Companion Diagnostics and Cancer Biomarkers

TLR4 Is a Novel Determinant of the Response to Paclitaxel in Breast Cancer

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Abstract

Overexpression of Toll-like receptor-4 (TLR4) in human tumors often correlates with chemoresistance and metastasis. We found that TLR4 is overexpressed in the majority of clinical breast cancer samples and in 68% of the examined breast cancer lines. TLR4 is activated by lipopolysaccharide (LPS) and other ligands including the widely used drug paclitaxel. LPS is frequently used to show a tumor-promoting role of TLR4 although this bacterial component is unlikely to be found in the breast cancer environment. We reasoned that paclitaxel-dependent activation of TLR4 is more relevant to breast cancer chemoresistance that could be mediated by activation of the NF-κB pathway leading to upregulation of prosurvival genes. To test this hypothesis, we correlated TLR4 expression with resistance to paclitaxel in two modified breast cancer lines with either depleted or overexpressed TLR4 protein. Depletion of TLR4 in naturally overexpressing MDA-MB-231 cells downregulated prosurvival genes concomitant with 2- to 3-fold reduced IC₅₀ to paclitaxel in vitro and a 6-fold decrease in recurrence rate in vivo. Conversely, TLR4 overexpression in a negative cell line HCC1806 significantly increased expression of inflammatory and prosurvival genes along with a 3-fold increase of IC₅₀ to paclitaxel in vitro and enhanced tumor resistance to paclitaxel therapy in vivo. Importantly, both tumor models showed that many paclitaxel-upregulated inflammatory cytokines were coinduced with their receptors suggesting that this therapy induces autocrine tumor-promoting loops. Collectively, these results show that paclitaxel not only kills tumor cells but also enhances their survival by activating TLR4 pathway. These findings suggest that blocking TLR4 could significantly improve response to paclitaxel therapy. Mol Cancer Ther; 12(8); 1676–87. ©2013 AACR.

Introduction

Chemoresistance is the major obstacle to successful treatment of metastatic cancers. The two well-recognized mechanisms of chemoresistance are overexpression of drug transporters that decrease intracellular concentration of the drug (1) and preexisting mutations that decrease drug binding to target proteins (2). In addition to these intrinsic mechanisms, therapeutic efficacy can be reduced by chemotherapy-induced upregulation of inflammatory and prosurvival genes (3–5). The goal of this study was to delineate the latter mechanism that enables tumor cells to escape cytotoxic therapy through acquisition of a more aggressive phenotype.

Chemotherapy induction of tumor-promoting pathways can be illustrated by response of malignant cells to taxanes. Paclitaxel, the active component of taxanes, is a common drug used against various solid tumors (6). Paclitaxel induces apoptosis by overstabilizing microtubules (7), which leads to cell arrest at the G₂–M phase (8). Although the therapeutic use of paclitaxel has been improved by formulating it as nanoparticles (nab-PXL; ref. 9), the overall response of patients with breast cancer even to this advanced therapy remains below 35% (10). The main reason for low response rate is tumor recurrence after cessation of therapy, which might relate to paclitaxel-mediated induction of inflammatory mediators including TNF-α (11), interleukin (IL)-1β (12), IL-8 (13), IL-6 (14), and VEGF-A (3, 4). In addition to inflammatory mediators, paclitaxel also upregulates prosurvival proteins including XIAP (15), Bcl-2 (16), Akt (17), and Bcl-xL (4, 15). Cumulatively, these studies suggest that tumor cells respond to paclitaxel by activating two conflicting pathways: one induced by overstabilizing microtubules which leads to apoptosis and the other induced by a currently undefined receptor that promotes tumor cell survival through upregulation of inflammatory and prosurvival genes.

