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Development of a recombinant human IL-15·sIL-15Rα/Fc superagonist with improved half-life and its antitumor activity alone or in combination with PD-1 blockade in mouse model

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ABSTRACT

Recombinant human interleukin-15 (IL-15) is a potent cancer immunotherapeutic candidate due to its excellent immune stimulating effects. Previous work demonstrated that IL-15 appeared with short half-life in circulation system, while the complex with its receptor can prolong the half-life as well as benefit its activities *in vivo*. Therefore, IL-15 complex was more favorably considered for clinical development. Herein we developed IL-15·sIL-15Rα/Fc, a complex comprising of IL-15 and the extracellular region of its receptor alpha subunit which fused to Immunoglobulin G (IgG1) Fc to further prolong the half-life in plasma. Through transient gene expression in HEK293 cells, we expressed the superagonist by co-transfection of plasmids encoding IL-15 and sIL-15Rα/Fc respectively, yielding 36 mg/L of product after purification. Pharmacokinetic study demonstrated that the combination profoundly prolonged the half-life of IL-15 to 13.1 h in mice, about 18 folds longer than that of IL-15 monomer which is around 0.7 h. The bioactivity of the superagonist was characterized by CTLL-2 cells proliferation assay *in vitro*, showing its capability of stimulating the expansion of memory $CD8^+$ T cells (cluster of differentiation) in mouse spleen. Using a HT-29 xenograft NOD-SCID mouse model, we observed tumor growth inhibition in all groups that received the superagonist, indicating its anti-tumor efficacy *via* stimulating infused human immune cells. In addition, combo cancer treatment by IL-15·sIL-15Rα/Fc and programmed death-1 (PD-1) antibody have shown stronger inhibitory effects as compared with treatment with either single molecule. Therefore, we developed IL-15·sIL-15Rα/Fc to be a long half-life potential cancer immunotherapy candidate that can be applied alone or in synergy with PD-1/PD-L1 blockade.

1. Introduction

Recombinant human interleukin-15 (IL-15) is a member of the four-helix bundle family of cytokines with strong natural killer (NK) cells and T lymphocytes stimulating activities [1,2]. In comparison with IL-2, IL-15 has more pleiotropic functions on stimulating both innate and adaptive arm of the immune system, which allow it to be an excellent candidate for tumor immunotherapy or as vaccine adjuvant [3–5]. Up to now, there are more than 70 clinical trials related to IL-15 in process (www.clinicaltrials.gov).

IL-15 functions through its receptor IL-15R which contains three subunits: α, β, and γ. IL-15 firstly binds to IL-15Rα to form a heterodimer complex, which subsequently engages IL-15Rβγ complex on the surface of target cells and induces downstream signals. However, the plasma half-life of natural IL-15 is very short, less than 1 h, due to its rapid renal clearance [6]. However, combining with IL-15Rα can effectively stabilize IL-15 and prolong its half-life [6–8]. It was reported that the circulating IL-15 exists actually as a heterodimeric complex with its soluble IL-15Rα (sIL-15Rα) [9,10]. Therefore, IL-15/sIL-15Rα complex is theoretically more favorable for clinical developments. There are already several IL-15 complexes with variants of receptor alpha, typically sIL-15Rα or sushi domain of sIL-15Rα [6,11–13].

Sushi domain, the N-terminal 77 amino acids containing IL-15 binding domain, binds to IL-15 to form a heterodimeric complex

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[12,14]. However, most of the extensive glycosylation site(s) of sIL-15Rα are located beyond the sushi domain, and contribute to the better stability of full length sIL-15Rα than sushi domain [15]. We analyzed the half-lives of all formats of complexes based on published reports, and it was reported that the half-life of RLI with sushi domain was the shortest of 3 h, IL-15·sIL-15Rα heterodimer was a better one of 4 h, and ALT-803 with sushi domain and Fc fusion was the longest of 7.7 h [6,16,17]. To yield a complex with longer half-life than all previously designed, in this study, we developed a superagonist IL-15·sIL-15Rα/Fc complex in which IL-15 non-covalently binds to sIL-15Rα/Fc fusion, and investigated its pharmacokinetics and biological activities.

Concepts of immune-enhancement and immune-normalization in tumor immunotherapy have been intensively discussed recently. The former involves the immune stimulators such as IL-15, and the latter mainly the specific inhibition of checkpoint molecules such as programmed death 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) [18]. Nivolumab, a fully human IgG4 anti-PD-1 monoclonal antibody (mAb), has already achieved great clinical success as a representative checkpoint inhibition antibody drug [19,20]. The combination of immune-enhancement and immune-normalization could lead to complementary or even synergistic effects in cancer therapy. In a mouse model of colorectal carcinoma, the combination of RLI and anti–PD-1 mAb reactivated the terminally exhausted $CD8^+$ T lymphocytes and thereby improved the survival of mice as compared to RLI alone [21]. In this study, we also evaluated the effect of combining IL-15·sIL-15Rα/Fc and anti-PD-1 mAb to provide evidence that the combination strategy could stimulate strong host immunity against cancer.

