Therapeutic efficacy of bifunctional siRNA combining TGF-β1 silencing with RIG-I activation in pancreatic cancer

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Running Title: Bifunctional siRNA against pancreatic cancer

Precis: This study shows how the potency of a therapeutic siRNA can be increased with a parallel strategy to combinatorially activate an RNA helicase that triggers inflammatory responses to double-stranded viral RNA, with implications in understanding how to effectively reprogram the tumor microenvironment to destroy tumor cells.

Key words: Pancreatic cancer; RIG-I, immunotherapy, siRNA, TGF-β
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ABSTRACT

Deregulated transforming growth factor-β (TGF-β) signaling in pancreatic cancer promotes tumor growth, invasion, metastasis, and a potent immunosuppressive network. A strategy for disrupting this tumor-promoting pathway is silencing TGF-β by small interfering RNA (siRNA). By introducing a triphosphate group at the 5’ end of siRNA (ppp-siRNA) gene silencing can be combined with immune activation via the cytosolic helicase retinoic acid-inducible gene I (RIG-I), a ubiquitously expressed receptor recognizing viral RNA. We validated RIG-I as a therapeutic target by showing that activation of RIG-I in pancreatic carcinoma cells induced IRF-3 phosphorylation, production of type I interferon (IFN), the chemokine CXCL10, as well as caspase-9 mediated tumor cell apoptosis. Next, we generated a bifunctional ppp-siRNA that combines RIG-I activation with gene silencing of TGF-β1 (ppp-TGF-β) and studied its therapeutic efficacy the orthotopic Panc02 mouse model of pancreatic cancer. Intravenous injection of ppp-TGF-β reduced systemic and tumor-associated TGF-β levels. In addition, it induced high levels of type I IFN and CXCL10 in serum and tumor tissue, systemic immune cell activation and profound tumor cell apoptosis in vivo. Treatment of mice with established tumors with ppp-TGF-β significantly prolonged survival, as compared to ppp-RNA or TGF-β siRNA alone. Furthermore, we observed the recruitment of activated CD8+ T cells to the tumor and a reduced frequency of CD11b+ Gr-1+ myeloid cells. Therapeutic efficacy was dependent on CD8+ T cells whereas NK cells were dispensable. In conclusion, combing TGF-β gene silencing with RIG-I signaling confers potent anti-tumor efficacy against pancreatic cancer by braking tumor-induced CD8+ T cell suppression.
INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related death and is characterized by early metastasis and resistance to chemotherapy and irradiation. The identification of deregulated molecular pathways in pancreatic cancer and the development of novel targeted therapies had so far little impact on clinical outcome (1). Prognosis of patients with pancreatic cancer has remained extremely poor with a 5-year survival rate of less than 5%. A key event in tumor progression of pancreatic cancer is deregulation of TGF-β signaling (2). Under normal conditions, TGF-β maintains tissue homeostasis by controlling cellular proliferation, differentiation, survival and cell adhesion. Deregulated TGF-β signaling allows tumors to usurp homeostatic effects for promoting tumor growth, invasion, metastasis, and tumor angiogenesis (2). Moreover, TGF-β has immunosuppressive effects such as inhibition of cytotoxic T cells and NK cells, induction of FoxP3+ regulatory T cells, and shifting antigen-presenting cell function towards tolerance (3, 4). Both tumor cells and immune cells, such as regulatory T cells and myeloid-derived suppressor cells (MDSC), contribute to enhanced TGF-β production in cancer patients. Elevated TGF-β levels in serum and tumors correlate with poor prognosis in tumor patients (5, 6). Thus, TGF-β has generated interest as a target for novel anti-cancer agents. Anti-TGF-β compounds have shown efficacy in preclinical studies and some of these have moved into clinical investigation for melanoma, brain tumors, colorectal, renal and pancreatic cancer (7-11).

Tumor infiltration with T cells represents a positive prognostic factor for pancreatic carcinoma, indicating that immune surveillance may occur despite locally active immunosuppressive mechanisms (12). However, tumor-infiltrating T cells frequently lack effector function due to the hostile tumor microenvironment, which is enriched with immunosuppressive factors. Identifying targets and compounds for immune activation is thus of utmost importance for effective immunotherapy. In this respect, in addition to TGF-β inhibition,
activation of innate immune effector mechanisms via pattern recognition receptors is a promising strategy for breaking tumor-mediated immunosuppression (13).

Eukaryotic cells have evolved pattern recognition receptors for the detection of viral nucleic acids in order to trigger appropriate antiviral immune responses. Four members of the TLR family (TLR3, 7, 8 and 9) are located in the endosomal membrane predominantly in immune cells, whereas the RIG-I-like helicases RIG-I and melanoma differentiation-associated gene-5 (MDA-5) are located in the cytosol of immune and non-immune cells. Recent work suggests that RIG-I may represent a novel target for cancer immunotherapy (14, 15). RIG-I is a cytosolic sensor of viral RNA detecting a triphosphate group at the 5′-end generated by viral RNA polymerases (16, 17). Upon activation, RIG-I initiates a signaling cascade mediated by IRF-3, IRF-7, and NF-κB, leading to an anti-viral response program characterized by the production of type I IFN (IFN-α and IFN-β) and other innate immune response genes, such as the chemokine CXCL10 (18). Moreover, RIG-I signaling induces apoptosis in susceptible cells. In vitro transcribed 5′-triphosphate RNA (ppp-RNA) can be applied as RIG-I ligand to trigger pro-apoptotic signaling via the intrinsic mitochondrial pathway (14, 15). Of note, RIG-I-mediated apoptosis occurs predominantly in tumor cells, as non-malignant cells are protected from pro-apoptotic signaling via Bcl-xL (14).

