

TLR-4 Signaling Promotes Tumor Growth and Paclitaxel Chemoresistance in Ovarian Cancer

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Abstract

Evidence suggests that an inflammatory profile of cytokines and chemokines persisting at a particular site would lead to the development of a chronic disease. Recent studies implicate bacterial infection as one possible link between inflammation and carcinogenesis; however, the crucial molecular pathways involved remain unknown. We hypothesized that one possible upstream signaling pathway leading to inflammation in carcinogenesis may be mediated by Toll-like receptors (TLR). We describe for the first time an adaptive mechanism acquired by ovarian cancer cells that allows them to promote a proinflammatory environment and develop chemoresistance. We propose that the TLR-4-MyD88 signaling pathway may be a risk factor for developing cancer and may represent a novel target for the development of biomodulators. Our work explains how bacterial products, such as lipopolysaccharide, can promote, directly from the tumor, the production of proinflammatory cytokines and the enhancement of tumor survival. In addition, we provide new evidence that links TLR-4 signaling, inflammation, and chemoresistance in ovarian cancer cells. (Cancer Res 2006; 66(7): 3859-68)

Introduction

Chronic infection and inflammation are considered to be some of the most important epigenetic and environmental factors contributing to tumorigenesis and tumor progression (1, 2). Individuals with ulcerative colitis, a chronic inflammatory disease of the colon, have a 10-fold higher likelihood of developing colorectal carcinoma. Similarly, inflammatory conditions of the liver, such as chronic hepatitis and cirrhosis, are well-established risk factors for the development of hepatocellular carcinoma (3). Ovarian endometriosis, a condition that promotes a proinflammatory environment within the ovary on a cyclical basis, predisposes women to specific types of epithelial ovarian carcinomas (4-6). Much progress has been made in the understanding of how inflammatory cells, through the production of cytokines and chemokines, drive this process (2); in addition, there is convincing evidence suggesting that an inflammatory profile of cytokines and chemokines persisting at a particular site would lead to the development of a chronic disease (7).

Although the adaptive immune system prevents tumor growth through immunosurveillance, the innate immune system is thought to promote tumor growth through inflammation-dependent mech-

anisms (2, 8). Recognition of bacterial and/or viral products by innate immune cells, such as macrophages, may induce an inflammatory response associated with tumor promotion (3). Indeed, it has been observed that bacterial infection following surgery can promote growth of metastases in experimental animals and human patients (3). *Helicobacter pylori* infection is the world's leading cause of stomach cancer, and *H. pylori* is established as a definite carcinogen for the development of gastric cancer.

The innate immune system recognizes the presence of bacterial pathogens through the expression of a family of membrane receptors known as Toll-like receptors (TLR). The *Toll* gene was initially found to be important in dorsoventricular polarization during embryogenesis in *Drosophila* (9). Subsequently, several mammalian homologues have been identified and characterized (10). These TLRs play an important role in immunosurveillance and responses towards commensal and pathogenic microorganisms (11). TLRs are transmembrane proteins with a conserved intracellular signaling domain homologous to the interleukin-1 receptor (IL-1R) domain and a specific leucine-rich repeat extracellular domain (12). TLRs recognize not only microbial-associated pathogen-associated molecular patterns (PAMP) expressed by bacteria and viruses but also host-derived PAMPs, such as stress proteins (13). TLRs generally signal through a MyD88-dependent manner, leading to a proinflammatory response. Signaling via MyD88 involves the early activation of nuclear factor- κ B (NF- κ B), which leads to the production of proinflammatory cytokines (14). However, MyD88-independent TLR signaling can also occur. MyD88-independent signaling involves the activation of the late phase of NF- κ B and also activation of IFN regulatory factor 3, which leads to the production of type I IFN (IFN α/β), IFN-inducible gene products, and an immune regulatory response (15).

TLR-4 upon recognition of its ligand lipopolysaccharide (LPS) can induce the production of proinflammatory cytokines, including IL-6, tumor necrosis factor- α (TNF- α), and IL-12, through MyD88 and potentially through NF- κ B activation. Although the link between Gram-negative bacteria, LPS, TLRs, TNF- α , NF- κ B, and inflammation has been well characterized, the association between TLRs and cancer remains unknown.

In the present study, we show a link between TLR-4-MyD88 signaling, inflammation, tumor growth, and chemoresistance. We describe the expression of TLR-4 in epithelial ovarian cancer (EOC) cells and the differential effect of TLR-4 ligation by LPS and paclitaxel in MyD88-positive (MyD88+) and MyD88-negative (MyD88⁻) EOC cells. In addition, we show that in EOC cells, signaling through TLR-4 and MyD88 represent a major source of proinflammatory cytokines, which promote tumor growth. Furthermore, we have identified a new mechanism for the acquisition of paclitaxel chemoresistance and therefore a novel target for the development of molecularly directed therapy in paclitaxel-resistant ovarian cancer.

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Materials and Methods

Reagents. LPS isolated from *Escherichia coli* (0111:B4), carboplatin, and paclitaxel were purchased from Sigma Chemical Co. (St. Louis, MO). The rabbit polyclonal antibody to TLR-4, clone H-80, was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Patients and samples. Tissue and ascites samples were collected from stage III/IV ovarian cancer patients. Tissues were cut in small aliquots and snap frozen in liquid nitrogen. All patients signed consent forms, and the use of patient samples was approved under Yale University's Human Investigations Committee (HIC #10425).

Cell lines. Human EOC cell lines A2780 and CP70 (gifts from Dr. T.C. Hamilton; ref. 16) were propagated in RPMI plus 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA) at 37°C in a 5% CO₂ atmosphere. Primary EOC cells were isolated from malignant ovarian ascites and cultured as previously described (17). EOC cells were isolated from tumors as previously described (17, 18). The normal ovarian surface epithelial cell line immortalized with telomerase was cultured as previously described (19). Purity of the EOC cells was 100% as determined by immunostaining for cytokeratin antigen.

Immunohistochemistry. Twenty-four samples of ovarian cancer tissues were evaluated for immunocytochemistry. The expression and cellular localization of TLR-4 and MyD88 by EOC cells was done as previously described (20). In short, sections of tumor samples (5 µm) were blocked with either 10% horse or goat serum in PBS for 1 hour at room temperature. Following three washes with PBS, samples were incubated overnight at 4°C with either the anti-TLR-4 (Santa Cruz Biotechnology) or the anti-MyD88 antibody. Mouse IgG1 or rabbit serum served as negative controls. After three washes with PBS, specific staining was detected by incubating with either a peroxidase-conjugated horse anti-mouse antibody (1:1,000 dilution) or a peroxidase-conjugated goat anti-rabbit antibody (1:1,000 dilution) for 1 hour followed by a 5-minute incubation with 3,3'-diaminobenzidine substrate (Vector Laboratories, Burlingame, CA). Cells and tissue sections were then counterstained with hematoxylin (Sigma Chemical) before dehydration with ethanol and HistoSolve (Shandon, Inc., Pittsburgh, PA). Slides were then mounted with Permount (Fisher Scientific, Pittsburgh, PA) and visualized by light microscopy.

Cell viability assay. Cell viability was evaluated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. The values from the treated cells were compared with the values generated from the untreated cells and reported as percent viability. Each experiment was done at least thrice.

Protein preparation. Protein was extracted as previously described (17). Briefly, cell pellets were lysed in a cold solution of 1% NP40 and 0.1% SDS in PBS, with freshly added protease inhibitor cocktail (20 µL/mL; Sigma Chemical) and 2 mmol/L phenylmethylsulfonyl fluoride (Sigma Chemical). Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), and proteins were stored at -40°C until further use.

