

PGE2 Released by Pancreatic Cancer Cells Undergoing ER Stress Transfers the Stress to DCs Impairing Their Immune Function



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ABSTRACT

This study shows that pancreatic cancer cells undergoing cell death by valproic acid (VPA) treatment activated dendritic cells (DCs) more efficiently than those treated with trichostatin A (TSA), as demonstrated by CD86 and CD80 surface expression. Surprisingly though, DCs cultured in the presence of supernatant derived from VPA-treated cancer cells showed a reduced allostimulatory capacity and an increased release of IL10 and IL8 cytokines in comparison with those exposed to TSA-treated cell culture supernatant. Searching for molecular mechanisms leading to such differences, we found that VPA treatment dysregulated choline metabolism and triggered a stronger endoplasmic reticulum (ER) stress in pancreatic cancer cells than TSA, upregulating CCAAT/enhancer-binding protein homologous protein, and activated cyclooxygenase-2, thus promoting the

release of prostaglandin (PG) E2. Interestingly, dysfunctional DCs cultured in the presence of VPA-treated cells culture supernatant showed a higher level of intracellular reactive oxygen species, 4-hydroxy-*trans*-2-nonenal protein adducts, and ER stress, as evidenced by the upregulation of spliced X-box binding protein 1 (XBP1s), effects that were reduced when DCs were exposed to supernatant of cancer cells treated with Celecoxib before VPA. Celecoxib prevented PGE2 release, restoring the function of DCs exposed to VPA-treated cells culture supernatant, and a similar effect was obtained by silencing XBP1s in DCs treated with VPA-treated cells culture supernatant. These results suggest that PGE2 could be one of the yet unidentified factors able to transfer the stress from cancer cells to DCs, resulting in an impairment of their function.

Introduction

Harnessing the immune system against tumors is the only way to obtain complete tumor eradication also in the course of chemotherapies (1, 2). A plethora of studies have investigated this topic showing that cellular processes such as autophagy (1, 3, 4), endoplasmic reticulum (ER) stress, and the activation of the unfolded protein response (UPR) arm inositol-requiring enzyme (IRE) 1 α , protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (5) may influence the immunogenicity of dead cancer cells. UPR and autophagy, among other effects, may promote the exposure and/or release of damage-associated molecular patterns (DAMPs), including heat shock proteins, calreticulin, ATP, and High Mobility Group Box

1 (6, 7), which may facilitate phagocytosis and induce dendritic cells (DC) activation. However, an effective anticancer treatment must also overcome cancer-mediated immune suppression, which paradoxically may, in some circumstances, be potentiated by ER stress and UPR activation (8). Targeting the constitutive ER stress triggered in cancer cells by intrinsic or extrinsic insults has been shown to counteract immune suppression, besides reducing the adaption to stressful conditions and thus reducing cancer cell survival (8, 9).

The activation of transcription factors such as NF- κ B (10) and STAT3 (11) contributes to the protumorigenic effects of UPR signaling promoting the production of proinflammatory and immune suppressive cytokines, chemokines, and prostaglandins (PG; ref. 12). The release of these molecules in the tumor microenvironment may skew macrophage polarization toward M2/tumor-associated macrophages (13, 14) or promote the formation of myeloid-derived suppressor cells (MDSC; refs. 15, 16). These cells not only induce immune suppression but also support tumor growth, that is, by increasing angiogenesis and drug resistance (17, 18). Moreover, several tumor-released factors induce the transdifferentiation of fibroblasts into myofibroblasts, which further contributes to tumor survival and progression (19). Interestingly, one of the strategies through which cancer cells subvert immune response is by activating ER stress in myeloid cells such as macrophages and DCs, through the release of not completely identified soluble factors (20, 21). These findings, sometimes contradictory, suggest that UPR must be finely tuned both in cancer and in immune cells, in order to stimulate an anticancer immune response, particularly when cancer cells undergo anticancer therapies that influence ER stress and UPR.

Posttranslational modifications such as methylation and acetylation are very common in cancers, and their targeting represents a possible strategy to selectively kill cancer cells. Histone deacetylase inhibitors (HDACi), which affect the acetylation of histones and not histone

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substrates, are among those promising anticancer agents (22, 23). They have been shown to be effective also against aggressive cancers such as glioblastoma (24) or pancreatic cancer (25). In addition, HDACis, particularly those inhibiting class I HDAC, have been reported to induce ER stress in cancer cells by several means, that is, by inducing acetylation of GRP78/binding immunoglobulin protein (BIP) or by increasing intracellular reactive oxygen species (ROS; refs. 26, 27). In a previous study, we have found that valproic acid (VPA) and trichostatin A (TSA) efficiently reduced cell survival of pancreatic cells, although VPA showed a broader cytotoxic effect against the cell lines tested (25). In this study, we investigated whether pancreatic cancer cells undergoing these HDACi treatments could stimulate DCs and correlated their immune modulating effects with the activation of UPR in cancer cells as well as in DCs exposed to cancer-released soluble factors.

Materials and Methods

Cell culture and reagents

The human pancreatic cancer cell line Panc1, obtained from the American Type Culture Collection, and PaCa44 from Dr. von Bulow (University of Mainz, Germany) were cultured as monolayers in RPMI 1640 (Sigma Aldrich, R0883), 10% FBS (Sigma-Aldrich, F7524), L-glutamine (Aurogene, AU-X0550), and streptomycin (100 µg/mL) and penicillin (100 U/mL; Aurogene, AU-L0022) in 5% CO₂ saturated humidity at 37°C and regularly tested for *Mycoplasma*. Cells were always detached using Trypsin-EDTA solution (Euroclone). VPA (P4543), TSA (T8552), GSK 2606414 (GSK; 516535) and Celecoxib (cele; PZ0008) were all purchased from Sigma-Aldrich. PGE2 (PGE2; sc-201225) was purchased from Santa Cruz Biotechnology.

PaCa44 and Panc1 cells were plated in 6-well plates at a density of 2×10^5 cells/well in 2 mL. The following day, when the cells were in the exponential growth phase, cells were treated with TSA (0.5 µg/mL; ref. 28) and VPA (10 mmol/L) and left untreated as control CT. In some experiments before adding VPA, cells were pretreated for 1 hour with GSK (0.5 µmol/L; ref. 29) or cele (5 µmol/L). After 24 hours, supernatant were collected and stored at -80°C. They were subsequently used to detect ATP and PGE2 and added to DC cell cultures. In some experiments, PGE2 (10^{-9} mol/L) was added to the cultures.

Immature DC generation

Human peripheral blood mononuclear cells (PBMC) from buffy coats of healthy donors were isolated by lymphocyte cell separation medium (Cedarlane, CL5020), according to previous studies (30).

Monocytes were isolated from PBMCs by immunomagnetic cell separation using anti-CD14-conjugated microbeads according to the manufacturer's instructions (Miltenyi Biotec, 1300-50-201), as previously reported (31).

Immunofluorescence staining and FACS analysis

For immunofluorescence, immature DCs were incubated with supernatants from PaCa44 and Panc1 cells cultured with TSA and VPA or left untreated (CT) or with adherent Panc1 cells treated with TSA and VPA or left untreated (CT). After 24 hours, cells were washed and incubated for 30 minutes at 4°C with CD86 antibody (Miltenyi Biotec, 130-094-878) and CD80 antibody (Miltenyi Biotec, 130-117-683). Cells were analyzed with FACSCalibur, using CELLQuest software (BD Biosciences). DCs were gated according to their forward scatter and side scatter properties. At least 10×10^3 events were acquired for each sample.

