Immunomodulatory antitumor effect of interferon-beta combined with gemcitabine in pancreatic cancer

AMBER BLAAUBOER[1,2], PETER M. VAN KOETSVELD[2], DANA A.M. MUSTAFA[3], JASPER DUMAS[3], FADIME DOGAN[2], SUZANNE VAN ZWIENEN[2], CASPER H.J. VAN EIJCK[1] and LEO J. HOFLAND[2]

Departments of [1]Surgery, [2]Internal Medicine, Division of Endocrinology and [3]Pathology, The Tumor Immuno-Pathology Laboratory, Erasmus Medical Center, 3015 CN Rotterdam, The Netherlands

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Abstract. Resistance to gemcitabine is common and critically limits its therapeutic efficacy in patients with pancreatic cancer. Interferon-beta (IFN-β) induces numerous antitumor effects and synergizes with gemcitabine treatment. The immunomodulatory effects of this treatment regimen have not yet been described. In the present study, the antitumor effect of IFN-β combined with gemcitabine was investigated in immune competent mice. Mouse KPC3 cells were used in all experiments. Treatment effects were determined with cell proliferation assay. Reverse transcription-quantitative PCR was used to measure gene expression. For *in vivo* experiments, cells were subcutaneously injected in immune competent mice. For immune profiling, NanoString analysis was performed on tumor samples of treated and untreated mice. Baseline expression of *Ifnar-1* and *Ifnar-2c* in KPC3 cells was 1.42±0.16 and 1.50±0.17, respectively. IC_{50} value of IFN-β on cell growth was high (>1,000 IU/ml). IFN-β pre-treatment increased the *in vitro* response to gemcitabine (1.3-fold decrease in EC_{50}; P<0.001). *In vivo*, tumor size was not statistically significant smaller in mice treated with IFN-β plus gemcitabine (707±92 mm^3 vs. 1,239±338 mm^3 in vehicle-treated mice; P=0.16). IFN-β alone upregulated expression of numerous immune-related genes. This effect was less pronounced when combined with gemcitabine. For the first time, to the best of our knowledge, the immunomodulatory effects of IFN-β, alone and combined with gemcitabine, in pancreatic cancer were reported. Prognostic markers for predicting effective responses to IFN-β therapy are urgently needed.

Correspondence to: Professor Leo J. Hofland, Department of Internal Medicine, Division of Endocrinology, Erasmus Medical Center, 80 Wytemaweg Street, 3015 CN Rotterdam, The Netherlands
E-mail: l.hofland@erasmusmc.nl

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Introduction

Pancreatic cancer is one of the most aggressive malignancies and highly resistant to currently applied cancer therapies (1). Even after curative-intent surgery, cure is exceedingly rare, as demonstrated by a 5-year overall survival (OS) of less than 20% (2). Effective adjuvant systemic therapies are necessary to improve survival outcomes.

Previously, the PRODIGE 24/CCTG PA.6 trial reported an impressive survival benefit with adjuvant modified-FOLFIRINOX (mFOLFIRINOX) compared with adjuvant gemcitabine (median OS 54 vs. 35 months; P<0.001) (3). However, due to the high toxicity rate of this treatment regime, gemcitabine is still being recommended, in particular for patients with poor or declining performance status.

Resistance to gemcitabine is a major impediment of successful treatment and is primarily due to molecular mechanisms limiting the intracellular uptake and metabolic activation of gemcitabine, and thus, its overall efficacy (4).

Type I IFNs (IFN-α and -β) have been proposed as potential adjuvant to gemcitabine treatment in order to improve survival outcomes in patients with pancreatic cancer (5,6). Type I IFNs are pleiotropic cytokines that were originally identified as viral replication suppressor. Further characterization of their biological effect revealed a wide range of antitumor effect; for instance, direct inhibitory effects on tumor cells, anti-angiogenesis, enhanced immunogenicity of tumors as well as other immune stimulatory effects (7,8). Thereby, type I IFNs induce synergistic effects on pancreatic cancer cells when co-treated with gemcitabine *in vitro* (9,10). Recently, the chemo-sensitizing effect of IFN-β in pancreatic cancer, formed in immune deficient mice, was confirmed and upregulation of gemcitabine transporter-coding genes was identified (9).

Both IFN-α and -β act via the type I IFN receptor (IFNAR) complex, of which IFNAR-1 and IFNAR-2c are the most important subunits (11). Binding to this receptor activates the JAK-STAT signaling pathway, which subsequently initiates the transcription of numerous interferon-stimulated genes (ISGs) that are responsible for mediating the biological activities of type I IFNs.

To accomplish an effect of type I IFNs, the presence of the IFNAR complex is necessary (11,12). Previously, expression

of IFNAR-1 and IFNAR-2c in 91.5 and 68.1% of pancreatic cancer tissues, respectively, was reported (13).

Although IFN-α and -β bind to the same receptor and induce signals through similar mechanisms, they have different binding affinities. Previous studies reported a 50-fold higher receptor-binding affinity for IFN-β than IFN-α, resulting in more potent, elicited at much lower concentrations, antitumor effects (10,14).

Despite strong evidence of the more potent and safer antitumor effects of IFN-β compared with IFN-α, only a few studies focused on adjuvant IFN-β therapy in pancreatic cancer. In addition, the immunomodulatory effects of IFN-α and -β are less described and primarily investigated in IFN-α. Hence, in the present study, the potential immunomodulatory effect of IFN-β towards pancreatic cancer cells was revealed for the first time. The unique KPC3 cell line was used, derived from the clinically relevant KPC mouse model, which mimics the immune phenotypic features, aggressiveness, and gemcitabine-resistant character of human pancreatic cancer (15,16). Thereby, the present study focused on expression of gemcitabine transporter- and activating-coding genes, as potential target to increase gemcitabine efficacies.

Materials and methods

Cells and culture conditions. The mouse KPC3 pancreatic cell line is derived from a primary pancreatic ductal adenocarcinoma tumor of a female KrasG12D/+;Trp53R172H/+; Pdx-1-Cre (KPC) mouse and was kindly provided by Dr van Montfoort (Leiden University Medical Center, Leiden, The Netherlands) (15). Origin of cells was confirmed using short tandem repeat profiling (Powerplex Kit; Promega Corporation). Cells were cultured in RPMI-1640 medium supplemented with 5%fetal calf serum (FCS), penicillin ($1x10^5$ U/l), and L-glutamine (2 mmol/l). FCS was purchased from Sigma-Aldrich; Merck KGaA. Media and other supplements were obtained from Gibco; Thermo Fisher Scientific, Inc. FCS was purchased from Sigma-Aldrich; Merck KGaA. Cells were cultured in a 5% CO_2 atmosphere at 37˚C and routinely validated as Mycoplasma-free. Culture conditions were as previously described in detail (17).