On the basis of the transcriptional profile induced by paclitaxel, we postulated that the likely candidate for transmitting paclitaxel signaling is Toll-like receptor-4 (TLR4). This hypothesis is based on multiple lines of evidence. First, paclitaxel-induced prosurvival transcription largely overlaps with that of lipopolysaccharide (LPS).
a natural ligand of TLR4 (18). Paclitaxel activates TLR4 by binding to human or mouse MD2, an adaptor protein that confers LPS responsiveness of the TLR4 cascade (19). Second, paclitaxel activates several pro-oncogenic signaling pathways including NF-κB (20), mitogen-activated protein kinase (MAPK; ref. 21), and phosphoinositide-3-kinase (PI3K; ref. 22). This effect of paclitaxel has been shown in many human cancers including breast (20), prostate (23), ovarian (17), colon (24), lung, and pancreatic (25) tumors. Third, paclitaxel increases expression of inflammatory cytokines in both tumor lines (12, 20, 26) and human patients with cancer (14). Fourth, TLR4 is likely to transduce paclitaxel signals because anti-TLR4 siRNA downregulates the expression of inflammatory cytokines induced by paclitaxel (15, 26). Finally, overexpressed TLR4 in human tumors correlates with resistance to paclitaxel therapy (26), recurrence (27), clinical stage and grade (28), and poor patient survival (17). Taken together, this evidence suggests that TLR4 expression in human breast cancer contributes to resistance to paclitaxel therapy due to transcriptional upregulation of prosurvival genes.

To test this hypothesis, we determined TLR4 expression in a broad panel of breast cancer lines, correlated cell responsiveness to paclitaxel with TLR4 expression, and generated two isogenic lines with either reduced or increased TLR4 expression. These new breast cancer models enabled us to analyze the role of TLR4 in paclitaxel resistance using a variety of in vitro and in vivo approaches. The results strongly suggest that TLR4-mediated increase of cytokines and their receptors protects tumors from chemotherapy through activation of autocrine loops in neoplastic cells that may, in turn, activate paracrine pathways in the tumor environment.

Materials and Methods

Materials

LPS derived from *Escherichia coli* 055:B5, TRI reagent, and protease inhibitors were purchased from Sigma-Aldrich. Dulbecco’s modified Eagle’s medium (DMEM) and supplements were from Lonza. SB225002 and PD98059 were purchased from EMD Millipore.

Plasmids and study drugs

Human TLR4 CDS ligated into pCDNA3.1 plasmid (TLR4-pCDNA3.1) was purchased from Addgene. TLR4 short hairpin RNA (shRNA) plasmid (TLR4-psiRNA) was purchased from InvivoGen. The pKT2-ires-PURO plasmid was a generous gift from Dr. Wilber (Southern Illinois University, Springfield, IL). Paclitaxel albumin-bound nanoparticles (nab-PXL) were obtained from Abraxis BioScience (now Celgene). Paclitaxel (Taxol) was obtained from Bristol-Meyer Squibb.

Antibodies

Primary antibodies were goat anti-hTLR4 (Imgenex); rabbit anti-Bcl-2, anti-Bcl-xl, anti-p-Akt and anti-Akt (Cell Signaling); rabbit anti-p65, anti-p50 and anti-p-p50 (Santa Cruz Biotechnology); rabbit anti-p-p65 (Epitomics), rabbit anti-active caspase-3 (R&D Systems), and mouse anti-β-actin (JLA20; Developmental Studies Hybridoma Bank). Secondary horseradish peroxidase (HRP)-conjugated anti-goat, anti-rabbit, and anti-mouse antibodies were from Jackson ImmunoResearch Laboratories.

Human breast cancer tissues and cell lines

Human tumor and normal breast cancer tissues were obtained from ILSBio. Luciferase-tagged MDA-MB-231 and HCC1806 cells were cultured in 10% DMEM with standard additives at 37°C in 10% CO2. All cell lines were authenticated by American Type Culture Collection and tested for mycoplasma using a kit from Roche Diagnostics GmbH.

Generation of stable sublines with modified TLR4

To suppress TLR4 expression, MDA-MB-231 cells were stably transfected with TLR4-psiRNA or scrambled shRNA followed by selection with zeocin (30 μg/mL). To overexpress TLR4, HCC1806 line was transfected with human TLR4 CDS subcloned in pKT2-ires-PURO vector or empty plasmid followed by selection with puromycin (1 μg/mL). Changes in TLR4 expression were determined by quantitative reverse transcriptase (qRT)-PCR and Western blotting. Modified sublines were designated as 231ΔTLR4 and 231ΔTLR4+ or 1806ΔTLR4 and 1806ΔTLR4+ for MDA-MB-231 and HCC1806 sublines, respectively.

RT-PCR and qRT-PCR

RNA was extracted by TRI reagent and reverse transcribed using Revert Aid cDNA synthesis kit. Primers were designed on the basis of CDS of human targets found in NCBI database. All primer sequences are listed in Supplementary Table S1. Targets were amplified for 35 cycles followed by gel analysis and imaging. For qRT-PCR, cells were treated with LPS (100 ng/mL) or nab-PXL (10 nmol/L) followed by transcript analysis using GoTaq-Green Master Mix (Promega) and Real-Time PCR machine (Applied BioSystems). Data were normalized to β-actin, and relative mRNA expression was determined using the ΔΔCt method (29).

