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Activation of TLR4 Is Required for the Synergistic Induction of Dual Oxidase 2 and Dual Oxidase A2 by IFN-γ and Lipopolysaccharide in Human Pancreatic Cancer Cell Lines

Yongzhong Wu,* Jiamo Lu,* Smitha Antony,* Agnes Juhasz,* Han Liu,† Guojian Jiang,* Jennifer L. Meitzler,* Melinda Hollingshead,‡ Diana C. Haines,‡ Donna Butcher,‡ Krishnendu Roy,† and James H. Doroshow*†

Pancreatitis is associated with release of proinflammatory cytokines and reactive oxygen species and plays an important role in the development of pancreatic cancer. We recently demonstrated that dual oxidase (Duox2), an NADPH oxidase essential for reactive oxygen species–related, gastrointestinal host defense, is regulated by IFN-γ–mediated Stat1 binding to the Duox2 promoter in pancreatic tumor lines. Because LPS enhances the development and invasiveness of pancreatic cancer in vivo following TLR4–related activation of NF-κB, we examined whether LPS, alone or combined with IFN-γ, regulated Duox2. We found that upregulation of TLR4 by IFN-γ in BxPC-3 and CFPAC-1 pancreatic cancer cells was augmented by LPS, resulting in activation of NF-κB, accumulation of NF-κB (p65) in the nucleus, and increased binding of p65 to the Duox2 promoter. TLR4 silencing with small interfering RNAs, as well as two independent NF-κB inhibitors, attenuated LPS- and IFN-γ–mediated Duox2 upregulation in BxPC-3 cells. Induction of Duox2 expression by IFN-γ and LPS may result from IFN-γ–related activation of Stat1 acting in concert with NF-κB–related upregulation of Duox2. Sustained extracellular accumulation of H2O2 generated by exposure to both LPS and IFN-γ was responsible for an ∼50% decrease in BxPC-3 cell proliferation associated with a G1 cell cycle block, apoptosis, and DNA damage. We also demonstrated upregulation of Duox expression in vivo in pancreatic cancer xenografts and in patients with chronic pancreatitis. These results suggest that inflammatory cytokines can interact to produce a Duox-dependent prooxidant milieu that could increase the pathologic potential of pancreatic inflammation and pancreatic cancer cells. The Journal of Immunology, 2013, 190: 1859–1872.

A substantial body of evidence suggests that chronic inflammation of the pancreas plays an important role in the subsequent development of pancreatic cancer and that the pathogenesis of exocrine cancers of the pancreas may be intimately related to the release of proinflammatory cytokines and cytokine–related reactive oxygen formation (1–4). Recently, the role of repetitive bouts of asymptomatic pancreatic inflammation in tumor development has been emphasized, as well as the critical role of anti-inflammatory interventions to enhance the repair of inflammation–related tissue injury and reduce subsequent tumorigenesis (5). Pancreatic cancer cells were demonstrated to produce reactive oxygen species (ROS) in a growth factor–dependent fashion, and these reactive species play an important role in the proliferative capacity of these cells (6–8). Therefore, it is possible that, during repeated bouts of pancreatitis, cytokine–related ROS production could increase genetic instability (9, 10) while decreasing the tumor-suppressor functions of essential protein phosphatases (11), thus enhancing the possibility of malignant transformation.

Although it has been known for more than two decades that tumor cells can produce a significant flux of H2O2 (12), only more recently has it become clear that much of the reactive oxygen formation emanating from human tumors may originate from members of the recently described family of epithelial NADPH oxidases (Noxs): reduced NAD phosphate oxidases (13, 14). Dual oxidase (Duox)2 is one of the seven members of the Nox gene family; although originally described as an H2O2-producing enzyme in the thyroid that plays a critical role in thyroid hormone biosynthesis (15), Duox2 also was found in bronchial epithelium and throughout the gastrointestinal tract (16, 17). In airway mucosal cells, Duox2 plays an important role in the generation of H2O2 for host defense against a variety of pathogens (18–20); under the stress induced by an infectious agent, Duox2 expression is regulated by several inflammatory stimuli, including IFN-γ, flagellin, and rhinovirus (16, 20). Duox2–induced ROS also appear to play a role in the antibacterial response in the gut (21, 22). However, the expression of Duox2 is significantly increased in human colon biopsies, as well as in isolated intestinal epithelial cells, from patients with inflammatory bowel disease (both Crohn’s disease and ulcerative colitis) compared with healthy...
control subjects (21, 23), suggesting that an unchecked ROS response to pathogens could contribute to the tissue injury observed in these chronic inflammatory disorders.

Previous work from our laboratory revealed that the proinflammatory cytokine IFN-γ initiates a Duox2-induced ROS cascade in human pancreatic cancer cells (24). Furthermore, several recent studies demonstrated that proinflammatory components of the bacterial cell wall, including LPS, mediate Nox-dependent ROS generation during the inflammatory response in the airway and gastrointestinal tract, in part due to direct interactions between members of the Nox family and TLR4, the critical downstream target that recognizes LPS from Gram-negative bacteria (25, 26). TLR4-related signaling was recently suggested to play a role in the pathogenesis of acute pancreatitis in model systems, as well as in the clinic (27–29). Because LPS-related TL4 signaling was also shown to play a critical role in modulating the invasive potential of human pancreatic cancer lines (30), as well as the transition from pancreatic inflammation to pancreatic cancer in genetically engineered mouse models (31), we sought to determine whether LPS, alone or in combination with IFN-γ, might regulate Duox2-mediated ROS generation in pancreatic cancer cells.

Hence, in this study, we evaluated the effects of IFN-γ and LPS on Duox2 expression and function, as well as the mechanism(s) by which these two proinflammatory agents regulate Duox2 levels in human pancreatic cancer cell lines. We found that, although both agents significantly increase Duox2 expression individually, the combination synergistically enhances the expression of Duox2 and its associated maturation factor, DuoxA2, leading to a significant increase in both extracellular and intracellular ROS production. The dramatic increase in ROS that we observed for the combination of LPS and IFN-γ depended critically on the upregulation of, and signaling through, the TLR4 pathway, ultimately resulting in enhanced binding of NF-κB (p65) to the Duox2 promoter. We also found that ROS production under our experimental conditions was sufficient to activate the DNA damage-repair pathway while arresting cells in G1, producing a significant increase in apoptosis, and inhibiting cellular proliferation. To determine whether Duox2 might contribute to inflammatory states in vivo, we examined its expression both in tissue specimens from patients with chronic pancreatitis and in pancreatic cancer xenografts; in both cases, we found that Duox2 expression levels were markedly upregulated. These results suggest that the pro-oxidant state generated by cytokine-mediated Duox2 upregulation could hinder recovery from inflammatory stress-related tissue injury and, thus, may contribute to the development and pathologic behavior of pancreatic cancer cells.

