to the control with respect to the retention time and the main peak profile. The main peak constituted ~98–99% of the total peak area with ~1% aggregate components. MutIL-15/Fc, heated at 55 °C for 8–12 h showed a minimal amount of aggregation (~8% of the total) with ~92% of the monomer peak still intact. When heated to a temperature of 70 °C for 3 h, the product showed no significant change in the elution time (RT ~10.8 min). The major peak constituted ~78% of the total peak area. Approximately 21% of the aggregate components were detected in the

heated sample. When the duration of heating was further increased to ~6 h at 70 °C the monomer component was reduced to ~68% of the total. The aggregates, eluting near the 8–10 min retention time, consisted of dimer/trimer and traces of higher M_W components constituting ~31% of the total. Estimated M_W for the major component was ~95–96 kDa with an R_H of 4.9 ± 0.2 nm and Pd of 1.001.

A bioactivity assay was also performed for the above stressed MutIL-15/Fc samples (Table 3). When the MutIL-15/Fc was treated to high pH or heated at 55 °C for 8–12 h, these samples showed a bioactivity comparable to the non-stressed control within the limits of assay variability. When the main peak area decreased and aggregation increased in the stressed samples under the 70 °C, the bioactivity of these samples also decreased. These data suggest that the biological function of MutIL-15/Fc is correlated to its molecular structure.

The stress effects on the Fc receptor binding activity of MutIL-15/Fc were also studied. The representative sensorgrams obtained for the binding of a 2 μ M solution of MutIL-15/Fc control and a heated sample to Fc γ RIIIa receptor are shown in Figure 6B. Another sample of MutIL-15/Fc, which was treated to high pH (pH 10), showed a binding pattern similar to the non-stressed control (data not shown). The data obtained from the kinetic runs were analyzed using BIA evaluation program and a bivalent model for the MutIL-15/Fc binding (Table 4). The control MutIL-15/Fc sample exhibited binding to Fc γ RIIIa, with a K_{D1} of 5.04 μ M and a K_{D2} of 9.12 μ M, respectively, whereas the heated MutIL-15/Fc sample showed the binding to Fc γ RIIIa, with a K_{D1} of 16.3 μ M and a K_{D2} of 20.7 μ M, respectively, indicating that the heated sample of MutIL-15/Fc has a weaker (31–44%) binding affinity to the Fc γ RIIIa receptor compared to the non-stressed control. K_{D1} and K_{D2} values for the unheated sample were within the range of values reported for IgGs^{13–16} and there was only two fold difference between the first and second dissociation constants. Earlier reports from different laboratories indicated increased avidity with lowered dissociation constant for multimer IgG compared to monomer IgG. Our results indicate that for MutIL-15/Fc the presence of aggregates slightly increased K_{D1} and K_{D2} . This is in contrast to the earlier reports for IgG.^{15,16} The impact of the cytokine part on the Fc γ interaction is not known at present. More detailed investigation on the Fc γ R interaction of the separated (purified) aggregates is like that published earlier for IgG¹⁶ is required to understand the effect of molecular aggregation on the Fc:Fc γ R interaction of Fc-cytokine conjugates. The bioactivity of the above samples was also examined (Figure 6C). The IC_{50} values were 30.12 ng/ml for the control and 80.11 ng/ml for the heated sample, respectively. The bioactivity of the heated sample was 38% relative to the control. These data suggested that both the bioactivity of MutIL-15/Fc and its Fc port binding to Fc γ receptor are affected by stress-induced molecular changes.

DISCUSSION

MutIL-15/Fc was constructed by recombination of a mutant human IL-15 gene with a human Ig Fc region gene. The antagonistic effect of MutIL-15/Fc against IL-15 activation was analyzed by an IL-15-mediated HT-2 cell proliferation assay. The inhibitory effect of MutIL-15/Fc on IL-15-induced cell proliferation was mutually dependent on the concentration of IL-15 and MutIL-15/Fc in the growth medium. These data confirmed MutIL-15/Fc inhibiting IL-15 induced proliferative responses of T cells. IL-2 and IL-15 are two prominent members of the family of T-cell growth factors with pleotropic and redundant effects. Because they share IL-2R β and γ_c , IL-2 and IL-15 have long been presumed to trigger the same signaling pathways in target cells, which may then be interpreted differently depending on cell type. IL-2 and IL-15 activate the Janus (JAK) kinases-1 and -3 leading to activation of the STAT-5 transcription factor, and also the MAPK and phosphatidylinositol 3-kinase (PI3K) pathways, leading to cell growth and

