

IL-2-loaded Polypeptide Nanoparticles for Enhanced Anti-cancer Immunotherapy

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Electronic Supplementary Information

Abstract Interleukin 2 (IL-2) is widely used as an active immunotherapeutic agent in clinical metastatic cancers. However, its therapeutic concentrations do not last long due to its short half-life. Thus, only a transient proliferation of the anti-cancer CD8⁺ T cells can be achieved, resulting in poor efficacy. Therefore, the aim of this work was to create a system that promotes CD8⁺ T cell proliferation at the tumor site using IL-2 persistently present and activates an anti-cancer immune response. This goal was achieved by the design of the IL-2-loaded polypeptide nanoparticles (P-IL-2) where methoxy poly(ethylene glycol) block poly-[(N-2-hydroxyethyl)-aspartamide] phenylboronic acid was used to encapsulate IL-2 through boron-nitrogen coordination with poly(L-lysine). P-IL-2 significantly prolonged the circulation time of IL-2 and achieved a selective drug release at the tumor site in the presence of high levels of reactive oxygen species, thus activating an anti-cancer immune response and exerting a better anti-cancer effect. The half-life of P-IL-2 was 3.15-fold higher than that of IL-2, and the quantity of CD8⁺ T cells after using P-IL-2 was 1.89-fold higher than that after using IL-2. In addition, the combination of P-IL-2 and anti-CTLA-4 monoclonal antibody resulted in an enhanced immune activation. Hence, this work provides a new approach to improve the efficacy of IL-2 in anti-cancer immunotherapy.

Keywords Polypeptide; Phenylboronic acid; Boron-nitrogen coordination; Interleukin 2; ROS-responsive

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INTRODUCTION

Interleukin 2 (IL-2) is a T cell growth factor,^[1] acting as both a stimulator and a regulator.^[2,3] It is used in clinical immunotherapy thanks to the medicinal benefits in stimulating the proliferation of primary anti-cancer immunocytes (CD8⁺ T cells and NK cells).^[4–6] Recombinant IL-2 was approved by the Food and Drug Administration (FDA) in 1992 as the first immunotherapeutic agent against metastatic renal cell carcinoma.^[7] High doses of IL-2 are beneficial in patients diagnosed with melanoma and renal cell carcinoma, prolonging their overall survival.^[8–12]

Despite these findings, the use of IL-2 therapy at high doses in oncology is limited for two reasons: (1) IL-2 has a short circulating half-life *in vivo*,^[13,14] so it requires frequent

injections and high doses to maintain its efficacy. Multiple applications of IL-2 at high doses frequently cause dose-dependent negative consequences such as pulmonary edema and vascular leak syndrome, the latter being the most common and severe complication, as well as other systemic organ damage.^[15–18] (2) High concentrations of IL-2 promote T cell proliferation, but when the *in vivo* concentrations of IL-2 decrease, it only leads to Tregs proliferation.^[19–23] Some scientists tried to solve the above two key issues by designing a PEGylated IL-2 (NKTR-214)^[24–26] system allowing the use of lower doses of IL-2 and longer stimulation of T cells to ultimately obtain a better anti-cancer efficacy. However, the preparation is complex, and the instability of the site of PEG modification may cause product heterogeneity, which limits the therapeutic effect and clinical application. Thus, a system ensuring a long half-life of IL-2 in an easy approach to enhance the anti-cancer effects is urgently needed.

This work described the preparation of a novel type of IL-2-loaded polypeptide nanoparticles (P-IL-2), where methoxy poly(ethylene glycol) block poly-[(N-2-hydroxyethyl)-aspart-

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amide] phenylboronic acid (mPEG-*b*-PHEA-PBA, P-PBA) was used to encapsulate IL-2 through boron-nitrogen coordination with poly(L-lysine) (PLL). The shell composed of poly(ethylene glycol) and polypeptide successfully prolonged the blood circulation time of IL-2 and achieved a ROS-responsive drug release,^[27–30] resulting in a long-term effect on CD8⁺ T cell proliferation. In addition, to avoid unexpected upregulation of Tregs by IL-2, the immunosuppressive tumor microenvironment was reduced by combining P-IL-2 with anti-CTLA-4 monoclonal antibody.^[31–33] The results showed that the half-life of P-IL-2 was 3.15-fold than that of IL-2, and the quantity of CD8⁺ T cells treated with P-IL-2 was 1.89-fold higher than that treated with IL-2. Moreover, the combination of P-IL-2 and anti-CTLA-4 resulted in a sustained and remarkable increase in CD8⁺ T cells as well as a significant decrease in Tregs.

EXPERIMENTAL

Materials and Instruments

N^ε-benzyloxycarbonyl-L-lysine-*N*-carboxyanhydride (Lys-NCA) and γ -benzyl-L-aspartate-*N*-carboxyanhydride (BLA-NCA) were purchased from Chengdu Enlai Biological Technology CO., Ltd. (Chengdu, China). Carbonyldiimidazole (CDI), 4-(hydroxymethyl) phenylboronic acid pinacol ester and 4-dimethylaminopyridine (DMAP) were purchased from Energy Chemical (Shanghai, China). Anhydrous *N,N*-dimethylformamide (DMF) and anhydrous dichloromethane were purchased from Energy Chemical (Shanghai, China). Other chemical reagents were purchased from Xilong Science Co., Ltd. (Shantou, China). Recombinant human IL-2 (IL-2) was provided by the Changchun Institute of Biological Products Co., Ltd. (Changchun, China). The anti-CTLA-4 monoclonal antibody was purchased from Bio X Cell (Clone 9D9, BE0164). The antibodies used for flow cytometry are listed in Table S1 (in the electronic supplementary information, ESI). ¹H nuclear magnetic resonance (¹H-NMR) spectra were characterized by Bruker AV-300 NMR spectrometer. The molecular weight was measured by gel permeation chromatography (GPC, SA). The dynamic laser scattering (DLS) and zeta potential of the nanoparticles were performed on a Zeta Potential/BI-90Plus particle size analyzer (Brookhaven, USA). The unloaded IL-2 was measured by an IL-2 ELISA kit (Thermo Fisher, USA). The transmission electron microscope (TEM) images were obtained using the JEOL JEM-1011 (Tokyo, Japan). The fluorescence intensity of the serum samples was measured with a microplate reader (Bio-Rad, Hercules, CA, USA). The lymphocytes were characterized by flow cytometry (BD FACS Celesta, USA). Cell populations were identified and quantified using FlowJo software (Becton Dickinson and Company, USA). Histological alterations were observed using an optical microscope (Nikon Eclipse Ti, Optical Instruments Corporation, Ardmere, PA, USA).