Materials and Methods

Materials

Recombinant human IFN-γ (cat. no. 285-IF) was from R&D Systems. Ab against human β-actin (cat. no. A3853) was acquired from Sigma-Aldrich. Our Stat1 (p84/p91; sc-346) Ab was obtained from Santa Cruz Biotechnology. Abs against components of the NF-κB complex, including p65 (cat. no. 6956), IκBα (cat. no. 34812), p-Stat1 (Ser 727) (cat. no. 9177S), and p-Stat1 (Tyr 701) (cat. no. 34812), were purchased from Cell Signaling Technology. Human TLR4 silencer select small interfering RNA (siRNA; cat. no. 4390825), silencer negative control siRNA (siRNA; cat. no. AM4635), human Duox2 primer (cat. no. Hs00204187_m1), human Duox2A2 primer (cat. no. Hs01595310_m1), human TLR4 primer (cat. no. Hs00152939_ml), human MyD88 primer (cat. no. Hs00182082_ml), human actin (cat. no. Hs9999903_m1), and TaqMan Universal PCR mix (cat. no. 4364340) were from Applied Biosystems (Foster City, CA). The QuikChIP Kit (cat. no. 331001K), anti-human TLR4 Ab (cat. no. IMG-6307A), anti-human TLR4 FITC Ab, and the Cell Surface TLR Staining Flow Kit (cat. no. 100999K) were from Immunex. 2′,7′-Dichlorodihydrofluorescein diacetate (CM-H₂-DC-FDA) was obtained from Molecular Probes (Carlsbad, CA). 1-Pyridinedicarboxylic acid, ammonium salt (PDTC) (cat. no. 548000) and BAY11-70829 (cat. no. 196870) were from EMD Chemicals (Gibbstown, NJ). MTTL (cat. no. M2128) was from Sigma-Aldrich. Our mouse anti-human Duox mAb, S-12, was developed by Creative Biolabs (Port Jefferson Station, NY) and characterized as described previously (24). Although the pancreatic cancer cell lines used for these experiments do not contain measurable Duox1 mRNA, because the Ab cross-reacts with Duox1, we have referred to the protein that it detects as “Duox.” The three human pancreas tissue microarrays (BIC14011, PA485, PA1001) were obtained from US Biomax (Rockville, MD).

Cell lines, cell culture, and mice

The human pancreatic cancer cell lines BxPC-3 (cat. no. CRL-1687), AsPC-1 (cat. no. CRL-1682), and MIA-PaCa (cat. no. CRL-1420) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium (cat. no. SH30255.01; HyClone) with 1% FBS and 10% FBS CPFC-P1 pancreatic cancer cells (cat. no. CRL-1918) were also obtained from the American Type Culture Collection and were cultured in IMDM with 10% FBS. The identity of each cell line was confirmed using Identifiler STR genotyping (Applied Biosystems). For starvation conditions, cells were propagated overnight in the same medium without FBS. We used starvation conditions because, although the induction of Duox2 expression by IFN-γ was observed under both serum-containing and serum-starved conditions, Duox2 induction was stronger after starvation. Cells were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO₂ in air. In control experiments, we found that the basal levels of expression of all of the Nox homologs (Nox1-5, Duox1, Duox2) in each of the pancreatic cancer cell lines used for these studies were very low, often at or near the lower limit of detection of our real-time RT-PCR assays (data not shown). All mice (female athymic nu/nu NCr) used in the study were obtained from the Animal Production Program (Frederick National Laboratory for Cancer Research, Frederick, MD). National Cancer Institute-Frederick is accredited by American Association for the Accreditation of Laboratory Animal Care International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals.

RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR assay

Total RNA was extracted from IFN-γ- or LPS-treated or untreated cells with the RNeasy Mini Kit (cat. no. 74104; QIAGEN), according to the manufacturer’s instructions. Two micrograms of total RNA was used for cDNA synthesis, using SuperScript II reverse transcriptase (cat. no. 18080-044) and random primers (cat. no. 48190-011; Invitrogen) in a 20-μl reaction system, with the following cycles: 25°C for 5 min, 42°C for 50 min, and 75°C for 5 min. After the reaction was complete, the RT-PCR products were diluted with diethylpyrocarbonate/H₂O to 100 μl for real-time PCR. Real-time RT-PCR was performed on 384-well plates in a 20-μl reaction system containing 2 μl diluted cDNA, 1 μl primer mixture, 7 μl H₂O, and 10 μl TaqMan 2× reaction mixture. PCR was carried out under default cycling conditions, and fluorescence was detected with the ABI 7900HT Sequence Detection System (Applied Biosystems). Triplicate determinations were performed for each sample that was used for real-time PCR; the mean value was calculated, and the data in the figures represent the results of three independent experiments. Relative gene expression was calculated as the ratio of the target gene to the internal reference gene (β-actin) multiplied by 10^β based on Ct values.