ATP detection

Extracellular ATP levels were measured in the PaCa44 and Panc1 supernatants, recovered as mentioned previously, by the ATPlite Luminescence Assay System (PerkinElmer) according to the manufacturer's instructions, and analyzed by VICTOR Multilabel Plate Reader (PerkinElmer).

Mixed lymphocyte reaction

PBMCs, isolated as mentioned previously, were labeled with 10 µmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Thermo Fisher Scientific) for 15 minutes at 37°C and then extensively washed before seeding. DCs pretreated with 25% (vol) supernatants of PaCa44 and Panc1 cells treated with TSA, VPA, or untreated (CT) were irradiated and, after washing, were seeded in 96 plates with CFSE-labeled PBMCs in 1/10 and 1/50 ratio (PBMCs/DCs). After 5 days, cells were washed in PBS and stained with Fixable Viability Dye eFluor780 (eBioscience–Thermo Fisher Scientific) following the manufacturer's instruction. Samples were acquired with a FACSCalibur and LSR Fortessa (BD Becton Dickinson) and analyzed with CELLQuest and FlowJo software (version 10.5.3), respectively.

Chemiluminescent immunometric assay

DCs were cultured for 24 hours with 25% vol/vol of supernatant of TSA or VPA-treated PaCa44 or Panc1 cells or left untreated (CT). Cells were then centrifuged, and the supernatants were collected to measure IL10 and IL8 by Magnetic Luminex assay performed by R&D systems, according to the manufacturer's instructions, as previously reported (32).

Western blot analysis

Following treatments, pancreatic cancer cells (1×10^6), detached with trypsin and EDTA, and DCs were washed in 1X PBS, lysed in RIPA buffer [150 mmol/L NaCl, 1% NP-40, 50 mmol/L Tris-HCl (pH 8), 0.5% deoxycholic acid, 0.1% SDS, protease, and phosphatase inhibitors], and centrifuged at 14,000 rpm for 20 minutes. The protein concentration was measured by using the Bio-Rad Protein Assay (BIO-RAD laboratories GmbH), and then, equal amounts of protein lysates were subjected to electrophoresis on 4% to 12% NuPage Bis-Tris gels (Life Technologies, N00322BOX) according to the manufacturer's instructions. Then, the gels were transferred to nitrocellulose membranes (BioRad, Hercules, 162-0115) for 2 hours in Tris-Glycine buffer. The membranes were blocked in 1X PBS–0.1% Tween20 solution containing 3% of BSA, probed with specific antibodies, and developed using ECL Blotting Substrate (Advansta, K-12045-D20). Rabbit polyclonal antibodies CCAAT/enhancer-binding protein homologous protein (CHOP; 1:1,000; 15204-1-AP; ProteinTech), BIP (1:5,000; 11587-1-AP; ProteinTech), and spliced X-box binding protein 1 (XBP1s; 1:1,000; NBP1-77681SS; Novus) were used. Mouse monoclonal anti-β-actin (1:10,000; Sigma-Aldrich, A2228) was used as marker of equal loading. Goat polyclonal anti-mouse IgG-horseradish peroxidase (HRP; Santa Cruz Biotechnology Inc., sc-2005) and anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc., sc-2004) were used as secondary antibodies. All the primary and secondary antibodies used in this study were diluted in a PBS–0.1% Tween 20 solution containing 3% BSA.

Metabolites extraction and high-performance liquid chromatography–mass spectrometry

Metabolite extraction and separation were performed according to Gevi and colleagues (33).

Mass spectrometry analysis

Mass spectrometry (MS) analysis was carried out on an electrospray hybrid quadrupole time-of-flight instrument MicroTOF-Q (Bruker-Daltonik) equipped with an ESI ion source, as previously described (34) with some modifications; electrospray capillary voltage was set at 4,500 V (–) ion mode, nebulizer set at 27 psi, and the nitrogen drying gasket to a flow rate of 6 L/min. Dry gas temperature was set at 200°C.

Data elaboration

Data files and metabolite assignments were processed by MAVEN.52 (available at <http://genomics-pubs.princeton.edu/mzroll/index.php?show=download>) and were performed upon conversion of raw files into mzXML format through MassMatrix. The linear concentration range of the calibration curve was 0.1 to 0.00001 µg/µL. The majority of the correlation coefficients (*R*) were higher or equal to 0.99. The limit of detection varied from 100 fmol to 1 pmol. The linearity of the proposed LC/MS method was evaluated with regard to the amount of metabolite injected and to metabolite concentrations.

RNA extraction and semiquantitative RT-PCR

Cells were harvested in TRIzol reagent (Invitrogen), and total RNA was isolated following the manufacturer's instructions. The first strand cDNA was synthesized from 2 µg of total RNA with MuLV reverse transcriptase kit (Applied Biosystems) and used for semiquantitative RT-PCR with Hot-Master Taq polymerase (Eppendorf s.r.l.) and gene-specific oligonucleotides. PCR products were resolved on 2% agarose gel and visualized with ethidium bromide staining using UV light. The 28S mRNA sequence was used as control for efficiency of RNA extraction and transcription. Densitometric analysis was applied to quantify mRNA levels compared with control gene expression. Primers for COX-2-for: TTCAAATGAGATTGTGGAATAAT; COX-2-rev: AGATCATCTCTGCCTGAGTATCTT.

PGE2 assay

Supernatants from Pac44 and Panc1 cells treated as above reported were used for the determination of PGE2 concentration by the Prostaglandin E2 Assay (R&D Systems) according to the manufacturer's instructions and analyzed by VICTOR Multilabel Plate Reader at an absorbance of 450 nm with a wavelength correction at 540 nm to correct for the optical imperfections in the plate.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

PaCa44 and Panc1 cells plated in a 96-well plate at 1×10^4 cells/well were treated with VPA and TSA or left untreated. The following day, 20 µL of 5 mg/mL MTT (Sigma Diagnostic) in $1 \times$ PBS was added to wells, according to the manufacturer's instruction. The optical density was determined with a microculture plate reader (Bio-Rad Microplate Reader) at 590 nm. Absorbance values were normalized to the values for the untreated cells to determine the percentage of survival. The experiments were performed in triplicate and repeated at least three times.

Measurement of intracellular reactive oxygen species production

To measure ROS production in DCs treated or untreated with PaCa44 supernatants, the 2',7'-dichlorofluorescein diacetate (DCFDA; Molecular Probes) was used, as previously described (35). DCs (1×10^6) were cultured in the presence of supernatant from VPA- or TSA-treated and untreated PaCa44. After 24 hours of cultures, DCs were washed with prewarmed PBS, incubated at 37°C with 10 µmol/L

DCFDA for 15 minutes in PBS, and then analyzed in FL-1 by a FACSCalibur flow cytometer (BD). For each analysis, 10,000 events were recorded.

Measurement of 4-hydroxy-trans-2-nonenal protein adducts

4-Hydroxy-trans-2-nonenal (4-HNE) protein adducts were measured in DCs to determine whether toxic lipid peroxidation aldehydes would be produced in response to the treatments. DCs (1×10^6) were cultured in the presence of supernatant obtained from VPA-treated, TSA-treated, or untreated PaCa44 cells. 4-HNE protein adduct levels were measured by an OxiSelect HNE-His Adduct ELISA kit, according to the manufacturer's instructions (Cell Biolabs, Inc.). On a 96-well plate, protein samples and standards (10 µg/mL) were absorbed for 2 hours at room temperature. Protein was probed with anti-HNE Histidine (His) antibody, followed by HRP secondary antibody. Protein levels were compared with a standard curve produced 4-hydroxynonenal modified bovine serum albumin standards.

