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Preclinical Development

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Selective Targeting of Interferon γ to Stromal Fibroblasts and Pericytes as a Novel Therapeutic Approach to Inhibit Angiogenesis and Tumor Growth

Ruchi Bansal[1], Tushar Tomar[1], Arne Östman[3], Klaas Poelstra[1,2], and Jai Prakash[1,2,3,4]

Abstract

New approaches to block the function of tumor stromal cells such as cancer-associated fibroblasts and pericytes is an emerging field in cancer therapeutics as these cells play a crucial role in promoting angiogenesis and tumor growth via paracrine signals. Because of immunomodulatory and other antitumor activities, IFNγ, a pleiotropic cytokine, has been used as an anticancer agent in clinical trials. Unfortunately only modest beneficial effects, but severe side effects, were seen. In this study, we delivered IFNγ to stromal fibroblasts and pericytes, considering its direct antifibrotic activity, using our platelet-derived growth factor-beta receptor (PDGFβR)-binding carrier (pPB-HSA), as these cells abundantly express PDGFβR. We chemically conjugated IFNγ to pPB-HSA using a heterobifunctional PEG linker. *In vitro* in NIH3T3 fibroblasts, pPB-HSA-IFNγ conjugate activated IFNγ-signaling (pSTAT1α) and inhibited their activation and migration. Furthermore, pPB-HSA-IFNγ inhibited fibroblasts-induced tube formation of H5V endothelial cells. *In vivo* in B16 tumor-bearing mice, pPB-HSA-IFNγ rapidly accumulated in tumor stroma and pericytes and significantly inhibited the tumor growth while untargeted IFNγ and pPB-HSA carrier were ineffective. These antitumor effects of pPB-HSA-IFNγ were attributed to the inhibition of tumor vascularization, as shown with α-SMA and CD-31 staining. Moreover, pPB-HSA-IFNγ induced MHC-II expression specifically in tumors compared with untargeted IFNγ, indicating the specificity of this approach. This study thus shows the impact of drug targeting to tumor stromal cells in cancer therapy as well as provides new opportunities to use cytokines for therapeutic application. *Mol Cancer Ther; 11(11); 2419–28. ©2012 AACR.*

Introduction

In the past decade, the complexity of the tumor microenvironment has been extensively studied, and this knowledge has contributed to the development of new therapies for cancer (1). Apart from cancer cells, solid tumors contain large amounts of tumor stroma comprising a variety of cell types such as cancer-associated fibroblasts (CAF), pericytes, endothelial cells, infiltrated immune cells, and cancer stem cells. Among them, CAFs are the major cell type that play a crucial role in tumorigenesis and metastasis (1, 2) by secreting various cytokines and growth factors (e.g., VEGF, HGF, SDF-1α), which act in a paracrine/exocrine fashion on other cell types, thereby activating tumor-inducing processes (2–4). In addition to CAFs, pericytes are another important cell type, having phenotypic characteristics of mesenchymal cells and fibroblasts. These pericytes stabilize endothelium by surrounding the blood vessels and support angiogenesis by secreting VEGF (1). Both stromal fibroblasts and pericytes, collectively referred here as stromal cells, express high levels of platelet-derived growth factor-beta receptor (PDGFβR) and its expression in tumor stroma has been inversely correlated with the survival rate in patients with different types of cancer (5, 6). Also, studies have shown that inhibition of the functions of these stromal cells using a PDGFβR inhibitor (imatinib) leads to inhibition of angiogenesis and thereby reduction in tumor growth (7, 8). These data indicate the key role of the tumor stromal cells in tumor development; therefore, selective targeting to stromal cells for cancer therapeutics is of great interest and could provide highly attractive strategies to treat cancer.

Among potent anticancer agents, IFNγ has been shown to possess multiple potent antitumor properties. IFNγ is a

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immunomodulatory cytokine produced by immune cells (mainly natural killer cells and subsets of T cells) and is physiologically involved in promoting innate and adaptive immune responses (9). It interacts with the IFNγ receptor and activates the JAK-STAT1 signaling pathway, which regulates transcription of various genes. IFNγ, apart from its physiologic functions, has been extensively explored as a therapeutic cytokine for various diseases such as immunodeficiency diseases, chronic inflammatory diseases, fibrosis, tumors, and atypical mycobacterial infections in pre-(clinical) studies (10–12). However, most clinical trials failed (13–16) and its clinical application is limited because of its side effects on nontarget cells as IFNγ receptors are present on almost all cell types. The antitumor response of IFNγ has been shown to be mainly associated with its immunologic effects, but also nonimmunologic effects such as direct killing of tumor cells and inhibition of proliferation of endothelial cells have been proposed. In addition, IFNγ has been shown to display strong antifibrotic effects in different fibrosis models in lung, liver, and kidneys (17–20) by inhibiting activation and proliferation of fibroblasts.

As stromal cells highly contribute to angiogenesis and tumor growth, we hypothesized that interference in the tumor-promoting activities of these cells by the local delivery of IFNγ might inhibit the tumor growth. We have designed a PDGFβR-recognizing drug carrier (pPB-HSA) composed of PDGFβR-binding cyclic peptides (pPB) conjugated to human serum albumin (HSA; ref. 21, 22) for specific targeting to PDGFβR-expressing tumor stromal cells. Furthermore, we have shown that pPB-mediated targeting of IFNγ to hepatic stellate cells, expressing high levels of PDGFβR during liver fibrosis, completely abolished advanced liver cirrhosis in mice (23). In this study, we delivered IFNγ to stromal fibroblasts and pericytes using pPB-HSA carrier to impair angiogenesis thereby inhibiting the tumor growth, whereas avoiding IFNγ-mediated off-target effects. To effectuate this, we conjugated IFNγ to pPB-HSA and examined the synthesized conjugate for its therapeutic efficacy *in vitro* and *in vivo*.

Materials and Methods

Cell lines

Murine NIH3T3 fibroblasts, B16-F10 melanoma cells, and RAW264.7 macrophages were obtained from American Type Culture Collection. H5V heart capillary endothelial cell line was kindly provided by Dr. A. Vecchi (Mario Negri, Institute for Pharmacological Research, Milan, Italy) to UMCG Groningen. RAW264.7, NIH3T3, H5V, and B16 cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and antibiotics. No authentication for cell lines was done by the authors.

Synthesis and characterization of pPB-HSA-IFNγ conjugate

The synthesis procedure of pPB-HSA-IFNγ conjugate has been described earlier (24). The brief methodology has been provided in the supplementary methods. The pPB-HSA-IFNγ conjugate was characterized using Western blot analyses and the biologic activity was assessed with a nitric oxide release assay in RAW cells as described earlier (24).