2. Materials and methods

2.1. Plasmids construction

The human IL-15-expressing plasmid and IL-15sRα/Fc-expressing plasmid were constructed as shown in Fig. 1. The DNA fragments of human IL-15 and IL-15sRα were synthesized by GenScript (Najing, China) according to the NCBI references NM_000585.4 and NM_002189.3 respectively. The expression cassette was driven by a CAG promoter, and the signal peptide named tPA2 (DNA sequence: atggatgcaatgaagagagggctctgctgtgtgctgctgctgtgtggagcagtcttcgtttcgccc-agccaggaaatccatgcccgattcagaagaggagccagagcg) was used for leading protein secretion. All comparts of the plasmid were amplified using PrimeSTAR Max (Takara; Tokyo, Japan) by PCR. All plasmids were purified by an endo-free maxi-prep kit (D6926, OMEGA Bio-tek; Norcross, GA, USA) according to the manufacturer's instructions and then stored at −20 °C.

2.2. Cell culture and transient expression of superagonist

The suspension HEK293E cells were cultured in a 1:1 mixture medium of Freestyle293 (Gibco; Waltham, MA, USA) and SFM4HEK293 (HyClone; Logan, Utah, USA) supplemented with 2% FBS and 100 μg/mL of G418 (Gibco) in shake flasks (Corning; Corning, NY, USA) at 37 °C with 125 rpm rotation and 5% CO_2. Cell growth and viability were monitored using a cell counter (Countstar; Shanghai, China). For transfection, cells reached exponential stage were spun down at 1000 rpm, 5 min and re-suspended at a density of 3×10^6 cells/mL. Then the IL-15-expression plasmid in combination with sIL-15Rα/Fc-expression plasmids were mixed at 1:1 and co-transfected using linear polyethylenimine (PEI) with a molecular weight of 25 kDa (Polysciences; Warrington, PA, USA) as we described previously [22]. Supernatants were harvested after 6 days to collect the secreted IL-15·sIL-15Rα/Fc superagonist. At the same time, sIL-15Rα/Fc was produced as a control by transfecting HEK293 cells with sIL-15Rα/Fc-expression plasmid alone.

2.3. Purification of IL-15·sIL-15Rα/Fc superagonist

The HEK293E cell culture medium was centrifuged and filtered by 0.22 μm filter to remove cells and debris, then loaded to a HiTrap protein A Sepharose Fast Flow column (GE Healthcare Life Sciences; Piscataway, NJ, USA). The column was pre-equilibrated with 20 mM phosphate buffer (PB), pH 7.2. After sample loading at 1 ml/min, the column was washed with 10 column volumes (CV) of 20 mM PB, pH 7.2, followed by 7 CV of washing buffer (0.1 M Na-citrate, pH 5.0) to remove non-specifically bound proteins [23]. The protein was eluted with 0.1 M Na-citrate, pH 3.0, and then immediately neutralized to pH 7.2 using 1 M Tris − HCl, pH 9.0. The preparation was concentrated and buffer exchanged into Phosphate-buffered saline (PBS), pH 7.4, by using an Amicon Ultra-15 centrifugal concentrator (30 kDa cutoff, Millipore; Billerica, MA, USA). The protein product was sterile filtered using a 0.22 μm filter (Corning Life Sciences; Lowell, MA, USA). Separately expressed sIL15Rα-Fc was purified using the same protein A affinity chromatography method as described above. Protein

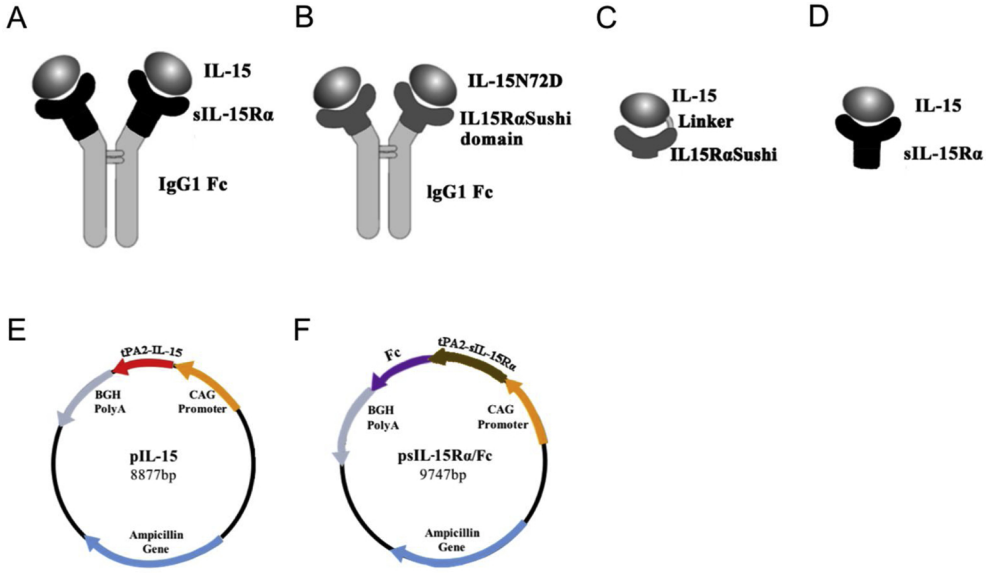

Fig. 1. Scheme of IL-15·sIL-15Rα/Fc complexes and plasmids construction. Scheme of superagonist developed in this study (A) and ALT-803 [33] (B), RLI [11] (C), IL-15·sIL-15Rα heterodimer [6] (D). (E) Plasmid encoding recombinant IL-15. (F) Plasmid encoding sIL-15Rα/Fc.

concentration was determined by BCA kit (Beyotime; Shanghai, China) at 562 nm according to manufacturer's instructions. Endotoxin levels in the final products were evaluated with end-point chromogenic Tachypiens Amebocyte Lysate (TAL) obtained from Bioendo (Xiamen, Fujian, China) according to manufacturer's instructions.

