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Self-fused concatenation of interferon with enhanced bioactivity, pharmacokinetics and antitumor efficacy

Jin Hu[1]†*, Jianquan Shi[2]†, Yeshuang Yuan[3]†, Shengjie Li[1]†, Bo Zhang[1], Haitao Dong[4], Qing Zhong[1], Xie Qiu[1], Xiaoyin Bai[1], Yingxing Li[1]

[1]Department of Medical Research Center, State Key Laboratory of Complex Severe and Rare Diseases, Peking Union Medical College Hospital, Chinese Academy of Medical Science and Peking Union Medical College, Beijing 100730, China

[2]Department of Intensive Care Unit, Beijing Chest Hospital, Capital Medical University, Beijing Tuberculosis and Thoracic Tumor Research Institute, Beijing 101149, China

[3]Department of Rheumatology, Beijing Hospital, National Center of Gerontology, Institute of Geriatric Medicine, Clinical Immunology Center, Graduate School of Peking Union Medical College, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China.

[4]Department of stomatology, State Key Laboratory of Complex Severe and Rare Diseases, Peking Union Medical College Hospital, Chinese Academy of Medical Science and Peking Union Medical College, Beijing 100730, China

†These authors contributed equally to this work.

*Corresponding author, email: ncuskhujin@163.com (Jin Hu)

1. Materials

All reagents and kits for experiments, unless otherwise stated, were purchased from New England Biolabs or Gibco. Daudi B and OVCAR-3 cells were purchased from the cell bank of the Chinese Academy of Medical Sciences. BALB/c-nude mice were purchased from Vital River Laboratories (Beijing, China) and accommodated in the Laboratory Animal Center of Peking Union Medical College Hospital accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

2. Biosynthesis and purification of IFN concatemers

The genes encoding interferon (IFN) (NCBI GI 386795) concatemers (including monomer, dimer and trimer of IFN that are marked as 1 × IFN, 2 × IFN and 3 × IFN, respectively) with a 6×His tag at the C-terminus were synthesized by Sangon Biotech (Shanghai, China) and inserted into pET-25b (+) vector (Novagen) using standard molecular techniques. The IFN subunits were gapped by the flexible linker GGGGS. After confirmed by DNA-sequencing, the constructed plasmids were transformed into *Escherichia coli* BL21(DE3) strain and cultured in Luria Bertani media containing 100 μg/mL ampicillin at 37 °C, shaking at 220 rpm. When the optical density at 600 nm reached 0.5-0.8, isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the culture at a final concentration of 300 μM at 20 °C for 12 h. Cells were collected via centrifugation at 4000 × g for 15 min and resuspended in 10 mM PBS, pH 7.4. After being lysed by a Continuous Flow Cell Disrupter (JNBIO, Guangzhou, China) twice with an ultrahigh pressure of 1200 bar, the extracts were centrifuged at 16000 × g for 30 min, the supernatant containing protein were collected and applied to a 5 mL HisTrap column (GE Healthcare) on an AKTA Purifier 10 system with a UV detector at 280 nm. The equilibration, washing and elution buffer consisted of 50 mM Tris, 500 mM NaCl, 10% glycerol containing 5 mM, 30 mM and 250 mM imidazole, respectively. The eluted proteins were finally loaded on a HiPrep 26/10 desalting column (GE Healthcare, USA) to exchange buffer into 50 mM Tris, 150 mM NaCl, pH 7.4. The purification processes and purities of IFN concatemers were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The concentrations of proteins were evaluated by Nanodrop (Thermo Fisher Scientific).

