



OPEN

Efficacy and antitumor activity of a mutant type of interleukin 2

Rada Dehghan^{1,7}, Arezoo Beig Parikhani^{1,7}, Sirous Zeinali², Mohamadali Shokrgozar³, Amir Amanzadeh³, Soheila Ajdary⁴, Reza Ahangari Cohan⁵, Yeganeh Talebkhan⁶ & Mahdi Behdani¹✉

Interleukin-2 (IL-2) is an important cytokine in survival, expansion, function of CD8+ T cells and natural killer cells in immunotherapy of melanoma and renal cell carcinomas. Its severe toxicity following binding to its high affinity IL-2 receptor alpha (IL-2R α) has restricted its application in cancer patients. In the present study, we investigated the antitumor efficacy and cytotoxicity of a mutated human IL-2 previously designed by selective amino acid substitutions, and its reduced affinity towards high-affinity IL-2R α (CD25) was approved compared to the wild type IL-2 (wtIL-2). Furthermore, their ability to induce PBMC cell proliferation, and interferon-gamma secretion was compared. The mutant IL-2 also represented higher antitumor activity and more efficient cytotoxicity than wild type hIL-2. The developed mutant IL-2 can be an alternative tool in IL-2 associated immunotherapy of various cancers.

Abbreviations

ConA	Concanavalin A
FDA	Food and Drug Administration
HD-IL-2	High-dose human interleukin-2
IL-2	Human interleukin-2
IL-2R	Interleukin-2 receptor
IFN	Interferon
IPTG	Isopropyl β -D-1-thiogalactoside
Mab	Monoclonal antibody
PBMC	Peripheral blood mononuclear cell
rIL-2	Recombinant wild-type human interleukin-2
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
TNF	Tumor necrosis factor
Treg	Regulatory T cells
VLS	Vascular leak syndrome
wtIL-2	Wild-type human interleukin-2

Interleukin-2 (IL-2), a member of the γ -chain cytokines with molecular weight of 15 kDa comprising 133 amino acids and four alpha-helices, is primarily produced by activated CD4+ and CD8+ T cells^{1–6}. It has been considered an important cytokine in survival, growth, proliferation, and differentiation of T cells and natural killer (NK) cells^{4,7}. It plays crucial roles in induction of cellular and humoral immunity through proliferation and activation of antigen-activated T cells, stimulation of NK cells, and homeostasis^{5,8–10}. Aldesleukin (Proleukin[®]), a recombinant IL-2 analogue, is the first FDA-approved cytokine for immunotherapy of metastatic renal cell carcinoma and malignant melanoma^{1,4,7,8}. However, intravenous administration of IL-2 has been associated with toxicity^{11,12}.

Stimulatory and regulatory functions of IL-2 are mediated by binding to multi-subunit IL-2 receptor (IL-2R) complex consisting of IL-2R α (CD25), IL-2R β (CD122), and IL-2R γ (CD132) subunits. IL-2 receptors consist of three forms; low-affinity (IL-2R α alone), intermediate-affinity (IL-2R $\beta\gamma$), and high-affinity (IL-2R $\alpha\beta\gamma$)^{1,6,13,14}.

¹Venom and Biotherapeutics Molecules Laboratory, Department of Medical Biotechnology, Biotechnology Research Center, Pasteur Institute of Iran, 1316543551 Tehran, Iran. ²Molecular Medicine Department, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran. ³National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran. ⁴Department of Immunology, Pasteur Institute of Iran, Tehran, Iran. ⁵Department of Nanobiotechnology, New Technologies Research Group, Pasteur Institute of Iran, Tehran, Iran. ⁶Department of Medical Biotechnology, Biotechnology Research Center, Pasteur Institute of Iran, 1316543551 Tehran, Iran. ⁷These authors contributed equally: Rada Dehghan and Arezoo Beig Parikhani. ✉email: ytgaroosi@gmail.com; behdani@pasteur.ac.ir