Cells and Animals

The murine colorectal cancer CT26 cells were purchased from BeNa Culture Collection (Beijing, China). They were routinely cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), penicillin (100.0 U/mL), and streptomycin (100.0 U/mL) and incubated at 37 °C under 5% CO₂. Female BALB/c mice aged 6–8 weeks (average body weight: 17–19 g) were

purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Female SD rats aged 7–8 weeks (average body weight 220–250 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China). All animal procedures were conducted according to the guidelines of Jilin University's Institutional Animal Care and the experiments were authorized by the Jilin University's Animal Ethics Committee.

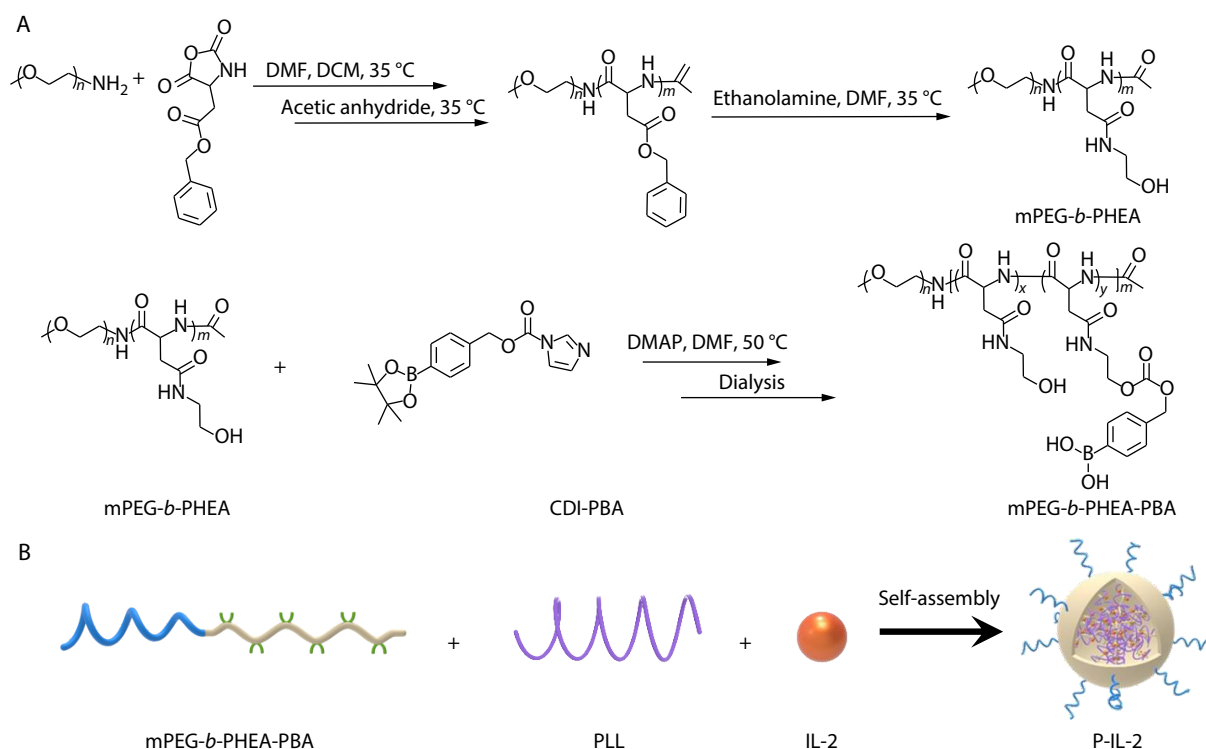
Synthesis of Poly(L-lysine) (PLL)

PLL was synthesized according to a previous report,^[34] and the synthetic route is shown in Fig. S1 (in ESI). First, *n*-hexamine (39.6 mg) and recrystallized Lys-NCA (3.0 g) were dissolved in anhydrous DMF and stirred at 35 °C for 48 h. The mixture was subjected to precipitation with cold ethyl ether and vacuum drying, and the white solid poly(benzyloxycarbonyl-L-lysine) (PBLL) was obtained. Then, PBLL (1.5 g) was dissolved in 15.0 mL of trifluoroacetic acid, and 4.5 mL of HBr/acetic acid was slowly added at 0 °C and stirred for 1 h at 25 °C. The product was precipitated with cold ethyl ether, then redissolved in DMF and dialyzed using a dialysis bag (MWCO=3500 Da) for 2 days. The white solid PLL was obtained after freeze-drying, and the structure was determined by ¹H-NMR using D₂O as the solvent. GPC measurements were performed on a Waters GPC system using poly(ethylene glycol) (PEG) as standard and phosphate buffer (0.2 mol/L, pH 7.4) as the eluent (35 °C, flow rate: 1.0 mL/min).

