Resistance of cyclooxygenase-2 expressing pancreatic ductal adenocarcinoma cells against γδ T cell cytotoxicity

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Abbreviations: BrHPP, bromohydrin-pyrophosphate; Cox, cyclooxygenase; n-BP, nitrogen-containing bisphosphonates; PAg, phosphorylated antigen; PDAC, pancreatic ductal adenocarcinoma; PG, prostaglandins; RTCA, Real Time Cell Analyzer; TCR, T cell receptor.

The prostaglandin (PG) synthetase cyclooxygenase 2 (Cox-2) promotes tumorigenesis, tumor progression, and metastasis in a variety of human cancer entities including pancreatic ductal adenocarcinoma (PDAC). In this study, we demonstrate that in PDAC cells such as Colo357 cells, enhanced Cox-2 expression and increased release of the Cox-2 metabolite prostaglandin E2 (PGE2) promotes resistance against γδ T cell-mediated lysis. Co-culture with activated γδ T cells induced an upregulation of Cox-2 expression in Colo357 cells, and thereby an enhanced PGE2 release, in response to tumor necrosis factor α (TNFα) secretion from γδ T cells. The PGE2-mediated inhibition of γδ T cell cytotoxicity against Cox-2-expressing PDAC cells can be partially overcome by Cox-2 inhibitors. Our results show that differences between PDAC cells in regards to sensitivity to γδ T-cell cytotoxicity can be due to distinct levels of Cox-2 expression associated with varying amounts of PGE2 release. While γδ T cell cytotoxicity against PDAC cells expressing low levels of Cox-2 can be effectively enhanced by tribody [(Her2)2 V9] with specificity for V9 T cell receptor and HER-2/neu on PDAC cells, a combination of tribody [(Her2)2 × V9] and Cox-2 inhibitor is necessary to induce complete lysis of Cox-2 high expressing Colo357. In conclusion, our results suggest that the application of tribody [(Her2)2 × V9] that enhances γδ T-cell cytotoxicity and Cox-2 inhibitors that overcome PGE2-mediated resistance of PDAC cells to the cytotoxic activity of γδ T cells might offer a promising combined immunotherapy for pancreatic cancer.

Introduction

Arachidonic acid is released from plasma membrane phospholipids and is further converted by cyclooxygenase (Cox) enzymes Cox-1 and Cox-2 to prostaglandins (PG), prostacyclins and thromboxanes.1 Cox-1 is constitutively expressed in most mammalian tissues, whereas prostaglandin endoperoxidase synthase enzyme 2 (PTGS2, better known as Cox-2) is induced by a variety of pro-inflammatory stimuli.1,2 Cox-2 is overexpressed in diverse tumor entities including ducal pancreatic adenocarcinomas (PDAC), which is mostly associated with a more aggressive tumor stage and worse prognosis.3-5 Cox-2 overexpression leads to an enhanced production of prostaglandin E2 (PGE2), which is involved in tumor progression as well as in tumor evasion of immunosurveillance.6 The release of PGE2 by cancer cells induces and recruits regulatory T cells to the tumor site and suppresses immune responses of CD8+ αβ T cells and natural killer (NK) cells as well as maturation of dendritic cells.2,7,8 Similar to αβ T cells, the function of the numerically small population of γδ T lymphocytes is also inhibited by PGE2 secreting and Cox-2 expressing mesenchymal stem cells involved in the development of tumor-promoting cancer stroma.9,10 PGE2 binds to prostaglandin E2 and E4 receptors expressed on activated γδ T cells, and thereby inhibits T cell receptor (TCR)-activated cytotoxic activity of γδ T cells by cyclic adenosine monophosphate (cAMP)-mediated protein kinase A type I-dependent signaling.26 However, such an enhanced PGE2 synthesis could be potently decreased by the Cox-2 inhibitor celecoxib in a pancreatic cancer xenograft mouse model.11 Although selective Cox-2 inhibitors such as celecoxib and DuP697 showed anti-proliferative activity toward different tumor entities and against several PDAC cell lines in vitro as well as in vivo, celecoxib as a single agent failed to exert anti-proliferative or antitumor effects toward pancreatic adenocarcinomas resected from patients and implanted in nude mice in a xenograft model, despite a decrease in PGE2 synthesis.11-13 Nevertheless, selective Cox-2 inhibitors have been
PDAC is a highly malignant gastrointestinal tumor characterized by the presence of a dense desmoplastic stroma composed of extracellular matrix and diverse non-neoplastic inflammatory cells mostly displaying an immunosuppressive phenotype. The overall 5-year survival rate is less than 5%. The poor prognosis of PDAC is due to the absence of specific symptoms and a rapid progression of this extremely aggressive disease, essentially limiting therapeutic options. Moreover, PDAC is characterized by a profound resistance toward current chemotherapeutic treatments.

