Reg3β Deficiency Impairs Pancreatic Tumor Growth by Skewing Macrophage Polarization

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Abstract

The lectin Reg3β provides crucial protection to various tissues against inflammation, a potential risk factor for pancreatic ductal adenocarcinoma. Reg3β is also overexpressed in serum and pancreatic juice from patients with this cancer, but its function in this context remains to be elucidated. In this study, we investigated the role of Reg3β in tumor development in an orthotopic mouse model of pancreatic cancer. Reg3β deletion in mice drastically impaired pancreatic tumor growth, correlating with decreased angiogenesis and increased apoptosis of tumor cells. Moreover, Reg3β deficiency resulted in an alteration of the tumoral immune microenvironment, reflected by a decrease in the M2/M1 ratio of tumor-associated macrophages and an upregulation of CD3+ cell infiltration. Addition of Reg3β to prestimulated RAW 264.7 or primary macrophages enhanced M2 polarization through the activation of STAT3 signaling pathway. Conditioned media from Reg3β-M2-polarized primary macrophages inhibited apoptosis and prolonged the viability of Panc02 tumor cells. Our studies reveal a novel role for Reg3β as a tumor promoter in pancreatic adenocarcinoma through the regulation of tumor stroma. Thus, inhibition of this protein may be a useful strategy in treatment of pancreatic cancer. Cancer Res; 73(18): 5682–94. © 2013 AACR.

Introduction

The Reg protein family is a group of small secretory proteins and is classified as a member of the calcium-dependent lectin super family (1). It comprises several secretory protein products from four genes (Reg 1, 2, 3, and 4). The type III subclass consists of three members: Reg3α, Reg3β, and Reg3γ (2). In this article, we focus on the Reg3β protein, also known as PAPI or HIP.

The Reg3β protein is overexpressed in pancreatic and intestinal tissues during inflammation (3–5). Our group has previously shown that Reg3β has an anti-inflammatory role, which is in agreement with its strong induction during the course of inflammatory diseases such as pancreatitis and inflammatory bowel diseases (5, 6). In a rat model of acute pancreatitis, an infusion of anti-Reg3β-specific antibodies worsened the pancreatic inflammatory response (7). In another study, Reg3β knockout mice exhibited enhanced pancreatic inflammation in vivo (8).

However, Reg3β expression is not restricted to inflammation. It has also been linked to the regenerative processes of damaged tissues and to different types of gastrointestinal cancers, such as hepatocellular carcinoma, pancreatic adenocarcinoma, and colorectal carcinoma (9–11). Because Reg3β was first reported, it has been attributed diverse physiologic roles, some of which have a direct relationship with cancer, such as a dual mitogenic and antiapoptotic function (12).

Pancreatic ductal adenocarcinoma (PDAC) is a malignant disease of the exocrine pancreas with a 5-year survival rate less than 5% (13). Because of its aggressive growth, most cases are diagnosed in advanced stages. This makes curative therapy impossible and leads to a poor prognosis and high mortality rates (14). The presence of stromal desmoplasia is a hallmark of PDAC, forming a unique microenvironment that comprises many cell types such as proliferating fibroblasts, pancreatic stellate cells, and inflammatory cells. In particular, infiltration of tumor-associated macrophages (TAM) is related to unfavorable prognosis (15).

TAMs originate from blood monocytes recruited from the tumor vasculature. Their activation can be polarized toward a classic proinflammatory phenotype, M1, or toward an alternative, anti-inflammatory M2 response. M1 macrophages are characterized by a proinflammatory cytokine profile and expression of MHC class II molecules, and are potent killers of pathogens and tumor cells. M2 macrophages are not only characterized by reduced proinflammatory cytokine secretion, but also by elevated expression of anti-inflammatory cytokines.
TAMs closely resemble M2 macrophages and show mainly protumoral functions, promoting cell survival, proliferation, and tissue repair (16).

Reg3β is overexpressed in PDAC (10, 17), but its role in the malignant transformation of this disease has not yet been elucidated. It is known that Reg3β treatment prevents TNF-α-induced NF-κB activation in macrophages, thus showing the protein's capacity to inhibit the expression of molecules related to the M1 phenotype (5). In this way, the possibility that Reg3β modulates the proinflammatory microenvironment that surrounds the pancreatic tumor is an attractive hypothesis. In this study, we investigated the role of Reg3β in tumor development in an orthotopic mouse model of pancreatic cancer. We observed that Reg3β deficiency notably impaired pancreatic tumor growth through alteration of the tumor microenvironment composition.

Materials and Methods

Mice and cell lines

C57BL/6 mice were obtained from Charles River Inc., Reg3β−/− mice were generated as previously reported from the C57BL/6 strain (18). Animals were housed in a controlled environment and fed with a standard laboratory pelleted formula (A04; Panlab) and tap water ad libitum. All procedures were carried out in accordance with a protocol approved by our Institutional Animal Care and Use Committee.

