HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2010 June 10.

Published in final edited form as:

Oncogene. 2009 December 10; 28(49): 4353–4363. doi:10.1038/onc.2009.289.

TLR4 signaling induced by lipopolysacharide or paclitaxel regulates tumor survival and chemoresistance in ovarian cancer

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Abstract

Toll-like receptors (TLRs) expressed on immune cells trigger inflammatory responses. TLRs are also expressed on ovarian cancer (OvCa) cells, but the consequences of signaling via the TLR4/MyD88 pathway in these cells are unclear. Here, TLR4 and MyD88 expression in OvCa tissues (n=20) and cell lines (OVCAR3, SKOV3, AD10, A2780 and CP70) was evaluated by RT-PCR, Western blots and immunohistochemistry. Cell growth, apoptosis, NF-κB translocation, IRAK4 and TRIF expression and cJun phosphorylation were measured following tumor cell exposure to the TLR4 ligands, lipopolysacharide (LPS) or Paclitaxel (PTX). Culture supernatants were tested for cytokine levels. TLR4 was expressed in all tumors, tumor cell lines and normal epithelium. MyD88 was detectable in tumor tissues and in 3/5 OvCa lines but not in normal cells. In $MyD88^{+}$ SCOV3 cells, LPS or PTX binding to TLR4 induced IRAK4 activation and cJun phosphorylation, activated the NF-κB pathway and promoted IL-8, IL-6, VEGF and MCP-1 production and resistance to drug-induced apoptosis. Silencing of TLR4 in SCOV3 cells with siRNA resulted in p-cJun downregulation and a loss of PTX resistance. In PTX sensitive, $MyD88^{neg}$ A2780 cells, TLR4 stimulation upregulated TRIF, and TLR4 silencing eliminated this effect. Thus, TLR4/MyD88 signaling supports OvCa progression and chemoresistance, promoting immune escape.

Keywords

ovarian cancer; paclitaxel; TLR4; MyD88

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INTRODUCTION

Epithelial ovarian cancer (EOC) has the highest mortality rate among patients with gynecologic malignancies (Brewer *et al*, 2003). Prognosis remains poor, because most cases of EOC are diagnosed at an advanced stage characterized by high grade tumors which invariably metastasize. Treatments for primary EOC include surgical cytoreduction followed by adjuvant chemotherapy generally with a paclitaxel (PTX) and cisplatin or carboplatin (Marupudi *et al*, 2007). Ovarian cancer is extremely sensitive to this drug combination, and most patients show an initial response to chemotherapy. However, chemoresistance is responsible for the high recurrence rate of ovarian cancer (Berek *et al*, 1999). While the mechanisms responsible for PTX resistance are not well understood, the prevailing hypothesis is that chemoresistant tumor variants acquire growth-promoting characteristics (Duan *et al*, 2003).

Microtubules are the best characterized cellular target of PTX (Blagosklonny and Fojo, 1999), which inhibits their depolymerization both *in vitro* and *in vivo*. In addition, PTX blocks the cell cycle in mitosis and induces activation of pro-apoptotic signaling (Taxman *et al*, 2003). In murine macrophages, PTX has been reported to mediate endotoxin/lipopolysacharide (LPS)-like effects, including production of TNF-α, phosphorylation of MAP kinases and the release of nitric oxide (Kawasaki *et al*, 2001). PTX was thus identified as a TLR4 ligand in murine macrophages (Takeda *et al*, 2003). A recent report suggests that development of resistance to PTX in human ovarian cancer may occur through the mechanism involving the TLR4 (Kelly *et al*, 2006).

Human TLRs comprise a large family of at least 11 proteins characterized by a member-specific activation and complex downstream signaling (Chuang and Ulevitch, 2001; Takeda *et al*, 2003; Takeuchi *et al*, 1999; Zhang *et al*, 2004). TLRs are predominantly expressed on various immune cells, serving as cell surface sensors for pathogens and promoting local inflammation. More recent studies report their expression on a broad variety of tissues, including the female reproductive tract (Girling and Hedger, 2007), tumor tissues and tumor cell lines, including ovarian cancer (Kelly *et al*, 2006; Szczepanski *et al*, 2009). While the TLR4 presence on tumor cells has been associated with the extent of inflammatory infiltrates (Backhed and Hornef, 2003; MacRedmond *et al*, 2005), the functional significance of TLR expression on tumor cells remains poorly understood. Bacterial LPS, the main TLR4 ligand, shares the receptor with PTX, despite the lack of a structural similarity between these two ligands (Byrd-Leifer *et al*, 2001; Fitzpatrick and Wheeler, 2003). TLR4 ligation with either LPS or PTX induces a range of effects in human tumor cells, depending on the cell type, culture conditions and ligand concentrations (He *et al*., 2007; Kelly *et al*, 2006).

TLR4 ligation activates at least two signaling pathways: a MyD88-dependent or a TRIF-dependent pathway (Tanimura *et al*, 2008). The former recruits TIRAP and MyD88 for NF-κB activation leading to the upregulation of anti-apoptotic signaling (Kreuz *et al*, 2004). The MyD88 independent pathway involves TRAM and instead of MyD88 recruits TRIF and TRAF3, leading to induction of IRF-3 (Interferon regulatory factor-3) and late NF-κB activation (Takeda *et al*, 2007). Despite considerable insights into the TLR4 signaling

pathway, biologic effects of TLR4 ligation by LPS or PTX in the tumor microenvironment remain controversial.

Here, we explore the mechanisms responsible for the regulation of cellular responses following binding of LPS or PTX to TLR4 in ovarian tumor cells. Investigating TLR4 expression in benign and malignant ovarian tumors *in situ* and its signaling in human ovarian cancer cell lines, we demonstrate that TLR4 plays a significant role in growth of ovarian tumor cells and their resistance to PTX.

MATERIALS AND METHODS

Reagents

Cell culture reagents, including RPMI 1640 medium, phosphate-buffered saline (PBS), heat-inactivated fetal calf serum (FCS), streptomycin, penicillin, recombinant Trypsin like enzyme (TripleSelect) and the Trypan-blue dye were purchased from Invitrogen. LPS was purchased from Sigma-Aldrich and PLX from Bedford Laboratories.

Tumor cell lines

Human ovarian cancer cell lines OVCAR-3, SKOV3, AD10, A2780 and CP70 were provided by Dr. S. Khlief, NIH, Bethesda, MD and were cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS, 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2 in air. Tumor cell lines were tested for *Mycoplasma* and confirmed to be negative.

Samples

Samples of benign tumors (n=3), borderline tumors (n=4) and ovarian cancer (n=20) were collected with the approval of the Ethics Committee of the University of Medical Sciences in Poznan, Poland.

RNA isolation and RT-PCR

Total RNA was isolated from cancer cells as previously described (Szczepanski *et al*, 2009) using Trizol reagent (Invitrogen) and reverse transcribed to cDNA. cDNA transcripts were then amplified using the following primers specific for TLR4:F:5′-CTGCAATGGATCAAGGACCA-3′, R : 5 ′-TCCCACTCCAG GTAAGTGTT-3′ (Lauzon *et al*, 2006). PCR reaction was performed using the following conditions: denaturation at 95°C for 45s, annealing at 60°C for 45s, and extension at 72°C for 60s.