Whole-cell and nuclear extract preparation and Western blot analysis

For preparation of whole-cell extracts, cell pellets from BxPC-3, AsPC-1, and CPFC-P1 cells, treated or not with IFN-γ and/or LPS, were lysed with 1× RIPA Lysis Buffer (cat. no. 20-188; Millipore, Temecula, CA), with the addition of a phosphatase inhibitor tablet (cat. no. 04-906-837001) and a protease inhibitor tablet (cat. no. 11-836-153001; both from Roche). The NE-PER Nuclear and Cytoplasmic Extraction Kit (cat. no. 78833; Thermo Scientific) was used to prepare nuclear extract from cells treated with IFN-γ and/or LPS. The protein concentrations of both whole-cell and nuclear extracts were measured using the BCA Protein Assay Kit (Pierce). Cell extracts were mixed with an equal volume of 2× SDS protein gel–loading buffer (Bio-Rad). The QuickPage 2D Polyacrylamide Gel Electrophoresis was loaded onto a 4–20% Tris glycine gel (cat. no. EC9028; Invitrogen), and the proteins were separated and electrophotographically transferred to nitrocellulose membranes using I Blot gel transfer stacks (cat. no. IB 3010-
FIGURE 1. LPS acts synergistically with IFN-γ to induce Duox2 and DuoxA2 expression, enhancing both intra- and extracellular production of ROS. (A) Induction of Duox2 expression by exposure to IFN-γ (25 ng/ml), LPS (1 μg/ml), or the combination for 24 h; quantitative, real-time RT-PCR analysis of relative Duox2 expression was normalized to β-actin (upper panel). Error bars represent standard deviations; data are from triplicate experiments *p < 0.05, Duox2 expression in IFN-γ– or LPS-treated BxPC-3 cells versus solvent-treated controls, ***p < 0.001, LPS and IFN-γ pretreatment versus either agent alone. Expression of the Duox protein by Western blot analysis in 50 μg of whole-cell extract from BxPC-3 cells treated with solvent, IFN-γ (25 ng/ml), LPS (1 μg/ml), or both IFN-γ and LPS for 24 h (lower panel); the Western blot shown is representative of three independent experiments. (B) Expression of both Duox2 and DuoxA2 following treatment with IFN-γ (25 ng/ml), LPS (1 μg/ml), or the combination for 24 h was quantitated by real-time RT-PCR normalized to β-actin. Error bars represent standard deviations; data represent the means of three independent experiments. ***p < 0.001, combination of LPS and IFN-γ pretreatment versus either agent alone for both Duox2 and DuoxA2. (C) Extracellular H2O2 production was detected using the Amplex Red H2O2/peroxidase assay. BxPC-3 cells were grown in serum-free medium with IFN-γ and/or LPS for 24 h. Cells were then collected, and 2 × 10^4 live BxPC-3 cells were mixed with 100 μl of the Amplex Red reaction mixture containing 50 μM Amplex Red and 0.1 U (Figure legend continues)
of HRP/ml of Krebs-Ringer phosphate buffer with 1 µM ionomycin for 1 h. H$_2$O$_2$ values were calculated by interpolation from a standard curve, using 0–2 µM H$_2$O$_2$. Data are shown as mean ± SD (n = 16). *p < 0.05, IFN-γ or LPS pretreatment versus solvent, **p < 0.001, combination of IFN-γ and LPS versus either agent used independently. (D) Production of intracellular ROS by IFN-γ- and/or LPS-treated BxPC-3 pancreatic carcinoma cells as detected by flow cytometry. Starved BxPC-3 cells were treated with solvent, IFN-γ (25 ng/ml), LPS (1 µg/ml), or both IFN-γ and LPS for 24 h; intracellular ROS production was measured at the end of the incubation by FACS, using the redox-sensitive dye CM-H$_2$DCF-DA. Ionomycin (1 µM) was added for 5 min following completion of IFN-γ and LPS treatment, prior to resuspension of the cells in fresh medium and quantitation of fluorescence. The data shown are representative of three independent experiments. (E) Effect of Duox2 siRNA on ionomycin-enhanced extracellular H$_2$O$_2$ production in BxPC-3 cells pretreated with IFN-γ or the combination of IFN-γ and LPS. The cells were treated with IFN-γ or IFN-γ and LPS, and H$_2$O$_2$ production was quantitated with ionomycin exposure exactly as described in (C), with the exception that control siRNA or siRNA targeting Duox2 were transfected into BxPC-3 cells 24 h prior to exposure to IFN-γ or IFN-γ and LPS. Data are mean ± SD (n = 3). *p < 0.05, IFN-γ versus IFN-γ and LPS pretreatment following transient transfection with either control or Duox2 siRNA.
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RNA and protein extraction from tumor xenographs

In addition to the methods described above for cell lines, a Polytron System PT 1200E homogenizer equipped with a 5–12-mm-diameter dispersing head (KINEMATICA, Lucerne, Switzerland) was used to homogenize the tumor tissue mixed with a suitable amount of either 1× RIPA buffer for protein extraction or RLT buffer for RNA extraction. A 350-μl volume of tumor homogenate was processed directly for isolation of RNA. For protein lysate preparation, the tumor homogenate in RIPA buffer was centrifuged at 13,000 rpm and 4°C for 10 min; the supernatant was used for Western blot analysis and to determine protein concentration.

Immunohistochemical analysis of Duox expression in pancreatic tissue arrays from patients with chronic pancreatitis

Immunohistochemistry using our Duox mAb was performed on three human pancreatic tissue microarrays. Immunohistochemistry staining was performed as follows: slides were deparaffinized with distilled water, and Ag retrieval was performed using DAKO target retrieval solution (cat. no. S1699) for 20 min at 95°C, followed by 20 min of cooling. Slides were rinsed in distilled water, transferred to TBST buffer (cat. no. S3006; DAKO) for 5 min, and incubated in peroxidase blocking solution (3% H2O2) for 10 min at RT. Duox Ab, at a dilution of 1:250 (diluted in DAKO diluent; cat. no. S0809), was used for 60 min at RT; slides were then rinsed in TBST, and secondary Ab was applied (DAKO EnVision+ Dual Link System-HRP [cat. no. K4063], Ready-To-Use) for 30 min at RT. The slides were rinsed in TBST (three times, 2 min each); 3,3′-diaminobenzidine was used for 10 min for visualization, and the slides were rinsed in TBST. Hematoxylin was used as a counterstain. Positive control tissues (normal human colon and cell pellets) and a negative control reagent (normal mouse IgGl isotype control, 10 μg/ml for 60 min [cat. no. 550878; BD Biosciences], diluted in DAKO diluent [cat. no. S0809]) were also examined.

Statistical analysis

Results are expressed as the mean ± SD from at least triplicate experiments. Statistical differences between mean values of control and treated samples were assessed using the Student t test; p < 0.05 was considered statistically significant.