3. Physicochemical characterization

For liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) assay, the samples were separated by a 10 min gradient elution at a flow rate of 0.5 mL/min with the ACQUITY UPLC system connected with a SYNAPT-G2-Si mass spectrometer (Waters, USA). Mobile phase A was 0.1% formic acid aqueous solution, and mobile phase B was 100% acetonitrile containing 0.1% formic acid. The analytical column was a Protein BEH C4 silica capillary column (2.1 mm ID, 100 mm length; made in Ireland) packed with C-4 resin (300 Å, 1.7 µm; Waters, USA). Aliquots of 2 µL analytes were loaded into an autosampler for nanoelectrospray ionization and analyzed on a Q-TOF mass spectrometer (SYNAPT G2-Si; Waters, USA) instrument optimized for high-mass protein analysis. The measurements were performed with capillary 3000V and data were collected over the expected m/z range. Once having acquired raw native electrospray mass spectra, the raw spectrum can be deconvoluted by MaxEnt 1 (Waters, USA) to generate a spectrum (relative intensity versus mass) where all the charge-state peaks of a single species have been collapsed into a single (zero-charge) peak.

For dynamic light scattering (DLS) analysis, the proteins were filtered with 0.22 μm pore size and carried out by a Malvern Zetasizer Nano-zs90 with a laser wavelength of 633 nm and a scattering angle of 90° at 25 °C. The data were analyzed with Zetasizer software 6.32.

For circular dichroism (CD) spectra assays, the proteins were diluted into 0.1 mg/mL in H_2O and performed on Pistar π-180 (Applied Photophysics Ltd, UK) instrument with a wavelength range from 190 nm to 260 nm.

4. *In vitro* bioactivity

Daudi B cells were cultured in 15% (v/v) fetal bovine serum (FBS) containing RPMI-1640 media at 37 °C under a humidified, 5% CO_2 atmosphere. 50 μL of cells were seeded in a 96-well plate (Corning) at a density of 5,000 per well. 50 μL of proteins diluted in fresh media at the same mass concentration (2, 5, 10, 20, 50, 100, 200, 500, 1000 and 10000 pg/mL) or the same molar concentration (0.031, 0.062, 0.13, 0.25, 0.50, 1.0, 2.5, 5.0, 10, 25 and 50 pM) were added into the wells. After incubating for 72 h, the inhibition ability of cell proliferation was evaluated by MTT assay following the instructions of Cell Proliferation Assay kit (Promega). The bioactivity of Intron A was also tested as a control. The well with cells and the well with media were defined as 100% and 0% of cell viability. The data fitting and IC_{50} calculation were analyzed by GraphPad Prism 5.0 software.

5. *In vivo* pharmacokinetics

Female BALB/c nude mice of 6 weeks old were randomly distributed into three groups (n = 3 per group) and *i.v.* injected with IFN concatemers at a dosage of 1 mg/kg mice body weight. 10 μL of blood samples were rapidly collected from the tail vein and placed into heparin-coated tubes when reached the given times (1, 5, 15, 30 min and 1, 3, 6, 24, 48 h). The samples were then centrifuged at 3000 × g for 15 min, and the plasmas were separated from the blood. The concentration of IFN in plasma was detected by IFN ELISA kit (PBL Interferon Source) under the instructions of the manufacturer. Pharmacokinetic parameters and data fitting in a two-compartment model were analyzed by DAS 3.0.

6. *In vivo* antitumor efficacy and biological safety

The human ovarian carcinoma cell line OVCAR-3 was cultured in 10% (v/v) FBS containing DMEM media. Before *in vivo* study, we also assayed the anti-tumor activity towards OVCAR-3. The protocols are the same as step 4, except the initial number of OVCAR-3 per well is 2,000. Female BALB/c nude mice of 6 weeks old were *s.c.* implanted 5×10^6 OVCAR-3 cells suspended in free media in the left dorsal area. When the tumor grew to an average size of 50 mm^3 (~30 days), mice were randomly assigned to 4 groups (n=6 or 8) and *i.v.* injected with saline and IFN concatemers at a dosage of 1 mg/kg mice body weight every three days until mice of the saline group were all sacrificed. The tumors and body weights of mice were measured on the day of treatment, and the mice would be killed if the tumor volume were over 1000 mm^3 or the body weight loss was over 15%. Tumor volume was measured using the equation: volume = (length × width × width)/2. The data were analyzed via GraphPad Prism software 5.0.