Immunostaining

Tumor cells deposited on glass slides were fixed in 2% (w/v) paraformaldehyde in PBS for 15 min, permeablized with 0.1% (w/v) Triton X in PBS, washed and blocked with 2% (w/v) bovine serum albumin (BSA; Sigma) in PBS for 4 min. Polyclonal goat anti-human TLR4 antibodies (Abs) (Santa Cruz Biotechnology; 1:100) or polyclonal rabbit anti-human MyD88 Abs (Cell Signaling Technology; 1:100) served as primary reagents. Cy3 (1:500)- or FITC (1:200)- labeled donkey anti-rabbit IgG served as secondary Abs, (Jackson ImmunoResearch). To eliminate non-specific Ab binding, slides were incubated in 10%

normal donkey serum for 1 h prior to incubation with the primary Abs for 1 h at room temperature (RT) in a moist chamber. Slides were then washed in PBS and incubated with the secondary Abs for 45 min at RT in the dark. In control samples, primary Abs were omitted or goat serum was used instead of primary Abs. Sections were mounted in a medium with DAPI (Vector Laboratories) to visualize cell nuclei. Slides were evaluated with the Olympus Provis (Olympus Japan) fluorescence microscope under 400 × magnification, and Adobe Photoshop 6.0 was used for digital image analysis.

Immunohistochemistry

Paraffin sections of tumor tissues were deparaffinized, microwaved while immersed in 0.01 M citrate buffer pH 6.0 for 20 min, washed with PBS, and incubated overnight at 4°C with polyclonal rabbit anti-human TLR4 Abs (Abcam; 1:50) or with polyclonal goat anti-human MyD88 Abs (Abcam; 3 μg/ml). After washing, tissues were incubated with horseradish peroxidase (HRP)- labeled anti-rabbit or anit-goat Ab, respectively, for 1 h, followed by 3, 3'diaminobenzidine (DAB; Dako). Tissue section were counterstained with hematoxilin (Sigma) and mounted in glycerol jelly. In control sections, primary Abs were omitted. The IHC results for TLR4 and MyD88 expressed in tissues were scored by two-independent investigators (MS, EE) based on the level of staining intensity as none (−), weak (+), moderate (++), or strong (+++).

Incubation of tumor cells wit TLR4 ligands

In all experiments testing effects of LPS or PTX on tumor cells, LPS was used at the concentration of 10 μg/ml and PTX at 1 or 20 μM.

Annexin V binding

A flow-based Annexin V (ANX-V) assay was used to measure ovarian tumor cell apoptosis as previously described (Martin *et al*, 1995). Briefly, cells were treated with PTX, LPS or medium for 24 h, washed in PBS, resuspended in ANX V-binding buffer and stained with 1 ug/mL FITC-conjugated ANX V (Sigma) for 15 min on ice in the dark. Tumor cell apoptosis was evaluated by flow cytometry, gating on forward angle scatter (FSC) and side angle scatter (SSC) to identify ANX V+ cells as previously described (Szczepanski et al, 2009).

CFSE-based proliferation assays

Tumor cells were labeled with 1.5 μM CFSE (carboxyfluorescein diacetate succinimidyl ester; Invitrogen) and stimulated with LPS or PTX. Cells were incubated for 5 days and CFSE dilution was measured by flow cytometry. The data were analyzed using the ModFit LT for Win32 software provided by Verity Software Housen, Inc., Topsham, ME, USA. The program calculates the proliferation index (PI) based on the dilution levels of CFSE detected in dividing cells (Strauss *et al*, 2007).

Cytokine and chemokine production

Cytokines and chemokine production by tumor cells was determined using the immunobead-based multiplex assays (Luminex®). Supernatants of tumor cells seeded in 12-well plates at

5×10^5 cells/well in 1 mL of medium ± LPS or ± PTX were collected after 48 h incubation. The levels of IL-1β, IL-2, IL-4, IL-6, IL-8, GM-CSF, VEGF, fibroblast growth factor (FGF), IP-10, MIG-1, RANTES, MCP-1 and MIP-1 were measured using panels of capture Ab-coated beads and the labeled detection Abs, which were pre-tested and qualified by the manufacturer to ensure the absence of cross-reactivity. The assay sensitivity varied from 5 to 15 pg/mL.

Western blots analysis

Ovarian cancer cells were stimulated with LPS or PTX for different periods of time and lysed in the presence of a protease inhibitor cocktail (Pierce Chemical Co). Proteins separated by SDS-PAGE were electrotransfered to polyvinylidene difluoride (PVDF) membranes. The following Abs: anti-MyD88, anti-cJun, anti-IRAK 4, anti-TRIF (all from Cell Signaling Technology) and anti-TLR4 (Abcam) were used for detection, and horseradish peroxidase-conjugated secondary antibodies (Pierce Chemical Co; 1:150,000 dilution) for development of reactions in a chemiluminescent detection system (Pierce Chemical). β-actin antibodies were used as controls for equal protein loading.

NF-κB assays

Cancer cells were plated overnight in 96-well plates at 15×10^3 cells per well. Following 2 h incubation with LPS or PTX, cells were fixed and stained for translocation of the NF-kB p65 subunit using rabbit anti-p65 Abs (Santa Cruz Biotechnology) and FITC-labeled donkey anti-rabbit IgG. Cells were counted and stained with Hoechst 33342 dye and the cells analyzed in a Cellomics ArrayScan HCS reader as previously described (Siervo-Sassi *et a.*, 2003). To measure the p65 subunits binding activity, nuclear and cytoplasmic extracts were prepared using a TransFactor extraction kit (Clontech). DNA binding by p65 was detected by ELISA using a TransFactor Prolfiling kit (Clontech) (Bayry *et al*, 2004). To visualize translocation of the p65 subunit of NF-κB to cell nuclei ± LPS or ± PTX, tumor cells (1×10^4) stained using rabbit anti-p65 Abs were examined by confocal microscopy as described (Szczepanski *et al*, 2009). Cells with evidence of NF-κB p65 translocation were counted by examining at least 200 tumor cells in an inverted Olympus Fluoview 1000 laser scanning confocal microscope under an oil immersion objective.

Silencing of TLR4 using siRNA

TLR4 expression was temporarily silenced in tumor cells using small interference RNA (siRNA) as previously described (Eskan *et al*, 2008; Huang *et al*, 2005). To determine the optimal working concentration of TLR4 siRNA, 2×10^5 cells were seeded in wells of 6-well plates, cultured in medium, harvested at confluence and resuspended in the transfection medium containing increasing siTLR4 concentrations (4μl, 8μl, 10μl, or 12μl). For TLR4 silencing, cells were incubated with siRNA (8–10 μl) for 6 h in an atmosphere of CO_2 in air at 37°C and then allowed to recover in medium for 24 h. Scrambled siRNA, with no homology to known human sequences was used as control. The transfected cells were treated with LPS or PTX as described above. In parallel cultures, the frequency of transfected cells was evaluated by fluorescence microscope, using non-targeting siRNA-FITC. Cultures with transfection efficiency >80% were used for further studies. Viability of

transfected tumor cells was tested by trypan-blue exclusion, and the expression of targeted genes in tumor cells was tested on day 4, 8 and 12 by RT-PCR and Western blots.