After trypsinization, KPC3 cells were plated in 24-well plates at the appropriate density in order to obtain 80% confluency at the end of the experiment. The next day, incubations were started in quadruplicate and control cells were vehicle-treated. Medium and compounds were refreshed after 3 days. All cell culture experiments were carried out at least twice in quadruplicate. Mouse recombinant IFN-β-1a (Bio-Connect) and gemcitabine (Sigma-Aldrich; Merck KGaA) stock dilutions were diluted in distilled water.

Cell proliferation assay. Effects of IFN-β and/or gemcitabine on cell growth were assessed by measuring the total DNA amount per well, as a measure of cell number. After treatment, media were removed and plates were stored at -20˚C until DNA measurement. Measurement of total DNA was performed with the bisbenzimide fluorescent dye (Hoechst 33258; Sigma-Aldrich; Merck KGaA) as previously described in detail (17).

Reverse transcription-quantitative (RT-q) PCR. RNA isolation, cDNA synthesis and RT-qPCR were performed as previously described (9), but using different primers (Table SI). Two housekeeping genes were used to normalize *mRNA* levels using the Vandesompele method: hypoxanthine-guanine phosphoribosyl transferase 1 (*Hprt1*) and glucuronidase beta (*Gusb*) (Gibco; ThermoFisher Scientific, Inc.) (18).

Mice. A total of 28 male C57BL/6 mice (8-10 weeks old) were purchased from Charles River Laboratories. All mice were housed in groups of seven and maintained at room temperature on a daily 12-h light/12-h dark cycle in ventilated cages with autoclaved bedding. Water and autoclaved laboratory rodent diet were provided *ad libitum*. All mouse experiments were controlled by the animal welfare committee of the Erasmus University Medical Center (Rotterdam, The Netherlands) and approved (approval no. AVD101002017867) by the national central committee of animal experiments, in accordance with the Dutch Act on Animal Experimentation and European Union (EU) Directive 2010/63/EU.

In vivo experiments. Mice were randomized in four groups (n=7 each) and subcutaneously injected in the flank with 100,000 low passage (passage number 3) KPC3 cells in 100 μl PBS/0.1% BSA (Gibco; Thermo Fisher Scientific, Inc.). Cultured KPC3 cells were harvested at 80% confluency and only single-cell suspensions of greater than 90% viability were used for injection. Tumor size and body weight were measured twice weekly. Tumor volume was calculated as (width^2 x length)/2 using a caliper. Treatment was started when tumor volumes reached ~50 mm^3. Mice in the control group and in the IFN-β monotherapy group received daily an intraperitoneal (i.p.) injection of 100 μl of distilled water or 10,000 units IFN-β. Mice randomized to the gemcitabine monotherapy group received two times a week (day 2 and 5) an i.p. injection of 50 mg/kg gemcitabine. Mice in the combination group received upon start of the treatment, daily an injection of 10,000 units IFN-β i.p. and, on day 2 and 5, an i.p. injection of 50 mg/kg gemcitabine. The effect of gemcitabine monotherapy in this model has been previously described (19).

Necropsy procedures. Mice were sacrificed by cervical dislocation under 5% isoflurane anesthesia when tumor volume reached 1,000 mm^3 or when the wellbeing of the mice could no longer be maintained. Tumors were resected during necropsy and tumor volumes were measured. Tumors were divided into two parts and subsequently snap-frozen in liquid nitrogen and fixed overnight at 4˚C in freshly prepared 4% formaldehyde solution, and prepared for paraffin sectioning (5-μm thick).

NanoString analysis. RNA was extracted according to the manufacturer's instructions using the RNeasy Plus Micro kit (Qiagen). RNA samples were diluted in RNA free water and stored at -80˚C. The 2100 Bioanalyzer (Agilent Technologies, Inc.) was used to measure RNA Quality Control. Total RNA concentrations were corrected to include fragments seized between 300 and 4,000 nucleotides. A total of 200 ng RNA was hybridized to the PanCancer IO 360 Panel (NanoString Technologies, Inc.) at 67˚C for 17 h. The advanced analysis module (version 2.0) of nSolver™ software (version 4.0;