The novel ppp-siRNA strategy offers the advantage of combining RIG-I-mediated immune activation with RNAi-mediated gene silencing within a single molecule. In the study by Poeck et al. a bifunctional ppp-siRNA silencing the anti-apoptotic molecule Bcl-2 resulted in efficient tumor cell apoptosis in melanoma (15). To date, little is known about RIG-I expression in other cancer types. We hypothesized that dual targeting of immune suppression via RIG-I activation and TGF-β silencing promotes efficacy against pancreatic cancer. We studied RIG-I expression and signaling in human pancreatic carcinoma cell lines. In addition, the therapeutic
efficacy of a bifunctional ppp-siRNA combining ppp-RNA-mediated RIG-I signaling with siRNA-mediated TGF-β silencing was assessed in the aggressive Panc02 mouse model of pancreatic carcinoma.
MATERIAL and METHODS

Cell lines and cytokine stimulation

The Panc02 cell line was established from a tumor that was induced by 3-methylcholanthren in the pancreas of C57BL/6 mice and was a kind gift of Prof. Christiane Bruns, Chirurgische Klinik, LMU Munich (19). The human pancreatic adenocarcinoma cell lines PANC-1, MIAPaCa-2, and BxPC-3 cell lines were obtained from American Type Culture Collection and were used within 6 months after resuscitation (ATCC, Manassas, VA, USA). PaTu8988t cell line was obtained from the German Collection of Cell Lines (DSMZ, Braunschweig, Germany). IMIM-PC1 was kindly provided by Prof. Patrick Michl (Department of Gastroenterology and Endocrinology, University of Marburg, Marburg, Germany) (20). Cancer cell lines were cultured in DMEM supplemented with 10% FCS (Gibco BRL, Berlin, Germany), 2 mM L-glutamine, 100 U/l penicillin and 0.1 mg/ml streptomycin (PAA, Pasching, Austria). Each cell line was routinely tested for mycoplasma contamination by MycoAlert™ Mycoplasma Detection Kit (LONZA, Cologne, Germany). IFN-α was used at concentrations of 100 IU/ml for murine and 1000 IU/ml for human cell lines (Miltenyi, Bergisch Gladbach, Germany).

siRNAs and transfection

siRNAs against TGF-β1, RIG-I, Puma and Noxa were designed according to published guidelines and were purchased from Eurofins MWG Operon (Ebersberg, Germany) (21). The matching 5’-triphosphate-modified siRNA was transcribed using the correlating DNA template that contained the T7 RNA polymerase promoter sequence. In vitro transcription of ppp-RNA was done using the MEGAshortscript™ T7 Kit (Ambion, Austin, Texas) according to the manufacturer’s protocol (14, 15). All sequences can be found in supplementary Table 1. Tumor cells were transfected with siRNA in OptiMEM (Gibco, BRL) using Lipofectamine™ 2000 (Invitrogen,
Karlsruhe, Germany). For in vivo administration, 50 µg of RNA was complexed with in vivo-JetPEI® (Peqlab, Erlangen, Germany) at an N/P ratio of 6 in 5% glucose solution for tail vein injection.

**Mice, tumor engraftment, and therapy**

Six week old female C57BL/6 mice were obtained from Harlan-Winkelmann (Borchen, Germany), *Trif*<sup>−/−</sup> and *Tlr7*<sup>−/−</sup> mice in a F6 C57BL/6 background originated from S. Akira and B. Beutler (22, 23). Experiments were performed according to ethical guidelines approved by the local government. Orthotopic tumors were induced by surgical implantation of 2x10<sup>5</sup> Panc02 cells into the pancreas as described (24). Therapy started on day 10, when sacrificed control mice showed visible tumor nodules (5-8 mm diameter), and was administered twice weekly over three weeks. Serum was obtained 6 h after the first RNA injection for cytokine analysis, and 48 h after second and fifth injections for TGF-β measurement. Survival of mice was monitored daily. Distressed mice were sacrificed. Where indicated CD8<sup>+</sup> T cells or NK cells were depleted one day prior to RNA treatment with 500 µg of anti-CD8 mAb (clone YTS 169.4) or anti-NK1.1 mAb (clone PK136) (BioXCell, West Lebanon, USA). Depletion efficacy was assessed by FACS analysis of peripheral blood and was > 98% after 24 h.

