Enhanced TLR4 Expression on Colon Cancer Cells After Chemotherapy Promotes Cell Survival and Epithelial–Mesenchymal Transition Through Phosphorylation of GSK3β

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Abstract. *Background: Phosphorylation of glycogen synthase kinase 3β (GSK3β) by phosphatidyl-inositide 3-kinase (PI3K)/protein kinase B (AKT) or inhibition of GSK3β with small-molecule inhibitor attenuates cell survival and proliferation and increases apoptosis in most cancer cell lines. In this study, we investigated the role of phosphorylated GSK3β activated by enhanced toll-like receptor 4 (TLR4) expression in drug-treated colon cancer cells as a model of post-chemotherapy cancer cells. Materials and Methods: The effect of TLR4 stimulation on metastasis and apoptosis in drug-exposed colon cancer cells was determined by real-time polymerase chain reaction (PCR) and immunoblotting. Results: Despite the induction of apoptosis after treatment with oxaliplatin and 5-fluorouracil, lipopolysaccharide (LPS) stimulation via increased TLR4 in drug-treated cancer cells effectively inhibited apoptosis through up-regulation of expression of anti-apoptosis-related B-cell lymphoma 2 (BCL2) family proteins [X-linked inhibitor of apoptosis protein (XIAP), BCL2, and survivin] and drug-resistance proteins [multidrug-resistance protein 1 (MDR1), multidrug resistance-associated protein (MRP)1/2/3]. LPS-mediated signaling in drug-treated cancer cells elevated the expression of phosphorylated GSK3β, extracellular signal–regulated kinase (ERK), and the p65 subunit of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB). Pharmacological inhibition of GSK3β (using SB216763) reduced phosphorylation of GSK3β, re-activated caspase-dependent apoptosis, and blocked the expression of cancer stem cell markers and invasive characteristics in LPS-stimulated drug-treated cells. In addition, the ERK-specific inhibitor, PD98059, triggered the apoptosis of TLR4-activated drug-exposed colon cancer cells, whereas there was no effect on the expression of epithelial–mesenchymal transition markers or GSK3β phosphorylation. Conclusion: These results suggest that TLR4-induced GSK3β and ERK phosphorylation independently controls cancer cell survival and regulation of GSK3β and ERK after chemotherapy, making TLR4 a critical target for reducing drug resistance and metastasis in patients with colon cancer.*

Toll-like receptor 4 (TLR4) is expressed in various types of human cancer, including prostate, pancreatic, liver, colon, and breast cancer (1-5). TLR4, the receptor for lipopolysaccharide (LPS), primarily induces inflammatory cytokines in immune cells, but it is also involved in carcinogenesis and cancer cell survival (1). Furthermore, LPS can modify cytokine levels of the tumor microenvironment to promote tumor growth, invasion and metastasis *in vitro* and *in vivo* (6). Adjuvant chemotherapy with 5-fluorouracil (5-FU) and leucovorin is commonly adopted to reduce the risk of recurrence in patients with advanced colon cancer after surgery (7). 5-FU induces TLR4 expression in colon cancer cells and synergistically enhances apoptotic cell death. In addition, the drug significantly induces the expression of TLR4 regardless of stimulation with LPS (8). TLR4 and interleukin (IL-6) up-regulation is a common feature of colorectal cancer and high expression of TLR4 and myeloid differentiation (MyD) 88-mediated signaling are associated with liver metastasis and poor prognosis (6). Based on these results, the exact role of increased TLR4 expression after anticancer drug treatment of cancer cells is still unclear and the role of LPS-induced TLR4 signaling after chemotherapy is also controversial.

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GSK3β is a ubiquitously expressed serine/threonine kinase that regulates glycogen synthesis (9). The activity of GSK3β is regulated by the phosphorylation of *N*-terminal serine-9 (p-GSK3β^{Ser9}) (14). The phosphorylation of GSK3β is necessary to induce inflammatory responses in LPS-stimulated macrophages through increased nuclear translocation and accumulation of β-catenin (10). LPS-mediated GSK3β phosphorylation, or inactivation, in macrophages enhances production of the pro-inflammatory cytokine interferon-β (11). The dephosphorylated and active form of GSK3β in resting cells regulates cell activation, differentiation, and survival (12). GSK3β, as a tumor suppressor, also inhibits a diverse group of pro-oncogenic substrates, such as cyclin D1, c-JUN, c-MYC and cAMP response element-binding protein (CREB) *via* induction of phosphorylation (13). In addition, inactivation by phosphorylation of GSK3β has been reported in many cancer cells (15, 16).

Many studies have demonstrated that pharmacological inhibitors of GSK3β, such as thiadiazolidinone or SB216763, can prevent proliferation and induce apoptosis of tumor cells, including multiple myeloma and ovarian cancer cell lines (17, 18). The phosphorylation of GSK3β by protein kinase B (PKB), also known as AKT, was also found to cause a dose- and time-dependent decrease in cell proliferation after TLR4 stimulation, while inhibition of GSK3β with lithium chloride reversed LPS-mediated growth inhibition in normal intestinal enterocytes (19). The levels of active GSK3β expression in colon cancer cells and patients with colorectal cancer are higher than in their normal counterparts (20), whereas the inactive form, phosphorylated GSK3β^{Ser9}, is frequently detected at high levels in normal tissues (20). Inhibition of GSK3β leads to attenuated proliferation and increased apoptosis by blocking the nuclear factor-κB (NF-κB) activation in pancreatic and colon cancer cells (21, 22). GSK3β is also significantly more activated in drug-resistant *versus* responsive patients after treatment with 5-FU (23). Based on these results, the precise role of GSK3β in different types of cancer and under diverse conditions remains unclear.

In the present study, we investigated whether an increase in TLR4 expression in drug-treated cancer cells regulates colon cancer cell survival after exposure to LPS. We also examined subsequent GSK3β-mediated activation of downstream factors in drug-treated colorectal cancer cells, including NF-κB and mitogen-associated protein kinase (MAPK), that control the epithelial–mesenchymal transition (EMT), and identified mechanisms for resistance to chemotherapeutic agents after treatment with LPS.

Materials and Methods

Cell lines and reagents. The human colorectal cancer cell lines HCT-116, HCT-8 and HT-29 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). These cells were maintained in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), streptomycin, and glutamine at 37˚C in 5% CO_2. LPS, oxaliplatin and 5-FU were purchased from Sigma-Aldrich (St. Louis, MO, USA). LY294002 (PI3K/AKT inhibitor), PD98059 (ERK inhibitor), and SB216763 (GSK3β inhibitor) were purchased from Calbiochem (San Diego, CA, USA).

Analysis of apoptosis by flow cytometry. Cells (1.5×10^5 cells/ml) were cultured in 6-well plates and pre-treated with either oxaliplatin (5 μM) or 5-FU (10 μM) singly for 8 h and then treated with LPS (500 ng/ml) for an additional 18 h. For comparison, cells were treated either with oxaliplatin (5 μM) or 5-FU (10 μM) alone for 8 h, subsequently washed and then incubated with complete medium for an additional 18 h. The percentages of cells undergoing apoptosis were determined by flow cytometry using fluorescein isothiocyanate (FITC)-labeled annexin-V (BD Biosciences, San Diego, CA, USA) and 7-aminoactinomycin D (7-AAD) (BD Biosciences). Cells were harvested, rinsed with phosphate-buffered saline (PBS), and resuspended in 100 μl of 1× annexin-V binding buffer. Next, 3 μl of annexin-V-fluorescein isothiocyanate (FITC) and 3 μl of 7-AAD were added, and cells were incubated at room temperature for 15 min in the dark with gentle vortex. The stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) equipped with CellQuestpro software (BD Biosciences).