Western blot analysis

Cells seeded in a 6-well plate at the density of 0.5 × 10^6 adhered overnight before treatment with 10 nmol/L nab-PXL for 0, 12, 24, and 48 hours followed by lysis in 150 μL buffer containing 50 mmol/L Tris-HCL, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton-X100, 0.1% SDS, and protease inhibitors. Lysates were cleared at 13,000 rpm for 10 minutes, boiled, separated on 12% SDS gels, and transferred to a nitrocellulose membrane followed by overnight incubation with primary antibodies against TLR4, Bcl-2, Bcl-xl, NF-xB p50, p-p50, NF-xB p65, p-p65, Akt, p-Akt, ERK1/2, p-ERK1/2, or β-actin. Protein bands were visualized by a Fujifilm LAS-3000 camera after a 1-hour incubation with HRP-conjugated secondary antibodies and development with ECL (Fierce).
Cytotoxic assay
Cells seeded at the density of 50,000 cells per well in 24-well plates were treated with 0 to 100 nmol/L of paclitaxel, nab-PXL, or plain medium. After 48 hours, viable cells were enumerated to determine the IC_{50}. Each condition was tested in duplicate and reproduced 3 times.

Fluorescence-activated cell-sorting analysis of apoptosis and cell cycle
Apoptotic cells were visualized using Annexin V Detection Kit according to the manufacturer’s instructions (BD Pharmingen). Briefly, nab-PXL–treated cells (10 nmol/L) for 48 hours were incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI). After a 15-minute incubation, samples were analyzed by a Becton Dickinson FACS Calibur flow cytometer. Cell-cycle phase distribution was determined in 70% alcohol-fixed cells stained with 10 μg/mL of PI, sorted by Fluorescence-activated cell sorting (FACS), and analyzed using the CellQuest Software (Becton Dickinson).

Measurement of cytokine concentrations by ELISA
Conditioned medium (CM) was collected from MDA-MB-231 and HCC1806 lines treated with 10 nmol/L of nab-PXL or plain medium. Concentrations of IL-6, IL-8, and TNF-α were determined by ELISA kits purchased from Peprotech. All experiments were carried out in duplicate and reproduced 3 times. Results are presented as mean pg of cytokine ± SD normalized per 10^6 cells.

Animal studies
Tumor growth of orthotopically implanted cell lines was monitored as described previously (3, 4). Briefly, 4 × 10^6 cells suspended in 50% Matrigel were implanted into the mammary fat pad (MFP) of 4- to 6-week-old female SCID mice (Taconic). Every 2 to 3 days, perpendicular tumor diameters were measured by digital caliper and used to calculate tumor volume according to the formula: volume = D_h^2π/6, where D equals larger diameter and d equals smaller diameter. Animal care was in accordance with institutional guidelines.

Statistical analysis
Statistical analyses were conducted using GraphPad Prism software. Results are expressed as mean ± SD. Combination Index (CI) indicating synergism of 2 drugs was calculated by CompuSyn software (CompuSyn Inc.). Statistical significance for continuous variables and categorical covariants was determined by Student paired t test and χ² test, respectively. P ≤ 0.05 was considered significant.

Results

TLR4 expression and function in human breast cancer tissues and cell lines
We first determined the frequency of TLR4 expression in human breast cancer clinical samples and established lines. qRT-PCR analysis of 20 malignant breast tumors and 5 normal mammary tissues showed significantly (P = 0.01) upregulated TLR4 expression in invasive breast cancer as compared with nonmalignant breast (Fig. 1A). Screening of breast cancer lines by endpoint and qRT-PCR showed that 11 of 16 (68%) lines had moderate to high TLR4 expression (Fig. 1B and C). The MDA-MB-231 line had the highest expression (53-fold above the level in negative lines) whereas HCC1806 line did not express TLR4. Western blotting confirmed TLR4 protein expression in MDA-MB-231 and HCC1806 lines (Fig. 1B, insert). These 2 lines were selected to represent TLR4-positive and -negative breast cancer.