Results

Combination of LPS and IFN-γ synergistically induces Duox2 expression and reactive oxygen production

BxPC-3 cells were treated with IFN-γ and/or LPS to determine the potential of each, as well as the combination, to induce Duox2 expression. LPS alone significantly increased Duox2 mRNA levels in BxPC-3 cells, albeit to a lesser degree than IFN-γ; however, the combination of LPS and IFN-γ increased Duox2 expression ~6-fold compared with IFN-γ alone (p < 0.001; Fig. 1A, upper panel). In control experiments, we found that decreasing the LPS concentration to 100 ng/ml did not alter the synergistic induction of Duox2 expression with IFN-γ that we observed; Duox2, rather than Duox1, was the predominant oxidase affected by the combination of IFN-γ and LPS (Supplemental Fig. 3). Western blot analysis confirmed that LPS- and IFN-γ–induced Duox2 mRNA expression correlated well with Duox protein levels (Fig. 1A, lower panel). The simultaneous exposure of cells to both LPS and IFN-γ induced a substantially higher level of Duox protein expression than did either LPS or IFN-γ (Fig. 1A). In like manner...
(Fig. 1B), we found that the addition of LPS to IFN-γ significantly increased the expression of the Duox2 maturation factor, DuoxA2, which plays a critical role in the formation of the active Duox2 membrane oxidase (32). To determine whether these levels of Duox2 and DuoxA2 correlated with functional oxidase activity, we examined the production of ROS using two techniques. In the presence of 1 μM ionomycin for 1 h, which is known to activate Duox 2 (13), BxPC-3 cells previously exposed for 24 h to IFN-γ and/or LPS produced significant amounts of extracellular H₂O₂ (Fig. 1C), as detected with Amplex Red reagent; cotreatment with LPS and IFN-γ resulted in significantly more extracellular H₂O₂ production than did exposure to either agent alone. To confirm our results obtained with Amplex Red, we performed flow cytometric quantitation of intracellular ROS production using the redox-sensitive dye CM-H₂DCF-DA. These studies clearly demonstrated that intracellular ROS levels were increased following exposure of BxPC-3 cells to LPS and/or IFN-γ and that the ROS levels observed were consistent with the expression levels of Duox2 and DuoxA2 following exposure to LPS or IFN-γ, alone or in combination (Fig. 1D). Although intracellular ROS production in BxPC-3 cells following ionomycin treatment was modestly increased by LPS alone and was increased to a greater degree by IFN-γ, exposure to the combination of LPS and IFN-γ resulted in substantially greater ROS production than did exposure to either agent alone (Fig. 1D). Finally, we found that ionomycin-enhanced H₂O₂ production by BxPC-3 cells exposed to LPS and IFN-γ could be decreased significantly following pretreatment with Duox2-specific siRNA (Fig. 1E). Taken together, these data are consistent with the synergistic induction of functional Duox2 and DuoxA2 by the combination of LPS and IFN-γ.

FIGURE 3. Silencing TLR4 attenuates IFN-γ- and LPS-mediated enhancement of TLR4 and Duox2/DuoxA2 expression in BxPC-3 cells. (A) Inhibition of TLR4 expression by TLR4 siRNAs. Control siRNA or two human TLR4-specific siRNAs targeting different domains of TLR4 were transiently transfected into BxPC-3 cells; 24 h following transfection, cells were incubated in serum-free medium with or without IFN-γ (25 ng/ml) or LPS (1 μg/ml) for another 24 h. RNA was then extracted and subjected to real-time RT-PCR, and relative TLR4 levels were normalized to β-actin. Error bars represent SD; data are from triplicate samples. (B) Silencing TLR4 produced no effect on IFN-γ-induced Stat1 expression. The identical RNA samples used in (A) were subjected to real-time RT-PCR assay with human Stat1-specific primers, and Stat1 expression relative to β-actin was calculated. (C) Silencing of TLR4 attenuated IFN-γ- and LPS-induced Duox2 expression. The same RNA samples used for the experiments shown in (A) were subjected to real-time RT-PCR using human Duox2-specific primers, and Duox2 expression relative to β-actin was calculated. (D) Silencing TLR4 attenuated IFN-γ- and LPS-induced DuoxA2 expression. The RNA samples used for the experiments in (A) were subjected to real-time RT-PCR assay, using human DuoxA2 primers; DuoxA2 expression relative to β-actin was calculated. (E) Control or TLR4-specific siRNAs were transiently transfected into BxPC-3 cells. At 24 h after transfection, cells were incubated in serum-free medium with or without IFN-γ (25 ng/ml) or LPS (1 μg/ml) for a subsequent 24 h, and 50 μg of whole-cell extract was subjected to Western blot analysis, using specific Abs as indicated. These results are representative of three separate experiments. ***p < 0.001, versus BxPC-3 cells treated with a control siRNA.
IFN-γ and LPS upregulate TLR4 and MyD88 in association with enhanced Duox2 expression levels

It was reported that exposure to IFN-γ augments the expression of TLR4 and its accessory components, MD-2 and MyD88, in human monocytes and macrophages (33). In those experiments, exposure of IFN-γ-primed monocytes to LPS enhanced the phosphorylation of IRAK, NF-κB DNA-binding activity, and TNF-α and IL-12 production. Furthermore, Abreu et al. (34) reported that intestinal epithelial cells expressed low levels of TLR4 and MD-2 and were hyporesponsive to LPS; however, in the same cells, the proinflammatory cytokines IFN-γ and TNF-α increased TLR4 and MD-2 expression and sensitized epithelial cells to LPS-dependent IL-8 secretion. We sought to determine whether our observed synergistic induction of Duox2 by LPS and IFN-γ occurred by the same mechanism. We used real-time RT-PCR to evaluate the expression of TLR4 and its adaptor protein MyD88 after exposure of BxPC-3 cells to solvent, LPS, and/or IFN-γ. IFN-γ induced a significant, ∼3–4-fold increase in TLR4 and MyD88 expression in BxPC-3 cells, and the combination of LPS and IFN-γ increased TLR4, but not MyD88, levels further (Fig. 2A). Western blot analysis confirmed that the increase in IFN-γ-induced TLR4 and MyD88 mRNA correlated with an increase in protein expression; we also found, as previously described (24), that treatment of BxPC-3 cells with IFN-γ enhanced the phosphorylation of Stat1 at tyrosine 701 (Fig. 2B). Finally, we evaluated the surface expression of TLR4 after IFN-γ exposure by flow cytometry using an FITC-labeled human TLR4 Ab. BxPC-3 cells demonstrated expression of TLR4 on the cell surface that was enhanced by IFN-γ, resulting in a substantial right shift of the fluorescent signal (Fig. 2C).