Quantitative real-time polymerase chain reaction (PCR). Total cellular RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was made from 2 μg total RNA using oligo(dT) (Bioneer, Daejeon, Korea) and reverse transcriptase (Bioneer). Quantitation of mRNA levels was measured using an ABI7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and SYBR Green Master Mix kit (Takara, Tokyo, Japan) with specific primer sets for: X-linked inhibitor of apoptosis protein (*XIAP*) (upstream primer, 5'-GTG CCA CGC AGT CTA CAA ATT CTG G; downstream primer, 5'-CGT GCT TCA TAA TCT GCC ATG GAT GG), B-cell lymphoma 2 (*BCL2*) (upstream primer, 5'-GGA TTG TGG CCT TCT TTG AG; downstream primer, 5'-CAG CCA GGA GAA ATC AAA CAT); and survivin (upstream primer, 5'-GAT TTG AAT CGC GGG ACC CGT TG; downstream primer, 5'-TCA AGA CAA AAC AGG AGC ACA GT). A specific primer set for β-actin (upstream primer, 5'-ATC CAC GAA ACT ACC TTC AA; downstream primer, 5'-STC CAC ACG GAG TAC TTG C) was used as a control. The relative mRNA quantification was calculated using the arithmetic formula $2^{-\Delta\Delta Cq}$, where ΔCq was the difference between the threshold cycle of a given target cDNA and an endogenous reference cDNA.

Detection of PI3K, GSK3β, and ERK activity using each inhibitor. HCT-116 cells (1.5×10^5 cells/ml) were exposed to either oxaliplatin (5 μM) or 5-FU (10 μM) for 8 h, subsequently washed, and then treated with LPS (500 ng/ml) for 18 h. Cells treated with oxaliplatin (5 μM) or 5-FU (10 μM) for 8 h, subsequently washed and then incubated with complete medium for an additional 18 h were used as a control, while cells treated with dimethylsulfoxide (DMSO) were used as a negative control. For preventing PI3K, GSK3β, and ERK activity, drug-exposed colon cancer cells were pre-treated with LY294002 (PI3K inhibitor, 25 μM), PD98059 (ERK inhibitor, 10 μM), or SB216763 (GSK3 inhibitor, 10 μM) for 2 h. Total cell lysates were

immunoblotted with antibodies against phospho-PI3K, PI3K, phospho-AKT, AKT, phospho-ERK, ERK, phospho-GSK3β, and GSK3β. β-Actin served as an internal control.

Western blotting. Cells were washed in PBS and lysed in NP-40 buffer (Elpis Biotech, Daejeon, Korea) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Protein phosphorylation states were preserved through the addition of phosphatase inhibitors (Cocktail II, Sigma-Aldrich) to the NP-40 buffer. Protein concentrations were determined using a BCA assay kit (Pierce, Rockford, IL, USA). Proteins (10 μg/sample) were resolved through sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (Millipore Corp., Billerica, MA, USA). Membranes were blocked with 5% skim milk prior to western blot analysis. Chemiluminescence was detected using an ECL kit (Advansta Corp., Menlo Park, CA, USA) and the multiple Gel DOC system (Fujifilm, Tokyo, Japan). The following primary antibodies were used: MyD88, E-cadherin, N-cadherin, snail family transcriptional repressor 1 (SNAIL), vimentin, alpha-smooth muscle actin (α-SMA), transcription factor 8 (TCF8)/Zinc finger E-box-binding homeobox 1 (ZEB1), octamer-binding transcription factor 4 (OCT4), sex determining region Y-box 2 (SOX2), multidrug-resistance protein 1 (MDR1), multidrug resistance-associated protein 1 (MRP1), MPR2, MRP3, Survivin, XIAP, BCL2, BCL-XL, caspase-9, caspase-3, caspase-7, poly (ADP-ribose) polymerase (PARP), phospho-p65 (Ser^{536}), p65, phospho-GSK3β (Ser^{9}), GSK3β, phospho-PI3K (Tyr^{458}), PI3K, phospho-AKT (Ser^{473}), AKT, phospho-ERK1/2 (Thr^{202}/Tyr^{204}), ERK1/2 (Tyr^{925}), phospho-p38-MAPK (Thr^{180}/Tyr^{182}), p38-MAPK, phospho-c-Jun *N*-terminal kinase (JNK), (Thr^{183}/Tyr^{185}), JNK, and β-actin (Cell Signaling Technology, Beverly, MA, USA).

Statistical analysis. Data are expressed as the mean±standard deviation (SD). Statistical analysis was conducted using one-way analysis of the variance. A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Increased expression of TLR4 in drug-treated colon cancer cells inhibits apoptosis through induction of anti-apoptotic and drug-resistance proteins. Three different human colon cancer cell lines (HCT-116, HT-29, and HCT-8) were analyzed to detect changes in TLR4 expression after treatment with 5-FU and oxaliplatin. The mRNA and protein levels of TLR4 in drug-treated colon cancer cells were significantly enhanced at an early time point in the presence of LPS stimulation (Figure 1A and B). Apoptosis of drug-treated colon cancer cells was enhanced at 48 h in a dose-dependent manner (data not shown). However, apoptotic cell death was blocked in HCT-116 cells subsequently activated with LPS 8 h after treatment with 5-FU or oxaliplatin (Figure 1C and D). Next, we investigated whether post-treatment with LPS modulates apoptosis-related proteins and sensitivity of colon cancer cells to anticancer drug. The expression of mRNA and anti-apoptotic proteins in drug-treated HCT-116 cells, including survivin, *BCL2*, and *XIAP* were enhanced after stimulation with LPS (Figure 2A). The mRNA levels of stem cell markers, including OCT4 and SOX2, were significantly up-regulated in colon cancer cells after post-drug treatment with LPS (Figure 2B). Drug resistance-related *MDR1, MRP1, MRP2*, and *MRP3* gene expression increased profoundly compared with untreated cells as measured by quantitative real-time PCR (Figure 2B). The protein levels of transcriptional factors for stem cells and drug resistance-related genes were also enhanced in cancer cells after post-drug treatment with LPS (Figure 2B). These results suggest that TLR4-mediated signaling plays an important role in survival and modulates sensitivity of cancer cells to chemotherapeutic agents.