The murine pancreatic adenocarcinoma cell line Panc02, syngeneic to C57BL/6 mice, was kindly provided by Dr. I. Melero (Centre for Applied Medical Research, University of Navarra, Pamplona, Spain). The murine macrophage cell line RAW 264.7 was obtained from Sigma. The cells were grown at 37°C in RPMI medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO2.

Orthotopic pancreatic tumor mouse model

Forty 9-week-old mice, 20 C57BL/6 wild-type (Reg3β+/+), and 20 knockout (Reg3β−/−), with an average weight of 20 g were used for implantation of the murine pancreatic adenocarcinoma cell line Panc02. The mice were anesthetized intraperitoneally with a combination of ketamine and xylazine. Panc02 cells (3 × 106) were injected with a 30G needle in the tail of the pancreas and the peritoneum and skin were closed with 5-0 prolene sutures.

The mice were evaluated for changes in body weight and signs of discomfort or morbidity. Animals were sacrificed 4 weeks after tumor cell injection. Plasma and peritoneal lavage were obtained and the tumors were isolated and weighed. The final tumor volume was measured as 0.5 × length × width × depth. Metastases were evaluated by visual inspection of spleen, liver, intestine, and peritoneum.

Primary macrophage isolation

Eight milliliter of cold harvest medium was injected into each mouse through the peritoneal wall. Fluid from the peritoneum was aspirated and the peritoneal exudates cells were centrifuged. The cell pellet was resuspended in cold media, plated, and incubated at 37°C, 5% CO2 for 2 hours. The remaining cells were washed and the attached ones were used for in vitro assays or for direct assessment of their phenotype.

In vitro assays

M1- and M2-polarized macrophages were obtained by culturing RAW 264.7 cells and primary macrophages with appropriate stimuli. M1 cells were polarized by stimulating cells 2 hours with lipopolysaccharide (300 ng/mL; Sigma). M2-macrophages were polarized by stimulating macrophages with a combination of 20 ng/mL interleukin (IL)-4 (PeproTech) plus 10 ng/mL IL-13 (Invitrogen). Macrophages were differentiated into M1 or M2 phenotypes in the absence or presence of 500 ng/mL Reg3β. In some experiments, macrophages were preincubated with a STAT3-specific inhibitor, AG490, at a concentration of 30 μmol/L, 2 hours before the treatment of appropriate stimuli. Human Reg3β was produced in the yeast Pichia pastoris according to the recommendations of the manufacturer (Dynabio). Reg3β recombinant protein was assayed for endotoxin with a commercial kit from Chemicon International with negative results. The dose of Reg3β treatment was chosen according to a dose-response induction previously conducted on RAW 264.7 cells.

For conditioned media (CM) studies, Panc02 cells were incubated with media alone (Control), Reg3β alone (Reg3β), or conditioned media obtained from: untreated macrophages (m0 conditioned media), untreated macrophages plus Reg3β (m0+Reg3β), Reg3β-treated macrophages (m0+Reg3β conditioned media), ILs-treated macrophages (m0+ILs conditioned media), or Reg3β+ILs-treated macrophages (m0+Reg3β+ILs conditioned media). Cells were incubated for 24 hours to measure apoptosis and proliferation, or for 48 hours to determine viability.

Real-time qRT-PCR

Total RNA was isolated from RAW 264.7, primary macrophages, and pancreatic tumors using the TRIzol reagent (Invitrogen). Reverse transcription was conducted on a 1 μg RNA sample using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The reaction was incubated at 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes, and was then stored at −80°C. Subsequent PCR amplification was conducted in the iCycler iQ Multi-Color Real-Time PCR (Bio-Rad Laboratories) using iQ SYBR Green Supermix and the corresponding mouse primers (see Supplementary Table S1). Reactions were carried out in duplicate and threshold cycle values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. The ratio of the relative expression of target genes to GAPDH was calculated by the ΔCt formula.

Immunohistochemistry

Antibodies for immunohistochemical analysis were as follows: rat monoclonal anti-CD31 (1:25 dilution; Abcam), rat anti-Gr1 (1:20 dilution; BD Biosciences), rabbit anti-CD3 (1:50 dilution; Abcam), rat anti-F4/80 (1:50 dilution; Abcam), rabbit anti-cleaved caspase-3 (1:100 dilution; Abcam), mouse anti-proliferating cell nuclear antigen (anti-PCNA; 1:100 dilution; Abcam).
Viability Assay (Promega). Brieﬁng, cellular viability (1:250 dilution; Abcam).

Pancreatic tumors were ﬁxed and embedded in parafﬁn. Antigen retrieval was conducted by incubating forms with formic acid solution 20% for PCNA detection, 10 μg/mL proteinase K solution for CD3 detection or with 10 mmol/L sodium citrate for F4/80, Gr1, CD3, and cleaved caspase-3, VEGF, ARG1, and iNOS detection. After blocking, the tissue sections were incubated overnight with the antibodies at the dilutions described earlier. Sections were then incubated with the appropriate biotinylated secondary antibody followed by conjugated horseradish peroxidase–streptavidin. Slides were counterstained with hematoxylin and mounted with diethylphtalate in Xylene (Sigma). Images were taken with a Nikon Eclipse E1000 microscope (Nikon) using Cell^B imaging software (Olympus America). For caspase-3, ARG1, iNOS, and F4/80 analysis, the percentage of the thresholded area occupied by 3,3'-diaminobenzidine (DAB) staining was measured using the ImageJ program. To avoid experimenter bias, immunohistochemical analysis and counting were conducted by two independent blinded raters.

