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IL-15:IL-15 receptor alpha superagonist complex: High-level co-expression in recombinant mammalian cells, purification and characterization

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Abstract

IL-15, a promising cytokine for treating cancer and viral diseases, is presented *in trans* by the IL-15 receptor (IL-15R) alpha-chain to the IL-15R $\beta\gamma_c$ complex displayed on the surface of T cells and natural killer (NK) cells. We previously reported that an asparagine to aspartic acid substitution at amino acid 72 (N72D) of IL-15 provides a 4–5 fold increase in biological activity compared to the native molecule. In this report, we describe Chinese hamster ovary (CHO) cell expression of a soluble complex (IL-15N72D:IL-15R α Su/Fc) consisting of the IL-15 N72D superagonist and a dimeric IL-15R α sushi domain-IgG1 Fc fusion protein. A simple but readily scalable affinity and ion exchange chromatography method was developed to highly purify the complex having both IL-15 binding sites fully occupied. The immunostimulatory effects of this complex were confirmed using cell proliferation assays. Treatment of mice with a single intravenous dose of IL-15N72D:IL-15R α Su/Fc resulted in a significant increase in CD8⁺ T cells and NK cells that was not observed following IL-15 treatment. Pharmacokinetic analysis indicated that the complex has a 25-hour half-life in mice which is considerably longer than <40-minute half-life of IL-15. Thus, the enhanced activity of the IL-15N72D:IL-15R α Su/Fc complex is likely the result of the increased binding activity of IL-15N72D to IL-15R $\beta\gamma_c$, optimized cytokine *trans*-presentation by the IL-15R α Su domain, the dimeric nature of the cytokine domain and its increased *in vivo* half-life compared to IL-15. These findings indicate that this IL-15 superagonist complex could serve as a superior immunostimulatory therapeutic agent.

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Conflict of interest

K. Han, X. Zhu, B. Liu, E. Jeng, L. Kong, W.D. Marcus, P.A. Chavaille, C.A. Romero, P.R. Rhode, and H.C. Wong are employees and/or shareholders of Altor BioScience Corporation.

Keywords

Interleukin-15 superagonist; IL-15 receptor α fusion protein; CHO cell expression; NK cells; CD8⁺ T lymphocytes

1. Introduction

Interleukin-15 (IL-15) is a critical factor for the development, proliferation and activation of effector NK and CD8⁺ memory T cells [1]. IL-15 and IL-2 are related cytokines capable of stimulating immune cells via interactions with shared signaling receptor components, IL-2/IL-15R $\beta\gamma_c$, and unique α chain subunits (IL-2R α and IL-15R α) that are required for high affinity binding to the $\beta\gamma_c$ receptor complex. Based on its ability to provide durable immune responses in a subset of patients, IL-2 has been approved by the FDA for use in treatment of metastatic renal cell carcinoma and malignant melanoma. However, the broad effectiveness of IL-2 as an anti-cancer therapeutic has been questioned based on its pivotal role in the maintenance of CD4⁺CD25⁺ T-regulatory cells and in activation-induced cell death (AICD), a process that leads to the elimination of stimulated T cells and induction of T-cell tolerance, thereby limiting memory T cell responses. In contrast, IL-15 inhibits IL-2-induced AICD and supports long lasting CD8⁺ T cell memory and effector responses against diseased cells [2], suggesting that IL-15 may have significant advantages over IL-2 for the treatment of cancer [3]. Indeed, a recent NCI review listed IL-15 as the most promising product candidate among twelve immunotherapy drugs that could potentially cure cancer [4]. However, there are several limitations in developing IL-15-based approaches that include difficulty in producing large amounts of this cytokine in standard mammalian cell expression systems, low potency and short serum half-life. To contend with one of these shortcomings, we have identified a novel IL-15 mutant, IL-15N72D, with increased affinity to bind IL-15R $\beta\gamma_c$ and enhanced biological activity [5].

IL-15 has a novel mechanism of action in which IL-15 and IL-15R α are coordinately expressed by antigen-presenting cells (monocytes and dendritic cells) [6], and IL-15 bound to IL-15R α is presented *in trans* to neighboring NK or CD8⁺ T cells expressing only the IL-15R $\beta\gamma_c$ receptor [7]. Soluble IL-15R α and IL-15 are able to form high-affinity heterodimeric complexes in solution with the N-terminal IL-15R α fragments, containing the so-called “sushi” domain (Su), which bears most of the structural elements responsible for cytokine binding. In fact, we have found that the IL-15 and IL-15 R α interaction domains could serve as a functional protein scaffold for generating multivalent or multispecific binding molecules [8]. Additionally, soluble IL-15:IL-15R α complexes are fully capable of modulating immune responses via the IL-15R $\beta\gamma_c$ complex [9–11]. It has been shown in some studies that the biological activity of IL-15 could be increased 50-fold by administering preformed complexes of IL-15 and soluble IL-15R α through a mechanism that is likely due in part to the longer half-life of the complex compared to IL-15 alone [9,11]. Recent studies in mouse tumor models have also demonstrated that the efficacy of IL-15 can be dramatically increased by pre-associating it with soluble IL-15R α either in a single chain format or as an IL-15R α /Fc fusion [12–14]. These responses were found to be mediated by either NK cell or T cells, including tumor-resident effector cells, depending on the tumor model [12–14].

Based on these findings, we evaluated whether it is possible to co-express the IL-15N72D superagonist and dimeric IL-15R α Su/Fc fusion protein to achieve a high level of production of an IL-15N72D:IL-15R α Su/Fc complex in recombinant CHO cells and to develop a simple approach to purify this IL-15 superagonist complex. The goal of these studies was to

produce significant quantities of this complex in a biologically active form to support clinical development of this IL-15-based immunotherapy.

2. Materials and methods

2.1. Construction of vectors for protein complex expression

The IL-15R α Su/Fc fusion gene was constructed by overlap PCR amplification of DNA templates encoding the sushi domain of human IL-15R α (aa1-66 of human IL-15R α) and the human IgG1 Fc fragment. The signal peptide-IL-15R α Su coding region [8] and human IgG1-Fc gene fragment [15] were amplified using the primer pairs: BA494: 5'-GACTTCAAGCTTAATTAAGCCACCATGGACAGACTTACTTCTTC-3'; BA550R: 5'-GTGAGTTTTGTGACAAGATTTTCGGCTCTCTAATGCATTTGAGACTGGGGGTTG-3', and BA550F: 5'-GAGCCGAAATCTTGTGACAAAACCTCAC-3'; BA393R: 5'-GTAATATTCTAGACGCGTTCATTATTTACCAGGAGACAGGGAGAGGCTCTTC-3', respectively. The resulting IL-15R α Su/Fc fusion gene was ligated into a puromycin-resistant expression vector pMSGV-1 [16] to construct the expression vector pMSGV-IL-15R α Su/Fc.

The coding sequence of IL-15N72D [5] was cloned into a modified retrovirus expression vector pMSGV-1 [16] that carries the neomycin resistance gene after an IRES region to construct the expression vector pMSGV-IL-15N72D.

2.2. Co-expression of IL-15N72D:IL-15R α Su/Fc fusion complex in CHO cells

To co-express IL-15N72D and IL-15R α Su/Fc fusion proteins (see Fig. 1), pMSGV-IL-15R α Su/Fc and pMSGV-IL-15N72D were co-transfected into CHO cells followed by selection in medium containing 2 mg/mL G418 (Hyclone, Logan, UT) and 10 μ g/mL of puromycin (Hyclone, Logan, UT). The IL-15R α Su/Fc fusion protein was also expressed individually in CHO cells for use in loading of recombinant human wild-type IL-15 (IL-15wt) as a control. For production of the fusion proteins, the recombinant CHO cells were grown in serum free defined medium (SFM4CHO, Hyclone, Logan, UT) at 37°C. When the viable cell density of the cultures reached a maximum, the incubation temperature was shifted down to 30°C for accumulation of the soluble complex. Culture supernatants were then harvested when the viable cell density of the cultures reached approximately 10% viable cells.