We next determined the functionality of TLR4 in these lines. Cells were treated with 100 ng/mL of LPS followed by quantitative analysis of TLR4 targets such as IL-6, IL-8, TNF-α, and MCP-1. LPS induced ~100-fold higher response in MDA-MB-231 as compared with HCC1806 cells. The effect in MDA-MB-231 cells was mediated specifically via TLR4 because 94% of the response was suppressed by an anti-TLR4 antibody (Supplementary Fig. S1). In contrast, a minor response in HCC1806 cells was TLR4-independent suggesting that other TLRs might be responsible for this effect. Indeed, HCC1806 cells express functional TLR2 and TLR9 (Supplementary Fig. S2) that might weakly respond to TLR4 ligands.

We next asked whether MDA-MB-231 and HCC1806 differentially respond to paclitaxel, a mimetic of LPS (30, 31). Cells were treated for 48 hours with nab-PXL followed by qRT-PCR analysis of IL-6, IL-8, TNF-α, and MCP-1 mRNA. As shown in Fig. 1D, TLR4+ MDA-MB-231 cells responded to paclitaxel in a dose-dependent manner with robust upregulation of cytokines ranging from a 6.5-fold for IL-8 (<0.01) to 9-fold for TNF-α (<0.05). This effect was mediated by TLR4 as indicated by a 95% reduction in cytokine expression in the presence of anti-hTLR4 antibody (Fig. 1E). Minor increase in cytokines was also noted in HCC1806 cells, albeit this response was not affected by anti-hTLR4 antibody (Fig. 1F and G).

TLR4 expression in breast cancer lines correlates with resistance to paclitaxel
Functional TLR4 may significantly enhance inflammation in the tumor environment leading to increased chemoresistance (32, 33) and metastasis (34). To test whether paclitaxel -induced inflammatory responses protect cells from chemotheraphy, we measured IC_{50} of paclitaxel in TLR4+ MDA-MB-231 and TLR4-negative HCC1806 cells. As shown in Fig. 2A and B, TLR4+ MDA-MB-231 was 4-fold more resistant to paclitaxel as compared with HCC1806 (IC_{50} of 12.5 vs.3.25 nmol/L; P = 0.003). This difference was equally observed in paclitaxel- and nab-PXL–treated cells indicating that this effect is formulation-independent. We also tested 4 additional lines with either high or low TLR4 expression. As shown in Supplementary Table S2, the mean IC_{50} for lines enriched with TLR4 was 2-fold higher (P = 0.037) than for those with low level of this receptor. This finding supported the role of TLR4 in chemoresistance although the firm
To this end, we generated and functionally characterized isogenic derivatives of MDA-MB-231 and HCC1806 with differential TLR4 expression. Compared with controls, the selected MDA-MB-231 clones had 80% reduced TLR4, whereas HCC1806 clones had 10,000-fold higher TLR4 expression (Supplementary Fig. S3). Modified TLR4 expression corresponded to changes in both LPS- and paclitaxel-induced upregulation of inflammatory cytokines. Downstream TLR4 mRNA and protein targets were significantly (50%–60%) reduced in 231TLR4− and 5- to 25-fold upregulated in 1806TLR4+ lines treated with TLR4 ligands. These clones designated 231TLR4− or 1806TLR4+ along with their controls 231Ctrl and 1806Ctrl, were used to analyze the TLR4 role in paclitaxel resistance.

After validating functionality of an ectopically expressed TLR4, we used the modified lines to determine differences in cytotoxic response to paclitaxel. Figure 2C and D shows that 231TLR4− cells were more sensitive to nab-PXL (2.5-fold; \( P < 0.04 \)) than 231Ctrl cells, whereas 1806TLR4+ line was nearly 3-fold more resistant to paclitaxel than 1806Ctrl cells. In addition to genetic manipulation, the role of TLR4 in conferring resistance to paclitaxel was also shown by a monospecific anti-hTLR4 antibody (Fig. 2E), specific intracellular and extracellular TLR4 inhibitors, TAK-242 and
LPS-EK\textsuperscript{ultra} (Fig. 2F and G). These structurally unrelated agents targeting TLR4 share the capacity to increase response to paclitaxel by 2.0- to 2.5-fold. Collectively, these data show that TLR4 expressed in human tumor cells regulates both expression of inflammatory cytokines and sensitivity to paclitaxel.