**Immunoblot**

Panc02 cells and pancreatic tumor tissue samples were lysed in radioimmunoprecipitation assay (RIPA) buffer. Total protein was loaded onto a 10 or 12% SDS-PAGE in a Mini Cell (Bio-Rad). The proteins were transferred to nitrocellulose membranes using a Trans-Blot SD semi-dry Electrophoretic Transfer Cell (Bio-Rad). Membranes were blocked and incubated with corresponding primary and secondary antibodies. The membranes were developed using the enhanced chemiluminescence (ECL) reagents from Bio-Rad. The antibodies used were the following: rabbit anti-cleaved caspase-3 (1:500 dilution; Abcam), mouse anti-PCNA (1:500 dilution; Millipore), mouse α-tubulin (1:5,000 dilution; Sigma), mouse anti-ARG1 (1:200 dilution; BD Biosciences), rabbit anti-iNOS (1:200 dilution; BD Biosciences), rabbit anti-phospho-STAT3 (Tyr 705; 1:800 dilution; Cell Signaling Technology), and rat anti-F4/80 (1:250 dilution; Abcam).

**Cellular viability**

Panc02 viability was studied with the CellTiter-Blue Cell Viability Assay (Promega). Briefly, Panc02 cells were seeded, treated with appropriate conditioned media, and incubated for 48 hours at 37°C, 5% CO2, in a humidified environment. After incubation, media was discarded and cells were exposed to the MTS mixture for 1 to 4 hours. The absorbance at 490 nm was recorded using an ELISA plate reader (Labsystems).

**Gelatin zymography**

Fifty microgram proteins were separated in 8% SDS-PAGE copolymerized with 0.1% gelatin. After electrophoresis, the gels were washed and incubated in 50 mmol/L Tris–HCl buffer (pH 7.5), 10 mmol/L CaCl2, and 0.02% NaN3. The gels were stained in 0.1% amido black in acetic acid/methanol/water (1:36) for 30 minutes and destained in the solvent, followed by a final wash in distilled water.

**Arginase activity**

Lysates from pancreatic tumor tissue were incubated with 10 mmol/L MnCl2 and 50 mmol/L Tris–HCl (pH 7.5). After enzyme activation, 25 μL of the lysate was removed and added to 25 μL 1 mol/L arginine (pH 9.7) in a new PCR plate and incubated for 1 hour at 37°C. Five microliter of each sample was added in duplicate to a 96-well ELISA plate along with 5 μL of each standard. Urea determination reagent from BioAssay Systems Quantichrome Urea Assay Kit (Bioassay Systems) was used in accordance with the manufacturer’s protocol.

**VEGF immunoassay**

Concentration of VEGF in peritoneal cavity, plasma, and tumor samples was measured using a commercially available ELISA Kit (R&D Systems) in accordance with the manufacturer’s instructions. The intensity of the reaction was revealed with tetramethylbenzidine. Then, the optical density was measured at 450 nm using the iEMS Reader MF and Ascent software (Labsystems). All samples were run in duplicate, and a standard curve was established for each assay.

**Caspase-3/7 activity**

Caspase-3/7 activity was measured using a homogeneous luminescent method (Caspase-Glo 3/7 Assay; Promega) in accordance with the manufacturer’s instructions. The luminescence intensity was determined using an Orion micro plate luminometer (Berthold Detection Systems).

**Analysis of blood vessel density**

Pancreatic tumors were ﬁxed in formalin and stained with anti-CD31, a marker of neovascularization, as described earlier. For assessment of the tumor blood vessel count, the mean number of CD31-positive vessels was calculated from four ×20 magniﬁcation ﬁelds per section of 4 different animals.

**Statistical analysis**

All experiments were carried out at least in triplicate. Representative data are shown. Results were tested for statistical signiﬁcance using the nonparametric Mann–Whitney U test with GraphPad Prism version 4.0 software. P < 0.05 was considered statistically signiﬁcant.