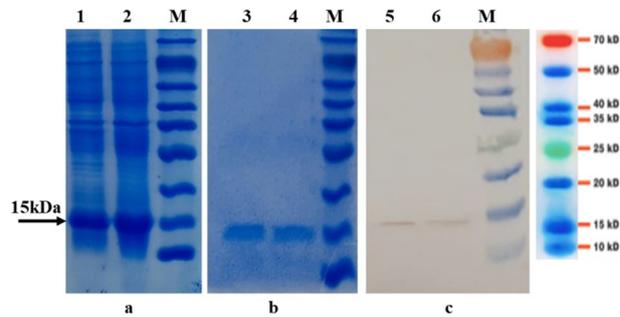


Figure 1. Analysis of protein expression: IPTG Induced bacterial lysate (a), Purified Protein (b) and western blotting analysis (c). #1, 3, 5: wtIL-2; #2, 4, 6: Mutant IL-2; M: Protein molecular weight marker. The mutant IL-2 protein was made by three amino acid substitutions (K35A, E61A, and F42A). The full-size original gels and the blot are presented in Supplementary Fig. S1a, S1b and S1c, respectively.

The alpha subunit of the receptor, the adaptor molecule in high affinity receptors, plays an important role in affinity towards IL-2 while β and γ subunits participate in signal transduction. Activated effector CD4⁺ T cells, CD8⁺ T cells, and regulatory T cells (Treg) constitutively express high-affinity receptor subunits^{1,4}. In contrast, intermediate affinity receptors lacking CD25 subunit are usually expressed in naïve CD8, CD4/CD8 memory T cells, and NK cells^{4,6,10,13–15}.

Low efficacy of IL-2 therapy in cancer patients is due to the short serum half-life, which requires administration of high doses of IL-2 (600,000 IU/kg; 0.037 mg/kg, every 8 h for a maximum of 14 doses) and consequently, severe toxicity and vascular leak syndrome can occur^{1,4,16,17}. In addition, the interaction of IL-2 with its alpha receptor and expansion of regulatory T cells will activate immunosuppressive responses and reduce T cell-mediated anti-tumor activities^{1,6,9,18}.

To overcome this problem, several variants of IL-2 were designed in which binding to IL2-R α was reduced without affecting the affinity of IL-2 towards IL-2R β or γ compared to the native IL-2^{1,4,19}. It has been well documented that mutations in two amino acids, R38 and F42, play important roles in reducing the affinity of IL-2 to CD25^{3,19}. In vitro treatment of human peripheral blood mononuclear cells (PBMCs) with IL-2 variants has reduced the production of inflammatory cytokines, including IL-1 β , TNF α/β , and IFN- γ ^{1,19}. This approach has shown promising results in reducing the IL-2 toxicity compared to the wild type IL-2 and can be effectively applied in immunotherapies^{3,19}.

In the present study, the efficacy and cytotoxicity of a mutant form of IL-2 with reduced binding to the alpha subunit which was previously designed by selective amino acid substitutions, was evaluated compared to the wild type human IL-2 (hIL-2).

Results

Protein expression and purification. After expression and purification of recombinant wtIL-2 and mutant IL-2, the proteins were analyzed on 12% SDS-PAGE (Fig. 1a,b and Supplementary Fig. 1a and 1b), and respective 15kDa proteins were observed. The size of the proteins was confirmed by Western blotting analysis using an HRP-conjugated anti-His antibody (Fig. 1c and Supplementary Fig. 1c).

Proliferation effect of recombinant IL-2 proteins on PBMCs. Cell proliferation assay was performed to determine the efficiency of wild and mutant IL-2 proteins on proliferation of PBMCs using Alamarblue. Both recombinant IL-2 proteins induced PBMC proliferation. However, a comparison of calculated EC50 values (133.3 and 944.8 ng/ml for wild and mutant IL-2, respectively) indicated that wtIL-2 resulted in a significantly higher proliferation than the mutant protein (Fig. 2) ($P < 0.0001$).

Effect of recombinant IL-2 proteins on IFN- γ production. To compare the effect of wild and mutant IL-2 proteins on IFN- γ secretion by T cells, PBMCs were stimulated with recombinant IL-2 proteins, and subsequently, IFN- γ level was measured by ELISA. The results revealed that IFN- γ secretion was significantly increased in response to both IL-2 proteins compared to the untreated control cells ($P < 0.0001$). Moreover, wtIL-2 induced significantly higher levels of IFN- γ compared to mutant IL-2 ($P < 0.05$) (Fig. 3).

Cytotoxicity assay. In a Calcein-AM mediated cytotoxicity assay, IL-2 stimulated effector PBMCs were co-cultured with K562 cell line. Cytotoxicity analysis revealed that increasing the effector to target cell ratio increased the cytotoxicity effect in comparison with the control group, particularly in ratios of 1:1 and 1:5, in which the observed cytotoxicity was significantly higher for cell complexes received mutant IL-2 protein rather than wild IL-2 ($P < 0.0001$). This finding demonstrated mutant IL-2 as a more effective molecule in inducing the cytotoxicity effect of PBMCs against K562 cell line (Fig. 4).