2.4. CTLL-2 cell proliferation assay

Based on its T lymphocytes proliferation stimulating ability, the bioactivity of IL-15·sIL-15Rα/Fc was determined using a colorimetric CTLL-2 cell proliferation assay [24–26]. Briefly, CTLL-2 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1000 U/mL IL-2. For proliferation assay, cells were washed with IL-2 free medium (assay medium) and seeded into 96-well plate at 50 μL/well. IL-15·sIL-15Rα/Fc proteins were diluted to an initial concentration of 75 ng/mL and serially down to 2.93 ng/mL, while the positive control, recombinant human IL-15 monomer produced from *E. coli*, was diluted from 1.3 ng/mL down to 0.045 ng/mL in the assay medium. The diluted samples were added in sextuplicate at 50 μL per well to 96-well plate containing CTLL-2 cells to achieve a final density of 10^4 cells/100 μL/well. After 48 h's incubation at 37 °C with 5% CO_2 and 95% humidity, cells viabilities was measured using Cell Counting Kit-8 (Donjindo; Kumamoto, Japan). The plate was read at wavelength of 450 nm with reference wavelength of 600 nm. The background readings in the wells with blank medium were subtracted from the sample wells read outs. The data was then analyzed using Graphpad Prism software and the four-parameter fit logistic equation to calculate the EC_{50} values.

2.5. Spleen lymphocyte stimulation

To investigate *in vivo* activities of the superagonist, 3 doses (10, 30 or 300 μg per mouse) were injected subcutaneously to 8-week-old female BALB/c mice (Charles River Lab; Beijing, China) to detect their $CD8^+$ T memory cell stimulatory effect [27]. IL-15 monomer produced from *E. coli* (1 μg per mouse) and PBS were used as controls. Due to its longer half-life, IL-15·sIL-15Rα/Fc was administered only once on day 1, while IL-15 monomer was injected every day consecutively for 3 days, day 1, 2, and 3 [28]. On day 5, mice were sacrificed and the single cell suspensions of splenocytes were isolated as previously described [29]. The cells were washed and re-suspended in PBS containing 0.1% BSA, then stained with the following panel of conjugated antibodies: Armenian hamster-anti-mouse CD3-PE-Cy5, rat-anti-mouse CD8-APC, and rat-anti-mouse CD44-PE. Afterwards, cells were fixed and membranes permeabilized to allow staining of mouse-anti-Ki67-FITC. All fluorescence conjugated antibodies and cell fix/perm solutions were purchased from BD Pharmingen (Franklin Lakes, NJ, USA). After staining, the cells were washed twice to remove unconjugated antibodies and measured by flow cytometry BD LSRFortessa (BD Biosciences; San Jose, CA, USA), and the data was analyzed by FlowJo software (Tree Star; Ashland, OR, USA).

2.6. Pharmacokinetic evaluation by ELISA assays

We performed animal experiments with approval by the Animal Care and Use Committee of Shanghai Jiao Tong University. To characterize the half-lives of superagonist and IL-15 monomer, 8-week-old Balb/c mice were treated with 15 μg of IL-15·sIL-15Rα/Fc or 2.5 μg of IL-15 (equimolar of IL-15 molecules) intraperitoneally. Mice were bled over time after treatment with 0.5, 2, 8, 24, 48 h after treatment for IL-15·sIL-15Rα/Fc, and 0.5, 1, 2, 4, 8 h for IL-15 monomer. The plasma levels of proteins were measured using a human IL-15-specific ELISA kit (Sigma-Aldrich, St. Louis, MO, USA) and an human IL-15/IL-15 Rα Complex DuoSet ELISA kit (R&D, Minneapolis, MN, USA) respectively. The serum half-life of IL-15 was calculated using the medical calculator provided by Cornell University per the given instructions (http://www-users.med.cornell.edu/~spon/picu/calc/halfcalc.htm).

2.7. In vivo antitumor experiments with xenograft mice model

To study the antitumor effect of IL-15·sIL-15Rα/Fc, we developed an xenograft mouse model by transplanting HT-29 human colon cells to NOD-SCID mice (male, 10 weeks old) obtained from Charles River Lab (Beijing, China). HT-29 cells were cultured in Mocoy's 5 A Medium from Gibco, containing 10% FBS and 1% P/S at 37°C with 5% CO_2. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation to serve human T lymphocytes with procedures previously we described [8,30]. Thirty-five NOD-SCID mice were injected with 3.0×10^6 HT-29 cells subcutaneously using a volume of 100 μL at day 0 of the experiment, and subsequently received 7.0×10^6 fresh human PBMCs intravenously on the same day. Six days later, all the mice were randomly divided into 7 groups (n = 5) for the following treatments with antitumor reagents. The dosages for each injection and the administration frequencies were designed referring to half-lives of each molecule as following: IL-15·sIL-15Rα/Fc: 10 μg, anti-PD-1 antibody: 200 μg, combination: 10 μg of superagonist and 200 μg of mAb, sIL15Rα/Fc control: 8 μg (equimolar with 10 μg of superagonist), and PBS control on days 6, 9, 12, 15, 19 and 23 [31,32]. All the above drugs were injected intraperitoneally in a volume of 100 μL. Tumor sizes were measured every 3–4 days until mice were sacrificed on day 28, and tumors were removed and processed.