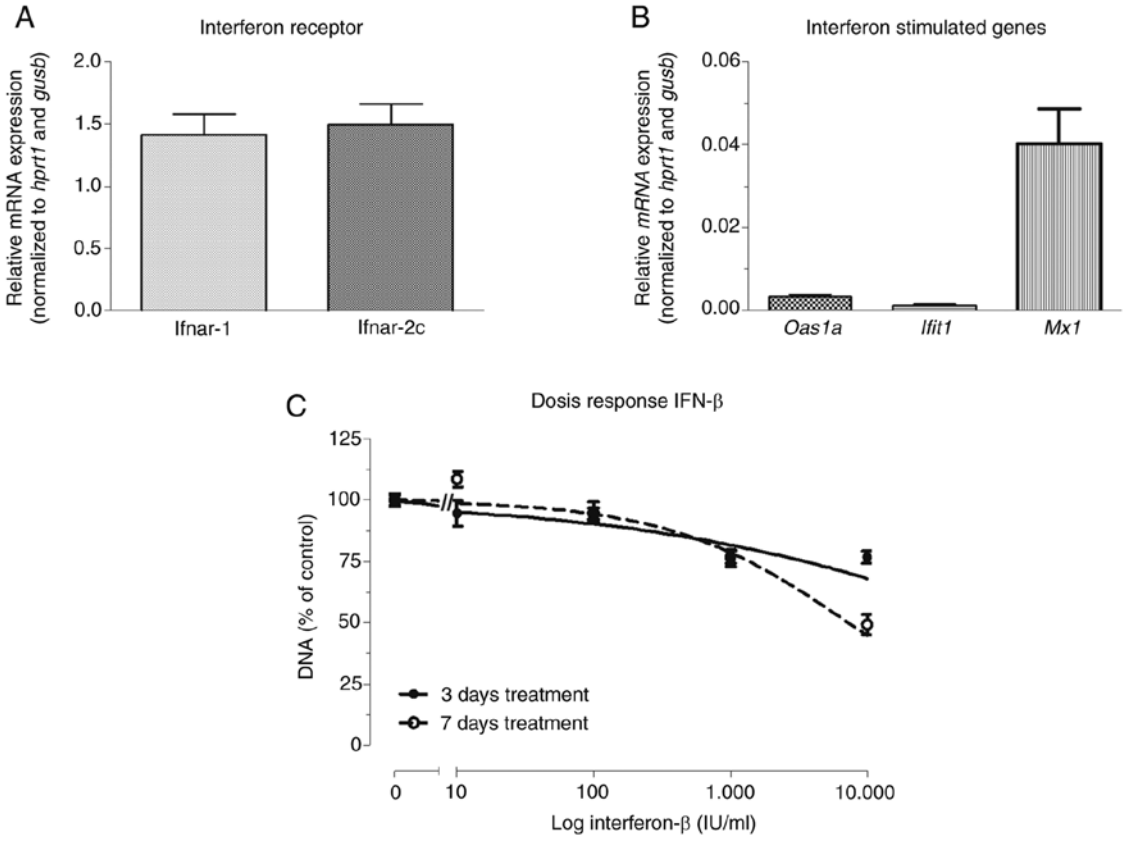

Figure 1. IFN-β sensitivity in KPC3 cells *in vitro*. (A) Baseline *mRNA* expression of the IFN receptor subunits *Ifnar-1* and *Ifnar-2c* and (B) baseline *mRNA* expression of the interferon-stimulated genes *Oas1a*, *Ifit1* and *Mx1*. (C) Dose response curves of IFN-β on cell amount (as measured by total DNA content per well), after 3 and 7 days of treatment. Values represent the mean ± SEM of at least two independent experiments in quadruplicate and are shown as the percentage of control. IFN-β, interferon-β; Ifnar-1, interferon alpha and beta receptor subunit 1; Ifnar-2c, interferon alpha and beta receptor subunit 2; Oas1a, 2'-5' oligoadenylate synthetase 1a; Ifit1, interferon induced protein with tetratricopeptide repeats 1; Mx1, MX dynamin like GTPase 1.

NanoString Technologies, Inc.) was used for data analysis. A total of 8 out of 11 housekeeping genes were selected for normalization with the geNorm algorithm embedded in the advanced analysis module (Table SII). Expression threshold was calculated as twice of the average expression of the negative controls. Gene expression below the threshold in more than 80% of the samples were excluded from further analysis. Normalized data were log2 transformed. Differentially expressed genes were identified with simplified negative binomial models, mixture negative binomial models, or log-linear models based on the convergence of each gene. Adjusted P-values were calculated with the Benjamini Hoghberg method. Genes were considered differentially expressed when the adjusted P<0.05.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 3.0 (GraphPad Software, Inc.). The half maximal effective concentration (EC_{50}) on cell growth was calculated using non-linear regression curve fitting program. Effect of IFN-β was set on 100% and used as control to analyze the combined effect of IFN-β pre-treatment and gemcitabine in *in vitro* experiments. One-way ANOVA followed by Tukey's multiple comparisons test was used for comparisons between treatment groups. In all analyses, P<0.05 was considered to indicate a statistically significant difference. Data are indicated as the mean ± SEM.

Results

IFN-β sensitivity in KPC3 cells in vitro. Relative *mRNA* expression of the IFN receptor-coding genes, *Ifnar-1* and *Ifnar-2c*, were comparable in KPC3 cells (1.42±0.16 and 1.50±0.17, respectively) (Fig. 1A). Baseline expression of ISGs in untreated KPC3 cells was relatively low (Fig. 1B). The growth-inhibitory effect of IFN-β was not very potent, as shown by an EC_{50} >1,000 IU/ml and maximal inhibition of 51% after 7 days (P<0.001 vs. control) (Fig. 1C).

Effect of IFN-β pre-treatment on the response to gemcitabine in vitro. Next, it was analyzed whether IFN-β pre-treatment could sensitize KPC3 cells, reflected by a decrease in the EC_{50} value of gemcitabine. Although 72 h after 1,000 IU/ml IFN-β pre-treatment alone had no statistically significant effect on the cell amount, gemcitabine sensitivity was slightly increased, as shown by a 1.3-fold decrease in EC_{50} value compared with untreated control cells (EC_{50} 1.5 ng/ml vs. EC_{50} 1.1 ng/ml respectively; P<0.001) (Fig. 2A and C). Expression of *Oas1a* and *Ifit1* was strongly upregulated following 72 h after 1,000 IU/ml IFN-β treatment (51- and 13-fold increase respectively; both P<0.001 vs. untreated cells), whereas expression of *Mx1* was not affected by IFN-β treatment (Fig. 2B).

Baseline expression of transporter- and metabolizing-coding genes in KPC3 cells was low and even