In vivo antitumor activity of recombinant IL-2 proteins. To evaluate the antitumor activity of the expressed IL-2 proteins, tumor-bearing C57BL/6 mice were treated with 1 mg/kg of wild and mutant forms of

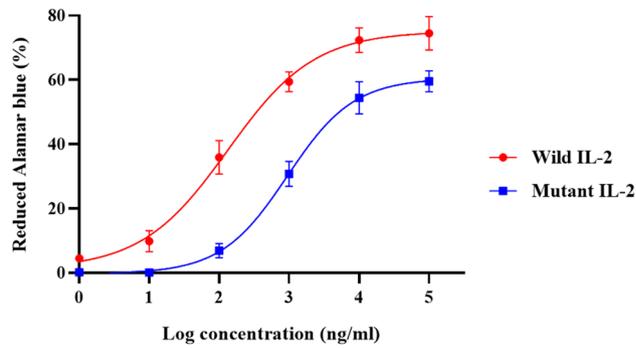


Figure 2. PBMC colorimetric proliferation assay. All experiments were done in triplicate. Data are represented as mean ± SD.

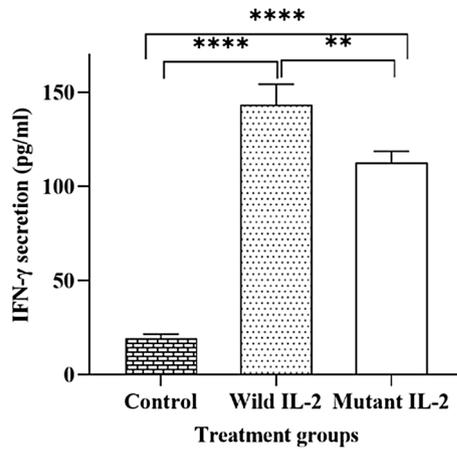


Figure 3. Assessment of IFN-γ secretion in IL-2 treated PBMCs. The results are mean ± SD values of triplicate experiments. Data comparisons were analyzed using ANOVA test (** $P < 0.05$; **** $P < 0.0001$).

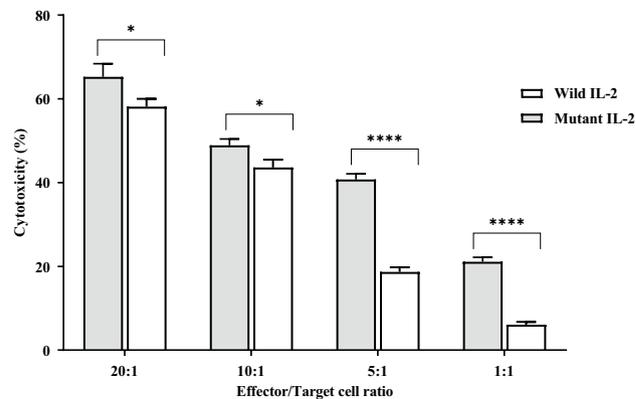


Figure 4. IL-2 cellular cytotoxicity effect in different effector and target cell ratios. All experiments were done in triplicate. Data analysis was done with t-test and represented as mean ± SD (* $P < 0.03$; **** $P < 0.0001$).

IL-2 protein. The tumor size was measured for approximately one month after tumor transplantation, and tumor volume was calculated. It was observed that tumor growth in animals treated with wild or mutant IL-2 proteins was significantly inhibited compared to the PBS treated control mice (Fig. 5). Observations revealed that tumor volume of the mutant IL-2-treated mice was significantly decreased in comparison with the wild IL-2 treated group ($P < 0.001$).