3. Results

3.1. IL-15·sIL-15Rα/Fc encoding plasmids construction

It has been widely accepted that plasma IL-15 exists mainly in the form of heterodimer with sIL-15Rα, which stabilizes IL-15 as well as promotes its trans-presenting immune functions [9,10]. In this study, we aimed to develop a novel molecule with stable IL-15 activity and much longer circulating half-life than previously reported complexes. Therefore, we designed a superagonist with structures shown in Fig. 1A, which employed the full sIL-15Rα instead of sushi domain, as well as fused to Fc fragment. Compared with previously constructed IL-15 complexes as shown in Fig. 1B, C, and D, our structure theoretically should have prolonged half-life due to the effect of both sIL-15Rα and IgG1 Fc. We then constructed two mammalian expression plasmids pIL-15 and psIL-15Rα/Fc which encoding IL-15 and sIL-15Rα/Fc respectively (Fig. 1E&F).

3.2. Expression and purification of IL-15·sIL-15Rα/Fc

To produce the superagonist in HEK293 mammalian cells, we co-transfected plasmids pIL-15 and psIL-15Rα/Fc of equal molar into HEK293 cells, which expressed and assembled the superagonist IL-15·sIL-15Rα/Fc intracellularly. The secretion of the superagonist was led by the signal peptide tPA2 as indicated in 'Materials and Methods'. Using one-step protein A affinity chromatography, we purified the superagonist from the culture medium of transfected cells (Fig. 2A). Using the same procedures, we expressed and purified sIL-15Rα/Fc homodimer as control (Fig. 2B) for experiments afterwards. The concentrations of purified protein fractions were detected by BCA assay, based on which the yields of IL-15·sIL-15Rα/Fc superagonist and sIL-15Rα/Fc homodimer were calculated to be 36 mg/L and 29 mg/L respectively. The highest expression level of IL-15 complexes was reported by Chertova et al. They generated high concentrations of IL-15·IL-15Rα equivalent to 70 mg/L of IL-15 monomer. However, they used stable cell line and expression was evaluated by ELISA assay but not after purification [6]. Moreover, the protein A chromatography was much easier than previous reported methods using HPLC [6].

Due to the fact that IL-15·sIL-15Rα/Fc complex consists non-covalently bound IL-15 and sIL-15Rα/Fc [34], we compared denatured SDS-PAGE to native PAGE to get a more detailed view of the complex. As shown in Fig. 2C, there were several bands in SDS-PAGE indicating the