2.3. Purification procedure

The recombinant CHO cell culture medium was centrifuged and filtered to remove cells and debris before the supernatant was adjusted to pH 8.0 with 1 M Tris-HCl, pH 8.0. The soluble IL-15N72D:IL-15R α Su/Fc fusion protein complex was purified using a two-step affinity and ion exchange chromatography-based process.

Since the IL-15N72D:IL-15R α Su/Fc complex contains the IgG1-Fc domain, an rProtein A Sepharose Fast Flow (GE Healthcare Life Sciences, Piscataway, NJ) column was used as the first step in the purification process. Prior to sample loading, the column was washed with 5 column volumes (CV) of 20 mM Tris-HCl, pH 8.0, sanitized with 5 CV of 0.1 N NaOH for 1 h, and then equilibrated with 7 CV of 20 mM Tris-HCl, pH 8.0. The supernatant was loaded onto the 11 mL column at 2 mL/min, and the column was then washed with 8 CV of 20 mM Tris-HCl, pH 8.0, followed by 7 CV of washing buffer (0.1 M Na-citrate, pH 5.0) to remove non-specifically bound proteins. The protein was then eluted with 0.2 M Na-citrate, pH 4.0 and the pH of collected peak fractions was immediately adjusted to pH 3.5 using 0.2 M citric acid; the eluted protein was held at this low pH for 30 minutes as a standard viral clearance step. After the low pH hold step, the pH of the eluted preparation was adjusted to pH 7.7 by using 2 M Tris-HCl, pH 8.0. The preparation was concentrated and buffer

exchanged into 20 mM Tris-HCl, pH 8.0 by using an Amicon Ultra-15 centrifugal concentrator (30 kDa cut-off, Millipore, Billerica, MA) before sterile filtration using a 0.22 μ m filter (Corning Life Sciences, Lowell, MA).

The protein preparation was then applied to a Q Sepharose Fast Flow (QSFF; GE Healthcare Life Sciences, Piscataway, NJ) ion exchange column. A 5 mL column was washed with buffer A (20 mM Tris-HCl, pH 8.0), sanitized by 5 CV of 0.1 N NaOH for 1h, and then equilibrated with buffer A. The protein concentration in the preparation was first adjusted to <1 mg/mL with 20 mM Tris-HCl, pH 8.0 and was then loaded onto the QSFF column at a rate of 1 mL/min. The protein was then eluted from the column using a three-step-gradient process as follows: 20 mM Tris-HCl, pH 8.0, 130 mM NaCl for four CV as the first step, 20 mM Tris-HCl, pH 8.0, 300 mM NaCl for four CV for the second step and 20 mM Tris-HCl, pH 8.0, 1 M NaCl for two CV as the last step. Protein peak fractions were collected, buffer exchanged into PBS (Hyclone, Logan, UT), and filtered using a 0.22 μ m filter. Protein concentration was determined by UV spectrophotometer at 280 nm using an extinction coefficient of 1 $A_{280\text{nm}} = 0.79$ mg/mL. This extinction coefficient was calculated based on the deduced amino acid sequence of the IL-15N72D:IL-15R α Su/Fc complex.

Individually expressed IL-15R α Su/Fc was purified using rProtein A affinity chromatography as described above for assembling of complex in solution with IL-15N72D or IL-15wt produced in *E. coli* and refolded [5]. These *in vitro* assembled complexes were used as standards for biological activity evaluation and estimation of degree of occupancy of the IL-15 binding sites in co-expressed complexes.

2.4. Gel electrophoresis and size exclusion chromatography (SEC) analysis

Purified proteins were analyzed by different types of gel electrophoresis methods, which included NuPAGE 12% Bis-Tris gel (under reduced and non-reduced conditions), 4–20% Tris-glycine gel (native condition), and IEF pH3-10 gel (for pI determination). All supplies were from Invitrogen (Carlsbad, CA). Experimental methods were performed as described by the manufacturer. Superdex 200 HR 10/30 (GE Healthcare Life Sciences) chromatography with PBS (Hyclone, Logan, UT) as the running buffer was used to examine purity and to estimate molecular mass of the proteins.

2.5. N-terminal amino acid sequence and glycosylation analysis

Protein bands of interest were separated on SDS-PAGE gels, blotted onto PVDF membrane and stained by Ponceau S solution. N-terminal amino acids sequencing was performed using the Edman degradation method (Molecular Structure Facility, UC Davis, Davis, CA).

To examine whether the fusion complex was glycosylated, 50 μ g of the highly purified protein after the ion exchange chromatography was digested with 2 μ L of N-Glycosidase F, *C. meningosepticum* (Calbiochem, La Jolla, CA) in a total volume of 50 μ L in PBS at room temperature for 48 h and then was subjected to electrophoresis in NuPAGE 12% Bis-Tris gel under a reduced condition.

2.6. Determination of IL-15N72D occupancy of the purified IL-15N72D:IL-15R α Su/Fc complex

Purified IL-15R α Su/Fc was loaded with IL-15wt (produced in *E. coli* and refolded, provided by J. Yovandich, NCI, Fredrick, MD) at various ratios for 15 h at 4°C. After incubation, the IL-15wt:IL-15R α Su/Fc complex was purified using rProtein A affinity chromatography as described above. This purified complex was evaluated using two ELISA formats, one (anti-human IgG Fc capture and anti-IL-15 detection) which detects the intact complex and the other (anti-human IgG Fc capture and anti-human IgG Fc detection) which detects only the

IL-15 α Su/Fc fusion protein. The ratio between the intact IL-15wt: IL-15 α Su/Fc complex and IL-15R α Su/Fc protein levels reflects the occupancy rate of the IL-15 binding sites of the complex. [Occupancy rate (%) = the intact complex (ng/mL) / IL-15R α Su/Fc (ng/mL) \times 100%]. Fully occupied complex (pre-associated of IL-15R α Su/Fc and IL-15wt at a 1:3 ratio) was then used as a standard to quantitate the occupancy rate of purified IL-15N72D:IL-15R α Su/Fc fusion protein complexes after purification.

2.7. Determination of IL-15 biological activity

An *in vitro* cell proliferation assay using the IL-15-dependent 32D β cell line was employed to assess the IL-15 biological activities of the purified complex and IL-15wt proteins as previously described [5].

2.8. Pharmacokinetic evaluation

The pharmacokinetic profile of IL-15N72D:IL-15R α Su/Fc complex and IL-15wt were evaluated in female CD-1 mice (4 mice/time point, Harlan, Indianapolis, IN) as previously described for IL-2 [17]. Serum levels of the IL-15N72D:IL-15R α Su/Fc complex were assessed with the two ELISA formats described above. IL-15wt levels were assessed by ELISA using anti-IL-15 capture (MAB647; R&D Systems, Minneapolis, MN) and anti-IL-15 detection (BAM247; R&D Systems, Minneapolis, MN). IL-15N72D:IL-15R α Su/Fc levels from each ELISA format were fit with a one-compartment model using PK Solution 2.0 (Summit Research Services, Montrose, CO). Data from mice treated with IL-15wt were best modeled as a two-compartment model.