Statistical analysis

Data were summarized by descriptive statistics (the mean and standard deviation for continuous variables; the frequency and percentage for categorical variables). All statistical analyses were performed using the Student's t test, and p values< 0.05 were considered significant

RESULTS

Expression of TLR4 and MyD88 in ovarian cancer tissues and cell lines

Expression of TLR4 was assessed *in situ* on paraffin sections of normal ovarian tissue adjacent to tumor (n=10), benign cysts (n=3), borderline tumors (n=4) and malignant tumors (n=20). Table 1 summarizes the pathologic characteristics of tissues. Figure 1 presents the representative IHC results for TLR4 and MyD88 expression in tissues. TLR4 was found to be expressed in normal ovarian epithelium (Figure 1A1), benign cysts (Figure 1A2), borderline tumors (Figure 1A3) and malignant tumors (Figure 1A4). However, the intensity of staining for TLR4 in malignant tumors was considerably greater than that in normal tissues or benign tumors. Normal ovarian epithelium did not express TLR signaling adapter protein, MyD88, (Figure 1A5). A variably strong or weak signal was observed in cancer tissues. The TLR4 and MyD88 expression was next evaluated in human ovarian cancer cell lines: OVCAR3, SKOV3, AD10, A2780 and CP70. Positive immunoreactivity for TLR4 was observed in all of the evaluated cell lines by staining of cell smears (Figure 1B2) and by Western blots or RT-PCR (Figure 1C). No staining was observed when IgG1 was used as a negative control (Figure 1B1). SKOV3, OVCAR3 and AD10 cells were $MyD88^{+}$, whereas A2780 and CP70 were $MyD88^{neg}$ as demonstrated by immunofluorescence (Figure 1B3 and Figure 4). Western blots further confirmed the expression of MyD88 in OVCAR3, SKOV3 and AD10 cells, but not in A2780 and CP70 cells (Figure 1C).

Two ovarian cancer cell lines A2780 and SKOV3 were chosen for further studies. The A2780 cell line was derived from a primary untreated and PTX-sensitive cancer (Behrens *et al*, 1987). In contrast, the SKOV3 cell line was obtained from the ascites of a patient with advanced, metastatic ovarian cancer. SKOV3 is resistant to most cytotoxic drugs (Cuello *et al*, 2001).

LPS induces proliferation in SKOV3 but not A2780 cells

Tumor cells (1×10^4 cells/well) were labeled with CFSE, incubated with increasing concentrations of LPS (0 to 15 μg/mL) for 5 days and analyzed for proliferation. As shown in Figure 2A, *left*, LPS used at the optimal concentration of 10 μg/mL induced proliferation of SKOV3 cells (PI=12.9) relative to untreated cells (PI=8.3) but had no effect on proliferation of A2780 cells. In contrast to LPS, PTX inhibited proliferation in both cell lines (Figure 2B, *left*). In the presence of PTX the cell viability decreased as the dose of PTX increased (1–20 μM). A2780 cells were found to be significantly more sensitive to PTX than SKOV3 (Figure 2B, *right*).

ANX-V binding to tumor cells incubated with LPS or PTX

ANX-V binding to tumor cells before and after exposure to LPS and PTX was measured to evaluate apoptosis. In control cultures, spontaneous apoptosis ranged from 2 to 8%, and it remained unchanged after stimulation with LPS alone (Table 2). PTX induced significant apoptosis levels in A2780 cells after 24 h and 48 h incubation. In contrast, SKOV3 cells were relatively resistant to PTX- induced apoptosis (Table 2).

NF-κB signaling

Translocation of the p65 subunit of NF-κB to cell nuclei, and NF-κB p65 binding activity were evaluated within 2 h of tumor cells stimulation with LPS or PTX. The baseline translocation of p65 subunit (Figure 3A) occurred in 23 ± 5% (mean ±SD) SKOV3 cells, and in 36 ± 9% A2780 cells. After LPS or PTX addition, the percentage of SKOV3 cells with the p65 subunit translocation significantly increased (Figure 3B). Also, the mean fluorescence intensity (MFI) of nuclear NF-κB was significantly increased in SKOV3 cells after exposure to LPS or PTX, whereas no changes were observed in A2780 cells (Figure 3C). Binding activity of p65 to cellular DNA in SKOV3 cells further indicated that LPS or PTX induced activation of the NF-κB pathway (Figure 3D). In contrast, no NF-κB activation was observed in A2780 cells upon LPS or PTX addition (Figure 3B–D).

TLR4 signaling pathways in ovarian carcinoma cell lines

The observed differential responses of the two cell lines to LPS or PTX signaling could reflect the presence or absence of MyD88 in tumor cells (Kelly *et al*, 2006). As shown in Figure 1E SKOV3 cells constitutively expressed MyD88, while A2780 cells did not. In addition, signaling downstream of TLR4 in response to LPS or PTX was found to be different in the two cell lines (Figure 5). SKOV3 cells constitutively expressed high levels of IRAK4, and activation by LPS or PTX slightly increased its expression. In A2780 cells no signal for IRAK4 was detectable before or after activation with LPS or PTX. Strongly increased expression of phosphorylated cJun upon LPS or PTX binding to TLR4 was observed only in SKOV3. In A2780 cells only TRIF was up-regulated upon the ligation of TLR4. These data suggest that the LPS- or PTX-induced signals are processed differently in A2780 vs. SKOV3 cells. In PTX-sensitive A2780 cells, both LPS-mediated and PTX-mediated signals were TRIF-dependent but cJun independent, while in PTX-resistant SKOV3 cells, both types of signals engaged cJun (Figure 5).

LPS and PLX induce cytokine production in SKOV3 but not A2780 cells

Supernatants of both cell lines exposed to LPS or PTX for 48 h were analyzed for levels of inflammatory cytokines and growth factors. SKOV3 cells constitutively secreted a wide range of cytokines and chemokines including: IL-6, IL-8, VEGF and MCP-1. In contrast, A2780 did not produce or produced low levels of these cytokines. LPS significantly increased secretion of IL-6, IL-8, VEGF and MCP-1 in SKOV3 cells but not in A2780 cells (Figure 4). Similarly, PTX resulted in a significant up-regulation of IL-6, IL-8, VEGF and MCP-1 in SKOV3 cells but not in A2780 cells (Figure 4). Levels of other cytokines (IL-1β, IL-2, IL-4, GM-CSF, FGF, IP-10, MIG-1, RANTES, and MIP-1) were not significantly up-regulated in LPS or PTX-treated SKOV3 or A2780 cells.