TLR4 promotes survival of paclitaxel-treated tumor cells by increasing levels of antiapoptotic proteins

Increased chemoresistance typically correlates with upregulation of prosurvival proteins (17, 26, 32). We therefore hypothesized that TLR4 promotes survival of paclitaxel-treated cells by activating NF-\kappa B pathway known to transcribe prosurvival genes (35, 36). To test this hypothesis, cells were treated with 10 nmol/L of nab-PXL for 0 to 48 hours followed by Western blot analysis of phosphorylated and total p50, p65, Akt, Bcl-xL, and Bcl-2. Depletion of TLR4 in MDA-MB-231 substantially decreased expression of both phosphorylated and nonphosphorylated p50 and p65, as well as, p-Akt, Bcl-xL, and Bcl-2 (Fig. 3A). Conversely, TLR4 overexpression in HCC1806 cells increased phosphorylation of NF-\kappa B concomitant with upregulation of Bcl-2 and Bcl-xL (Fig. 3B).

We next examined the consequences of TLR4-dependent biochemical changes in major prosurvival proteins for developing resistance to paclitaxel therapy. Paclitaxel induces apoptosis by overstabilizing microtubules which leads to cell arrest in the G2–M phase (1). Therefore, we hypothesized that TLR4 might alter distribution of tumor cells through cell-cycle phases. To test this hypothesis, cells were treated with nab-PXL (10 nmol/L) for 48 hours, stained with PI, and analyzed for phase distribution by FACS. As shown in Fig. 3C, TLR4 knockdown significantly increased the number of cells arrested at the G2–M phase as evidenced by 58% of 231TLR4- cells detected in this phase after paclitaxel treatment as compared with 35% in the control line (\(P < 0.05\)). An opposite distribution was observed in the HCC1806 model (65% in control vs. 34% in 1806TLR4\textsuperscript{+}, Figs. 3D and Supplementary Fig. S4).

These results suggested that tumor cells with reduced TLR4 are more susceptible to paclitaxel-induced apoptosis as compared with TLR4-overexpressing cells. To test this hypothesis, we quantified paclitaxel-induced apoptosis in MDA-MB-231 and HCC1806 lines. Cells were treated as described above followed by quantification of apoptotic cells identified by double-staining with PI and Annexin V. Figure 3E shows that percentage of apoptotic...
cells significantly increased in 231TLR4- as compared with 231Cntrl (43% vs. 29%, \( P < 0.05 \)). An identical experiment in HCC1806 model independently confirmed causality between TLR4 and drug-induced apoptosis: only 28% of 1806TLR4+ cells were apoptotic as compared with 40% in 1806Cntrl (Fig. 3F; \( P < 0.05 \)). Similar results were obtained by identifying apoptotic cells using anti-active caspase-3 antibody. As shown in Supplementary Fig. S4, 43% of 231TLR4- cells were positive for caspase-3 as compared with 20% in control line (2.2-fold increase, \( P < 0.05 \)). Similarly, 1806TLR4+ cells showed a 2.1-fold decreased apoptosis compared with 1806Cntrl cells (\( P < 0.05 \)). These results suggest that paclitaxel-mediated activation of the TLR4 pathway concordantly diminishes the number of cells arrested in G2–M phase and those undergoing apoptosis. This, in turn, may significantly increase tumor recurrence after cessation of therapy.

**Paclitaxel-activated TLR4 upregulates both inflammatory ligands and receptors potentially creating prosurvival autocrine loops**

In macrophages, the hallmark of LPS-activated TLR4 signaling is coincident induction of inflammatory cytokines and matching receptors. Generation of these autocrine loops amplifies intracellular pathways that enhance proliferation, migration, and survival of pathogen-fighting immune cells. Because paclitaxel is an LPS mimetic (30), we hypothesized that a similar effect can be
produced by taxanes on TLR4+ cancer cells. To test this hypothesis, we compared the expression of both cytokines and matching receptors in control and paclitaxel-treated MDA-MB-231 and HCC1806 cell lines. Cells were treated with nab-PXL followed by qRT-PCR analysis of about 100 inflammatory cytokines and matching receptors. Using this approach, we identified a substantial number of targets upregulated by nab-PXL by at least 1.5-fold (Fig. 4A). 1806Cntrl line lacking TLR4 was the least responsive line with 17.9% of upregulated receptors, whereas the broadest responses (50% upregulated targets) were obtained in the 231Cntrl cells that have the highest expression of TLR4. This response was TLR4-dependent because both the number of ligands and receptors upregulated >1.5-fold in 231Cntrl versus 231TLR4- cells.

