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A potent *in vivo* anti-tumor efficacy of novel recombinant type I interferon

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Abstract

Purpose—Anti-proliferative, antiviral, and immunomodulatory activities of endogenous type I interferons (IFN1) prompt the design of recombinant IFN1 for therapeutic purposes. However, most of designed interferons exhibited suboptimal therapeutic efficacies against solid tumors. Here we report evaluation of the *in vitro* and *in vivo* anti-tumorigenic activities of a novel recombinant interferon termed sIFN-I.

Experimental Design—We compared primary and tertiary structures of sIFN-I with its parental human IFN α -2b, as well as affinities of these ligands for IFN1 receptor chains and pharmacokinetics. These IFN1 species were also compared for their ability to induce JAK-STAT signaling and expression of the IFN1-stimulated genes and to elicit anti-tumorigenic effects. Effects of sIFN-I on tumor angiogenesis and immune infiltration were also tested in transplanted and genetically engineered immunocompetent mouse models.

Results—sIFN-I displayed greater affinity for IFNAR1 (over IFNAR2) chain of the IFN1 receptor and elicited a greater extent of IFN1 signaling and expression of IFN-inducible genes in

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Conflict of interest

Guang-Wen Wei is the Chairman of the Board, Xin-Yuan Liu is a Board member, and Rong-Bing Guo is a senior scientist of Sichuan Huiyang Life Science and Technology Corp. Chengdu, Sichuan, China that produces sIFN-I. Kang-Jian Zhang's travel was in part supported by the Sichuan Huiyang Life Science and Technology Corp. Other authors claim no conflict of interest.

human cells. Unlike IFN α -2b, sIFN-I induced JAK-STAT signaling in mouse cells and exhibited an extended half-life in mice. Treatment with sIFN-I inhibited intratumoral angiogenesis, increased CD8⁺ T cell infiltration, and robustly suppressed growth of transplantable and genetically engineered tumors in immune-deficient and immune-competent mice.

Conclusions—These findings define sIFN-I as a novel recombinant IFN1 with potent preclinical anti-tumorigenic effects against solid tumor thereby prompting the assessment of sIFN-I clinical efficacy in humans.

Keywords

Type I interferon; cancer; IFNAR1; recombinant interferon; anti-tumor effects

INTRODUCTION

Type I interferons (IFN1) family of anti-viral cytokines comprises thirteen different subtypes of IFN α , as well as IFN β , IFN ϵ , IFN κ , IFN ω etc (1–3). Potent anti-proliferative, pro-apoptotic, anti-angiogenic and immunomodulatory effects of IFN1 prompted their use for anti-cancer treatment (reviewed in (4, 5)). However, after more than 40 years of trials, the use of IFN1 against tumors is limited by the suboptimal ratio between clinical efficacy and the severity of its side effects (6), as well as limited response rate, which is often attributed to the downregulation of IFN1 receptor (7). This heterodimeric receptor complex encompassing the IFNAR1 and IFNAR2 chains mediates all effects of IFN1 on cells (8–10). Levels of IFN1 receptor were indeed shown to correlate with IFN1-induced growth arrest (11) and apoptosis in the tumor samples (12, 13).

The levels of IFN1 receptor on cell surface are largely regulated by the ubiquitin-mediated internalization and degradation of IFNAR1 (10, 14–18). Downregulation of IFNAR1 can be accelerated in some cancers (19–22) thereby limiting the anti-tumorigenic effects of IFN1. Remarkably, although activation of the JAK-STAT pathway is required for both anti-viral and anti-tumor effects of IFN1, lower receptor density still allows efficient antiviral responses while impeding ability of IFN1 to suppress cell proliferation (23). Schreiber and colleagues have proposed that responses to IFN1 could be classified as “robust” (such as anti-viral effects) or “tunable” (such as anti-proliferative or pro-inflammatory), the latter being much more sensitive to receptor density (24). Indeed, high cell surface receptor density and maximal receptor occupancy by relatively high doses of ligands are required to mount an efficient anti-proliferative effect (24, 25).

Furthermore, the affinity of IFN1 subtypes for the extracellular domain of IFNAR1 correlates with ability of these subtypes to elicit specific anti-proliferative effect (26–29). Thus, anti-tumorigenic efficacy of IFN1 may be optimized by increasing cell surface receptor density and/or by designing novel recombinant IFN1 species that display a greater affinity for IFNAR1. A number of IFN1 variants were generated and shown to be effective against tumor cells. For example, a mutant derivative of IFN α -2, IFN α -YNS exhibited tight binding to IFNAR1 and elicited potent pro-apoptotic activity and anti-proliferative/anti-angiogenesis effects *in vivo*; this mutant surpassed IFN α -2 in anti-tumorigenic activity in a breast cancer xenograft (28, 30).

Yet, another approach to increase efficacy of IFN1 treatment is to improve its pharmacokinetics and biological activities. Various efforts in this direction including the use of IFN α -2b-albumin fusion protein (31), antibody armed with IFN1 (32), and peggylation of IFN1 (33). Furthermore, given that many of anti-tumorigenic effects of IFN1 are mediated by the stromal cells, generation of an elegant transgenic mouse model that expresses human IFNAR1 and IFNAR2 subunits, and can be used for transplantation of human tumors resulted in improved ability to test the anti-tumorigenic effects of IFN1 (34).