Synthesis of Methoxy Poly(ethylene glycol) Block Poly-[(*N*-2-hydroxyethyl)-aspartamide]

Phenylboronic Acid (mPEG-*b*-PHEA-PBA, P-PBA)

P-PBA was synthesized according to a previous research,^[35] and the synthetic route is shown in the Scheme 1(A). First, 4-(hydroxymethyl) phenylboronic acid pinacol ester (3.0 g, 1.0 equiv.) was dissolved in 30 mL of anhydrous dichloromethane, carbonyldiimidazole (2.5 g, 1.2 equiv.) was added and the mixture was stirred at 25 °C for 3 h. Next, the mixture was diluted in 150 mL of ethyl acetate, washed with brine (three times) and deionized water (three times), dried with Na₂SO₄ overnight, then dried under vacuum and the white solid CDI-PBA was obtained. The structure was determined by ¹H-NMR using CDCl₃ as the solvent. Then, methoxy poly(ethylene glycol) block poly(γ -benzyl-L-aspartate) (mPEG-*b*-PBLA) was synthesized through ring-opening polymerization of BLA-NCA with mPEG-NH₂ as the initiator. Briefly, mPEG-NH₂ (*M*_n=5000 Da, 3.0 g) with the azeotropic removal of water with toluene was dissolved in 3.0 mL of anhydrous DMF; BLA-NCA (7.4 g) and 60.0 mL of anhydrous dichloromethane were subsequently added to the above solution and stirred at 35 °C for 3 days. Acetic anhydride (0.6 g) was added to the mixture and left for another 12 h to react. The mixture was precipitated with cold ethyl ether, the white solid mPEG-*b*-PBLA was obtained, and the structure was determined by ¹H-NMR using TFA-d as the solvent. Next, mPEG-*b*-PBLA (5.5 g) was dissolved in 50.0 mL of anhydrous DMF, ethanolamine (3.0 g) was added and the mixture was stirred at 35 °C overnight. The solution was precipitated with cold ethyl ether, then the precipitate was redissolved in DMF and dialyzed with deionized water (MWCO=3500 Da) for 3 days. The white solid mPEG-*b*-PHEA was obtained after freeze-drying, and the structure was determined by ¹H-NMR using TFA-d/D₂O (1:9, V:V) as the solvent. Finally, mPEG-*b*-PHEA (1.5 g), CDI-PBA



Scheme 1 Preparation of P-IL-2. (A) Synthesis of methoxy poly(ethylene glycol)-block-poly-[(N-2-hydroxyethyl)-aspartamide] phenylboronic acid (mPEG-b-PHEA-PBA); (B) Preparation of P-IL-2.

(1.2 g) and DMAP (443.5 mg) were dissolved in 15.0 mL of anhydrous DMF and the mixture was stirred at 50 °C for 24 h. The solution was precipitated with cold ethyl ether, then the precipitate was redissolved in DMF and dialyzed with deionized water (MWCO=3500 Da) for 3 days. The white solid P-PBA was obtained after freeze-drying, and the structure was determined by $^1\text{H-NMR}$ using DMSO-d_6 as the solvent. GPC measurements were performed under the same measurement conditions used for PLL described above.

Synthesis of P-IL-2

The preparation of P-IL-2 is shown in Scheme 1(B). Briefly, IL-2 (1.0 mg/mL) was added to different amounts of P-PBA (16.0 mg/mL) solutions and stirred on ice for 3 min to obtain the P-PBA/IL-2. Then, different amounts of PLL were added to the P-PBA/IL-2 solutions and stirred on ice for 5 min. The solutions were purified with Amicon® Ultra Centrifugal Filters (MWCO=30000 Da, 3000 g for 5 min), washed with deionized water for three times, and then the unloaded IL-2 in the ultrafiltrate was measured by IL-2 ELISA kit. The drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following equations:

$$\text{DLC} = W_1/W_2 \times 100\% \quad (1)$$

$$\text{DLE} = W_1/W_3 \times 100\% \quad (2)$$

where W_1 , W_2 and W_3 represent the weight of the loaded IL-2, IL-2-loaded nanoparticles and feeding IL-2, respectively. The particle size and zeta potential of P-PBA/IL-2 and P-IL-2 were measured on a particle size analyzer, and the image of P-IL-2 was obtained by TEM.

Stability and ROS Responsiveness of P-IL-2

P-IL-2 was dissolved in PBS buffer (pH 7.4) or PBS buffer (pH 7.4)

containing 10% FBS, then placed in a constant temperature incubator shaker (37 °C, 100 r/min), 1.0 mL of P-IL-2 solution was extracted at pre-established intervals (1, 3, 6 and 24 h), and the diameters were measured by DLS to understand the stability and dispersibility. As for the ROS-responsive measurement, P-IL-2 was dissolved in PBS buffer (pH 7.4) with or without 100.0 $\mu\text{mol/L}$ H_2O_2 , and the suspension was placed in a constant temperature incubator shaker (37 °C, 100 r/min) for 6 h, then the diameters were measured by DLS.

Pharmacokinetics

The pharmacokinetics of P-IL-2 was investigated using 6 female SD rats, which were randomly divided into two groups ($n=3$) and treated with an intravenous injection of Cy5-labeled IL-2 or Cy5-labeled P-IL-2. The Cy5-labeled IL-2 was prepared as follows: 1.0 mg of Cy5-NHS ester was dissolved in 400.0 μL of DMSO, then 10.0 mg IL-2 (1.0 mg/mL) was added and stirred at 4 °C overnight. The mixture was placed into a dialysis bag (MWCO=7000 Da), dialyzed with deionized water for 48 h, and the blue Cy5-labeled IL-2 solution was obtained. The loading process of Cy5-labeled IL-2 was the same as the loading process of IL-2 in the preparation part of P-IL-2. Before the pharmacokinetic experiment, both Cy5-labeled IL-2 and Cy5-labeled P-IL-2 concentrations were quantified to the same concentration (0.3 mg/mL) by UV-Vis, and these two drugs (1.5 mg/kg based on IL-2) were intravenously injected according to each mouse weight. Blood samples were collected from the retro-orbital sinus at indicated times (0.25, 0.5, 1, 2, 4, 8 and 12 h) after injection, then centrifuged at 2000 g for 5 min to separate the serum. A quantity of 100.0 μL of serum was added to each well, and the fluorescence intensity of each well was evaluated at appropriate wavelengths ($\lambda_{\text{ex}}=605 \text{ nm}$, $\lambda_{\text{em}}=665$

nm) by a microplate reader. The PK Solver was used to compute the half-life ($t_{1/2}$) of the drugs by non-compartmental analysis.