In vitro binding of the IFNγ conjugate to mouse 3T3 fibroblasts

Cells were cultured overnight in Lab-Tek (Nunc) and incubated with pPB-HSA-IFNγ (1 μg/mL) for 2 hours. To block the PDGFβR-mediated binding, anti-PDGFβR immunoglobulin G (IgG; Santa Cruz Biotechnology) was added 1 hour before adding IFNγ conjugate. Then, cells were stained with anti-pPB antibody.

In vitro effects of the IFNγ conjugate in mouse 3T3 fibroblasts

Cells (3×10^4 cells/24-well and 7.5×10^4 cells/12-well plate) were cultured for overnight and starved with 0.5% FBS containing medium for 24 hours. Cells were then incubated with 5 ng/mL human recombinant TGFβ1 (Roche) with or without IFNγ (16 nmol/L), pPB-HSA-IFNγ (equivalent to 16 nmol/L IFNγ), and pPB-HSA (molar equivalent) for 48 hours. Subsequently, cells were stained for collagen-I or α-SMA and were analyzed for gene expression (Supplementary Materials and Methods).

The IFNγ signaling p-STAT1α was analyzed using Western blot analysis in 3T3 fibroblasts, 24 hours after incubating with different compounds as mentioned above. Western blot analysis was carried out from the cell lysates using rabbit monoclonal anti-pSTAT1α antibody (1:1000, Cell signaling technology Inc.) and β-actin (1:5000, Sigma) as detailed in Supplementary Materials and Methods.

Wound-healing assay

NIH3T3 cells were grown for 24 hours and starved overnight in 0.5% FBS containing medium. A standardized scratch was made using a 200 μL pipette tip fixed in a holder. Then, cells were incubated with IFNγ (16 nmol/L), pPB-HSA-IFNγ (equivalent to 16 nmol/L IFNγ), or pPB-HSA (molar equivalent). Digital pictures of wounds were captured at $t = 0$ hour and $t = 24$ hours and were analyzed by NIH-ImageJ software to calculate the area of the scratch wound and represented as the percentage of wound healed relative to the controls.

To study the indirect effect of fibroblasts (3T3) on tumor cells (B16), 3T3 cells (1×10^5) were grown for 24 hours, starved for overnight, and then incubated with TGFβ (5 ng/mL) with or without IFNγ (16 nmol/L), pPB-HSA-IFNγ (equivalent to 16 nmol/L IFNγ), or pPB-HSA for 24 hours. Thereafter, cells were washed thrice and incubated with fresh starved medium for 24 hours. This conditioned medium was put on B16 cells (5×10^4, cultured for 48 hours) to carry out the wound-healing assay as described above.

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In vitro matrigel tube-formation assay

The fibroblast-mediated paracrine effects of IFNγ and pPB-HSA-IFNγ conjugate on endothelial cells (H5V) were examined using the matrigel tube-formation assay (25). In brief, 3T3-conditioned medium collected after different treatments as mentioned above was added to H5V cells (4×10^4) plated on the matrigel-coated 8-chamber slides (Lab-Tek). VEGF (10 ng/mL, Peprotech) was used as a positive control and added directly to H5V cells. After 20 hours incubation, tubes were visualized, counted, and represented as relative percentage of tube formation.

Subcutaneous B16 tumor mouse model

All animals (male C57BL/6 mice, 20–22 g, Harlan) received *ad libitum* normal diet and 12-hour light—dark cycle. Experimental protocols were approved by the Animal Ethics Committee (University of Groningen). Subcutaneous tumors were induced by injecting B16 cells (1×10^6 cells/100 μL PBS/mouse) in the left flank. Tumor size was measured using a digital vernier caliper and tumor volume was established using the following formula ($a \times b^2/2$), where a and b denote the length and width of a tumor, respectively.

To determine the therapeutic efficacy, on day 5 after tumor cell injection (when tumors were formed) mice were randomized into 4 groups and were injected intravenously with 5 μg IFNγ/mouse/day ($n = 5$), pPB-HSA-IFNγ (equivalent to 5 μg IFNγ, $n = 5$), pPB-HSA (molar equivalent to pPB-HSA-IFNγ, $n = 4$), or vehicle (PBS, $n = 5$) on days 5, 7, 9, 11, 13, 15, and 17. IFNγ amount in the conjugate was analyzed by Western blot analysis. The IFNγ dose was based on our previous studies in liver fibrosis and literature (26, 27). Animals were sacrificed and blood, tumors, and other organs were collected for further analysis. To examine the IFNγR signaling (pSTAT1α) *in vivo*, 20 μg of protein from tumor lysates were analyzed by Western blot analysis using anti-pSTAT1α, STAT1α, and β-actin antibodies.

For the biodistribution of pPB-HSA-IFNγ in B16 tumor-bearing mice with tumor size of approximately 2,000 mm^3, a single dose (5 μg/mouse) of pPB-HSA-IFNγ was injected intravenously 15 minutes before sacrifice. Cryosections from tumors and other tissues were stained with anti-pPB IgG for *in vivo* localization.

Immunohistochemistry and immunofluorescence

Cryosections (4 μm) of tumors and organs were cut and the staining protocol was followed as described earlier (23). Antibodies with their dilution and the detailed method have been described in Supplementary Methods.

Statistical analyses

Data are presented as the mean ± SEM. Multiple comparisons between different groups were carried out by 1-way ANOVA with Bonferroni posttest unless otherwise mentioned in the figure legends.

Results

Expression of IFNγR-II and PDGFβR receptors in subcutaneous B16 tumors and other tissues in mice

We initially compared IFNγR-II and PDGFβR expression in B16 tumors and other organs and found that both receptors were highly expressed in tumor stroma (Supplementary Fig. S1). In other organs, IFNγR-II was also strongly expressed but PDGFβR expression was low as compared with tumors. In addition, many immune cells especially macrophages strongly express IFNγR-II (28). Systemic administration of IFNγ will therefore elicit effects in multiple cells in many different organs and immune cells, and distribution to tumors will be relatively low. The high PDGFβR expression on tumor stromal cells in tumors relative to all other tissues supports our notion for the suitability of this receptor for cell-selective targeting of IFNγ.