Knockdown by siRNA

XBPI knockdown was performed by specific siRNA transfection (Santa Cruz Biotechnology, sc-38627) into DCs using Lipofectamine 2000 (Invitrogen, 11,668,027) according to the manufacturer's instructions. Briefly, at the day of transfection, monocytes were seeded into 6-well plates at a density of 3×10^6 cells/well in 1.25 mL of RPMI medium without antibiotics. Next, 30 pmol of siRNA combined with 10 µL of Lipofectamine 2000 were added to the cells. Transfection reactions were performed in serum-free OptiMEM medium (Life Technologies, 31985062). Control siRNA-A (Santa Cruz Biotechnology, sc-37007) was used as a scrambled control. After 72 hours, cells and supernatant were recovered for further analysis.

Injection of conditioned medium from pancreatic cancer cells treated with VPA in C5BL/6 mice

Cell conditioned medium was prepared by cell culture supernatant from pancreatic cancer cells treated with TSA, VPA, or untreated (CT). Conditioned media were concentrated 10-fold by using Vivaspinn concentrator (10,000 MW; Sartorius).

One hundred microliters of 10-fold concentrated conditioned medium from VPA- or TSA-treated and untreated pancreatic cancer cells were injected intraperitoneally in 10- to 12-week-old C57BL/6 mice. Three mice for each group were used. Eight hours after conditioned medium injection, mice were killed, and livers were surgically removed, passed through a 40-µm cell strainer, washed once in PBS, and processed for Western blot analysis as above reported.

Densitometric analysis

Densitometric analysis was performed by using the Image J software, which was downloaded from the NIH web site (<http://imagej.nih.gov>).

Statistical analysis

Data are represented by the mean $\pm$ SD of at least three independent experiments.

Ethics approval and consent to participate

This research involving human subjects has been performed in accordance with the Declaration of Helsinki and has been approved by the ethic committee of Policlinico Umberto I, Rome, Italy (847/19).

All experiments on mice were conducted in accordance with the ethical standards and according to the Declaration of Helsinki. A veterinary surgeon was present during the experiments. The animal care both before and after the experiments was performed only by

trained personnel. Mice were bred under pathogen-free conditions in the animal facilities of the University of Rome "Tor Vergata" and were handled in compliance with European Union and institutional standards for animal research. The work was conducted with the formal approval of the local ["Organismo Preposto al Benessere degli animali" (O.P.B.A.), University of Rome Tor Vergata] animal care committee.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Results

VPA-treated pancreatic cancer cells culture supernatant induces a higher CD86 and CD80 expression on DCs than TSA-treated pancreatic cancer cells culture supernatant

We investigated whether the supernatant of pancreatic cancer cells PaCa44 and Panc1 treated with VPA or TSA HDACs could activate DCs. We found that DCs exposed for 24 hours to VPA-treated cells culture supernatant showed a higher expression of CD86 and CD80 on their surface than those treated with TSA-treated cell culture supernatant (Fig. 1A and B, E–H). These results suggest that VPA-treated cells culture supernatant was able to trigger a stronger DC activation. Similar results were obtained when pancreatic cells treated with VPA or TSA were cocultured with DCs for 24 hours (Fig. 1C–H). Next, we evaluated whether the stronger DC activation induced by VPA cells culture treatment could correlate with a higher release of ATP by cancer cells, a DAMP involved in DC activation (1). As shown in Fig. 1I, VPA-treated PaCa44 and Panc1 cells produced a higher amount of ATP than those treated with TSA. Interestingly, the release of ATP can be promoted by autophagy (3). In our previous study, we showed that VPA stimulated autophagy more efficiently than TSA in these pancreatic cancer cells (25). These findings suggest that VPA-treated pancreatic cancer cells supernatant is able to induce a stronger DC activation compared with TSA, in correlation with a higher release of ATP.

VPA-treated pancreatic cancer cells culture supernatant reduces DCs allostimulatory capacity and increases the release of IL10 and IL8 cytokines

We next evaluated whether the higher CD86 and CD80 expression on the surface of DCs exposed to VPA-treated pancreatic cancer cells culture supernatant could be followed by an increased capacity to trigger T-cell proliferation in mixed lymphocyte reaction (MLR). Unexpectedly, these DCs displayed a reduced allostimulatory capacity compared with those treated with TSA-treated cell culture supernatant (Fig. 2A). To exclude the carry-over effect of VPA, we exposed DCs to the same concentration of this HDACi contained in the supernatant of pancreatic cancer cells and found that it did not influence the allostimulatory capacity in comparison with the control DCs (Fig. 2A). Furthermore, a higher amount of IL10 and IL8 (Fig. 2B) was released by DCs exposed to VPA-treated cell supernatants. These cytokines are known to promote immunosuppression, angiogenesis, and to recruit leukocytes in the tumor bed to fuel inflammation (1, 36, 37).

These results suggest that VPA-treated pancreatic cancer cells culture supernatant, despite inducing high expression of CD86 and CD80 activation markers on DC surface, impaired their immune function.

VPA alters choline metabolism, induces ER stress, upregulates CHOP, and activates cyclooxygenase-2 (COX-2) in pancreatic cancer cells, promoting the release of PGE2.

Searching for the molecular mechanisms leading to the immune suppressive effect induced by VPA-treated cells culture supernatant, we found that it increased the expression of CHOP in both Panc1 and PaCa44 cells (Fig. 3A) and that, differently from it, BIP was upregulated by both VPA and TSA in both cell lines. The different impact on the expression of BIP and CHOP ER stress molecules could correlate with a stronger and proapoptotic ER stress induction by VPA (38).

Next, to investigate the mechanism through which VPA induced a stronger ER stress in pancreatic cancer cells, a lipid metabolomic assay was performed on the supernatant of these cells following VPA or TSA treatment. Interestingly, we found that VPA dysregulated choline metabolism (Fig. 3B), effect previously shown to induce an exaggerated ER stress with CHOP upregulation in other cell types (39, 40). Previous studies have suggested that CHOP upregulation could activate COX-2, leading to the production of PGE2 (41), molecule that negatively influences immune response (12). Therefore, we next evaluated the expression of COX-2 and the release of PGE2 by VPA- and TSA-treated cancer cells. As shown in Fig. 3C and D, both were upregulated by VPA treatment in PaCa44 and Panc1 cell lines (Fig. 3C) and could be involved in the immune suppressive effects induced by VPA supernatant in DCs.

PERK inhibition by GSK2606414 downregulates CHOP and counteracts PGE2 production induced by VPA