2.9. Lymphocyte stimulation

C57BL/6 mice (male, 6 wks of age, Harlan, Indianapolis, IN) were injected intravenously with a single dose of IL-15N72D:IL-15R α Su/Fc fusion complex at 1 mg/kg or human IL-15wt at 0.28 mg/kg (molar equivalent dose), respectively, or PBS as a negative control. Four days after treatment, pooled blood (5 mice per group) and splenocytes were collected. PBMCs were isolated from the blood using histopaque (Sigma, St. Louis, MO). The PBMC and splenocytes were then stained with PE-labeled anti-CD19, PE-labeled anti-CD335 (NKp46), FITC-labeled anti-CD4 and FITC-labeled anti-CD8 antibodies (BioLegend, San Diego, CA). The stained cells were analyzed on a FACScan flow cytometer (BD Bioscience, San Jose, CA). All animal studies were performed following Altor's IACUC approved protocols.

3. Results and discussion

We have previously reported that an asparagine to aspartic acid substitution at amino acid 72 of human IL-15 helix C (N72D) provided a 4–5 fold increase in IL-15 biological activity compared to the native molecule in proliferation assays with cells bearing the human IL-15R $\beta\gamma_c$ complex [5]. This increased activity is due to improved interactions of the IL-15N72D mutein with the human IL-15R β chain [5]. We also found that the activity of IL-15N72D following *in vitro* association with a soluble dimeric IL-15R α /IgG fusion protein was further enhanced to 10–25 fold compared with that of native IL-15 [5], consistent with the results shown previously with other IL-15/IL-15R α complexes [9–13]. These findings illustrate the potential of IL-15:IL-15R α /IgG as an IL-15 based human therapeutic. However, production of such complexes has been largely limited to *in vitro* preassociation of *E. coli*-produced IL-15 and mammalian produced IL-15R α molecules. Active IL-15 is currently generated as an insoluble, inclusion body in recombinant *E. coli* and requires a tedious and inefficient process of solubilization, refolding and purification [18]. For clinical development purposes, such a production method is impractical and cost

prohibitive. Thus, a better production and purification method for such a protein complex is needed to support the projected demand for its clinical development.

3.1. Co-expression of IL-15N72D and IL-15R α Su/Fc fusion gene in CHO cells

Previous studies have shown that recombinant IL-15 is poorly expressed by mammalian cells [18]. However, it has been reported that intracellular complex formation with IL-15R α prevents IL-15 degradation in the ER [6]. Hence, we postulated that IL-15 could be produced at a higher level if it is co-expressed with IL-15R α . It is known that soluble IL-15R α fragment, containing the so-called “sushi” domain (Su) at the N terminus, bears most of the structural elements responsible for cytokine binding. Soluble IL-15R α Su (without its transmembrane domain) and IL-15 are able to form very stable heterodimeric complexes in solution (K_d of complex = 100 pM [19]) and these complexes are capable of modulating (*i.e.* either stimulating or blocking) immune responses via the IL-15R $\beta\gamma_c$ complex [9–11,19]. Thus, we chose to generate a complex consisting of IL-15N72D and an IL-15R α Su/Fc fusion protein (see Fig. 1). The IL-15R α Su domain was genetically fused to the human IgG1-Fc region to facilitate its purification and dimerization via interchain disulfide bonds. To co-express IL-15N72D and the IL-15R α Su/Fc, two individual retrovirus-based expression vectors, pMSGV-IL-15R α Su/Fc and pMSGV-IL-15N72D, were constructed and co-transfected into CHO cells. The recombinant CHO cells were selected based on the neomycin and puromycin resistance elements provided by the two expression vectors, and individual producing cell lines were then generated using limited dilution cloning. We were able to identify a clone that is capable of producing approximately 100 mg/L of IL-15N72D:IL-15R α Su/Fc complex, based on ELISA, in a serum-free, defined medium. This result demonstrated that IL-15 could be expressed at high levels in mammalian cells if it is co-expressed with the IL-15R α Su domain.

3.2. Purification and characterization of the IL-15N72D:IL-15R α Su/Fc complex

When IL-15R α Su/Fc and IL-15N72D were co-expressed and assembled intracellularly in recombinant CHO cells, four different forms of proteins were expected in the cell culture supernatants: 1) dimeric IL-15R α Su/Fc molecule fully occupied with two IL-15N72D subunits, 2) dimeric IL-15R α Su/Fc molecule partially occupied with one IL-15N72D subunit, 3) a small amount of free homodimeric IL-15R α Su/Fc molecule with no IL-15 bound, and 4) free IL-15N72D. Since IL-15N72D lacks an Fc region, we first used a rProtein A-based affinity purification step to separate the free IL-15N72D from all of the Fc-bearing fusion proteins in the culture supernatant.

An ion exchange chromatography method was then developed to separate various forms of the IL-15R α Su/Fc complex. The calculated isoelectric point (pI) of the IL-15R α Su/Fc dimeric molecule is 8.5. As expected, this protein in 20 mM Tris-HCl, pH 8.0 solution was subsequently found to not bind to QSF resin. Additionally, the calculated pI of IL-15N72D is 4.5. We, therefore, predicted that the overall charge of the partially occupied IL-15N72D:IL-15R α Su/Fc (*i.e.* dimeric IL-15R α Su/Fc + one IL-15N72D molecule) and the fully occupied IL-15N72D:IL-15R α Su/Fc (dimeric IL-15R α Su/Fc + two IL-15N72D molecules) are different. This is consistent with IEF gel analysis of the Protein-A-purified preparations, which showed two major groups of complexes with pIs between 5.8–6.3 and 6.6–6.9 corresponding with the expected pIs of the fully occupied and partially occupied complexes, respectively (Fig. 2A). The heterogeneity among pI bands of each protein group is likely due to the degree of subunit glycosylation (see below) and C-terminal lysine variants in the IgG1 chain [20]. Thus, buffers with different ionic strengths were employed to separately elute the partially occupied and fully occupied complexes from the QSF. Using 130 mM NaCl, 20 mM Tris-HCl, pH 8.0, a single protein fraction (Q step 1) was eluted from QSF and found to contain mainly the partially occupied complex based on

ELISAs determining the fractional occupancy of the IL-15R α Su/Fc molecule (see Section 2.6). In the subsequent step using 300 mM NaCl, 20 mM Tris-HCl, pH 8.0, two protein fractions designated as Q1c and Q2c were further eluted from the QSFF. ELISA analyses performed on these preparations indicated that Q1c fraction contained a mixture of partially occupied (10% of total) and fully occupied (90%) complexes whereas Q2c fraction contained only the fully occupied complex (data not shown). These findings are consistent with IEF gel analysis of the purified protein preparations (Fig. 2B). Proteins eluted from Q step 1 have broad pIs ranging from 5.8 to 6.9; proteins of pIs 6.6 to 6.9 representing the partially occupied complex. Fraction Q1c of Q step 2 elution mainly contained protein with pIs ranging from 5.8 to 6.3 (*i.e.* fully occupied complex) but with small amounts of contaminant protein with pIs of 6.6 to 6.9. The Q2c fraction contained only proteins with pIs ranging from 5.8 to 6.3.

In SEC analysis, the purified IL-15N72D:IL-15R α Su/Fc Q2c preparation was found to elute as a single molecule with high purity (Fig. 3). The estimated molecular weight of the homodimer was approximately 114 kDa, which was larger than the 92 kDa molecular weight calculated based on the deduced amino acid sequence of IL-15N72D and IL-15R α Su/Fc fusion proteins. This is likely due to the glycosylation of the proteins produced by mammalian cells. This is supported by the results from our de-glycosylation studies as described below.