Characterization of pPB-HSA-IFNγ conjugate

Western Blot analysis of the synthesized PDGFβR-targeted IFNγ conjugate (see diagram in Fig. 1A) using anti-HSA and anti-IFNγ antibodies showed coupling of about 2 IFNγ per pPB-HSA molecule. Nitric oxide release assay in murine RAW264.7 monocytes showed that there was no loss of biologic activity of pPB-HSA-IFNγ as also shown earlier (24). A clear binding of pPB-HSA-IFNγ to 3T3 cells was observed, which was strongly inhibited by anti-PDGFβR IgG, showed its PDGFβR-related specificity (Fig. 1B). Upregulation of PDGFβR expression on 3T3 fibroblasts after activation with TGFβ (Fig. 1C) favors the binding of the construct to the activated fibroblasts and pericytes, known to express high PDGFβR (2, 4).

Modification of IFNγ might cause a loss of activity, however activation of pSTAT1α signaling and MHC-II expression in 3T3 cells by pPB-HSA-IFNγ clearly indicate a full retention of the IFNγ-related activity after chemical modification (Fig. 1D).

pPB-HSA-IFNγ inhibits fibroblasts activation

Furthermore, we investigated the inhibitory effects of IFNγ conjugate on fibroblast activation. Both IFNγ and pPB-HSA-IFNγ substantially inhibited TGFβ-induced activation of 3T3 fibroblasts as shown by protein and gene expression of α-SMA (Fig. 2A and B). In addition, they inhibited TGFβ-induced protein and gene expression of collagen-I and fibronectin-I ($P < 0.01$; Supplementary Fig. S2A–S2C). In contrast, no inhibitory effects of pPB-HSA rules out the possibility of PDGFβR-blocking effects. Furthermore, IFNγ or pPB-HSA-IFNγ significantly inhibited the migration of fibroblasts, as shown with wound-healing assay (Fig. 2D). Earlier, we have shown that targeted IFNγ inhibits the PDGF-BB-induced proliferation of fibroblasts (24). In our wound-healing assay, however, absence of apoptosis and TGFβ-related proliferation stresses that inhibition of wound healing was mainly caused by inhibition of cell migration (Supplementary Fig. S2C and S2D). These results show that both IFNγ

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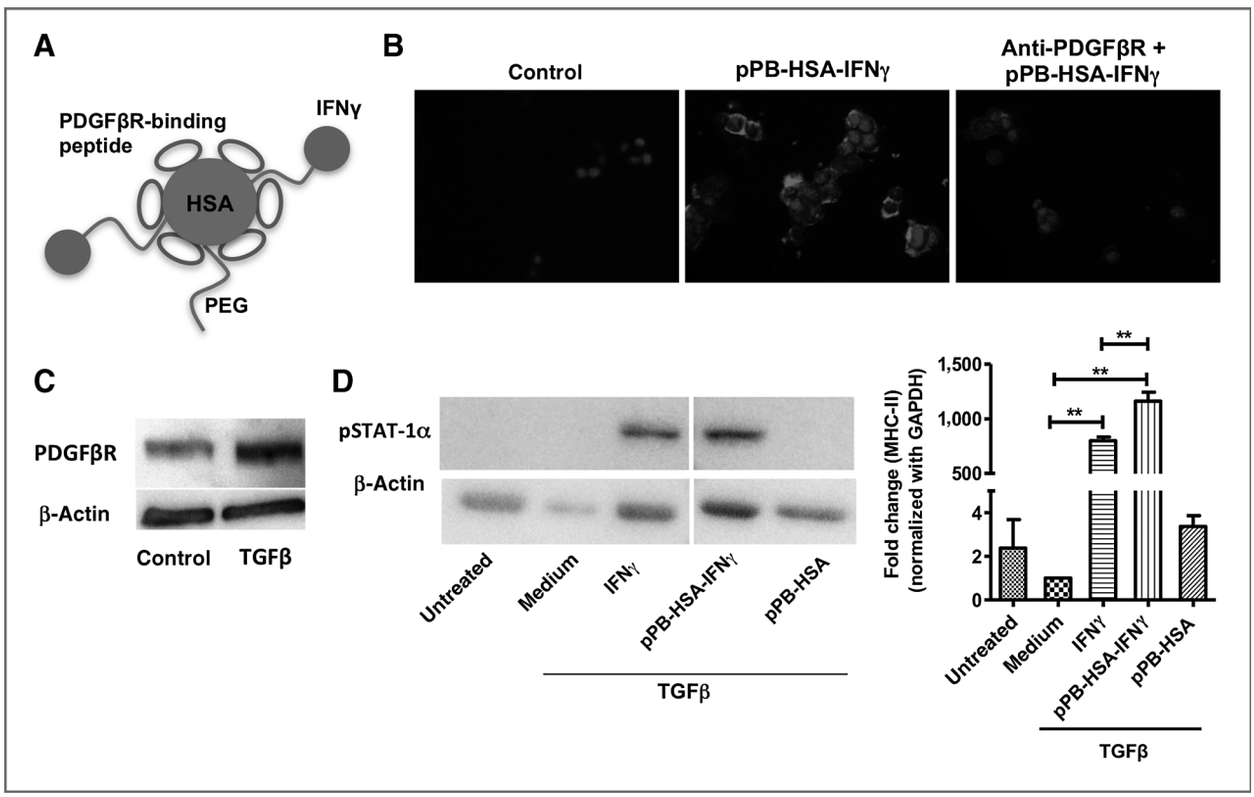

Figure 1. Structure and *in vitro* characterization of pPB-HSA-IFNγ conjugate. A, a diagrammatic structure of pPB-HSA-IFNγ conjugate. B, fluorescent photographs showing binding of pPB-HSA-IFNγ to mouse 3T3 fibroblasts using anti-pPB immunostaining, which was blocked by anti-PDGFβR antibody. C, representative bands from the Western blotting showing increased PDGFβR expression in TGFβ-activated 3T3 fibroblasts. D, Western blot analyses of pSTAT1α and qPCR analysis of MHC-II in 3T3 cells after the treatment with TGFβ (5 ng/mL) with or without IFNγ (16 nmol/L), pPB-HSA-IFNγ (equivalent to 16 nmol/L IFNγ), or pPB-HSA alone. All samples were blotted at the same time and blots were analyzed with the same exposure time. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mean + SEM; $n = 3$; **, $P < 0.01$.

and PDGFβR-targeted IFNγ can block the activation and migration of fibroblasts.