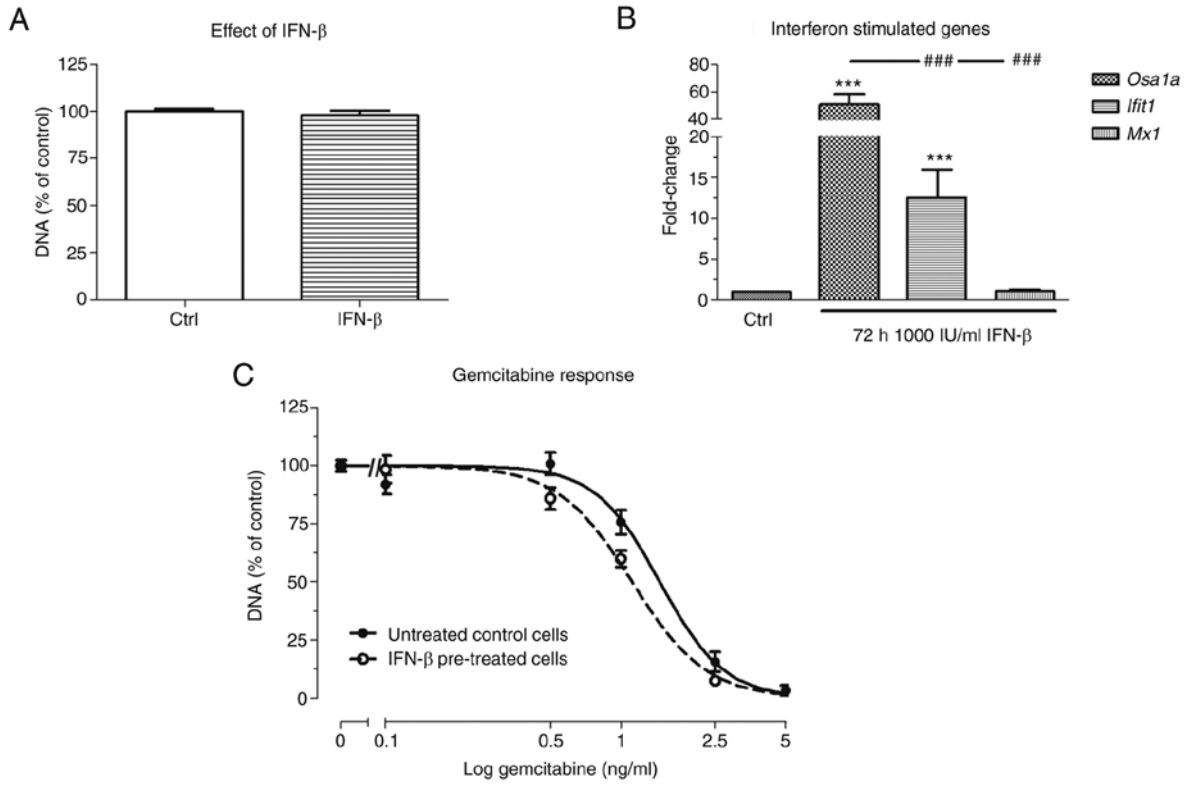

Figure 2. Effect of IFN-β pre-treatment on gemcitabine response in KPC3 cells. Cells were pre-treated for 72 h with 1,000 IU/ml IFN-β, followed by 72 h gemcitabine monotherapy. (A) Effect of 72 h 1,000 IU/ml IFN-β monotherapy. (B) Fold change in *mRNA* expression of *Oas1a*, *Ifit1*, and *Mx1* between no treatment and after 72 h 1,000 IU/ml IFN-β. (C) Overall gemcitabine response in untreated control cells (solid line) vs. 72 h IFN-β pre-treated cells (dotted lines). Controls represent vehicle control for non-IFN-β pre-treated and treatment with IFN-β for IFN-β pre-treated cells. Values represent the mean ± SEM of at least two independent experiments in quadruplicate and are shown as a percentage of control. ***P<0.01 vs. control and $^{###}$P<0.01 vs. other gene. IFN-β, interferon-β; Oas1a, 2'-5' oligoadenylate synthetase 1a; Ifit1, interferon induced protein with tetratricopeptide repeats 1; Mx1, MX dynamin like GTPase 1.

undetectable for *Slc28a1* and *Slc28a3* (Fig. S1). Expression was not upregulated after IFN-β treatment (Fig. S1).

In vivo validation of IFN-β combined with gemcitabine in immune-competent mice. To study the immunomodulatory antitumor effect of IFN-β, low-passage KPC3 cells were subcutaneously injected in C57BL/6 mice. Mice were randomized into four treatment arms: daily H_2O (control), daily 10,000 units IFN-β, twice weekly 50 mg/kg gemcitabine, or the combination of daily 10,000 units IFN-β plus twice weekly 50 mg/kg gemcitabine (Fig. 3A). All mice were sacrificed at day 21, after which tumors were collected for analysis. None of the treatment arms resulted in a statistically significant tumor growth inhibition over time, although lowest tumor volumes were observed in the combined treatment group, suggesting an additive effect rather than a synergistic effect (Fig. 3B-D). After 21 days of treatment, tumor volume was 707±92 mm^3 in the combination treated-mice compared with 1,239±338 mm^3 in the vehicle treated-mice (P=0.16) (Fig. 3C). Tumor volumes in gemcitabine and IFN-β mono-treated mice were 1,162±232 and 962±271 mm^3, respectively (both P>0.05 vs. untreated mice).

No significant weight loss was observed in any of the treatment groups, indicating that all drugs were well tolerated (Fig. 3E). Expression of transporter- and metabolizing-coding genes of gemcitabine was low in untreated KPC3 tumors and were not affected by any treatment (data not shown).

Effect of IFN-β on expression of immune-related genes. To specifically address the immunomodulatory capacity of IFN-β, a targeted gene expression array was performed on tumor samples of treated and untreated mice. Differentially expressed genes upon treatment compared with untreated mice are revealed in Fig. 4A. After adjusting for multiple testing, only a few genes were significantly altered by the different treatment groups. Specifically, gemcitabine downregulated expression of *Itpk1*, *Igf2r*, *Bmp2*, *Dusp1*, and *Cdkn1a*, but increased *Tgfbr1*. IFN-β only upregulated *Gdp2*, and in combination-treated tumors, *Itpk1* and *Lilra5* were respectively down- and upregulated (Fig. 4A, Table SIII).

Without adjusting P-values, an extensive number of genes was found to be differentially expressed (Fig. 4B and Table SIII). In total, 35 genes were upregulated by IFN-β, while 38 genes were downregulated. In gemcitabine-treated tumors, 30 and 69 genes were respectively up- and downregulated. A further 32 genes were upregulated by combination therapy, whereas 56 genes were downregulated (Fig. 4B). Top ten up- and downregulated genes are summarized in Table I. Gemcitabine and combination therapy commonly affected several genes (*Snca*, *Lilra5*, *Fgfl3*, *Siglecf*, *Prkaa2*, *Selp*, *Hk2*, *Erol1*, *Ndufa412* and *Vegfa*), while IFN-β alone induced a different gene expression profile (Table I).