**Results**

**Pancreatic tumor growth is impaired in Reg3β−/− mice**

To evaluate the contribution of Reg3β in regulating tumor growth, we implanted Panc02 cells into the tail of the pancreas of Reg3β−/− and Reg3β+/+ mice. The mice were sacriﬁced 28 days after tumor cell injection, when all Reg3β−/− animals (n = 20) had developed pancreatic tumors. Notably, tumor growth was signiﬁcantly reduced or absent in mice lacking Reg3β in comparison with their wild-type counterparts (Fig. 1A). In fact, 11 Reg3β−/− mice of a total of 20 had no macroscopically visible tumor; this was then conﬁrmed by histologic analysis. Furthermore, they presented no local spread of
tumor cells, whereas in the Reg3β<sup>+/−</sup> group tumor nodules were found in liver and/or intestine (data not shown). Tumor volume and weight in the remaining 9 Reg3β<sup>−/−</sup> mice that developed tumors were also significantly reduced compared with Reg3β<sup>+/−</sup> mice (Fig. 1B). Representative images of hematoxylin and eosin–stained sections of tumors collected from
Reg3β+/+ and Reg3β−/− mice presented some typical features of pancreatic cancer (Fig. 1C). To determine the localization of Reg3β in pancreatic tumors, immunohistochemical staining was conducted with an anti-Reg3β antibody. In accordance with previous reports, control mice had no Reg3β expression and the expression of this protein in Reg3β+/+ mice was confined to pancreatic acinar cells surrounding the tumor. As expected, Reg3β−/− mice did not express Reg3β (Fig. 1D).

Reg3β−/− mice developing tumors present no changes in proliferation, while apoptosis of tumor cells is increased

The decreased tumor volume observed in Reg3β−/− mice led us to examine proliferation and apoptosis of tumor cells. Immunohistochemical analysis of PCNA showed that Reg3β−/− mice developing pancreatic tumor proportionally contained the same levels of PCNA when compared with tumors from Reg3β+/+ (Fig. 2A). We also measured mRNA levels for the proliferation marker Ki67 and detected no changes (Fig. 2B). In addition, the levels of apoptotic cells were also studied in the same series of tumors by immunostaining and immunoblot of activated caspase-3. A significant increase was found in the number of apoptotic nuclei present in Reg3β−/− mice compared with their wild-type counterparts (Fig. 2C and D).

Reg3β deficiency impairs tumor vascularization

Tumor specimens obtained at the time of necropsy were processed for immunohistochemistry studies to localize blood vessels. Representative images of CD31-positive endothelial cells from sections are shown in Fig. 3A. Staining for this vascular endothelial marker revealed a significant decrease in the density of blood vessels in Reg3β−/− mice that developed tumors compared with Reg3β+/+ mice. Moreover, CD31 mRNA expression confirmed these results (Fig. 3B).

We also measured VEGF levels in tumors by immunohistochemistry and ELISA. Tumors from Reg3β−/− mice showed significantly less VEGF staining than tumors growing in Reg3β+/+ mice (Fig. 3C). Similarly, ELISA results showed a marked decrease in VEGF levels in tumors from Reg3β−/− mice in comparison with Reg3β+/+ mice (Fig. 3D). We next measured VEGF content in the peritoneal fluid and plasma of these mice. Reg3β deficiency had a pronounced effect on VEGF content in peritoneal cavity and plasma (Fig. 3D), reducing its levels to those of controls. Finally, we measured proangiogenic matrix metalloproteinase 9 (MMP9). Using conventional gelatin zymography, we compared MMP9 activity in pancreatic tumors from Reg3β+/+ mice and Reg3β−/− mice, and found greatly reduced MMP9 activity in Reg3β−/− mice (Fig. 3E).

Reg3β deficiency modifies immune stroma composition in mice

A number of immune cells have been attributed a tumor-promoting role, including TAMs and granulocytes (19). To determine the contribution of these cells in the stromal reaction, we characterized cells expressing F4/80 and Gr1 antigen respectively in both Reg3β+/+ and Reg3β−/− mice. As shown in Fig. 4A, the same extent of macrophage infiltration was observed in the tumors growing in Reg3β−/− mice and those in Reg3β+/+ mice. Real-time quantitative reverse transcriptase PCR (qRT-PCR) analysis of F4/80 confirmed the results obtained in the immunohistochemistry study (Fig. 4D). No significant differences were found about Gr1

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Figure 2. Proliferation and apoptosis in tumors from Reg3β−/− and Reg3β+/+ mice. A, left, representative photographs of tumor sections from Reg3β+/+ and Reg3β−/− mice stained with anti-PCNA antibody. A, right, PCNA immunostaining quantification represented as number of positive cells. B, real-time qRT-PCR analysis for KI67 in tumors from Reg3β+/+ and Reg3β−/− mice. Results are expressed as KI67 mRNA levels relative to GAPDH mRNA. Bars represent mean values of each group ± SE. Each determination has been done in triplicate. C, left, representative photographs of cleaved caspase-3 immunostaining in tumors sections from Reg3β−/− and Reg3β+/+ mice. C, right, caspase-3 immunostaining quantification represented as percentage of marked surface. D, Western blot analysis of cleaved caspase-3 expression in Reg3β+/+ and Reg3β−/− mice. *, P < 0.05. Scale bar, 100 μm.
expression (Fig. 4B) although a tendency toward a decrease was observed in Reg3β−/− mice assayed by qRT-PCR (Fig. 4E).

To complete the analysis of immune cell subtypes, we examined infiltration of T lymphocytes into the Reg3β−/− and Reg3β+/− mice. CD3 expression had a tendency to be upregulated in Reg3β−/− mice, whereas Reg3β+/− mice lost T-cell response. This tendency was significant in Reg3β−/− mice developing tumors (Fig. 4C and F).