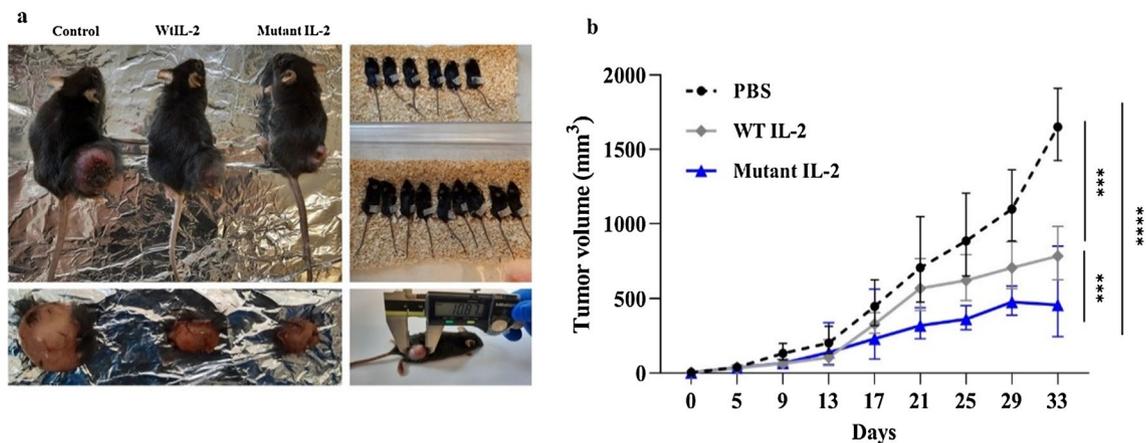


Figure 5. Assessment of in vivo antitumor activity: TC-1 cells (mouse lymphoblast B lymphocytes) were subcutaneously (s.c) injected into the right flank of the mice. 1 mg/kg of recombinant purified IL-2 proteins were injected at the tumor site two times per week for one month. **(a)** Macroscopic tumor sizes in individual mice from three tested groups; **(b)** Inhibited tumor volume in wild or mutant IL-2-treated mice vs. PBS treated control group. Data are represented as mean \pm SD of tumor volume in treated groups (5 mice/group). (** $P < 0.001$, **** $P < 0.0001$).

Discussion

Interleukin 2 plays a crucial role in immunotherapy of metastatic melanoma and renal cell carcinoma⁴. However, short half-life, severe toxicity, and activation of Tregs, which occur through binding to the high-affinity receptor limits its administration in clinic^{1,19}. These side effects may be enhanced by the secretion of several inflammatory cytokines such as IL-1 β , TNF- α , TNF- β , and IFN- γ in IL-2 stimulated PBMCs, which cause hypotension by producing nitric oxide as a mediator of vasodilatation^{17,19}. In addition, these cytokines are involved in binding of IL-2 activated leukocytes to the endothelium by increasing the expression of adhesion molecules, yielding to pathogenic conditions such as vascular leak syndrome^{19,20}.

Several approaches have been used to reduce the cytotoxicity of IL-2, including alterations in the route of administration and its combination with other medicines. Administration of anti-TNF antibodies in a murine model^{3,21} or the use of nitric oxide synthase inhibitors such as N-methyl arginine in dogs received intravenous IL-2 injection could decrease IL-2 side effects and hypotension²². Application of interleukin-2 variants is another approach that has attracted very attention during the last decades^{1,3,4,6}.

In our previous study, potential amino acid substitutions which would reduce the affinity of IL-2 protein towards IL-2R α subunit were identified based on computational studies²³. Molecular dynamic simulations revealed that mutant 2 variant of IL-2 possessing triple mutations of K35A, E61A, and F42A had a significant reduced affinity to IL-2R α which has been confirmed through flow cytometry analysis using anti-CD25 monoclonal antibody. This effect is in agreement with previous studies indicating preferential stimulation of cytotoxic CD8+ T and NK cells, reduced interaction with Tregs and endothelial cells, lower toxicity, and improved immunity in comparison to wIL-2^{1,3,6,19,24–26}. In the present study, the biological activity of a newly designed mutant form of human IL-2 with reduced affinity to CD25 was investigated and compared to the wild IL-2.

First, the effect of the mutant IL-2 on the proliferation of PBMCs and their IFN- γ secretion was examined. The results showed a reduced proliferation rate and IFN- γ secretion level compared to the wild IL-2 molecule. The weaker interaction of mutant IL-2 towards α -subunit could yield a lower proliferation effect on PBMCs, consistent with previous studies^{19,27}.

Our finding is in line with that of Heaton et al. which reported low production of IFN- γ by PBMCs stimulated with a mutant IL-2 (R38A and F42K). They reported that this mutant molecule binds to the intermediate-affinity IL-2R and could induce cytotoxic effects on PBMCs¹⁹. Since IL-1 β , TNF- α , and IFN- γ cytokines are believed to mediate cytotoxicity of IL-2 based immunotherapies^{4,19}, mutant IL-2 with reduced binding to the high-affinity IL-2R may be more effective and less toxic for cancer treatment^{19,28–30}.