Effects of TLR4-specific siRNA

To further probe the role of TLR4 in cancer cells, SKOV3 and A2780 cells were pre-treated with siRNA specific for TLR4 or scrambled siRNA. Tumor cells silenced by siRNA lacked the expression of TLR4 at the mRNA and protein levels for at least 8 days when tested by RT-PCR and Western blots on day 4, 8 and 12 (Figure 6A). The effects of gene silencing on ANX-V binding evaluated on day 4, consistently showed that SKOV3 cells became highly sensitive to PTX after TLR4 silencing. In contrast, PTX-sensitive A2780 cells, showed no change in the frequency of ANX-V^+ cells after TLR4 silencing (Figure 6B). Cytokine production in response to LPS and PTX stimulation was significantly inhibited in SKOV3 cells in the presence of TLR4-specific siRNA (compare Figure 6C to Figure 4). No changes in cytokine production were observed in A2780 cells.

TLR4 silencing was accompanied by the significant ($p<0.05$) down-regulation of phosphorylated cJun expression in SKOV3 cells at 30 and 60 min after treatment with LPS or PTX (Figure 6D). In contrast, no such change was observed in A2780 cells. There was no significant change in expression of IRAK4 in either cell line; however, TRIF up-regulation evident in LPS and PTX-treated A2780 cells, was no longer evident following TLR4 silencing. This confirms that the TRIF pathway is dependent on TLR4 signaling in A2780 cells. The data suggest that PTX mediates its biologic effects through TLR4 activation in SKOV3 cells and in A2780 cells.

DISCUSSION

The presence of TLRs on cancer cells has been reported only recently (Kelly *et al*, 2006; Szczepanski *et al*, 2007), and the role of these receptors in cancer progression and resistance to therapy remains questionable, because of inconsistent results available for distinct members of the TLR family of receptors in various tumor types (Fukata and Abreu, 2008; He *et al*, 2007; Molteni *et al*, 2006). In respect to TLR4, evidence supporting its involvement in cancer growth and sensitivity to apoptosis mediated by drugs or immune cells is available for ovarian carcinoma (Kelly *et al*, 2006), head and neck cancer (Szczepanski *et al*, 2009), and lung cancer (He *et al*, 2007). In ovarian carcinoma, Kelly and colleagues (2006) first reported that the TLR4 expression is related to PTX resistance. Our study using two TLR4+ cell lines, A2780 and SKOV3, derived from primary and metastatic tumors, respectively, shows that TLR4 signaling promotes tumor growth and drug resistance in metastatic ovarian carcinoma but not in primary tumors.

In SKOV3 cells, TLR4 was functional, as LPS induced proliferation, activation of NF-κB, p65 binding to DNA and production of pro-inflammatory cytokines, (IL-6, IL-8) and VEGF as well as chemokine MCP-1. In A2780 cells, LPS induced an increase in TRIF expression after 30 min, and more clearly after 60 min, suggesting that TLR4 signaling in these cells is also dependent on LPS. The two cell lines differed in their response to PTX, which has been reported to signal via TLR4 (Byrd-Leifer *et al*, 2001; Kelly *et al*, 2006). PTX induced TRIF up-regulation and death in A2780 cells, while SKOV3 cells were resistant to the drug. Thus, in A2780, but not in SKOV3, TLR4 was responsive to PTX signaling. The mechanism(s) responsible for PTX resistance in ovarian cancer is not completely understood (Duan *et al*, 1999) and may involve a loss of p53 function (Petty *et al*, 1998). SKOV3 cells which lack

the p53 gene (Cuello *et al*, 2001) are resistant to DNA-damaging agents and unable to initiate apoptosis (Cimoli *et al*, 2004; Hollstein *et al*, 1997), while A2780 cells which express wt p53 are chemosensitive. A2780 cells become resistant to cytotoxic drugs after transfection with a mutated p53 (Cimoli *et al*., 2004; Gallardo *et al*, 1996). However, the p53 status alone is not the only determinant of sensitivity/resistance to cytotoxic drugs. Because the understanding of cellular and molecular mechanisms by which PTX exerts its effect on ovarian cancer is of critical clinical importance, we tested the hypothesis that ovarian carcinoma sensitivity to PTX depends on the integrity of signaling via TLR4.

It has been reported that MyD88, is an essential down-stream component of the TLR4 signaling cascade mediating PTX resistance (Jordan and Wilson, 2004; Kelly *et al*, 2006). In our study, SKOV3 cells, which are resistant to PTX, expressed MyD88, up-regulated IRAK-4 expression and mediated cJun phosphorylation upon exposure to PTX. Moreover, in SKOV3 cells, PTX signaling via TLR4 induced the NF-κB pathway activation and nuclear translocation associated with the increased transcription of a number of different genes and production of pro-inflammatory cytokines. These effects of NF-κB activation in tumor cells have been previously linked to upregulation of anti-apoptotic protein expression and an increase in cell proliferation (Landen *et al*, 2008). It is thus possible to speculate that *in vivo*, utilization of TLR4 by cancer cells, leading to NF-kB activation, and proinflammatory cytokine production allows them to progress and metastasize and acquire resistance to chemotherapy. In contrast, in A2780 cells, which do not express MyD88, the lack of this adaptor protein caused the engagement of the TRIF pathway in response to TLR4 stimulation. However, A2780 cells remain sensitive to PTX, suggesting that the induction of apoptosis by PTX is independent of TRIF signaling and is probably mediated via the interaction of PTX with an alternative receptor, (Wang *et al*, 2006), which is fully functional in the absence of TLR4 signaling following its siRNA silencing.

If TLR4 signaling provides a survival benefit to tumor cells and alters their sensitivity to PTX, then its silencing via siRNA should help in identifying the molecular mechanisms responsible for LPS- and PTX-mediated effects. Indeed, silencing of TLR4 expression in SKOV3 resulted in sensitization of SKOV3 cells to PTX-induced apoptosis, and this sensitization was accompanied by the inhibition of cytokine production in response to PTX and LPS. Among the downstream MAP kinases activated by LPS or PTX in SKOV3 cells, cJun appears to play an important role in ovarian cancer resistance to chemotherapy. The loss of cJun phosphorylation upon PTX binding to TLR4 was associated with a loss of resistance to PTX in SKOV3 cells. Thus, up- or down-regulation of cJun phosphorylation downstream of TLR4 appears to be a major molecular mediator of PTX-induced as well as LPS-induced signaling. The effects of TLR4 silencing on SKOV3 but not A2780 cells suggest that this signaling pathway is utilized by metastatic tumor cells to promote their own survival. The development of PTX resistance appears to be associated with the expression of functional TLR4 in SKOV3 cells. We show that cJun phosphorylation is one of the signals downstream of TLR4 that participates in developing PTX resistance in ovarian carcinoma.

Interestingly, activation of cJun has been reported to be a major stimulator of the IL-6 gene regulation (Wang *et al*, 2006), linking molecular with cellular effects of PTX signaling via TLR4. In many human cancers, IL-6 production and its increased circulating levels have

emerged as a biomarker of poor prognosis (Penson *et al*, 2000). In ovarian cancer, increased levels of IL-6 in patients' sera were linked to tumor progression (Hefler *et al*, 2003), resistance to apoptosis (Yusuf et al, 2003) and chemoresistance (Duan et al, 1999a). We have observed significantly increased production of IL-6 by SKOV3 cells after PTX and LPS binding to TLR4. In addition to IL-6, PTX induced the release of other cytokines in SKOV3 cells, which might be considered as tumor promoting factors. IL-8 has been reported to be overexpressed in ovarian cancer and its level associated with decreased patient survival and poor clinical outcome (Kassim *et al*, 2004; Lokshin *et al*, 2006). VEGF, in addition to inducing angiogenesis, is also an immunosuppressive cytokine which contributes to promoting ascites formation through stimulation of vascular permeability and promoting angiogenesis (McClure *et al*, 1994; Senger *et al*, 1983). The production of these cytokines after PTX binding to TLR4 in SKOV3 suggests that this mechanism operative *in vivo* could significantly contribute to tumor cell survival and their chemoresistance.