**Protein preparation and Western blot**

Tumor tissue was snap frozen in liquid nitrogen and homogenized using mortar and pestle under constant liquid nitrogen cooling. Cells were lysed (Bio-Plex Cell Lysis Kit, BioRad, Munich, Germany), protein concentrations of supernatants were analyzed by Bradford assay (BioRad) and adjusted for whole protein concentration. For Western blotting, cells were lysed in Laemmlli buffer and boiled for 10 min. Cell lysates were separated using a 10% SDS-PAGE. RIG-I was
detected with anti-RIG-I mAb (clone ALME-1, ENZO Life Sciences GmbH, Lörrach, Germany) followed by HRP-coupled secondary antibody (Santa Cruz Biotechnology, Heidelberg, Germany). Anti-pIRF-3 mAb was from New England BioLabs (Frankfurt, Germany). Blots were visualized using ECL substrate (GE Healthcare, Munich, Germany).

**ELISA**

Cytokine levels in supernatants, serum or tumor lysates were quantified by ELISA for IFN-α and IFN-β (PBL Interferon source, Lörrach, Germany), CXCL10 (R&D Systems, Wiesbaden, Germany), TGF-β (eBiosciences, Frankfurt, Germany), and TNF-α (BD Biosciences, Heidelberg, Germany).

**Flow cytometry**

Lymphocyte activation in spleens was assessed 12 h after in vivo administration of RNA. Spleens were removed and processed into a single cell suspension for staining. Following antibodies were used: anti-CD3ε (clone 145-2C11), anti-CD4 (clone RM4-5), anti-CD8α (clone 53-6.7), anti-CD19 (clone 1D3), anti-NK1.1 (clone NKR-P1B, NKR-P1C, all BD Biosciences), and anti-CD69 (clone H1.2F3, Caltag Laboratories). MDSC were analyzed with: anti-CD11b (clone M1/70), anti-Gr-1 (clone RB6-8C5), anti-Ly6G (clone 1A8), anti-Ly6C (clone Al-21), anti-CD11c (clone HL3), anti-CD80 (clone 16-10A1), and anti-Sca-1 (clone D7). For the characterization of MDSC-subpopulations, Gr-1⁺CD11b⁺ cells were further subdivided by differential expression of the Ly6G. Briefly, Gr-1⁺CD11b⁺Ly-6Ghi cells were defined as polymorph nuclear (PMN)-MDSCs, whereas Gr-1⁺CD11b⁺Ly-6Glo/neg cells were classified as the monocytic (MO)-MDSCs. For MHC-I staining anti-human HLA-A,B,C (clone G46-2.6) or anti-mouse H-2Db (clone KH95, both BD Biosciences) were used. Samples were acquired on a
FACSCanto II (BD) and data analyzed using FlowJo software (Version 8.5.3, Tree Star Inc., Ashland, Oregon).

Detection of apoptosis

Apoptosis was determined using annexin V-APC (Invitrogen, Karlsruhe, Germany) and propidium iodide staining by flow cytometry. Activation of caspase-9 in tumor cells was analyzed by flow cytometry using the caspase-9 and caspase 3/7 FLICA Kits (Immunochemistry, Biomol, Hamburg, Germany). Caspase-9 western blot was done using mouse anti-caspase-9 mAb detecting both the pro-form and active form (New England Biolabs GmbH, Frankfurt am Main, Germany). Activated caspase-9 in tumor lysates was quantified by colorimetric analysis using the Caspase-9 Activity Detection Kit (Abcam, Cambridge, UK). TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) and mounted with Vectashield® w/DAPI (Vector Laboratories, Burlingame, USA) for nuclei visualization. Stained tissues were visualized by confocal fluorescence microscopy (Leica TCS SP5, Wetzlar, Germany).

Quantitative RT-PCR

Total mRNA was isolated using the RNeasy Kit (Qiagen, Hilden, Germany). cDNA was transcribed using Protoscript First Strand DNA Synthesis Kit (New England BioLabs). qRT-PCR was performed using the LC 480 Probes Master Kit and Light Cycler 480 instrument (Roche Diagnostics, Penzberg, Germany). Primers were designed with the Universal Probes library (Roche). The copy numbers of each sample were correlated to HPRT.

Histology
H&E staining was performed according to common protocols. For immunohistology, paraffin-embedded specimens were cut at 3 µm. After deparaffinization and rehydration, heat pre-treatment was done with ProTaqs V Antigen Enhancer (Quartett, Immunodiagnostika & Biotechnologie GmbH, Berlin, Germany). The staining was performed using mouse anti-RIG-I mAb (Enzo Life Sciences AG). Detection was accomplished by Real Detection System APAAP (Dako, Carpinteria, CA, USA) and counter stained with haematoxylin Gill’s Formula (Vector Laboratories). Lymphocyte infiltrates in Panc02 tumors were analyzed with mAb anti-CD3 (clone 17A2) and anti-CD8 (clone 53-6.7, all BD Pharmingen). Images were obtained by fluorescence microscopy (Axiovert 2000, Carl Zeiss, Jena, Germany) and processed with Adobe Photoshop CS4 for adjustment of contrast and size.