IL-2 is a vital cytokine for effective maintenance and control of T cell responses and expansion of the CD8+ T cell population in in vivo conditions^{24,31}. However, IL-2 administration to cancer patients has increased Treg cells at the tumor site which play a fundamental role in immune system inhibition and anti-tumor responses^{6,24}. Our results indicated that despite a lower proliferation rate and IFN- γ production, the mutant IL-2 could induce a more cytotoxic effect in PBMCs against K562 cells than the wIL-2. To examine the toxicity of the molecule in in vivo conditions, mice were transplanted with TC-1 cells to induce tumoral tissue. The results revealed that local administration of recombinant IL-2 proteins could control the tumor growth, which was more prominent with mutant IL-2 than the wIL-2.

Levin et al., designed an IL-2 superkine with a decreased affinity toward IL-2R α subunit²⁵. They showed that superkine could induce superior expansion of cytotoxic T cells, improve anti-tumor responses in in vivo conditions, and elicit proportionally lesser expansion of Tregs and pulmonary oedema. In a similar study, Chen et al., reported that FSD13 mutant form of IL-2 was effective in NK92 cell line proliferation and activation of CD8+ T cells without considerable side effects or damages in lungs or liver of tested animals⁶. Our results are consistent

with previous studies which used mutagenesis to reduce the binding of IL-2 molecule to the high-affinity IL-2R to improve its therapeutic effects^{1,4,6,25,32}.

In conclusion, this study indicated that the mutant IL-2 (K35A, E61A, and F42A) could be a promising agent for IL-2-based immunotherapy of cancer. Further physiological studies regarding lung oedema as well as kidney and liver function are required to be evaluated.

Materials and methods

Ethics statement. All experiments and procedures were approved by the ethics committee of Pasteur Institute of Iran (IR.PII.REC.1400.034) and performed in accordance with the approved guidelines and regulations. Animal studies were performed in accordance with the ARRIVE reporting guidelines³³. Human blood samples were obtained from one of the authors as a volunteer according to the same proposal approved by the ethics committee of Pasteur Institute of Iran and in accordance with the Declaration of Helsinki and other relevant guidelines, including the ethical guidelines for medical and health research on human subjects by the Iranian government. The informed consent was obtained before human blood sampling.

Cloning, expression and purification of wild and mutant hIL-2. The amino acid sequence of the wild IL-2 was obtained from drug bank (Accession No. DB00041). In our previous study, IL-2 mutant molecules were designed through computational approaches. In brief, the amino acid residues involved in electrostatic interactions between IL-2 and IL-2R α subunit were identified and alanine mutations were applied at selected positions and the variant with the lowest affinity towards IL-2R α (M2 molecule, K35A, E61A, and F42A) was identified using docking, molecular dynamics simulation, umbrella sampling, and Gibbs energy calculations²³. The two IL-2 encoding gene fragments were separately subcloned into pET28a expression vector at *NcoI* and *HindIII* restriction sites. The identity of the gene fragments was confirmed through restriction digestion and sequencing.

E. coli BL21 (DE3) strain was transformed with recombinant vectors and inoculated into 200 ml Luria Bertani (LB) medium. The expression of wild and mutant hIL-2 proteins was induced with 0.5 mM isopropyl β -D-1-thiogalactoside (IPTG) at OD_{600nm} of 0.5 and incubated for additional 6 h. The bacterial pellet was resuspended in lysis buffer I (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM Imidazole; pH8.0). The cells were sonicated at 100% amplitude (30 s pulses with 10 s intervals) and centrifuged at 10,000 \times g for 20 min. The pellet, containing inclusion bodies, was solubilized in lysis buffer II (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM Imidazole, 8 M Urea; pH8.0) and placed under agitation for 1 h. After centrifugation (10,000 \times g for 30 min), the supernatant was loaded to Ni-NTA agarose column (ABT, Spain) at a flow rate of 1 ml/min. Refolding of the proteins was carried out through a gradient of urea concentration (from 8 M to 0) using the refolding buffer (50 mM NaH₂PO₄, 500 mM NaCl, 20 mM Imidazole; pH8.0). Histidine-tagged protein was eluted by the elution buffer (50 mM NaH₂PO₄, 500 mM NaCl, 250 mM imidazole; pH8.0). Protein concentration was determined by UV adsorption at 280 nm using a spectrophotometer (BioTeK, USA). The purity of the eluted protein was analyzed on 12% SDS-PAGE and Coomassie Brilliant Blue staining.