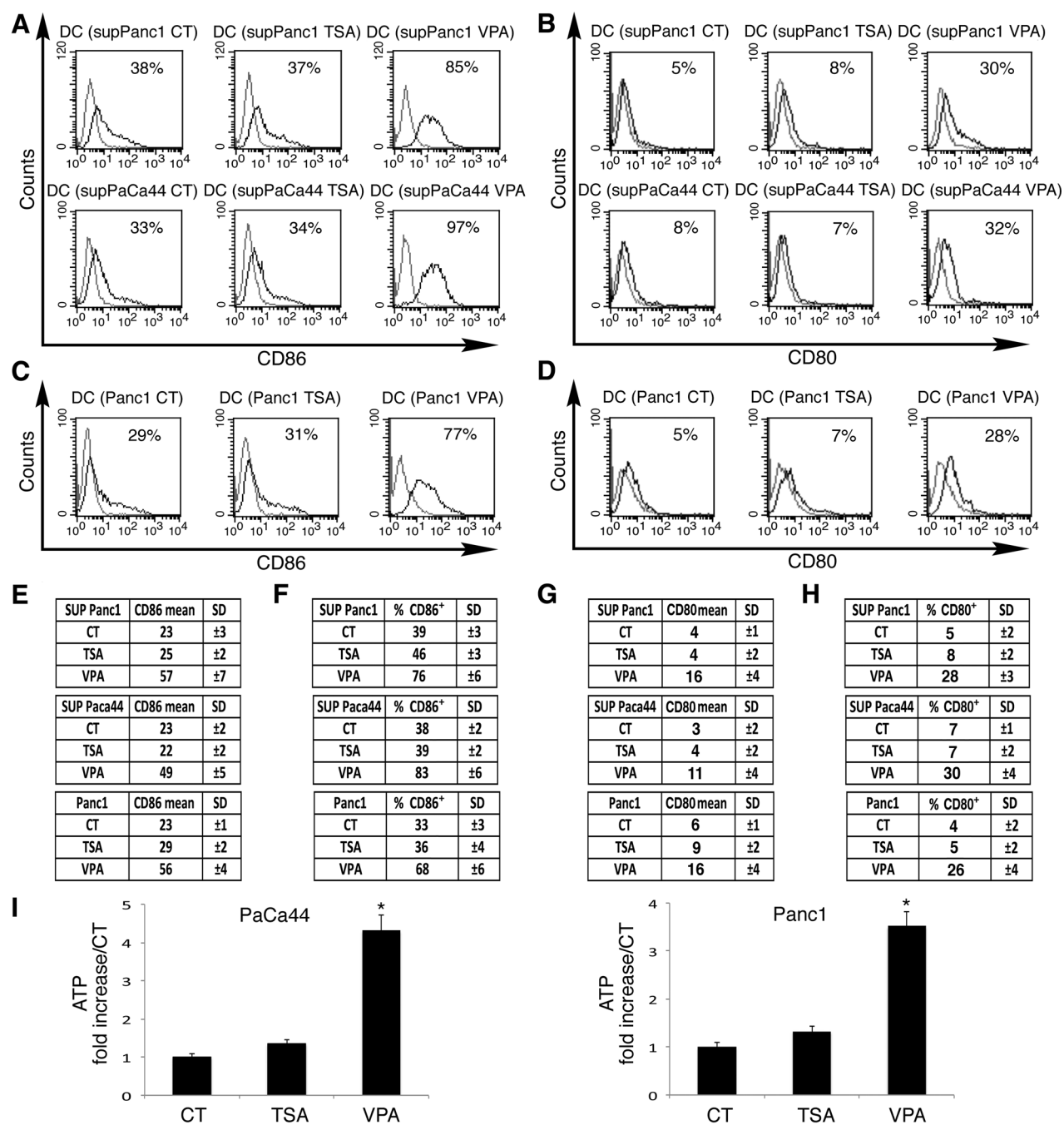
As PERK is the main UPR sensor involved in CHOP upregulation (38, 42), we next investigated whether pretreatment of pancreatic cancer cells with GSK2606414 (GSK) PERK inhibitor before exposure to VPA could reduce its expression and PGE2 secretion. We observed that such pretreatment counteracted both CHOP upregulation (Fig. 4A) and PGE2 release induced by VPA (Fig. 4B). Similar to GSK, celecoxib was also able to reduce the release of PGE2 induced by VPA (Fig. 4C), suggesting that the activation of PERK–CHOP–COX-2 axis was promoting the release of PGE2 by pancreatic cancer cells treated with this HDACi.

Celecoxib prevented DC dysfunction induced by VPA supernatant

PGE2 has been previously reported to act as a negative DAMP (43). Accordingly, here we found that cele reduced the production of IL10 and IL8 cytokines by DCs exposed to VPA-treated cells culture supernatant (Fig. 5A) and restored their capacity to stimulate T-cell proliferation in MLR (Fig. 5B). All together, these data indicate that ER stress and the activation of PERK upregulated CHOP and COX-2 expression in VPA-treated pancreatic cancer cells, promoting the release of PGE2 that induced DC dysfunction. To exclude that cele could interfere with cell death induction by VPA, we pretreated cancer cells with this drug and found that it did not influence VPA cytotoxicity, as evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 5C).

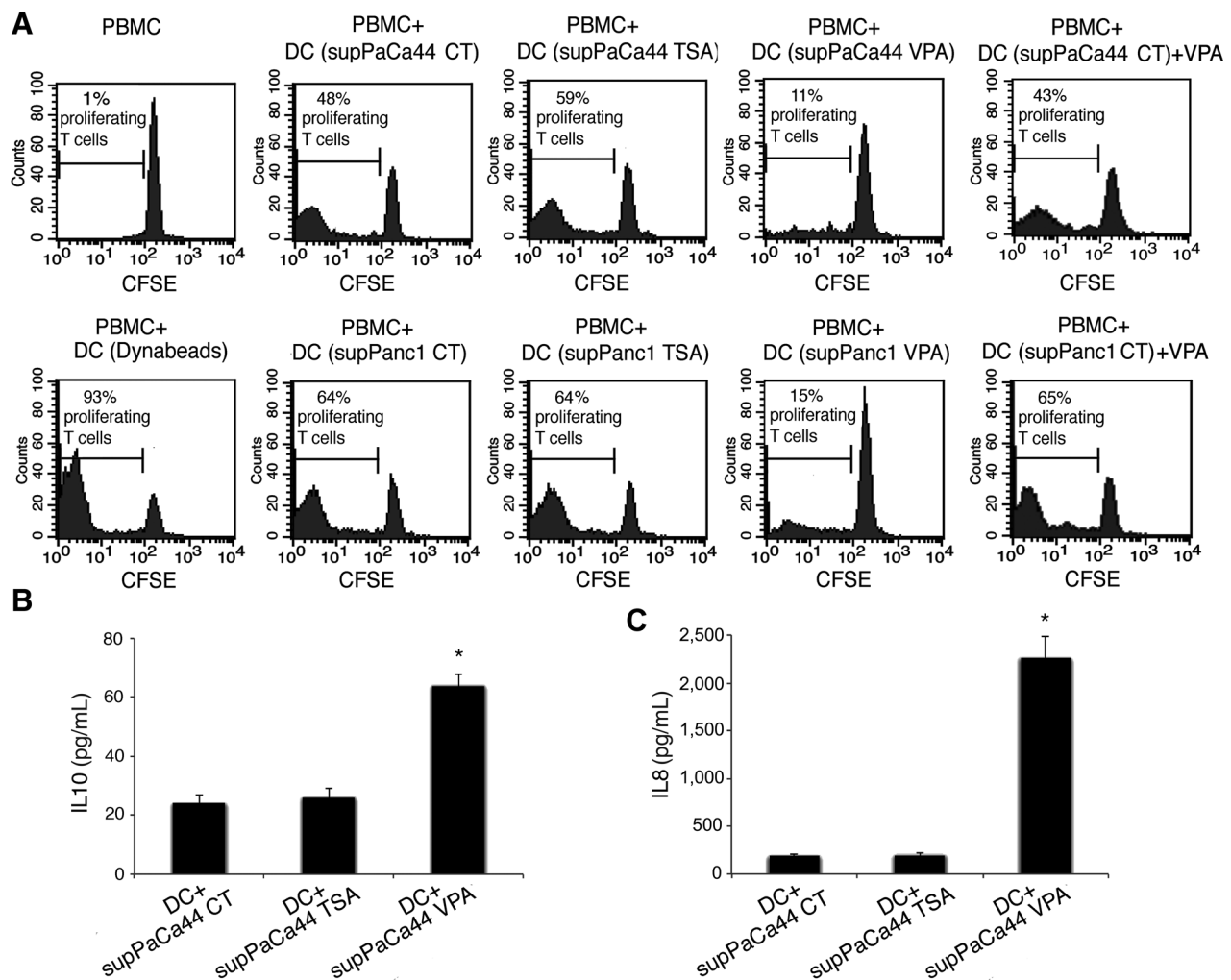
VPA-treated cancer cells supernatant activates XBP1s UPR sensor and increases ROS and 4-HNE protein adducts in DCs, promoting their dysfunction.