Statistical analysis

Data present means + SD (in vitro data) or SEM (in vivo data). Significant differences were analyzed using two-tailed Student's t-test. Multiple comparisons were analyzed by one-way ANOVA including Bonferroni correction. Survival curves were analyzed with Mantel-Cox test. Statistical analysis was performed using GraphPad Prism software (version 5.0a); p-values <0.05 were considered significant.
RESULTS

Pancreatic carcinoma cells express functional RIG-I

RIG-I expression was studied in human PanIN lesions, primary pancreatic adenocarcinomas and metastases by immunohistochemistry. We found strong cytosolic staining for RIG-I in premalignant lesions and in tumor cells in 10 out of 10 specimens (suppl. Fig. 1). We also assessed RIG-I expression by qRT-PCR and Western blot in various human pancreatic cancer cell lines, including PANC-1, PaTu8988t, MIAPaCa-2, IMIM-PC-1 and BxPC-3 that were cultured in the absence or presence of IFN-α. All human pancreatic cancer cell lines tested expressed basal levels of RIG-I protein that were upregulated upon IFN-α treatment (Fig. 1A). We next assessed RIG-I signaling in pancreatic cancer cells in response to treatment with the RIG-I ligand ppp-RNA. We observed phosphorylation of the nuclear transcription factor IRF-3 in all cell lines (Fig. 1B). Furthermore, RIG-I stimulation induced the secretion of CXCL10 (IP-10) and IFN-β (Fig. 1C and D). The cell line MIAPaCa-2 lacked IFN-β production due to a deletion of the IFN-β gene (25) but showed IRF-3 phosphorylation and secreted CXCL10, indicative of intact RIG-I signaling. In addition, FACS analysis revealed upregulation of MHC-I surface expression in all cell lines (Fig. 1E). To confirm that these effects were mediated by RIG-I, we silenced RIG-I in PANC-1 cells with siRNA prior to ppp-RNA stimulation. Phosphorylation of IRF-3, CXCL10 secretion and MHC-I expression were significantly reduced in RIG-I-silenced cells (Fig. 1F and G). Together, these data show that human pancreatic cancer cells express functional RIG-I and validate RIG-I as a potential therapeutic target.

We next investigated RIG-I expression in the murine pancreatic carcinoma cell line Panc02, which forms highly aggressive tumors in C57BL/6 mice. RIG-I expression was low in Panc02 cells under basal conditions but strongly upregulated upon IFN-α treatment (Fig. 2A). As observed with human cell lines, treatment with ppp-RNA resulted in phosphorylation of IRF-3 as
well as secretion of CXCL10 and IFN-β and upregulation of MHC-I expression (Fig. 2B and C). Again, RIG-I silencing confirmed the critical role of RIG-I signaling for these effects (Fig. 2D). Thus, the Panc02 model enabled us studying the therapeutic potential of ppp-RNAs in vivo in immune competent mice.

**RIG-I signaling induces apoptosis in pancreatic carcinoma cells**

In previous studies, RIG-I signaling was shown to induce apoptosis in melanoma cells via the intrinsic, caspase-9-dependent pathway involving upregulation of the pro-apoptotic BH3-only proteins Noxa and Puma (14, 15). We next assessed whether this pro-apoptotic pathway is also active in pancreatic cancer cells. Treatment with ppp-RNA strongly induced apoptosis in both human and murine pancreatic carcinoma cells as determined by PARP cleavage (data not shown) and annexin V binding, which was strongly reduced in RIG-I silenced tumor cells (Fig. 3A and B). In line with activation of the intrinsic apoptosis pathway we observed activation of caspase-9 and caspase-3/7 (Fig. 3C and D). Moreover, ppp-RNA induced upregulation of Puma and Noxa in pancreatic cancer cells (Fig. 3E). Interestingly, siRNA-mediated silencing of Puma significantly inhibited apoptosis induction. Together these results demonstrate that pancreatic cancer cells are sensitive to pro-apoptotic RIG-I signaling and confirm a role of pro-apoptotic BH3-only proteins in RIG-I-induced apoptosis.

**Bifunctional ppp-TGF-β combines TGF-β gene silencing with RIG-I activation in vitro**

To assess whether RIG-I activation and RNAi-mediated silencing of TGF-β can be combined in a single molecule, we designed a siRNA targeting TGF-β1 and the corresponding ppp-siRNA by in vitro transcription using a DNA template of the same sequence containing the T7 RNA polymerase promoter sequence (16). Unmodified siRNA carrying a free 5’-OH group
(OH-TGF-β) and ppp-modified siRNA (ppp-TGF-β) reduced TGF-β to a similar extent in Panc02 cells on mRNA and protein levels (Fig. 4A). Thus, silencing activity was not impeded by the ppp-modification. Moreover, ppp-TGF-β induced upregulation of CXCL10, IFN-β and MHC-I as well as apoptosis of Panc02 cells to a similar extent as control ppp-RNA (Fig. 4B-D). Of note, silencing of TGF-β with OH-TGF-β by itself had no influence on the viability of Panc02 cells. Similar results were obtained with a ppp-siRNA targeting TGF-β in human PANC-1 cells (suppl. Fig. 2). Thus, ppp-TGF-β effectively combines RNAi-mediated TGF-β silencing with ppp-RNA-mediated RIG-I activation in pancreatic cancer cells.