**Effect of Reg3β on TAM polarization in vitro**

We therefore hypothesized that Reg3β may not act directly by altering the levels of TAM infiltration but rather by inhibiting its M1 polarization and by enhancing the M2 phenotype. To test this possibility, we analyzed the expression of M1 and M2 markers after stimulating RAW 264.7 macrophages with Reg3β in vitro. Macrophages stimulated with lipopolysaccharide or IFN-γ assume a M1 proinflammatory phenotype characterized by a high-expression level of iNOS, a high antigen-presenting capacity, and production of proinflammatory cytokines such as TNF-α and IL-1β (20).

In the presence of proinflammatory lipopolysaccharide, Reg3β significantly inhibited the generation of M1 markers such as IL-1β, IL-6, TNF-α, and iNOS (Fig. 5A). In addition, Reg3β was also able to enhance the alternative differentiation of macrophages in vitro. RAW macrophages were differentiated into alternative M2 macrophages with recombinant IL-4 and IL-13 in the absence or presence of Reg3β. The M2 macrophages marker ARG1 was strongly induced by the IL-4/IL-13 combination. This effect was significantly amplified by Reg3β. A similar regulation of other M2 response genes such as chitinase 3-like 3 (Chi3l3), mannose receptor (Mr), and IL-10 was found. The same results were obtained when primary peritoneal macrophages were used (Fig. 5B). Moreover, in the presence of IL-4/IL-13 combination, Reg3β enhanced expression of angiogenic factor VEGF in the cultured macrophages.

Figure 3. Tumor neovascularization is altered in Reg3β−/− mice. A, left, representative images of tumor blood vessel staining conducted with an anti-CD31 antibody. A, right, the number of vessels is significantly reduced in Reg3β−/− mice that developed tumors compared with Reg3β+/− mice. Vessel count was evaluated from four fields per section of 4 different animals under ×20 magnification. B, real-time qRT-PCR analysis for CD31 in tumors from Reg3β−/− and Reg3β+/− mice. C, left, representative images of VEGF staining by immunohistochemistry in tumors from Reg3β−/− and Reg3β+/− mice. C, right, the number of VEGF-positive cells is significantly reduced in Reg3β−/− mice that developed tumors compared with Reg3β+/− mice. Cell counts were evaluated from four fields per section of 4 different animals under ×20 magnification. D, levels of VEGF measured by ELISA in tumor, plasma, and ascitic fluid samples from Reg3β−/− and Reg3β+/− mice. E, gelatinolytic activity of MMP9 in pancreatic tumor homogenates of Reg3β−/− and Reg3β+/− mice. In all cases, mRNA induction levels were normalized to GAPDH mRNA expression. Bars represent mean values of each group ± SE. *, P < 0.05 versus Reg3β+/− mice. Each determination was carried out in triplicate. Scale bar, 100 μm.
Figure 4. Effects of Reg3β deficiency on immune cell tumor microenvironment. A, left, immunohistochemistry with anti-F4/80 was used to assess macrophage recruitment in Reg3β+/+ and Reg3β−/− mice. A, right, the percentage of thresholded stained area was quantified. B, left, immunohistochemistry with anti-Gr1 was used to assess granulocytes recruitment in Reg3β+/+ and Reg3β−/− mice. B, right, the number of positive cells for Gr1 was quantified. C, left, immunohistochemistry with anti-CD3 was used to assess T lymphocyte recruitment in tumors grown in Reg3β+/+ and Reg3β−/− mice. C, right, the number of positive cells for CD3 was quantified. D, real-time qRT-PCR analysis for F4/80 in tumors from Reg3β+/+ and Reg3β−/− mice. E, real-time qRT-PCR analysis for Gr1 in tumors from Reg3β+/+ and Reg3β−/− mice. F, real-time qRT-PCR analysis for CD3 in tumors from Reg3β+/+ and Reg3β−/− mice. NS/NS, nonsignificant versus Reg3β+/+ mice, considering all Reg3β−/− mice or only the Reg3β−/− mice developing tumors. *, P < 0.05 versus Reg3β+/+ mice, considering only the Reg3β−/− mice developing tumors. Black dots represent those mice with pancreatic tumor growth, whereas gray dots indicate those Reg3β−/− mice that did not develop tumors. In all cases, mRNA induction levels were normalized to GAPDH mRNA expression. Bars represent mean values of each group ± SE. Scale bar, 100 μm. Each determination was carried out in triplicate.
Furthermore, a direct functional effect of the Reg3β-M2-polarized macrophages on the pancreatic cancer cells was determined by incubating Panc02 cells with conditioned media from Reg3β-M2-polarized primary macrophages. Panc02 cell viability was increased after incubation with conditioned media from M2-polarized macrophages in accordance with the tumor-promoting role of M2 macrophages. This effect was further increased when incubated with conditioned media from Reg3β-M2-polarized macrophages. 