To detect the *in vivo* biological safety of IFN concatemers, the histomorphology, hematology and clinical biochemistry were assayed during and after the treatment. Mice were killed on day 33 and major tissues including tumors, hearts, kidneys and livers were collected and fixed with 4%

paraformaldehyde. The tissues were embedded in paraffin and sliced up into 5 μm thickness of sections. The sections were mounted onto glass slides and stained with hematoxylin-eosin (H&E) according to the standard procedures. The stained slides were imaged with a Nikon Eclipse 90i. Blood was collected from the retro-orbital sinus of mice after being treated for six times (on day 21). The hematological parameters of complete blood, including the number counts of white blood cells (WBC), red blood cells (RBC), platelets (PLT) and the concentration of hemoglobin (HGB) were performed with a Hematology Analyzer (SYSMEX). The biochemical indicators of serum, including lactate dehydrogenase (LDH) and creatine kinase isoenzymes (CK-MB) for heart function, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) for liver function, creatinine (CRE) and blood urea nitrogen (BUN) for kidney function, were detected by Automatic Biochemical Analyzer (HITACHI).

7. Statistical analysis

Data were analyzed using GraphPad Prism software 5.0 and were shown as the mean ± standard deviation. Comparisons of the data including *in vitro* bioactivity, *in vivo* pharmacokinetics and antitumor efficacy were carried out using Student's t test and one-way ANOVA. A P value less than 0.05 was considered significant. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

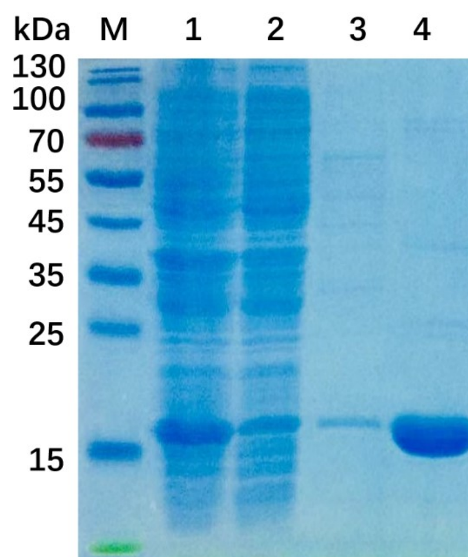

Fig. S1 SDS-PAGE analysis of 1 x IFN purification. M: protein marker; lane 1: crude *E. coli* lysate after ultrasonication; lane 2: flow through; lane 3: 20 mM imidazole wash; lane 4: 200 mM imidazole wash.

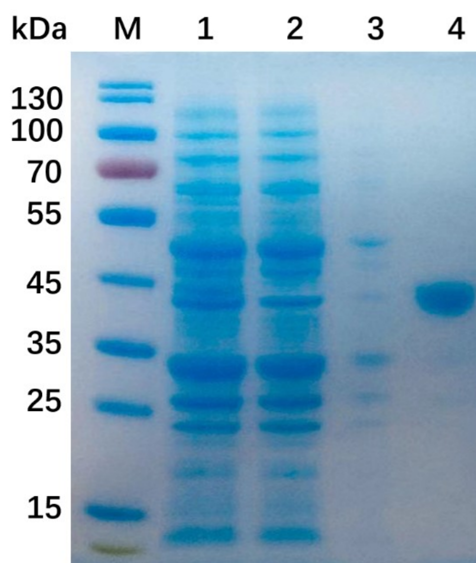

Fig. S2 SDS-PAGE analysis of 2 x IFN purification. M: protein marker; lane 1: crude *E. coli* lysate after ultrasonication; lane 2: flow through; lane 3: 20 mM imidazole wash; lane 4: 200 mM imidazole wash.