For separation of the cytoplasmic and nuclear fractions, cell pellets were processed using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions.

SDS-PAGE and Western blots. Twenty-microgram protein was denatured in sample buffer [2.5% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.15 mol/L Tris (pH 6.8), and 0.01% bromophenol blue] and subjected to 12% SDS-PAGE as previously described (17). Antibodies used: rabbit anti-TLR-4 (Santa Cruz Biotechnology; 1:1,000), rabbit anti-MyD88 (eBiosciences, San Diego, CA; 1:1,000), mouse anti-NF-κB (Santa Cruz Biotechnology; 1:1,000), mouse anti-DNA topoisomerase 1 (BD Biosciences, San Jose, CA; 1:500), and rabbit anti-actin (Sigma Chemical; 1:10,000). Proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology).

RNA isolation and reverse transcription-PCR. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription was done on 5 µg of total RNA using the First Strand cDNA Synthesis kit (Amersham Biosciences, Buckinghamshire, United Kingdom) according to the manu-

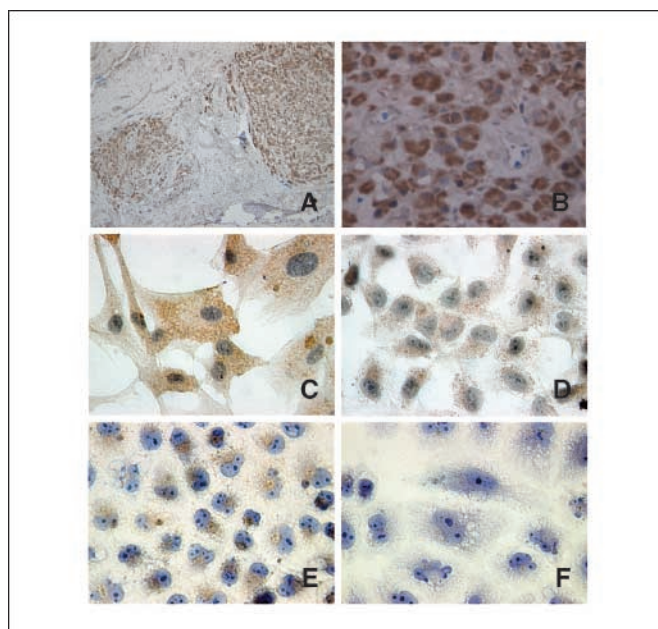


Figure 1. TLR-4 expression in ovarian cancer samples. Paraffin sections of ovarian cancer tissues (A and B) and EOC cell lines isolated from malignant ascites and tumor samples (C-E) were immunoassayed for TLR-4 (brown staining) as described in Materials and Methods. F, negative control (isotype-matched control). Magnification, ×10 (A) and ×40 (B-F).

facturer's instructions. The primers used for amplification of human TLR-4 is as follows: forward primer, TGGATACGTTTCCTTATAAG; reverse primer, GAAATGGAGGCACCCCTTC. Thirty cycles of PCR were done at 95°C for 15 seconds, 54°C for 45 seconds, and 72°C for 60 seconds. The size of the product was 449 bp.

Caspase-Glo 3/7 assay. Ten micrograms of protein in a 50-µL total volume was mixed with 50 µL of equilibrated Caspase-Glo 3/7 reagents (Promega). After incubating at room temperature for 1 hour, luminescence was measured using TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA). Blank values were subtracted, and fold increase in activity was calculated based on activity measured from untreated cells. Each sample was measured in triplicates.

Cytokine and chemokine studies. Chemokine production was determined using the Human Cytokine Array kit, III (for cell culture supernatants; RayBiotech, Atlanta, GA) as previously described (20). The intensity of the signals was quantified by densitometry using a digital imaging analysis system and 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY). The signal intensities were normalized against the positive controls on each array membrane, which were given the arbitrary unit of 1. Any expression levels below 0.2 unit were considered nonsignificant.

The concentrations of the IL-6, GRO-α, MCP-1 were evaluated by ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). A panel of 10 cytokines, proinflammatory and anti-inflammatory, was simultaneously evaluated using the Fast Quant Human II kit (Whatman/Schleicher and Schuell, Keene, NH) according to the manufacturer's instructions. The signal was detected using the Genepix 4000 microarray reader, and the results were analyzed using the Genepix 3.0 software (Whatman/Schleicher and Schuell).

RNA interference. EOC cells were transiently transfected with a GFP-expressing plasmid containing silencing RNA (siRNA) directed against MyD88 (psiRNA-hMyD88, Invivogen, San Diego, CA). Briefly, 1.5×10^5 cells were seeded in 60-mm dishes and cultured overnight until 40% to 60% confluent. Cells were then transfected for 18 hours with 2 µg of DNA using Fugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN). Ratio of Fugene to DNA was 3:1. Following transfection, cells were allowed to recover in growth media for 24 hours before treatment.

MyD88 transfections. Transfection was done using a plasmid containing the full-length cDNA of human MyD88 (pUNO-hMyD88, InvivoGen). CP70 and A2780 cells were grown in 75-mm² surface tissue culture flasks until they reached 50% confluence; 12 μ L of the Fugene 6 transfection reagent was added into 4 mL of serum-free media for each transfection. After 5 minutes of incubation at room temperature, 1 μ g of the plasmid pUNO-hMyD88 was added to the serum-free media containing Fugene 6 transfection reagent, and the mixtures were incubated at room temperature for another 15 minutes. The growth media in each flask were discarded, and the corresponding Fugene 6/plasmid/serum-free media mixture was added, and the flasks were incubated overnight at 37°C 5% CO₂. The transfection media were replaced with fresh growth media the second day, and the cells were allowed to recover for 24 hours after transfection before treatments.

Laser capture microdissection. Ovarian cancer specimens ($n = 24$) obtained in the operating room were snap frozen in liquid nitrogen and stored in cryovials at -80°C. For laser capture microdissection, a small fraction of tissue was embedded in ornithine carbamyl transferase at -20°C. Eight-micrometer sections were cut with a microtome and fixed on Leica glass foiled PEN membrane slides. The specimens were fixed in 95% ethanol and H&E stained. Dehydration was done by immersing the slides in 100% ethanol followed by HistoSolve (xylene substitute) and air-drying for 10 to 15 minutes.

Using the Leica Laser Capture Microdissection System (Leica Microsystems, Bannockburn, IL), 6,000 ovarian cancer cells were selected and collected in PCR Eppendorf tubes containing sample buffer used for preparation of samples for Western blot analysis. Samples underwent five cycles of thawing and freezing followed by 10 minutes at 95°C. Afterward, the samples were stored at -20°C until used for Western blot analysis.

Statistical analysis. Data are expressed as mean \pm SD (21). Statistical significance ($P < 0.05$) was determined using one-way ANOVA with the

Bonferroni correction. Survival curve of the patients were done by the method of Kaplan-Meier (22), and the significance of the difference was estimated by log-rank test.