IFN-β monotherapy markedly upregulated expression of several immune-related genes. As a consequence, 7 out of 9 immune-related pathways were upregulated in IFN-β-treated

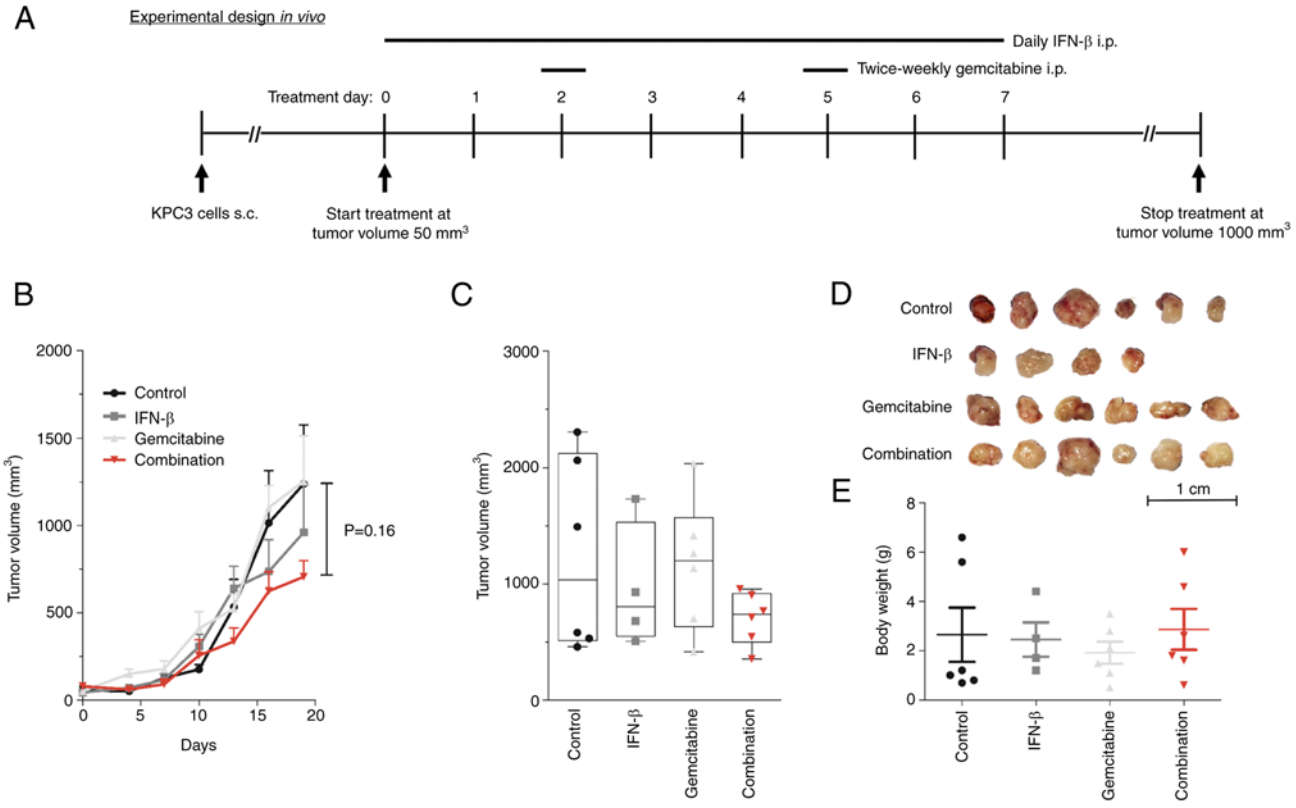

Figure 3. *In vivo* antitumor effects of IFN-β alone and in combination with gemcitabine, in immune competent mice. (A) Experimental design for *in vivo* experiments. KPC3 mouse pancreatic cancer cells (1x10^5/100 μl PBS/0.1% BSA) were subcutaneously injected in C57BL/6 mice. Treatment was started when tumor volumes reached 50 mm^3. Groups of mice received daily an i.p. injection of IFN-β (10,000 units), two times a week (at day 2 and 5) an i.p. injection of gemcitabine (50 mg/kg), or the combination of IFN-β plus gemcitabine. Mice in the control group received daily an i.p. injection of 100 μl distilled water. (B) Time course of change in tumor volume. (C) After 21 days of treatment, mice were sacrificed and tumor volumes were measured. (D) Tumor images after 21 days of treatment. (E) Body weight difference from start until end of treatment. Values represent the mean ± SEM. IFN-β, interferon-β; i.p, intraperitoneal.

tumors compared with untreated mice (range pathway score 1.67-0.54) (Fig. 4C and D). By contrast, gemcitabine induced a suppressive effect on these pathways (range pathway score-1.11-0.39), but when co-treated with IFN-β, 6 out of 9 immune-related pathways were increased (range pathway score 0.96-0.43).

Expression of IFN signaling pathway regulators (*Ifi35*, *Irf1*, *Irf7* and *Stat2*) and four ISGs (*Gbp2*, *Gbp3*, *H2-d1* and *Uba7*) were induced by IFN-β alone. Moreover, expression of several ISG chemokines (*Ccl19*, *Cxcl10* and *Cxcl11*) were increased in these tumors (Table II). In combination-treated tumors, the regulatory factors *Ifi35* and *Trim21* were upregulated as well as two well-known pro-apoptotic ISGs (*Mx1* and *Oas1a*). By contrast, gemcitabine downregulated IFN-related genes (*H2-K1*, *H2-T23*, *Ifi203*, *Igf2r* and *Irf2*). Numerous genes involved in antigen presentation were stimulated by IFN-β; for instance, *Tap1*, *Tap2*, *Psmb9* and *Psmb10*. This effect was less pronounced in combination-treated tumors and also affected a different set of genes (*Trim21*, *Ctsw* and *Lag3*). Remarkably, the myeloid compartment pathway was downregulated by IFN-β, but stimulated by gemcitabine and combination therapy through upregulation of *Clec7a*, *Lilra5*, and *P2ry13*.

Analysis reported no evident differences in pro-tumor pathways, such as angiogenesis and metastasis, and were equally inhibited in all treatment groups compared with untreated tumors (Fig. 4C).

Discussion

Pancreatic cancer is a highly aggressive malignancy with limited treatment outcomes. Despite promising recent advances in systemic therapies, for instance mFOLFIRINOX, gemcitabine is still being recommended for patients with a poor or declining performance status (3).

Type I IFN-based adjuvant therapies have been widely studied in pancreatic cancer, primarily due to their potential synergistic effects when co-treated with gemcitabine (5,6,9,10). Thereby, type I IFNs induce several direct antitumor effects, including apoptosis and cell growth arrest as well as critical immune stimulatory effects on various immune cells (7,8).

To date, studies have primarily focused on the direct antitumor effects of the less effective IFN-α. The immunomodulatory effects of IFN-α and -β are less described in pancreatic cancer and primarily investigated in IFN-α. To the best of our knowledge, this is the first study evaluating the antitumor effects of the more promising IFN subtype IFN-β, alone and combined with gemcitabine, on pancreatic cancer cells in immune competent mice. The unique KPC3 pancreatic cancer cell line was used, generated from the clinically relevant and non-immunogenic KPC mouse model (15).