Here, we characterized anti-tumorigenic properties of a novel recombinant IFN1 derived from human IFN α -2b and other IFN1 subtypes by mutagenesis and termed “super-compound interferon-I” (sIFN-I). Compared to IFN α -2b, sIFN-I exhibited higher anti-HIV activity in severe combined immunodeficient mice reconstituted with human peripheral blood leukocytes (35). Current studies revealed that sIFN-I exhibits increased affinity for IFNAR1 and has greatly improved pharmacokinetics and signaling in human and mouse cells. sIFN-I robustly inhibits intratumoral angiogenesis and suppresses growth of transplantable and genetically engineered tumors in immune deficient and immunocompetent mice. We discuss the direct and indirect mechanisms of potent anti-tumorigenic effects of sIFN-I and potential perspectives of its use in human cancer treatment.

METHODS AND MATERIALS

Cytokines

The novel recombinant super-compound interferon (sIFN-I) and interferon IFN α -2b were provided by Sichuan Huiyang Life Sci & Tech Corporation and Shanghai Huaxin Biotech, respectively. Human IFN β (#: 10704-HNAS) and murine IFN β (#: 50708-M02H), M-CSF (#: 11792-H08H), recombinant type I interferon receptor subunit extracellular domain IFNAR1-EC (#: 13222-H08H) and IFNAR2-EC (#: 10359-H08H) were purchased from Sino Biological Inc, Beijing. Recombinant B18R protein (Vaccinia Virus-Encoded Neutralizing Type I Interferon Receptor) was purchased from eBioscience (#: 14-8185).

Protein crystallization, data collection and structure determination

Crystals of super interferon (sIFN-I) were grown by the hanging-drop vapor diffusion method (3 mg/mL protein concentration) at 20 °C with, in the buffer of 1.2 M Li₂SO₄, 0.1 M 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11.1, 0.02 M MgCl₂. Before data collection, the crystals were equilibrated in a solution containing paraffin oil for a few seconds, and then flash-cooled in a liquid nitrogen stream at -173 °C. Original Data collection to 2.6 Å resolutions was conducted by using the synchrotron radiation from beamline BL5A at a photon factory in Tsukuba, Japan. Primary structural determination was achieved by a combination of molecular replacement method. The position of the sIFN-I was found by molecular replacement using PHASER with the crystal structure of IFN α (Protein Data Bank name: IB5L) used as the search model. The final sIFN-I structure was refined by using molecular modeling techniques and a computerized optimization program, CNS1.1.

Surface Plasmon Resonance assay

Based on surface plasmon resonance technology, binding affinities of both IFN α -2b and super interferon (sIFN-I) toward recombinant extracellular (EC) domain of type I interferon receptor subunit IFNAR1-EC or IFNAR2-EC were measured using the Biacore T100 Protein Interaction Array system (General Electric HealthCare Co.). For immobilization of the receptor subunit via binding the carboxylated dextran surface of the chip via amino groups in protein, a CM5 sensor chip was incubated with the IFNAR1-EC subunit and IFNAR2-EC subunit, at 20 and 50 μ g/mL, respectively. The two tested Interferons were then injected perpendicularly to ligands at different concentrations within the range of 100–3000 nM for IFN α -2b/IFNAR1 binding, 50–1000 nM for sIFN-I/IFNAR1 binding and 3.125–80 nM for both of them on IFNAR2 binding. During IFNs/IFNAR2 binding, a five-second regeneration procedure with 2 M NaCl was added between each step of concentration. Data was analyzed by using Biacore T100 software. Dissociation constants K_D were determined from the rate constants according to the Equation $K_D = k_d/k_a$ (k_d : dissociation; k_a : association).

Cells, cell culture and reagents

Human amnion epithelium WISH cells, all human (A549, HeLa, HT-29, SMMC-7721) and murine (MC38, LLC, B16F10) cancer cell lines were cultured in their complete conditional medium, primary murine melanoma cell line YUMM was cultured as reported previously (36). Lentiviral shRNA targeting sequences were used for knocking down expression of IFNAR1 in WISH cells. For the construction of A549-IFNAR1-KO cells, the *IFNAR1* gRNA targeting sequences were inserted into the Cas9/gRNA target vector LentiCRISPR (37). Lentivirus was packaged and used to infect parental A549 cells. The IFNAR1-negative cell clones were selected with 0.2 μ g/mL puromycin and then confirmed by FACS assay and immunoblot. Detailed information about the cell lines and cell culture, shRNA and sgRNA sequences are provided in Supplementary materials and methods and Supplementary Table 1.

Preparation of cell suspensions from murine spleen, lymph nodes, liver and small intestinal epithelial tissues

Spleen, lymph nodes (including inguinal, brachial, axillary, bilateral superficial cervical, and mesenteric lymph nodes), liver and small intestinal epithelial tissue were isolated from C57BL/6 mice. Briefly after organs were mechanically disaggregated, primary splenocytes and liver cells were obtained and resuspended in PBS after depletion of red blood cells. For isolation of small intestinal epithelial tissue cells (IEC), the intestinal tube of 3 cm length distant from the connection with stomach was cut out and the interior side was washed from one end by using syringe and sterile PBS. Cells were scraped off with the edge of a cover glass, counted and collected for further cell culture or mRNA extraction by Trizol.