Our data suggest that TLR4 signaling represents a potential therapeutic target for ovarian cancer. A better understanding of the molecular mechanisms contributing to drug resistance is necessary for the development of novel strategies that are most likely to eliminate drug resistance and thus improve prognosis and survival of patients with ovarian cancer.

ACKNOWLEDGMENTS

We thank B. Hilldorfer for technical assistance and The Centre for Biologic Imaging at the University of Pittsburgh for assistance in performing confocal microscopy. This study was supported in part by NIH grants PO1 CA109688 (TLW) and NO1-HB37165 (TLW) and by Heidi L. Browning Ovarian Cancer Research Scholar Fund. Dr. Szajnik was supported by the fellowship from the PACT Program of the National Heart Lung and Blood Institute (NHLBI).

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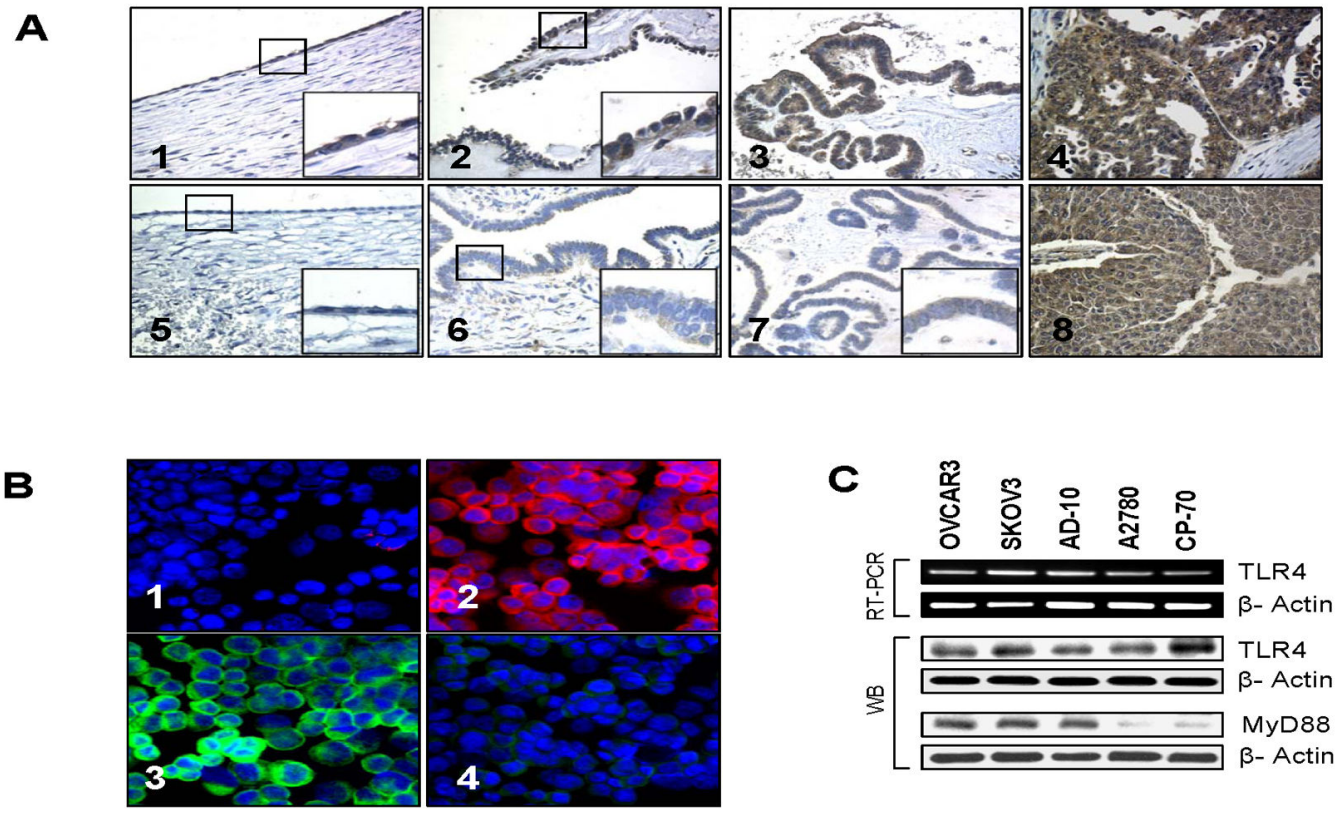

Figure 1. TLR4 and MyD88 expression in ovarian tumor tissues and cell lines

A) Immunostaining for TLR4 in a normal ovarian tissue adjacent to tumor (1), benign tumor (2), borderline tumor (3) and ovarian cancer (*Adenocarcionoma serosum* FIGO III) (4) (Mag. × 200, Inset × 600); Immunostaining for MyD88 in ovarian tissues adjacent to tumor (5), benign tumor (6), borderline tumor (7), ovarian cancer (*Adenocarcionoma serosum* FIGO III) (8) (Mag. × 200, Inset × 600. **B)** Immunofluorescence for TLR4 protein in an ovarian cancer cell line (SKOV3) (2) Negative staining for TLR4 using isotype control IgG (1). Immunofluorescence for MyD88 in ovarian carcinoma cell lines, SKOV3 (3) and A2780 (4) (× 400); **C)** Expression of TLR4 mRNA and Western blot for TLR4 and MyD88 in ovarian cancer cell lines.