LPS stimulation prevents apoptosis signaling in drug-treated cancer cells through phosphorylation of GSK3β and ERK. The PI3K/AKT signaling pathway is activated by TLR4 in response to LPS binding and plays a central role in the growth and progression of colorectal cancer (24). The PI3K/AKT pathway is also responsible for inactivation of GSK3β by phosphorylation (25, 26). Next, we investigated whether exposure to LPS alters the activity of PI3K/AKT, resulting in phosphorylation of GSK3β and MAPK in drug-treated HCT-116 and HCT-8 cells. Increased TLR4 in drug-treated colon cancer cells induced phosphorylation of MyD88, PI3K, AKT, and the p65 subunit of NF-κB after stimulation with LPS (Figure 3A). Exposure of drug-treated colon cancer cells to LPS enhanced the phosphorylation of ERK and GSK3β, but prevented the activation of JNK and p38 MAPK (Figure 3B). Inhibition of GSK3β by SB216763, an ATP-competitive inhibitor of GSK-3, promoted phosphorylation of PI3K/AKT in drug-exposed colon cancer cells after LPS stimulation; however, the expression of phosphorylated ERK was sustained. In addition, the expression of the active form of NF-κB, the phosphorylated p65 subunit, was blocked by SB216763 (Figure 3C). We also examined the effect of TLR4-mediated signaling pathway on inhibition of caspase-dependent apoptosis. Although 5-FU and oxaliplatin induced the cleavage of caspase-3 or caspase-7 for apoptosis signal transduction, LPS stimulation inhibited apoptosis of drug-treated colon cancer cells (Figure 4A). Meanwhile, pre-treatment with SB216763 attenuated the prevention of LPS-induced cell survival in drug-treated colon cancer cells (Figure 4B). These results suggest that up-regulation of TLR4 by anticancer drugs and TLR4-mediated GSK3β phosphorylation or inactivation are closely related to inhibition of apoptosis and survival in cancer cells.

LPS-induced activation of ERK and NF-κB prevents apoptosis of drug-treated cancer cells. To further understand the signaling mechanisms involved in blocking apoptosis, we examined whether PI3K/AKT and ERK signaling are required to prevent apoptosis of drug-treated HCT-116 cells and are related to GSK3β after stimulation with LPS. Pre-treatment with PI3K/AKT inhibitor LY294002 prevented the

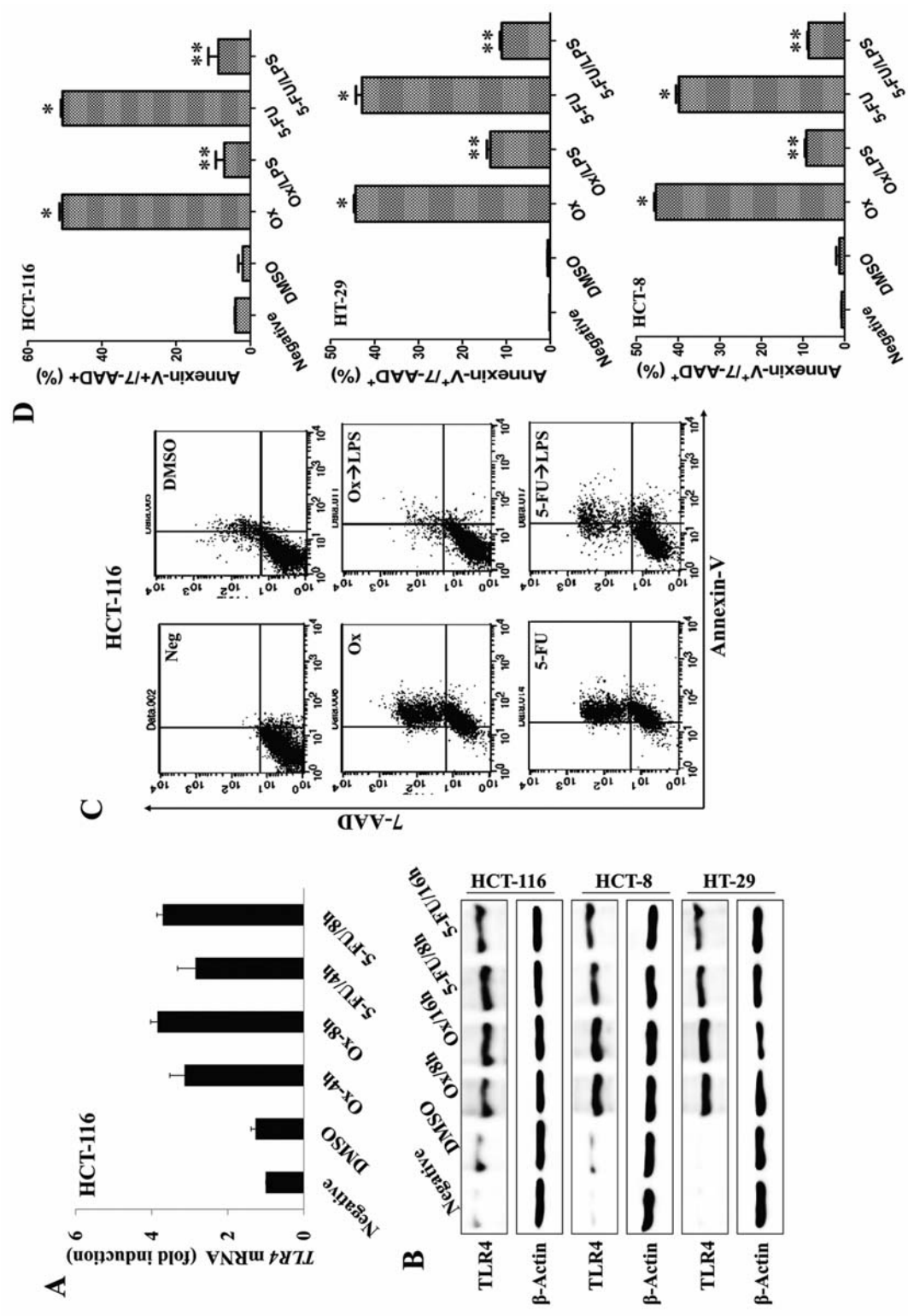

Figure 1. *Increased toll-like receptor 4 (TLR4) expression in drug-treated colon cancer cells prevents apoptosis. Three different human colon cancer cell lines (HCT-116, HT-29, and HCT-8) (1.5×10^5 cells/ml) were cultured in 6-well plates overnight. Cells were exposed to either oxaliplatin (Ox; 5 μM) or 5-fluorouracil (5-FU) (10 μM) for 4 or 8 h for mRNA level analysis, and for 8 or 16 h for protein level analysis. HCT-116, HT-29, and HCT-8 cells (1.5×10^5 cells/ml) were cultured in 6-well plates overnight. Total RNA and proteins were extracted from cell lysates, and then real-time polymerase chain reaction (A) and western blots (B) for TLR4 were performed. C: Cells were exposed to either oxaliplatin (5 μM) or 5-FU (10 μM) for 8 h, then subsequently washed, and treated with lipopolysaccharide (LPS) (500 ng/ml) for 18 h. For comparison, cells were treated either with oxaliplatin (5 μM) or 5-FU (10 μM) for 8 h, subsequently washed and then incubated with complete medium for an additional 18 h. To detect the degree of apoptosis, cells were stained with fluorescein isothiocyanate (FITC)-labeled annexin-V and 7-aminoactinomycin D (7-AAD) and analyzed by flow cytometry. D: The percentage of annexin-V$^+$/7-AAD$^+$ cells, signifying apoptotic cells. *p<0.001 (DMSO-treated cancer cells versus drug-treated cancer cells). **p<0.005 (drug-treated cancer cells versus LPS-stimulated drug-treated cancer cells). Data are presented as the mean±standard deviation of three determinations. DMSO: Dimethyl sulfoxide.*