For western blotting, proteins were transferred to nitrocellulose membrane. After blocking the membrane with 3% w/v skim milk in phosphate-buffered saline (PBS) overnight (o/n) at 4 °C, the membrane was washed with PBS supplemented with 1% Tween-20 for 5 min (3 times). The membrane was treated with 1:2000 dilution of HRP-conjugated anti-His antibody, and the protein bands were visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB) solution as the substrate.

PBMC proliferation assay. Alamarblue assay was used to evaluate the proliferative responses of lymphocytes to the recombinant proteins. Heparinized peripheral blood was obtained from a healthy volunteer after approval by the Ethics committee of Pasteur Institute of Iran (IR.PII.REC.1400.034) according to the declaration of Helsinki and other relevant guidelines. PBMCs were isolated on a Ficoll-Hypaque (Lymphoprep, Nyegaard, Norway) density gradient approach¹⁷. PBMCs were washed and resuspended in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen, UK), 2 mM L-glutamine, and 10% fetal calf serum (FCS, Sigma). The cells were seeded at a density of 2×10^5 cells/well and stimulated with 2 μ g/well of concanavalin A (Con A, Sigma). Negative control cells were treated with medium without any mitogen. The cells were incubated at 37 °C, 5% CO₂ for 24 h. Subsequently, wtIL-2 or mutant IL-2 proteins were added to the cells in a range of 0.01 nM to 10 μ M and incubation continued for 48 h. Then, Alamarblue reagent was added to the wells and incubated for 16 h. Replicates of each experiment were analyzed on a microplate reader (BioTek ELx808, USA) at 570 and 600 nm. The percentage of reduction in Alamar blue was calculated according to the Manufacturer's recommendations (Thermo Fisher Scientific, USA).

IFN- γ assay. Isolated PBMCs were seeded at a density of 2×10^6 cells/ml in 24-well plates, treated with 1 μ g IL-2 proteins and incubated at 37 °C for 48 h. The supernatants were harvested and IFN- γ secretion level was measured using the Human IFN- γ ELISA kit (Karmania Pars Gene, IRAN).

Cytotoxicity assay. Cytotoxicity assay was performed using Calcein-AM bio-detergent. Isolated PBMCs from Healthy donor were served as effector cells (E) against the target cell line (T), K562 (Human leukemia cell). In brief, PBMCs were stimulated with 1 μ g mutant IL-2 or wtIL-2 for 48 h. Then, 1×10^6 K562 cells/ml were labeled with 2 μ M Calcein-AM (R&D Systems, USA) in complete RPMI-1640 medium, washed twice with the medium and incubated for 30 min at 37 °C. PBMCs were co-cultured with labeled K562 cells in specific cell ratios (T/E ratios of 1:1, 1:5, 1:10, 1:20) 96-well plates and incubated under 5% CO₂ at 37 °C for 12 h in triplicate. The culture supernatants were transferred into a black 96-well plate and fluorescence was measured using a

fluorometer (BioTeK, USA) at 490 nm excitation and 520 nm emission wavelengths. Target cells within complete medium and the cells treated with 1% Triton X-100 were considered as negative and positive controls representing spontaneous and maximum fluorescence release, respectively. Cytotoxicity was calculated according to the following formula³⁴:

$$\text{Cytotoxicity (\%)} = [\text{test release} - \text{spontaneous release}] / [\text{maximum release} - \text{spontaneous release}] \times 100$$