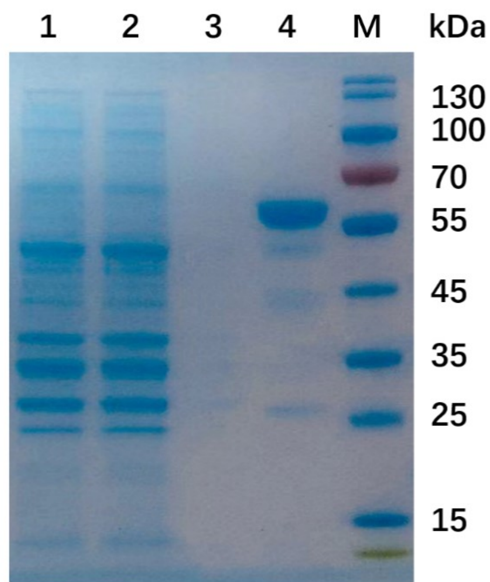

Fig. S3 SDS-PAGE analysis of 3 x IFN purification. M: protein marker; lane 1: crude *E. coli* lysate after ultrasonication; lane 2: flow through; lane 3: 20 mM imidazole wash; lane 4: 200 mM imidazole wash.

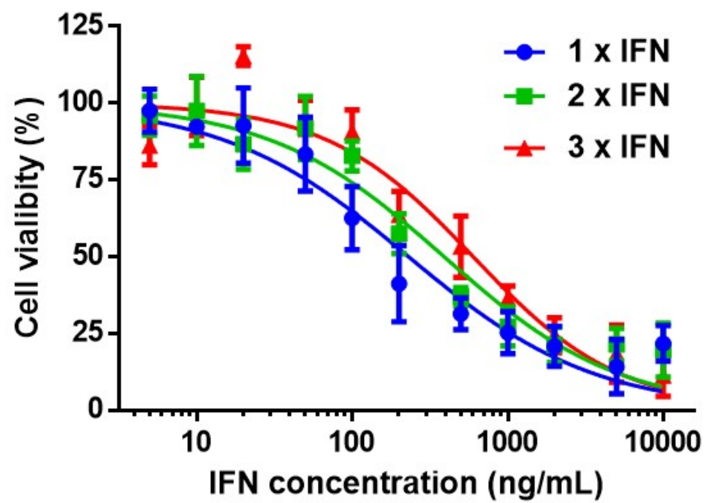

Fig. S4 *In vitro* anti-tumor activity of IFN concatemers for OVCAR-3 cells.