It has been reported that not yet completely identified soluble factors can transfer ER stress from cancer cells to myeloid immune cells such as DCs (20) and that such ER stress activation in immune cells results in an impairment of their function (44). Therefore, we next investigated whether VPA-treated cells supernatant could activate ER stress/UPR in DCs. We focused on XBP1s, as this molecule has been

**Figure 1.**

Pancreatic cancer cells treated by VPA upregulate CD86 expression on DCs in correlation with a higher release of extracellular ATP. Supernatants are collected from PaCa44 and Panc1 cells treated for 24 hours with TSA and VPA or left untreated as control CT. CD86 and CD80 expression were analyzed by FACS analysis in DCs cultured with supernatants (**A** and **B**) or Panc1 treated cells (**C** and **D**). A representative experiment showing the percentage of CD86- and CD80-positive cells is reported, in **A-D**. Gray peaks represent the isotype controls, and black peaks represent CD86- and CD80-stained DCs. The means of the values of the mean fluorescence intensity (MFI) and of the percentage of CD86- and CD80-positive DCs plus SD are also reported in **E** to **H**. In **I**, histograms represent the mean plus SD of the amount of ATP level measured in the supernatant of PaCa44 and Panc1 treated with TSA and VPA or left untreated (CT) of three independent experiments. *, $P < 0.05$.

PGE2 Transfers the Stress from Cancer to DCs

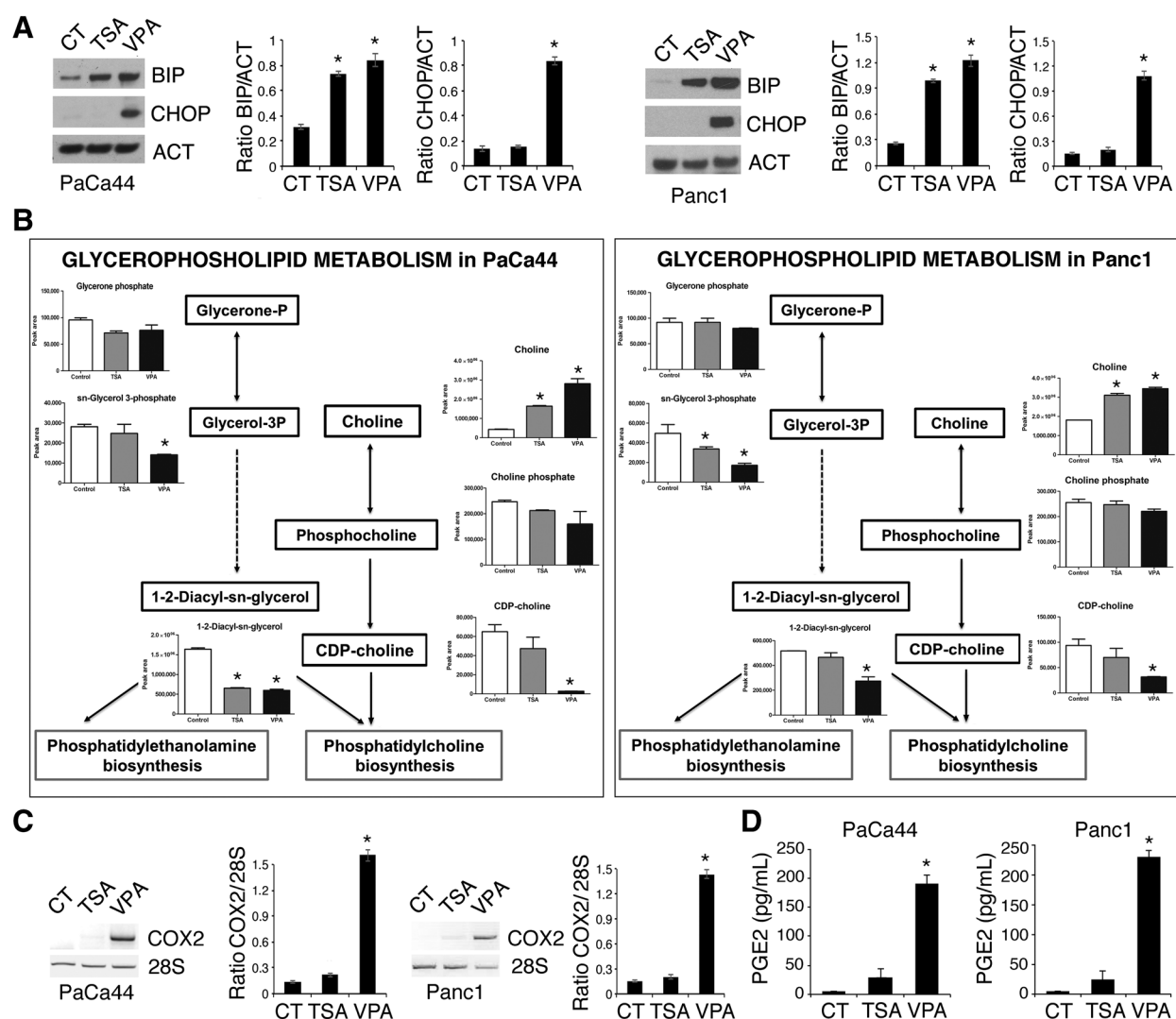
**Figure 2.**

DCs cultured in the presence of VPA-treated pancreatic cancer cell supernatant show a reduced T-cell allostimulatory capacity and release a higher amount of IL10 and IL8. DCs cultured with supernatant of PaCa44 and Panc1 treated with TSA and VPA or left untreated (CT) or with CT supernatant plus VPA were irradiated and used to stimulate allogeneic PBMCs in an MLR assay. **A**, Results showing the percentage of proliferating PBMCs are reported. **B**, Histograms representing the mean plus SD of the IL10 and IL8 released by DCs of three independent experiments are shown in **B** and **C**. *, $P < 0.05$.

reported to impair the function of stressed DC-infiltrating ovarian cancer (45). As shown in **Fig. 6A**, we found that DCs exposed to VPA-treated cells supernatant showed a higher XBP1s expression level than those exposed to TSA-treated cells supernatant. More importantly, XBP1s was less expressed when DCs were exposed to the supernatant of cancer cells pretreated with cele before VPA. These results suggest that PGE2 was involved in transferring the stress from pancreatic cancer cells to DCs. On the other hand, the supplementation of PGE2 to TSA-treated cell culture supernatant increased XBP1s expression level in DCs compared with TSA-treated cells supernatant only (**Fig. 6B**), further confirming the role of PGE2 in transferring ER stress in DCs. To investigate the induction of ER cell stress *in vivo*, 10-fold concentrated supernatant of pancreatic cancer cells treated with VPA was injected intraperitoneally in C57BL/6 mice. As shown in **Fig. 6C**, concentrated VPA-treated cells supernatant was able to induce XBP1s upregulation in cells isolated from liver, an organ very sensitive to ER stress, according to previous studies (20).

In the above reported study (45), XBP1s activation was found to be fueled by intracellular ROS, lipid peroxidation, and accumulation of unsaturated aldehyde 4-HNE protein adducts in DCs derived from ovarian cancer-bearing hosts (45–47). According to these findings, we found that ROS (**Fig. 7A**) and 4-HNE protein adducts (**Fig. 7B**) accumulated in DCs exposed to VPA-treated cells supernatant and that, as for XBP1s expression, these effects were reduced in DCs exposed to the supernatant of cancer cells pretreated with cele before VPA (**Fig. 7A** and **C**), whereas they were increased by adding PGE2 to TSA supernatant (**Fig. 7A** and **C**).