Figure 4. PXL upregulates inflammatory cytokines and receptors in TLR4+ lines. A, number of inflammatory genes upregulated >2-fold in 231Cntrl, 231TLR4, 1806Cntrl, and 1806TLR4+ cells in PXL-treated (10 nmol/L, 48 hours) versus untreated cells. * and **, P values < 0.05 and < 0.01 versus control determined by χ² test. B, fold increase of upregulated inflammatory genes in 231Cntrl, 231TLR4+, 1806Cntrl, and 1806TLR4+ lines after PXL treatment. *, P < 0.05 versus control as determined by Mann-Whitney U test comparing number of ligands and receptors upregulated >1.5-fold in 231Cntrl versus 231TLR4- cells. C, main cytokines and corresponding receptors upregulated >2.0-fold in 231Cntrl, 231TLR4+, 1806Cntrl, and 1806TLR4+ cells.
degree of their upregulation correlated with TLR4 expression in both MDA-MB-231 and HCC1806 models (Fig. 4A and B). Importantly, some targets became detectable only after exposure to paclitaxel (Supplementary Tables S3 and S4), suggesting that this therapy not only intensifies inflammation but also qualitatively changes the tumor environment.

Significantly, several upregulated cytokines in both breast cancer models were coincided with matching receptors, potentially creating autocrine growth-promoting loops (Fig. 4A and C). This is best illustrated by the changes in HCC1806 line in which ectopic expression of TLR4 increased the number of matching pairs by 328% (16.6% and 54.5% in 1806Cntrl and 1806TLR4, respectively; Fig. 4A). Using this profiling, we identified several new ligand–receptor pairs induced by TLR4 as indicated by significant differences between the control and TLR4-manipulated lines (Fig. 4C, P < 0.05). In the 231Cntrl line, the highest upregulated pairs were: CXCL2-CXCR2 (11.40 ± 1.89 and 9.43 ± 1.60-fold increase for ligand and receptor, respectively), CCL20-CXCR3 (9.95 ± 1.53 and 8.02 ± 0.62-fold), and IL-8-IL-8R (7.46 ± 0.55 and 6.76 ± 1.93-fold). Pairs upregulated in 1806TLR4 cells included CSF1-CSF1R, BAFF-BAFFR, and INHBB-ACVR2B. This study, therefore, suggests that treatment with nab-PXL creates a broad inflammatory response that fundamentally alters the tumor milieu, and consequently, the response to therapy.

**Paclitaxel-activated cytokine-receptor autocrine loops contribute to drug resistance through increased phosphorylation of AKT and ERK1/2**

We next examined whether drug-upregulated cytokine-receptor pairs contribute to chemoresistance. We tested whether neutralizing antibodies or specific pharmacologic inhibitors of cytokines or receptors increase tumor cell sensitivity to paclitaxel. Of 7 most upregulated pairs in MDA-MB-231 (Fig. 4C), we assessed the effect of interrupting a putative autocrine loop for 5 pairs (IL-6, IL-8, MCP-1, CXCL1, and CXCL2). While neutralization of IL-6 or IL-8 had no significant effect on nab-PXL toxicity, blockade of CCR4 and CXCR2 receptors was highly synergistic with paclitaxel treatment (Fig. 5A and B). Analysis of synergism of blocking CCR4 and CXCR2 by CompuSyn software yielded highly significant CI values for both inhibitors of 0.59 and 0.52, respectively (Fig. 5C). Both pathways activate MEK that phosphorylates extracellular signal–regulated kinase (ERK)1/2 (37). Indeed, combination of MEK inhibitor PD98059 (100 μmol/L) with nab-PXL was highly synergistic (CI = 0.45) resulting in 69% inhibition compared with about 50% caused by nab-PXL alone (Fig. 5C and D). Synergy with nab-PXL was also evident by enhanced phosphorylation of ERK1/2 and AKT (Fig. 5E). Collectively, these results indicate that CXCL1/2-CXCR2 and MCP-1-CCR4 loops functionally contribute to survival of tumor cells during chemotherapy.

**Expression of TLR4 in breast cancer cells dictates their responses to paclitaxel therapy in vivo**

Substantial differences in susceptibility of breast cancer lines to paclitaxel in vitro suggested a prominent role of TLR4 in chemoresistance in vivo. To test this hypothesis, we measured tumor growth rate of orthotopically implanted lines with differential TLR4 expression. All 6 tested clones of 231TLR4 grew substantially slower than control 231Cntrl line (Fig. 6A). The period before exponential tumor growth of 231TLR4 clones doubled in most mice with some requiring more than 110 days compared with only 10 to 15 days in control mice (Fig. 6B). Expectantly, all 1806TLR4 clones (n = 4) grew much faster than control lines (P > 0.02; Fig. 6C).