Preparation of murine bone marrow-derived macrophages

Bone marrow cells were flushed from the femurs and tibias of sacrificed C57BL/6 mice and then depleted for red blood cells using red cell lysing solution. The cells (1×10^7 cells/well) were cultured in 6-well plates in medium supplemented with 20 ng/mL macrophage colony-

stimulating factor (M-CSF). Nonadherent cells were carefully removed, and fresh conditional medium was added every 2 days. On day 5, the adherent murine BMM cells were collected for further treatment.

Mice

Female nude mice (6–8 weeks old) and female C57BL/6 mice (8 weeks old) were purchased from Shanghai SLAC Company. C57BL/6 *Ifnar1*^{+/+} or *Ifnar1*^{-/-} mouse (strain: B6.129S2-*Ifnar1*^{tm1Agt/Mmjax}) were purchased from The Jackson Laboratory. More detailed information for nude mice models and syngenic transplantable model is provided in Supplementary Materials and Methods. The experiments and animal procedures conducted at Shanghai Institute of Biochemistry and Cell Biology were approved by the Institution Animal Care and Use Committee (IACUC, protocol recording code: IBCB0029REV1). Experiments and all animal procedures conducted at the University of Pennsylvania were approved by the IACUC (protocols # 803995). Female C57BL/6 mice harboring *Tyr::CreER*^{T2}; *Braf*^{CA/+}; *Pten*^{f/f} alleles (which, upon tamoxifen treatment, were converted into *Braf*^{V600E/+}; *Pten*^{-/-} specifically in melanocytes) were kindly provided by Drs. McMahon and Bosenberg. Induction of malignant melanoma by tamoxifen treatment was carried out as previously described (22, 38).

Pharmacokinetics (PK) animal experiments

For PK studies, female C57BL/6 mice (8-week old; Shanghai SLAC Co.) were injected intraperitoneally with sIFN-I or IFN α -2b. All mice in each IFN treated group (n= 9, further divided into three subgroups) simultaneously received a dose of 50 μ g/kg in PBS. Blood samples from each group were collected after 5 min, 15 min, 30 min, 1 h, 1.5 h, 2 h, 4 h, 6 h, 12 h and 24 h from the retro-orbital sinus (subgroup I: 5 min, 1 h, 4 h and 24 h; subgroup II: 15 min, 1.5 h and 6 h; subgroup III: 30 min, 2 h and 12 h). Serum was obtained by centrifugation at 10,000 rpm for 10 min at 4°C, and was stored at -80°C. Untreated mice (n=3) served as negative control. To determine half-life of the two interferons in serum, the concentration values, determined from ELISA measurements (VeriKine TM Human IFN Alpha ELISA Kit, #: 41100, PBL Assay Science Inc.), were plotted against time post injection and numerically fitted using WinNonlin version 6.2 software (Pharsight, St Louis, MO, USA) as described elsewhere (39). Non-compartmental models were assumed. Data (including standard deviations) and curve fits were finally plotted with Graphpad Prism 5.

FACS assays

A549 *IFNAR1*^{-/-} cells (3×10^5) were seeded into 6-well plates. After 24 h, the cells were dissociated with cell dissociation buffer (#: 13151-014, Life Technologies) and 1,500 rpm centrifugation for 5 min in FACS tube, washed with 1x PBS for one time. Then, cells were stained with the self-made mouse anti-human IFNAR1 antibody (1:1000 diluted in 1% BSA-PBS) for 30 min at room temperature. After washing with PBS, cells were stained with AF488 conjugated goat anti-mouse IgG (1:1000 diluted in 1% BSA-PBS) for 30 min. Cells stained with IgG isotype and secondary Ab only were used as negative control were then analyzed.

For detection of cell populations in spleen from tumor bearing mice (*Braf*^{V600E/+}, *Pten*^{-/-}), splenocytes were suspended after red cell lysis. Then, cells were incubated with Fc Blocker antibody for 15 min at room temperature. Subsequently, specific antibodies (listed in Supplementary Table 2) were added and staining was continued for 20 min on ice. After a washing step, cells were stained with 0.5 µg/mL DAPI and were then analyzed immediately. Flow cytometry data acquisition was performed by LSRFortessa machine (BD, NJ, USA), and analysis was performed using FlowJo software.

Immunological and other techniques

Immunoblots, immunofluorescent analysis and other immunological techniques using antibodies listed in the Supplementary information and have been described in our previous publications (15–17). For details on the methods for RNA extraction, cDNA synthesis, quantitative PCR, the information of the synthesized primers, H&E staining, cellular senescence detection of paraffin sections, immunofluorescent analysis of frozen sections, for cell viability assay on human and mouse cells and illustrator image processing, data analyzing, and statistics were described in Supplementary Materials and Methods and our previous publications (22).