The immune suppressive role of XBP1s accumulation in DCs exposed to VPA supernatant was then evaluated by silencing this molecule by specific siRNA. We found that XBP1 knockdown (**Fig. 7C**) rescued DC allostimulatory capacity (**Fig. 7D**) and reduced IL10 and IL8 cytokine release by DCs exposed to VPA-treated cells supernatant (**Fig. 7E**), confirming the role of XBP1s in DC immune dysfunction. All together, these results suggest that PGE2 released by

**Figure 3.**

VPA activates ER stress upregulating CHOP, alters choline metabolism, and induces the transcription of COX-2 leading to the production of PGE2 in pancreatic cancer cells. **A**, The expression of BIP and CHOP of PaCa44 and Panc1 cells treated for 24 hours with TSA and VPA or left untreated as control CT is evaluated in a Western blot analysis. Actin was used as loading control. A representative experiment out of three is shown. Histograms represent the mean plus SD of the densitometric analysis of the ratio of BIP/actin and CHOP/actin. *, $P < 0.05$. **B**, The analysis of glycerophospholipid metabolism in supernatants of PaCa44- and Panc1-treated cells is reported. *, $P < 0.05$. **C**, mRNA expression of COX-2 expression of PaCa44- and Panc1-treated cells is evaluated by semiquantitative RT-PCR. A representative experiment out of three is shown. 28S was used as control. Histograms represent the mean plus SD of the densitometric analysis of the ratio of COX-2/28S. *, $P < 0.05$. **D**, Histograms represent the mean plus SD of the amount of PGE2 released in the supernatants in three independent experiments. *, $P < 0.05$.

pancreatic cancer cells stressed by VPA treatment transferred the stress to DCs, increasing ROS and 4-HNE protein adducts, activating XBP1s, and impairing their immune function.

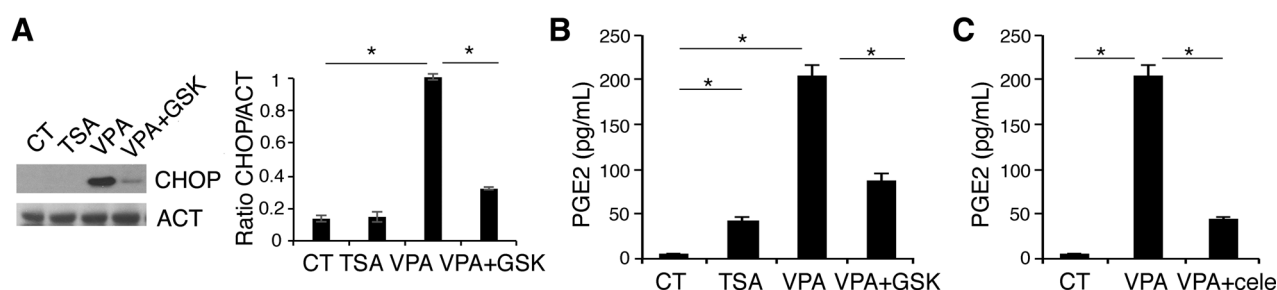
Discussion

This study unveils that PGE2 released by pancreatic cancer cells undergoing ER stress following VPA treatment was able to transmit the stress to DCs, increasing ROS and 4-HNE protein adducts and activating XBP1 to induce their dysfunction.

Although ER stress may be exacerbated by several chemotherapies, cancer cells are constitutively stressed due to insults such as the presence of oncogenes, nutrient shortage, and hypoxia. Therefore,

due to their condition of basal stress, they may constitutively release immune suppressive molecules such as PGE2. ER stress and UPR activation in cancer cells have been shown to foster an inflammatory microenvironment that not only leads to an impairment of immune response but subverts immune cell function in a way that they support instead of fighting cancer (8). However, UPR is, first of all, an adaptive response that helps cancer cells to survive in the face of stress. It may, for example, autophagy or induce an upregulation of chaperone expression, improving protein folding. Therefore, targeting UPR represents a promising strategy to selectively kill cancer cells, as these cells are more stressed than normal cells (9), and to concomitantly counteract the establishment of a status of chronic inflammation (48). The latter process is promoted by the activation of transcription factors

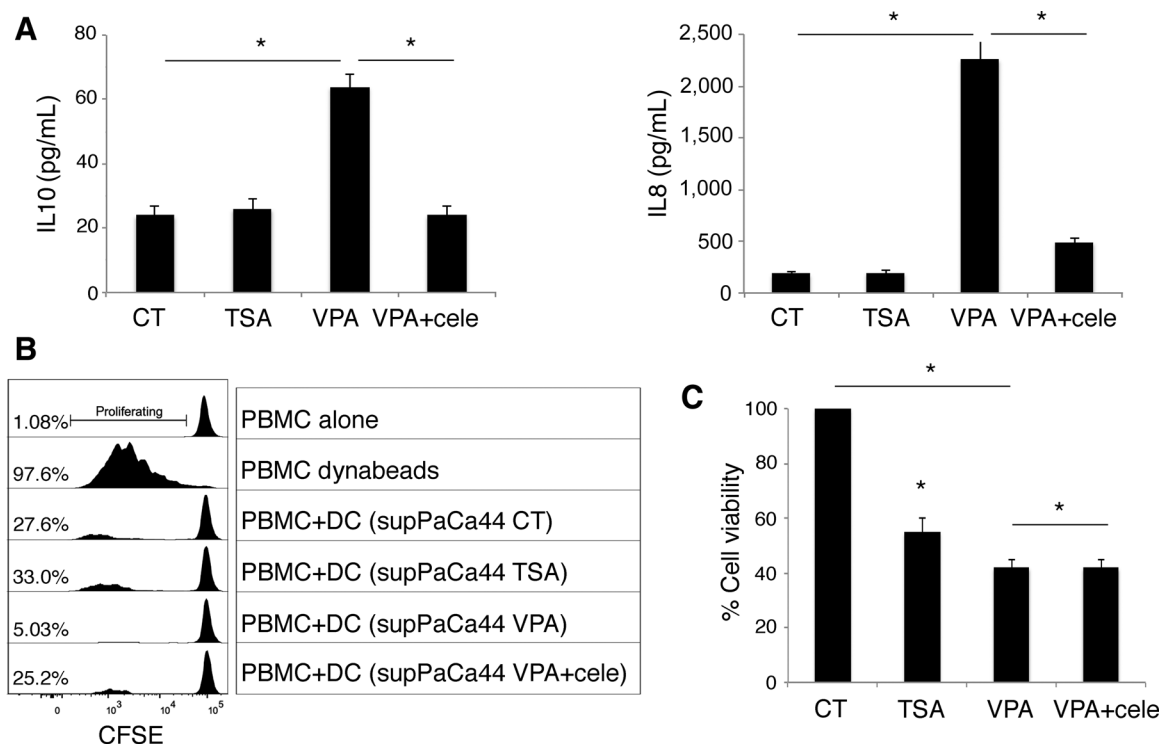
PGE2 Transfers the Stress from Cancer to DCs