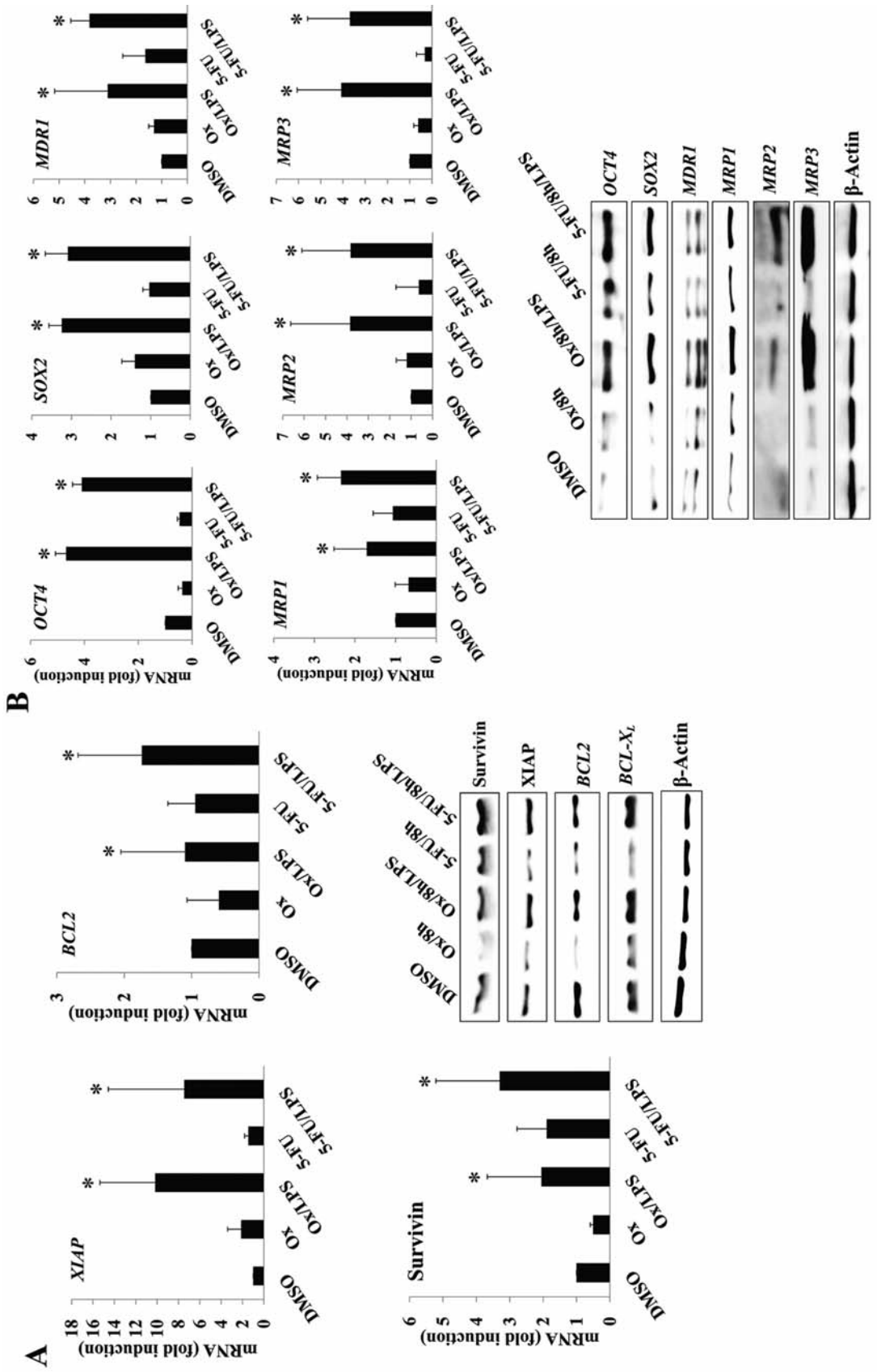

Figure 2. *Lipopolysaccharide (LPS) stimulation inhibits apoptosis of drug-exposed colon cancer cells through the induction of anti-apoptotic and drug-resistance proteins. HCT-116 cells (1.5×10^5 cells/ml) were cultured in 6-well plates overnight. Cells were exposed to either oxaliplatin (Ox; 5 μM) or 5-fluorouracil (5-FU) (10 μM) for 4 h (for mRNA level) or 8 h (for protein level), subsequently washed, and then treated with LPS (500 ng/ml) for 18 h. For comparison, cells were treated either with oxaliplatin (5 μM) or 5-FU (10 μM) for 4 h (for mRNA level) or 8 h (for protein level), subsequently washed and then incubated with complete medium for an additional 18 h. Total RNA and proteins were extracted from cell lysates, and real-time polymerase chain reaction and western blots for X-linked inhibitor of apoptosis protein (XIAP), B-cell lymphoma 2 (BCL2), BCL-X_L, survivin (A), and octamer-binding transcription factor 4 (OCT4), sex determining region Y-box 2 (SOX2), multidrug-resistance protein 1 (MDR1), multidrug resistance-associated protein 1 (MRP1), MRP2, and MRP3 (B), were performed. β-Actin served as an internal control. *$p<0.05$ (drug-treated cancer cells versus LPS-stimulated drug-treated cancer cells). Data are presented as the mean±standard deviation of three determinations. Results are representative of three independent experiments.*