Our recent studies revealed that PDAC cells differ in their Cox-2 expression and PGE2 release by PDAC cells in the absence of γδ T cell lines established from different healthy donors (n = 10) and PDAC patients (n = 15). To demonstrate that the different sensitivity of the PDAC cells to γδ T cell cytotoxicity was not due to differences between individual γδ T cell lines established from different donors, γδ T cell lines from the same 4 representative donors (2 healthy and 2 PDAC patient) were used for the lysis of the various PDAC cells as indicated.

**Differential Cox-2 expression and PGE2 release by PDAC cells**

Next, we examined whether PDAC cells differ in their Cox-2 expression, thereby releasing different amounts of PGE2 which could be one explanation for the different sensitivity toward γδ T cell lysis. The expression of Cox-2, the inducible enzyme in the PGE2 producing pathway, was analyzed in all PDAC cells by flow cytometry as well as by Western Blot. Regarding the mean fluorescence intensity determined by cytometric fluorimetric analysis, we observed a very low expression of intracellular Cox-2 in PancTu-I as well as in Panc89 cells compared to Colo357 cells, which highly expressed intracellular Cox-2 (Fig. 2A and B). These results were verified with an additional Cox-2 antibody by Western Blot analysis confirming the highest Cox-2 expression level in Colo357 cells (Fig. 2C). The parallel analysis of Cox-1, the constitutively active enzyme of the PGE2 producing pathway, revealed a similar expression in all PDAC cell lines (Fig. 2D).

Next, we investigated whether the differences in intracellular Cox-2 expression were reflected by differential release of PGE2 from PDAC cells. In line with the low intracellular Cox-2 expression, PancTu-I and Panc89 cells secreted very low amounts of PGE2 (< 0.5 ng/mL), whereas Cox-2 high expressing Colo357 cells released relatively high amounts of PGE2 (> 6 ng/mL) (Fig. 2E).
Taken together, the 3 different PDAC cell lines differed significantly from each other regarding Cox-2 expression and PGE2 release.

**PGE2 inhibited γδ T cell-mediated lysis of PDAC cells abolished by Cox-2 inhibitors**

The enhanced release of PGE2 by Colo357 cells being in line with the reduced sensitivity toward γδ T cell lysis (Fig. 1) prompted us to examine the effect of PGE2 on the γδ T cell-mediated lysis of PDAC cells. Different concentrations (0.1–10 μg/mL) of PGE2 (dissolved in DMSO) were added to the 3 different PDAC cell lines directly after seeding of the PDAC cells for 20 h (in comparison to DMSO as a control) and before BrHPP-stimulated γδ T cell lines were added and cell lysis was determined by RTCA assay (data not shown). Additionally, we tested the effect of PGE2 on PDAC cells alone showing that PGE2 has no effect on the impedance, and thus on the cell death of PDAC cells, in the absence of γδ T cells (data not shown). However, the presence of 0.1–1 μg/mL PGE2 strongly reduced the lysis of Panc89 as well as of PancTu-I cells by BrHPP-stimulated γδ T cell lines compared to the stimulation in the absence of PGE2 and in the presence of medium or DMSO control.
Colo357 (Fig. 3C). These results fit well with the indicated high release of PGE2 by PancTu-I and Panc89, the weak lysis of Colo357 cells was virtually unaltered by additional treatment with PGE2 (Fig. 3C). These results fit well with the indicated high release of PGE2 by Colo357 (Fig. 2E) and demonstrate that an additional application of PGE2 to the already high level PGE2-producing Colo357 only slightly affected the nearly complete resistance against γδ T cell-mediated lysis (Fig. 3C).