pPB-HSA-IFNγ inhibits fibroblast-mediated activation of endothelial cells

Tumor-associated stromal fibroblasts and pericytes activate endothelial cells in a paracrine manner by secreting cytokines and thereby induce angiogenesis (1). In our fibroblasts-induced angiogenesis *in vitro* model, we found that the conditioned media derived from TGFβ-stimulated fibroblasts but after removal of stimuli enhanced tube formation compared with that of unstimulated media (Fig. 3A and B), which was similar to that achieved with VEGF, an endogenous angiogenesis-inducing growth factor. Interestingly, conditioned media derived from 3T3 cells treated with IFNγ or pPB-HSA-IFNγ significantly diminished the TGFβ-induced tube formation capability of fibroblasts ($P < 0.01$; Fig. 3). Of note as the conditioned media lacked all the added stimuli, no direct effect of IFNγ or its construct on endothelial cells was exhibited. As TGFβ did not cause any proliferative effect on fibroblasts and also the treatments did not cause any changes in proliferation and apoptosis of fibroblasts (Supplementary Fig. S2), the paracrine effects of 3T3 cells were only dependent on the change in the activation state of the cells. These data indicate that selective inhibition of fibroblasts activation with our targeted IFNγ construct may inhibit endothelial cells activation and thereby angiogenesis.

pPB-HSA-IFNγ specifically accumulates in stromal fibroblasts and pericytes *in vivo*

To show the tumor stroma targeting *in vivo*, we investigated the accumulation of pPB-HSA-IFNγ in tumors and various organs, 15 minutes after intravenous injections. Using anti-pPB immunostaining, we found that pPB-HSA-IFNγ rapidly accumulated in tumors especially in tumor stroma (Fig. 4A) where PDGFβR was highly expressed (see Supplementary Fig. S1). pPB-HSA-IFNγ was also found in livers where pPB staining was localized in the sinusoidal lumina. In other organs such as kidneys, heart, and lungs, there was almost no staining detectable (Fig. 4A), which correlates with the low PDGFβR expression in these organs (see Supplementary Fig. S1). As pericytes surrounding tumor endothelium express high PDGFβR, we carried

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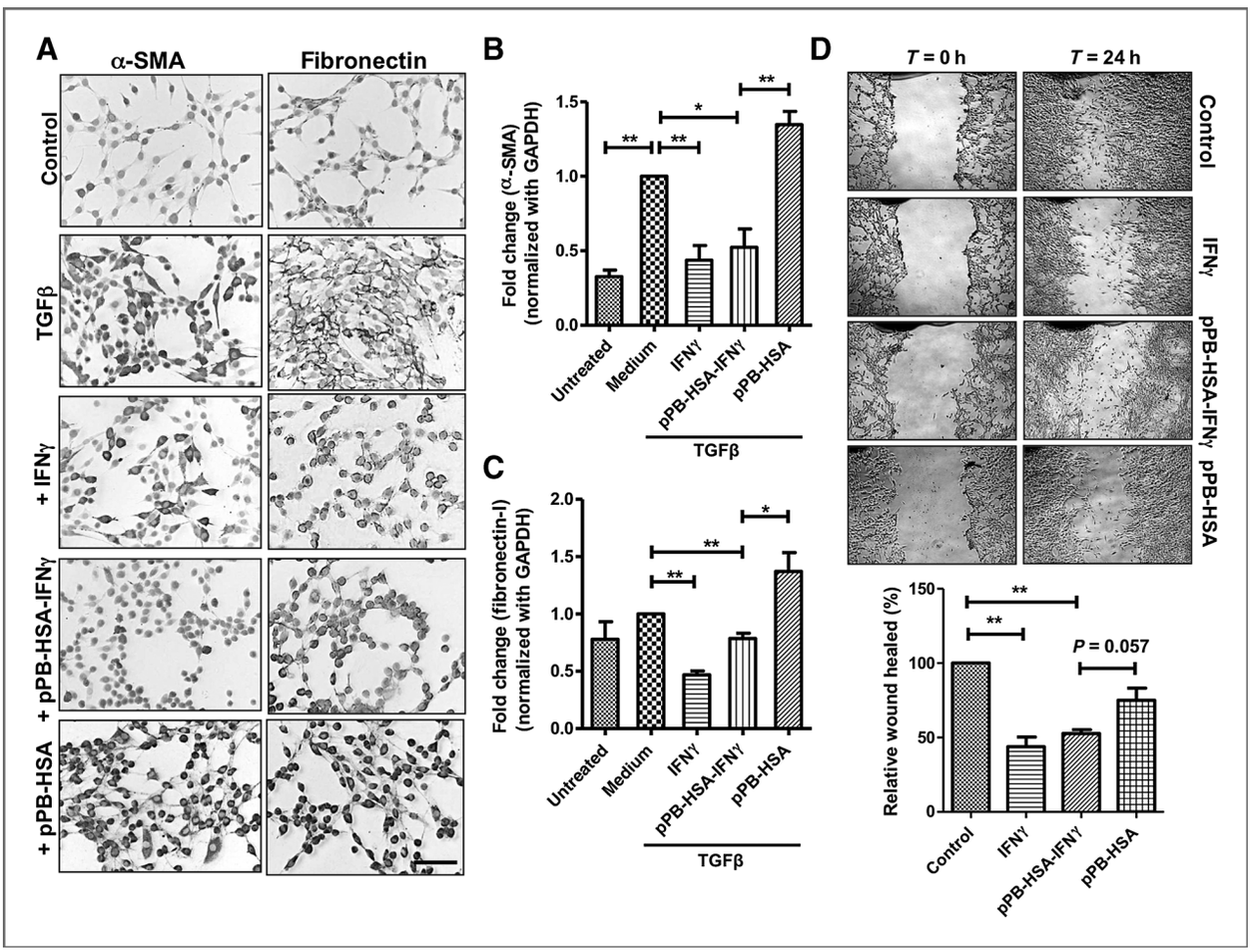

Figure 2. *In vitro* inhibitory effects of pPB-HSA-IFNγ in mouse 3T3 fibroblasts. A, representative microphotographs showing α-SMA and fibronectin-I staining in 3T3 fibroblasts, incubated with TGFβ (5 ng/mL) with or without IFNγ (16 nmol/L), pPB-HSA-IFNγ (equivalent to 16 nmol/L IFNγ), or pPB-HSA alone. Scale bars, 100 μm. QPCR analysis of α-SMA (B) and fibronectin-1 (C) in 3T3 fibroblasts. D, representative microscopic images and analysis of wound-healing assay in 3T3 fibroblasts 24 hours after the incubation with IFNγ (16 nmol/L), pPB-HSA-IFNγ (equivalent to 16 nmol/L IFNγ), or pPB-HSA (equivalent). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mean + SEM; $n = 3$; *, $P < 0.05$; **, $P < 0.01$.

out coimmunostaining for PDGFβR and pPB-HSA-IFNγ (anti-pPB), and found a colocalization of the conjugate with pericytes (Fig. 4B). These results show that pPB-HSA-IFNγ conjugate specifically accumulates into PDGFβR-expressing tumor stromal fibroblasts and pericytes.

pPB-HSA-IFNγ reduces tumor growth *in vivo* by inhibition of angiogenesis

In B16-F10 subcutaneous tumor-bearing mice, treatment with pPB-HSA-IFNγ significantly reduced the progression of this malignant tumor (Fig. 4C) whereas PBS, IFNγ, or pPB-HSA did not inhibit it. The enhanced antitumor effect of the targeted construct was attributed to an increased tumor uptake of pPB-HSA-IFNγ as also shown by the activation of the IFNγ signaling (pSTAT1α) in tumors from pPB-HSA-IFNγ-treated animals ($P < 0.05$ vs. PBS) as compared with other treatment groups (Fig. 5A).