Anti-cancer Efficiency *In vivo*

The CT26 tumor model was established on day -8 by the subcutaneous injection of 5.0×10^5 CT26 tumor cells dissolved in 100.0 μ L of sterile PBS buffer (pH 7.4) on the shaved right lower abdomen of Balb/c mice. The mice were randomly divided into 5 groups ($n=4$) when the tumor volume reached approximately 60.0 mm³ to 80.0 mm³, and were treated with the following drugs: PBS, anti-CTLA-4, IL-2, P-IL-2 and P-IL-2 + anti-CTLA-4. IL-2 (1.0 mg/kg) and P-IL-2 (1.0 mg/kg based on IL-2 concentration) were intravenously injected on day 0, 3, 6, 9 and 12. The anti-CTLA-4 monoclonal antibody (100.0 μ g) in PBS was intraperitoneally injected on day 0, 4, 8, and 12. Tumor volume and body weight were recorded every other day. The tumor volume (V ; mm³) was calculated using the following equation:

$$V = a \times b^2 / 2 \quad (3)$$

where a (mm) is the longest axis and b (mm) is the shortest axis of the tumor.

The tumor suppression rate (TSR) was calculated as follows:

$$\text{TSR} \% = [(V_c - V_x) / V_c] \times 100\% \quad (4)$$

where V_c is the mean tumor volume in the PBS group and V_x is the mean tumor volume in the treatment groups.

Flow Cytometry Analysis

All mice were euthanized, and their tumors and spleens were collected at the end of the anti-cancer experiment ($n=4$) to analyze the lymphocytes. The spleens were mechanically ground and filtered, and the red blood cells were lysed in ACK lysis buffer. The tumor dissociation buffer was used to digest the tumor to generate a cell suspension. Cells were filtered through a nylon filter, a single cell suspension was prepared and surface staining was performed using fluorescence conjugated antibodies. Then, the samples were intracellularly stained with FXP3 antibody and analyzed by flow cytometry.

Histological and Immunohistochemical Analysis

Mice were euthanized at the end of the anti-cancer efficiency test to analyze the histological alterations in the tumors and major organs. The major organs (heart, liver, spleen, lung and kidney) and tumors were collected and fixed with 4% paraformaldehyde for 72 h. Then, they were cut into 5 μ m-thick slices and stained with hematoxylin and eosin (H&E) or TUNEL according to a standard protocol. Images of the slides were captured using a camera connected with an optical microscope.

Systemic Toxicity Evaluation *In vivo*

The blood of the mice ($n=4$) was collected after various treatments for the analysis of serum biochemistry. Liver function parameters (alanine aminotransferase -ALT-, aspartate aminotransferase -AST-) and kidney function parameters (creatinine -CRE- and blood urea nitrogen -BUN-) were measured following the manufacturer's protocol.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism8 (GraphPad Software, CA, USA). One-way ANOVA followed by Tukey's multiple comparisons was used for the comparison of multiple groups. The results are presented as mean $\pm$ standard

deviation (SD). A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Synthesis of Poly(L-lysine) (PLL)

The ¹H-NMR spectrum of PLL is shown in Fig. S2 (in ESI). The peak c at $\delta=4.19$ ppm was the characteristic peak of $-\text{CH}-$ in the main chain of PLL, the peak a at $\delta=0.72$ ppm was the characteristic peak of $-\text{CH}_2-$ in the initiator, and the area ratio of peak c to peak a was 25.3/3.1, indicating that the polymerization degree of PLL was 25. The M_n of PLL was approximately 4944 (PDI=1.29) according to the GPC spectrum in Fig. S3 (in ESI).

Synthesis of Methoxy Poly(ethylene glycol) Block Poly-[(N-2-hydroxyethyl)-aspartamide] Phenylboronic Acid (mPEG-*b*-PHEA-PBA, P-PBA)

According to the ¹H-NMR spectrum of mPEG-*b*-PBLA in Fig. S5 (in ESI), the area ratio of peak f ($\delta=7.08$ ppm) to peak b ($\delta=3.78$ ppm) was 185.1:452.0, indicating that the polymerization degree of mPEG-*b*-PBLA was 37. Then the mPEG-*b*-PHEA was obtained through the aminolysis of mPEG-*b*-PBLA with ethanolamine. The ¹H-NMR spectrum of mPEG-*b*-PHEA showed that the benzene ring of the mPEG-*b*-PBLA side chain ($\delta=6.50$ ppm to 8.50 ppm) was completely removed (Fig. S6 in ESI). Subsequently, P-PBA was obtained after the reaction of mPEG-*b*-PHEA and CDI-PBA, and the ¹H-NMR spectrum of P-PBA is shown in Fig. 1(A). The area ratio of peak h ($\delta=5.11$ ppm) to peak c ($\delta=4.58$ ppm) was 28.1:37.2, indicating that P-PBA was successfully synthesized, and the grafting rate of the phenylboronic acid side groups was 37.8%. The M_n of P-PBA was approximately 5078 (PDI=1.04) according to the GPC spectrum in Fig. S7 (in ESI).