Since Cox-2 expression goes along with the release of PGE2, we tested whether Cox inhibitors were able to increase γδ T cell-mediated lysis of PDAC cells. First, we studied the effect of different concentrations (5, 10, 50 and 200 μM) of the Cox-1/2 inhibitor Indomethacin and the selective Cox-2 inhibitor DuP697 on PDAC cells alone to exclude toxic effects on the tumor cells potentially induced by these inhibitors. None of the tested concentrations of the inhibitors (dissolved in DMSO) revealed an influence on PDAC cell growth except 200 μM DuP697, which caused a growth inhibition on all PDAC cells, and 50 μM DuP697, which reduced Panc89 cell growth (data not shown).

Accordingly, pretreatment of PDAC cells with 50 μM Indomethacin or of PancTu-I with 50 μM and Panc89 with 10 μM DuP697 revealed no effect on the lysis of these cells by BrHPP-stimulated γδ T cell lines (data not shown), whereas the lysis of Colo357 was strongly enhanced by γδ T cell lines in the presence of Indomethacin or 50 μM DuP697 (Fig. 3D and E). Moreover, we observed no difference between Cox-1/2 and Cox-2 inhibitors on the enhanced lysis suggesting that only Cox-2 expression influences the cytotoxic activity of γδ T cells toward PDAC cells.

To analyze whether the Cox-inhibitors effect PGE2 production by Colo357, the tumor cells were cultured alone or co-cultured with BrHPP-stimulated γδ T cell lines in the presence of the indicated Cox-inhibitor and PGE2 release was determined in the supernatants. Interestingly, PGE2 release by Colo357 alone was not reduced after the addition of the indicated concentrations of Cox-inhibitors. However, the co-culture with BrHPP-stimulated γδ T cell lines enhanced the PGE2 release dramatically which could be significantly reduced in the presence of Cox-2 inhibitors (Fig. 4). In line with the enhanced PGE2 release, Cox-2 expression in Colo357 cells was drastically enhanced after co-culturing them with BrHPP-stimulated γδ T cell lines (data not shown), which underlines the results by others that Cox-2 expression correlates with PGE2 production.21

These data demonstrate that an enhanced PGE2 release induced by an intensified expression of Cox-2 in Colo357 cells co-cultured with γδ T cell lines decreased γδ T-cell cytotoxicity that could be abolished by Cox-2 inhibitors.

**TNFα released by γδ T cells enhanced Cox-2 expression in Colo357 PDAC cells**

In light of our observations that Colo357 PDAC cells co-cultured with γδ T cells displayed enhanced production of PGE2 and intracellular Cox-2 expression taken together with reports by others that Cox-2 expression can be stimulated by various antigens and cytokines,1,6,22 we next set out to investigate the role of cytokines on the regulation of Cox-2 expression in Colo357 cells. We focused on tumor necrosis factor α (TNFα) as well as interferon γ (IFNγ) because γδ T cell lines produce both cytokines after activation with PAg, such as BrHPP.23,24 We found that TNFα, but not IFNγ, induced a dose-dependent and significant upregulation of intracellular Cox-2 expression in Colo357, but not in PancTu-I or Panc89 cells (Fig. 5A, data not shown). When IFNγ was added to Colo357 cells, Cox-2 expression decreased significantly. However, concomitant stimulation of
Colo357 cells with TNFα applied together with IFNγ led to a similar increase of Cox-2 expression as TNFα stimulation alone (Fig. 5A). In accordance with these results, application of the TNFα-blocker Infliximab during co-culture of Colo357 cells and TNFα producing γδ T cell lines clearly prevented the increase of Cox-2 expression, an effect that was not observed after treatment with control Abs (Fig. 5B).