We further explored the effect of the targeted construct on stromal cells and found that α-SMA-positive cells (fibroblasts and pericytes) were markedly less prevalent in targeted IFNγ-treated tumors compared with control tumors (Fig. 5B). Also, there was a significant reduction ($P < 0.01$) in the pericyte population in pPB-HSA-IFNγ-treated mice, as shown with reduction of α-SMA staining around the blood vessels (Fig. 5B). In line with our *in vitro* tube formation assays, we found a significant reduction ($P < 0.01$ vs. IFNγ or pPB-HSA) in angiogenesis with the construct, as shown with the quantitative analysis of CD31-stained lumen area of tumor blood vessels (Fig. 5C). In addition, we carried out cleaved caspase-3 staining in tumors and found that neither free IFNγ nor pPB-HSA-IFNγ-induced apoptosis (data not shown), excluding a possibility of direct proapoptotic effect of the compounds on tumors. As IFNγ is a proinflammatory cytokine and tumor inhibitory effects of the conjugate could be immune-mediated, we carried out CD68 (a common

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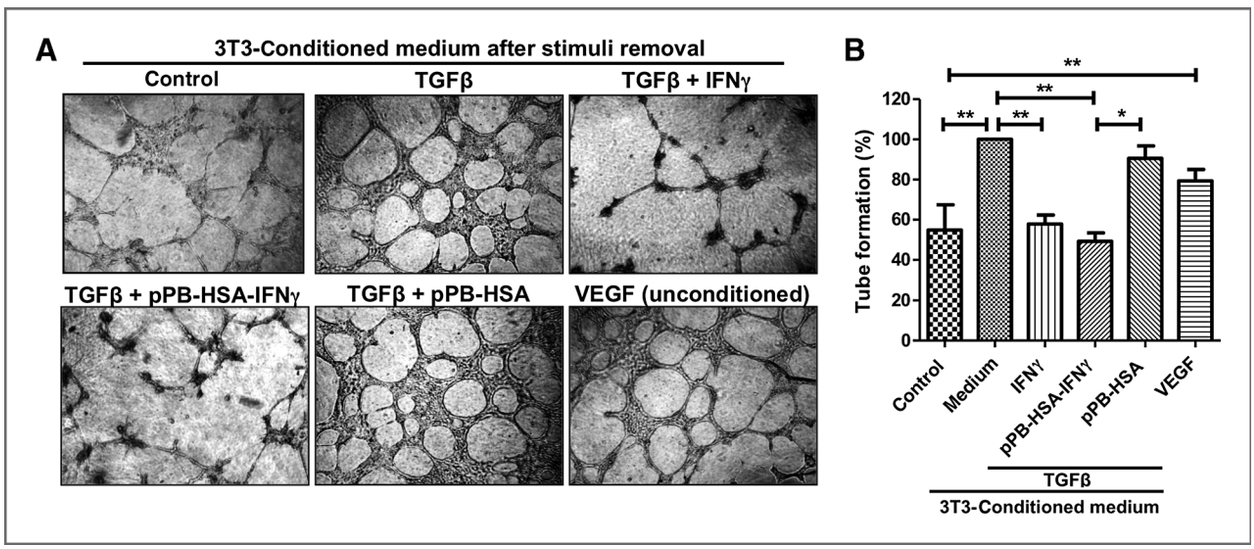

Figure 3. *In vitro* inhibition of paracrine effect of activated fibroblasts on endothelial cells. A, representative pictures of endothelial cell tube formation after incubation with conditioned medium from 3T3 cells that were treated with medium alone (control), TGFβ (5 ng/mL) with or without IFNγ (16 nmol/L), pPB-HSA-IFNγ (equivalent to 16 nmol/L IFNγ), or pPB-HSA. Magnification, ×40. B, tubes were counted 24 hours following incubations. VEGF (10 ng/mL) was used directly on H5V cells as a positive control. Mean + SEM; $n = 3$; *, $P < 0.05$; **, $P < 0.01$.

marker for monocytes, macrophages, kupffer cells, dendritic cells), CD4, and CD8 (markers for T-lymphocytes) stainings on the tumor tissues and found no significant differences among different treatment groups (Supplementary Fig. S3). These data show that the most of beneficial effects of targeted IFNγ are attributed to the direct inhibition of stromal fibroblasts- and pericyte-supported blood vessel formation.

To examine the effect of targeted IFNγ on other organs, we carried out MHC-II immunostaining in tumors and liver, lungs, and kidneys and carried out semiquantitative analyses. We found that targeted IFNγ-induced MHC-II expression significantly more in tumors compared with other treatments (Fig. 6). In other organs, there was no significant increase with any of the treatments. In the biodistribution study, we observed the distribution of the conjugate in liver sinusoids, but absence of liver inflammation (detected by CD68 immunostaining; Supplementary Fig. S3) in the conjugate-treated livers rules out the possibility of side effects in liver. Furthermore, we examined the body weight and blood parameters in all groups and found no adverse effects of the treatments (Supplementary Table S1 and Supplementary Fig. S4).

Taken together, these results show that selective targeting of IFNγ to tumor stroma inhibits tumor growth indirectly by inhibition of angiogenesis. The targeted construct displayed significantly more potent antitumor activity than native IFNγ, with no significant side effects in other organs.

Discussion

The present study reveals that specific targeting of IFNγ to stromal fibroblasts and pericytes through a PDGFβ receptor-recognizing carrier leads to inactivation of these key cell types in tumors and thereby reduces the tumor growth *in vivo*. Epithelial-derived tumors are generally characterized by the generation of mesenchymal-derived stromal cells, including intratumoral and peritumoral fibroblasts and tumor vasculature-associated pericytes. The paracrine signals induced by these cells have been implicated in tumor growth, angiogenesis, invasion, and metastasis (2, 3). Selective targeting of antifibrotic compounds to these cells, as shown with IFNγ in the present study, may therefore pose a novel approach for the development of a new potential anticancer therapy.