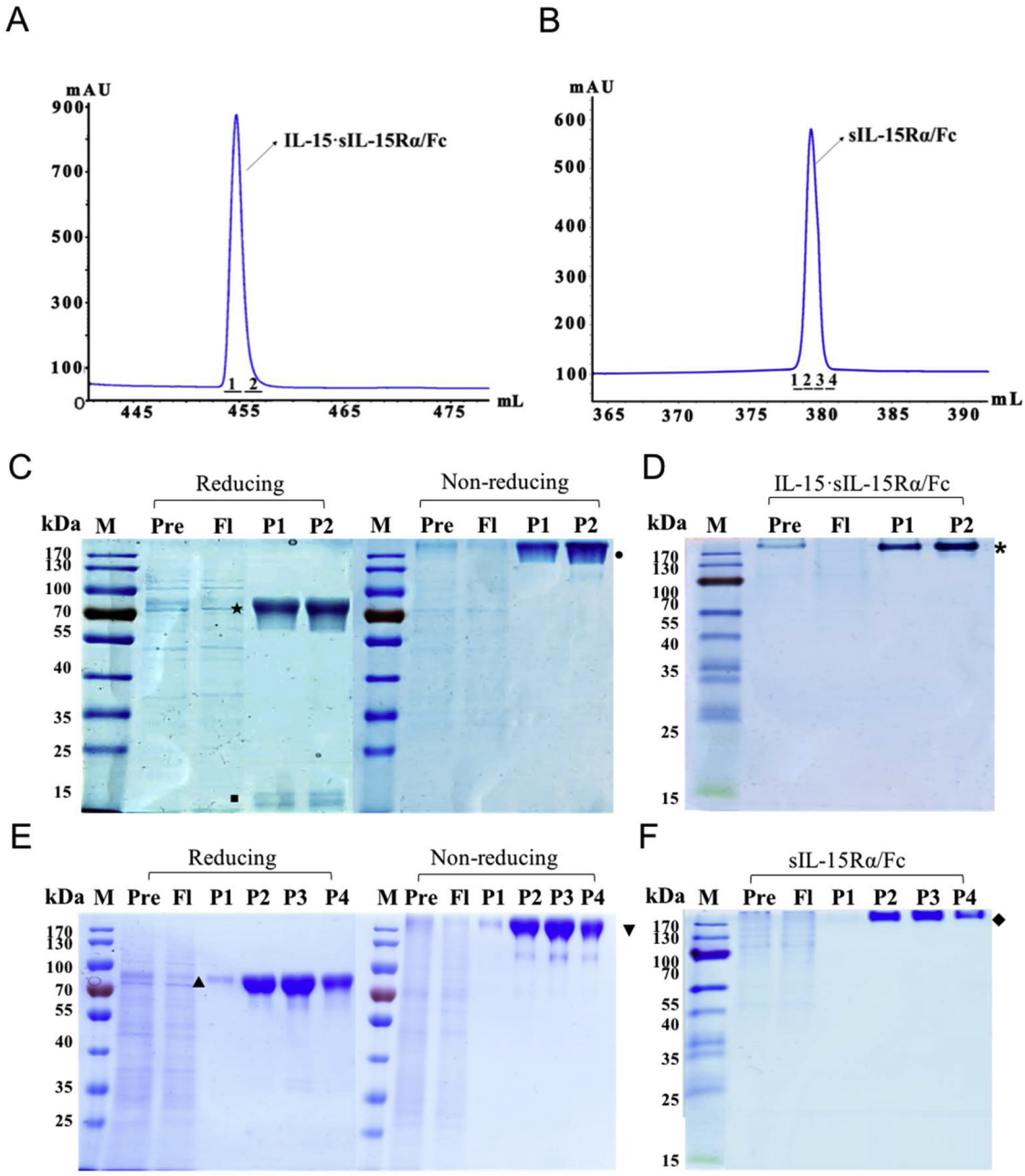

Fig. 2. Purification and characterization of purified IL-15·sIL-15Rα/Fc. IL-15·sIL-15Rα/Fc (A) or sIL15Rα/Fc (B) were purified by protein A affinity chromatography. (C) Reducing (★) and non-reducing (●) SDS-PAGE analysis of purified IL-15·sIL-15Rα/Fc superagonist. The released IL-15 monomer (reducing) was indicated by (■). Lane M: Marker; lane Pre: pre-column loading sample; lane Fl: flow through; lane P1-P2: eluted fractions. (D) Native-PAGE of IL-15·sIL-15Rα/Fc (*); (E) Reducing (▲) and non-reducing (▼) SDS-PAGE of purified sIL-15Rα/Fc stained by Coomassie blue. Lane M: Marker; lane Pre: pre-column loading sample; lane Fl: flow through; lane P1-P4: eluted fractions. (F) Native-PAGE of sIL-15Rα/Fc (◆).

dissociated monomers of IL-15 (bands around 17 kDa, reducing) and sIL-15Rα/Fc (around 80 kDa, reducing), or complexes of IL-15·sIL-15Rα/Fc (around 200 kDa, non-reducing). While in the native PAGE, there was only one band of complex observed (Fig. 2D). The estimated 200 kDa molecular weight of the superagonist was larger than the 114 kDa molecular weight calculated based on the deduced amino acid sequence of IL-15 and sIL-15Rα/Fc fusion proteins. This is likely due to the glycosylation of the proteins produced by mammalian cells as observed in other reports [12].

We performed the same analysis with sIL-15Rα/Fc homodimer as a control, which showed only one band of dimer in native PAGE and non-reducing or monomer in reducing SDS-PAGE (Fig. 2E&F). The results demonstrated that the structure of the superagonist was rather stable. It also indicated that the binding between IL-15 and its receptor is highly related to the tertiary conformation of the molecules, which could be disrupted by reducing regents such as DL-Dithiothreitol (DTT) and β-Mercaptoethanol (β-ME), but not detergents such as Sodium dodecyl sulfate (SDS).

3.3. In vitro bioactivity of IL-15·sIL-15Rα/Fc

To evaluate the bioactivity of the superagonist IL-15·sIL-15Rα/Fc, we performed CTLL-2 cell proliferation assay as described in 'Materials and Methods'. As shown in Fig. 3, the superagonist stimulated the proliferation of CTLL-2 cells from 10 pM, and the EC_{50} was calculated to be around 30 pM. The activity of IL-15·sIL-15Rα/Fc was equivalent or

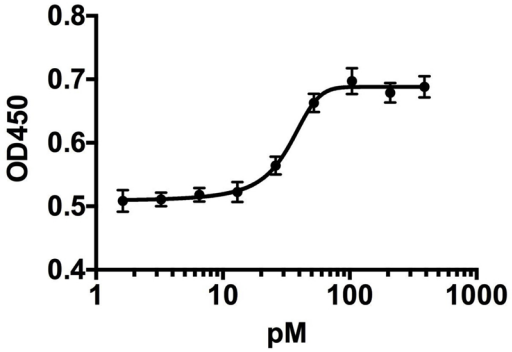

Fig. 3. CTLL-2 cells proliferation assay of purified superagonist to evaluate its bioactivity. The EC_{50} was calculated by the four-parameter fit logistic equation to be 30 pM.

better than other reported IL-15 complexes, which with EC_{50} values between 25 pM and 3 nM [16].