Statistical analysis

Comparisons between experimental groups were performed using the Student's t-test and GraphPad Prism 5 software (GraphPad Prism software Inc.). All data were shown as Mean ± SEM. Statistically significant differences are indicated in figures by single (p<0.05), double (p<0.01) or triple (p<0.001) symbols (such as * or #).

RESULTS

sIFN-I differs from IFNα-2b in spatial structure and receptor binding affinity

Primary structural analysis showed that sIFN-I has 89% amino acid sequence homology with IFNα-2b (Figure 1A). The crystal structure of sIFN-I was solved at 2.6 Å resolution; the resulting structure showed that sIFN-I is mainly composed of 6 helices (A, B, C, D, E, F as shown in Figure 1A) and two distinct loops (AB and BC). This structure was generally comparable to the one previously reported for IFNα-2b (40). Nevertheless, a difference between these proteins was noted in the structure of AB loop (residues 25-33: SPFSCLKDR) and BC loop (residues 44-52: DGNQFQKAQ) (Figure 1A and Supplementary Figure 1A). Given previously published data regarding putative role of these loops in the interaction with the ligands (30), we next sought to determine relative affinities of sIFN-I for the receptor chains IFNAR1 and IFNAR2.

Surface Plasmon Resonance assay indeed demonstrated different receptor binding affinities for sIFN-I and IFNα-2b. Under the condition of steady-analysis model used in this experiment, sIFN-I exhibited greater affinity for the extracellular domain of IFNAR1 (KD 6.003×10^{-7} mol/L (0.6 µM)) than IFNα-2b (KD 2.835×10^{-6} mol/L (2.8 µM)) (Figures 1B and 1D). Affinity constant of the extracellular domain of IFNAR2 chain analyzed by the dynamic-analysis model exhibited KD for sIFN-I of 2.192×10^{-8} mol/L (21.9 nM) and KD for IFNα-2b of 1.843×10^{-9} mol/L (1.84 nM). Compared to IFNα-2b, sIFN-I displayed a

higher affinity to IFNAR1 (4.72 folds) but lower affinity for IFNAR2 (11.9-fold) (Figure 1C–D). These properties distinguish sIFN-I from other IFN1 variants such as IFN-YNS and IFN-YNS- α 8tail which exhibit increased affinities to both IFNAR1 and IFNAR2 (24). In fact, with weaker binding towards IFNAR2 but stronger binding to IFNAR1, sIFN-I is mostly reminiscent of properties reported for IFN α -21 (29) that shared 95 % homology with sIFN-I (Supplementary Figure 1B)

sIFN-I requires IFNAR1/IFNAR2 for activating the JAK-STATs pathway

We next compared signaling elicited by sIFN-I and IFN α -2b in human A549 or HeLa cells. A similar extent of STAT1, STAT2 and STAT3 tyrosine phosphorylation was detected after administering both IFN1 types. However, IFN α -2b-induced signaling was more sensitive to inhibition by the vaccinia virus-derived B18R protein mimicking soluble IFN1 receptor and known to inhibit IFN1 pathway via ligand squelching (41) in both cell lines (Figures 2A–B). This result suggests that sIFN-I may exhibit an enhanced signaling capacity under signaling limiting conditions.

Recombinant IFN1 proteins were shown to opportunistically bind other receptors besides IFNAR1/2 such as the opioid receptors (42–44). Thus, we sought to determine whether signaling by sIFN-I depends on canonical IFNAR1/2-JAK-STAT pathway. Experiments in human fibrosarcoma 2fTGH cells (sensitive to IFN1) and derivative clones lacking IFNAR2 (U5A) or JAK1 (U4A) revealed that both IFNAR2 and JAK1 are required for sIFN-I-induced phosphorylation of STAT1 and STAT3 (Figure 3A).

Consistent with these results, sIFN-I did not induce the expression of interferon-stimulated genes (ISG) such as *ISG15* or *CCL5* in either U4A or U5A cells (Figure 3B). Furthermore, RNAi-mediated knockdown of IFNAR1 attenuated sIFN-I-induced phosphorylation of STAT1/STAT3 and expression of *TRAIL* in WISH cells (Figure 3C–D, Supplementary Figure 2) suggesting an important role of IFNAR1 in sIFN-I signaling. To corroborate these data, we used CRISPR/Cas9 approach to knock out IFNAR1 in human A549 cells (Supplementary Figure 3). A robust phosphorylation of STAT1 observed in response to IFN γ (which utilizes Type II IFN receptor (45)) in selected *IFNAR1*^{+/−} or *IFNAR1*^{−/−} clones demonstrated that these cells do not harbor defects in JAK signaling. Importantly, STAT1 phosphorylation in IFNAR1 deficient clones was not induced by sIFN-I (Figure 3E). These data suggest that sIFN-I signals through the IFNAR1/IFNAR2-JAK pathway in human cells.

sIFN-I can act on mouse cells and exhibits distinct pharmacokinetics and tissues responses *in vivo*

Poor sensitivity of mouse IFN1 receptor to human IFN1 species and suboptimal pharmacokinetics of IFN1-based agents pose a challenge for efficient testing of biological effects of human IFN1 (34). Notably, treatment of primary mouse cells with sIFN-I revealed that activity of this ligand in induction of ISGs (*Irf7* and *Isg15*) is superior to that of human IFN α -2b. All these effects were dependent on IFN1 receptor status as evident from the lack of sIFN-I-induced gene expression increase in *Ifnar1* knockout mice (Figure 3F).