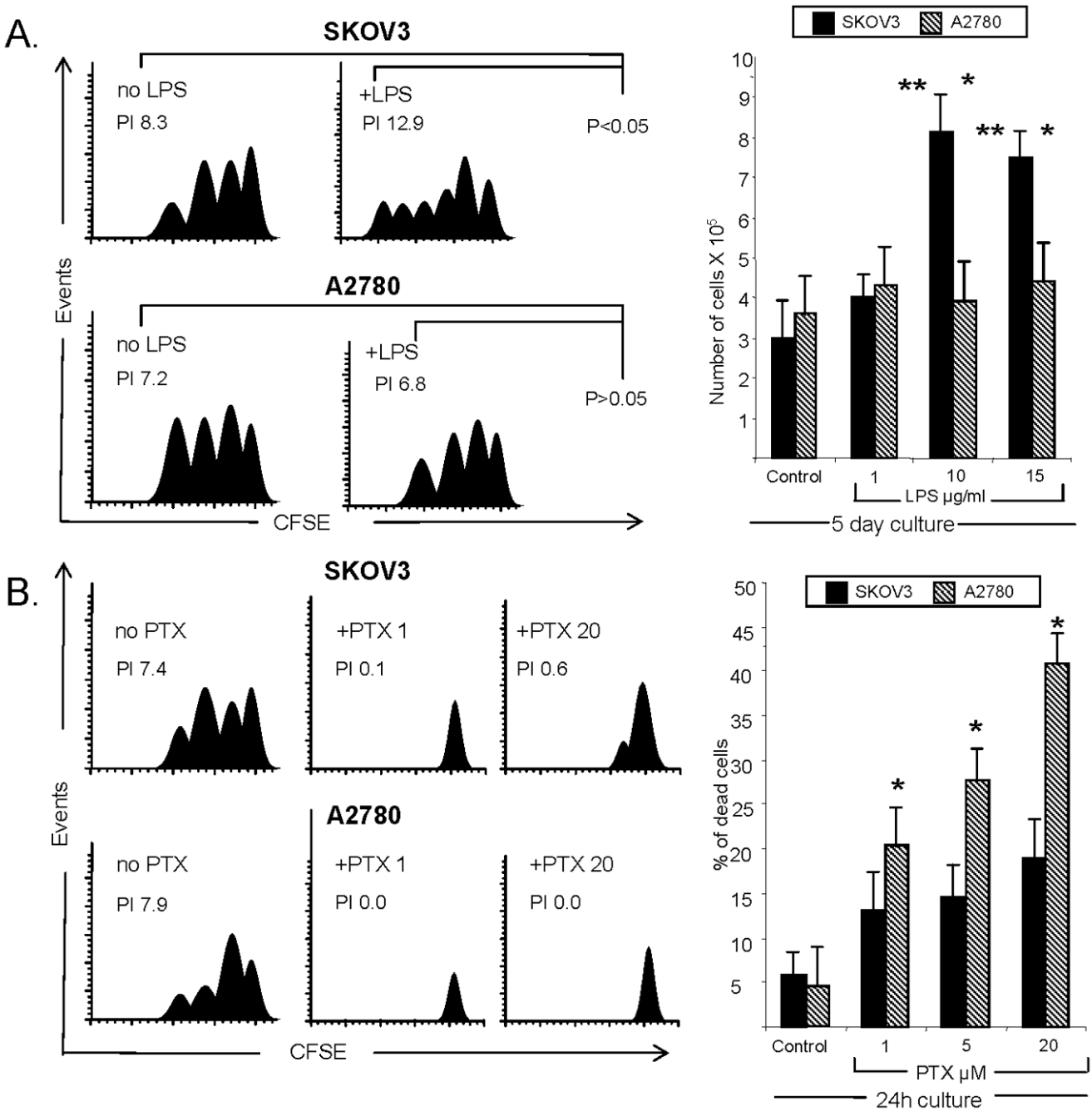

Figure 2. Proliferation of tumor cells in response to LPS

A) SKOV3 and A2780 cells were labeled with CFSE and incubated with 1–15 µg/mL of LPS (A) or with PTX (1–20 µg/mL) (B) for 5 days, at which time, the proliferative index (PI) in (A) or cell death in (B) were evaluated. Control cultures contained no LPS or no PTX. *Left*, one representative CFSE proliferation experiment of five performed is shown. *Right*, the data are mean cell counts ± SD from three independent experiments performed with each cell line. Single asterisk indicates significant differences ($p<0.05$) between two cell lines. The double asterisk indicates differences between experimental and control

cultures. PI=proliferative index. **B)** The same CFSE-labeled cell lines were incubated with PTX at the concentration of 1 and 20 μM

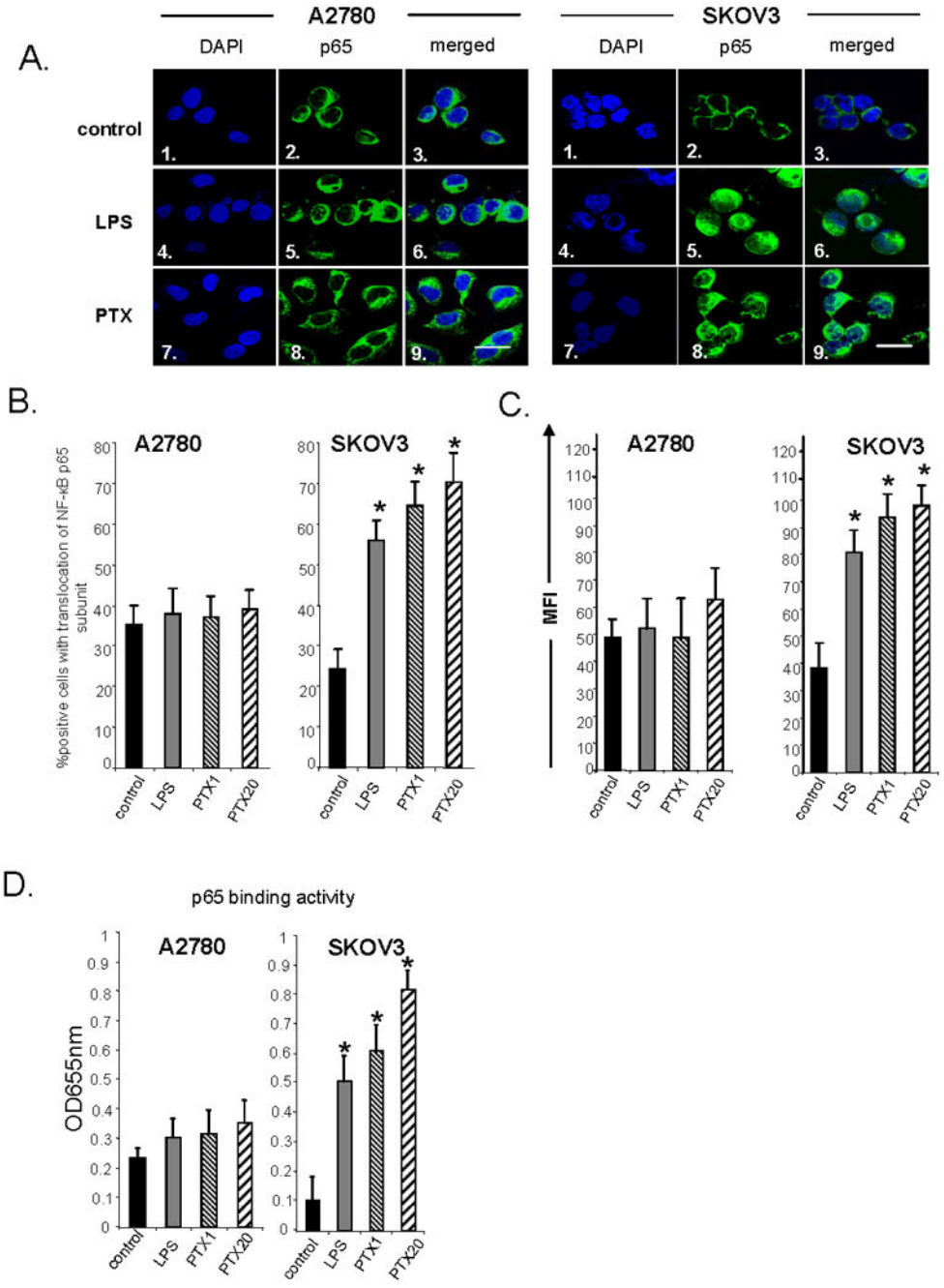

Figure 3. Effects of LPS or PTX on NF-κB activation in ovarian cancer cells

A) Tumor cells plated overnight were treated with LPS (10μg/mL) or PTX (1 or 20 μM) for 2 h and then stained as described in Materials and Methods. 1 to 3, control cells in medium only. In 1, 3, 4, 6, 7 and 9 nuclei are stained blue with DAPI; in 2, 5 and 8, the p65 subunit of NF-κB in the cytosol is stained green; 3, 6 and 9 are overlays showing that the p65 subunit has not translocated to the nucleus. Bar= 20μm. **B)**. Mean percentages ± SD of the cells with translocation of the NF-κB p65 subunit. At least 200 cells were randomly counted. Results are representative of three independent experiments performed with each