3.4. Prolonged half-life of IL-15·sIL-15Rα/Fc

The half-life of circulating IL-15 in blood is critical for its *in vivo* bioactivities. To evaluate the half-life of the superagonist, 25 mice were treated i.p. with 15 μg of IL-15·sIL-15Rα/Fc, and the control group was treated with 2.5 μg of IL-15 monomer from *E. coli*. The protein concentrations were estimated by BCA assay to avoid the interference of glycosylation on dosages, therefore the two groups of mice received equimolar of IL-15 molecules according to the amino acid sequences based molecular weights of the two drugs. Mice were bled at time points described in 'Materials and Methods' and plasma were collected and tested concentrations of the injected agents. The pharmacokinetics curves were plot as shown in Fig. 4. Plasma concentrations of both IL-15·sIL-15Rα/Fc and IL-15 climbed to peaks and then decreased over time, but that of IL-15·sIL-15Rα/Fc decreased more slowly. With regard to the maximum serum levels, IL-15·sIL-15Rα/Fc peaked 2 h after administration at a concentration of ˜200 ng/mL after administration, whereas IL-15 peaked at a concentration of ˜78 ng/mL around 30 min. When the amount of IL-15 dropped to be lower than 1 ng/mL at 8 h, IL-15·sIL-15Rα/Fc was still as high as 17 ng/mL at 48 h post injection. The half-lives were calculated to be about 13.1 h for IL-15·sIL-15Rα/Fc and 40 min (0.7 h) for IL-15 monomer, indicating the combination with sIL-15Rα and Fc have significantly prolonged the half-life of IL-15 by more than 18 folds.

3.5. In vivo bioactivity assay of IL-15·sIL-15Rα/Fc

Before *in vivo* bioactivity assay of the purified IL-15·sIL-15Rα/Fc, its endotoxin level was examined to be 0.013 EU/μg, so qualified for the following dosages of injections [35]. Since IL-15 is functional to

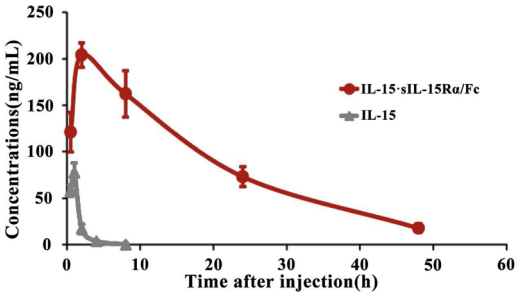

Fig. 4. Pharmacokinetics properties of IL-15·sIL-15Rα/Fc and IL-15 monomer control. The pharmacokinetics curves were drawn and half-lives of therapeutic molecules were calculated to be 13.1 h for IL-15·sIL-15Rα/Fc and 40 min (0.7 h) for IL-15 monomer control.

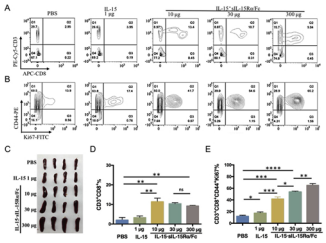

Fig. 5. T lymphocytes stimulatory effect of IL-15·sIL-15Rα/Fc *in vivo*. (A) Representative flow cytometry results of splenocytes differentiated by PE-Cy5-CD3 and APC-CD8. (B) Representative flow cytometry profile of $CD3^+CD8^+$ T cells differentiated by CD44-PE and Ki67-FITC. (C) Spleens separated from experimented mice treated with IL-15·sIL-15Rα/Fc, IL-15 or PBS control, n = 4 for each group. The statistical frequencies of $CD3^+CD8^+$ (D) and $CD3^+CD8^+CD44^+Ki67^+$ (E) of groups were counted respectively. The statistical significance was calculated by two-tailed students' *t*-test and indicated as *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; or 'ns': no significant difference observed.

promote $CD8^+$ T memory cells expansion, $CD3^+CD8^+CD44^+$ subsets of splenocytes were selected to evaluate the potency of the prepared superagonist, using IL-15 monomer as a reference. We found that IL-15·sIL-15Rα/Fc increased the proportion of $CD3^+CD8^+$ cells which was failed to be boosted by IL-15 monomer in our previous study [36], indicating the strong immune stimulatory ability of the superagonist (Fig. 5A). Furthermore, the single injection of IL-15·sIL-15Rα/Fc at all the investigated dosages (10, 30, and 100 μg) significantly elevated the percentages of $CD3^+CD8^+CD44^+Ki67^+$ cells to a much higher level than that of IL-15 monomer control group with 3 injections (1 μg each) (Fig. 5B). In addition, we found that spleens of 30 and 100 μg groups enlarged dramatically due to the rapid accumulation of immune cells (Fig. 5C). The statistics of FACS data revealed that 10 μg was enough to observe the response of $CD8^+$ T cells, but higher dosage can further stimulate the proliferation of the memory subset of $CD8^+$ T cells (Fig. 5D&E). Combining all the results above, we selected the dosage of 10 μg to further evaluate the antitumor efficacy of IL-15·sIL-15Rα/Fc on xenograft mouse model.