We further compared pharmacokinetics of sIFN-I and IFN α -2b in mice after intraperitoneal injection of these agents. To this end, blood samples were taken at fixed time points after IFN administration and IFN concentrations in serum were assessed by ELISA followed by numerical analysis using WinNonlin6.2 software (Figure 4A–B). The pharmacokinetic parameters of sIFN-I and IFN α -2b after administration at the same dose are summarized in Supplementary Table 3. At 15 minutes after injection, the mean serum peak concentration ($C_{\max}$) for IFN α -2b was 16730 pg/mL. However, the $C_{\max}$ of sIFN-I with 9915 pg/mL was delayed to 1 hour after administration. Despite the $C_{\max}$ differences between sIFN-I and IFN α -2b, the area under concentration versus time curve [AUC (0– ∞)] for sIFN-I and IFN α -2b exhibited comparable value (27425 and 24648 pg per hour/mL, respectively) at the same dosage. Such PK data suggested volume distribution (V_z -F) of sIFN-I (4384 mg/kg) is more extensive than that of IFN α -2b (2055 mL/kg) at steady state. In other words, the tissue concentrations of sIFN-I were higher than that of IFN α -2b. Consistent with these data, the induction of expression of IFN-induced genes *Irf7* and *Isg15* in mouse lymph nodes, spleen, liver and intestinal epithelial cells was notably greater after treatment of mice with sIFN-I compared to IFN α -2b (Figure 4C–F) treatment. In all, these data suggest that, compared to IFN α -2b, sIFN-I exhibits a greater distribution in mouse tissues and accordingly elicits a greater IFN-stimulated genes induction in these tissues.

sIFN-I inhibits growth of solid tumors

We next compared the anti-tumorigenic properties of sIFN-I and of IFN α -2b. These agents administered at the doses of 50 μ g–150 μ g/mice were reasonably well tolerated by the A549 or HT-29 tumor-bearing immunocompromised mice; these mice did not exhibit body weight loss during the course of treatment (Supplemental Figure 4). Whereas a modest inhibition of tumor growth was elicited by IFN α -2b, administration of sIFN-I robustly suppressed this growth and led to a stable disease (Figure 5A and Supplementary Figure 4). Analysis of tumor tissues revealed that sIFN-I treatment increased cell senescence markers (senescence-associated β -galactosidase) and dramatically decreased the rate of cell proliferation (assessed by Ki67 staining). Accordingly, an increased expression of p53 tumor suppressor protein as well as cyclin-dependent kinase inhibitors p21 and p27 was found in tumor tissues from mice treated with sIFN-I (Supplemental Figure 5).

When tested for growth inhibition *in vitro*, both IFN α -2b and sIFN-I exhibited robust effects on human WISH cells at the dose of 1 μ g/mL (Figure 5B). A greater dose (50 μ g/mL) was required to detect modest inhibitory effect of either of these IFN1 agents on growth of A549, HT-29 human cancer cells and MC38 mouse cancer cell line. Under these conditions, sIFN-I was slightly more efficient than IFN α -2b while growth of some of human (SPC-A4) or mouse (YUMM) cancer cell lines (MC38, YUMM) *in vitro* was not inhibited by IFN1 even at 50 μ g/mL (Figure 5C). Given that IFN1 can act on tumor vascularization and anti-tumor immunity (45), it is plausible that these indirect mechanisms may contribute to potent anti-tumorigenic effects of sIFN-I observed *in vivo*.

sIFN-I suppresses angiogenesis and stimulates anti-tumor immunity

Treatment of C57BL/6 mice bearing a syngeneic B16F10 melanoma with sIFN-I but not IFN α -2b resulted in suppression of tumor growth (Supplementary Figure 6A). sIFN-I also

suppressed tumor growth in mice burdened with syngeneic colorectal (MC38) or lung (LLC) adenocarcinomas (Supplementary Figure 6B–C). These results suggest that sIFN-I can elicit its anti-tumorigenic activities in immunocompetent hosts.

To further understand the antitumor effects of sIFN-I on tumor host, we tested its action in immunocompetent C57BL/6 mice inoculated with syngeneic murine melanoma cell line YUMM (*Braf*^{V600E/+}/*Pten*^{-/-}/*Cdkn2a*^{-/-}). Administration of sIFN-I into tumor bearing *Ifnar1*^{+/+} mice led to a dramatic suppression of growth of transplanted tumor. Importantly, when *Ifnar1* knockout animals were chosen as hosts, tumors grew more aggressively and did not respond to treatment with sIFN-I (Figure 6A). Given this *Ifnar1*-dependent difference in responses to sIFN-I and the fact that YUMM cells were poorly sensitive to growth inhibition by sIFN-I *in vitro* (Figure 5B–C), these results suggest that sIFN-I can suppress tumor growth through affecting tumor stromal compartment.