Preparation and Characterization of P-IL-2

The first step was to determine whether IL-2 could be successfully loaded through the boron-nitrogen coordination between the phenylboronic acid groups in P-PBA and the amino groups in IL-2. The ratios of P-PBA to IL-2 were selected as 4:1, 8:1, 16:1 and 32:1 for the IL-2 loading experiments. However, the nanoparticle dispersions with the ratios of 4:1, 8:1 and 16:1 ratio were heterogeneous (Fig. S8A in ESI). Although a homogeneous dispersion of nanoparticles was obtained with the ratio of 32:1, the particle size changed significantly after being placed in PBS buffer (pH 7.4) for 1 h (Fig. S8C in ESI). Moreover, the DLEs were unsatisfactory in all ratios (DLE < 70%, Table S2 in ESI). The above results indicated that the force between P-PBA and IL-2 was not strong enough to load IL-2 and maintain the morphological stability of nanoparticles. PLL was added and IL-2 was entangled by its boron-nitrogen coordination with P-PBA to improve the stability of nanoparticles to achieve more efficient encapsulation of IL-2 and obtain compact IL-2 nanoparticles. A variety of nanoparticles loaded with IL-2 were prepared by varying the weight ratio of P-PBA, PLL, and IL-2 (32:3:1, 32:2:1, 32:1:1) and DLEs, particle sizes and zeta potentials were characterized. Table S2 (in ESI) shows that IL-2 was efficiently loaded and realized high DLE at various ratios (93.8% $\pm$ 0.3% for 32:3:1, 93.5% $\pm$ 1.1% for 32:2:1, and 92.7% $\pm$ 0.4% for 32:1:1), and the particle size distributions were uniform (Fig. S8D in ESI). The zeta potential of

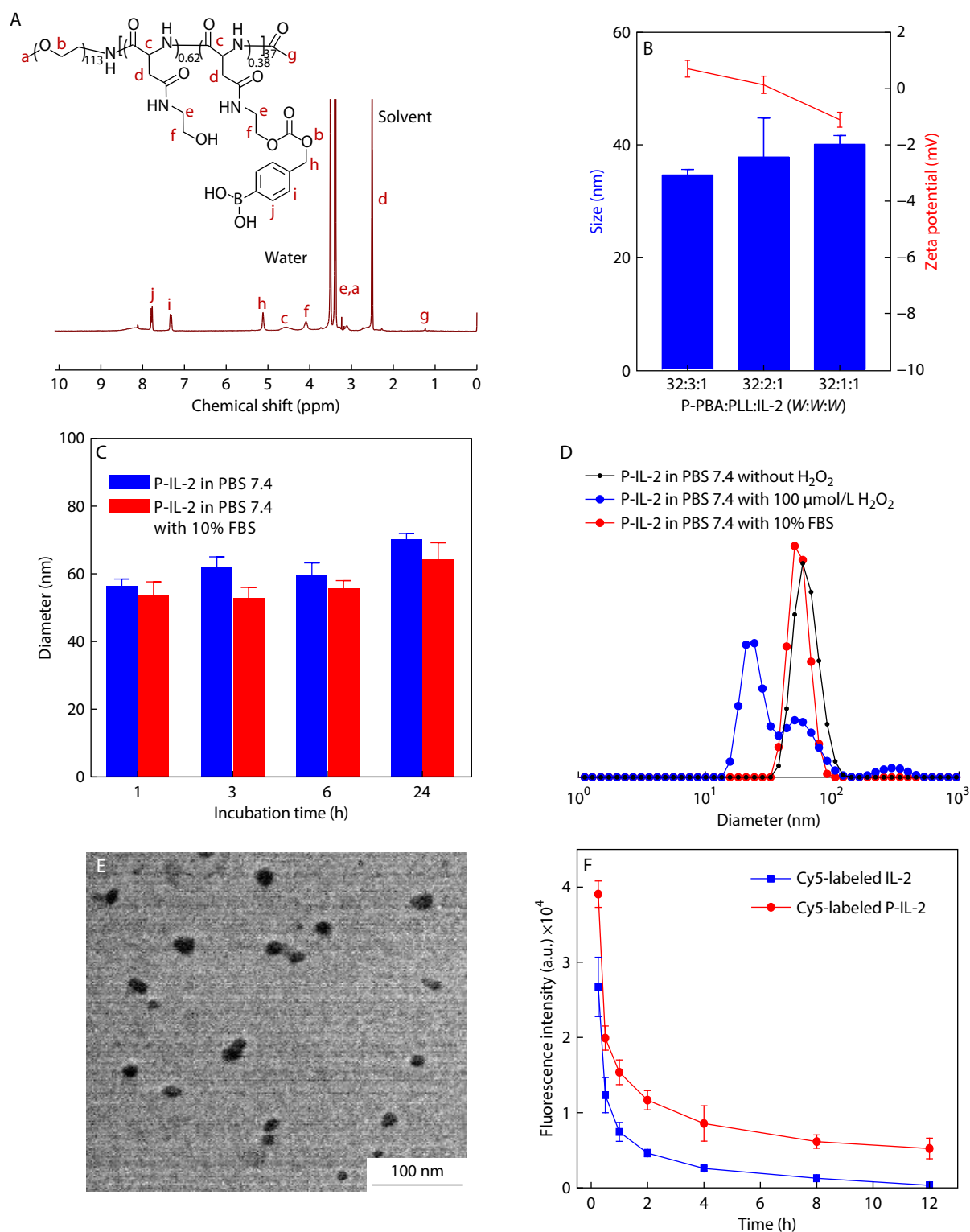


Fig. 1 Characterization of P-IL-2. (A) ¹H-NMR spectrum of P-PBA; (B) Particle size and zeta potential of P-PBA:PLL:IL-2 with different weight ratios; (C) Stability of P-IL-2 in PBS buffer (pH 7.4) or PBS buffer (pH 7.4) with 10% FBS; (D) Particle sizes of P-IL-2 after 6 h incubation in PBS buffer (pH 7.4) with or without 100.0 μmol/L H₂O₂, and in PBS buffer (pH 7.4) with 10% FBS; (E) TEM image of P-IL-2 (Scale bar: 100 nm); (F) Pharmacokinetic results of Cy5-labeled IL-2 and Cy5-labeled P-IL-2. Results are presented as mean±SD.