3.6. Antitumor efficacy of IL-15 sIL-15Rα/Fc alone and in combination with anti-PD-1 mAb

HT-29 xenograft mouse model was constructed to evaluate the cancer chemotherapeutic effects of the IL-15·sIL-15Rα/Fc. It was reported that the blockade of PD-1/PD-L1 could synergize with several IL-15 complexes containing IL-15, sIL-15Rα, or sushi domain of sIL-15Rα to stimulate and maintain the NK and T cells functions in tumors [37]. Here we also evaluated the antitumor efficacy of the combination of IL-15·sIL-15Rα/Fc and anti-PD-1 mAb, which we prepared from a previous developed stable CHO cell line KPSG-6 according to procedures we reported [23]. Anti-PD-1 mAb was purified by protein A affinity chromatography, and the K_D of anti-PD-1 mAb binding ability was detected to be 9.15 nM (supplementary Fig. S1 & S2). As shown in Fig. 6A, HT-29 xenograft transplantation, PBMCs treatment and immunotherapy with IL-15·sIL-15Rα/Fc or anti-PD-1 mAb were conducted on NOD-SCID mice. Both IL-15·sIL-15Rα/Fc and anti-PD-1 mAb inhibited tumor growth significantly in comparison with PBS as a control. The antitumor effect of IL-15·sIL-15Rα/Fc was contributed to IL-15 component since tumor growth profile of sIL-15Rα/Fc group was close to that of PBS and reached 2000 mm^3 at the end of the experiment (day 28). The most important observation was that anti-PD-1 mAb can dramatically boost the antitumor effect of IL-15·sIL-15Rα, and their combination limited the growth of tumors by about 50% in comparison with PBS group. The combination strategy also showed additive anti-tumor effect over single treatment (Fig. 6B). The difference of tumor growth curves between groups were highly consistent with the tumor size which was

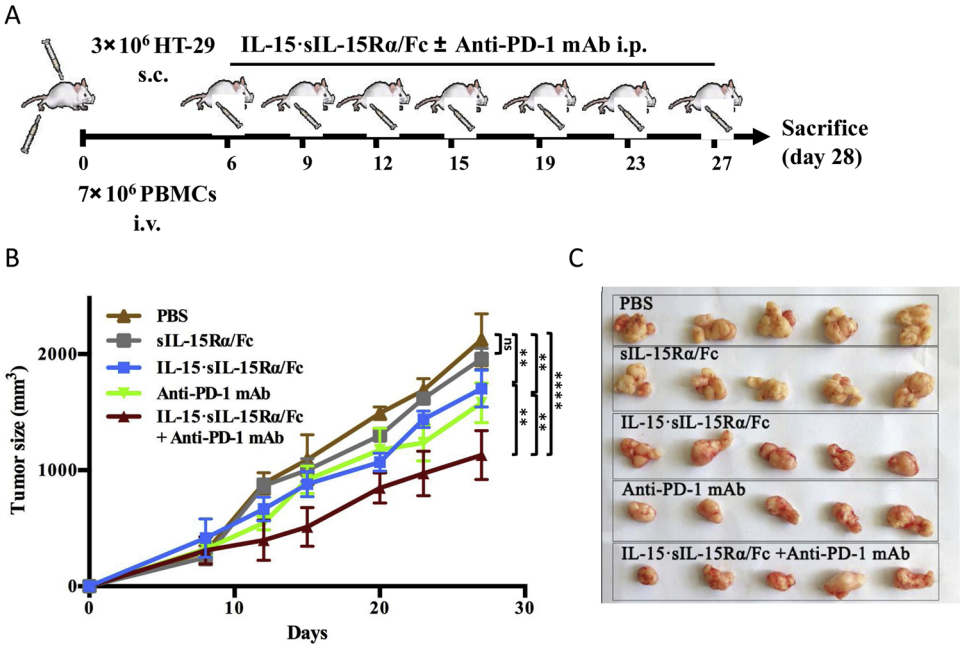

Fig. 6. Xenograft immunotherapy by the superagonist IL-15·sIL-15Rα/Fc. (A) Administration schedule of IL-15·sIL-15Rα/Fc and anti-PD-1 mAb treatment in NOD-SCID mice with colon tumor. Immunostimulatory IL-15·sIL-15Rα/Fc (10 μg) and anti-PD-1 mAb (200 μg) alone or in combination were administered on days 6, 9, 12, 15, 19, 23 and 27, and the antitumor activity against the xenografted human colon cancer mediated by transferred allogeneic human PBMCs were observed. (B) The progression curves of subcutaneous tumor volumes depicted in mean ± SEM with n = 5 for each group. Statistical significance between groups at the end time point was calculated by two-tailed students' *t*-test and indicated as *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; or 'ns': no significant difference observed. (C) Tumors were removed after sacrifice and photographed by groups.

measured after mice sacrifice as shown in Fig. 6C. In the xenograft mouse model, human T lymphocytes from PBMCs were the key element to kill cancer cells, and the superagonist IL-15·sIL-15Rα/Fc has successfully stimulated the expansion and immunotherapeutic activity of T cells. The synergy of immunostimulation and anti-PD-1 mAb mediated immune checkpoints blockade further benefits each other with a maximized outcome of tumor inhibition.