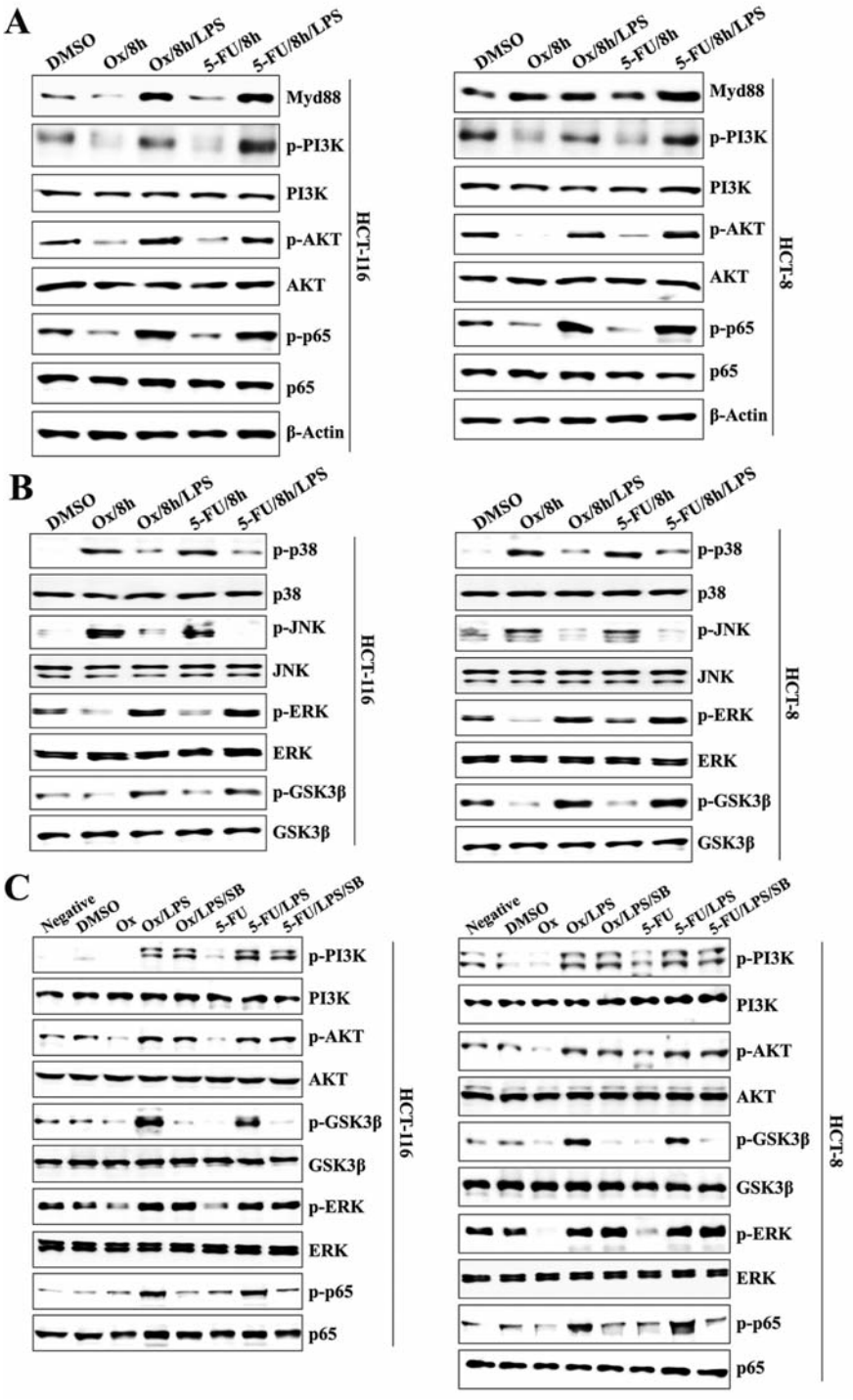

Figure 3. *Increased toll-like receptor 4 (TLR4) expression in drug-treated colon cancer cells induces the activation of phosphatidyl-inositide 3-kinase (PI3K), protein kinase B (AKT), and p65 and the phosphorylation of glycogen synthase kinase 3β (GSK3β) and extracellular signal–regulated kinase (ERK) after stimulation with lipopolysaccharide (LPS). HCT-116 and HCT-8 cells (1.5×10^5 cells/ml) were cultured in 6-well plates overnight. Cells were exposed to either oxaliplatin (Ox; 5 μM) or 5-FU (10 μM) for 8 h, subsequently washed, and then treated with LPS (500 ng/ml) for 18 h. For comparison, cells were treated either with oxaliplatin (5 μM) or 5-FU (10 μM) for 8 h, subsequently washed and then incubated with complete medium for an additional 18 h. Total protein was subjected to western blot analysis with antibodies against MyD88, phospho-PI3K, PI3K, phospho-AKT, AKT, phospho-p65, p65 (A) and phospho-p38, p38, phospho-c-Jun N-terminal kinase (JNK), JNK, phospho-ERK, ERK, phospho-GSK3β, and GSK3β (B). β-Actin served as an internal control. C: Drug-exposed colon cancer cells were pre-incubated with 10 μM SB216763 (SB) for 2 h and then treated with 500 ng/ml LPS for 18 h. Total proteins were extracted from cell lysates, and western blots for the indicated antibodies were performed. Results are representative of three independent experiments.*

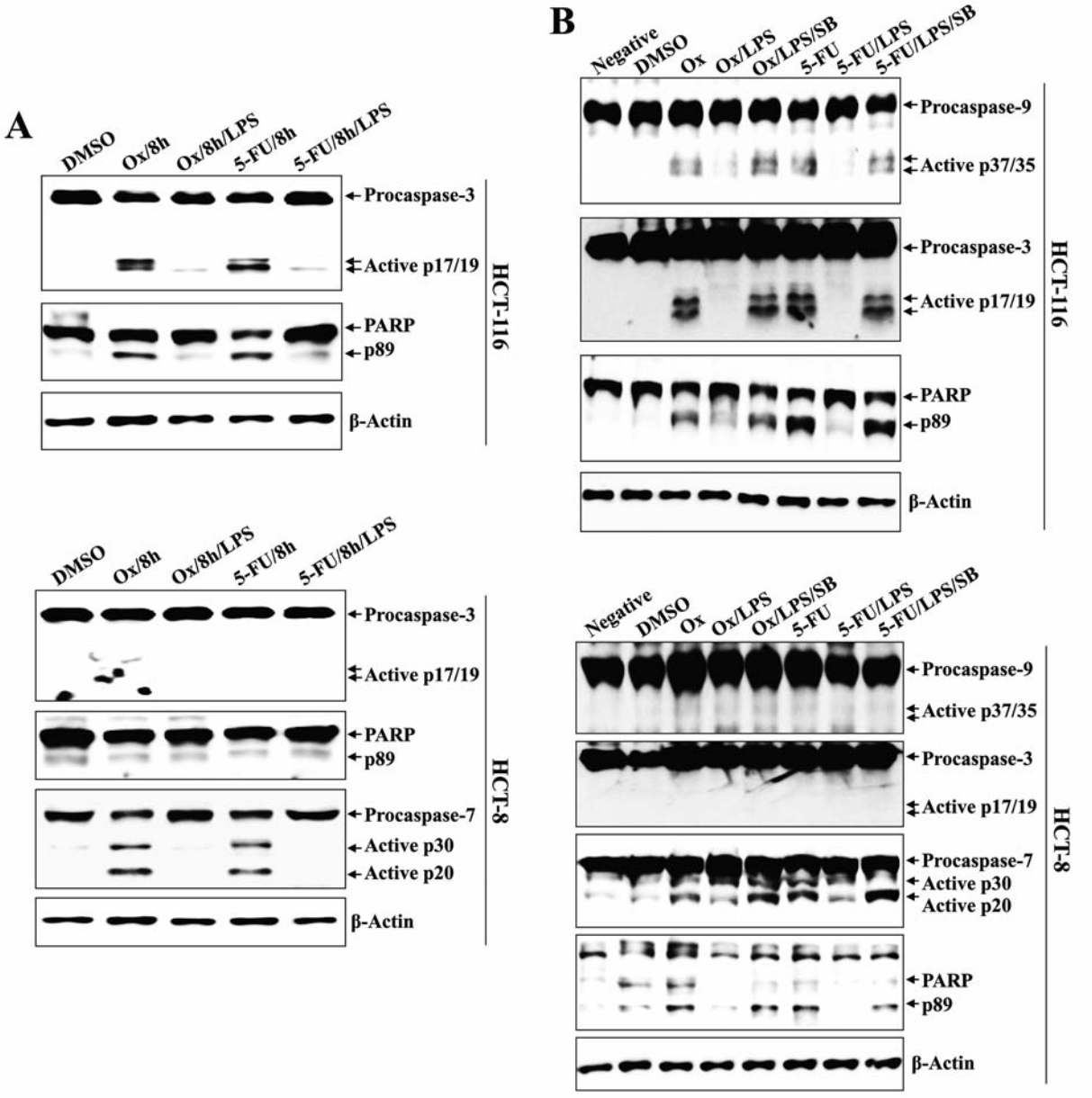

Figure 4. *Lipopolysaccharide (LPS) stimulation blocks apoptosis signaling in drug-exposed cancer cells through glycogen synthase kinase 3β (GSK3β). HCT-116 or HCT-8 cells (1.5×10^5 cells/ml) were cultured in 6-well plates overnight. Cells were exposed to either oxaliplatin (Ox; 5 μM) or 5-fluorouracil (5-FU) (10 μM) for 8 h, subsequently washed, and then treated with LPS (500 ng/ml) for 18 h. For comparison, cells were treated with either with oxaliplatin (5 μM) or 5-FU (10 μM) for 8 h, subsequently washed and then incubated with complete medium for an additional 18 h. A: Total protein was subjected to western blot analysis with antibodies to caspase-3, caspase-7, and poly (ADP-ribose) polymerase (PARP). B: Drug-exposed colon cancer cells were pre-incubated with 10 μM SB216763 (SB) for 2 h and then treated with 500 ng/ml LPS for 18 h. Total proteins were extracted from cell lysates, and western blots for caspase-9, caspase-3, caspase-7, PARP. β-Actin served as an internal control. Results are representative of three independent experiments.*