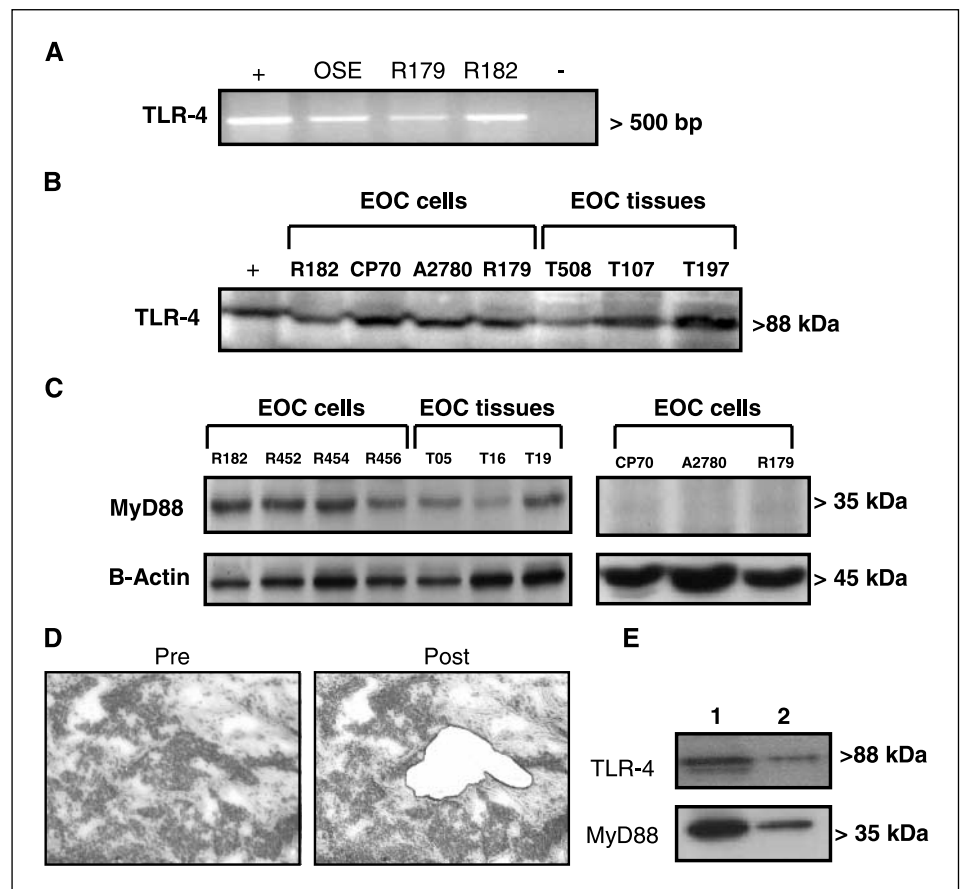
Results

EOC expression of TLR-4 and the signaling adapter protein MyD88. Our first objective was to determine whether TLR-4 is expressed in EOC cells. Therefore, we evaluated TLR-4 expression in paraffin sections of ovarian cancer tissues. As shown in Fig. 1A and B, positive immunoreactivity for TLR-4 was observed in the tumor cells but not in the neighboring nondysplastic cells. The staining was localized in the cytoplasm as well as on the cell surface. We then evaluated TLR-4 expression in EOC cell lines isolated from malignant ovarian ascites and from ovarian cancer tissues. Positive immunoreactivity for TLR-4 was observed in all of the evaluated EOC cell lines (Fig. 1C-E). The staining pattern was similar to the one observed on the paraffin sections. No staining was observed when mouse IgG1 was used as negative control (Fig. 1F).

To confirm the specificity of these findings, the expression of TLR-4 by EOC cells and tumors were further evaluated by reverse transcription-PCR and Western blot analysis. As shown in Fig. 2, EOC cells and tumors expressed TLR-4 both at the mRNA (Fig. 2A) and protein level (Fig. 2B). In addition, the mRNA message for TLR-4 was found to be expressed in a normal ovarian surface epithelium (OSE) cell line immortalized with telomerase (ref. 19; Fig. 2A).

To determine whether TLR-4 expressed in EOC cells had the potential to signal and therefore be functional, we evaluated the

Figure 2. Expression pattern of TLR-4 and MyD88 in EOC cells. A, evaluation of TLR-4 mRNA expression by reverse transcription-PCR from OSE and EOC cell lines. B and C, Western blot analysis for TLR-4 and MyD88 protein expression from EOC cell lines and ovarian cancer tumor samples. +, positive control; -, negative control (no reverse transcription). D, laser capture microdissection of ovarian cancer cells from tissue sections before (Pre) and after dissection (Post). E, Western blot analysis for the expression of TLR-4 and MyD88 by ovarian cancer cells following laser microdissection. Lane 1, positive control (macrophages); lane 2, 6,000 EOC cells microdissected by laser. Representative of 10 tumors evaluated.