Figure 5. Effects of Reg3β on M1:M2 polarization in vitro. A, M1 macrophage marker expression levels (iNOS, TNF-α, IL-1β, and IL-6) and M2 macrophage markers expression levels (ARG1, Ch33, MR, and IL-10) were evaluated by real-time qRT-PCR in lipopolysaccharide or IL-4/IL-13-stimulated RAW 264.7 cells in the absence or presence of Reg3β. B, M1 macrophage marker expression levels (iNOS), M2 macrophage marker expression levels (ARG1), and VEGF expression levels were evaluated by real-time qRT-PCR in lipopolysaccharide or IL-4/IL-13-stimulated mouse primary macrophages in the absence or presence of Reg3β. In all cases, mRNA induction levels were normalized to GAPDH mRNA expression. Bars represent mean values of each group ± SE; *P < 0.05 versus C; †P < 0.05 versus respective stimuli. C, left, percentage of Panc02 cell viability when incubated with appropriate stimuli; middle, Western blot analysis of cleaved caspase-3 and PCNA expression in Panc02 cells incubated with appropriate stimuli; right, caspase-3/7 activity was measured using the caspase-3/7 Glo assay system in Panc02 cells incubated with appropriate stimuli. Data represent fold change compared with control.

In viability and apoptosis experiments, Reg3β was added to the medium alone or to macrophage conditioned media (CM) as control groups. Each determination was carried out in triplicate. Bars represent mean values of each group ± SE; *P < 0.05 versus control; †P < 0.05 versus macrophage conditioned media; #P < 0.05 versus mϕ+Reg3β conditioned media.
from Reg3β-M2-polarized macrophages (Fig. 5C), indicating a direct effect of Reg3β-M2-polarized macrophages on pancreatic tumor cell growth. Concordantly, conditioned media from Reg3β-M2-polarized macrophages inhibited Panc02 apoptosis as measured by Western blotting of cleaved caspase-3 and enzymatic activity of caspase-3/7 (Fig. 5C). Cell proliferation, measured through PCNA levels, remained unaltered.

To rule out the possibility that the Reg3β present in the medium might act directly on Panc02 cells, thus preventing apoptosis, we also incubated Panc02 cells with Reg3β. No effects in cell viability or apoptosis were observed in comparison with medium alone. To further explore whether acini-produced Reg3β could have any effect on tumor cells surrounded by macrophages, we stimulated Panc02 cells with primary macrophage conditioned media with or without subsequent addition of Reg3β. Again, no differences were observed in terms of Panc02 cell viability, proliferation, or apoptosis between Reg3β+CM and conditioned media alone. These results confirm that Reg3β does not exert a direct antiapoptotic effect on Panc02 cells in this setting. Therefore, the Reg3β-induced antiapoptotic effects observed may be indirectly mediated through M2-polarized macrophages.

M2 TAM polarization is reduced in Reg3β−/− mice

On the basis of the in vitro results, we explored whether Reg3β may promote pancreatic tumorigenesis in vivo by inducing a phenotypic switch in macrophages to a protumoral phenotype. We examined the expression of target genes responsible for controlling M1 and M2 response in Reg3β+/+ and Reg3β−/− mice following tumor formation. mRNA levels from both M2 macrophage markers ARG1 and Chi3L3 were significantly increased in Reg3β+/+ mice (Fig. 6A) compared with Reg3β−/− mice. In contrast, levels of M1 macrophage markers iNOS and TNF-α were higher in Reg3β−/− mice. It is known that arginase and iNOS compete for arginine, which serves as the substrate for both these enzymes. The activation of arginase can therefore lead to arginine depletion, causing decreased iNOS activity (21). Thus, the relative ratio of ARG1 to iNOS is frequently used as a functional readout to differentiate M2 from M1 macrophages (22). In our case, the M2:M1 ratio clearly showed that macrophages from Reg3β+/+ mice were predominantly polarized to a M2 phenotype. In contrast, Reg3β−/− mice also presented macrophages that switched to a M1 phenotype (Fig. 6B). Accordingly, arginase activity measured in tumor lysates was significantly reduced in tumors from Reg3β−/− mice (Fig. 6C).

To characterize the macrophage phenotype present in these tumors, we conducted immunohistochemistry studies of macrophage marker F4/80, M2-marker ARG1, and M1-marker iNOS in serial slices of tumors from Reg3β+/+ and Reg3β−/− mice. As shown in Fig. 6D, macrophages from Reg3β+/+ mice did not show iNOS immunostaining but all were positive for ARG1. On the other hand, macrophages from Reg3β−/− mice were positive for markers, iNOS and ARG1. Moreover, immunoblot assay of F4/80, ARG1, and iNOS confirmed that Reg3β deficiency shifted the polarization of TAMs in Reg3β−/− mice (Fig. 6E). These results were confirmed by qRT-PCR in primary macrophages isolated from peritoneal fluid of Reg3β+/+ and Reg3β−/− mice. Macrophages from Reg3β+/− mice (Reg3β+/− males) exhibited a more predominant M1 phenotype, characterized by increased iNOS expression and decreased expression of ARG1 and VEGF. This result contrast with the M2 polarization present in Reg3β+/+ male (Fig. 6F).