A trend towards smaller tumor volumes in combination-treated mice was observed, suggesting an additive effect rather than synergistic effect. Moreover, tumors displayed a differential gene expression profile upon treatment. In

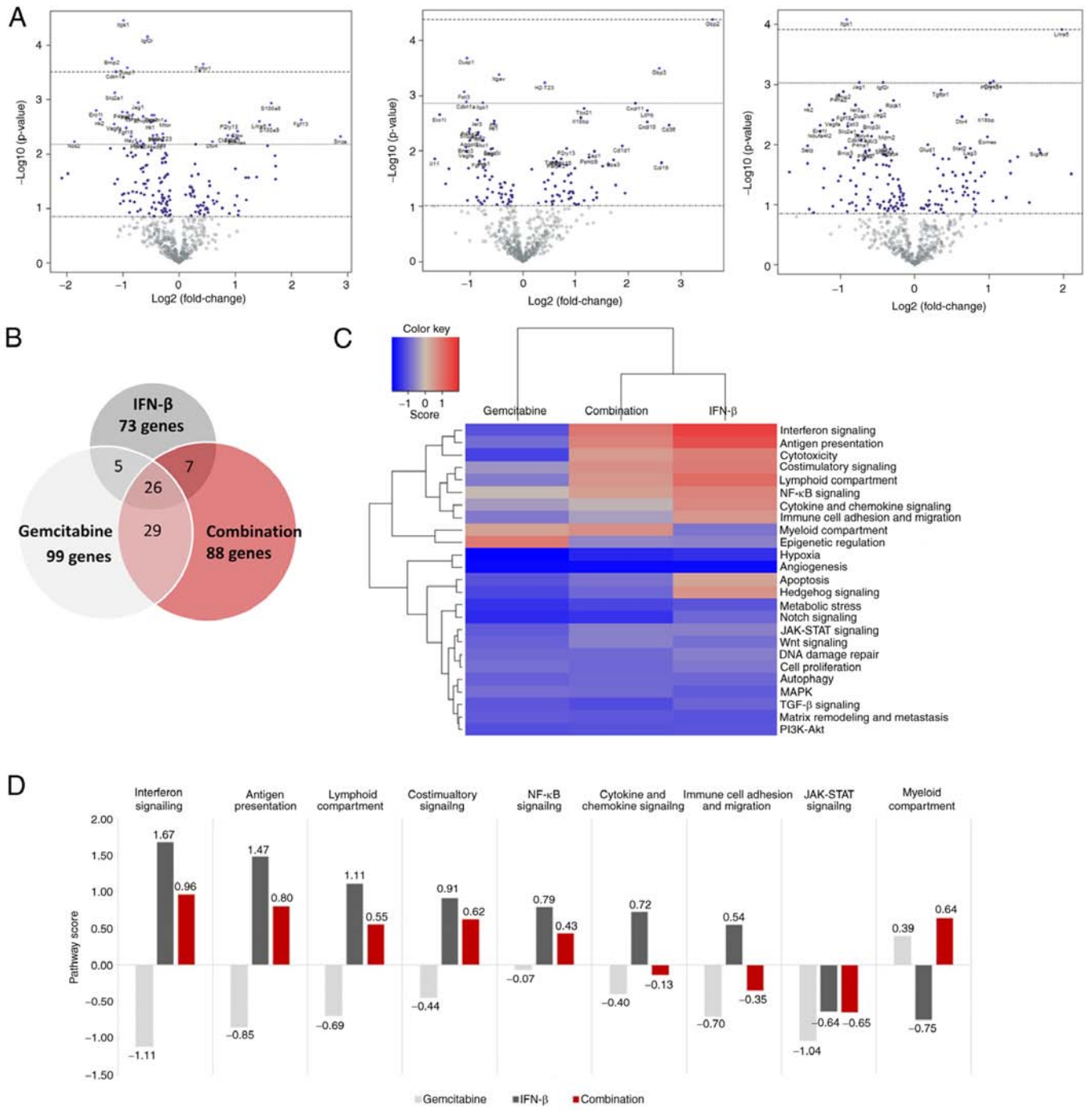

Figure 4. Nanostring analysis PanCancer IO 360 panel. (A) Volcano plot displays-log10(p-value) and log2 fold change of each gene with the selected covariate. Highly statistically significant genes fall at the top of the plot above the horizontal lines, and highly differentially expressed genes fall to either side. Horizontal lines indicate various P-value thresholds. The 40 most statistically significant genes are labelled in the plot. (B) Venn diagram shows the number of up- and downregulated genes in the three treatment arms compared with control. Overlapping areas represent the number of genes that were altered by both treatment arms. (C) Heatmap displays directed global significance scores of each sample. Directed global significance statistics measure the extent to which gene sets of a gene are up- or downregulated with the variable. Red denotes gene sets whose genes exhibit extensive overexpression with the covariate, blue denotes gene sets with extensive under expression. (D) Pathway scores of immune-related pathways. Increasing pathway scores corresponds to increasing expression. IFN-β, interferon-β.

particular, IFN-β alone induced expression of numerous immune-related genes, such as chemokines (*Ccl19*, *Cxcl10* and *Cxcl11*) and antigen processors (Tap1 and Tap2). Thereby, expression of *Irf7* was upregulated, which primarily regulates the immunomodulatory capacities of IFN-β, as well as other IFN signaling pathway regulators (e.g., *Ifi35*, *Irf1* and *Stat2*) (20,21). Notably, *Irf1*, which promotes expression of ISGs, and *Irf7* are key factors in the positive feedback regulation of IFN-β production and potentiate ISG expression as they directly target ISGs, even in the absence of IFN signaling (22-24). It should be emphasized, however, that only *mRNA* expression was evaluated. Further studies should demonstrate that IFN-β treatment indeed results in effective antitumor immune responses.

Table I. Top ten up- and down-fold regulation.