Consistent with this possibility, compared to untreated animals or treated *Ifnar1*^{-/-} mice, tumors from sIFN-I-treated *Ifnar1*^{+/+} mice contained fewer blood vessels and were less positive for endothelial marker CD31 (Figure 6B–C). Furthermore, these tumors contained a greater number of CD3⁺CD8⁺ cytotoxic lymphocytes (Figure 6B–C). These results support a notion that sIFN acts on tumor stromal compartment and may impede tumor growth via inhibiting tumor angiogenesis and increasing tumor infiltration by CD3⁺CD8⁺ cytotoxic lymphocytes (indicative of reversing tumor immunosuppression) in the IFNAR1-dependent manner.

Having observed a robust therapeutic effect of sIFN-I in transplanted tumors, we sought to determine whether this agent can also be active in genetically engineered models. To this end, we induced melanoma tumors in *Braf*^{V600E/+}; *Pten*^{-/-} mice and started the treatment after establishing tumors with the average size of 51 mm³ in both groups. Administration of sIFN-I to notably suppressed growth of these tumors (Figure 6D). When all control mice receiving vehicle had to be sacrificed for humane reasons (i.e. tumor size reaching the limit required by IACUC), animals receiving sIFN-I exhibited either stable disease or partial/complete tumor regression (Figure 6D–E).

In this model, sIFN-I did not noticeably affect infiltration of tumors with CD31-positive cells. However, consistent with tumor regression, we observed significant increase of infiltrating cytotoxic lymphocytes in tumors treated with sIFN-I in genetically engineered mouse melanomas (Figure 6F and Supplemental Figure 7). Furthermore, sIFN-I notably suppressed metastases of genetically engineered melanoma into the lymph nodes (Figure 6G). These results strongly suggest that sIFN-I exhibit a potent anti-tumorigenic effect against primary tumors and metastatic disease.

DISCUSSION

Endogenous IFN1 plays an important role in protection against tumors due to their anti-proliferative, anti-angiogenic and immunostimulating activities (2). The response rate and therapeutic efficacy of IFN1-based pharmaceutical agents is limited, especially in solid tumors (4, 6) because oncogene signaling, tumor microenvironment stress, unfolded protein

response and inflammation can decrease the levels of IFNAR1 available for ligand interaction (19–21, 46, 47). Besides developing means to reverse downregulation of IFNAR1, additional solutions for optimizing IFN1 therapy can be based on the observation that anti-tumorigenic efficacy of diverse IFN1 subtypes parallels affinity of these types for IFNAR1 (48, 49). Here we describe sIFN-I, a novel recombinant IFN1 exhibiting increased affinity for IFNAR1 and potent anti-tumorigenic properties.

Intriguingly, while tightly binds to IFNAR1, sIFN-I exhibits a lesser affinity for IFNAR2 (normally a chain with greater affinity for endogenous ligands (48) compared to its “parental” molecule IFN α -2b (Figure 1), which is different from the other reported interferon variants such as IFN-YNS and IFN-YNS- α 8tail (24). The latter variants displayed enhanced ligand binding affinity to both IFNAR1/2, and also showed enhanced anti-proliferation activity for cancer cells *in vitro* (28). Whereas *in vitro* activities of sIFN-I are relatively underwhelming, sIFN-I exerts its potent anti-tumor effect *in vivo* (Figure 5A and 6, Supplementary Figure 4A).

sIFN-I elicits notable activation of STAT proteins and ensuing induction of ISGs (Figures 2 and 3F); importantly, all these effects of sIFN-I depend on integrity of the IFNAR1/IFNAR2-JAK pathway (Figure 3). Furthermore, tumor-bearing mice lacking *Ifnar1* are poorly responsive to anti-tumorigenic activities of sIFN-I (Figure 6). These results suggest that despite (or because of) potentially altered ligand-IFNAR1-IFNAR2 complex, sIFN-I robustly activates this receptor and downstream IFN1 signaling pathway.

Remarkably, compared to human IFN α -2b, the effects of sIFN-I appear to transcend the species differences. Data presented here reveal that sIFN-I elicits the IFN1-stimulated gene induction responses in primary mouse cells and mice *in vivo* (Figures 4–5). Furthermore, in terms of pharmacokinetics in mouse, sIFN-I exhibited longer half-life and lower peak drug concentration in serum compared to IFN α -2b (Figure 4). Intriguingly, there was a two-step serum increase for sIFN-I; this phenomenon was not observed for IFN α -2b injected into mice. These differences could be attributed to the different binding model for sIFN-I towards plasma protein or lipoprotein in blood which lead to re-release of sIFN-I from the sIFN-I/plasma protein or sIFN-I/lipoprotein dynamic binding complex (50). Such possibility would be consistent with two peaks in concentration-time curve for serum concentration of 2'5'-OAS (a well-known downstream markers of the pharmacodynamic activity of interferon) observed in blood after sIFN-I subcutaneous injection for the healthy volunteers (51). Altered pharmacokinetic characteristics of sIFN-I may contribute to greater ISG induction and improved anti-tumorigenic activities *in vivo* (Figure 5) and, furthermore, may potentially cause lesser side effects. These possibilities in humans will be revealed by clinical trials of sIFN-I in Singapore (CTC1300056) and USA (NCT02464007) that are currently conducted in patients with solid tumors.