Treatment of tumor-bearing mice with ppp-TGF-β leads to immune activation and TGF-β silencing \textit{in vivo}

Next, we examined the immune stimulatory activity of ppp-TGF-β in the Panc02 pancreatic cancer model. Ten days after orthotopic tumor induction, mice were treated intravenously with RNAs and cytokine production was measured in serum. Injection of ppp-RNA and ppp-TGF-β induced high serum levels of CXCL10, IFN-α and moderate levels of TNF-α (Fig. 5A). In addition, we observed a potent systemic immune activation as evidenced by a strong upregulation of CD69 expression on B cells, CD4+ and CD8+ T cells, as well as NK and NKT cells (Fig. 5B). Of note, intermediate levels of immune activation were also observed for siRNA against TGF-β. However, these were strongly reduced in TLR7−/− mice, indicative of a previously described off-target effect of unmodified siRNA (suppl. Fig. 3) (26, 27). In contrast, immune activation in response to ppp-TGF-β treatment was not affected in mice lacking either TLR7 or TRIF (TLR3 signaling).
A hallmark of pancreatic cancer is the expansion of myeloid-derived suppressor cells (MDSC) that effectively suppress CD8\(^+\) T cell responses (28-30). Both, TGF-\(\beta\) blockade and type I IFN have been reported to reduce the suppressive function of MDSC (31, 32). We therefore investigated the effect of ppp-TGF-\(\beta\) treatment on MDSC in spleens of mice with Panc02 tumors. Strikingly, we observed a reduction in CD11b\(^+\) Gr-1\(^+\) MDSC numbers by 50%. This reduction was due to increased apoptosis of MDSC, as shown by enhanced caspase-9 activation (Fig. 5C). A similar trend was observed for OH-TGF-\(\beta\), but lacked significance. Furthermore, ppp-TGF-\(\beta\) induced a shift from Ly6G\(^+\) PMN-MDSC to Ly6C\(^+\) MO-MDSC and upregulation of CD11c, CD80 and Sca-1 expression (suppl. Fig. 4). Similar phenotypic changes of MDSC have been found in tumor-bearing mice treated with a TLR9 ligand or recombinant IFN-\(\alpha\) and were associated with a reduced suppressive function (32).

High serum levels of TGF-\(\beta\) correlate with poor prognosis and resistance to therapy in pancreatic cancer patients (33). We previously reported elevated TGF-\(\beta\) serum levels in mice with Panc02 tumors (34). To document the influence of Panc02 tumors on TGF-\(\beta\) serum levels, we analyzed serum samples on days 0, 14 and 25 after tumor induction. TGF-\(\beta\) serum levels were increased in tumor-bearing animals and correlated with tumor burden (Fig. 5D). In vivo administration of both, OH-TGF-\(\beta\) or ppp-TGF-\(\beta\) significantly reduced serum TGF-\(\beta\) levels in mice with early and late stage pancreatic tumors (Fig. 5E). Together, these results confirm the in vivo activity of ppp-TGF-\(\beta\) in regards to systemic TGF-\(\beta\) silencing and RIG-I activation in mice with orthotopic pancreatic cancer.

**Systemic treatment of mice with ppp-TGF-\(\beta\) induces a Th1 cytokine profile, CD8\(^+\) T cell activation and apoptosis in tumor tissue**
Next, we addressed the question whether systemic treatment with ppp-TGF-β results in TGF-β silencing and RIG-I activation in pancreatic tumor tissue in vivo. Mice with orthotopic Panc02 tumors were treated with RNAs on days 12 and 14 after tumor induction, and tumors were removed 12 hours later for ex vivo analysis. Both, OH-TGF-β and ppp-TGF-β significantly reduced TGF-β on mRNA and protein levels (Fig. 6A). Moreover, bifunctional ppp-TGF-β induced upregulation of CXCL10 and IFN-β expression in tumor tissue (Fig. 6B). To further characterize the cytokine milieu in tumors, we measured levels of IL-4, IL-5 and IFN-γ expression by qRT-PCR. Interestingly, OH-TGF-β and ppp-TGF-β significantly reduced levels of IL-4 and IL-5. In addition, ppp-TGF-β enhanced IFN-γ expression, indicative of a shift from a Th2 towards a Th1 immune response (Fig. 6C). No difference in FoxP3 expression, a marker expressed by regulatory T cells, was observed between treatment groups (Fig. 6C).

H&E and TUNEL staining of tumor sections revealed profound tumor cell apoptosis in mice treated with ppp-TGF-β (Fig. 7A and B), which correlated with increased caspase-9 activity in tumor lysates (Fig. 7C). Immunohistology and FACS analysis revealed increased numbers of tumor-infiltrating CD8+ T cells and upregulation of the activation marker CD69 (Fig. 7D). Together, these data demonstrate potent anti-tumor activity of ppp-TGF-β treatment in vivo.

**Therapy with ppp-TGF-β controls pancreatic tumor growth in a CD8+ T cell-dependent manner**

Finally, we assessed the in vivo efficacy of ppp-TGF-β treatment in regards to survival in mice with orthotopic Panc02 tumors. We treated mice with RNAs twice weekly for three weeks, starting on day 10 after tumor induction. All mice without treatment or treated with non-silencing OH-RNA had to be sacrificed due to progressive tumor growth within 40 days after tumor
induction (median survival 31 and 29 days, respectively). Treatment with OH-TGF-β or ppp-RNA without silencing activity significantly prolonged survival (median survival 43 and 39 days, respectively). Most efficient tumor control was achieved by bifunctional ppp-TGF-β with a median survival time of 49 days (OH-RNA vs. ppp-TGF-β < 0.0001; OH-TGF-β vs. ppp-TGF-β < 0.05). Complete tumor regressions, confirmed at autopsy on day 100 after tumor induction, were 0%, 6%, 6% and 33% for OH-RNA, ppp-RNA, OH-TGF-β and ppp-TGF-β, respectively (Fig. 7E).