In reducing SDS-PAGE (Fig. 2C), the purified IL-15N72D:IL-15R α Su/Fc preparation was found to contain three proteins with molecular weights of 40 kDa, 16 kDa and 13 kDa. However, after a digestion with N-Glycosidase F, only two proteins, with molecular weights of ~37 kDa and 13 kDa, were detected (Fig. 2D). These molecular weights closely match the calculated molecular weights of IL-15R α Su/Fc and IL-15 or IL-15N72D. This suggests that these two proteins were glycosylated during mammalian cell production and the IL-15N72D was produced in two major glycosylation forms with molecular weights of 13 kDa and 16 kDa. Based on IEF gel analysis, deglycosylation also reduced the number of the charge variants in the purified complex (data not shown). The relative abundance of these IL-15N72D species in the different purification fractions shown in Fig. 2C is consistent with levels of complex occupancy determined by ELISA and IEF gel analysis.

The IL-15N72D and IL-15R α Su/Fc were separated in reducing SDS-PAGE and the N-terminus amino acid sequences of these proteins were determined using the Edman degradation method. Approximately 15 N-terminal amino acid sequences were obtained for IL-15R α Su/Fc and IL-15N72D, respectively. The determined N-terminal amino acid sequences of these proteins matched their amino acid sequences deduced from the coding regions of the two genes. The amino acid sequences for the two major bands that appeared on reduced SDS-PAGE at 13 and 16 kDa were confirmed to be IL-15N72D. This sequence confirmation again provided the evidence of glycosylation of IL-15N72D in mammalian cells.

3.3. Pharmacokinetics and in vitro and in vivo biological activities of the IL-15N72D:IL-15R α Su/Fc complex

3.4.1 Pharmacokinetic properties—It has previously reported that IL-15 and *in vitro* assembled IL-15:IL-15R α /Fc complex had a 1 h and 20 h serum half-life, respectively, in mice when these proteins were injected intraperitoneally [11]. To assess whether IL-15 and the co-expressed, purified IL-15:IL-15 α Su/Fc complex behaved similarly when administered intravenously, their pharmacokinetic parameters were determined in CD-1 mice. Intravenous administration was chosen because this is likely the route of drug delivery to be used for the IL-15:IL-15 α Su/Fc complex in humans. Female mice were injected intravenously with 1.0 mg/kg IL-15:IL-15 α Su/Fc or 0.28 mg/kg IL-15 (a molar equivalent

dose) and blood was collected at various time points from 15 min to 8 h for IL-15 and 30 min to 72 h for IL-15N72D:IL-15 α Su/Fc post injection. Serum concentrations of IL-15N72D:IL-15 α Su/Fc were evaluated using two ELISA formats, one (anti-IL-15 Ab detection) which detects the intact complex and the other (anti-human IgG Fc Ab detection) which detects only the IL-15 α Su/Fc fusion protein (see Section 2.6). Concentrations of IL-15 were evaluated with a standard IL-15-specific ELISA.

The predicted fit and actual data for IL-15:IL-15 α Su/Fc and IL-15 following the single intravenous bolus injections are shown in Figure 4. The estimated half-life of IL-15:IL-15 α Su/Fc using anti-IL-15 Ab-based or anti-human IgG Fc Ab-based ELISAs was about 25 or 18 h, respectively. These results indicate that the fusion protein was not cleaved and the IL-15 did not significantly disassociate from the IL-15 α Su/Fc molecule *in vivo*. The clearance (Cl) of IL-15:IL-15 α Su/Fc ranged from 0.059 to 0.051 mL/h and the volume of distribution at steady state (V_{ss}) ranged from 2.1 to 1.3 mL depending on the assay format. In comparison, IL-15 had an absorption half-life of 0.24 h and a terminal half-life of 0.64 h. The Cl of IL-15 was 49 mL/h, and the V_{ss} was 18.4 mL. These results indicate that IL-15:IL-15 α Su/Fc displays a >24 fold longer terminal half-life and is cleared >800 fold slower than IL-15.

3.4.2. In vitro and in vivo biological activities—The biological activity of the co-expressed and purified IL-15N72D:IL-15 α Su/Fc complex was evaluated using an IL-15 dependent 32D β cell proliferation assay. For this assay we also generated an *in vitro* assembled (IVA) IL-15N72D:IL-15 α Su/Fc complex (IL-15N72D:IL-15 α Su/Fc IVA) by mixing IL-15N72D and IL-15 α Su/Fc at a 1:1 ratio for 30 min at 4°C. As shown in Figure 5, the IL-15N72D:IL-15 α Su/Fc complex had equivalent biological activity as IL-15N72D:IL-15 α Su/Fc IVA to support growth of 32D β cells. The IL-15N72D:IL-15 α Su/Fc complex exhibited an EC₅₀ of 15.61 pM and the IL-15N72D:IL-15 α Su/Fc IVA displayed an EC₅₀ of 15.83 pM. This demonstrates that the co-expressed IL-15N72D:IL-15 α Su/Fc complex is appropriately processed intracellularly and retains full IL-15 activity after purification. Thus, our method represents a better approach for generating cGMP-grade clinical material than current strategies employing *in vitro* assembly individually produced and in some cases refolded proteins.

The IL-15N72D:IL-15 α Su/Fc complex and IL-15wt were also compared for their ability to induce the expansion of NK cells and CD8⁺ T cells in C57BL/6 mice. As shown in Figure 6, IL-15wt has no significant effect on the expansion of NK and CD8⁺ cells four days after a single intravenous dose of 0.28 mg/kg. In contrast, the IL-15N72D:IL-15 α Su/Fc complex significantly promoted NK and CD8⁺ T cell proliferation in the blood and spleen, which led to lymphocytosis in blood and splenomegaly (Figs. 6 and 7). These findings are consistent with previous reports that IL-15:IL-15 α complexes significantly increased the biological activities of IL-15 *in vivo* [10–14]. This enhanced activity of the IL-15N72D:IL-15 α Su/Fc complex is likely the result of a combination of the increased binding activity of the N72D mutein to the IL-15R $\beta\gamma_c$ complex [5], optimized cytokine *trans*-presentation by the IL-15 α chain *in vivo* (through the FcR receptors on dendritic cells and macrophage), the dimeric nature of the cytokine domain (increased avidity of binding to IL-15R $\beta\gamma_c$) and its increased *in vivo* half-life compared to IL-15 (25 h vs. <40 min).

4. Conclusions

Taken together, we have demonstrated that the IL-15N72D and IL-15 α Su/Fc genes can be co-expressed in recombinant CHO cells and a fully occupied IL-15N72D:IL-15 α Su/Fc complex can be highly purified from cell culture supernatants using a simple and readily scalable purification method. The purified complex retains full biological activities both *in*

vitro and *in vivo*. We are currently employing this expression and purification approach to produce sufficient amounts of highly purified cGMP-grade IL-15N72D:IL-15R α Su/Fc material to support clinical development of IL-15-based immunotherapy.

Highlights

- > Soluble IL-15:IL-15R α superagonist complex was produced at high levels in CHO cells.
- > The highly purified superagonist complex has long serum half-life in mice.
- > This complex exhibits potent immunomodulatory activities on NK and CD8⁺ T cells.