Cell-specific targeting to stromal cells is an unexplored area of research and so far only small molecule inhibitors of Hedgehog, fibroblasts activation protein and PDGFR have been used to show the anti-stromal effects on tumor growth (8, 29, 30) and drug uptake (7, 31). Until now, IFNγ has been shown to possess no/moderate anticancer activity in experimental models (27). In fibrosis field, IFNγ has been well explored as an antifibrotic cytokine due to its direct effects on fibroblasts, and examined in clinical studies for idiopathic pulmonary fibrosis and liver fibrosis, though remained ineffective (13, 14). Main reasons for its clinical failure are its poor pharmacokinetics and severe side effects. IFNγR is highly expressed on immune cells (28) and numerous other cells in different organs, as shown in Supplementary Fig. S1, which leads to severe systemic adverse effects. Therefore, targeted delivery of IFNγ to specific key disease-inducing cells is prerequisite to enhance its therapeutic efficacy and to reduce its side effects.

Many attempts have been made to deliver IFNγ to tumors using liposomes, polymer gels, microspheres and nanoparticles (32–34). In these approaches, however, cell-selective targeting is lacking which might result in

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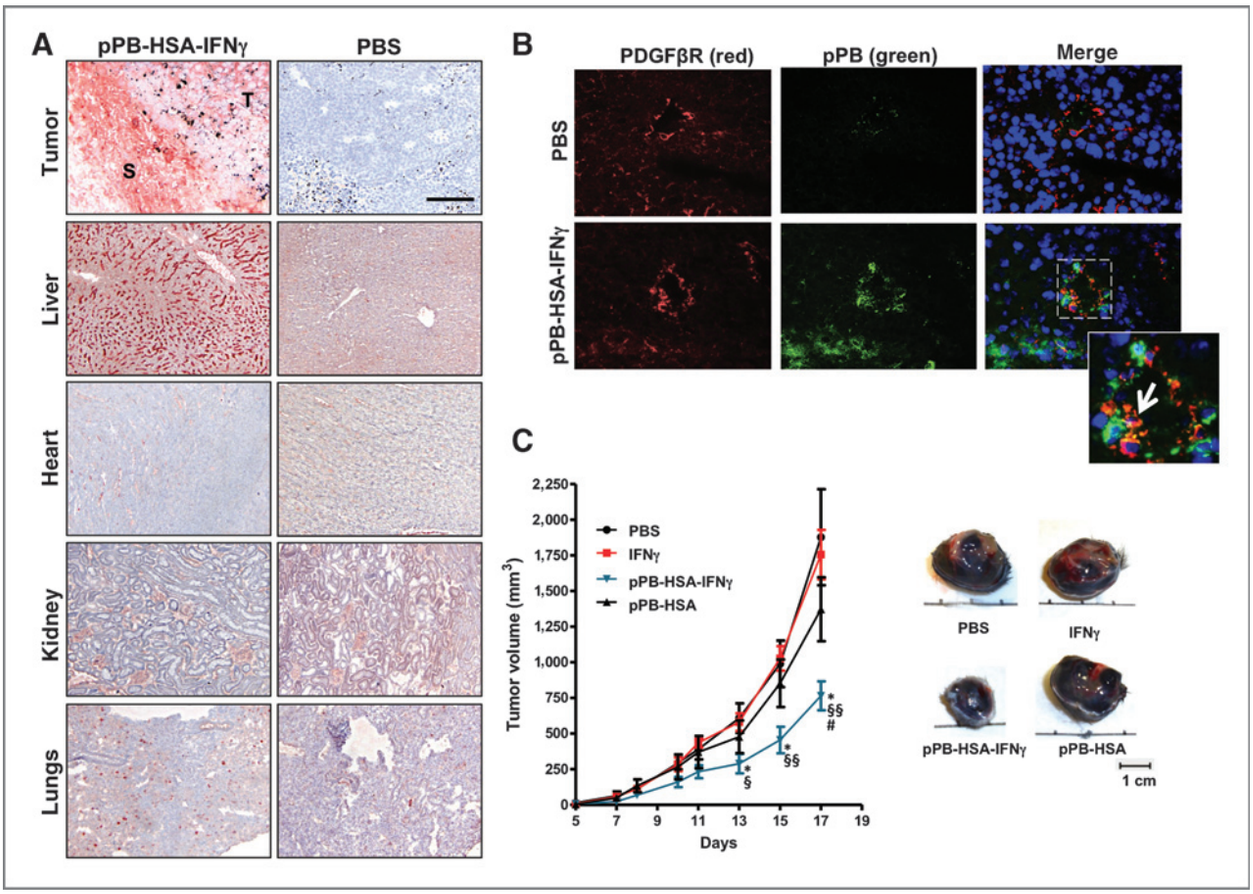

Figure 4. *In vivo* distribution and the therapeutic effects of pPB-HSA-IFNγ in subcutaneous B16 tumor. A, the anti-pPB immunostaining illustrates the distribution of the conjugate in tumors and different organs 15 minutes after the intravenous administration of pPB-HSA-IFNγ in B16-tumor bearing mice. Scale bar, 200 μm. B, representative immunofluorescent photographs depicting specific accumulation of pPB-HSA-IFNγ in pericytes. Anti-pPB (green), anti-PDGFβR (red), and nuclei counterstained with 4′, 6-diamidino-2-phenylindole (blue). Magnification, ×400. C, tumor growth curve of B16 tumors following intravenous treatment with PBS ($n = 5$), IFNγ ($n = 5$), pPB-HSA-IFNγ ($n = 5$), and pPB-HSA ($n = 4$). *, $P < 0.05$ versus PBS; §, $P < 0.05$ and §§, $P < 0.01$ versus IFNγ; #, $P < 0.05$ versus pPB-HSA; unpaired Student *t* test. Representative pictures of the isolated tumors at the end of the experiment.

systemic side effects in long-term treatment. Delivery of IFNγ specifically to tumor blood vessels using a GCNGRC peptide (NGR) has also been attempted to induce immune-mediated antitumor effects (27). However, IFNγ-NGR construct induced potent antitumor effects at very low doses (0.005 μg/kg), whereas nontargeted IFNγ induced little or no effect at the dose of 0.003 to 250 μg/kg. At higher doses, both untargeted and targeted IFNγ were ineffective because of induction of immune-mediated counter-regulatory mechanisms (27), and moreover, multiple treatments at low doses induced resistance to the therapy (35). In the present study, however, we applied a different approach and targeted IFNγ to both stromal fibroblasts and pericytes using a PDGFβR-targeting peptide. This strategy has many advantages over other approaches because (i) stromal fibroblasts and pericytes compose the largest component in a tumor providing a large area for targeting; (ii) these cells strongly participate in many tumor-promoting processes, inhibition of which may lead to hampering of tumor growth; (iii) PDGFβR expression is highly expressed on these stromal cells compared with tumor cells and normal tissues; (iv) stromal cells are likely to be more genetically stable and commonly present in multiple tumor types; (v) furthermore, the antitumor effects are mostly exhibited through its antifibrotic activity than immunomodulatory effects, and therefore chances of counter-regulatory mechanisms, as exemplified above, would be minimal.