inhibition of apoptosis.³¹ In some cases, IL-2 and IL-15 exert opposing effects. IL-15 triggers proliferation of CD8 memory cells, while IL-2 indirectly inhibits proliferation of CD8 memory cells.³² Activated, but not resting, T cells bear high affinity receptors for IL-2 and IL-15. The specificity of the IL-15-induced response is conferred upon the cell by its own IL-15R α subunit, just as IL-2R α makes the high affinity IL-2R specific for IL-2. The β and γ subunits of the IL-15R are shared by the IL-2R.³³ The similar and contrasting effects of IL-2 and IL-15 in cytokine-related therapies are summarized in two excellent reviews.^{6, 34} Although both IL-15 and IL-2 have similar proliferative activities on T cells, they display no primary sequence homology³⁰ and considered to be distinct from one another. Under similar conditions, the antagonistic effect of MutIL-15/Fc was specific for IL-15-induced activation. IL-2 or an IL-2/Fc recombinant fusion protein-induced HT-2 cell proliferation was not inhibited by MutIL-15/Fc. These data confirmed the specificity of this functional assay for determining MutIL-15/Fc antagonism against IL-15 responses.

Although the MutIL-15/Fc targets the high affinity IL-15R, the Fc portion of this fusion protein confers *in vivo* longevity.¹⁰ Fc γ receptor is constitutively expressed on almost all leukocytes, including lymphocytes B, T and natural killer cells. The abilities of Fc γ RIII to induce phagocytosis, ADCC, and inflammation have also been shown.³⁵ Parallel experiments employing SPR and bioassay and SEC-MALS provided direct evidence that molecular changes induced by heat stress affect Fc γ R binding as well as biological activity (IL-15 antagonism). The Fc:Fc γ RIIIa interaction is one mechanism of effector cell-mediated ADCC. The Fc γ RIIIa affinity may be correlated with the *in vitro* ADCC effect and may provide a tool for evaluation of the Fc-related effect. Together with the glycosylation analysis, these analytical methods provide very valuable tools for studying the effect of potential glycosylation changes during any process changes in manufacturing development.

We used the SEC-MALS methodology to evaluate potential changes in molecular properties as well as aggregation of the MutIL-15/Fc under differing stress conditions. Anomalies in the molecular weight estimates based on SEC or gel filtration have been reported in several cases. Protein-gel matrix interaction, protein conformation (misfolding or unfolding), etc., have been cited as factors that contribute to the anomalous behavior of protein migration in gel filtration. Anomalous Mw has been reported for a number of recombinant proteins.³⁶ Our laboratory has noticed that *Wolinella asparginase* elutes from SEC columns with a retention time corresponding to a Mw of about 50 kDa, whereas online SEC-LS estimated a Mw of about 138–140 kDa, corresponding to a tetrameric form (unpublished results) consistent with the literature-cited Mw of the asparginase enzyme.³⁷ Our laboratory also noticed anomalous behavior of recombinant human IL-15. SEC-MALS revealed a Mw of 18–20 kDa whereas SEC elution time showed a column retention time consistent with a protein of Mw 35–40 kDa (unpublished results). The retarded movement in the gel filtration column could be attributable to protein gel matrix interaction. A discrepancy between the calculated molecular weight (31 kDa) for xXPA and its apparent molecular weight on SDS-PAGE (multiple bands from ~40 to 45 kDa) and gel filtration chromatography (~92 kDa) was reported.^{38,39} The authors concluded that xXPA under native conditions has an unusually extended shape, which can contribute to its aberrant behavior during gel filtration chromatography. An abnormally high apparent molecular weight by gel exclusion has been used as an indicator for intrinsically unfolded protein.⁴⁰ In our case, MutIL-15/Fc showed a Mw of ~95kDa by SDS-PAGE under non-reducing conditions, consistent with the MALS estimated Mw. These observations are indicative of conformation or extended shape related factors may be contributing to the faster movement of MutIL-15/Fc in the SEC column. The results summarized in Table 3 show the bioactivity, and the major component and aggregate estimated from integrated UV peak areas from SEC. The changes in molecular characteristics, including the percentage of monomer and the aggregate, are relevant to the

changes in bioactivity. The Fc:FcγR interaction and glycosylation analysis together with the measurement of effector mediated function and IL-15 antagonistic effect would be used to monitor the effect of any process change (upstream or downstream) on the functional activity. Such studies could pave the way for Fc engineering directed to improve Fc:FcγR interaction-mediated effector functions in case the early-phase clinical evaluation studies show promising therapeutic value.