Abbreviations

IL-15N72D	Human IL-15 aa 72 N to D variant
IL-15RαSu	human IL-15 receptor alpha-chain sushi domain

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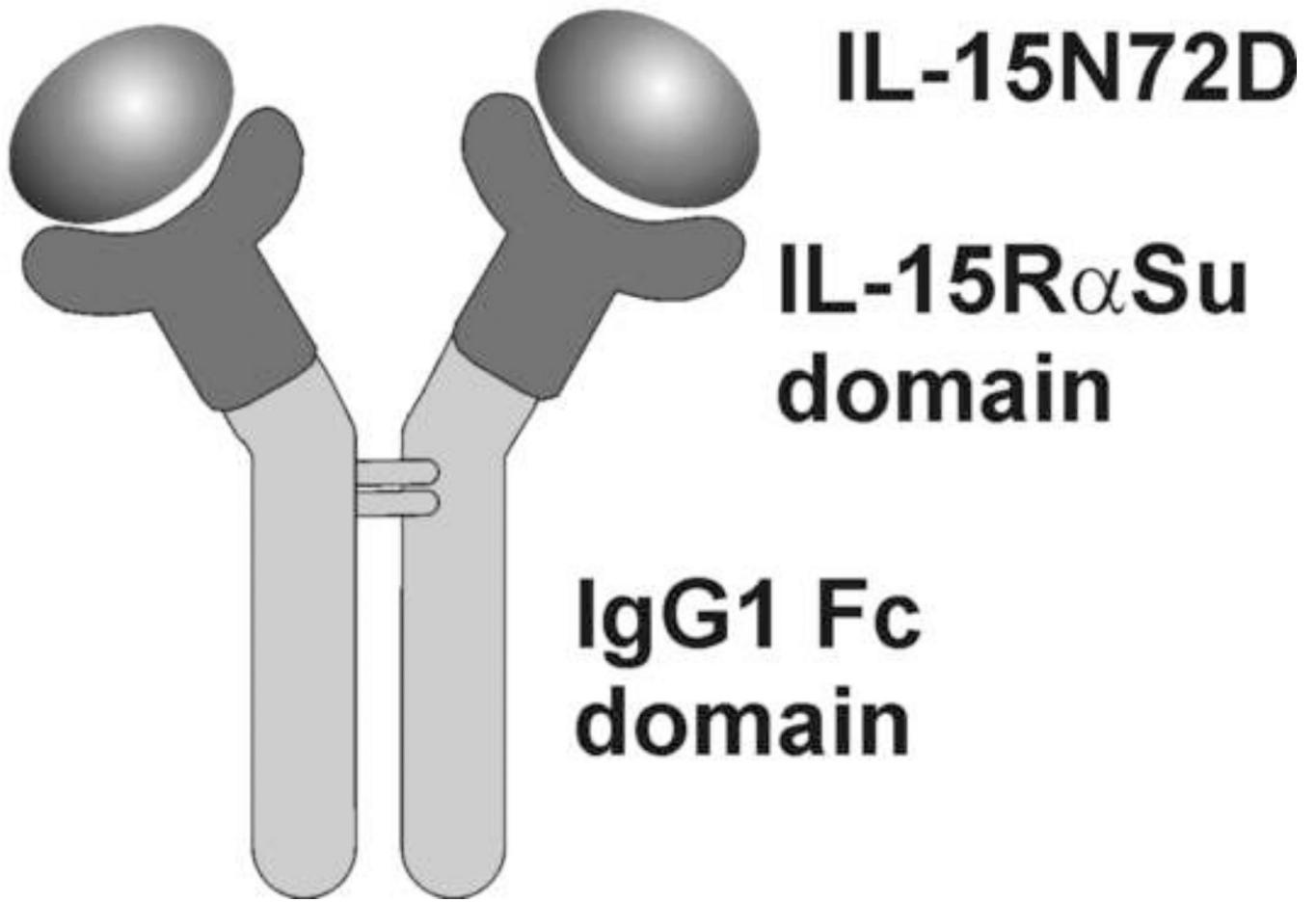


Fig. 1. Schematic drawing of the IL-15N72D:IL-15R α Su/Fc complex consisting IL-15N72D noncovalently associated with the dimeric IL-15R α Su/Fc fusion protein.

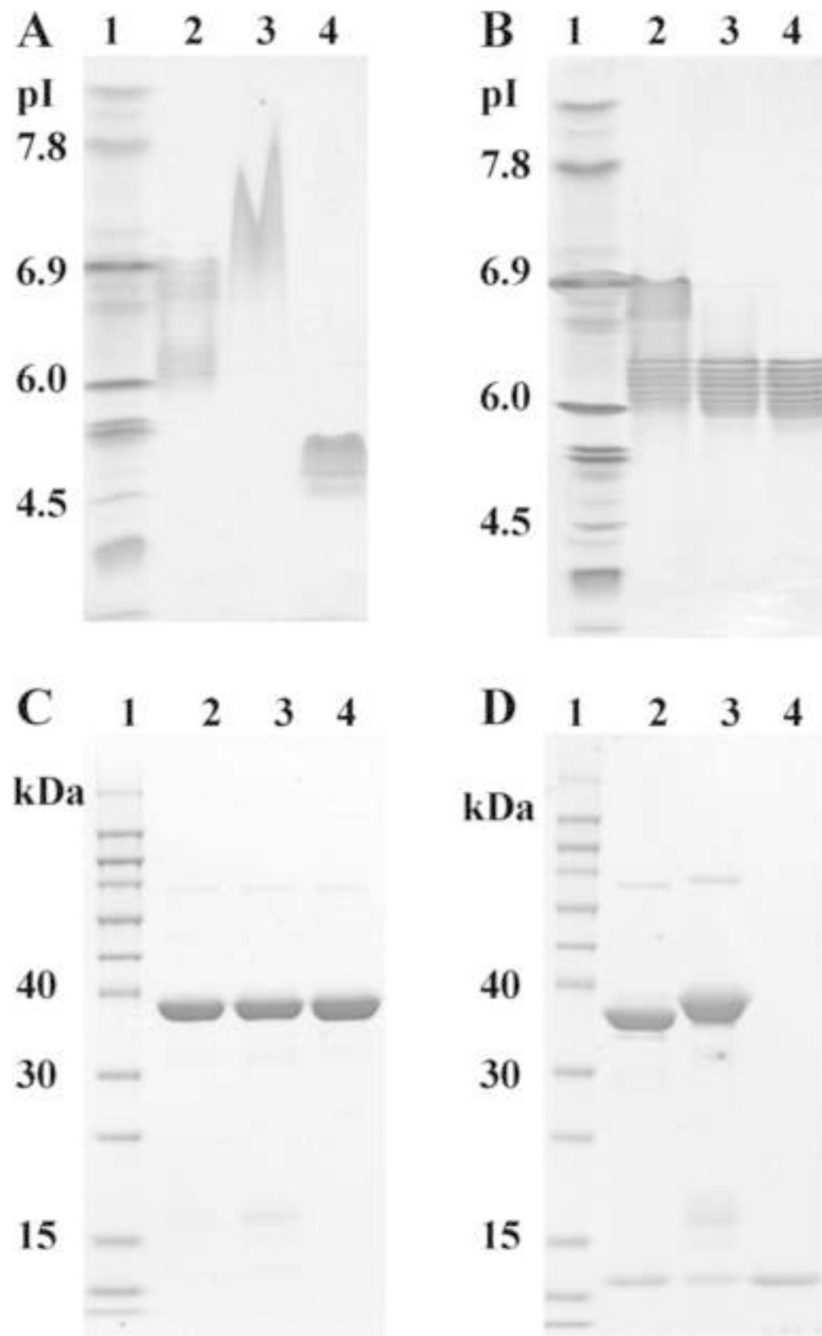


Fig. 2. Gel electrophoresis profiles of IL-15N72D:IL-15R α Su/Fc preparations. (A) IEF pH 3–10 gel analysis. Lane 1, IEF Marker. Lane 2, IL-15N72D:IL-15R α Su/Fc complex purified by rProtein A column. Lane 3, IL-15R α Su/Fc. Lane 4, IL-15wt. (B) IEF pH3-10 gel analysis. Lane 1, IEF Marker. Lane 2, IL-15N72D:IL-15R α Su/Fc complex purified by Q step 1 elution. Lane 3, Q1c by Q step 2 elution. Lane 4, Q2c by Q step 2 elution. (C) SDS-PAGE (reduced) analysis. Lane 1, MW maker. Lane 2, IL-15N72D:IL-15R α Su/Fc complex purified by rProtein A column. Lane 3, IL-15N72D:IL-15R α Su/Fc (Q2c) by Q step 2 elution. Lane 4, IL-15R α Su/Fc (from Q flow through). (D) SDS-PAGE (reduced) analysis

showing protein deglycosylation. Lane 1, MW markers. Lanes 2 and 3 show N-Glycosidase F digested and undigested IL-15N72D:IL-15R α Su/Fc protein, respectively. Lane 4, IL-15wt.