**Figure 4.**

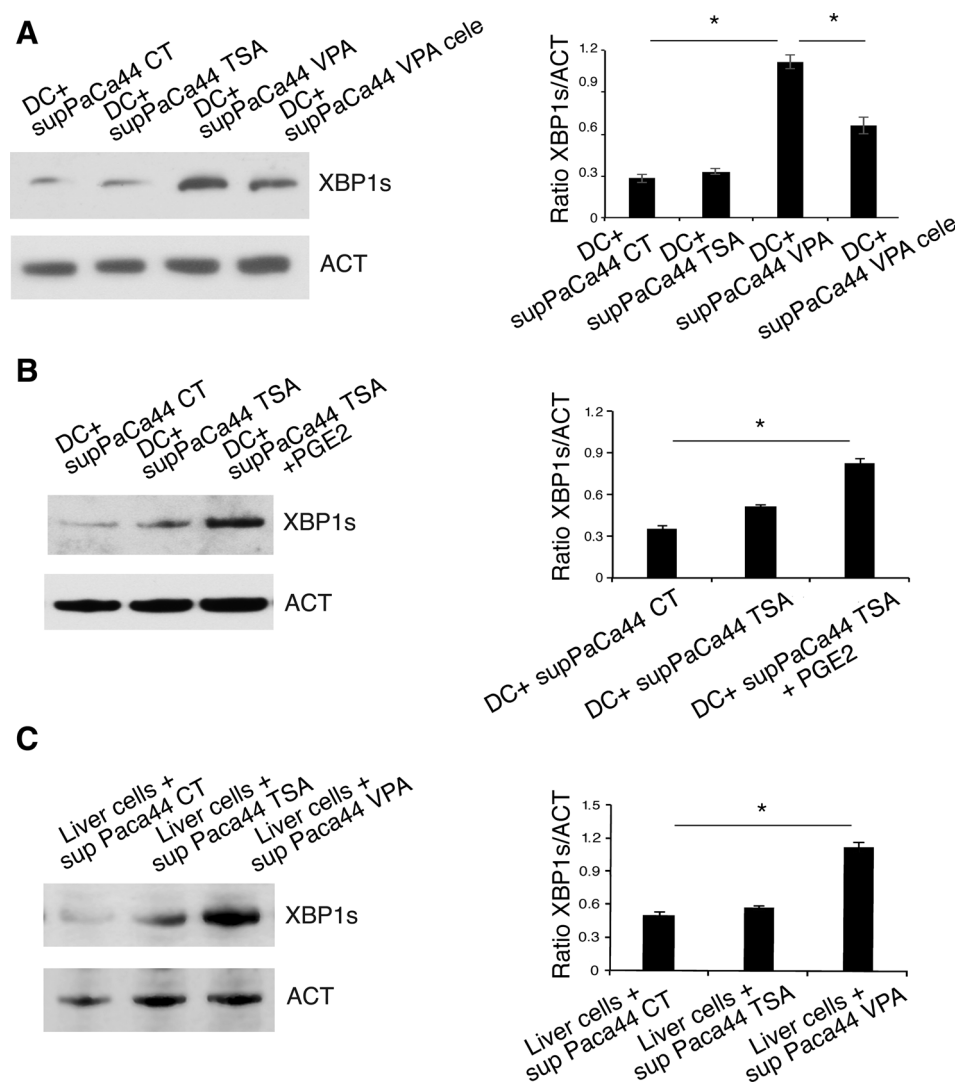
PERK-CHOP axis is involved in PGE2 production. **A**, The expression of CHOP in Paca44 pretreated or not with the GSK2606414 (GSK) PERK inhibitor before VPA treatment was analyzed by Western blot. Actin was used as loading control. A representative experiment out of three is shown. Histograms represent the mean plus SD of the densitometric analysis of the ratio of CHOP/actin. *, $P < 0.05$. **B** and **C**, The amount of PGE2 released in the supernatants of Paca44 pretreated or not with GSK (**B**) or cele (**C**) is shown. Histograms represent the mean plus SD of the amount of PGE2 released in the supernatants of three independent experiments. *, $P < 0.05$.

such as NF- κ B, STAT3, and MAPKs to which contributes the UPR signaling (49, 50). These pathways are involved in the release of proinflammatory cytokines such as IL6 or chemokines such as IL8 that recruit immune cells into the tumor bed, subverting their function and further sustaining inflammation (51). Interestingly, NF- κ B can activate COX-2 to induce the release of PGE2, and the latter may, in turn, reactivate NF- κ B in a positive feed-back loop that amplifies inflammation and immune suppression (52, 53).

Although the protumorigenic functions of UPR are well documented, several anticancer treatments have been reported to induce an immunogenic cell death by inducing ER stress and activating UPR. However, only the phosphorylation of eIF2 α seems to be required for an immunogenic cell death, as it promotes the translocation of calreticulin, an “eat me signal,” on the surface of cancer cells committed to undergo apoptosis (54, 55). The cell context, the duration, and the intensity of stress are likely to play a role in shifting the balance

**Figure 5.**

Celecoxib prevented the immune suppressive effects induced in DCs by PaCa44 VPA treatment. **A**, Production of IL10 and IL8 by DCs exposed to the supernatant from PaCa44 pretreated or not with cele before VPA exposure. Histograms representing the mean plus SD of the IL10 and IL8 released by DCs of three independent experiments are shown. *, $P < 0.05$. **B**, Allostimulatory function of DCs exposed to the supernatant from PaCa44 pretreated or not with cele before VPA exposure. FACS profiles show the percentages of proliferating PBMCs. **C**, Cell survival of PaCa44 cells treated with VPA in the presence or in the absence of cele as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay is shown. Histograms represent the mean plus SD of three independent experiments. *, $P < 0.05$.

**Figure 6.**

VPA-treated cancer cells induce XBP1s in DCs and in mice liver cells. **A**, Western blot analysis showing XBP1s expression level in DCs exposed to CT, TSA, and VPA supernatant obtained in the presence or in the absence of cele and **(B)** in DCs exposed to TSA supernatant plus PGE2. **C**, Western blot analysis showing XBP1s expression level in liver cells from intraperitoneally injected mice with concentrated CT, TSA, and VPA supernatant from Paca44 cells. Actin was used as loading control. A representative experiment out of three is shown. Histograms represent the mean plus SD of the densitometric analysis of the ratio of XBP1s/actin. *, $P < 0.05$.

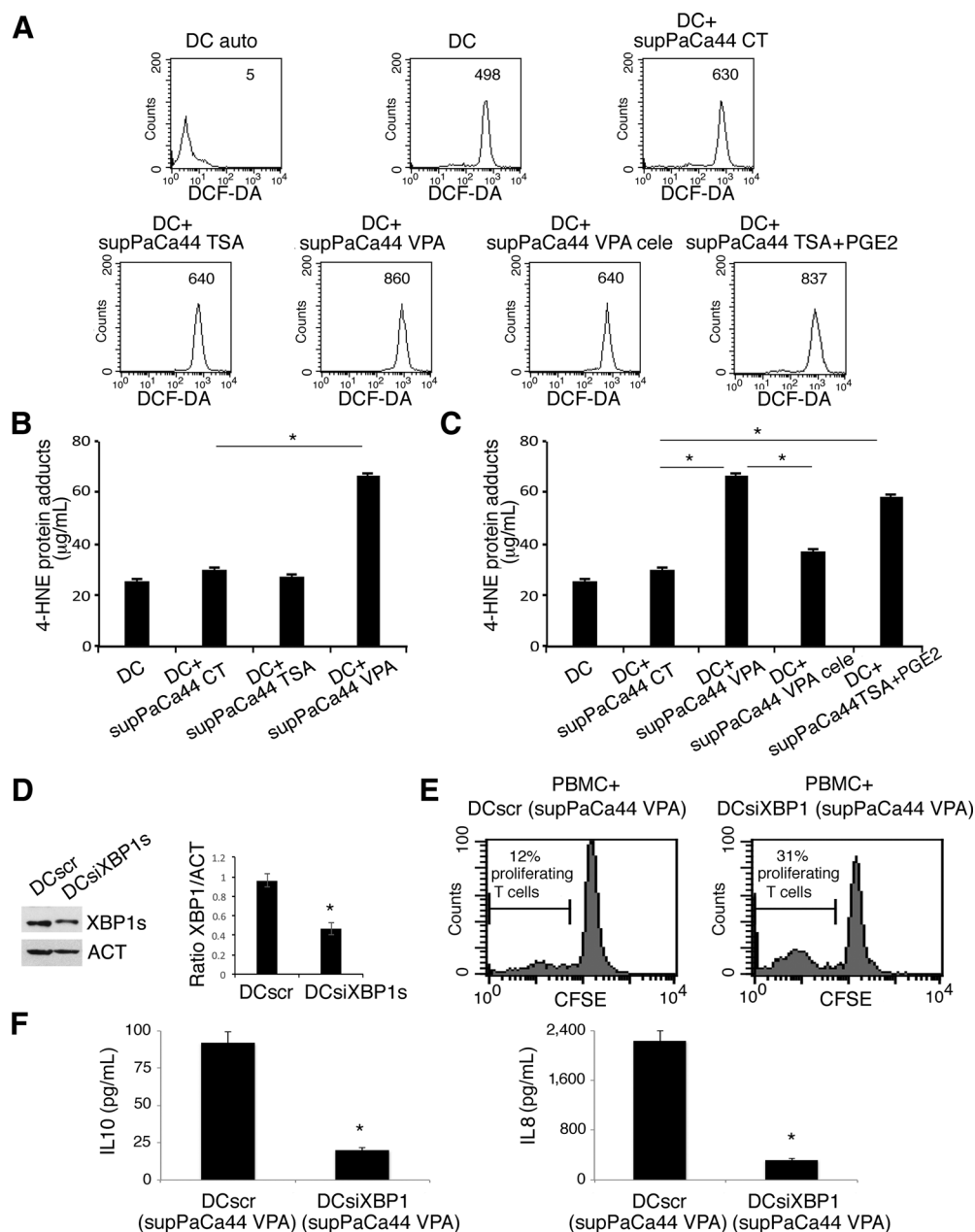
of UPR toward an immune activating or an immune suppressive process.