The inhibition by Infliximab demonstrates that TNFα released by activated γδ T cell lines accounts for the strong induction of Cox-2 expression in Colo357 cells.

Cox-2 inhibitor DuP697 together with [(Her2)2×Vγ9] overcome the resistance toward γδ T cell-mediated lysis of Colo357

To investigate whether the addition of the Cox-2 inhibitor DuP697 co-administered together with the tribody [(Her2)2×Vγ9] could overcome the resistance of Colo357 cells toward γδ T cell-cytotoxicity, we activated several γδ T cell lines from different healthy donors with BrHPP in the absence or presence of DuP697, [(Her2)2×Vγ9], or with the combination of both. As expected, γδ T cell lines only weakly lysed the tumor...
cells after activation with BrHPP. The additional treatment with DuP697 or [(Her2)2×Vγ9] strongly enhanced the cytotoxic activity of γδ T cells toward Colo357 cells (Fig. 6). Similar results were obtained with γδ T cell lines from PDAC patients (data not shown). In the absence of BrHPP, we observed no enhancing effect of DuP697, whereas [(Her2)2×Vγ9] with or without BrHPP similarly increased the cytotoxic effects γδ T cells toward Colo357 cells, as we previously showed.18 Interestingly, the combination of DuP697 and [Her2]2×Vγ9] most prominently enhanced the γδ T cell-mediated lysis of the naturally resistant Colo357 cells. Similar results were obtained by using γδ T cell lines derived from PDAC patients. We conclude that the killing of Cox-2 high PDAC cells by γδ T cell lines is more efficient in the presence of DuP697 together with [(Her2)2×Vγ9] than with [(Her2)2×Vγ9] alone.

Discussion

Our study indicates that the inhibition of the PGE2 pathway with Cox-2 inhibitor DuP697 together with [(Her2)2×Vγ9], an enhancer of γδ T cell cytotoxicity, abolished the resistance of the PDAC cell line Colo357 against γδ T cell-mediated lysis. γδ T lymphocytes have raised substantial interest for immunotherapy based on their capacity to kill (radio- and chemotherapy resistant) PDAC cells in an HLA-independent manner. We
previously reported that γδ T cells in PDAC tissues are predominantly extensively distributed in the ductal epithelium and the stroma close to the ductal epithelium, which demonstrates mobilization and infiltration to the tumor site. However, PDAC is characterized by the presence of dense desmoplastic stroma composed of extracellular matrix and diverse (immunosuppressive) cells dampening cytotoxic activity of γδ T cells.18,25 An efficient strategy to overcome immunosuppression by the stromal composition on γδ T-cell activity could be the usage of enhancers of γδ T-cell cytotoxicity. Recently, we demonstrated that [(Her2)₂×Vγ9] selectively enhanced the cytotoxicity of γδ T cells in vitro as well as in vivo upon transfer into immunocompromised mice.18 Although our prior study revealed promising results, heterogeneity between the various PDAC cell lines was observed. Several of the PDAC cell lines, such as Colo357 cells, were neither completely lysed by γδ T cells alone nor in combination with enhancers of their cytotoxicity suggesting that the malignant cells themselves actively promote resistance. Therefore, we analyzed the direct cross-talk between PDAC cells and γδ T cells in the absence of other cell types in vitro. We observed that