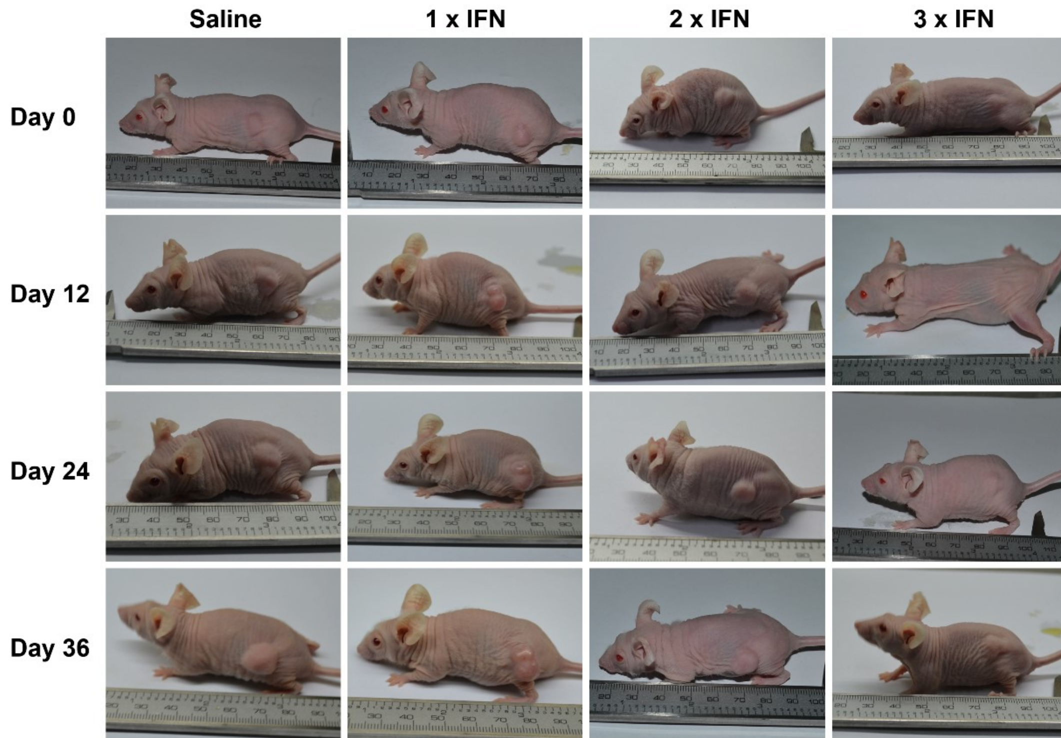

Fig. S5 Representative images for the tumor growth post administration.

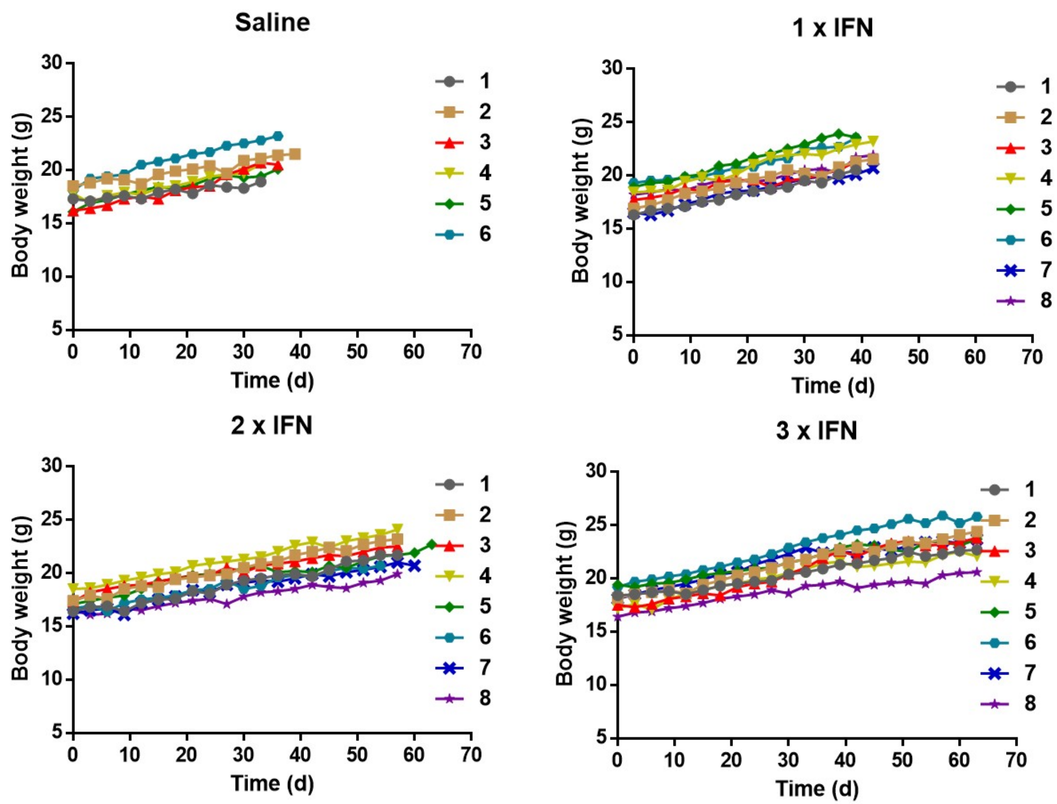

Fig. S6 The change of body weight during treatment.