cell line. **C)**. Mean fluorescence intensity (MFI) of nuclear NF-κB expression was determined in ovarian cancer cell lines that were either untreated or treated with LPS (10 μg/mL) or PTX (1 or 20 μM). Results are mean MFI ± SD of three independent experiments performed with each cell line. **D)**. The p65 subunit binding activity to nucleus in ovarian cancer cells untreated or treated with LPS or PTX. Asterisks indicate significant increase of p65 binding activity at $p<0.05$ in tumor cells stimulated with LPS or PTX.

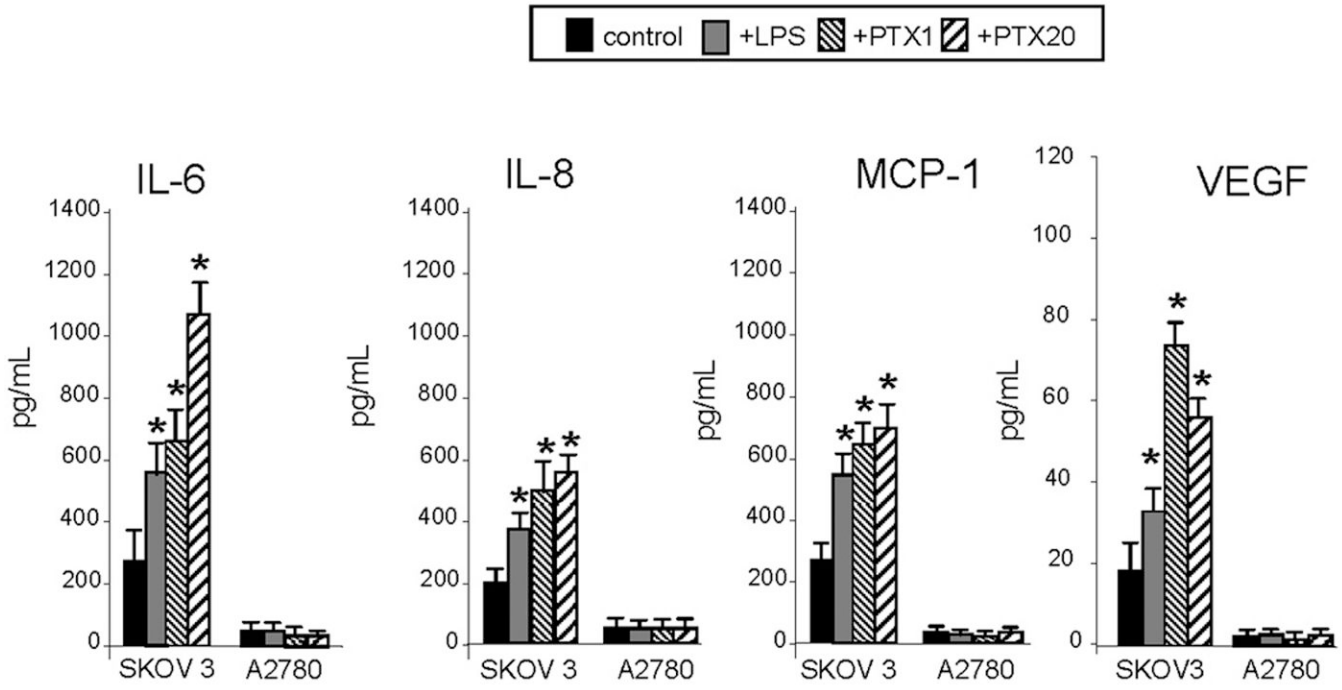

Figure 4. Levels of cytokines in tumor cell supernatants (SN)

SNs were collected from tumor cells cultured at density 5×10^5 cells/well after 48 h of treatment and tested for levels of various cytokines as described in Materials and Methods. Three independent experiments were performed. Asterisks indicate differences ($p<0.05$) between experimental and control cultures.

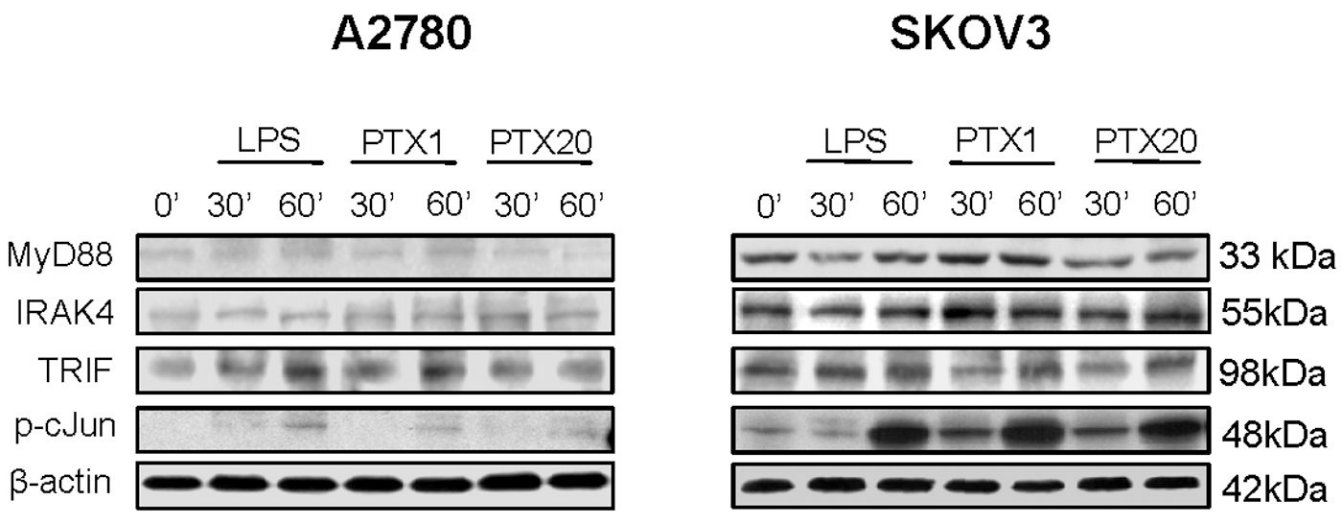

Figure 5. Alteration in TLR4 signaling in ovarian carcinoma cell lines treated with LPS or PTX

Western blot of the cells which were either untreated or treated with LPS or PTX for 30 and 60 min. Strong up-regulation of p-cJun was observed only in SKOV3 cells treated with LPS or PTX but not in A2780 cells, where only the TRIF pathway is activated in response to LPS or PTX signaling via the TLR4.