Increased infiltrations of tumors with activated CD8+ T cells led us to hypothesize that long-term tumor regression induced by ppp-TGF-β treatment may reflect the induction of an adaptive immune response against Panc02 tumor cells. We therefore analyzed the role of CD8+ T cells as well as NK cells in the treatment response by injecting either α-CD8 or α-NK1.1 depleting mAb prior to RNA therapy. Depletion of CD8+ T cells substantially reduced the therapeutic efficacy of ppp-TGF-β, whereas depletion of NK cells had no major effect on tumor control in this model (Fig. 7F). Thus, CD8+ T cells appear to be the main effector cells for ppp-TGF-β-induced tumor control.

We next evaluated toxicity of RNA treatment by monitoring blood cell counts, creatinine, urea, LDH and ALT serum levels. We observed a transient leukopenia in mice treated with either OH-TGF-β or ppp-TGF-β, which was completely reversible within 48 hours. No obvious signs of therapy-associated distress or organ toxicity were detected by serum chemistry or histopathology (suppl. Fig. 5 and data not shown).
DISCUSSION

Sequence specific degradation of viral RNA by RNAi and innate antiviral responses upon detection of viral nucleic acids by pattern recognition receptors, such as RIG-I, are two major antiviral defense mechanisms preceding the development of an adaptive immune response. The requirements for the elimination of virus-infected cells and tumor cells share many features. Here we employ both antiviral principles for therapy of pancreatic cancer. We demonstrate that functional RIG-I is expressed in human pancreatic cancer cells. Furthermore, a designed RNA molecule to perform both RIG-I activation and silencing of the immunosuppressive cytokine TGF-β induced tumor regression in a CD8⁺ T cell-dependent manner in an aggressive mouse model of pancreatic cancer. In this respect, RNA molecules that contained either the RIG-I ligand motif or the silencing capability alone were less effective supporting the concept that bifunctional siRNA is superior for effective tumor therapy.

A major hurdle in cancer immunotherapy is the profound immune suppression, both systemically and locally within the tumor microenvironment. RIG-I signaling leads to type I IFN responses with IFN-dependent gene products triggering innate and adaptive immune responses (18). These effects include systemic activation of NK cells and T cells, as well as activation of dendritic cells, which are critical for the induction and regulation of adaptive immune responses and play a key role in cancer immune surveillance (35). It is important to note that upon RIG-I activation type I IFN not only derives from immune cells but also from tumor tissue, as shown for human and murine pancreatic cancer cells in our work. Secretion of CXCL10 can attract lymphocytes to tumor tissue, and locally produced IFN-β can activate Th1 responses and tumor-infiltrating CTL thus enhancing their killing function. In this respect, upregulation of MHC-I expression by the tumor cells upon RIG-I activation may promote CTL-mediated tumor recognition and killing. In fact, we observed that treatment with ppp-TGF-β resulted in a Th1
cytokine profile in tumor tissue and a more vigorous tumor infiltration with activated CD8+ T cells. In addition, we found that tumor regression in response to ppp-TGF-β treatment was mediated by CD8+ T cells. This leads to the question of how ppp-TGF-β restored CD8+ T cell responses in tumors.

Soluble factors, such as TGF-β (5, 6), and immune cell populations, such as MDSC and regulatory T cells, have been demonstrated to play immunosuppressive roles in pancreatic cancer (30, 36). In our study treatment with ppp-TGF-β effectively reduced TGF-β levels in both serum and tumors. The frequency of regulatory T cells was not influenced, however, we have previously reported that TGF-β silencing results in a marked down-regulation of CD103 expression on regulatory T cells (37). As CD103 identifies a particularly suppressive subtype of regulatory T cells, treatment with ppp-TGF-β may counteract regulatory T cell-mediated CTL suppression. In addition, we observed that ppp-TGF-β significantly reduced numbers of CD11b+ Gr-1+ MDSC. Since MDSC are frequently found in pancreatic cancer tissue and potently suppress CD8+ T cells, this finding is particularly interesting (36). Moreover, MDSC underwent phenotypic changes, such as upregulation of CD11c, CD80 and Sca-1 expression. Interestingly, similar changes in MDSC have been reported in tumor-bearing mice treated with recombinant IFN-α and were found to correlate with a reduced T cell suppressive function (32). Thus, TGF-β silencing and type I IFN induction induced by ppp-TGF-β appear to have additive effects on breaking the immunosuppressive milieu created by pancreatic cancer cells and are capable of tipping the balance towards effective antitumor CTL responses.