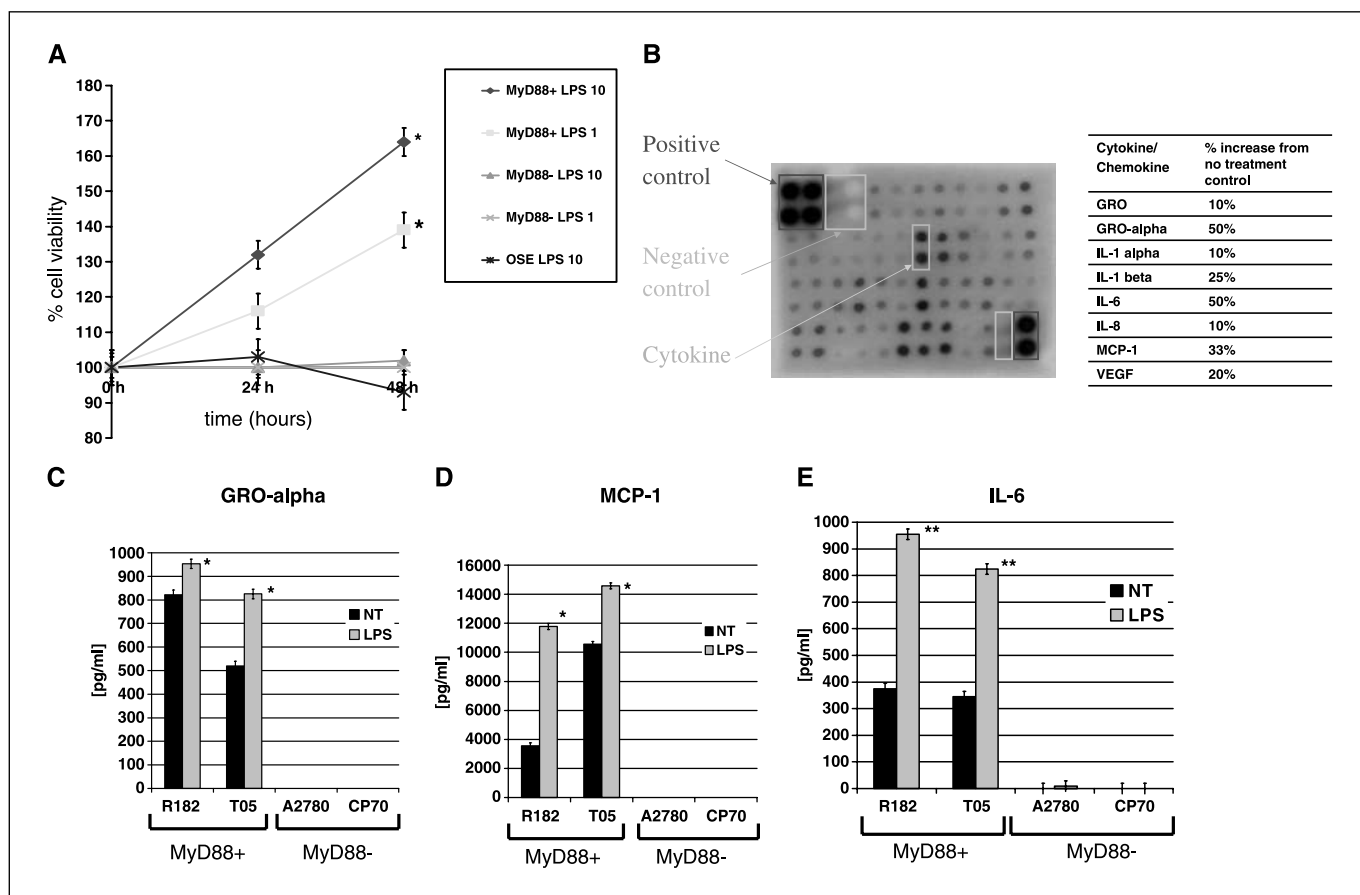


Figure 3. Differential response of MyD88⁺ and MyD88⁻ cells to ligation of TLR-4 with LPS. *A*, cell viability: EOC and OSE cells were treated with increasing concentration of LPS for 24 and 48 hours, and cell viability was determined using CellTiter 96 Aqueous One Solution Cell Proliferation Assay. Results for R182 (MyD88⁺) and A2780 (MyD88⁻). Similar results were obtained with other cell lines. *Points*, mean from at least three independent experiments; *bars*, SD. *, $P < 0.01$ relative to 0 hour. *B*, cytokine/chemokine expression profile of MyD88⁺ and MyD88⁻ EOC cells in response to TLR-4 ligation with LPS. MyD88⁺ EOC cells were treated with 10 $\mu\text{g}/\text{mL}$ LPS for 48 hours, and the levels of secreted cytokine/chemokine was detected using a human cytokine array as described in Materials and Methods. Representative array from no-treatment controls showing the constitutive secretion of cytokines/chemokines. % Increase in secretion of each cytokine/chemokine relative to no treatment controls. *C-E*, MyD88⁺ and MyD88⁻ EOC cell lines were treated with LPS as in (*A*), and the secreted levels of GRO- α (*C*), MCP-1 (*D*), and IL-6 (*E*) were measured from cell-free supernatants using ELISA assay. *Columns*, mean from at least three independent experiments; *bars*, SD. *NT*, no-treatment control. *, $P < 0.05$; **, $P < 0.01$, both relative to no treatment.

expression of the TLR signaling adapter protein, MyD88. Unlike TLR-4, which was ubiquitously expressed by all EOC cell lines and tumors evaluated, the expression of MyD88 was cell line and tumor specific (Fig. 2C). Furthermore, normal OSE cells, which expressed TLR-4, did not express MyD88 (data not shown).

We then evaluated whether the expression of TLR-4 and MyD88 could be determined in cancer cells isolated with laser microdissector. Thus, ovarian cancer cells were microdissected from 8- μm tissue sections using Laser Capture Micro dissector. Cells were collected in sample buffer, and TLR-4 and MyD88 expression was evaluated by Western blot. Figure 2D shows the area before and after microdissection. Figure 2E shows the presence of TLR-4 and MyD88 in microdissected ovarian cancer cells.

MyD88 expression is required for LPS-induced tumor growth. Once the expression of TLR-4 in EOC samples was established, we evaluated the biological function of this receptor. In addition, we also evaluated the significance of MyD88 status. Therefore, EOC cell lines expressing MyD88 (MyD88⁺) and those shown to lack MyD88 (MyD88⁻) were treated with increasing concentrations of LPS (one of the main ligands for TLR-4) for 24

and 48 hours, and cell viability was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay. Figure 3A shows the results for R182 (MyD88⁺) and A2780 (MyD88⁻). A significant increase in cell proliferation was observed at 24 hours ($P < 0.01$) and 48 hours ($P < 0.01$) in MyD88⁺ but not in MyD88⁻ EOC cells. Thus, LPS induced a time-dependent and dose-dependent increase in cell proliferation in cells expressing MyD88 but not in MyD88⁻ EOC cells. LPS had no effect on OSE cell viability, which are also negative for MyD88 expression (data not shown). These results suggest that the proliferative effect of LPS in EOC cells depends on the presence of MyD88.

TLR-4 ligation by LPS induced cytokine production in MyD88 expressing cell lines. One of the main characteristics of TLR-4 ligation by LPS in cells of the immune system is the induction of cytokine production, mainly of the proinflammatory type. Thus, our next objective was to evaluate whether TLR-4 ligation by LPS would have a similar effect in EOC cells. Thus, MyD88⁺ EOC cells were incubated in the presence or absence of 10 $\mu\text{g}/\text{mL}$ LPS for 48 hours, and cytokine secretion was evaluated in the cell-free culture supernatants using a human cytokine array. MyD88⁺ EOC cells constitutively secreted a wide range of

proinflammatory cytokines and chemokines, and this secretion was further enhanced by TLR-4 ligation with LPS (Fig. 3B).

The results from the cytokine array lead to the identification of specific cytokines/chemokines that were significantly affected by LPS treatment. To validate these results, we then did ELISA and compared the differential response between MyD88⁺ and MyD88⁻ EOC cells. Thus, EOC cell lines were treated for 48 hours with 10 μ g/mL LPS, and the levels of GRO- α , MCP-1, and IL-6 were determined. These cytokines were selected because their secretion was shown to be the most significantly affected by LPS treatment (Fig. 3B). Unstimulated MyD88⁺ EOC cells constitutively express all the cytokines tested. In addition, TLR-4 ligation significantly induced secretion of GRO- α ($P < 0.05$; Fig. 3C), MCP-1 ($P < 0.05$; Fig. 3D), and IL-6 ($P < 0.01$; Fig. 3E). In contrast, unstimulated MyD88⁻ EOC cells secreted undetectable or low levels of these proinflammatory cytokines, which were not affected by TLR-4 ligation with LPS (Fig. 3C-E). These findings suggest that the increased production of proinflammatory cytokines in response to LPS was dependent on MyD88 expression in EOC cells.

TLR-4 activates the NF- κ B pathway in MyD88 expressing EOC cells. NF- κ B is one of the main intracellular pathways mediating the induction of cytokine expression following TLR-4 activation. Therefore, we next determined if the ligation of TLR-4 induces NF- κ B activation in EOC cells. Thus, EOC cells were incubated in the presence or absence of LPS (10 μ g/mL) for 1, 2, and 4 hours, and the activation status of NF- κ B was determined by Western blot analysis. As shown in Fig. 4, in MyD88⁺ EOC cells, the p65 active form of NF- κ B was translocated to the nucleus 1 hour after treatment with LPS (Fig. 4A). This shows that in MyD88⁺ EOC cells, the ligation of TLR-4 by LPS induces NF- κ B activation. Interestingly, constitutive nuclear localization of p65 NF- κ B was observed in MyD88⁻ EOC cells, and its level was not affected by LPS treatment (Fig. 4B). Therefore, we determined the status of the NF- κ B inhibitor, I κ B- α , in these cells. Our results show I κ B- α degradation in MyD88⁺ cells in response to LPS. In contrast, constitutive degradation of I κ B- α was not observed in MyD88⁻ cells;