IL-2 nanoparticles slightly increased with the increase of PLL content (Fig. 1B, -1.1 ± 0.3 mV for 32:1:1, 0.1 ± 0.3 mV for 32:2:1, and 0.7 ± 0.3 mV for 32:3:1). The weight ratio of P-PBA:PLL:IL-2

was selected as 32:1:1 (W:W:W) to reduce the possibility of immunogenicity and toxic side effects caused by PLL,^[36] and the IL-2-loaded nanoparticles prepared at this ratio were named P-

IL-2. The particle size of P-IL-2 was further evaluated by TEM and it was approximately 25.0 nm (Fig. 1E), which was slightly smaller than that recorded by DLS due to the dehydration of the nanoparticles.

Stability and ROS Responsiveness of P-IL-2

Fig. 1(C) shows that the particle size of P-IL-2 does not show any significant change after incubation for 24 h in both PBS buffer (pH 7.4) and PBS buffer (pH 7.4) containing 10% FBS, revealing the good stability of P-IL-2. The ROS responsiveness of P-IL-2 was also measured. After 6 h of incubation in different environments, the particle size of P-IL-2 did not change in PBS, while it changed irregularly in the presence of 100 $\mu\text{mol/L}$ H_2O_2 due to the boron-nitrogen coordination destruction of P-PBA and PLL, indicating the ROS-responsiveness of P-IL-2 (Fig. 1D).

Pharmacokinetics

The pharmacokinetics of P-IL-2 was assessed by an intravenous injection of Cy5-labeled IL-2 or Cy5-labeled P-IL-2 into SD rats (Fig. 1F). The $t_{1/2}$ of the Cy5-labeled P-IL-2 group was 8.53 ± 1.56 h, which was 3.15-fold longer than that of the Cy5-labeled IL-2 group ($t_{1/2} = 2.71 \pm 0.21$ h). The results above indicated that P-IL-2 possessed a longer blood circulation ability than IL-2, which might enable P-IL-2 to realize long-term Teff cell proliferation effects.

In vivo Evaluation of the Anti-cancer Effect

The *in vivo* anti-cancer effect and systemic toxicity of P-IL-2 were further investigated in the CT26 murine colorectal cancer model (Fig. 2A). The tumors grew rapidly in the mice treated with PBS

and IL-2, and no significant difference in tumor size was observed between the PBS and IL-2 groups (Fig. 2B). IL-2 was rapidly removed from the bloodstream, so it failed to continuously promote Teff cell proliferation, resulting in a poor anti-cancer effect (with a TSR of 26.5%). In contrast, the P-IL-2 group realized a TSR of 52.54% on day 14 due to its extended circulation time *in vivo* and the ROS-responsive IL-2 release. The combination of anti-CTLA-4 with P-IL-2 was performed to further reverse the unexpected Tregs increase and immunosuppression of the tumor microenvironment and resulted in an increased TSR to 75.31%; the tumor volume in the P-IL-2 + anti-CTLA-4 group was 1.92-fold less than that in the P-IL-2 group, and 2.49-fold less than that in the anti-CTLA-4 group. Moreover, no significant weight loss was observed among the treated groups, indicating that these treatments are not systemically harmful (Fig. 2C).

The H&E and TUNEL staining were analyzed to further confirm the anti-cancer effect after different treatments (Fig. 2D). The H&E staining showed that the PBS group had large nuclei and more chromatin, indicating the rapid proliferation ability of tumor cells. A large area of necrosis occurred in the P-IL-2 + anti-CTLA-4 group compared with other groups. The TUNEL staining showed that the PBS group had high cell density and negligible apoptosis, and the IL-2, anti-CTLA-4, and P-IL-2 groups had partial apoptosis, while the P-IL-2 + anti-CTLA-4 group had the largest apoptosis area, which revealed the most effective anti-cancer effect of the combination of P-IL-2 and anti-CTLA-4.