A central aspect of ppp-RNA treatment is the induction of tumor cell apoptosis. Pancreatic cancer cells frequently acquire loss-of-function mutations of the gate keeper protein p53 which reduces their sensitivity towards pro-apoptotic signals (38). An elegant strategy to
circumvent this limitation is the exploitation of p53-independent apoptosis induction. We found that pancreatic carcinoma cell lines, including those with p53 mutations (PANC-1, BxPC-3 and MIAPaCa-2), were sensitive to ppp-RNA-mediated apoptosis. In line with findings in melanoma (14), we found that ppp-RNA triggers apoptosis via the mitochondrial pathway in pancreatic cancer cells involving upregulation of the BH3-only proteins Noxa and Puma with subsequent caspase-9 activation. Moreover, systemic treatment with ppp-TGF-β induced profound tumor cell apoptosis in vivo, whereas normal pancreas (as well as other organs, such as liver, kidney and lung) showed no signs of histopathology. These findings confirm previous reports that tumor cells are highly susceptible to ppp-RNA-induced apoptosis (14). The predilection for tumor cells as compared to healthy tissue is critical for avoiding toxicity and provides a therapeutic window for ppp-RNA treatment.

In conclusion, we identified RIG-I as a novel target for immunotherapy of pancreatic cancer. Combining RIG-I activation with TGF-β silencing via bifunctional ppp-siRNA brakes tumor-mediated immunosuppressive mechanisms and confers potent antitumor efficacy. Whether this strategy can be further improved, e.g. by combination with cytotoxic agents or immunization, is focus of ongoing studies. Further improvement can be expected by designing new delivery systems for selective tumor targeting and by assessment in genetically engineered mouse models of pancreatic cancer which allow studying effects on the tumor stromal compartment and metastatic spreading (39, 40).
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References


Figure legend

**Figure 1: Human pancreatic cancer cells express functional RIG-I**

(A) Different human pancreatic cancer cell lines were cultured in the absence or presence of 100 U/ml IFN-α or 1 µg/ml ppp-RNA for 12 h. Expression of RIG-I protein was determined by Western blot. (B) Tumor cells were stimulated with ppp-RNA (1 µg/ml) for 2 h and phosphorylated IRF-3 (pIRF-3) was assessed by Western blot. (C-E) Pancreatic cancer cells were treated with 1 µg/ml OH-RNA or ppp-RNA for 24 h. Release of CXCL10 (C) and IFN-β (D) was measured by ELISA. (E) MHC-I surface expression was measured by flow cytometry. MHC-I expression levels are shown as fold increase compared to cells treated with control OH-RNA. (F) PANC-1 cells were incubated with irrelevant (Ctrl.) or RIG-I-specific siRNA for 24 h and subsequently stimulated with OH-RNA or ppp-RNA (1 µg/ml) for 2 h. pIRF-3 was assessed by Western blot. Efficiency of RIG-I silencing was assessed by Western blot. (G) PANC-1 cells were incubated with irrelevant (Ctr.) or RIG-I-specific siRNA for 24 h and subsequently stimulated with 1 µg/ml OH-RNA or ppp-RNA. CXCL10 levels and MHC-I expression were analyzed after an additional 24 h. Means + SD from triplicates of one out of three and representative images of three independent experiments are shown. * indicates p<0.05

**Figure 2: Murine Panc02 pancreatic carcinoma cells express functional RIG-I.**

(A) Panc02 cells were cultured in the absence or presence of IFN-α for 12 h. Expression of RIG-I was determined by qRT-PCR and Western blot. (B) Phosphorylation of IRF-3 was assessed by Western blot after treatment with OH-RNA or ppp-RNA (1 µg/ml) for 2 h. (C) Panc02 cells were stimulated with 1 µg/ml OH-RNA, ppp-RNA or left untreated for 24 h. CXCL10 and IFN-β secretion were analyzed by ELISA. MHC-I surface expression was measured by flow cytometry.
and was expressed as fold increase compared to untreated cells. (D) Panc02 cells were incubated with irrelevant (Ctrl.) or RIG-I-specific siRNA for 24 h and subsequently stimulated with OH-RNA or ppp-RNA. CXCL10 levels in supernatants were measured by ELISA and MHC-I expression by flow cytometry. Means ± SD from triplicates of one out of three independent experiments are shown. * indicates p<0.05.

Figure 3: ppp-RNA induces apoptosis in murine and human pancreatic cancer cells.

(A) Different human pancreatic cancer cell lines were treated with OH-RNA or ppp-RNA (2 µg/ml each) for 48 h. Induction of apoptosis was measured via Annexin V/PI staining by flow cytometry. (B) PANC-1 and Panc02 cells were incubated with irrelevant (Ctrl.) or RIG-I-specific siRNA for 24 h and subsequently stimulated with OH-RNA or ppp-RNA. After additional 48 h apoptosis was studied by flow cytometry. (C) PANC-1 and Panc02 cells were treated with Lipofectamin, OH-RNAs or ppp-RNAs for 48 h as indicated and activation of caspase-3/7 and caspase-9 were measured by flow cytometry using corresponding FLICA kits. (D) Activation of caspase-9 in PANC-1 and Panc02 cells was assessed by western blot. (E) Expression levels of Puma and Noxa in Panc02 cells in response to ppp-RNA was assessed by qRT-PCR. Effect of Puma or Noxa silencing on apoptosis induction by ppp-RNA is shown. Representative data of three independent experiments are shown. Bars represent means ± SD from triplicates. * indicates p<0.05.

Figure 4: Bi-functional ppp-siRNA directed against TGF-β1 combines gene silencing with RIG-I signaling.