Figure 6. [(Her2)₂×Vγ9] together with Cox-2 inhibitors overcome the resistance of Colo357 toward γδ T cell-mediated lysis. After culturing Colo357 overnight, cells were left untreated (green line) or were co-cultured with phosphorylated antigen (PAg; 300 nM BrHPP) stimulated γδ T cell lines at an effector to target (E:T) cell ratio of 25:1 in the presence of 50 IU/mL IL-2 with medium (dark blue line), 1 μg/mL [(Her2)₂×Vγ9] (light blue line), 50 μM DuP697 (red line) or the combination of [(Her2)₂×Vγ9] and DuP697 (pink line). The cell index (as measured by electrical impedance) was analyzed in 5 min steps over 24 h and was normalized at the time of addition of substances and γδ T cell lines. Thereafter, cell index was measured in 1 min steps for 6 h. Five different individual experiments with Colo357 are shown. The arrow indicates addition of substances and/or γδ T cells.
the intracellular Cox-2 expression directly correlated with an increased PGE2 release by Colo357 cells and their resistance against γδ T cell-mediated lysis. Similar to the report of Martinet and colleagues, who demonstrated an inhibitory effect of PGE2 on γδ T-cell cytotoxicity, we observed that the addition of PGE2 to PDAC cells releasing scarce native PGE2, such as PANC-89 and PancTu-1 cells, robustly inhibited γδ T-cell-mediated lysis. In contrast, the addition of exogenous PGE2 to PDAC cells that already released high concentrations of native PGE2 only slightly modulated γδ T-cell cytotoxicity, suggesting that the endogenous release of PGE2 by Colo357 cells is sufficient to potently inhibit γδ T-cell cytotoxicity. This hypothesis was confirmed by the experiment with the inhibitors Indomethacin and DuP697 that partially abrogated the resistance of Colo357 against γδ T-cell mediated lysis. Furthermore, since the Cox-1/2 inhibitor Indomethacin and the selective Cox-2 inhibitor DuP697 had similar effects on γδ T-cell mediated cytotoxicity against PDAC cells we can rule out that Cox-1 is involved in this context, although Cox-1 reportedly also contributes to tumorgenesis. Cox-1 is constitutively expressed in many tissues, whereas inducible Cox-2 has been reported to be overexpressed in ~70% of human pancreatic cancer fulfilling an intrinsic role in tumor initiation, development and progression through the activation of the phosphatidylinositol 3 kinase (PI3K)/AKT pathway. While anti-Cox-2 therapy has been consistently shown to inhibit PGE2 synthesis in xenografted pancreatic tumors and in pancreatic cancer patients, conflicting findings have been reported in regards to the antitumor, anti-proliferative and anti-angiogenic effect of Cox-2 inhibitors in xenografted pancreatic carcinomas, a result that may be due to the usage of PDAC cell lines versus primary PDAC cells in these two contradictory reports. In our experiments, we did not focus on the direct toxic effect of Cox-2 inhibitors on PDAC cells but on the effect on γδ T-cell mediated cytotoxicity. Based on previous titration results, we used Cox-2 inhibitors at concentrations that were not toxic for PDAC cells but potently inhibited their PGE2 release. This allowed us to examine the effect of PGE2 on γδ T-cell mediated lysis of Colo357 in more detail. We observed that the co-culture of activated γδ T cell lines with Colo357 cells enhanced the PGE2 release, which could be significantly inhibited by the Cox-inhibitors Indomethacin and DuP697. As shown by others, PGE2 binds to specific G-protein-coupled receptors termed prostaglandin E receptor 2 (PTGER2, better known as EP2) and prostaglandin E receptor 4 (PTGER4, better known as EP4), both of which are expressed on activated γδ T cells. Activation of EP2 as well as the EP4 receptor induces adenylate cyclase and thereby the concomitant increase in the secondary messenger cAMP. Subsequently, cAMP mediates the dissociation of the regulatory and catalytic subunits of protein kinase A, which subsequently initiates the corresponding transactivation of the transcription factor cAMP responsive element binding (CREB). CREB proteins are essential regulators for T-cell function and cytokine production (e.g., of IFNγ). Moreover, PGE2-induced cAMP was described to also activate the raft-associated enzyme c-src tyrosine kinase (CSK), which negatively regulates the phosphorylating activities of the TCR signaling kinase LCK, the Z chain TCR associated