Both CAFs and pericytes are mesenchymal cell types and commonly express PDGFβR and α-SMA whereas pericytes present in normal tissues do not express α-SMA (2, 4). TGFβ-activated 3T3 fibroblasts, as shown in this study, had also high expression of these markers, depicting the characteristics of stromal fibroblasts. Inhibition of activation and migration of these cells as well as decrease in the production of extracellular matrix by IFNγ and pPB-HSA-IFNγ indicates the potent antifibrotic effects of these compounds. As expected, free and targeted IFNγ showed similar effects *in vitro* because of no constrains for binding to IFNγR. The real impact of stromal cells in a tumor is

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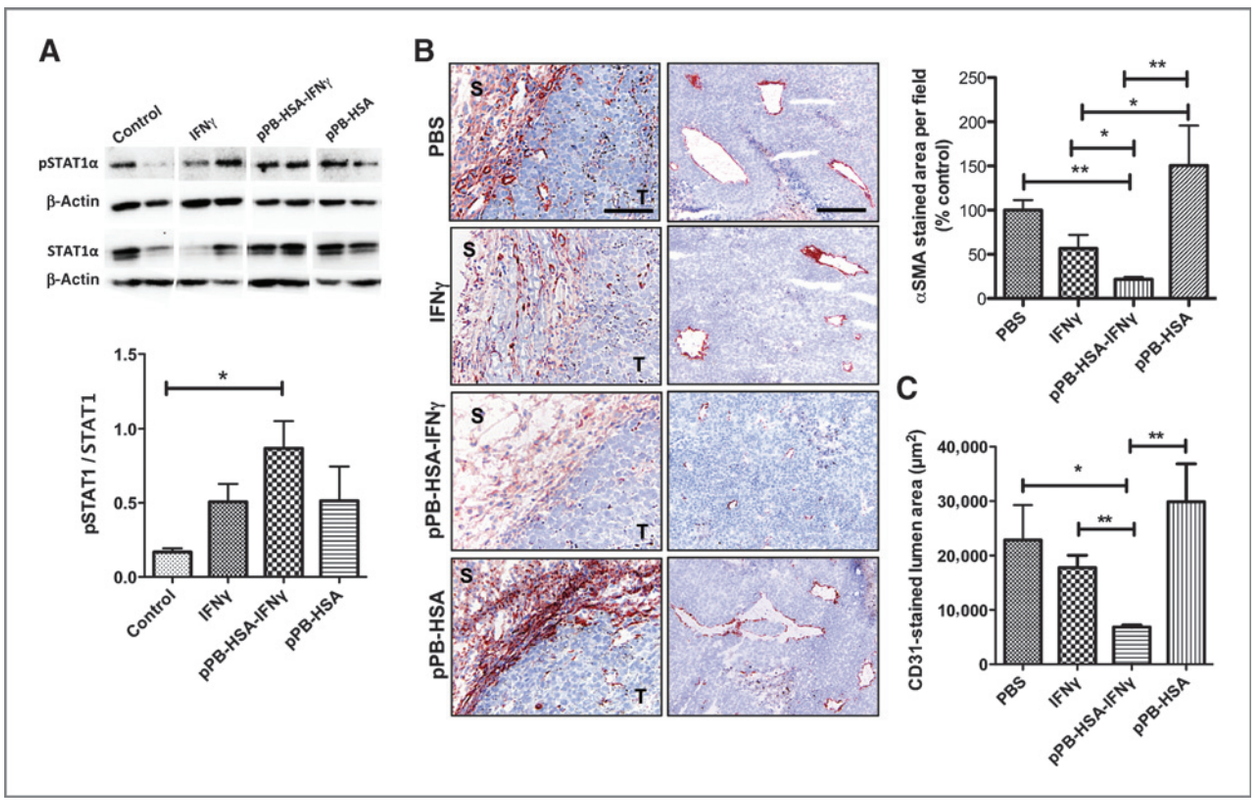

Figure 5. *In vivo* effect of pPB-HSA-IFNγ on stromal fibroblasts and pericytes in subcutaneous B16 tumor-bearing mice. A, Western blot analyses of pSTAT-1α in tumors for pSTAT-1α and STAT1α. The pSTAT1α and STAT1α bands were quantified and neutralized by their respective β-actin controls and then the ratio of pSTAT1α and STAT1α was calculated. All the electrophoresed samples were blotted at the same time and blots were analyzed with the same exposure time. $N = 4–5$ mice per group; *, $P < 0.05$. B, representative pictures showing immunostaining for αSMA, a marker for fibroblasts and pericytes, in stromal fibrous capsule (S), tumor (T), and around blood vessels. Scale bar, 200 μm. Quantitative analyses of α-SMA immunostaining in tumors using image analysis software. C, bar graph showing the lumen area of tumor blood vessels analyzed after CD31 immunostaining on tumor sections. Unpaired Student *t* test; *, $P < 0.05$; **, $P < 0.01$.

exerted by their strong paracrine actions through which they induce angiogenesis, invasion, metastasis, and tumorigenesis (2, 4). Through the paracrine mimicking *in vitro* experiments, we showed that treatment of fibroblasts with targeted IFNγ strongly inhibited the fibroblasts-induced tube formation of endothelial cells. These data support the notion that the selective inhibition of stromal cells *in vivo* may inhibit their paracrine action and thereby the tumor growth.