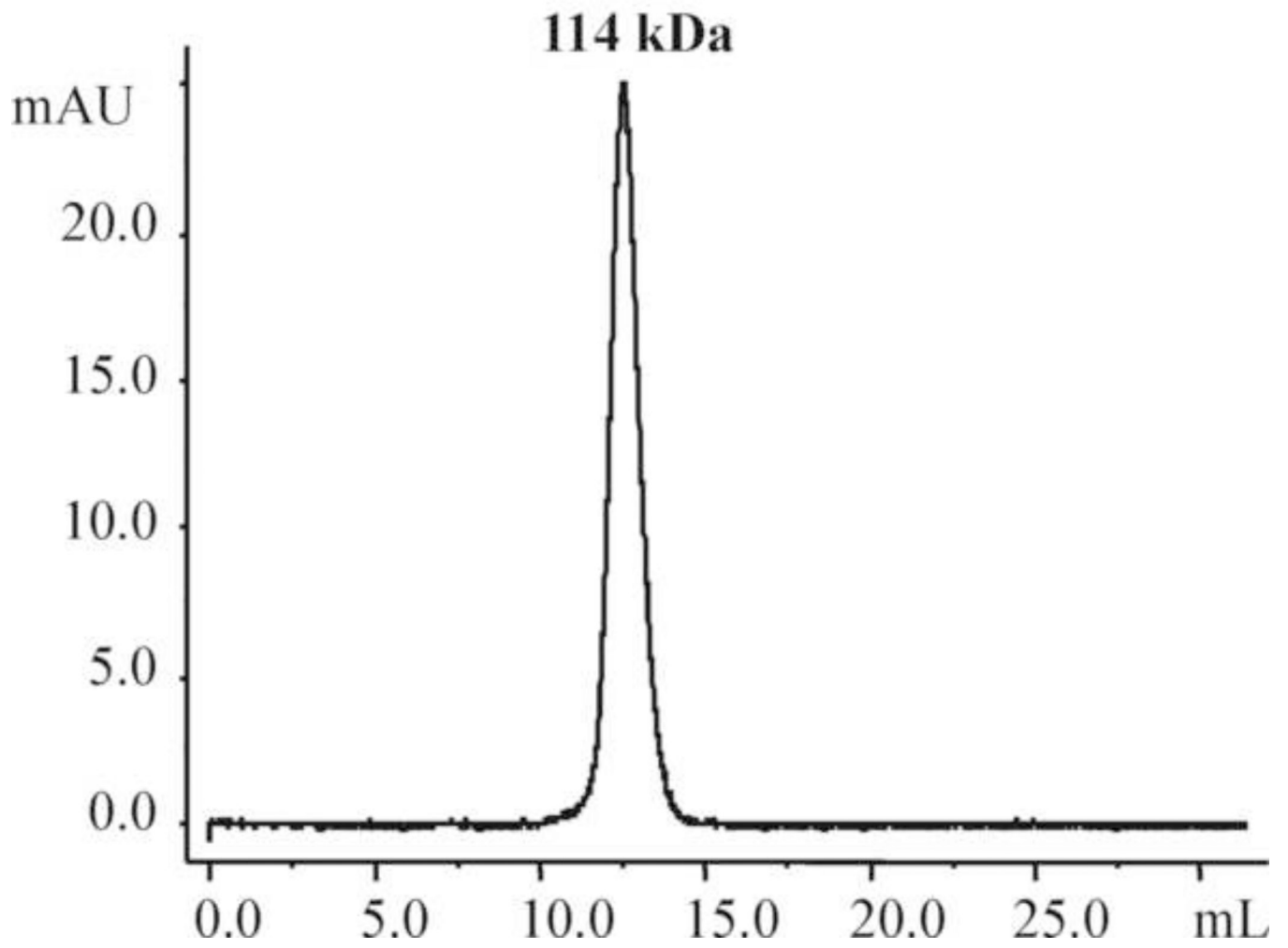


Fig. 3. SEC chromatogram using Superdex 200 HR 10/30 gel filtration column. The purified IL-15N72D:IL-15R α /Fc complex was eluted as a single peak.