kinase ZAP-70 and AKT in T cells. Although EP2 and EP4 receptors share the same signaling pathway, a distinguishing feature of the EP4 receptor is its activation of the PI3K signaling pathway resulting in subsequent nuclear factor κB (NF-κB) activation and thus TNFα release. Martinet and co-workers reported that PGE2 inhibits TCR-activated γδ T cell-cytotoxicity by a cAMP-mediated protein kinase A type I-dependent signaling. In addition, EP2- and EP4-specific agonists reduced intracellular IFNγ production in activated γδ T cells comparable to the addition of exogenous PGE2. An enhanced release of IFNγ often leads to reduced intracellular stores of IFNγ. To this end, Martinet and colleagues analyzed whether IFNγ modulates the Cox-2 expression or PGE2 secretion of mesenchymal stem cells, which are involved in the tumor stroma development and inhibit γδ T cell mediated cytotoxicity by an enhanced PGE2 release. In their experiments, they observed that 100 ng/mL IFNγ as well as 100 ng/mL TNFα only slightly enhanced intracellular Cox-2 expression in mesenchymal stem cells, whereas the combined addition of both cytokines significantly increased Cox-2 expression suggesting that both cytokines together produced by activated γδ T cells are necessary to induce Cox-2 expression in mesenchymal stem cells. Additionally, we observed that IFNγ alone rather diminished intracellular Cox-2 expression in Colo357 tumor cells, whereas TNFα significantly increased Cox-2 expression. Further, the combination of both cytokines enhanced Cox-2 expression in Colo357 cells to levels comparable to that of TNFα stimulation alone. TNFα produced by macrophages has been reported to enhance Cox-2 expression in tissues normally Cox-2 negative. In our study, the importance of TNFα in enhancing Cox-2 expression in Colo357 cells was supported by the effect of the anti-TNFα blockers Infliximab and Adalimumab (Fig. 5B and data not shown) that abrogated the enhanced intracellular Cox-2 expression in Colo357 cells mediated by exogenous TNFα. In line with the concept that Infliximab specifically reduced the TNFα-mediated Cox-2 up-regulation but not the constitutive expression of Cox-2, the addition of Infliximab in the cytotoxic assay only marginally affected the γδ T cell-cytotoxicity against Colo357 cells (data not shown). Besides the potential upregulation of Cox-2 expression in pancreatic tumors by cytokines, other factors such as genetic alterations during the pancreatic carcinogenesis or mutations in p53 and KRAS could play a role. The oncogene KRAS (also known as Ki-ras), for example, has been previously described to stimulate Cox-2 expression. EP receptor antagonists have been tested for their ability to suppress breast cancer metastasis. Although the targeted modulation of T-cell function by blocking specific EP receptor signaling seems to be a promising approach, there are several limitations in the usage of EP receptor antagonist for therapy. EP receptor antagonists display many compensatory and opposing roles, such as their ability to ameliorate not only Th1 responses but also Th17 responses. Moreover, these agents typically inhibit only one or 2 specific receptors, which might be not as efficient as Cox-inhibitors, which inhibit all downstream prostaglandins.
Several Cox-2 inhibitors such as Celecoxib (Celebrex®) have been included in several clinical protocols, e.g., in a clinical Phase II study combined with chemotherapy (paclitaxel, carboplatin) and radiotherapy for patients with inoperable stage IIIA/B non-small-cell lung cancer or with chemotherapy (gemcitabine) combined with topoisomerase I inhibitor irinotecan or not for patients with advanced or metastatic pancreatic adenocarcinoma.14,15,43,44 However, the application of Celecoxib over a prolonged period of time seems to be critical since it may cause cardiovascular side effects.12 Moreover, the effect of Celecoxib alone induced different effects on pancreatic cancer cells.12,45 In our experiments, we used different Cox inhibitors, which did not induce cell death or cell cycle arrest in PDAC cells in the used concentrations but inhibited the PGE2 release. Our data demonstrate that a combined immunotherapy with Cox-2 inhibitors together with the newly designed tribody [(Her2)2×V9] gives us a tool to efficiently induce the γδ T cell-mediated lysis of Colo357 PDAC cells.