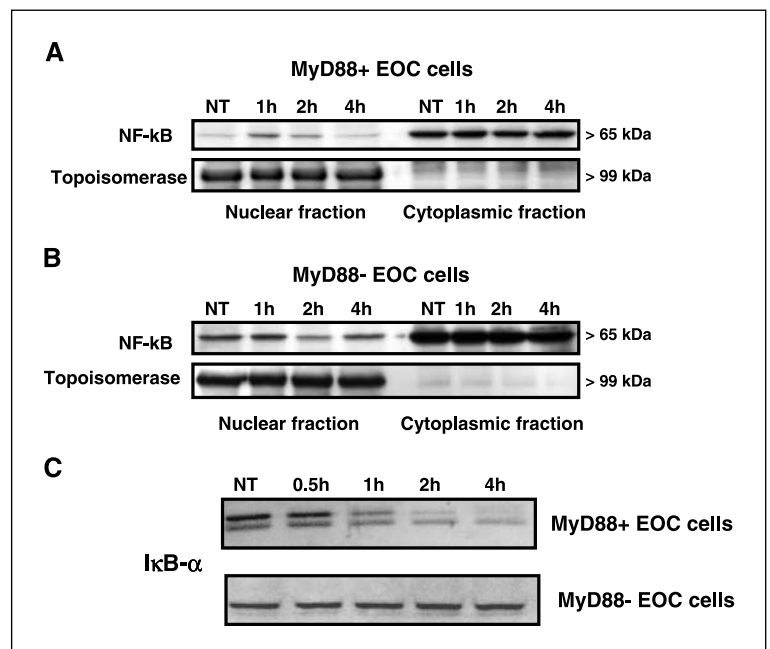
in addition, no change in its level was observed after LPS treatment (Fig. 4C). These results suggest that TLR-4 ligation by LPS results in early-phase activation of NF- κ B in MyD88⁺ but not in MyD88⁻ EOC cells.

MyD88 expression was associated with paclitaxel resistance. Paclitaxel has been reported to be a potential ligand to TLR-4 (23). Because our results showed that (a) TLR-4 is ubiquitously expressed in all EOC cell lines tested, (b) MyD88 expression is cell line specific; and (c) because paclitaxel resistance is a major characteristic of recurrent ovarian cancer, we evaluated whether MyD88 status affects the response of EOC cells to paclitaxel. Thus, EOC cells were treated with 2 μ mol/L paclitaxel for 24 hours, and apoptosis was determined by measuring the activity of caspase-3 and caspase-7 using Caspase-Glo 3/7 Assay. As shown in Fig. 5A, there was a significant increase in caspase-3/7 activity following paclitaxel treatment in MyD88⁻ EOC cell lines. Significant increase in caspase activity was also observed in normal OSE cells following paclitaxel treatment (data not shown). In contrast, no significant change in caspase-3/7 activity was detected in MyD88⁺ EOC cells. These results suggest that the MyD88 status of EOC cells determines their apoptotic response to paclitaxel.

MyD88 expression mediates Paclitaxel-induced production of proinflammatory cytokines. Because the previous results showed that TLR-4 ligation by LPS in MyD88⁺ EOC cells results in increased cytokine secretion, we then determined if TLR-4 ligation by paclitaxel would induce the same type of response. Thus, MyD88⁺ and MyD88⁻ EOC cells were treated with increasing concentrations of paclitaxel for 24, 48, and 72 hours, and levels of IL-6 secretion were determined by ELISA. As shown in Fig. 5B, in MyD88⁺ EOC cell lines, paclitaxel induced a significant increase in IL-6 production in a dose-dependent and time-dependent manner ($P < 0.01$). In contrast, paclitaxel treatment did not affect IL-6 levels in MyD88⁻ EOC cell lines.

To further characterize the effect of paclitaxel and LPS on the secretion of several cytokines and chemokines in MyD88⁺ and MyD88⁻ EOC cells, we used a high-throughput microarray system

Figure 4. Effect of TLR-4 ligation with LPS on NF- κ B activation in EOC cells: (A) MyD88⁺ and (B) MyD88⁻ EOC cells were treated with 10 μ g/mL LPS for 1, 2, and 4 hours, and the nuclear and cytoplasmic fractions were separated as described in Materials and Methods. The translocation of p65 from the cytoplasm into the nucleus was determined by Western blot analyses. Topoisomerase was included to show integrity of the fractions. C, levels of I κ B- α in MyD88⁺ and MyD88⁻ EOC cells following LPS stimulation. Note the decrease in I κ B expression in MyD88⁺ cells but not in MyD88⁻ EOC cells. Results from R182 (MyD88⁺) and A2780 (MyD88⁻). Similar results were obtained from other EOC cell lines.



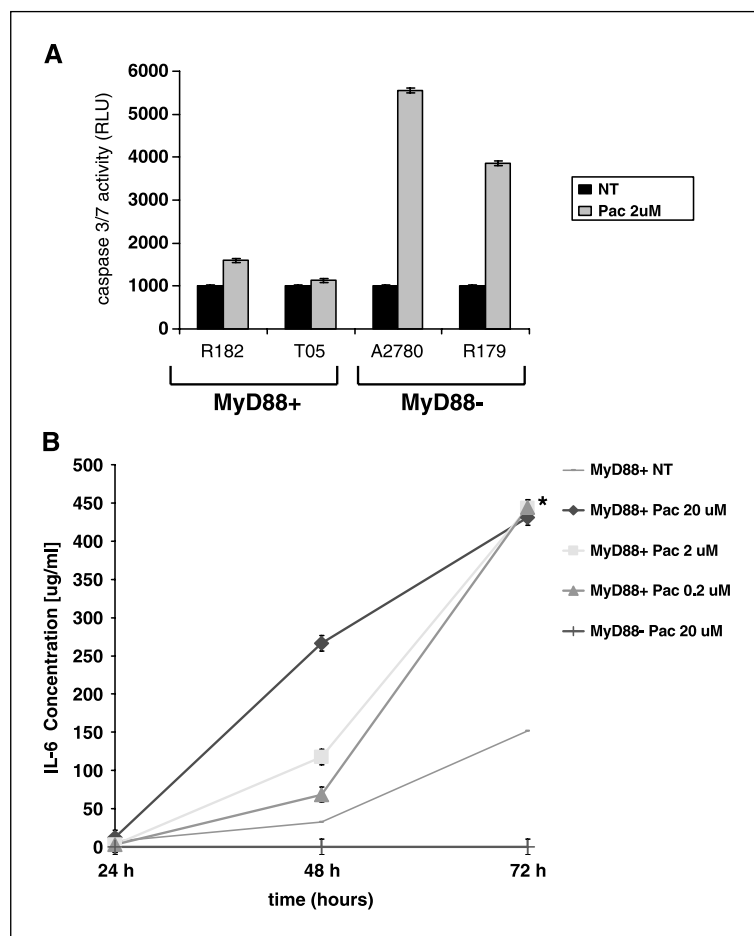


Figure 5. Effect of paclitaxel treatment in MyD88⁺ and MyD88⁻ EOC cell lines. **A**, apoptotic response: EOC cell lines were treated with 2 $\mu\text{mol/L}$ paclitaxel for 24 hours, and the levels of caspase-3/7 was measured using the Caspase-Glo 3/7 Assay. *Columns*, mean from at least three independent experiments; *bars*, SD. **B**, effect of paclitaxel treatment on IL-6 secretion in R182 EOC cells (MyD88⁺) and A2780 EOC cells (MyD88⁻) EOC cell lines. EOC cell lines were treated with increasing concentrations of paclitaxel, and IL-6 levels were measured after 24, 48, and 72 hours by ELISA. From R182 (MyD88⁺) and A2780 (MyD88⁻). Similar results were observed in other EOC cell lines tested. *Points*, mean from at least three independent experiments; *bars*, SD. *NT*, no-treatment control; *Pac*, paclitaxel. *, $P < 0.01$, relative to no treatment.