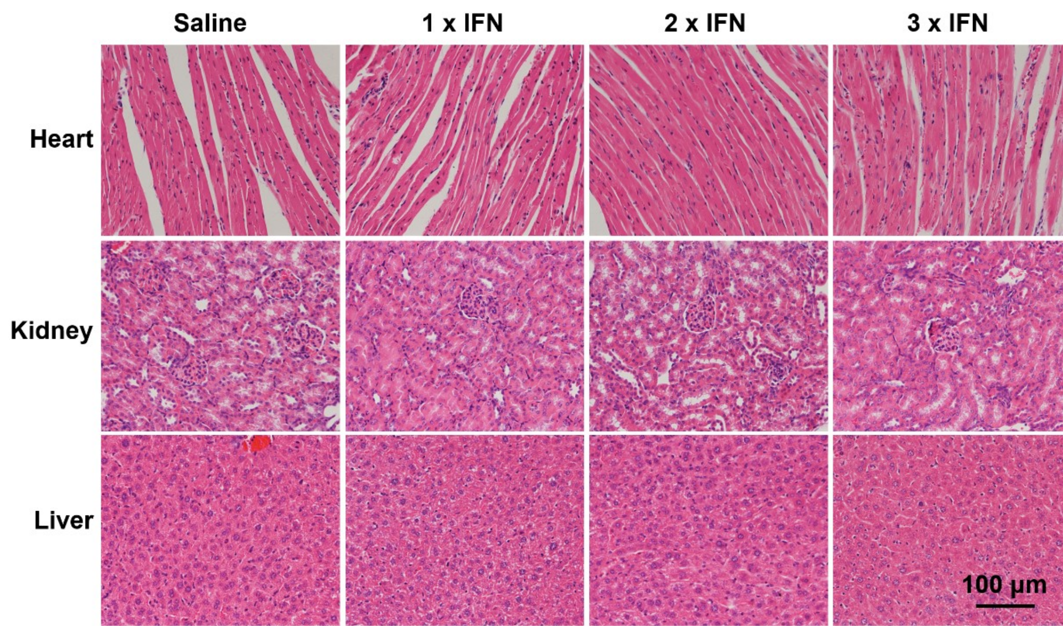

Fig. S7 H&E staining of heart, kidney and liver of mice at 33 days post treatment.

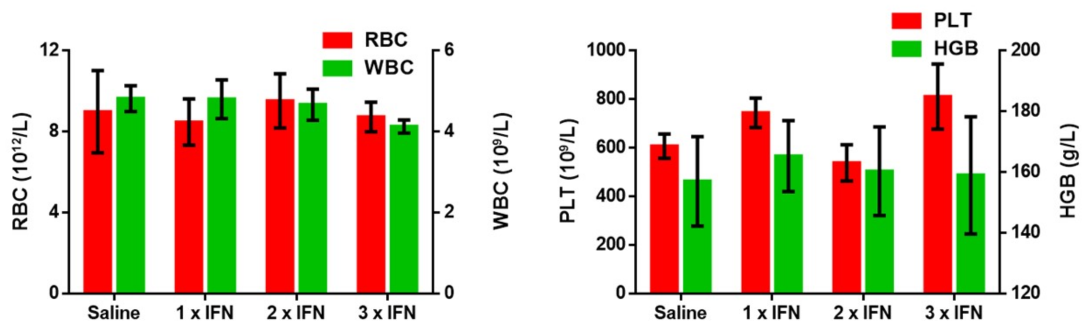

Fig. S8 Hematological parameters for mice at 21 days post administration. WBC, white blood cells; RBC, red blood cells; PLT, platelets; HGB, hemoglobin.