Gemcitabine vs. control				IFN-β vs. control				Combination vs. control			
Gene name	Log2 fold change	P-value	B.H. P-value	Gene name	Log2 fold change	P-value	B.H. P-value	Gene name	Log2 fold change	P-value	B.H. P-value
Snca	2.87	0.00479	0.0887	Gbp2	3.6	$4.27x10^{-5}$	0.0261	Snca	2.1	0.0307	0.266
Fgf13	2.17	0.00234	0.0731	Cd36	2.78	0.0034	0.116	Lilra5	1.98	0.000121	0.0369
Fcer1a	1.72	0.0288	0.22	Ccl19	2.63	0.0164	0.251	Fgf13	1.69	0.0135	0.197
Gzmb	1.72	0.0166	0.161	Gbp3	2.59	0.000323	0.0638	Siglecf	1.67	0.0122	0.193
Siglecf	1.71	0.0106	0.12	Cxcl10	2.36	0.00302	0.115	Prkaa2	1.38	0.0161	0.206
S100a8	1.64	0.00117	0.0731	Cxcl11	2.13	0.00139	0.0943	Sfrp1	1.29	0.0302	0.266
S100a9	1.61	0.00293	0.0745	Cd1d1	1.89	0.00804	0.196	Col11a1	1.28	0.0191	0.212
Prkaa2	1.57	0.00711	0.101	Fcer1a	1.76	0.0412	0.377	Clec7a	1.06	0.000881	0.095
Lilra5	1.43	0.0025	0.0731	Cpa3	1.72	0.0147	0.242	P2ry13	1.01	0.000932	0.095
Cpa3	1.38	0.0265	0.214	Nlrc5	1.63	0.0175	0.258	Eomes	0.978	0.00744	0.162
A2m	-2.09	0.0276	0.216	Il11	-1.68	0.014	0.241	Nkg7	-1.69	0.0286	0.266
Sfrp4	-1.98	0.0226	0.203	Ero1l	-1.58	0.00223	0.113	Selp	-1.44	0.0104	0.192
Nos2	-1.86	0.00582	0.094	Arg1	-1.47	0.0394	0.375	Tnfrsf11b	-1.44	0.0207	0.222
Ero1l	-1.47	0.00156	0.0731	Adm	-1.41	0.0212	0.267	Hk2	-1.42	0.00219	0.112
Hk2	-1.41	0.00227	0.0731	Fstl3	-1.12	0.000866	0.0882	Ero1l	-1.27	0.00483	0.139
Vegfa	-1.15	0.00281	0.0745	Bnip3	-1.09	0.00845	0.197	Ndufa4l2	-1.27	0.0059	0.152
Selp	-1.14	0.0355	0.244	Vegfa	-1.07	0.0102	0.202	Adm	-1.22	0.025	0.239
Ndufa4l2	-1.22	0.00788	0.105	Cdkn1a	-1.07	0.0013	0.0943	Vegfa	-1.1	0.00387	0.124
Itpk1	-0.989	$3.5x10^{-5}$	0.0211	Dusp1	-1.06	0.00021	0.0638	P4ha2	-1.02	0.0015	0.102
Bnip3	-0.984	0.00789	0.105	Slc2a1	-1.02	0.00446	0.134	Col5a1	-1.03	0.0401	0.295

B.P., Benjamini Hoghberg.

Table II. Up- and downregulation of immune-related genes.

	Gemcitabine vs. control	IFN-β vs. control	Combination vs. control
Antigen presentation	↑ *Ctsw, Cd276, Gzmb, H2afx, Hells, Sfxn1, Snca* ↓ *Atf3, Bnip3, Ccl4, Cd274, H2-K1, H2-M3, H2-T23, Parp4, Rad50, Srebf1, Tnks*	↑ *Cd1d1, Cxcl10, Cxcl11, Cd36, Cybb, H2-D1, H2-T23, Irf1, Lck, Ldhb, Parp9, Parp12, Psmb9, Psmb10, Tap1, Tap2, Tapbp, Uba7* ↓ *Bnip3, Gzme, Ill1, Itgav, Ldha, Pms2*	↑ *Trim21, Ctsw, Lag3, Snca* ↓ *Bnip3, Hdac4, Mdm2, Mlh1, Nkg7, Rad51c, Srebf1*
Costimulatory signalling	↑ *Cd274, Mtor, Rictor*	↑ *Il18bp, Lck, Psmb10, Psmb9* ↓ *Ptgs2*	↑ *Havcr2, Hck, Il18bp, Lag3*
Cytokine and chemokine signalling	↑ *Ccl4, Jak3, Rock1*	↑ *Ccl19, Cxcl10, Cxcl11, Il18bp, Stat2* ↓ *Il11*	↑ *Cxcr2, Il18bp, Stat2* ↓ *Rock1*
Immune cell adhesion and migration	↑ *Cd276, Clec5a, Clec7a* ↓ *Cd274, H2-K1, H2-M3, H2-T23, Pvr, Rock1, Selp*	↑ *H2-D1, H2-T23* ↓ *Itgav, Pvr*	↑ *Clec7a, Cybb* ↓ *Cdh1, Pvr ,Rock1, Selp*
Interferon signalling	↓ *H2-K1, H2-T23, Ifi203, Igf2r, Irf2*	↑ *Gbp2 , Gbp3, H2-D1, Ifi35, Irf1, Irf7, Stat2, Uba7*	↑ *Ifi35, Mx1, Oas1a, Trim21*
JAK-STAT signalling	↑ *Cdkn1a, Jak3, Lif, Mtor*	↑ *Stat2* ↓ *Cdkn1a , Il11, Lif*	↑ *Stat2* ↓ *Cdkn1a*
Lymphoid compartment	↑ *Eomes, Gzmb, Lck, Tbx21* ↓ *Cd274, Igf2r, Pvr*	↑ *cd1d1, cxcl10, cxcl11, eomes, ikzf3, stat2, tbx21* ↓ *F2rl1, gzme, pvr*	↑ *Eomes, Lag3, Mx1, Stat2* ↓ *Igf2r, Pvr*
Myeloid compartment	↑ *Clec5a, Clec7a, Fcer1a, Lilra5, P2ry13, S100a8, S100a9* ↓ *Areg, Ccl4, Cdkn1a, Ier3, Lmab3, Lif, Nos2*	↑ *Fcer1a, P2ry13* ↓ *Areg, Arg1, Cdkn1a, Ier3, Lif, Ptgs2*	↑ *Clec7a, Col11a1, Cybb, Hck, Lilra5, P2ry13* ↓ *Areg, Cdkn1a, Dll4, Ier3, Lamb3*
NF-kappaB signalling	↑ *Ltb*	↑ *Psmb10, Psmb9*	↓ *Tnfrsf11b*

Controversially, gemcitabine induced an immune suppressive effect, whereas addition of IFN-β to gemcitabine solely induced a subset of immune-related genes, which was less pronounced compared with IFN-β mono-treated tumors. Since gemcitabine promotes the infiltration of particularly M2 macrophages in the tumor microenvironment, it may have diminished the immune stimulating effect of IFN-β when administered together (25).