Previous published data suggested that sIFN-I can suppress the tumor growth in some isolated clinical cases in human patients (52). Our current data demonstrate greater efficacy of sIFN-I over IFN α -2b against human tumors xenotransplanted into immunocompromised mice (Figure 5A). Given a robust response of mouse tissues to sIFN-I, this response may at least in part be attributed to the effects of sIFN-I on mouse stromal cells. Indeed, in

immunocompetent syngeneic transplantation or genetically engineered mouse melanoma models, sIFN-I notably suppressed angiogenesis and/or increased tumor infiltration with cytotoxic lymphocytes. These anti-angiogenic and immunostimulatory effects of sIFN-I are likely to contribute to robust anti-tumorigenic efficacy of sIFN-I that elicit stable disease or/and tumor regression in very aggressive melanoma tumors (Figure 6). Detailed studies of the mechanisms underlying immunostimulatory and other effects of sIFN-I are ongoing. These studies will be instrumental in designing clinical trials in humans that will address clinical efficacy of sIFN-I alone or in combination with traditional, molecularly targeted or immune targeted therapies.

Supplementary Material

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Statement of translational relevance

Despite potent anti-tumorigenic properties of natural and pharmacological Type I interferons (IFN1), these agents achieved only a limited success in cancer therapy. This manuscript describes the molecular and biological characterization of de novo engineered and highly potent recombinant interferon (sIFN-I), which has evoked massive clinical interest and is currently undergoing clinical trials in patients with solid tumors in Singapore (CTC1300056) and USA (NCT02464007), as well as patients with HBV in China (2009L04155). Here we present data obtained both *in vitro* and *in vivo* settings; these data demonstrate that sIFN-I exhibits superior pharmacodynamics and pharmacokinetics characteristics compared to its parental human IFN α -2b species. Furthermore, studies conducted in cells and in animals harboring transplantable and genetically engineered tumor models reveal that sIFN-I evokes potent anti-tumorigenic effects at least in part by inhibiting stromal angiogenesis and by stimulating anti-tumor immunity.

Figure 1. sIFN-I exhibits altered binding affinities towards the receptor subunits compared to IFNα-2b

A. Protein sequence and structure comparison between sIFN-I (red) and IFNα-2b (green). Top, amino acid sequence alignment between IFNα-2b and sIFN-I. Bottom, the secondary structures on monomer including side view (left) and vertical view (right). Each monomer consists of 6 main segments of the helices (A, B, C, D, E, F) and the connecting peptide segments. Broken ellipses represent the AB or BC loop.

B. Comparison of the dissociation constants for sIFN-I (black) and IFNα-2b (blue) to immobilized IFNAR1-EC. The constants were determined by steady-analysis model.

C. Binding curves of IFNα-2b or sIFN-I to immobilized IFNAR2-EC. The constants were determined by dynamic-analysis model.

D. Quantification of the binding affinities toward the two receptor subunits between sIFN-I and IFNα-2b.

Figure 2. sIFN-I is capable of an increased signaling under limiting conditions
A. IFN signaling in A549 cells: 10-fold serial dilutions of recombinant B18R protein (1 to 100 ng/mL, final assay concentration) were prepared in media and combined with a constant amount (1 ng/mL, final assay concentration) of each IFN protein (sIFN-I or IFNα-2b) for 1 h at room temperature. The B18R/IFN complexes were transferred to cells and then incubated for 30 min. The phosphorylation and total signal of STAT1, STAT2, and STAT3 were detected by immunoblot and the p-STATs level were quantified compared to their corresponding total STAT proteins, +, means treatment with 100 ng/mL B18R protein.
B. IFN signaling in HeLa cells was analyzed as in Panel A.

Figure 3. sIFN-I elicits its signaling in an IFNAR1/2 dependent manner

A. Human fibrosarcoma 2fTGH cells (sensitive to IFN1) and derivative clones deficient in either JAK1 (U4A) or IFNAR2 (U5A) were treated with human IFN β , IFN α -2b or sIFN-I (10 ng/mL). The phosphorylation and total signal of STATs were detected by Western blot after 15-min treatment.

B. The induction of indicated IFN-stimulated genes in cells described in Panel A was detected by qPCR after 24-h treatment.

C. WISH cells with stable IFNAR1 knockdown expression were treated with human IFN α -2b or sIFN-I (10 ng/mL) or mouse IFN β (negative control) for 15 min. The phosphorylation of STAT proteins was detected by immunoblot.

D. Cells described in panel C were treated with indicated IFN for 24h and the induction of TRAIL mRNA was detected by qPCR.

E. A549 cells harboring various IFNAR1 status expression were treated with indicated IFNs (10 ng/mL) for 30 min. The phosphorylation of STAT1 and ERK were detected by immunoblot.