activation of GSK3β and the p65 subunit of NF-κB in LPS-stimulated drug-treated cancer cells, whereas it failed to suppress the phosphorylation of ERK (Figure 5A). Meanwhile, the ERK inhibitor (PD98059) re-activated the apoptosis signaling pathway in drug-treated HCT-116 cells through cleavage of caspase-3 and PARP, despite stimulation with LPS (Figure 5B). Although PD98059 blocked the activation of ERK and NF-κB for cancer cell survival, the expression of phosphorylated PI3K and GSK3β was sustained in LPS-exposed drug-treated HCT-116 cells

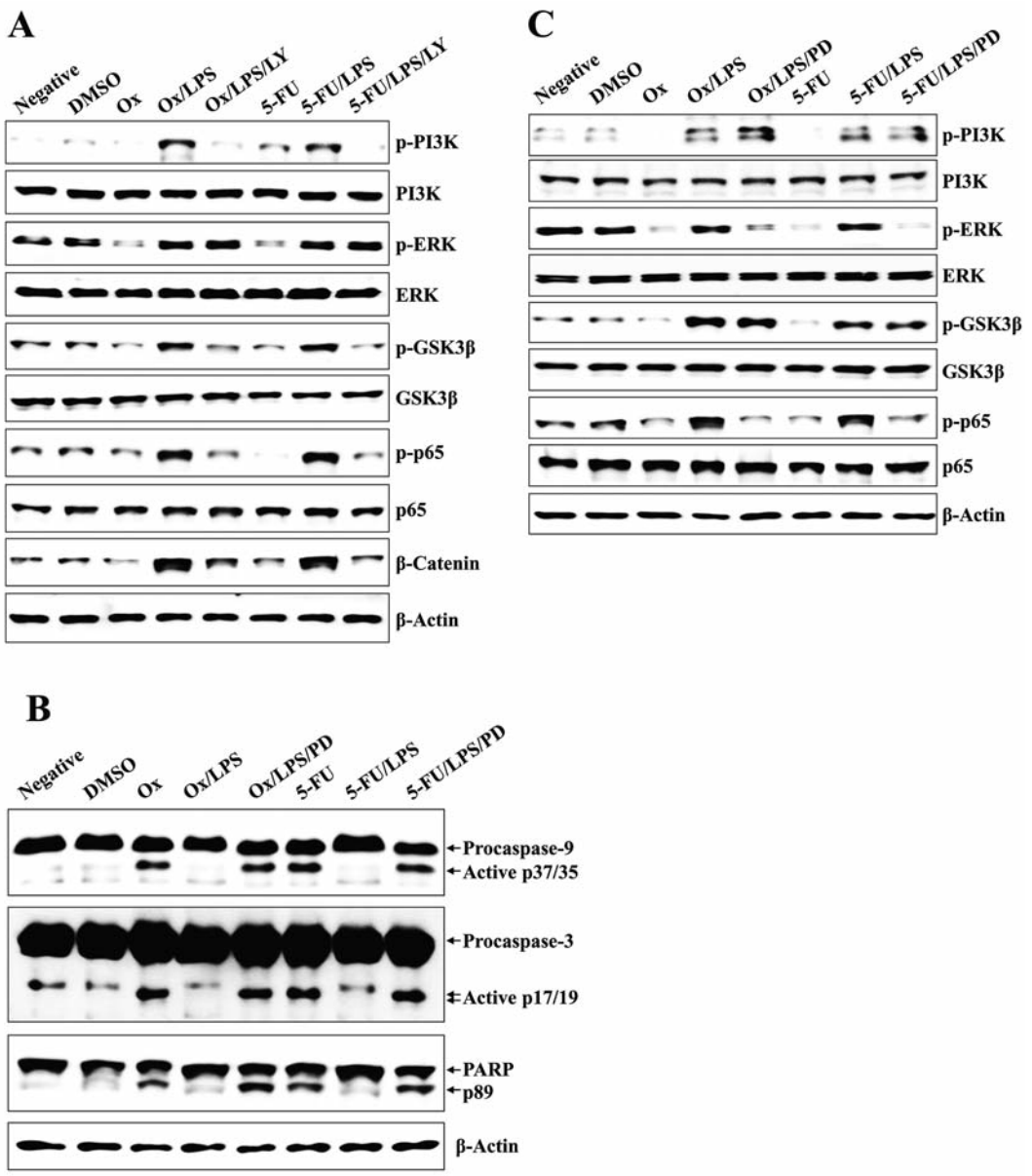

Figure 5. *Activation of phosphatidyl-inositide 3-kinase (PI3K)/protein kinase B (AKT) and extracellular signa-regulated kinase (ERK) by lipopolysaccharide (LPS) stimulation inhibits apoptosis of drug-treated cancer cells. HCT-116 cells (1.5×10^5 cells/ml) were cultured in 6-well plates overnight. Cells were exposed to either oxaliplatin (Ox; 5 μM) or 5-fluorouracil (5-FU) (10 μM) for 8 h, subsequently washed, and then treated with LPS (500 ng/ml) for 18 h. For comparison, cells were treated either with oxaliplatin (5 μM) or 5-FU (10 μM) for 8 h, subsequently washed and then incubated with complete medium for an additional 18 h. Drug-exposed colon cancer cells were pre-treated with 25 μM LY294002 (LY) (A) or with 10 μM PD98059 (PD) (B and C) for 2 h and then treated with 500 ng/ml LPS for 18 h. Total proteins were extracted from cell lysates, and western blots performed for p-PI3K, PI3K, p-ERK, ERK, phospho-glycogen synthase kinase 3β (GSK3β), GSK3β, p-p65, p65, and β-catenin (A), caspase-9, caspase-3, and PARP (B), and p-PI3K, PI3K, p-ERK, ERK, p-GSK3β, GSK3β, p-p65, and p65 (C). β-Actin served as an internal control. Results are representative of three independent experiments.*

(Figure 5C). These results suggest that LPS-mediated ERK/NF-κB activation is independent of the LPS-induced GSK3β anti-apoptosis signaling pathway in drug-treated colon cancer cells.