Efficacy of several anticancer therapies, including chemotherapies, partially depend on an intact type I IFN signaling for the promotion of both direct (tumor cell inhibition) and indirect effects (antitumor immune responses) (26). Thus, impaired IFN signaling may as a consequence contribute to therapy resistance in patients with cancer. KPC tumors respond poorly to gemcitabine therapy, particularly orthotopic tumors, which is consistent with clinic outcomes, as only 5-10% of gemcitabine-treated patients show an objective radiographic response at the primary tumor site (27). Regarding *in vivo* research in KPC mice, the most frequently used gemcitabine concentrations are 50 and 100 mg/kg (16,28,29). To avoid any potential toxicity, mice were treated with 50 mg/kg gemcitabine. As expected, no significant tumor inhibition was observed with gemcitabine alone. Moreover, type I IFN signaling was diminished in gemcitabine-treated tumors as several IFN-related genes were downregulated when compared with untreated tumors (for instance, *H2-K1*, *H2-T23*, *Ifi203*, *Igf2r* and *Irf2*). Meanwhile, combination therapy increased expression of IFN regulator factors (for instance, *Ifi35* and *Trim21*) as well as two well-known pro-apoptotic ISGs (*Mx1* and *Oas1a*), but was still insufficient to significantly inhibit tumor growth.

Over the years, numerous studies highlighted the significant role of immune host-mediated mechanisms in the response to type I IFNs, even in IFN-resistant tumor cells (30-33). However, most studies used high-dose intratumoral IFN-β concentrations (33-35). Although intratumoral IFN-β concentrations were not evaluated in the present study, the relatively low concentration IFN-β (i.p. administered) as well as the resistant character of KPC-3 cell to IFN-β therapy may have resulted in insufficient intratumoral levels to induce significant tumor growth inhibition. It is plausible that IFN-β will exert stronger antitumor responses in IFN-β sensitive tumors. In fact, it has been previously shown that IFN-β combined with gemcitabine synergistically reduced tumor volumes in immune deficient mice, bearing an IFN-β sensitive tumor, when compared with untreated mice (9). Moreover, the relatively short half-life time of IFN-β may limit sufficient circulating IFN-β concentrations. Strategies to increase the half-life time of IFN-β, such as IFN-based conjugates or PEGylated form of

IFN-β, have demonstrated promising results to achieve higher serum concentration, requiring lower and less frequent doses compared with the conventional IFNs (36,37).

While recombinant IFN therapies are generally given as exogenous pharmaceuticals, it is suggested that the autocrine and paracrine actions of endogenous type I IFNs on tumor growth control (both the direct and indirect effects) are much stronger. New IFN-related cancer treatment strategies, such as STING and RIG-I agonists, have emerged as promising and effective strategies to produce significant amounts of endogenous type I IFNs and are currently being examined in (pre-) clinical studies in various types of cancer, including pancreatic cancer (8). IFN-β gene therapy induced by viral vectors provides another promising strategy to achieve high intratumoral IFN-β levels and has demonstrated potent anti-tumor efficacy in several pre-clinical cancer models, including pancreatic cancer, with low toxicities (38-40). Discrepancies between treatment outcomes largely depend on differences in IFN-β sensitivity, highlighting the need for accurate biomarkers to predict IFN-β treatment response. Expression of the active IFN-receptor subunits (IFNAR-1 and IFNAR-2c) is required to form a high-binding affinity site and to initiate signal transduction leading to the induction of ISGs (11,41). However, despite expression of both receptor subunits, KPC3 cells responded poorly to IFN-β, suggesting other alterations involving the IFN downstream signaling pathway. In fact, IFN dysregulation can occur through several mechanisms such as loss or silencing of key signaling effector proteins and components (JAKs, STATs and IRFs) or through upregulation of negative regulators (SOCS1/3) (42,43). A careful evaluation of type I IFN signaling may provide important insights into sensitivity to IFN-β therapies and may possibly contribute to a more personalized medicine approach in the future.

Previously emerged evidence has suggested that type I IFNs may have dual roles in antitumor immunity, which may be even harmful and cause further adaptive resistance to therapies, including chemotherapy, radiotherapy and immune checkpoint blockade (44-47). Two hypotheses have been proposed for this controversial effect. First, continuous exposure of type I IFNs may upregulate PD-L1 expression in tumor cells, which subsequently promotes immune resistance trough interaction with PD-1+ immune effector cells (48). Thereby, prolonged type I IFN stimulation may induce an IFN-related DNA damage resistance signature (IRDS) that indicates an unfavorable response to DNA-damaging interventions such as chemotherapy and radiotherapy (44). Previously, IRDS scoring strategies have been used to identify patients with breast and lung cancer and showed higher expression of specific ISGs in poor responders to chemotherapy (49). These paradoxical findings are not yet fully understood and may also differ among types of cancer and depend on the subtype of type I IFNs (50).

In conclusion, for the first time the immunomodulatory potential of exogenous IFN-β combined with gemcitabine was revealed in the immune competent KPC3 mouse pancreatic model. The interplay between tumor cells and type I IFNs is complex and still not fully understood. The dynamic role of type I IFNs should be carefully considered to fully exploit its therapeutic value as anticancer drug. Further studies should focus on timing and duration of type I IFN administration as well as prognostic markers for predicting effective antitumor responses.

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Availability of data and materials

All data are available from the corresponding author on reasonable request. All raw gene expression array data are available on https://figshare.com/s/ec1cfa7d1d66f8528ec3.

Authors' contributions

AB, PMVK, CHJVE and LJH conceptualized the study. AB, PMVK, DAMM and LJH performed formal analysis. CHJVE acquired funding. AB, PMVK, JD and SVZ conducted investigation. AB, PMVK, DAMM, CHJVE and LJH provided the methodology. PMVK, CHJVE and LJH supervised the study. FD validated the data. AB, CHJVE and LJH performed visualization. AB wrote the original draft. PMVK, DAMM, FD, CHJVE and LJH wrote, reviewed and edited the manuscript. AB, PMVK, CHJVE and LJH confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was controlled by the animal welfare committee of the Erasmus University Medical Center (Rotterdam, The Netherlands) and approved (approval no. AVD101002017867) by the national central committee of animal experiments, in accordance with the Dutch Act on Animal Experimentation and European Union (EU) Directive 2010/63/EU.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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