It is emerging that, besides cancer cells, ER stress and UPR may be activated in immune cells such as macrophages and DCs, negatively influencing their immune response, particularly against cancer (44, 46, 56). For example, ER stress/UPR has been shown to sculpt tolerogenic myeloid immune cells in cancer (57), enhance the accumulation of MDSCs (58), or even reduce the lifespan of these cells isolated from patients with cancer (59). Moreover, it has been reported that the activation of XBP1s UPR sensor may strongly impair the immune function of DC-infiltrating ovarian cancer (45) or that the IRE1 α -XBP1 axis may upregulate the immune checkpoint inhibitor PD-L1 in macrophages (60, 61).

Intriguingly, ER stress in immune cells may be triggered by soluble factors released by stressed cancer cells (20, 21), although the nature of the molecules able to mediate this process has not been completely identified (20). The findings of the present study highlight for the first time that PGE2 is one of the molecules able to transmit the stress from cancer cells stressed by VPA to DCs (Fig. 8). PGE2 has been reported to be an immune suppressive molecule (12) and acts as an inhibitory DAMP when released by dying cancer cells (43). However, its capacity

to propagate the stress from cancer to immune cells has not been previously demonstrated. Identifying tumor-released factors that induce immune suppression or the pathways that regulate their release may help to discover new targets able to improve the outcome of anticancer treatments. In this study, we found that the inhibition of PGE2 release by cele could prevent the accumulation of ROS, 4-HNE protein adduct, and XBP1s accumulation in DCs exposed to VPA-treated cancer cells supernatant, restoring their immune function. The production of PGE2 as a consequence of CHOP upregulation has been previously shown to occur in other cancer cell types as a prosurvival mechanism (41). Although with a different role, here we found that the increased release of PGE2 was due to the activation of PERK-CHOP-COX-2 by VPA that induced a stronger ER stress than TSA, effect that correlated with a dysregulation of choline metabolism in pancreatic cancer cells. The finding that dysfunctional DCs exposed to VPA-treated cells supernatant displayed higher expression level of XBP1s as well as intracellular ROS and 4-HNE protein adducts is also in agreement with a previous study reporting that XBP1s activation can be fueled by ROS, lipid peroxidation, and 4-HNE protein adducts in dysfunctional DC-infiltrating ovarian cancers (45, 46). In such study, these effects could not be attributed to either immune suppressive/

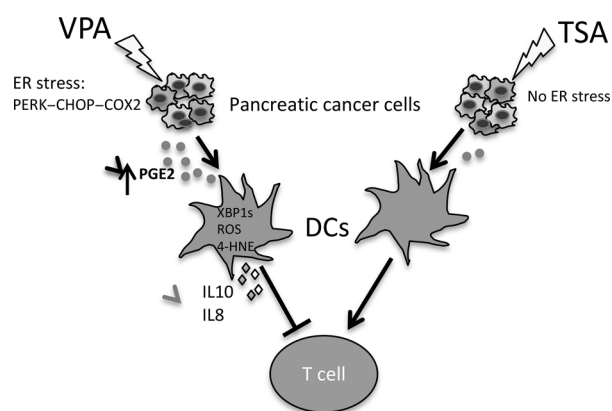
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**Figure 7.**

VPA-treated cancer cells induce the accumulation of ROS and 4-HNE protein adducts in DCs impairing their function. **A**, FACS profiles of intracellular ROS measured in DC following exposure to CT, TSA, and VPA supernatant obtained in the absence or in the presence of cele or TSA supernatant plus PGE2 using DCF-DA as staining. **B** and **C**, 4-HNE protein adducts in DC following exposure to VPA supernatant obtained in the absence (**B**) or in the presence of cele or TSA supernatant plus PGE2 (**C**). Histograms represent means plus SD of three independent experiments. *, $P < 0.05$. **D**, Western blot analysis showing the expression of XBPs in scramble (scr)-treated and XBP1-silenced (siXBP1) DCs exposed to VPA cancer cell supernatant. **E**, FACS profiles of PBMCs proliferation stimulated by scr-treated and XBP1-silenced DCs exposed to VPA cancer cell supernatants. **F**, IL10 and IL8 release by scr-treated or XBP1-silenced DCs exposed to VPA cancer cell supernatants. Histograms represent the mean plus SD of three independent experiments. *, $P < 0.05$.

inflammatory cytokines of the tumor microenvironment or hypoxia-mimicking conditions. In addition, the ability of VPA-treated cells supernatant to induce ER cells stress was corroborated by *in vivo* experiments. In conclusion, this study unveils for the first time that the activation of PERK-CHOP-COX-2 in stressed pancreatic cancer cells promotes the production of PGE2 that propagates the stress from cancer to DCs. This suggests that combining

PERK inhibitors or COX-2 inhibitors with anticancer treatments that exacerbate ER stress in cancer cells such as VPA may counteract cancer-induced immune suppression. Immune dysfunction can also be reverted by the inhibition of XBPs in DCs exposed to tumor-released factors, and this is particularly important in fighting aggressive cancers such as pancreatic cancers that display poor response to anticancer therapies.

**Figure 8.**

The molecular pathways activated by VPA and TSA in pancreatic cancer cells, the release of factors able to transfer the stress to DCs, and the consequences on DC immune function.

Authors' Disclosures

No disclosures were reported.

Authors' Contributions

M.S. Gilardini Montani: Supervision, validation, investigation. R. Benedetti: Investigation, methodology. S. Piconese: Supervision, investigation. F.M. Pulcinelli: Validation, investigation. A.M. Timperio: Investigation. M.A. Romeo: Software, investigation. L. Masuelli: Data curation, investigation. M. Mattei: Supervision, investigation. R. Bei: Data curation, supervision. G. D'Orazi: Supervision. M. Cirone: Conceptualization, funding acquisition, writing—original draft, writing—review and editing.

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Molecular Cancer Therapeutics

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