Reg3β enhances M2 polarization through STAT3 pathway activation

Because STAT3 has been found to regulate immune activation and macrophage function (23), it is tempting to speculate that this signaling pathway might have a role in macrophage polarization. In fact, few recent studies show different mechanisms of M2 polarization of TAMs involving STAT3 activation (24, 25). Thus, we hypothesized that Reg3β might exert its effects on TAMs through activation of STAT3. Mouse primary macrophages differentiated into alternative M2 phenotype showed STAT3 tyrosine phosphorylation and increased expression of M2 marker ARG1 (Fig. 7A). The addition of Reg3β clearly enhanced STAT3 activation and ARG1 expression. Also, macrophages were preincubated with a STAT3-specific inhibitor, AG490, before macrophage-M2 polarization. The increased STAT3 phosphorylation and ARG1 induction presented by Reg3β-M2-polarized macrophages was prevented in the presence of AG490, suggesting that Reg3β-M2 polarization is induced through STAT3 signaling. As shown in Fig. 7B, these results were confirmed using qRT-PCR by measuring ARG1 mRNA expression and SOCS3 mRNA expression as a key target of STAT3 activation.

Discussion

The novelty of our study is the identification of a new function for Reg3β released from pancreatic acinar cells in the context of pancreatic adenocarcinoma. Reg3β was discovered more than two decades ago and identified as a pancreatic secretory protein that is strongly overexpressed during acute pancreatitis (10). Since then, the observation that Reg3β is also upregulated in pancreatic cancer (17) as well as in extra-pancreatic locations during neoplastic transformation (11, 26–28) has suggested a role in cancer for this protein. Reg3β has been found to be overexpressed in serum samples and pancreatic juice from patients with PDAC (11, 17). However, the clinicopathologic significance of this expression in pancreatic tumors remains to be elucidated.

In the current study, we examined the role of Reg3β in pancreatic tumor progression in an orthotopic mouse model of pancreatic cancer. All Reg3β−/− mice presented highly vascularized primary tumors. The peritoneal cavity of these mice was full of ascites, and some tumor nodules were found in liver and/or intestine. In contrast, Reg3β−/− mice presented a reduced and poorly vascularized (or nonexistent) pancreatic tumor and none presented either nodules of metastatic growth or ascites.

The tumors that achieved growth in Reg3β−/− mice showed the same cell proliferation index as those growing in Reg3β+/− mice but presented increased apoptotic levels. Moreover, tumors from Reg3β−/− mice were very pale compared with tumors from Reg3β+/−, suggesting that the Reg3β deficiency might cause an antiangiogenic effect. Supporting this notion,
Figure 6. Effect of Reg3β on M1/M2 macrophage polarization in vivo. A, top, M2 macrophage marker expression levels (ARG1 and Chi3l3) were evaluated by real-time qRT-PCR in pancreas from Reg3β+/+ and Reg3β-/- mice. Bottom, M1 macrophage marker expression levels (iNOS and TNF-α) were evaluated by real-time qRT-PCR in pancreas from Reg3β+/+ and Reg3β-/- mice. Black dots represent mice with pancreatic tumor growth, whereas gray dots represent mice that did not develop tumors. B, M2:M1 ratio calculated from ARG1 (M2) and iNOS (M1) expression levels in pancreas from Reg3β+/+ and Reg3β-/- mice. *, P < 0.05 versus Reg3β+/+ mice. C, arginase enzymatic activity expressed as mg urea/dL in tumor homogenates from Reg3β+/+ and Reg3β-/- mice. **, P < 0.05 versus Reg3β+/+ mice. D, left, representative images of macrophage marker F4/80, M2-marker ARG1, and M1-marker iNOS immunohistochemistry in serial slices of tumors from Reg3β+/+ and Reg3β-/- mice showing peritumoral region. D, right, the number of positive cells for F4/80, ARG1, and iNOS was quantified as percentage of marked surface. E, Western blot analysis of F4/80, ARG1, and iNOS in tumor samples from Reg3β+/+ and Reg3β-/- mice. F, real-time qRT-PCR analysis of ARG1, iNOS, and VEGF expression in macrophages isolated from Reg3β+/+ and Reg3β-/- mice. *, P < 0.05 versus Reg3β-/- mice. In all cases, mRNA induction levels were normalized to GAPDH mRNA expression. Bars represent mean values of each group ± SE. Scale bar, 100 μm. T, tumor cells; A, acinar cells.
Reg3β−/− mice tumors revealed decreased blood vessel density and marked reductions of angiogenesis-related factor VEGF and MMP9 activity. Plasma and ascitic fluid samples of Reg3β−/− mice showed highly reduced VEGF levels in comparison with Reg3β+/+ mice. In this sense, several studies have shown a correlation between VEGF expression and systemic expansion of tumor mass in pancreatic cancer (29, 30). Our findings provide an explanation for the poor vascularization found in Reg3β−/− mice as well as the absence of metastasis, suggesting that Reg3β contributes to the angiogenic switch required for tumor progression during pancreatic carcinogenesis.