Panc02 cells were incubated with different RNAs (0.5 µg/ml) with or without silencing activity against TGF-β for 24 h or left untreated. (A) TGF-β levels were analyzed by qRT-PCR and
ELISA. (B) CXCL10 was measured in supernatants by ELISA and IFN-β expression by qRT-PCR (C) MHC-I expression and (D) viability were assessed by flow cytometry. Means + SD from triplicates of one out of three independent experiments are shown. * indicates p<0.05.

Figure 5: Bi-functional ppp-siRNA targeting TGF-β1 induces systemic immune activation and gene silencing in vivo.

On day 10 after Panc02 tumor induction mice were injected with 50 µg of the indicated RNAs complexed with jetPEI via the tail vein. (A) CXCL10, IFN-α and TNF-α serum levels were analyzed by ELISA after 6 h. (B) CD69 expression by splenic lymphocyte populations was determined by flow cytometry after 12 h. (C) Percentage and caspase-9 activation of CD11b+ Gr-1+ MDSC were measured by flow cytometry after 12 h. (D) TGF-β serum levels in mice during Panc02 tumor progression on days 0, 14 and 25 after tumor induction. (E) Influence of treatment with RNAs with and without TGF-β silencing activity on TGF-β serum levels at days 14 and 25. Mice were treated twice weekly and serum was taken 48 h after the second and fifth RNA injections. Pooled data of 3-7 mice per group are shown as means + SEM. * indicates p<0.05.

Figure 6: Treatment with ppp-TGF-β induces TGF-β silencing, type I IFN induction and a Th1 cytokine profile in tumor tissue.

Mice with Panc02 tumors were treated twice with 50 µg RNA as indicated. (A) Expression levels of TGF-β in tumor tissue were assessed by qRT-PCR and ELISA. (B, C) Expression levels of CXCL10, IFN-β, IL-4, IL-5, IFN-γ and FoxP3 in tumor tissue were measured by qRT-PCR. Data represent means + SEM of pooled data from 5 mice per group. * indicates p<0.05.
Figure 7: Treatment with ppp-TGF-β induces tumor cell apoptosis in vivo and prolongs survival of mice with Panc02 tumors in a CD8+ T cell-dependent manner.

(A-C) Mice with Panc02 tumors were treated with 50 µg of the indicated RNA on days 12 and 14 after tumor induction. Tumors were removed on day 15 for ex vivo analysis using H&E staining (A) and TUNEL staining (B) for detecting apoptotic tumor cells (green) in cryosections of tumor tissue. Nuclei were stained with DAPI (blue). Representative pictures of 5 tumors per group are shown. (C) Caspase-9 activity in tumor tissue was assessed by colorimetric analysis. (D) Number and CD69 expression of tumor-infiltrating CD8+ T cells as assessed by histology and flow cytometry, respectively. Data represent means + SEM from 5 mice. * indicates p<0.05. (E) Survival of mice with orthotopic Panc02 tumors treated with RNA twice weekly for three weeks was monitored. Treatment started on day 10 after tumor induction. Experiments were terminated after 100 days (all surviving mice were tumor-free). Pooled data with statistical analysis from four independent experiments with 9-20 mice per group are depicted. (F) Survival of mice with orthotopic Panc02 tumors treated with RNA in the absence or presence of depleting mAb against CD8 or NK1.1 was monitored. Data from 5 mice per group with statistical analysis are depicted.
Figure 2

A

- Untreated
- IFN-α

![Beta-actin expression](beta-actin.png)

![RIG-I expression](rig-i.png)

B

- OH-RNA
- ppp-RNA

![Beta-actin expression](beta-actin.png)

![piRF3 expression](pirf3.png)

C

- CXCL10 (ng/ml)
- IFN-β (ng/ml)
- MHC-I (fold increase)

![Graphs](graphs.png)

D

- OH-RNA
- ppp-RNA

![Graphs](graphs.png)
Figure 5

A

![Graphs showing cytokine levels](Figure5A)

B

![Bar charts showing CD69 expression in B Cells, CD4+ T Cells, and CD8+ T Cells](Figure5B)

C

![Bar charts showing CD11b/Gr-1+ and Caspase-9 expression in MDSC](Figure5C)

D

![Graph showing TGF-β levels over time](Figure5D)

E

![Graphs showing TGF-β levels on Day 14 and Day 25](Figure5E)
Figure 7

A

PBS
OH-TGF-β
ppp-TGF-β

B

PBS
OH-TGF-β
ppp-TGF-β

C

D

E

F

p-Values:
- ppp-TGF-β vs. Untreated: p<0.0001
- ppp-TGF-β vs. OH-RNA: p<0.0001
- ppp-TGF-β vs. ppp-RNA: p<0.01
- ppp-TGF-β vs. OH-TGF-β: p<0.0001

p-Values:
- ppp-TGF-β vs. Untreated: p<0.01
- ppp-TGF-β + αCD8 vs. Untreated: n.s.
- ppp-TGF-β + αCD8 vs. ppp-TGF-β: p<0.05
- ppp-TGF-β + αNK1.1 vs. ppp-TGF-β: n.s.
Therapeutic efficacy of bifunctional siRNA combining TGF-β1 silencing with RIG-I activation in pancreatic cancer

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