Antitumor activity. Fifteen female C57BL/6J mice (6–8 weeks, 20 g) were purchased from the animal resource center (Pasteur Institute of Iran) and maintained under standard housing conditions. The research protocols and all animal studies were approved by the Ethics committee of Pasteur Institute of Iran (IR.PII.REC.1400.034) and followed ARRIVE reporting guidelines³³. Lymphoblast B lymphocyte TC-1 (#ATCC: CRL-2785) cell line was obtained from the department of Cell Bank (Pasteur Institute of Iran) and cultured in DMEM medium supplemented with 10% Fetal bovine serum (FBS, Sigma) at 37 °C under 5% CO₂ atmosphere. On day 0, 1×10^6 cells in PBS were injected subcutaneously (*s.c*) into the right flank of the mice and tumor growth was daily examined until the tumor dimension reached 50 mm³. After scarification of the mice by cervical dislocation, solid tumor samples were thinly sliced (2 mm thick) and subcutaneously transplanted into the shaved right flank of 15 mice which were anesthetized with intraperitoneal (*i.p*) ketamine (10 mg/ml)/xylazine (10 mg/ml) (Sigma, USA) formulated in water and randomly divided into three groups (5 mice per group). Mutant and wild type groups were injected by the recombinant purified IL-2 proteins diluted in sterile PBS to a final concentration of 1 mg/kg at the tumor site, two times per week for one month. The control group received 200 µl PBS. Tumor size in each group was measured using a caliper and tumor volume was calculated by the following formula: $0.5 \times \text{length} \times \text{width}^2$ of the tumor³⁵.

Statistical analysis. Data analysis was performed with GraphPad Prism (v. 8.0). The significance level between two or more groups was determined by two-tailed unpaired t-test and One-way ANOVA analysis of the variance.

Received: 6 September 2021; Accepted: 21 March 2022

Published online: 30 March 2022

References

- Carmenate, T. *et al.* Human IL-2 mutein with higher antitumor efficacy than wild type IL-2. *J. Immunol.* **190**, 6230–6238 (2013).
- Ju, G. *et al.* Structure-function analysis of human interleukin-2. Identification of amino acid residues required for biological activity. *J. Biol. Chem.* **262**, 5723–5731 (1987).
- Tang, A. & Harding, F. The challenges and molecular approaches surrounding interleukin-2-based therapeutics in cancer. *Cytokine X*. **1**, 100001 (2019).
- Sim, G. C. *et al.* IL2 variant circumvents ICOS+ regulatory T-cell expansion and promotes NK cell activation. *Cancer. Immunol. Res.* **4**, 983–994 (2016).
- Malek, T. R. The biology of interleukin-2. *Annu. Rev. Immunol.* **26**, 453–479 (2008).
- Chen, X. *et al.* A novel human IL-2 mutein with minimal systemic toxicity exerts greater antitumor efficacy than wild-type IL-2. *Cell. Death. Dis.* **9**, 989 (2018).
- Rosenberg, S. A. IL-2: The first effective immunotherapy for human cancer. *J. Immunol.* **192**, 5451–5458 (2014).
- Rao, B. M., Girvin, A. T., Ciardelli, T., Lauffenburger, D. A. & Wittrup, K. D. Interleukin-2 mutants with enhanced α -receptor subunit binding affinity. *Protein. Eng.* **16**, 1081–1087 (2003).
- Sun, Z. *et al.* A next-generation tumor-targeting IL-2 preferentially promotes tumor-infiltrating CD8+ T-cell response and effective tumor control. *Nat. Commun.* **10**, 3874 (2019).
- Boyman, O. & Sprent, J. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat. Rev. Immunol.* **12**, 180–190 (2012).
- Tester, W. J. *et al.* A randomized Phase II study of interleukin-2 with and without beta-interferon for patients with advanced non-small cell lung cancer: An Eastern Cooperative Oncology Group study (PZ586). *Lung Cancer* **25**, 199–206 (1999).
- Mi, D., Ren, W. & Yang, K. Adoptive immunotherapy with interleukin-2 & induced killer cells in non-small cell lung cancer: A systematic review & meta-analysis. *Indian J. Med. Res.* **143**, S1–S10 (2016).
- Liao, W., Lin, J.-X. & Leonard, W. J. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. *Immunity* **38**, 13–25 (2013).
- Lin, J. X. & Leonard, W. J. Signaling from the IL-2 receptor to the nucleus. *Cytokine. Growth. Factor. Rev.* **8**, 313–332 (1997).
- Chinen, T. *et al.* An essential role for the IL-2 receptor in T reg cell function. *Nat. Immunol.* **17**, 1322–1333 (2016).
- Dutcher, J. P. *et al.* High dose interleukin-2 (Aldesleukin)-expert consensus on best management practices-2014. *J. Immunother. Cancer.* **2**, 26 (2014).
- Siegel, J. P. & Puri, R. Interleukin-2 toxicity. *J. Clin. Oncol.* **9**, 694–704 (1991).
- Cesana, G. C. *et al.* Characterization of CD4+ CD25+ regulatory T cells in patients treated with high-dose interleukin-2 for metastatic melanoma or renal cell carcinoma. *J. Clin. Oncol.* **24**, 1169–1177 (2006).
- Heaton, K. M., Ju, G. & Grimm, E. A. Human interleukin 2 analogues that preferentially bind the intermediate-affinity interleukin 2 receptor lead to reduced secondary cytokine secretion: Implications for the use of these interleukin 2 analogues in cancer immunotherapy. *Cancer Res.* **53**, 2597–2602 (1993).
- Rosenberg, S. A. *et al.* Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients. *Ann. Surg.* **210**, 474–484 (1989).
- Fraker, D. L., Langstein, H. N. & Norton, J. A. Passive immunization against tumor necrosis factor partially abrogates interleukin 2 toxicity. *J. Exp. Med.* **170**, 1015–1020 (1989).
- Kilbourn, R., Owen-Schaub, L. & Griffith, O. Interleukin-2 (IL-2) mediated hypotension in dogs is reversed by N-methyl-L-arginine (NMA), an inhibitor of nitric oxide (NO) formation. *Proc. Am. Assoc. Cancer Res.* **33**, 328 (1992).