Silencing of TLR4 attenuates IFN-γ- and LPS-mediated induction of TLR4 and Duox2/DuoxA2 expression

We used RNA interference to further define the role of TLR4 in the synergistic induction of Duox2 and DuoxA2 by LPS and IFN-γ in BxPC-3 cells. Control siRNA or two distinct human TLR4-specific siRNAs targeting different domains of TLR4 were transiently transfected into BxPC-3 cells. At 24 h following transfection, cells were cultured in serum-free medium with LPS and/
or IFN-γ for another 24 h. Cells were then collected and subjected to RNA extraction and real-time RT-PCR. Human TLR4 siRNAs were found to specifically suppress IFN-γ- and LPS-induced TLR4 expression (Fig. 3A) but had no inhibitory effect on IFN-γ–induced Stat1 expression (Fig. 3B). Human TLR4 siRNAs also significantly attenuated the expression of IFN-γ– and LPS–induced Duox2 (Fig. 3C) and DuoxA2 (Fig. 3D), whereas control siRNAs had no inhibitory effect on the expression of IFN-γ– and LPS–induced TLR4 or Duox2/DuoxA2. Western blot analysis confirmed that TLR4 siRNA had an inhibitory effect on LPS– and IFN-γ–induced TLR4 protein expression (Fig. 3E). Silencing of TLR4 also decreased LPS– and IFN-γ–induced Duox protein expression (Fig. 3E, lanes 4 and 8).

Intact NF-κB signaling is required for LPS– and IFN-γ–induced Duox2 expression

To expand our results with BxPC-3 cells, the AsPC-1 human pancreatic cancer cell line was also exposed to IFN-γ; as demonstrated in Fig. 4A, these cells also respond to IFN-γ by increasing the expression of Duox2. We also sought to determine whether LPS and IFN-γ act synergistically to induce Duox2 expression in AsPC-1 cells as they do in the BxPC-3 line. Starved AsPC-1 cells were treated with solvent or with LPS (1 μg/ml) and/or IFN-γ (25 ng/ml), and real-time RT-PCR was used to evaluate TLR4 and Duox2 expression. Although treatment of AsPC-1 cells with IFN-γ alone induced a significant level of Duox2 expression, in marked contrast to our results in BxPC-3 cells, exposure to LPS plus IFN-γ did not result in a further significant increase in Duox2 expression (Fig. 4A). Furthermore, IFN-γ, either alone or in combination with LPS, increased TLR4 expression by ~4-fold over the levels observed with solvent or LPS (Fig. 4B). Whole-cell extracts from AsPC-1 cells that had been treated for 24 h with LPS and/or IFN-γ were subjected to Western blot analysis. As shown in Fig. 4D, IFN-γ–activated Stat1 signaling in this cell line and augmented Duox expression. IFN-γ treatment also induced TLR4 expression; however, unlike BxPC-3 cells, cotreatment with LPS did not result in a further increase in Duox protein levels. We next compared NF-κB signaling in the two cell lines after priming the cells with either solvent or IFN-γ for 24 h and then exposing the cells to LPS for 1 h. In BxPC-3 cells, priming the cells with IFN-γ and treating for 1 h with LPS resulted in iκB degradation (Fig. 4C, compare lanes 1 and 4). In contrast, for AsPC-1 cells, exposure to LPS plus IFN-γ did not result in iκB degradation (Fig. 4E). Finally, we examined nuclear extracts prepared from BxPC-3 and AsPC-1 cells that had been primed with either solvent or IFN-γ for 24 h and then treated with LPS for 1 h. In both cell lines, IFN-γ priming resulted in a similar level of nuclear accumulation of Stat1 and IRF-1 (Fig. 4F), which was not dependent on LPS treatment. However, results for the transcription factor NF-κB differed in the two cell lines. In BxPC-3 cells, LPS exposure alone resulted in a clear nuclear translocation of NF-κB; priming with IFN-γ prior to treatment with LPS resulted in further enhancement of the nuclear accumulation of NF-κB. However, in AsPC-1 cells, the nuclear distribution of NF-κB was not influenced by either LPS or IFN-γ treatment (Fig. 4F). To evaluate the generality of our finding that NF-κB signaling could play an important role in regulating the expression of Duox2 in human pancreatic cancer cells, we also evaluated the effects of IFN-γ and LPS on CFPAC-1 human pancreatic cancer cells (Supplemental Fig. 1). In this line, similar to our observations with BxPC-3 cells, we found that exposure to IFN-γ and LPS produced a more-than-additive effect on Duox2 expression at both the RNA and protein levels that was
accompanied by activation of TLR4 and was associated with degradation of 1xBox (Supplemental Fig. 1). Furthermore, nuclear accumulation of NF-κB following LPS and IFN-γ exposure resembled our findings in BxPC-3, and not in AsPC-1, cells (Supplemental Fig. 2). These results suggest that an intact NF-κB–signaling pathway is necessary for the synergistic induction of Duox2 by LPS and IFN-γ in human pancreatic cancer cells.

**Inhibition of NF-κB diminishes LPS- and IFN-γ–induced Duox2 expression in BxPC-3 cells**

To investigate further whether LPS- and IFN-γ–mediated induction of Duox2 depended on NF-κB activation, we exposed BxPC-3 cells to two NF-κB inhibitors, PDTC (100 μM) and BAY 11-7082 (10 μM), for 30 min prior to LPS and/or IFN-γ. Both inhibitors significantly decreased LPS- and IFN-γ–mediated Duox2 induction (Fig. 5A), indicating that activation of NF-κB is required for Duox2 induction by LPS and IFN-γ. Neither NF-κB inhibitor, over the time course and concentrations tested, was cytotoxic, as measured by exclusion of trypan blue dye (data not shown).