(Whatman/Schleicher and Schull) to measure the levels of IL-6, IL-4, IL-8, IL-12, RANTES, and IFN- γ . Cytokine secretion was measured in the supernatants of EOC cells following 48 hours of incubation with either LPS (10 $\mu\text{g/mL}$) or paclitaxel (20 $\mu\text{mol/L}$). As shown in Fig. 6A-C, MyD88⁺ EOC cells displayed constitutive secretion of the proinflammatory cytokines IL-6, IL-8, and RANTES, and treatment with LPS or paclitaxel resulted in increased secretion of these proinflammatory cytokines. In contrast, MyD88⁻ EOC cells showed neither constitutive secretion nor LPS-induced or paclitaxel-induced IL-6, IL-8, or RANTES secretion. Interestingly, neither MyD88⁺ nor MyD88⁻ EOC cells secreted detectable levels of IL-4, IL-12, and IFN- γ , with or without treatment with either LPS or paclitaxel (Fig. 6D-F).

Expression of MyD88 in EOC cells is necessary for IL-6 production. To confirm that in MyD88⁺ EOC cells, the ligation of TLR-4 by either LPS or paclitaxel induces the production of cytokines, we inhibited its expression in MyD88⁺ R182 EOC cells using RNA interference (RNAi) technology. As shown above, wild-type (wt) R182 cells produce high levels of IL-6 in response to both LPS (10 $\mu\text{g/mL}$) and paclitaxel (2 $\mu\text{mol/L}$). However, following the inhibition of MyD88 expression by RNAi (Fig. 7B), treatment with LPS or paclitaxel resulted in decreased IL-6 secretion (Fig. 7A). These findings confirmed that the cytokine response in EOC cells following LPS and paclitaxel treatment involves signaling through MyD88.

Induction of MyD88 expression in EOC cells reverses chemosensitivity to paclitaxel. Because we observed a positive

correlation between MyD88 expression and resistance to paclitaxel-induced apoptosis (Fig. 5A), our next objective was to determine whether the induction of MyD88 expression in EOC might confer resistance to paclitaxel. Therefore, we induced the expression of MyD88 in A2780 and CP70 EOC cells, both MyD88⁻ and paclitaxel sensitive, by transient transfection with a plasmid vector containing the full-length cDNA of MyD88 (pUNO-hMyD88). After 24 hours of transfection, the cells were either left untreated or treated with 2 $\mu\text{mol/L}$ paclitaxel for 48 hours, and caspase-3/7 activity was determined. A significant increase in caspase-3/7 activity was observed in wt cells following paclitaxel treatment. In contrast, no changes in caspase-3/7 activity were observed in cells transfected with pUNO-hMyD88. Figure 7C and D shows the results for CP70 EOC cells. These findings show that MyD88 expression contributes to paclitaxel chemoresistance.

We then determined whether the expression of MyD88 confers resistance that is specific to paclitaxel. Thus, wt A2780 EOC cells (carboplatin sensitive) and A2780 EOC cells transfected with MyD88 were treated with 100 $\mu\text{g/mL}$ carboplatin or 2 $\mu\text{mol/L}$ paclitaxel for 48 hours. Apoptosis was determined by measuring caspase-3/7 activity. As shown in Fig. 7E, wt A2780 cells showed significant increase in caspase-3/7 activity following treatment with carboplatin or paclitaxel. Upon the introduction of MyD88, the cells retained its sensitivity to carboplatin but not paclitaxel. This suggests that the protective effect of MyD88 is specific for paclitaxel-induced apoptosis.

TLR-4 ligation by paclitaxel induces the expression of antiapoptotic proteins. TLR-4 ligation through NF- κ B and inflammatory molecules promotes cell survival by the induction of the expression of antiapoptotic proteins (24, 25). The expression of X-linked inhibitor of apoptosis (XIAP), a major inhibitor of caspase-3 and caspase-9, and the expression of Akt has been associated with tumor growth and chemoresistance in ovarian cancer cells (26–28). Therefore, we evaluated the expression of XIAP and phosphorylated Akt (pAkt) following paclitaxel treatment in MyD88⁺ cells. As shown in Fig. 8, a significant increase in pAkt was observed 4 hours after treatment with paclitaxel. Similarly, XIAP levels increased 2 hours after treatment. In contrast, no change on the levels of total Akt was observed (Fig. 8).

Correlation between MyD88 expression in ovarian cancer tissues and patients' progression-free survival. To determine whether our *in vitro* findings have clinical relevance, we did a pilot retrospective study to analyze MyD88 expression in tissues obtained from patients with stage IIIC ovarian cancer ($n = 12$) and determine whether there is a correlation between MyD88 expression and progression-free survival. Tissue collection was done before chemotherapy, and the expression of MyD88 in ovarian cancer tissues was determined by Western blot analysis. All patients received six cycles of paclitaxel/carboplatin regimen after surgery. As shown in Fig. 9, the mean time to recurrence for patients with tumors that expressed MyD88 was 23 months ($n = 5$). However, the mean time to recurrence for patients with tumors that did not express MyD88 was 42 months ($n = 7$). Patients whose tumors did not express

MyD88 had a statistically significant improved progression-free interval compared with patients whose tumors expressed MyD88 ($P = 0.03$).

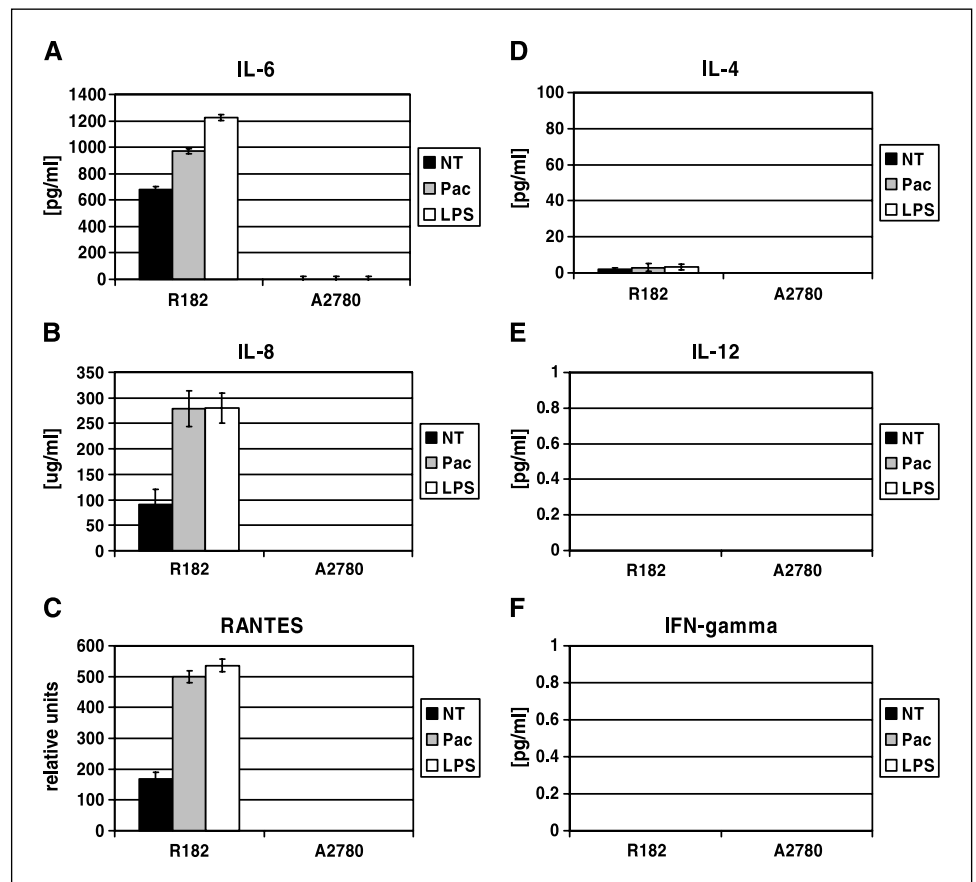
Discussion

Substantial evidence indicates that bacterial-induced and viral-induced inflammatory processes can mediate tumorigenesis. In the present study, we describe a specific defense mechanism used by the innate immune system, that cancer cells have imitated and adapted to generate proinflammatory cytokines, leading to their own proliferation and survival. Specifically we show for the first time the following: (a) the expression of TLR-4 in EOC cells, (b) the induction of tumor growth by TLR-4 ligation in MyD88⁺ EOC cells, (c) the production of chemokines and cytokines by MyD88⁺ EOC cells upon TLR-4 ligation, and (d) chemoresistance to paclitaxel mediated by the expression of MyD88.