Treatment with nab-PXL (10 mg/kg) produced drastically different results in isogenic lines with modified TLR4 expression (Fig. 6D–F). Within few weeks after paclitaxel treatment, 100% of control mice bearing MDA-MB-231 tumors had recurrence. In sharp contrast, only 1 of 6 mice (17%) with 231TLR4 tumors had recurrence 127 days posttreatment, whereas rest of the group was disease-free for experimental duration of 6 months (P > 0.0001 vs. control; Fig. 6D). Consistently, 100% of mice with 1806TLR4 clones recurred immediately upon cessation of therapy, whereas 40% of mice with tumors lacking TLR4 had no palpable masses after chemotherapy (Fig. 6F). The mean inhibition in the latter group was 95% as compared with 10% inhibition in TLR4-overexpressing group (P > 0.001; Fig. 6F). These results unequivocally show that TLR4 plays a major role in determining the response of tumor cells to paclitaxel therapy.

**Discussion**

TLR4 has been previously shown to be overexpressed in a variety of human cancers (17, 38) and to correlate with tumor progression (39), recurrence (27), metastasis (38, 40), and resistance to paclitaxel therapy (17, 26). However, a protumorigenic effect of TLR4 was mainly assessed using LPS (25, 27), a bacterial component that is absent in epithelial malignancies outside of the gastrointestinal tract. More relevant to cancer research is use of paclitaxel, an LPS mimetic (41) and a widely administrated chemotherapeutic drug (42). Paclitaxel-mediated resistance to therapy has been previously shown in ovarian cancer cells in vitro (15, 26). Here, we present extensive evidence showing the chemoresistance-promoting role of TLR4 in breast cancer using both in vitro and in vivo models. We show that TLR4 is overexpressed in clinical breast cancer, and it is specifically responsible for mediating chemoresistance to paclitaxel as evident in increasing IC50 in tumor cells in vitro and causing recurrence in breast cancer in vivo.

TLR4 is considered as a “double-edge sword” with some studies showing its contribution to chemoresistance (17) and others arguing its role in enhancing anticancer immunity (43, 44). Here, we show in 2 distinct breast cancer models that activated TLR4 pathway drastically...
diminishes therapeutic efficacy as evidenced by reduced apoptosis of treated cells in vitro and prominent recurrence of treated tumors in vivo. This effect is mediated by NF-κB–mediated transcription of inflammatory and pro-survival genes (Figs. 3 and 4). These findings indicate that TLR4 affords tumor evasion from paclitaxel therapy rather than enhancing its cytotoxic effect.

While this conclusion is consistent with the evidence from ovarian cancer models (17, 26, 32), our study shows several new findings underscoring the functional significance of TLR4 in breast cancer. We show that TLR4 is broadly expressed in clinical breast cancer as well as the in majority (68%) of examined breast cancer lines (Fig. 1). We also show that TLR4 is functional in human cancer cells as evidenced by upregulated cytokine expression after treatment with either LPS or paclitaxel (Fig. 1). Previous reports suggested that only mouse TLR4–MD2 complex transduces paclitaxel signaling (18, 19). In contrast, we found that both paclitaxel and nab-PXL phosphorylates NF-κB in TLR4+ human cancer cells followed by up to 15-fold upregulation of multiple targets induced in dose- and time-dependent manners. These data indicate that despite the biochemical difference between human and mouse TLR4–MD2 complexes (41), paclitaxel can enhance chemoresistance of TLR4+ breast tumors in a clinical setting.

The specificity of TLR4 in mediating tumor-promoting paclitaxel effects is shown here by genetic manipulation of TLR4 in 2 breast cancer lines and by reduced cytotoxicity and cytokine expression following treatment with a monospecific anti-hTLR4 antibody, an extracellular LPS lipid antagonist, and an intracellular inhibitor cyclohexene TAK-242. The fact that several structurally unrelated TLR4 inhibitors significantly increase sensitivity of tumor cells to paclitaxel strongly argues for important role of this receptor in conferring resistance. This is also supported by the evidence that paclitaxel promoted resistance in TLR4+ cells regardless of their genetic background (Supplementary Table S2), suggesting that TLR4 expression could be an independent prognostic marker for resistance to therapy. Mechanistically, TLR4-dependent chemoresistance could be mediated by prosurvival autocrine loops some of which have been identified in this study.