4. Discussion

Co-expression of IL-15 with the subunit of its receptor alpha (IL-15Rα) greatly enhances IL-15 stability and bioavailability [11]. In this study, we constructed a superagonist IL-15·sIL-15Rα/Fc, aiming to obtain a complex with high activity and prolonged half-life *in vivo*. We used transient co-expression of two plasmids in HEK293 cells and a single step of protein A affinity chromatography to produce the molecule. And the product was confirmed to be fully active *in vitro*, with an EC_{50} of 30 pM to stimulate CTLL-2 cells proliferation, which was comparable or even better than previously reported data of IL-15 complexes [16].

There could be three types of molecules in the co-expression product: non-occupied dimeric sIL-15Rα/Fc, partially occupied dimeric sIL-15Rα/Fc + one IL-15, and fully-occupied dimeric sIL-15Rα/Fc + two IL-15. In this study, we firstly optimized the expression by using different ratios of IL-15-expressing and sIL-15Rα/Fc-expressing plasmids (1:1, 3:1, 6:1). However, the excessive IL-15-encoding plasmids of ratio 3:1 or 6:1 did not improve the bioactivity of the final products. The total amount of assembled IL-15·sIL-15Rα/Fc was also decreased due to lower amount of receptor plasmids. The result demonstrated that with equimolar co-transfection it achieved the best yield and product activity (Fig. S3). Therefore, the current process with equimolar plasmids co-transfection and protein A affinity chromatography can produce the superagonist with the best yield and high quality.

Compared with several previously reported IL-15 based complex, IL-15·sIL-15Rα/Fc actually showed an extended half-life which was even longer than ALT-803 (7.7 h s.c.), contributing to longer peptide of sIL-15Rα and its extensive glycosylation. There were 1 occupied N-glycosylation site: N107, and 6 O-glycosylation sites: Thr2, Thr81, Thr86, Thr156, Ser158, and Ser160, in sIL-15Rα, which contributed significantly to improve molecular stability in circulation. In contrast, Sushi domain of ALT-803 contains only the N-terminal 77 amino acids of sIL-15Rα and lacks of N-glycosylation, but with only 1 site of O-glycosylation [15]. Therefore, the rich glycosylation of the superagonist would prevent the degradation and prolong its serum half-life. Considering the different distribution and clearance characteristics of three main administration methods: intravenous (i.v.), intraperitoneal (i.p.), and subcutaneous (s.c.), the 13.1 h serum half-life i.p. of IL-15·sIL-15Rα/Fc is rather long in comparison with reports about other complexes. IL-15 molecule contributes to half-life of the superagonist as well. It was reported that the mammalian expressed IL-15 contained 1 site of N-glycosylation at Asn79, which can partially prevent Asn77-deamidation and prolong the half-life [15]. While the *E. coli* expressed IL-15 should lack of glycosylation and have comparatively shorter half-life. However, half-lives of most of the IL-15 monomer, either mammalian or *E. coli* expressed, were between 30–40 min, as reported previously [12,16]. In this study we used *E. coli* expressed IL-15 monomer as control in pharmacokinetics study and its half-life was ˜40 min, close to the reported values.

The extended half-life greatly benefitted the *in vivo* immune stimulating efficacy of the superagonist. In this study, 10 μg of IL-15·sIL-15Rα/Fc (a single injection, equivalent to 2 μg of IL-15 monomer in molar ratio) increased memory $CD8^+$ T cells much more than 3 μg of IL-15 monomer (3 injections, 1 μg for each injection) as shown in Fig. 5, underlining the superiority of the complex.

IL-15 can play its antitumor effects by enhancing the infiltration of NK cells and $CD8^+$ T cells in tumor tissues [38]. It was reported that IL-15 complex treated early stage tumors but not ones at late stage due to the exhaustion of $CD8^+$ T cells, which could be reversed by PD-1 blockade [21]. And a recent study showed that an oncolytic virus expressing the superagoinst IL-15-IL-15Rα displayed superior antitumor activity when combined with anti-PD-1 antibody in a murine colon cancer model, leading to dramatic tumor regression and prolongs the survival of tumor bearing mice [39]. Therefore, we studied the anticancer effect of IL-15·sIL-15Rα/Fc alone as well as in combination with anti-PD-1 mAb. We used a widely used HT-29 human colon tumor model in NOD-SCID mice, so as to be comparable to human tumors to induce an immunological response by $CD8^+$ T cells [32]. Either IL-15·sIL-15Rα/Fc or anti-PD-1 mAb alone was capable to induce the growth delay of the xenograft, while the combination group showed stronger antitumor responses for their synergistic effects. The data was similar to previous studies about combination of other IL-15 complexes

and PD-1 blockade, indicating that PD-1 blockade could help IL-15·sIL-15Rα/Fc overcome the checkpoint inhibition of T cells, and on the other hand IL-15·sIL-15Rα/Fc promoted effector or memory $CD8^+$ T cell responses and enhances antitumor activity of PD-1 antagonists [21]. And collectively, our data illustrated that the superagonist IL-15·sIL-15Rα/Fc was well developed and promising for cancer immunotherapy, especially in combination with PD-1/PD-L1 pathway blockade reagents.

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical approval

This article does not contain any studies with human participants. All animal studies were evaluated and approved by the Animal Care and Use Committee of Shanghai Jiao Tong University.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.108677.

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