**NF-κB p65 binds to the human Duox2 promoter**

Having confirmed that IFN-γ exposure can upregulate TLR4 and MyD88 expression in BxPC-3 cells and is associated with a more-than-additive effect on Duox2 expression when combined with LPS, as well as accumulation of NF-κB p65 in the nucleus, we sought to determine whether a component of the NF-κB complex could bind to the human Duox2 promoter. We also wished to examine whether such binding would be enhanced by cotreatment with LPS and IFN-γ over that seen with either LPS or IFN-γ treatment alone. ChIP was used to investigate binding of the NF-κB p65 subunit in vivo under various experimental conditions. Using the TRANSFAC search program (35), we identified a canonical NF-κB binding site within 3 kb of the human Duox2 promoter between −2771 and −2763 bp from the transcription start site. The sequence was 5′-GGGAATTCCC-3′, which exactly matched the known NF-κB consensus motif (5′-GGGRNNTYCCC-3′) provided by TRANSFAC. Therefore, PCR primers were designed on the basis of the DNA sequence surrounding the potential NF-κB binding site. Equal amounts of DNA were used for the initial immunoprecipitation (Fig. 5B, top panel). Control IgG Ab produced a very weak background PCR signal, indicating immunoprecipitation, as well as that PCR reactions were specific (Fig. 5B, middle panel). With a p65-specific Ab (Fig. 5B, bottom panel), LPS and IFN-γ treatment produced a clear PCR product; furthermore, cotreatment with LPS and IFN-γ produced a much stronger PCR signal that was indicative of greater p65 binding to the endogenous human Duox2 promoter.

**LPS and IFN-γ treatment leads to extracellular H2O2 accumulation, inhibition of growth and cell cycle progression, and induction of apoptosis and DNA damage in human pancreatic cancer cells**

To examine potential consequences of LPS- and IFN-γ–mediated upregulation of Duox2 and DuoxA2, we examined the time course of extracellular H2O2 accumulation following a variety of stimulation conditions. Surprisingly, in the absence of ionomycin stimulation, extracellular H2O2 production by BxPC-3 cells steadily increased over time; after 4 h of incubation, extracellular H2O2 production for cells that had been exposed for 24 h previously to IFN-γ or the combination of LPS plus IFN-γ continued to generate H2O2 at substantial levels (Fig. 6A). When ionomycin was added to the reaction system, the differences among the treatment conditions became more pronounced (Fig. 6B); cells primed with LPS plus IFN-γ produced the highest amounts of extracellular H2O2 following a variety of stimuli. H2O2 generation was inhibited by exposure to IFN-γ and the combination of IFN-γ and LPS in BxPC-3 cells. BxPC-3 cells grown in serum-free medium were treated with ionomycin. H2O2 production by AsPC-1 cells was determined exactly as described for the BxPC-3 line in the absence of added ionomycin. The data shown are mean values from multiple determinations (n = 16). **(E)** H2O2 production by AsPC-1 cells was determined exactly as described for the BxPC-3 line in the absence of added ionomycin. The data shown are mean values from multiple determinations (n = 16). **(F)** Neither IFN-γ alone nor the combination of IFN-γ and LPS significantly inhibited cell growth in the AsPC-1 cell line under experimental conditions identical to those used for BxPC-3 cells. **###p < 0.001**, versus cells exposed to vehicle alone.
H₂O₂, consistent with their expression levels of Duox2 and DuoxA2. In contrast, consistent with the lower levels of Duox2 expression measured in AsPC-1 cells following treatment with IFN-γ and/or LPS, the production of H₂O₂ was lower in these cells (Fig. 6C) and was enhanced to a much lesser degree by the presence of the calcium ionophore (Fig. 6D). We next measured the effects of LPS and IFN-γ on cell growth in BxPC-3 and AsPC-1 cells using the MTT assay (Fig. 6E, 6F). On day 1, there was no significant difference among the various treatment groups. On day 2, LPS alone still demonstrated no inhibitory effect on cell proliferation; in contrast, IFN-γ alone significantly inhibited the growth of BxPC-3 cells. The combination of LPS and IFN-γ resulted in >50% retardation of cell growth by day 2 of exposure. In comparison, IFN-γ induced both Duox2 and DuoxA2 expression in AsPC-1 cells to a lesser degree than for the BxPC-3 line; IFN-γ alone or combined with LPS did not have an inhibitory effect on AsPC-1 cell growth (Fig. 6F). These data suggest that the sustained intra- and extracellular ROS production mediated by LPS- and IFN-γ-induced Duox2 expression in BxPC-3 cells may be sufficient (at least compared with the AsPC-1 line) to affect tumor cell proliferation. To evaluate potential mechanisms of cell death, we examined the effects of IFN-γ and LPS on cell cycle progression and apoptosis in BxPC-3 cells. As shown in Fig. 7A, exposure of BxPC-3 cells to IFN-γ and LPS produced a profound inhibition of cell cycle progression at G₁ that exceeded that of IFN-γ alone and which is consistent with an effect of H₂O₂ (36); the G₁ arrest was associated with a marked degree of both apoptotic cell death and loss of cell membrane permeability (Fig. 7B). Finally, to examine the possibility that Duox2-related reactive oxygen metabolism might contribute to enhanced genetic instability of our pancreatic cancer cells, we evaluated the effect of IFN-γ and LPS on the activation of phosphorylated γH2AX, a biomarker for DNA damage and repair (37). As shown in Fig. 7C, we found that both IFN-γ alone or the IFN-γ and LPS com-

FIGURE 7. Effects of IFN-γ, LPS, or the combination of IFN-γ and LPS on cell cycle progression, apoptosis, and DNA damage in BxPC-3 cells. (A) BxPC-3 cells that had been grown in complete media were treated with solvent, LPS (1 μg/ml), IFN-γ (25 ng/ml), or both LPS and IFN-γ for 24 h. Cells were collected, fixed with 70% ethanol, stained with propidium iodide, and subjected to analytical cytometry. The data were analyzed with ModFit LT software. (B) To examine the effects of LPS and/or IFN-γ on cell death in the BxPC-3 line, cells were exposed to each agent individually and in combination for 72 h; live and dead (floating) cells were collected for estimation of the degree of apoptosis using the Vybrant Apoptosis Kit #3 with 7-AAD viability staining solution. Labeled cells were analyzed by flow cytometry. Dead cells that cannot exclude 7-AAD were detected in the far red spectral range, depicted on the y-axis; apoptotic cells labeled with FITC conjugated to annexin V are shown with green fluorescence on the x-axis. (C) IFN-γ and LPS produce DNA double-strand breaks in BxPC-3 cells detected by an increase in phosphorylated γH2AX protein. Cells were treated with IFN-γ or the combination of IFN-γ and LPS for 72 h; the thiol-containing ROS scavenger N-acetyl-l-cysteine (NAC) or the cell-permeable H₂O₂-detoxifying enzyme PEG-catalase (PEG-CAT) was administered 30 min prior to the initiation of IFN-γ or IFN-γ plus LPS exposure to evaluate the effect of scavenging ROS on the DNA damage response. Western blot analysis was performed with 50 μg of cell lysate using the specific Abs, as indicated. The results shown are typical of three identical experiments.
bination induced a marked DNA damage response in BxPC-3 cells. Furthermore, the γH2AX signal was decreased by pre-treatment of the BxPC-3 cells with either the ROS scavenger N-acetyl-L-cysteine or by a formulation of the H$_2$O$_2$ detoxifying enzyme catalase (PEG-catalase) that crosses the tumor cell outer membrane. These studies indicate that ROS produced by IFN-γ in combination with LPS may produce DNA damage that activates a DNA repair response measurable by the expression of γH2AX.