Taken together, our results indicate that the usage of the tribody [(Her2)2×V9], which re-directs γδ T cells to target cancer cells and enhances their cytotoxic activity toward PDAC cells, administered together with Cox-2 inhibitors that reduce PGE2 production might be a promising approach to target γδ T cell-resistant Cox-2 expressing PDAC cells.

Materials and Methods

Isolation of PBMC and establishment of γδ T cell lines

Short-term γδ T cell lines were established from peripheral blood mononuclear cells (PBMC) of healthy donors or patients with PDAC. PBMC were isolated from leukocyte concentrates or from heparinized blood by Ficoll-Hypaque (#6115, Biochrom) density gradient centrifugation. Blood from healthy donors was provided by the Department of Transfusion Medicine or the Institute of Immunology, whereas heparinized blood from PDAC patients was obtained from the Department of General Surgery of the municipal hospital. Informed consent was obtained from all donors, and the research was approved by the relevant institutional review boards (code number: D 405/10, D404/14).

PBMC were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 25 mM HEPES, antibiotics, 10% FCS (complete medium), and stimulated with 5 μM Nitrogen-containing-bisphosphonate (n-BP) zoledronic acid (Novartis) to selectively activate VγVδ2 γδ T lymphocytes within the PBMC. Since initially VγVδ2 γδ T cells produced only low amounts of IL-2, 50 U/mL rIL-2 (Novartis) were added every 2 d over a culture period of 14 to 21 d. After the culture period, most short-term activated γδ T cell lines (termed γδ T cell lines) comprise > 97% VγVδ2 γδ T cells as analyzed by flow cytometry.

PDAC cell lines

Colo357, Pt45P1, PancTu-I and Panc89 cells were cultured in complete medium. All PDAC cells were kindly provided by Prof. Dr. Kalthoff, Section of Molecular Oncology, Kiel. The genotype of PDAC-cell lines was recently confirmed by short tandem repeats analysis. 0.05% trypsin/ 0.02% EDTA was used to remove adherent PDAC cells from flasks.

Flow cytometry

For the analysis of the purity of the γδ T cell lines, cells were stained with the following phycoerythrin (PE), allophycocyanin (APC), fluorescein isothiocyanate (FITC) or Per-CP fluorochrome-conjugated monoclonal antibodies (mAb): anti-CD3 (clone SK7; PE: #345765 or APC; #344812, BD Biosciences), anti-TCRγδ (clone 11F2; FITC: #347903 or APC: custom research conjugate, BD Biosciences), anti-TCRαβ (clone IP26, #306706, Biolegend), anti-TCRβVδ2 (clone Immu389, FITC: #PNIM1464, Beckman Coulter or clone B6, PerCP: #331410), anti-TCRVδ81 (clone TS8.2, #TCR2730, Thermo Fisher Scientific) or corresponding isotype controls (BD Biosciences or Biolegend).

For intracellular staining, 10^5-10^6 tumor cells were washed, permeabilized and fixed with Cytofix/Cytoperm kit (#554714, BD Biosciences) and stained with 20 μL anti-Cox-2-PE mAb (clone AS70/AS67, #334090, BD Biosciences) or the appropriate isotype controls. After washing, all samples were analyzed on a FACS Calibur flow cytometer (BD Biosciences) using Cell Quest Pro software. To analyze the modulation of intracellular Cox-2 expression, 1.5 x 10^5/500 μL of PDAC cells were seeded in complete medium in 24-well plates together with a final concentration of 10 or 100 ng/mL recombinant TNFα or IFNγ (#210-TA/CF or 285-IF, R&D Systems) or the combination of both for 24 h before intracellular Cox-2 expression was determined by flow cytometry. For blocking experiments during co-culture of Colo357 cells with γδ T cell lines (effector/target ratio of 5:1), the chimeric mAb Infliximab (Remicade®, Janssen Biologics B.V.) as well as the human mAb Adalimumab (Humira®, Abbott GmbH & Co KG), both of which bind TNFα, were used to prevent the activation of the TNFα receptor. As an irrelevant control human IgG (#1-001-A, R&D Systems) was added. The optimal concentrations of the mAb for inhibition were previously titrated using final concentrations of 2, 5, 10 and 50 μg/mL.