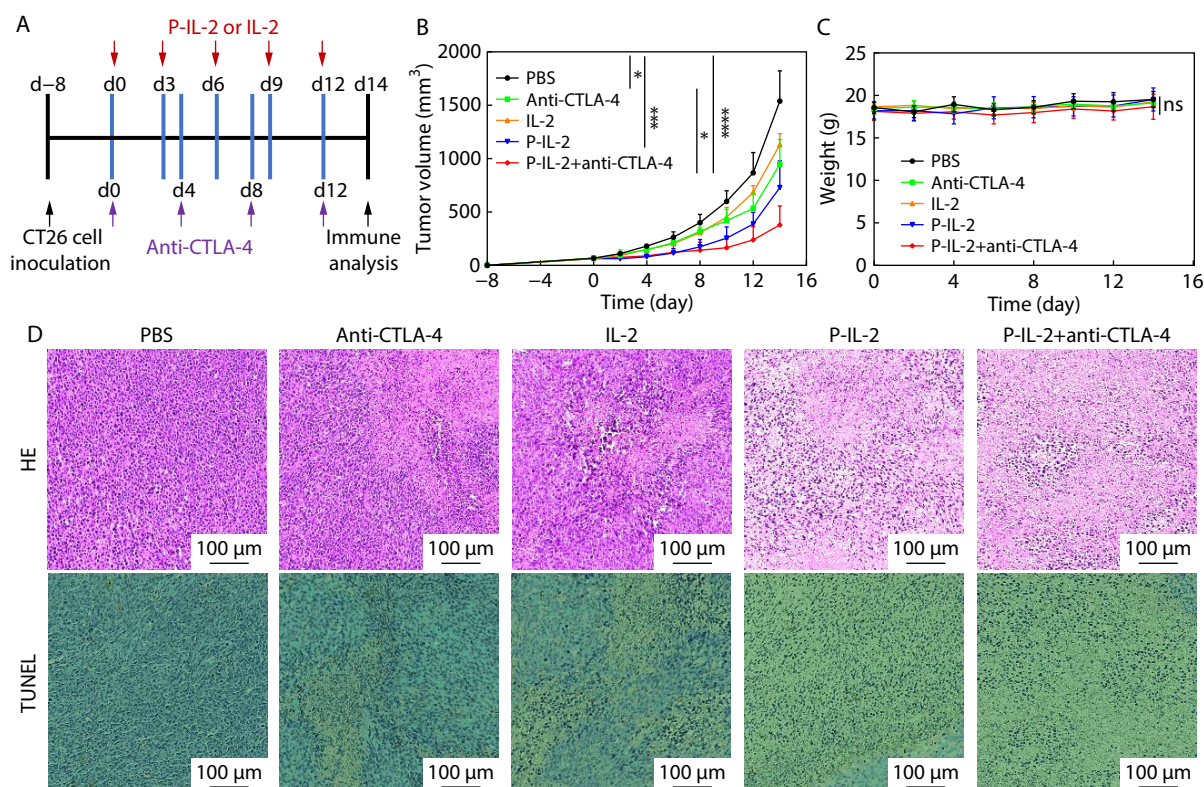


Fig. 2 Anti-cancer effect on CT26 tumor model. (A) Schematic illustration of the establishment of the CT26 tumor model and the therapeutic schedule; (B, C) Tumor growth curves and body weights of CT26 tumor-bearing mice; (D) H&E and TUNEL staining of tumors (Scale bar: 100 μm). Results are presented as mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns: not significant.

Immune Microenvironment Evaluation

The amount of CD8⁺ T cells in the P-IL-2 group was 3.75-fold higher than that of the PBS group, and 1.89-fold higher than that of the IL-2 group in the tumors (Fig. 3A), and the amount of CD8⁺ T cells in the spleen (Fig. 3D) was significantly increased in the P-IL-2 group compared to that in the IL-2 group or PBS group. Furthermore, in the tumor the amount of Tregs in the P-IL-2 + anti-CTLA-4 group was 29.91% lower than that in the P-IL-2 group, indicating that this combination strategy reversed the

immunosuppressive tumor microenvironment (Fig. 3C). These results indicated that P-IL-2 promoted CD8⁺ T cell proliferation persistently at the tumor site, induced an effective immune response and further enhanced the anti-tumor immune response and anti-tumor effect after the combination with anti-CTLA-4.

Systemic Toxicity Evaluation *In vivo*

The morphologies of the main organs (heart, liver, spleen, lung and kidney) showed no noticeable abnormality in all the treated

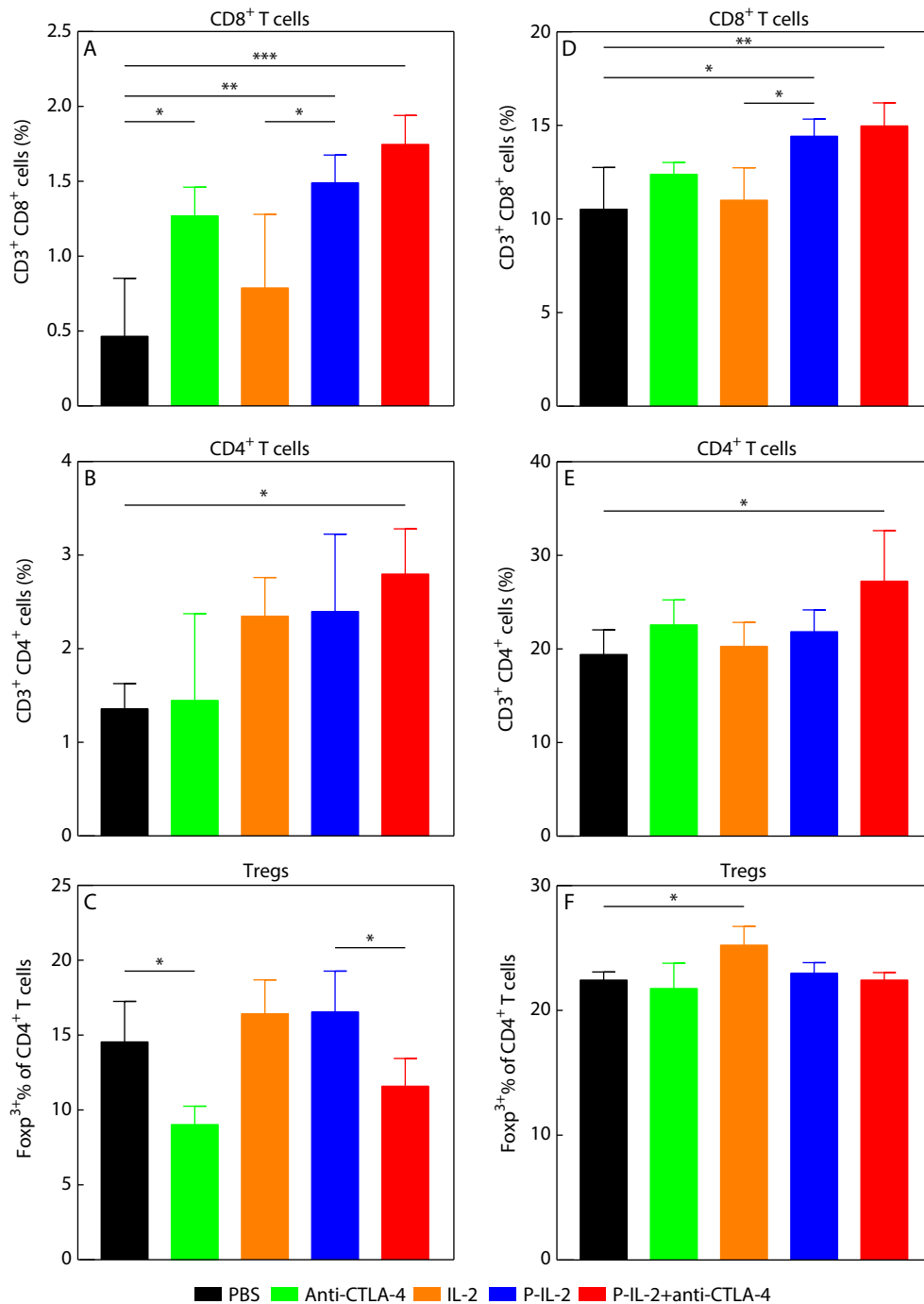


Fig. 3 Tumor and spleen immune microenvironment after various treatments. (A–C) The tumor and (D–F) spleen relative quantification of CD8⁺ T cells, CD4⁺ T cells and Tregs. Results are presented as mean ± SD; **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