LPS-induced phosphorylation of GSK3β modifies drug resistance and epithelial mesenchymal transition in drug-treated cancer cells. Next, we investigated whether GSK3β regulates sensitivity to anticancer drug and metastasis or

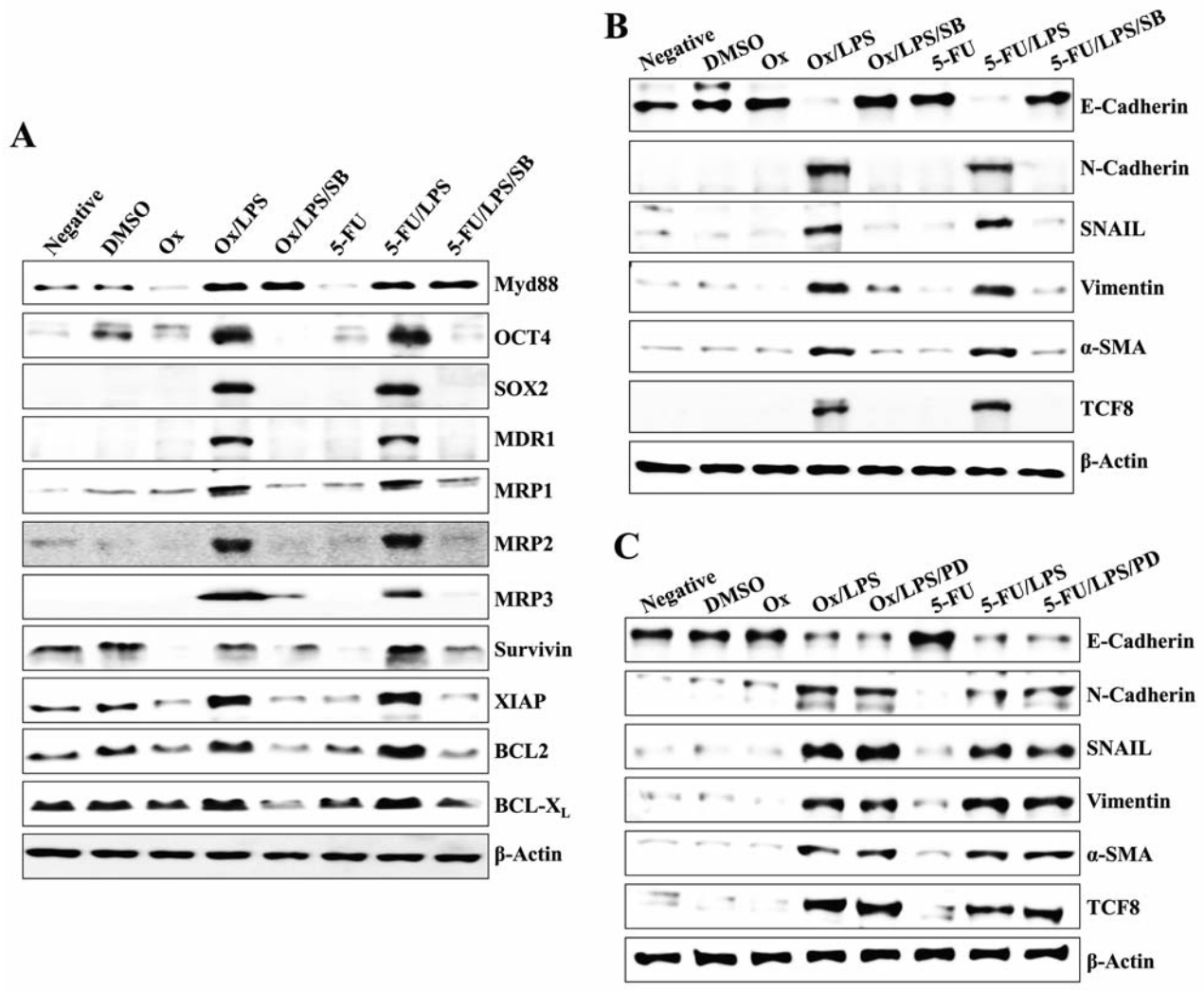

Figure 6. *Phosphorylation of glycogen synthase kinase 3β (GSK3β) by lipopolysaccharide (LPS) stimulation induces drug resistance and epithelial–mesenchymal transition in drug-exposed cancer cells. HCT-116 cells (1.5×10^5 cells/ml) were cultured in 6-well plates overnight. Cells were exposed to either oxaliplatin (Ox; 5 μM) or 5-fluorouracil (5-FU) (10 μM) for 8 h, subsequently washed, and then treated with LPS (500 ng/ml) for 18 h. For comparison, cells were treated either with oxaliplatin (5 μM) or 5-FU (10 μM) for 8 h, subsequently washed and then incubated with complete medium for an additional 18 h. Drug-exposed colon cancer cells were pre-incubated with 10 μM SB216763 (SB) (A and B) or 10 μM PD98059 (PD) (C) for 2 h and then treated with 500 ng/ml LPS for 18 h. Total proteins were extracted from cell lysates, and western blots performed for MyD88, octamer-binding transcription factor 4 (OCT4), sex determining region Y-box 2 (SOX2), multidrug-resistance protein 1 (MDR1), multidrug resistance-associated protein 1 (MRP1), MRP2, MRP3, survivin, XIAP, B-cell lymphoma 2 (BCL2), BCL-X_L (A) and E-cadherin, N-cadherin, snail family transcriptional repressor 1 (SNAIL), vimentin, alpha-smooth muscle actin (α-SMA), and transcription factor 8 (TCF8)/zinc finger E-box-binding homeobox 1 (ZEB1) (B and C). β-Actin served as an internal control. Results are representative of three independent experiments.*

invasion in drug-treated colon cancer cells after LPS stimulation. LPS stimulation in drug-treated HCT-116 cells enhanced the expression of cancer stem cell markers (OCT4 and SOX2) and drug-resistance related proteins (MDR1, MRP1, MRP2, and MRP3) (Figure 6A). However, inhibition of GSK3β with SB216763 suppressed the induction of those markers as well as expression of anti-apoptosis proteins including survivin, XIAP, BCL2, and BCL-XL (Figure 6A). TLR4 stimulation with LPS contributed to the expression of EMT-related mesenchymal markers (N-cadherin, SNAIL, vimentin, and α-SMA) in drug-treated colon cancer cells (Figure 6B). In addition, inhibition of GSK3β also prevented the aberrant expression of invasive phenotypes in 5-FU or oxaliplatin-treated cancer cells after treatment with LPS (Figure 6B). Meanwhile, exposure of cancer cells to PD98059 before drug treatment sustained the expression of

EMT-related mesenchymal markers after treatment with LPS (Figure 6C). These results suggest that inhibition of the GSK3β- and ERK-associated signaling pathways in drug-treated cells contributes to concomitant control of drug resistance and EMT processes.