Western blot analysis

For detection of Cox-2 in PDAC cells by Western Blot, PDAC cells were cultured for 24 h, trypsinized and lysed with TNE lysis buffer (50 mM Tris, 1% (v/v) NP-40, 150 mM NaCl, 2 mM EDTA and protease inhibitors sodium fluoride [10 mM] and Na3VO4 [1 mM]). Protein concentration was analyzed by Coomassie Protein Assay Reagent (#1856209, Thermo Fisher Scientific). Twenty μg of protein was separated by 10% SDS-PAGE, transferred to nitrocellulose membrane (#10600001, GE Healthcare) and detected with 1 μg/mL unconjugated anti-Cox-2 mAb 33/Cox-2 (G10203, BD Biosciences) followed by peroxidase (POD)-conjugated rabbit anti-mouse (0.24 μg/mL, #315-005-020, Dianova) and chemiluminescence detection reagent (ECL Plus, #RPN2106, GE Healthcare). As control for protein load, Ab-linked POD was detected using a primary Ab linked to horseradish peroxidase (HRP) followed by chemiluminescence detection reagent.
inactivated by 15% H2O2 and reprobed with anti-β-Actin mAb (AC-15, 0.29 μg/mL, #A5441, Sigma-Aldrich).

**Real time cell analyzer**

Five × 10^5 adherent PDAC cells were seeded in 96-well E-plates covered at the bottom with gold-electrodes (ACEA), which measured the impedance changes of attached PDAC cells. The impedance reflects cellular parameters such as cell number, size and shape, which was monitored with the Real Time Cell Analyzer (RTCA) single-plate (SP) system (ACEA) every 15 min for up to 24 h. The RTCA-SP system displays impedance in arbitrary cell index units, which correlates with cell number. After the PDAC cells reached linear growth phase, PGE2 (#2296/10, Tocris, R&D Systems), PAg Bromohydrin-pyrophosphate (BrHPP, selective Vg9Vd2 γδ T cell stimulator, Innate Pharma), tribody [(Her2)2×V9] consisting of 2 single chain variable fragments derived from trastuzumab (Herceptin) V-regions genetically fused to a Vγ9 Fab fragment via flexible linkers or 1:500 DMSO (#1.02952.1000, Merck) were pre-incubated over plates for 20 h, and were stimulated with either medium or the previously titrated and indicated concentrations of Cox-inhibitors Indomethacin or DuP697 in the absence or presence of BrHPP-stimulated γδ T cell lines (at an E/T ratio of 5:1) together with 12.5 U/mL rIL-2 for the last 4 h. After a total incubation time of 24 h, supernatants were collected and PGE2 was measured by Prostaglandin E2 Parameter Assay Kit in duplicates following the procedures outlined by the manufacturer (#SKGE004B, R&D Systems).

**Statistical analysis**

Since no violation of normal distribution assumption was found (Shapiro-Wilk test), all statistical comparisons were done parametrically by using t-tests. All statistical tests were 2-sided and the level of significance was set at 5%, not corrected for multiple testing.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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ELISA

To quantify PGE2 released by PDAC cells, 1.5 × 10^5 PDAC cells in 500 μL of complete medium were seeded into 24-well plates for 20 h, and were stimulated with either medium or the previously titrated and indicated concentrations of Cox-inhibitors Indomethacin or DuP697 in the absence or presence of BrHPP-stimulated γδ T cell lines (at an E/T ratio of 5:1) together with 12.5 U/mL rIL-2 for the last 4 h. After a total incubation time of 24 h, supernatants were collected and PGE2 was measured by Prostaglandin E2 Parameter Assay Kit in duplicates following the procedures outlined by the manufacturer (#SKGE004B, R&D Systems).

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