groups (Fig. 4A). The key liver and kidney functions, such as ALT (Fig. 4B), AST (Fig. 4C), BUN (Fig. 4D) and CRE (Fig. 4E), were assessed after the treatments, and no significant difference in

serum biochemistry among all the treated groups was observed, demonstrating the excellent biosafety performance of P-IL-2.

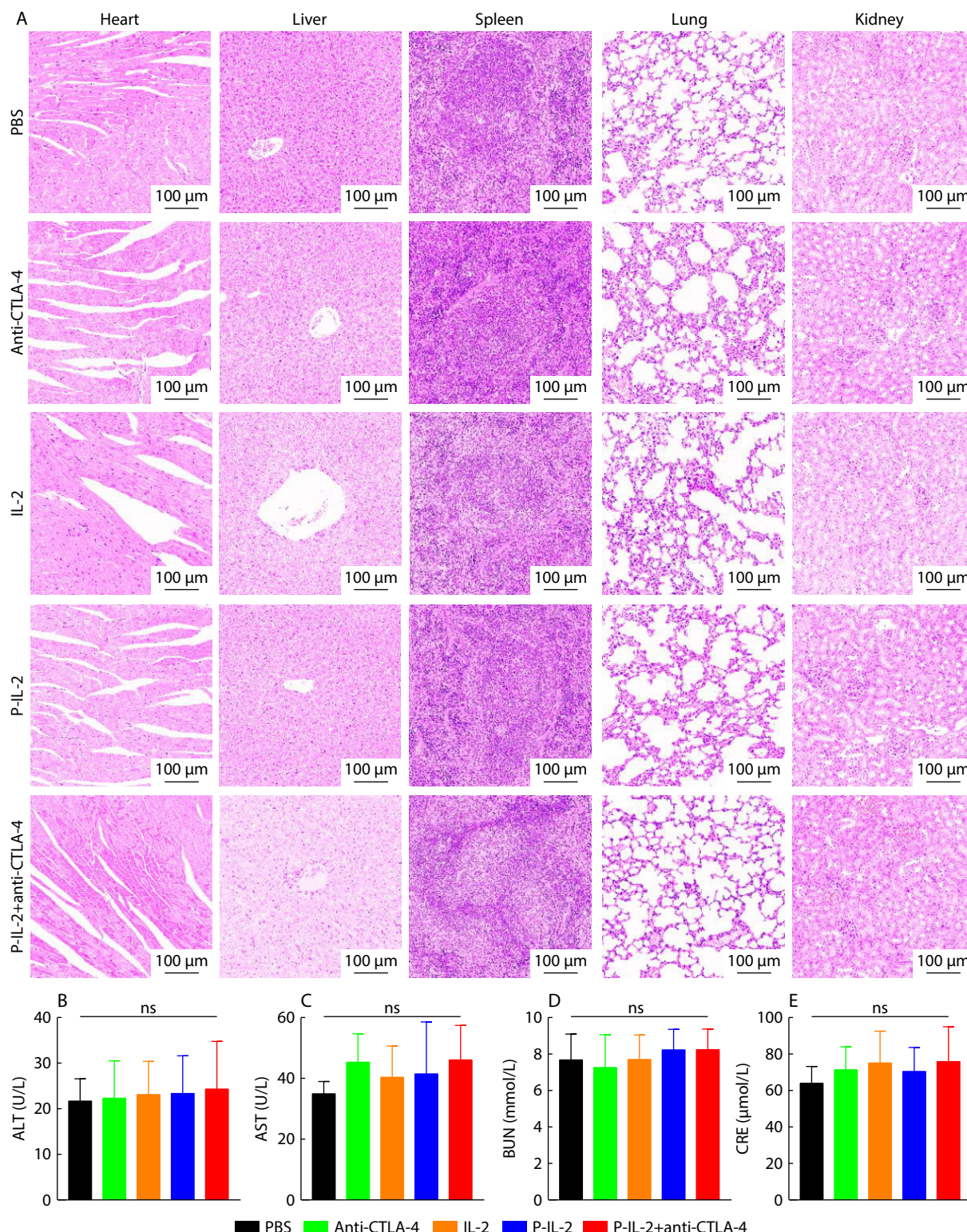


Fig. 4 Systemic toxicity after various treatments. (A) H&E staining of the major organs (heart, liver, spleen, lung, and kidney, Scale bar: 100 μm). Levels of the serum liver and kidney function markers: (B) ALT, (C) AST, (D) CRE and (E) BUN. Results are shown as mean±SD; ns: not significant.

CONCLUSIONS

IL-2-loaded polypeptide nanoparticles were developed using P-PBA to encapsulate IL-2 through boron-nitrogen coordination with PLL, which showed good stability and a ROS-responsive IL-2 release. P-IL-2 possessed a longer blood circulation time than IL-2, leading to a persistent proliferation of CD8⁺ T cells in treated tumors of the P-IL-2 group. As a result, the anti-cancer efficacy of P-IL-2 was significantly higher than that of IL-2, and the combination of P-IL-2 + anti-CTLA-4 monoclonal antibody resulted in an enhanced immune activation and anti-cancer efficacy without significant side effects. This work represents great potential for future clinical applications of IL-2.

Conflict of Interests

The authors declare no interest conflict.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://doi.org/10.1007/s10118-023-2898-2>.

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