**Growth of human pancreatic cancer cells as xenografts is associated with increased Duox2 expression**

In our current experiments, as well as our previous study (24), we found that Duox2 expression in human pancreatic cancer cells in culture is very low in the absence of cytokine exposure, and certain pancreatic lines (e.g., MIA-PaCa cells) are unresponsive to IFN-γ. We hypothesized that it might be possible to evaluate the relevance of our current investigations to the setting of tumor cell growth in vivo by examining whether the extracellular milieu of pancreatic tumor cells growing as xenografts could provide an environment that was sufficiently proinflammatory to alter Duox2 expression in the absence of treatment with an exogenous cytokine. As shown in Fig. 8, we found that during the first passage of BxPC-3 or AsPC-1 cells in athymic mice, when tumors reached 300 or 500 mg in size, respectively, Duox2 expression increased dramatically compared with the expression levels in the cells immediately prior to xenografting. Furthermore, for both the BxPC-3 and AsPC-1 lines, Duox2 expression in vivo approximated that following exposure to IFN-γ for 24 h in vitro (Fig. 8A, 8B, upper panels). In contrast, the level of Duox2 in MIA-PaCa cells, which is at the lower limit of detection by RT-PCR and does not respond to IFN-γ, did not change following growth of the tumor in vivo as a xenograft (Fig. 8C, upper panel). Tumors of 500 mg, rather than 300 mg, were used to study the AsPC-1 and MIA-PaCa xenografts to optimize the chance for upregulation of Duox2. Western blot analysis from lysates prepared from the same tumors (that had been split into quarters) used for RT-PCR confirmed the changes in expression that we found at the mRNA level (Fig. 8A–C, lower panels).

**Duox expression is increased in patients with chronic pancreatitis**

To evaluate the expression of Duox earlier in the process of pancreatic carcinogenesis, we performed immunohistochemical analyses using three human pancreatic tissue arrays that included normal pancreatic tissue from 10 patients (18 spots) and chronic pancreatitis specimens from 48 patients (49 spots). The specimens of normal pancreas had a low-level, diffuse, cytoplasmic signal that was most noticeable within pancreatic islets (Fig. 9A). None of the normal pancreas specimens had evidence of membrane staining. The majority of the pancreatitis specimens (34/48) had multifocal, markedly increased Duox staining in both cytoplasm and membrane that was most noticeable in duct(ule)s (Fig. 9B). Minimal inflammation and low numbers of ducts, as well as sampling variability, were considered the probable reasons for negative results in some of the pancreatitis cases (data not shown). Increased staining in patients with pancreatitis was often closely associated with areas of inflammatory cell infiltrates (Fig. 10).

**Discussion**

Oxidative stress plays a critical role in modulating the innate immune response to inflammatory stimuli (38, 39). Further, there is increasing evidence that the source of at least some of the ROS...
that accompany acute and chronic inflammation in many organs is one or more epithelial members of the Nox family of oxidases (in addition to the Nox2 found in granulocytes and macrophages) (3, 4, 40). Thus, in the experiments reported in this article, we sought to evaluate in pancreatic cancer cells the mechanism(s) by which proinflammatory stimuli regulate the expression of Duox2, the Nox family member that supplies a substantive amount of the H2O2 used for host defense by airway mucosal cells, as well as organs of the gastrointestinal tract (17, 41).

In a previous study, we demonstrated that IFN-γ upregulated both Duox2 and its critical maturation factor DuoxA2 in a Stat1-dependent fashion that involved both the Jak and p38-MAPK pathways (24). Specific binding of Stat1 to the Duox2 promoter plays an essential role in the upregulation of Duox2 in BxPC-3 human pancreatic cancer cells, as well as the subsequent Duox2-related production of a substantive flux of H2O2. It was observed that pancreatic cancer lines, like certain other epithelial tumors, express TLRs (30) and are capable of signal transduction through TLR-related pathways previously thought to be an exclusive characteristic of immune cells (42). Furthermore, Ochi et al. (31) recently reported that LPS accelerated tumorigenesis in the pancreas and that inhibition of TLR4 signaling significantly decreased the development of inflammation-related pancreatic tumors in vivo. These studies support our evaluation of the effect of LPS alone and in combination with IFN-γ on the formation of ROS by cancer cells of pancreatic origin, as well as our examination of Duox2 expression during the growth of pancreatic tumor models in vivo and in the process of pancreatic inflammation in man.

We found that the combination of IFN-γ and LPS produced a substantially greater-than-additive effect on Duox2 expression in BxPC-3 (Fig. 1) and CFPAC-1 (Supplemental Fig. 1), but not AsPC-1 (Fig. 4), pancreatic cancer cells. The major increase in expression of Duox2 and DuoxA2 that we observed was accompanied by a significant enhancement of functional oxidase activity measured by extracellular H2O2 formation and intracellular ROS production (Fig. 1). Although IFN-γ increased the expression of activated Stat1 to a similar degree in all three cell lines, the addition of LPS did not lead to any further change in Duox2 levels in AsPC-1 cells compared with the BxPC-3 and CFPAC-1 lines (Fig. 4).