Discussion

GSK3β phosphorylates proto-oncoproteins such as β-catenin to suppress carcinogenesis by interfering with the expression of several oncogenic transcription factors (27). Aberrant expression and activity of GSK3β is not related to WNT/β-catenin signaling and AKT activation, but does contribute to cancer cell survival and proliferation (20). GSK3β inhibition using SB216763 or knockdown of GSK3β using siRNA transfection reduces survival and proliferation of pancreatic cancer cells (21). Based on these reports, the role of GSK3β in cancer cells remains controversial and difficult to define according to cell type or condition. TLR4 expressed in cancer cells or tumor stromal cells reflects considerably increased risk of disease progression and poor prognosis in colorectal cancer (28, 29). However, the role of increased TLR4 expression in colorectal cancer after treatment with chemotherapeutic drug is still unknown. In this study, TLR4 stimulation with LPS of drug-treated cancer cells induced PI3K-mediated phosphorylation of GSK3β and the p65 subunit of NF-κB, inhibiting apoptosis, up-regulating expression of drug-resistant proteins, and promoting EMT processes. Stimulation with LPS in drug-treated colon cancer cells also phosphorylated and activated ERK to promote cell survival without disturbing the phosphorylation of GSK3β. Pharmacological inhibition of GSK3β attenuated survival and metastasis of drug-treated cancer cells after treatment with LPS. These results suggest that phosphorylation or inactivation of GSK3β by TLR4 stimulation of drug-exposed cancer cells induces drug-resistance and metastasis or invasion of cancer cells, and concomitantly inhibition of GSK3β and ERK signaling pathway, which is important in the control of acquired drug resistance and metastasis of cancer cells (Figure 7).

Although the level of TLR4 expression varies across colon cancer cell lines as well as across patients of different ages and sexes (30), elevated TLR4 expression is detected in all tumor components, including cancer cells and stromal layers (28). The TLR/MyD88 signaling pathway is one of key factors for development of colitis-associated cancer and is also related to the progression from adenoma to invasive carcinoma (31). MyD88 signaling facilitates the growth of intestinal polyps while the deletion of MyD88 limits polyp growth in mice (32). In addition, overexpression of TLR4 generates a higher level of PI3K/AKT activation, which in turn phosphorylates GSK3β and activates downstream signaling pathways (33). Pre-treatment with the PI3 kinase inhibitor, wortmannin, reduces GSK3β phosphorylation and protects against liver damage (26). Inhibition of GSK3 activity by SB216763 significantly reduces the activation of BCL2-like protein 4 (BAX) and caspase-3 in neural precursor cells and efficiently blocks cell death after trophic factor withdrawal (34). In contrast, although suppression of PI3K and GSK3β using a specific inhibitor ameliorated GSK3β inactivation or phosphorylation, treatment with LY294002 and SB216763 reactivated the caspase-dependent apoptosis signaling pathway in LPS-stimulated drug-exposed colon cancer cells. These results suggest that up-regulated TLR4 stimulation in drug-treated colon cancer cells triggers altered GSK3β-dependent signaling to promote cell survival.

LPS stimulation promotes the survival of cancer cells through the activation of JNK and p38 MAPK, and LPS-dependent PI3K/AKT activation stabilizes anti-apoptotic protein myeloid cell leukemia 1 (MCL1), resulting in prevention of apoptosis (35). TLR/MyD88 stimulation using LPS induces ERK phosphorylation to block the degradation of the oncoprotein c-MYC for cell survival (36). Stimulation of drug-treated colon cancer cells with LPS induced the

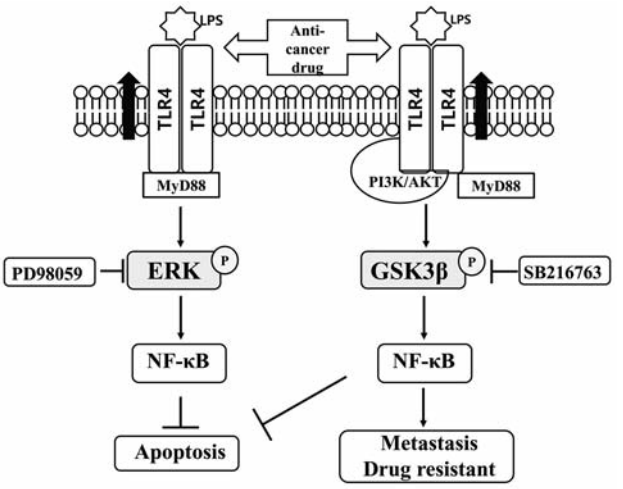

Figure 7. *Schematic diagram of intracellular signaling mechanism with enhanced toll-like receptor 4 (TLR4) expression in human colon cancer cells after chemotherapy. TLR4 stimulation with lipopolysaccharide (LPS) in drug-treated cancer cells activated phosphatidyl-inositide 3-kinase (PI3K)/protein kinase B (AKT) and subsequently phosphorylated glycogen synthase kinase 3β (GSK3β), leading to inhibition of apoptosis, induction of drug-resistant proteins, and promotion of epithelial–mesenchymal transition (EMT) processes. Stimulation with LPS in drug-treated colon cancer cells also induced the activation of extracellular signal–regulated kinase (ERK) to promote cell survival independently of LPS-mediated GSK3β phosphorylation. NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B-cells; PD98059: ERK inhibitor; MyD88: myeloid differentiation primary response gene 88; SB216763: selective GSK3 inhibitor.*

phosphorylation of ERK in this study. Pre-treatment with PD98059, an ERK-specific inhibitor, re-induced the cell death signaling pathway of the drug-exposed cancer cells after LPS stimulation through the re-activation of caspase-3 and caspase-9. Moreover, pre-exposure to LY294002 or SB216763 failed to attenuate the activation of ERK in drug-treated cancer cells stimulated by LPS. In addition, PD98059 also had no influence on the phosphorylation of PI3K and GSK3β. These results demonstrate that LPS-mediated ERK signaling activates the cell-survival signal concomitantly and GSK3β independently in drug-damaged cancer cells (Figure 7).

LPS-induced TLR4 signaling stimulates hepatocellular carcinoma cell invasion and EMT processes *in vitro* and *in vivo*. A high expression of TLR4 in hepatoma is strongly connected with both poor prognosis and survival (37). TLR4-positive hepatocellular carcinoma cell lines also display high migratory capacity and stem-like properties (38). Meanwhile, inhibition of GSK3β by lithium chloride, SB216763, and dominant-negative forms of GSK3β promotes cell invasiveness through the activation of the noncanonical WNT/β-catenin pathway (39). We also observed that LPS-mediated GSK3β phosphorylation of drug-treated cancer cells elevated the expression of drug resistance-related genes and anti-apoptotic proteins, as well as induced EMT phenotypes in this study. In addition, inhibition of GSK3β by SB216763 suppressed the activation of NF-κB, metastatic characteristics, drug resistance, and stem cell-like properties in LPS-stimulated drug-damaged cancer cells. These results suggest that TLR4 in cancer cells after anticancer therapy is a promising target molecule, because it is responsible for metastasis or invasive activity. Furthermore, phosphorylation of GSK3β by LPS in drug-treated cancer cells also contributes to the migratory activity and generation of cancer stem cells. Based on these results, we need to further investigate approaches for controlling phosphorylated GSK3β in order to prevent metastasis and drug resistance in patients with cancer after treatment with chemotherapeutic drug.

Expression of TLR4 is very low in normal cells, but increases in both precancerous colonic adenomas and colon cancer tissue (40). Our study demonstrates that TLR4 stimulation responds to LPS derived from intestinal microflora that may have some implications for the chemotherapeutic strategies against cancer. Taken together, characterization of TLR4-mediated GSK3β phosphorylation might be critical for preventing recurrence or metastasis after chemotherapeutic treatment. Analysis of downstream or connected signaling pathways may be a promising goal for defining the mechanism of progression of colorectal cancer.

Conflicts of Interest

The Authors declare that they have no conflicts of interest to disclose.

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