Cell-selective targeting *in vivo* is a challenging task mainly due to nonspecificity of a target receptor. In our approach, targeting to stromal cells through PDGFβR caused a rapid accumulation of pPB-HSA-IFNγ in tumor stroma and pericytes in subcutaneous tumors. Although PDGFβR is known to be expressed on many cell types in different organs, as a matter of fact its expression is mainly high during early developmental stages but quite low in normal tissues (36, 37). In many pathologic conditions, PDGFβR expression increases remarkably, especially in fibrotic diseases and in tumor stroma (2, 38). For the same reason, we found a negligible distribution of pPB-HSA-IFNγ in normal organs except in liver sinusoids, which is most likely due to its presence in circulation. We have shown in an earlier study that a pPB-HSA-doxorubicin conjugate was visible in liver sinusoids after 30 minutes of an intravenous injection but disappeared after 2 hours (22). Moreover, in the present study no significant induction in MHC-II and CD68 expression in livers with the conjugate clearly indicates no side effect in liver. These data further signifies the tumor specificity of the therapy.

The potential benefits of the targeted approach were observed *in vivo* where targeted IFNγ significantly reduced the tumor growth while untargeted IFNγ (at the equivalent dose) was ineffective. A substantial induction of pSTAT1α expression in tumors by pPB-HSA-IFNγ confirmed its IFNγ-mediated local effects. In contrast, free IFNγ or the carrier did not significantly enhance the pSTAT1α in tumors. Reduction in α-SMA expression in the tumor-associated fibrous tissue and around blood vessels with targeted IFNγ clearly showed the deactivation and/or reduction of fibroblasts and pericytes, which resulted in the antitumor effects. In addition, no increase in tumor macrophage or lymphocytes infiltration in the

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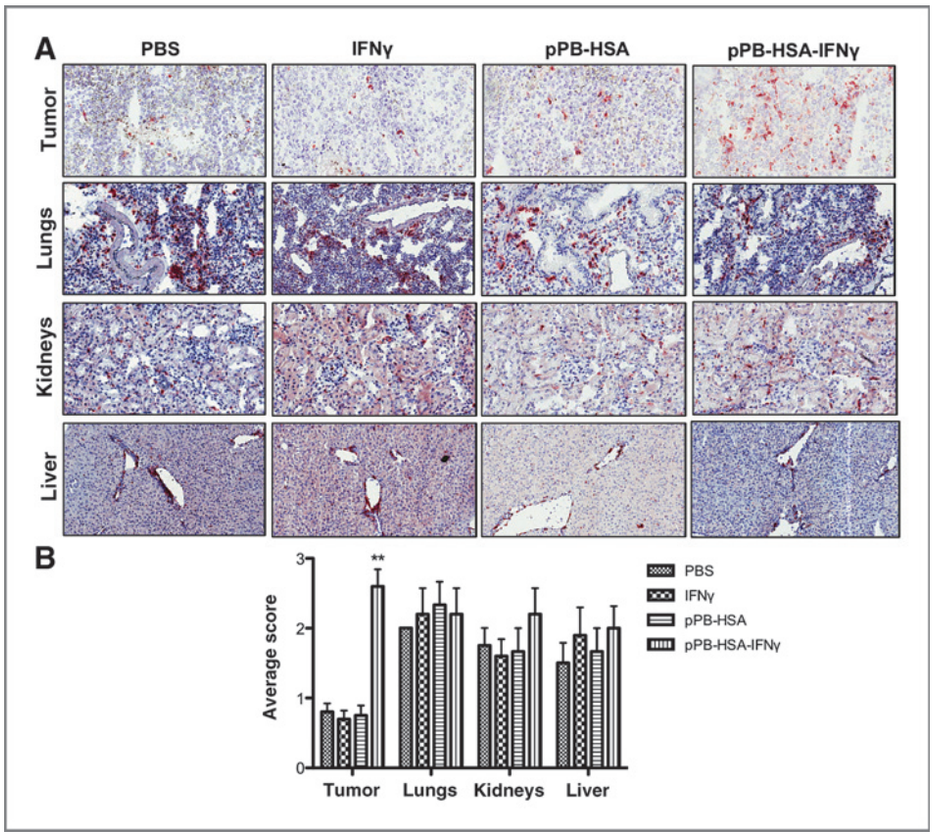

Figure 6. *In vivo* expression of MHC-II in tumors and different organs in B16 tumor-bearing mice. A, representative microscopic pictures of MHC-II staining in tumors and different organs. Magnification, ×200, except livers (×100). B, semiquantitative analyses of MHC-II staining showing the average score (+SEM) in tumors and other organs. $n = 4$–5 mice/group for tumors and $n \geq 3$ mice/group for other organs. Unpaired Student *t* test; **, $P < 0.01$ versus PBS, IFNγ, and pPB-HSA groups. The whole section was scored using the following scoring criteria. Negative section (score 0.5), occasionally positive cells (score 1), a significant number of positive cells with some negative area (score 2), and areas with strong positive cells but still negative areas (score 3). No tumor or any other organ was completely positive.

conjugate-treated animals further supports the direct effect on the targeted cells. As pericytes are directly involved in blood vessel maturation, contribution of pericyte inhibition for antitumor effects is more evident than that of fibroblast inhibition. However, it is difficult to delineate the role of different cell types for these effects.

Induction of systemic side effects by IFNγ has been a major reason for the failure in clinical trials (11). However, at the used doses we did not see any side effect of free IFNγ on body weight and hematologic parameters. Also in MHC-II staining analysis, untargeted IFNγ did not induce its expression in tumors and other organs while the conjugate induced it only in tumors (see Fig. 6). As IFNγ itself did not show side effects at the injected doses, no further improvements were expected from targeted IFNγ.

In conclusion, this study reveals a novel approach to deliver IFNγ to stromal fibroblasts and pericytes using our PDGFβR-targeting carrier. Blockade of the activation of these cells by targeted IFNγ construct leads to a reduction in tumor growth. These data may form a strong base to develop a novel therapeutic compound for the treatment of cancer as well as provide new opportunities to use cytokines as therapeutic compounds.

Disclosure of Potential Conflicts of Interest

K. Poelstra is a co-inventor of pPB-HSA patent and is a co-founder and CSO of Biorion Technologies, Netherlands and holds <5% stocks in the company. J. Prakash is VP Preclinical, Biorion Technologies and acts as an advisory member. No potential conflicts of interest were disclosed for other authors.

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Development of methodology: R. Bansal, T. Tomar, J. Prakash
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Bansal, T. Tomar, J. Prakash
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Bansal, T. Tomar, A. Ostman, K. Poelstra, J. Prakash
Writing, review, and/or revision of the manuscript: R. Bansal, A. Ostman, K. Poelstra, J. Prakash
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Prakash
Study supervision: K. Poelstra, J. Prakash

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