In summary, analytical methodologies are developed for characterization of the functional and molecular properties of MutIL-15/Fc for characterization and clinical product release, and stability monitoring to facilitate planned therapeutic evaluations of the MutIL-15/Fc. The information collected on the MutIL-15/Fc dose-dependence effect on the blockade of IL-15-induced *in vitro* cell proliferation may provide insight into the importance of the antagonist MutIL-15/Fc dosage selection under different treatment regimes. The potential therapeutic use of MutIL-15-Ig fusion protein in short-term triple therapy using rapamycin, IL-2/Fc and MutIL-15-Ig was emphasized in a recent article,⁴¹ which demonstrated prolonged survival of allogenic islets in cytomologus monkeys after short-term triple therapy. This result along with earlier reports clearly emphasis the potential possibility of further clinical development and the detailed characterization of the molecule during the manufacturing process changes will be critical for both manufacturing control as well as better understanding of the clinical data in terms of potential product structural alterations. The data highlights the importance of identification, optimization and qualification of analytical methodologies for characterization of complex biopharmaceutical drugs for establishing product lot-lot consistency, stability during the product storage as well as monitoring the stability of the drug under the clinical product delivery conditions.

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Abbreviations

ADCC	antibody dependent cytotoxicity
BSA	bovine serum albumin
CDC	complement dependent cytotoxicity
FBS	fetal bovine serum
Fuc	fucose
Gal	galactose
GalN	galactosamine
Glu	glucose
GluN	glucosamine

IL	Interleukin
IL-2/Fc	IL-2/Fc fusion protein
LS	light scattering
MALS	multi-angle light scattering
Man	mannose
MutIL-15/Fc	mutant IL-15/Fc fusion protein
PBS	phosphate buffered saline
PBST	phosphate buffered saline-0.05% Tween-20
RI	refractive index
RU	response units
SEC	size-exclusion chromatography
TMB	tetramethylbenzidine

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Figure 1. Molecular characterization of MutIL-15/Fc

A. Binding activity of MutIL-15/Fc determined by anti-Fc/anti-IL-15 dual probes ELISA. A 96-well Nunc plate was coated with goat anti-human IgG. MutIL-15/Fc or human IgG control diluted in serial dilutions were added to the above coated plate and incubated at 37 °C for 1 h. The bound MutIL-15/Fc was detected using an anti-IL-15. The details of the procedure are described in Materials and methods.

B. The molecular characteristics of MutIL-15/Fc analyzed using analytical size exclusion chromatography with online laser light scattering (LS), refractive index (RI) and UV-Vis detectors. The figure shows UV_{280nm}-LS (90° angle)-RI overlay of MutIL-15/Fc analyzed by SEC-MALS.

C. Kinetic analysis of MutIL-15/Fc binding to Fc γ receptor. Sensorgrams showing dose-dependent binding of MutIL-15/Fc on Fc γ RIIIa coupled to a CM-5 chip. Fc γ RIIIa binding

kinetics was performed as described in Materials and Methods. Serial twofold dilutions (eight) of MutIL-15/Fc starting from 2 μ M (curve 1) to 15.6 nM (curve 8), were injected.

Figure 2. N-glycan analysis of MutIL-15/Fc and IL-2/Fc

N-linked carbohydrates on the glycoprotein were cleaved using PNGase F. The cleaved carbohydrates were separated, lyophilized and labeled with 2-AA. The dried, labeled samples were dissolved in water and analyzed on a HPLC with a fluorescent detector. The details of the procedure are described in Materials and methods.

A. N-linked glycosylation profile of MutIL-15/Fc.

B. N-linked glycosylation profile of IL-2/Fc.

Figure 3.

MutIL-15/Fc dose-dependently inhibits IL-15-mediated HT-2 cell proliferation. HT-2 cells were seeded in a 96-well plate with medium containing 0.4, 0.6, or 1 ng/ml IL-15. An equal volume of culture medium containing varying concentrations of MutIL-15/Fc was added to a final MutIL-15/Fc concentration indicated in the figure. After 48 h incubation, MTS was added and incubation was continued for an additional 4 h. The plate was read at 490 nm. The final IL-15 concentration (ng/ml) used is as marked in the figure.

Figure 4. Specificity of MutIL-15/Fc antagonistic effect on IL-15-induced HT-2 cell proliferation

A. IL-15-induced dose-response curve is right-shifted by MutIL-15/Fc. HT-2-cells were seeded in 96-well plates in cytokine-free culture medium with or without MutIL-15/Fc. An equal volume of IL-15 containing culture medium was added to the seeded cells to the final concentration indicated. After 48 h incubation at 37 °C, 5% CO₂, MTS was added and incubated for an additional 4 h. The plate was read at 490 nm.

B. MutIL-15/Fc has no effect on IL-2/Fc-mediated HT-2 cell proliferation. HT-2-cells were seeded in 96-well plates in culture medium containing 20 μ g/ml of MutIL-15/Fc and no cytokine growth factors. An equal volume of IL-2/Fc containing culture medium was added to the seeded cells to the final concentration indicated. The final concentration of MutIL-15/Fc was 10 μ g/ml. After 48 h incubation at 37 °C and 5% CO₂, MTS was added and incubated for an additional 4 h. The plate was read at 490 nm.