F. Lymphocytes (lym), splenocytes (spl) and bone marrow-derived macrophages (BMM) were obtained from *Ifnar1* knockout (*Ifnar1*^{-/-}) or wild type (*Ifnar1*^{+/+}) mice. The similar numbers (6–10×10⁶) of these primary cells were cultured and treated with murine IFN β (10 ng/mL), hIFN α -2b (1 μ g/mL) or sIFN-I (1 μ g/mL) for 24 h, then the induction of *Irf7* and *Isg15* were quantified by qPCR. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$, vs mock group; #, $P<0.05$, ##, $P<0.01$, ###, $P<0.001$, vs IFN α -2b group.

Figure 4. sIFN-I displays different pharmacokinetics and distinct tissues responsiveness *in vivo*

A. Calibration curve of human IFNα ELISA used for pharmacokinetics assay.

B. C57BL/6 wild type mice were subjected to intraperitoneal injection in each group (n=3) with hIFNα-2b or sIFN-I. Serum was obtained at the indicated time point and the concentrations of serum IFNs were detected by ELISA assay. Comparison of the pharmacokinetic curves of hIFNα-2b or sIFN-I administered as in Panel A. Additional information is provided in the Supplementary Table 3.

C. C57BL/6 wild type mice were intraperitoneal injected with murine IFNβ (1 μg/mL), human IFNα-2b (1 μg/mL) or sIFN-I (1 μg/mL), respectively. Primary tissues were collected for gene expression detection after 6-h treatment. The induction of *Irf7* and *Isg15* mRNA in lymph nodes was quantified by qPCR.

D. Analysis of *Irf7* and *Isg15* mRNA was quantified by qPCR in spleen tissues of mice described in Panel C.

E. Analysis of *Irf7* and *Isg15* mRNA was quantified by qPCR in intestinal epithelial tissues of mice described in Panel C.

F. Analysis of *Irf7* and *Isg15* mRNA was quantified by qPCR in liver tissues of mice described in Panel C. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, vs mock group; #, $P < 0.05$, ##, $P < 0.01$, ###, $P < 0.001$, vs IFNα-2b group.

Figure 5. sIFN-I exhibits potent anti-solid tumor effects in xenotransplanted tumor models
 (A) A549 or HT-29 xenograft tumors were treated with intratumoral injection of sIFN-I or IFN α -2b (5 mg/kg) every other day for the indicated days; in the HT-29 model, 5 mg/kg Mitomycin (MMC) treatment as a positive control. The tumor volume was measured and calculated as follows: tumor volume (mm^3) = (length $\times$ width²)/2. (B-C) Indicated cancer cell lines (human lung cancer cells SPC-A4, A549, human colon adenocarcinoma cell HT-29, murine colorectal cancer cell line MC38 and murine primary melanoma cell line YUMM) and WISH cells were treated with 1 μ g/mL (B) or 50 μ g/mL (C) interferons for four days. Cell viability and proliferation was assessed by WST1 assay. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, vs mock group; #, $P < 0.05$, ##, $P < 0.01$, ###, $P < 0.001$, vs IFN α -2b group.

Figure 6. Anti-tumorigenic, anti-angiogenic and immunostimulating effects of sIFN-I in immunocompetent mouse models

A. YUMM (*Braf*^{V600E/+}; *Pten*^{-/-}; *CDKN2A*^{-/-}) cells were injected subcutaneously into *Ifnar1*^{+/+} and *Ifnar1*^{-/-} mice to establish transplantable tumor model. sIFN-I or IFN α -2b (5 mg/kg) were injected intraperitoneally every other day for the indicated days. The tumor volume was measured and calculated.

B. H&E and immunofluorescence staining of YUMM tumors isolated from mice after sIFN-I treatment. Arrows indicate the vessels in tumor tissue. Scale bar, 100 μ m.

C. Quantification on the positive CD31⁺ vessels number in the fields (n=7) and CD3⁺CD8⁺ T cells infiltrated in YUMM allograft tumor microenvironment (n=10).

D. Melanocyte-specific Cre activity was induced in adult mice (*Braf*^{CA/+}*Pten*^{f/f}) by topical application of 4-HT to shaved back skin. Melanoma growth was measured after intraperitoneal injection with sIFN-I every other day.

E. Volume of melanoma tumors that grew in *Braf*^{V600E/+}; *Pten*^{-/-} mice to initial volume ("-", blue circles). After that, mice were randomly assigned to two groups treated with PBS (black squares, left mouse in the inset) or sIFN-I (red triangles, right mouse at inset) for 32 days. p<0.001 between PBS and sIFN-I group.

F. Immunofluorescence staining of the tumor isolated from *Braf*^{V600E/+}; *Pten* / mice after sIFN-I treatment. Bottom, quantification on the average positive CD31⁺ vessels number in the fields (n=10) and the double positive CD3⁺CD8⁺ cells in the fields (n=10) presenting infiltrated effector T cells in tumors from *Braf*^{V600E}; *Pten* / mice. Scale bar: 100 μ m.

G. H&E staining of the tumors and superficial lymph nodes (n=12) isolated from *Braf*^{V600E/+}; *Pten* / mice after sIFN-I treatment. Bottom, quantification on the number of metastatic tumors in lymph node (LN). Scale bar, 100 μ m.