23. Beig Parikhani, A. *et al.* Human IL-2 α subunit binding modulation of IL-2 through a decline in electrostatic interactions: A computational and experimental approach. *PLoS One*. **17**, e0264353 (2022).
24. Carmenate, T. *et al.* Blocking IL-2 signal in vivo with an IL-2 antagonist reduces tumor growth through the control of regulatory T cells. *J. Immunol.* **200**, 3475–3484 (2018).
25. Levin, A. M. *et al.* Exploiting a natural conformational switch to engineer an interleukin-2 'superkine'. *Nature* **484**, 529–533 (2012).
26. Mott, H. R. *et al.* The solution structure of the F42A mutant of human interleukin 2. *J. Mol. Biol.* **247**, 979–994 (1995).
27. Liang, S. *et al.* Biological characterization of human interleukin-2 mutant proteins. Structure–activity relationship studies. *J. Biol. Chem.* **263**, 4768–4772 (1988).
28. Burdach, S. *et al.* Differential regulation of lymphokine production by distinct subunits of the T cell interleukin 2 receptor. *J. Clin. Invest.* **87**, 2114–2121 (1991).
29. Reem, G. H. & Yeh, N. H. Interleukin 2 regulates expression of its receptor and synthesis of gamma interferon by human T lymphocytes. *Science* **225**, 429–430 (1984).
30. Vilcek, J. *et al.* Regulation of IFN-gamma induction in human peripheral blood cells by exogenous and endogenously produced interleukin 2. *J. Immunol.* **135**, 1851–1856 (1985).
31. Beyer, M. *et al.* In vivo expansion of naïve CD4+ CD25(high) FOXP3+ regulatory T cells in patients with colorectal carcinoma after IL-2 administration. *PLoS One*. **7**, e30422 (2012).
32. Ahmadzadeh, M. & Rosenberg, S. A. IL-2 administration increases CD4+ CD25hi Foxp3+ regulatory T cells in cancer patients. *Blood* **107**, 2409–2414 (2006).
33. Percie du Sert, N. *et al.* The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *J. Cereb. Blood. Flow. Metab.* **40**, 1769–1777 (2020).
34. Neri, S., Mariani, E., Meneghetti, A., Cattini, L. & Facchini, A. Calcein-acetyoxymethyl cytotoxicity assay: Standardization of a method allowing additional analyses on recovered effector cells and supernatants. *Clin. Diagn. Lab. Immunol.* **8**, 1131–1135 (2001).
35. Xanthopoulos, J., Romano, A. & Majumdar, S. K. Response of mouse breast cancer cells to anastrozole, tamoxifen, and the combination. *J. Biomed. Biotechnol.* **2005**, 10–19 (2005).

Acknowledgements

This project was financially supported by Pasteur Institute of Iran. The authors wish to express their deep gratitude to all who provided support during the course of this research.

Author contributions

M.B., and Y.T. were chief investigators and revised the manuscript. R.D., and A.B.P. were responsible for the major experimental work and contributed in writing the manuscript. S.Z., and R.A.C. contributed in design of the study. A.A., and M.Sh. assisted in in vitro studies. S.A. contributed in in vivo studies.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-022-09278-7>.

Correspondence and requests for materials should be addressed to Y.T. or M.B.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2022