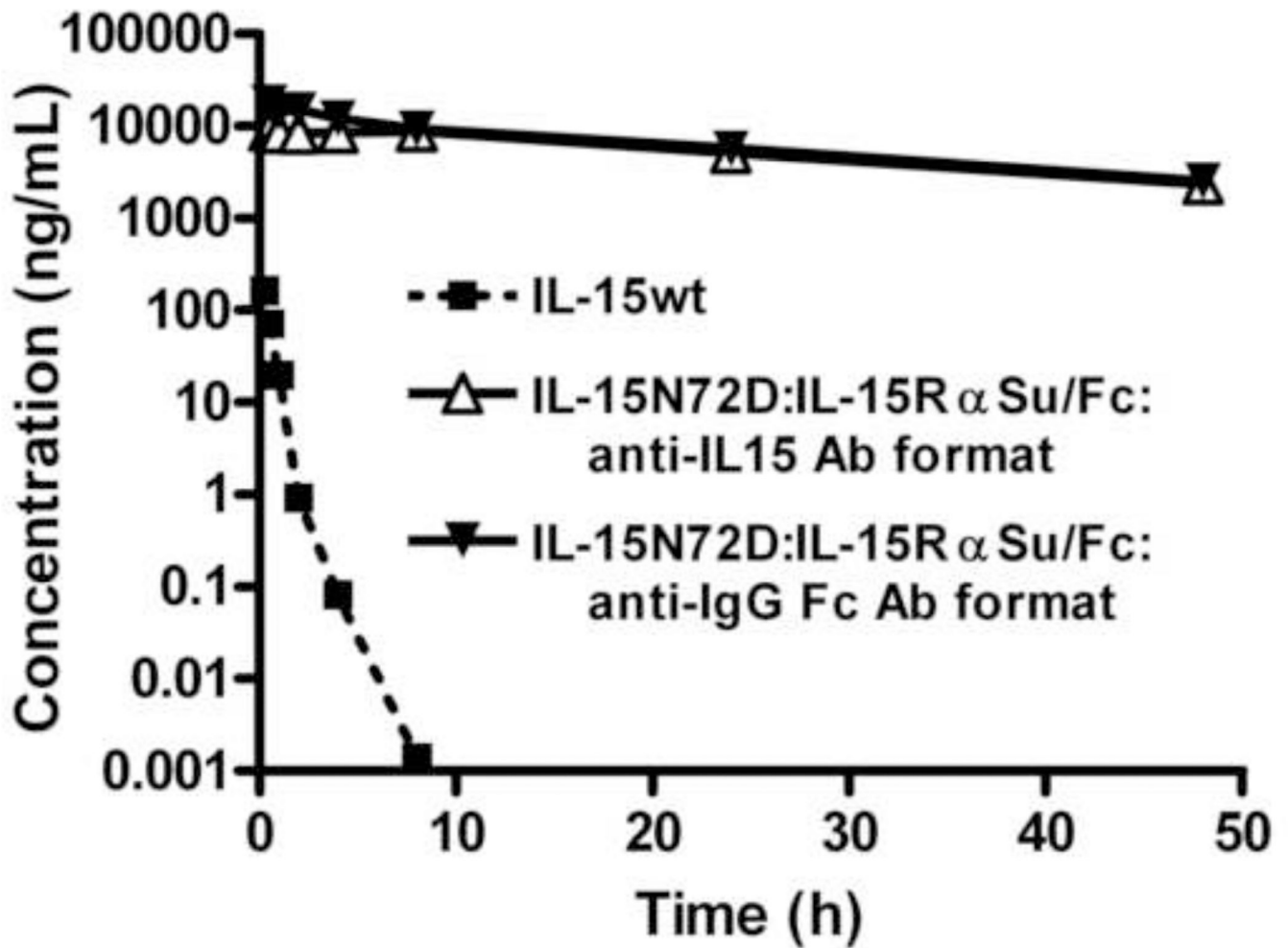


Fig. 4. Comparison of the pharmacokinetic profile of IL-15wt and IL-15N72D:IL-15R α Su/Fc complex following intravenous administration in CD-1 mice. The anti-IL-15 Ab ELISA measures the concentration of IL-15wt (\blacksquare). The anti-IL-15 Ab ELISA measures the concentration of the intact IL-15N72D:IL-15R α Su/Fc molecule (Δ), whereas the anti-human IgG Fc Ab ELISA measures serum concentration of the IL-15R α Su/Fc fusion protein (\blacktriangledown). The observed concentrations are represented by symbols and the model-fitted curves are represented by lines.

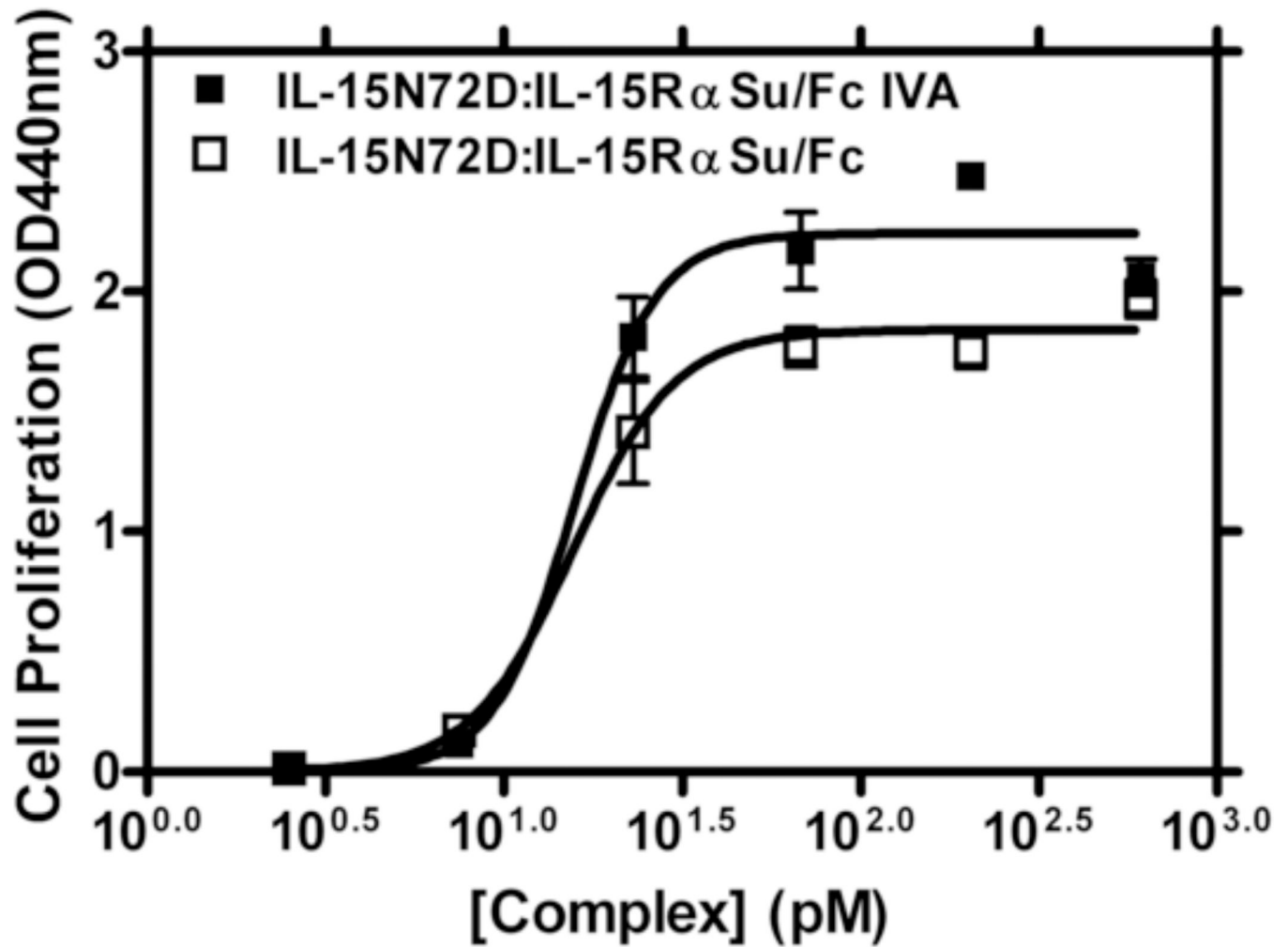


Fig. 5.

Comparison of the biological activity of the *in vitro* assembled IL-15N72D:IL-15R α Su/Fc (IL-15N72D:IL-15R α Su/Fc IVA) with IL-15N72D:IL-15R α Su/Fc. 32D β cells were incubated with increasing concentrations of the *in vitro* assembled IL-15N72D:IL-15R α Su/Fc (■) or IL-15N72D:IL-15R α Su/Fc (□) for 72 h prior to addition of WST-1 for 4 h. Cell proliferation was quantitated by absorbance reading at 440 nm to assess formazan levels. The data points shown are means (\pm standard error) of triplicate samples and the lines represent sigmoidal dose-response curve fit for EC₅₀ determination. The results are representative of at least three experiments.