C. MutIL-15/Fc has no effect on IL-2-mediated HT-2 cell proliferation. HT-2-cells were seeded in 96-well plates in culture medium containing 20 $\mu\text{g/ml}$ of MutIL-15/Fc and no cytokine growth factors. An equal volume of IL-2 containing culture medium was added to the seeded cells to the final concentration indicated. The final concentration of MutIL-15/Fc was 10 $\mu\text{g/ml}$. After 48 h incubation at 37 °C and 5% CO_2 , MTS was added and incubated for an additional 4 h. The plate was read at 490 nm.

Figure 5. Inter-assay variations of IC₅₀ values obtained from MutIL-15/Fc bioactivity assays performed over a 3-year period (trend analysis)

A. Distribution of IC₅₀ values of a reference standard lot examined at cell density of 5×10^4 /well. Experiments were performed on different days (#1, day 1; #2, day 15; #3–5, day 242; #6–8, day 256; #9–12, day 266; and #13–16, day 260).

B. Distribution of IC₅₀ values of a reference standard lot examined at cell density of 2.5×10^4 /well. Experiments #1–8 were performed on different days (#1, day 1; #2, day 111; #3, day 242; #4, day 253; #5, day 270; #6–7, day 629; and #8, day 660).

Figure 6. Bioactivity and Fc binding affinity of MutIL-15/Fc correlated with changes in molecular characteristics under stress conditions

A. Light Scattering Overlay of MutIL-15/Fc under different stress conditions.

B. Representative sensorgrams obtained for the Fc receptor binding of a 2 μ M solution of MutIL-15/Fc control and the heated sample.

C. Comparison of MutIL-15/Fc bioactivity between control and the heated sample.

Table 1Effect of MutIL-15/Fc on ED₅₀ of IL-15-mediated dose-dependent responses

MutIL-15/Fc (μg/mL)	ED ₅₀ for IL-15 Response (ng/mL)	ED ₅₀ ratio *
0	0.060	1
2.5	6.658	110.97
5	9.250	154.17
10	11.287	188.12
20	23.294	388.23

* ED₅₀ ratio = ED₅₀ (IL-15 in presence of MutIL-15/Fc) ÷ ED₅₀ (IL-15 Control)

Table 2

Potency assay results for MutIL-15/Fc stability in mock infusion study

Sample	Concentration	Estimated protein concentration	* Protein mass recovery (%)	** Relative activity
A: 45 minutes infusion				
Control	1.1 mg/ml	1.1 mg/ml	100	100
Low dosage	16.7 µg/ml	11.2 µg/ml	67	97
High dosage	730 µg/ml	738 µg/ml	101	98
B: 15 minutes infusion				
Control	1.1 mg/ml	1.1 mg/ml	100	100
Low dosage	16.7 µg/ml	11.1 µg/ml	66	109
High dosage	730 µg/ml	718 µg/ml	98	100

* Mass Recovery % = (Experimentally determined protein concentration ÷ Expected protein concentration) × 100%.

** Relative activity = (IC₅₀ of control ÷ IC₅₀ of test sample) × 100%. The activity values (IC₅₀) and relative activity (% of reference control) are calculated based on the experimentally determined protein concentration.

Table 3

Relationship between molecular characteristics and bioactivity of MutIL-15/Fc

Stress condition	Main peak %	Aggregation %	Relative bioactivity %
Control	98	<1.5	100
High pH for 12 h	99	1	117
55 °C for 12 h	92	8	96
70 °C for 3 h	78	21	ND
70 °C for 6 h	68	31	38

ND-not determined

Table 4

Kinetic parameters for the binding of MutIL-15/Fc to FcγRIIIa receptor

MutIL-15/Fc	Run	First binding			Second binding		
		ka1 (1/Ms)	kd1 (1/RUs)	Kp1 (μM)	ka2 (1/Ms)	kd2 (1/RUs)	Kp2 (μM)
Control	1	9.92×10 ³	0.049	4.92	7.78×10 ⁻⁵	2.97×10 ⁻³	9.08
	2	9.96×10 ³	0.051	5.16	8.69×10 ⁻⁵	3.34×10 ⁻³	9.15
	Average	9.94×10 ³	0.050	5.04	8.24×10 ⁻⁵	3.16×10 ⁻³	9.12
Heated at 70 °C for 6 h	1	1.63×10 ⁴	0.285	17.5	6.74×10 ⁻⁵	7.23×10 ⁻³	25.5
	2	0.95×10 ⁴	0.142	15.0	7.64×10 ⁻⁵	5.07×10 ⁻³	15.8
	Average	1.29×10 ⁴	0.214	16.3	7.19×10 ⁻⁵	6.15×10 ⁻³	20.7