It has been observed in animal studies that surgical removal of a primary tumor is often followed by rapid growth of previously dormant metastases, and LPS has been suggested to be responsible for this effect (29). Indeed, BALB/c mice receiving a tail vein injection of 4T1 mouse mammary carcinoma cells showed an increase in lung metastases following LPS injection (30). LPS is recognized by TLR-4, which is expressed by the cells of the innate immune system. Following its ligation, it has been shown to induce NF- κ B activation, cytokines/chemokines production, and inflammation (14).

TLRs represent a main receptor pathway, which can induce the expression of proinflammatory cytokines (15). TLRs are widely

Figure 6. Effect of paclitaxel and LPS treatment on cytokine secretion. The effect of paclitaxel (Pac) on (A) IL-6, (B) IL-8, (C) RANTES, (D) IL-4, (E) IL-12, and (F) IFN- γ in MyD88⁺ and MyD88⁻ EOC cell lines was determined using a cytokine microarray (Whatman/Schleicher and Schuell). Cell lines were treated with either 20 μ M/L paclitaxel or 10 μ g/mL LPS for 48 hours, and the levels of cytokine/chemokine secreted was measured by high-throughput microarray system as described in Materials and Methods. NT, no-treatment control; Pac, paclitaxel.



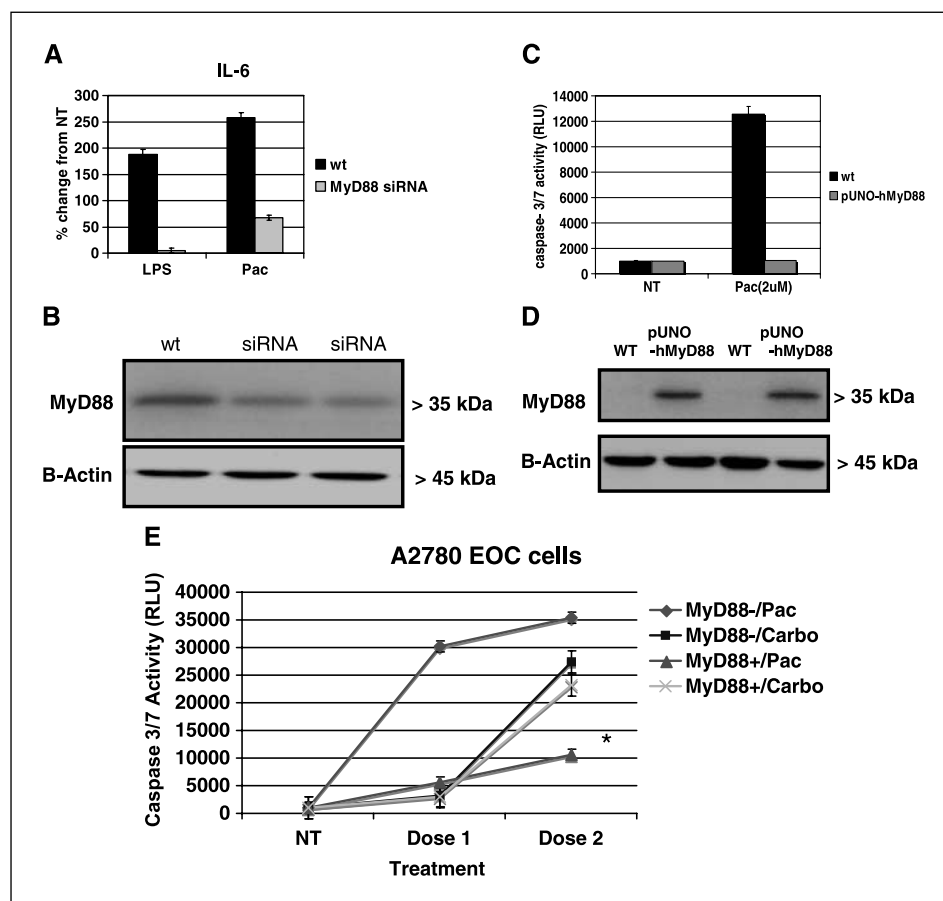


Figure 7. Effect of knocking down MyD88 on IL-6 secretion in response to LPS and paclitaxel. *A*, MyD88⁺ R182 cells were transfected with the siRNA for MyD88 and treated with 10 µg/mL LPS and 2 µmol/L paclitaxel for 48 hours. IL-6 was measured by ELISA as described in Materials and Methods and calculated as percent increase from no-treatment controls. *B*, Western blot analysis showing levels of MyD88 expression following siRNA-MyD88 transfection. *wt*, wild type; *Pac*, paclitaxel. *C*, MyD88 expression confers resistance to paclitaxel treatment. The CP70 MyD88-EOC cells were transfected with a plasmid vector containing the full-length cDNA of MyD88 (pUNO-hMyD88) and treated with 2 µmol/L paclitaxel for 48 hours. Caspase-3/7 activity was measured using the Caspase-Glo 3/7 assay. *D*, Western blot analysis showing MyD88 expression following transfection. *E*, MyD88 expression confers resistance to paclitaxel but not to carboplatin. The carboplatin-sensitive cells A2780 EOC cells (MyD88⁻) were transiently transfected with pUNO-hMyD88 (MyD88⁺) followed by treatment with carboplatin (dose 1 = 50 µg/mL, dose 2 = 100 µg/mL) or paclitaxel (dose 1 = 0.2 µmol/L, dose 2 = 2 µmol/L). *, *P* = 0.0001. *NT*, no treatment control; *wt*, wild type; *Pac*, paclitaxel.

expressed by cells of the immune system and, in response to microbial products or stress factors, initiate an inflammatory process (10). In addition, TLRs have been described in nonimmune cells, such as mucosal epithelium and trophoblast cells (20, 31). Similar to immune cells, the ligation of TLRs in nonimmune cells results in the expression and secretion of proinflammatory cytokines (20). In this study, we have described the presence of TLR-4 in all EOC cells tested and the differential expression of MyD88. We also showed that in MyD88⁺ EOC cells, ligation of TLR-4 by LPS enhances cell proliferation and induces the production of chemokines and proinflammatory cytokines. Interestingly, unstimulated MyD88⁺ cells constitutively secrete cytokines/chemokines. This suggests the presence of endogenous ligands of TLRs (i.e., apoptotic bodies and cellular debris from necrotic cells), which can also act through TLR-4 or other TLRs, which are also present in EOC cells.¹ The possible role of IL-1 on the constitutive secretion of cytokines/chemokines is another possibility that remains to be determined.