The divergent responses of these cell lines to cotreatment with LPS and IFN-γ prompted us to examine the underlying mechanisms that might explain these differences. Under baseline conditions, NF-κB is sequestered in the cytoplasm by the inhibitory activity of IκBα. The engagement of LPS to the TLR4 receptor results in the activation of IκBα kinases. Activated IκB kinases phosphorylate IκBα, leading to its ubiquitination and subsequent degradation. Once IκBα is degraded, NF-κB is released and translocates to the nucleus where it can bind to DNA sequences known as κB sites, resulting in the transcriptional activation of many inflammation-related genes (43). A critical role for the NF-κB pathway in the explanation of our findings is supported by the observation that pretreatment with inhibitors of NF-κB signaling, PDTC and BAY117082, significantly decreased Duox2 expression following exposure to IFN-γ and LPS (Fig. 5A). Furthermore, as shown in Fig. 4 and Supplemental Fig. 2, following IFN-γ priming, exposure to LPS in BxPC-3 and CFPAC-1 cells leads to degradation of IκBα and substantial translocation of NF-κB (p65) to the nucleus, whereas translocation of NF-κB (p65) to the nucleus.
is minimal in AsPC-1 cells under the same experimental conditions. It seems likely that this major difference in activation of NF-κB signaling may help to explain the absence of LPS-related enhancement of Duox2/DuoxA2 expression in the AsPC-1 line (Fig. 4D) versus that observed for BxPC-1 and CFPAC-1 cells.

The requirement for upregulation of TLR4-related signaling in the control of Duox2 expression by IFN-γ and LPS was substantiated by the RNA interference studies shown in Fig. 3; siRNAs targeting different sites on TLR4 decreased TLR4 expression and decreased Duox2 and DuoxA2 expression at the RNA level, as well as the expression of Duox protein, without altering the increase in activated Stat1 levels that we observed previously following exposure to IFN-γ alone (24). These results are consistent with prior studies demonstrating that LPS-induced reactive oxygen production in the THP-1 human monocyte line required TLR4-mediated signaling through the NF-κB pathway (44), as well as that a direct interaction between TLR4 and the Nox4 isoform is required to sustain LPS-mediated ROS generation and activation of NF-κB in HEK293 cells that express Nox4 (25). Our finding that exposure of BxPC-3 cells to IFN-γ and LPS leads to enhanced binding of NF-κB (p65) to the Duox2 promoter strongly suggests that two concomitant mechanisms interact to produce the marked increase in Duox2 expression that we observed (Fig. 1). LPS alone enhances p65 binding to the Duox2 promoter; the combination of LPS and IFN-γ increases p65 binding further. In complementary fashion, IFN-γ by itself appears to increase Duox2 expression by increasing the binding of Stat1 to the Duox2 promoter (24). We suggest that the combined transcriptional activation produced by p65 and Stat1 is responsible for the dramatic increase in Duox2 expression and function observed in these experiments.

Cytokine-induced Duox2 expression in BxPC-3 cells also significantly increased extracellular H2O2 production in the presence or absence of stimulation by ionomycin (Fig. 6). The H2O2 levels produced were of sufficient magnitude to decrease the proliferation of BxPC-3 cells by ~50% (Fig. 6E); decreased tumor cell growth was associated with a significant block in the G1 phase of the cell cycle and markedly enhanced apoptosis (Fig. 7A, 7B). In contrast, H2O2 production in the AsPC-1 line, which upregulates Duox2 following IFN-γ treatment to a lesser degree than BxPC-3 cells, was only modestly changed under these conditions (Fig. 6D) and was not associated with any change in proliferation. Exposure to IFN-γ and LPS also produced DNA double-strand breaks (as measured by an increase in phosphorylated γH2AX) in BxPC-3 cells that could be diminished by ROS scavengers (Fig. 7C). This degree of oxidant stress is consistent with a role for Duox2-mediated ROS in producing LPS-dependent injury to the extracellular matrix (30), as well as contributing to tumor cell genomic instability. These results also support recent studies suggesting that increased Duox2 expression in the intestinal epithelial cells of patients with Crohn’s disease and ulcerative colitis may have pathophysiologic significance (21).

We recently demonstrated that Duox2 can be highly expressed in human colorectal cancers in comparison with adjacent normal colonic tissue (14). Other investigators (45) reported a 10-fold increase in Duox2 expression in premalignant adenomatous polyps in the large intestine compared with adjacent normal tissue in the same individuals, further supporting a role for Duox2 in malignan transformation. A role for Duox2 in both human pancreatic cancer and chronic pancreatitis is supported by our in vivo experiments demonstrating the rapid upregulation of Duox2 expression in two human tumor cell lines (BxPC-3 and AsPC-1) grown as xenografts, but not for an IFN-γ-unresponsive line (MIA PaCa cells), as well as the increased staining for Duox2 in patients with chronic pancreatitis (Figs. 9, 10). Our results are concordant with an investigation by Fukushima et al. (46) that found Duox2 expression to be increased 13.6-fold in tissue adjacent to infiltrating pancreatic ductal carcinomas evaluated by mRNA expression–array analysis.

Finally, it is important to note that chronic pancreatic inflammation, in addition to being a premalignant condition, can result in inflammation-related pancreatic fibrosis, which can produce severe clinical consequences (1, 2). Recent studies in model systems suggest that inflammation can be interrupted by treatment with inhibitors of the Nox gene family, leading to the prevention of many of the ROS-related morphologic consequences of chronic inflammation in the pancreas (3).

In summary, our experiments demonstrate that the proinflammatory stimuli IFN-γ and LPS significantly enhance the transcription of Duox2 and DuoxA2, leading to the production of substantial amounts of extracellular H2O2 by human pancreatic cancer cell lines, and that H2O2 generation may depend on the activation of both Stat1- and NF-κB-signaling pathways. Furthermore, expression of Duox appears to be increased in human pancreatic cancer models in vivo and in chronic pancreatitis in man. In light of these data, support exists for the possibility that Duox2-related H2O2 production could provide an oxidative local milieu that enhances leucocyte recruitment and increases genetic instability and that is sufficiently proangiogenic to be conducive to malignant transformation (9, 47, 48). Our ongoing studies are focused on how an understanding of the pathophysiologic consequences of increased Duox2 expression in pancreatic cancer could lead to novel methods to interrupt proinflammatory cycles of oxidative injury in the gastrointestinal tract.

Disclosures

The authors have no financial conflicts of interest.

References