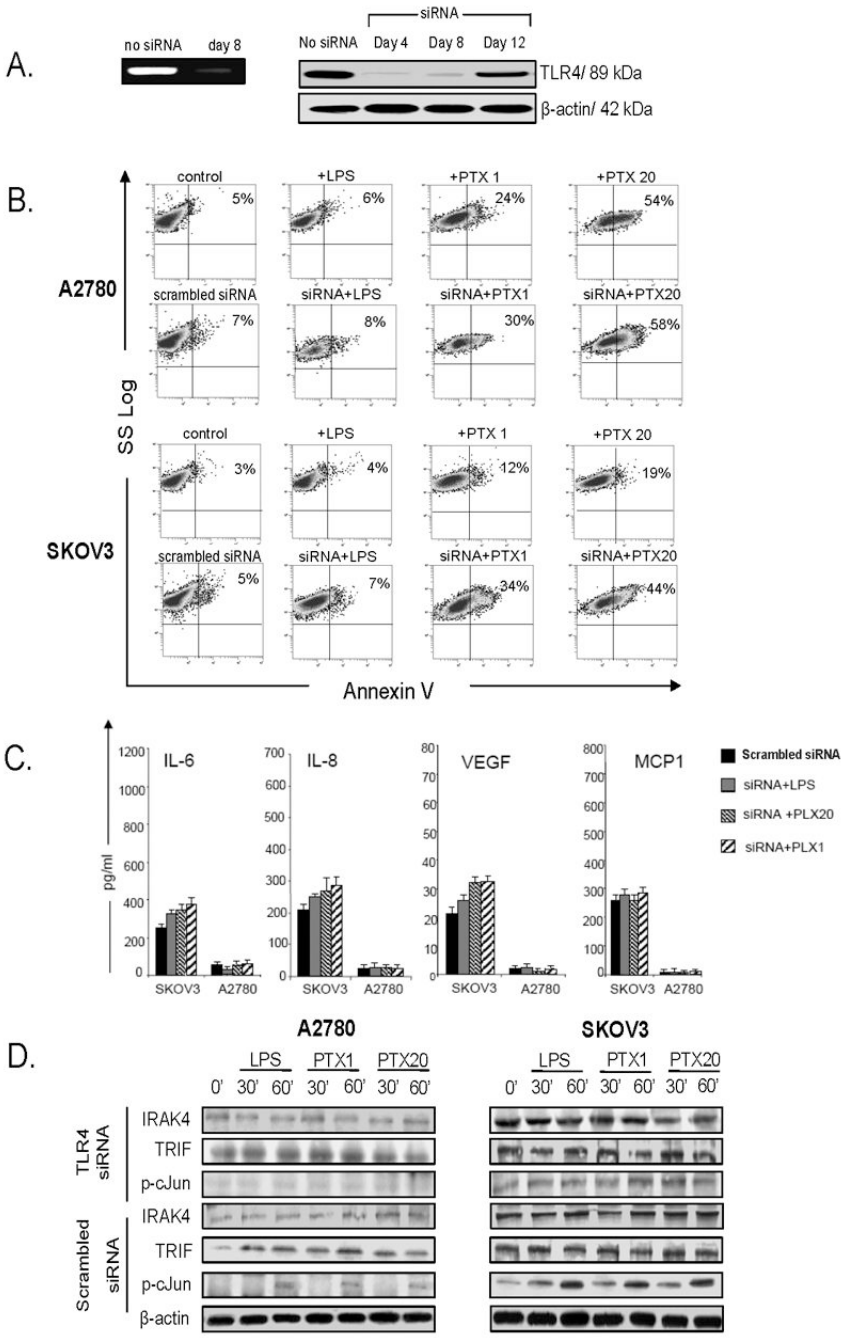

Figure 6. Silencing of TLR4 using siRNA specific for TLR4

A) TLR4 was silenced for at least 8 days as confirmed by RT-PCR and Western blot assays. **B)** Tumor cell apoptosis was detected by ANX V binding and flow cytometry. **C)** Levels of cytokines in tumor cell lines treated with scrambled siRNA or siRNA specific to TLR4 and then with LPS or PTX. The data are means ± SD from 3 experiments performed with each cell line. Asterisks indicate differences ($p<0.05$) between experimental and control cultures. **D)** TLR4 signaling pathway in ovarian cancer cells treated with control siRNA or siRNA specific to TLR4 and LPS or PTX. Tumor cell silenced with TLR4/siRNA or control siRNA

were treated with LPS or PTX for 30 or 60 min. No changes were observed in cJun in both cell lines. The data shown in A, B and D are from one representative experiment of 3 performed.

Table 1

Expression of TLR4 and MyD88 in ovarian cancer, benign tumors and normal controls[a]

Definite histology		FIGO[b]	Grade	TLR4	MyD88
Carcinomas n=20	*serous*	Ic	G3	+	+
		Ic	G3	+	++
		IIa	G3	+++	++
		IIIa	G3	+++	+
		IIIb	G3	++	++
		IIIb	G2	++	+
		IIIb	G3	++	++
		IIIb	G3	+++	++
		IIIc	G3	++	+++
		IIIc	G3	++	++
	endometrial	Ia	G3	+++	+
		IIa	G3	+++	+
		IIc	G3	++	+
		IIIa	G3	++	++
		IIIa	G1	+++	++
		IIIb	G2	++	+++
		IIIb	G1	+++	++
	clear cell	Ic	G3	++	+
		IIb	G3	+	++
	undifferentiated	IV	G3	+++	+++
Borderline tumors n=4	*serous*	Ia	NA	++	+
	serous	Ia	NA	+	-
	serous	IIa	NA	+	+
	mucinous	Ic	NA	++	−
Benign cysts n=3	*serous*	NA	NA	++	−/+
	serous	NA	NA	+	−
	serous	NA	NA	++	−

Definite histology		FIGO[b]	Grade	TLR4	MyD88
Normal ovary n=10	NA	NA	NA	+/++	−

[a] Immunohistochemistry results for TLR4 and MyD88 expression in ovarian cancer, borderline tumors, benign cysts and normal controls.

[b] Federation International of Gynecology and Obstetrics

Table 2

Apoptosis of ovarian cancer cells after LPS or PTX treatment [a]

	A2780		SKOV3	
	24h	48h	24h	48h
	% ANX V$^+$ cells			
Control	5 ±3	6 ±2	3 ±2	6 ±2
LPS	6 ±4	7 ±3	4 ±1	6 ±2
PTX1	23 ±7	44 ±11	12 ±5	18 ±8
PTX20	54 ±10	86 ±9	19 ±7	26 ±8

[a] Ovarian cancer cells were treated with LPS (10 ug/ml) or PTX (1 or 20 μM) for 24 h or 48 h, washed and examined for ANX V binding as described in Materials and Methods. The data are mean percentages of ANX V$^+$ cells ± SD obtained in three independent experiments.