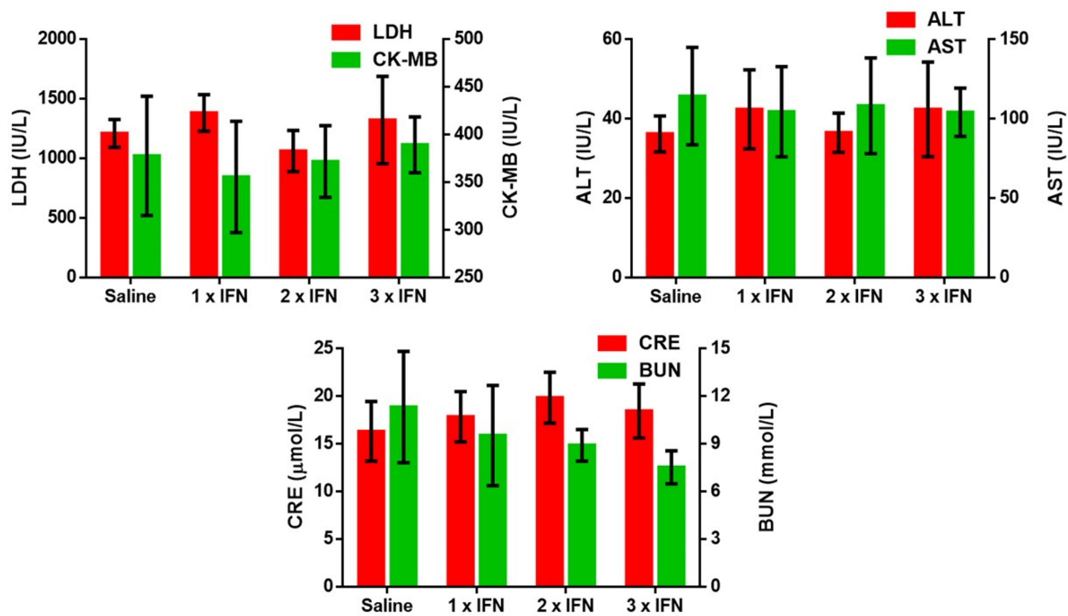

Fig. S9 Clinical biochemistry parameters for mice at 21 days post administration. Liver function markers: ALT, alanine aminotransferase; AST, aspartate aminotransferase. Heart function markers: LDH, lactate dehydrogenase; CK-MB, creatine kinase isoenzymes. Kidney function markers: CRE, creatinine; BUN blood urea nitrogen.

Table S1. The bioactivity retention of concatemers per unit of IFN.

	1 x IFN	2 x IFN	3 x IFN
IC50 (pg/mL)	20.82	29.36	49.98
Activity (%)	100%	70.91%	41.66%

Table S2. The relative number of IFN per protein.

	1 x IFN	2 x IFN	3 x IFN
IC50 (pM)	1.19	0.74	0.52
Activity (n)	1	1.6	2.3

Table S3. Pharmacokinetic parameters of IFN concatemers.

Parameters	1 x IFN	2 x IFN	3 x IFN
Distribution half-life t1/2α (h)	0.411 ± 0.287	0.618 ± 0.0879	0.854 ± 0.0263
Terminal half-life t1/2β (h)	1.26 ± 0.193	6.05 ± 2.22	13.3 ± 1.55
Central compartment volume of distribution V1 (L/g)	3.85 ± 2.18	2.57 ± 0.203	1.20 ± 0.0875
Clearance CL (L/h/g)	4.70 ± 0.240	2.11 ± 0.0218	0.737 ± 0.0672
Area under curve (0-t) (μg/L*h)	0.203 ± 0.0102	0.462 ± 0.00501	1.34 ± 0.124
Area under curve (0-∞) (μg/L*h)	0.213 ± 0.0106	0.474 ± 0.00487	1.36 ± 0.125
Elimination rate constant K10 (1/h)	1.47 ± 0.644	0.823 ± 0.0550	0.615 ± 0.0223
Central to peripheral rate constant K12 (1/h)	0.876 ± 0.692	0.353 ± 0.0868	0.192 ± 0.0488
Peripheral to central rate constant K21 (1/h)	0.639 ± 0.0661	0.142 ± 0.0501	0.0593 ± 0.00603