The microenvironment found in PDAC supports tumor growth and progression and the recruitment of immune cells including dendritic cells, T cells, granulocytes, and macrophages. Esposito and colleagues characterized the inflammatory cell infiltrate in this disease, reporting the macrophages as the major component (31). In the present study, deficiency of Reg3β changed neither the levels of macrophage recruitment observed in Reg3β+/+ mice nor granulocyte infiltration in tumors. On the other hand, a significant increase in CD3+ tumor-infiltrating T cells was observed in Reg3β−/− in the mice that developed tumors. The presence of CD3 in human cancers is associated with improved clinical outcomes (32, 33). Thus, increased levels of CD3 in Reg3β−/− mice developing tumors could help to explain the fact that these tumors are less developed than those growing in Reg3β+/+ mice and do not present metastases.

Studies of the relation between tumor cells and the surrounding stroma in pancreatic cancer have shown the existence of spatially distinct compartments of gene expression (31). Moreover, some of the genes that are considered over-expressed in pancreatic tumors actually come from the peritumoral tissue. For instance, proteomic profiling of pancreatic juice has identified higher levels of Reg3β in patients with pancreatic cancer and the source of elevated Reg3β was the pancreatic acinar cells adjacent to the infiltrating adenocarcinoma (34). In addition, the use of microarrays allowed gene expression patterns to be characterized in pancreatic acinar tissue adjacent to infiltrating PDAC. The most abundantly expressed gene was Reg3β itself, which increased over 100-fold (35).

A key observation in our previous in vitro studies is the fact that Reg3β inhibits the expression of proinflammatory molecules (5, 6, 36). In this regard, we found that in RAW 264.7 and primary macrophages Reg3β inhibited the expression of several proinflammatory markers related to the M1 phenotype in response to proinflammatory stimuli. Surprisingly, Reg3β also enhanced the alternative differentiation of macrophages by inducing M2 target genes.

Given that not only the number of macrophages but also their phenotypes regulate tumorigenesis, we further investigated whether the lack of Reg3β resulted in the polarization of macrophages in vivo. Recent studies have found upregulated expression of M2 markers in macrophages from patients with pancreatic cancer and in a murine pancreatic adenocarcinoma model (37, 38). Interestingly, these M2-polarized macrophages were associated with large tumor size and poor prognosis due to accelerated lymphatic metastasis (37, 39). Our results showed that M2 TAM markers ARG1 and Chi3L3 were clearly reduced in Reg3β−/− mice tumors compared with Reg3β+/+ mice. In contrast, levels of M1 TAM markers iNOS and TNF-α were higher in tumors from Reg3β−/− mice than in Reg3β+/+ mice, which displayed an enhanced M2:M1 ratio.
polarization analysis of isolated primary peritoneal macrophages from mice confirmed that Reg3β deficiency skewed Reg3β−/− macrophages toward an M1 phenotype. Altogether, these results provide evidence that Reg3β plays a role in TAM polarization in pancreatic cancer, contributing to the portfolio of molecules that determine the anti-inflammatory and growth-supporting M2-polarized macrophage phenotype. More than half of the Reg3β−/−-induced mice did not develop pancreatic tumors. Even more surprising was the finding that the pancreas of these mice presented an emerging M1 phenotype characterized by iNOS and TNF-α expression. In view of these interesting data, we hypothesize that the reduction in tumor development in Reg3β−/− mice may be due to the fact that the lack of this protein allows macrophages to maintain a proinflammatory phenotype. And this phenotype contributes to delaying or even inhibiting tumor development.

An antiapoptotic function of Reg3β under proinflammatory stimuli has been described earlier (40). To explore whether this function was involved in the current setting, we also analyzed if Reg3β on its own, and independently of its effects on stimulated macrophages, might directly affect apoptosis or cell viability in Panc02 tumor cells. The results obtained here suggest that, at least in part, Reg3β exerts its tumor-promoting role in an indirect way via M2 macrophages. Further studies are necessary to establish whether other cellular types and mechanisms are involved in this Reg3β oncogenic function.

Finally, our experiments show that STAT3 activation by Reg3β contributes to the induction of M2 macrophage polarization in vitro, as its inhibition abolishes Reg3β-induced expression of ARG1 and the STAT3 target gene SOCS3. These results provide an interesting preliminary mechanistic insight into the crucial role of Reg3β in macrophage polarization during pancreatic adenocarcinoma.

In summary, our study identifies a novel tumor-promoting role of Reg3β in the context of pancreatic cancer. We show that Reg3β secreted by pancreatic acinar cells acts in a paracrine manner on TAMs by skewing them toward the M2 phenotype through STAT3 activation and, thus, promoting angiogenesis and pancreatic tumor growth. Therefore, Reg3β inactivation may be a promising approach in the treatment of pancreatic cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Gironella, A. Fernández, D. Closa, J.L. Iovanna, E. Folch-Puy
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