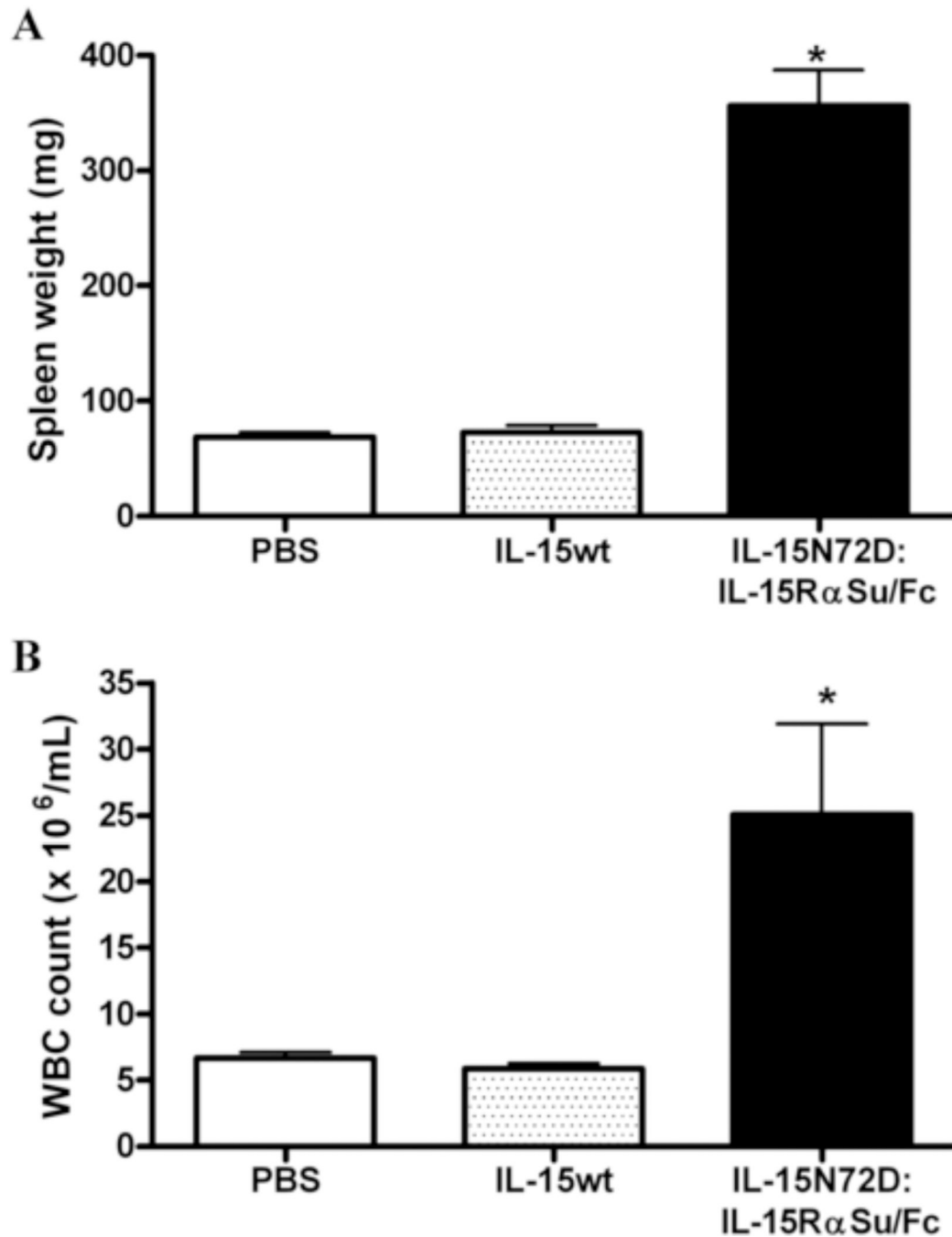


Fig. 6. Effects of IL-15wt and IL-15N72D:IL-15R α Su/Fc complex on spleen weight and white blood cell levels. C57BL/6 mice (5 mice per group) were injected intravenously with a single dose of IL-15N72D:IL-15R α Su/Fc fusion complex at 1 mg/kg IL-15wt at 0.28 mg/kg (molar equivalent dose), or PBS as a negative control. Spleen weights (Panel A) and white blood cell counts in blood (Panel B) were determined 4 days after injection. The bars represent the mean \pm standard error ($n = 5$). * $P > 0.05$ compared to PBS and IL-15wt. The results are representative of at least two experiments.

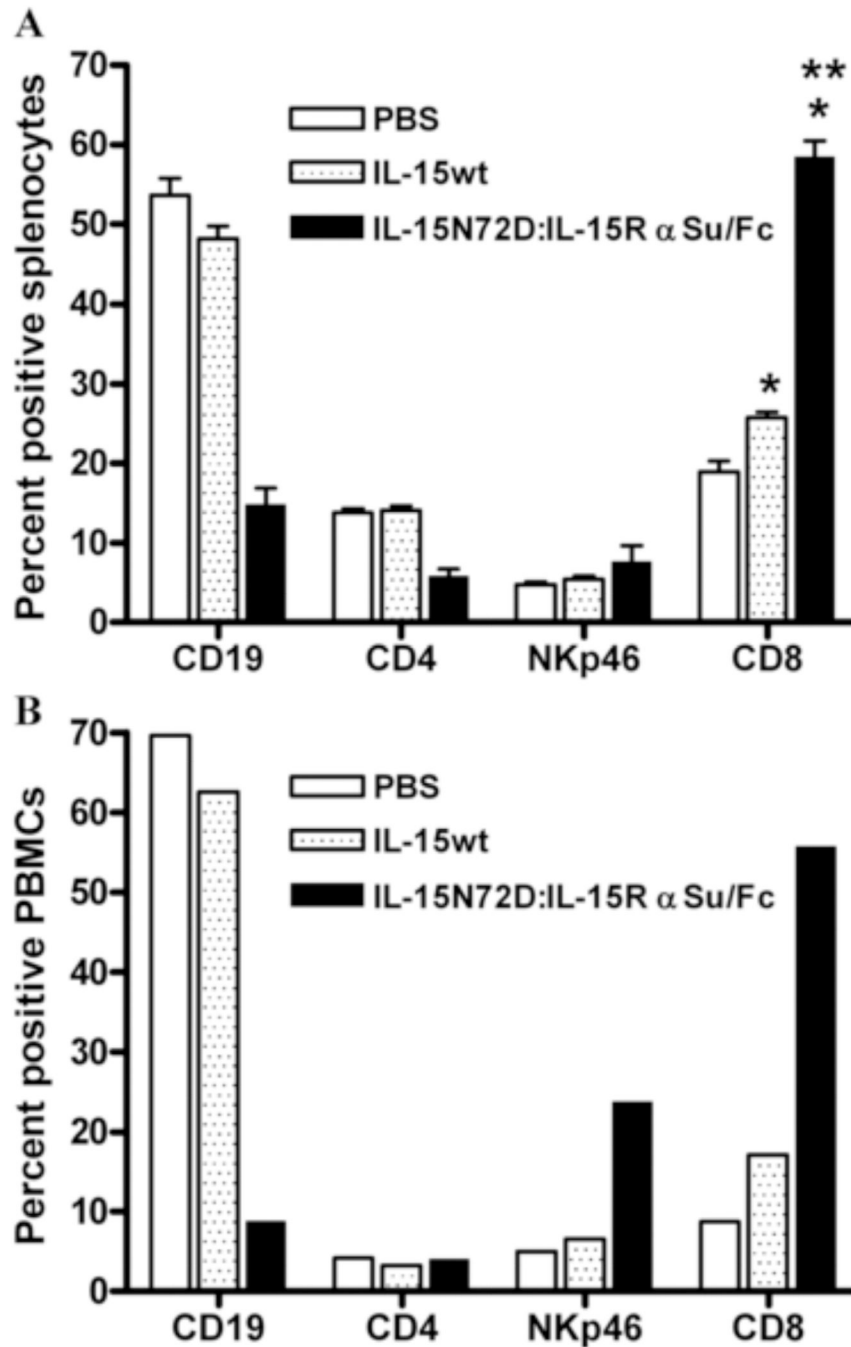


Fig. 7. Effects of IL-15wt and IL-15N72D:IL-15R α Su/Fc complex on mouse lymphocytes. C57BL/6 mice (5 mice per group) were injected intravenously with a single dose of IL-15N72D:IL-15R α Su/Fc fusion complex at 1 mg/kg, IL-15wt at 0.28 mg/kg (molar equivalent dose), or PBS as a negative control. The percentage of B cells (CD19), CD4 T cells (CD4), NK cells (NKp46) and CD8 T cells (CD8) were determined in splenocytes (Panel A: mean \pm standard error (n = 5)) and PBMCs (Panel B: levels in pooled blood (n = 5)) 4 days after injection. * $P > 0.05$ compared to PBS, ** $P > 0.05$ compared to IL-15wt. The results are representative of at least two experiments.