Figure 5. Inhibition of CXCR2 and CCR4 receptors synergistically increases sensitivity to nab-PXL in MDA-MB-231 cells. Cells were pretreated with 0 to 1 μmol/L of a CXCR2 inhibitor SB225002 (A) or a CCR4 receptor inhibitor C021 dihydrochloride (B) followed by 48-hour treatment with nab-PXL (10 nmol/L). IC50 was calculated as described in legend for Fig. 2. C, table showing the synergy between nab-PXL and inhibitors of CCR4, CXCR2, or MEK as represented by measured as CI. D, MDA-MB-231 cells were treated with MEK inhibitor (0–100 μmol/L) and nab-PXL (10 nmol/L) for 48 hours followed by calculating IC50. Data are presented as percentage of viable cells versus control ± SD from 2 experiments done in triplicate. E, MDA-MB-231 cells were treated with MEK inhibitor (0–100 μmol/L) and nab-PXL (10 nmol/L) for 48 hours. Phosphorylation of ERK1/2 and AKT was analyzed by Western blotting.
Supplementary Tables S3 and S4). Although paclitaxel-medi-iated increases in some cytokines have been previously reported in both human patients with breast cancer (14, 45) and experimental breast cancer models (3, 4), this is the first study that analyzed coexpression of inflammatory ligands and receptors in a TLR4-dependent manner. Two TLR4-regulated pathways that seem to be important in this regard are MCP-1*CCR4 and CXCL-1/-2*CXCR1/2. This is consistent with large body of evidence that shows strong correlations between MCP-1 (46), CCR4 (47), CXCL-1/-2 (48), and CXCR1/2 (49) with chemoresistance and metastasis. Importantly, in vivo cytokine-activated pathways have the potential to promote tumor cell survival through both autocrine and paracrine loops. TLR4 signaling in tumor cells may significantly reduce therapeutic efficacy by promoting chronic inflammation, angiogenesis, and recovery of damaged cells. Importantly, this pathway may also promote metastasis. This study, therefore, suggests that blocking TLR4 signaling during paclitaxel therapy is essential for increasing the responsiveness of primary tumors and preventing relapse at secondary sites.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Rajput, S. Ran
Development of methodology: S. Ran
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.D. Volk-Draper
Analysis and interpretation of data (e.g., statistical analysis, bio- statistics, computational analysis): S. Rajput, S. Ran
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.D. Volk-Draper, S. Ran
Study supervision: S. Ran

Figure 6. TLR4 determines sensitivity to nab-PXL in breast cancer models in vivo. A, the growth of 231TLR4− and control lines implanted in SCID mice was monitored twice weekly. Each point represents the mean tumor volume ± SE. **P < 0.01, P < 0.05. B, growth of a 231TLR4 clone showing significant delay in establishing tumor mass in all mice per group (n = 6) compared with controls. Each line represents tumor growth in individual mouse. C, growth of HCC1806TLR4− and HCC1806Cntrl tumors in SCID mice. TLR4 significantly increased tumor growth in all clones with ** and *** indicating P values < 0.05 and < 0.001, respectively. D, 231TLR4− and control tumors of 150 mm3 were treated with 10 mg/kg of nab-PXL intravenously for 8 days. Five of 6 mice (83.3%) bearing 231TLR4− tumors (yellow circles) had complete response (CR), whereas 0% CRs were achieved in all other groups. E, bioimaging of representative tumors from groups described in D. F, tumor growth of control and TLR4+ overexpressing HCC1806 lines.

In summary, we show that TLR4 increases resistance to paclitaxel in human breast cancer cells by activating the NF-kB pathway leading to transcription of inflammatory genes that alter the tumor environment through autocrine and paracrine loops. TLR4 signaling in tumor cells may significantly reduce therapeutic efficacy by promoting chronic inflammation, angiogenesis, and recovery of damaged cells. Importantly, this pathway may also promote metastasis. This study, therefore, suggests that blocking TLR4 signaling during paclitaxel therapy is essential for increasing the responsiveness of primary tumors and preventing relapse at secondary sites.
Grant Support

This work was supported by NIH grant R01CA140732 and by grants from the Illinois William E. McElroy Foundation awarded to S. Ran.

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Received October 19, 2012; revised May 16, 2013; accepted May 20, 2013; published OnlineFirst May 29, 2013.

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doi:10.1158/1535-7163.MCT-12-1019

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