One of the main cytokines induced in EOC cells following LPS stimulation is IL-6. In addition, following TLR-4 ligation, these cells also secreted the chemokines MCP-1 and GRO- α . It is now well documented that chemokines can dramatically alter the neoplastic process, not only by recruiting leukocytes that will enhance the inflammatory environment but also by having a

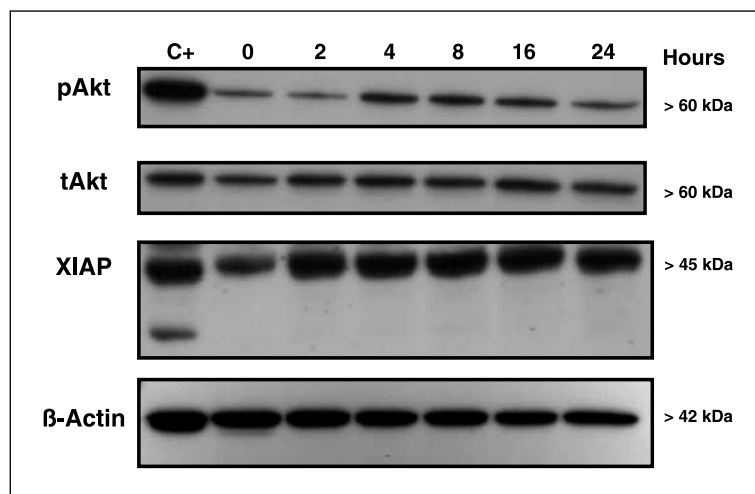
direct effect on nearby stromal and neoplastic cells (32). The induction of these chemokines by TLR-4 activation may, therefore, help to recruit inflammatory cells as well as to enhance tumor growth and neoplastic progression. Melanoma is one example in which chemokines have been shown to exert autocrine control over neoplastic proliferation (33, 34). Blocking GRO- α activity on the CXCR2 receptor attenuates melanoma cell proliferation *in vitro*, whereas overexpression of GRO- α and GRO- β enhances tumor colony formation and tumorigenicity in nude mice (34–36). In our study, ligation of TLR-4 by LPS induced a significant increase in GRO- α secretion, which may provide the stimuli for the proliferative effect observed on EOC cells following LPS treatment. Furthermore, the presence of proinflammatory cytokines and chemokines has been described as predictors of poor prognosis (37).

We observed differential response to LPS by EOC cells, which was due to the expression of the TLR intracellular signaling molecule MyD88. In the presence of MyD88, treatment with LPS results in the nuclear localization of NF- κ B, increase cell proliferation, and cytokine/chemokine production. These results provide preliminary evidence of the involvement of NF- κ B in TLR-4-MyD88 signaling pathway in EOC cells. However, the exact downstream signaling pathway after TLR-4-MyD88 activation still remains to be determined.

In the absence of MyD88, treatment with LPS failed to induce NF- κ B translocation to the nucleus and had no effect on cellular proliferation nor on cytokine/chemokine production. Interestingly, however, the MyD88⁻ A2780 EOC cell line constitutively express

¹ Our unpublished data.

Figure 8. Induction of antiapoptotic proteins by Paclitaxel in MyD88⁺ EOC cells. R182 EOC cells (MyD88⁺) were treated with paclitaxel (2 μ mol/L), cells were collected at different times, and cell lysates were prepared and evaluated for the expression of pAkt, total Akt (tAkt), and XIAP.



nuclear p65 NF- κ B, yet does not constitutively express any of the proinflammatory cytokines observed in MyD88⁺ cells. These results suggest that the effect of NF- κ B on cancer cells may be cell type specific, and the final output of NF- κ B activation (e.g., cytokine production) is the determining factor on tumor progression and differentiation. The significance of this constitutive NF- κ B nuclear localization in these cells, which is not related to the production of proinflammatory cytokines, is under investigation in our laboratory.

Differential response was also observed in EOC cells in response to paclitaxel. MyD88⁺ EOC cells responded to paclitaxel in similar manner as they did with LPS: they produced and secreted proinflammatory cytokines. In addition, these cells did not undergo apoptosis in response to paclitaxel. This is in contrast to MyD88⁻ cells, which underwent apoptosis and did not secrete cytokine/chemokine in response to paclitaxel.

Studies in mice by Ding et al. showed that similar to LPS, treatment with paclitaxel activates murine macrophages and induces the secretion of inflammatory cytokines including, TNF- α , IL-6, and IL-8. This effect of paclitaxel was shown to

be both TLR-4 and MyD88 dependent (23, 38). These results are supported by the *in vivo* observation showing a correlation between expression of MyD88 and progression-free survival. Although only a small number of patients were evaluated, the data indicate that the expression of MyD88 correlates with a poor survival. Our lab is actively evaluating whether the expression of MyD88 could be used as a marker to predict chemoresistance. Preliminary studies using laser capture microdissection suggest this possibility.²

In this study, we also show that in MyD88⁺ EOC cells, TLR-4 ligation is able to induce the activation of the Akt survival pathway and enhance the expression of the antiapoptotic protein XIAP. Both proteins have been shown to be highly expressed in ovarian cancer and are linked to the development of chemoresistance (17, 27, 39). Therefore, in MyD88⁺ EOC cells, the proapoptotic effect of paclitaxel is overcome by the induction of the antiapoptotic proteins (pAKT and XIAP) following TLR-4 ligation. Therefore, in these cells, treatment with paclitaxel does not induce apoptosis but induces the secretion of cytokines/chemokines. The correlation between TLR-4-MyD88 signaling and the up-regulation of pAkt and XIAP remains to be determined. NF- κ B may represent a potential link between these pathways.

During the past 20 years, multiple combinations of cytotoxic chemotherapeutics have been evaluated in patients with recurrent ovarian cancer. Our work provides new insight into a molecular mechanism that links inflammation and neoplastic development/progression. It suggests that an active TLR-4-MyD88 signaling pathway may be a risk factor for developing cancer and may be a novel target for the development of biomodulators aimed at reversing chemoresistance to paclitaxel.

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² D-S. Silasi et al., in preparation.

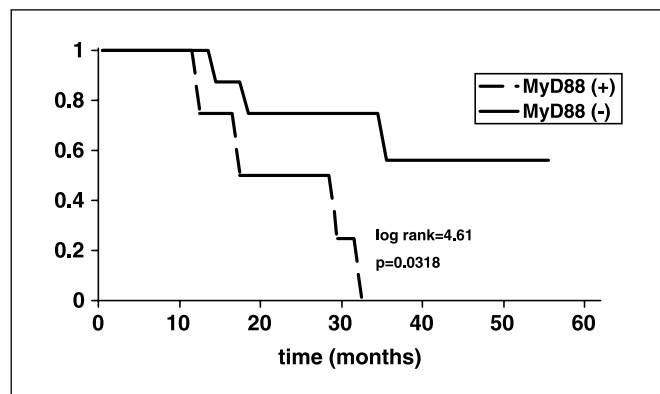


Figure 9. Correlation between MyD88 expression in ovarian cancer tissues and patients' progression-free survival. Tumor samples obtained from 12 ovarian cancer patients (stage IIIc) were evaluated for MyD88 expression by Western blot analysis, and the correlation with progression-free survival (Kaplan-Meier curve) was determined. Tissues that did not express MyD88 had a significantly better progression-free interval compared with patients whose tumors express MyD88 ($P = 0.03$).

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