Cancer Research

LPS-Induced TLR4 Signaling in Human Colorectal Cancer Cells Increases β1 Integrin-Mediated Cell Adhesion and Liver Metastasis

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Abstract

Infectious complications resulting from resection of colorectal cancer (CRC) elevates the risk of cancer recurrence and metastasis, but the reason for this risk relationship is unknown. Defining the mechanisms responsible may offer opportunities to improve outcomes in a majority of patients whose tumors are resected as part of their therapy. The complex formed between Toll receptor TLR4 and myeloid differentiation factor MD2 defines a major cell surface receptor for lipopolysaccharide (LPS), a gram-negative bacterial antigen that has been implicated in infectious complications after CRC resection. As the TLR4/MD2 complex is expressed on CRC cells, we hypothesized that LPS may promote liver metastasis in CRC by stimulating TLR4 signaling. In support of this hypothesis, we report here that LPS enhances liver metastasis of human CRC cells that express TLR4/MD2 after intrasplenic graft of immunocompromised nude mice. Compared with TLR4 nonexpressing, nonmetastatic CRC cells, we observed increased *in vitro* adherence to different extracellular matrices and human umbilical vein endothelial cells (HUVEC). Furthermore, we observed an increased likelihood of *in vivo* capture within hepatic sinusoids after LPS treatment. No differences were apparent in phosphorylation of p38 and MAPK isoforms, but in metastatic CRC cells expressing surface TLR4 treatment with LPS increased Ser473 phosphorylation of AKT kinase. We showed that enhanced adherence elicited by LPS in these cells could be blocked at three different levels, using Eritoran (TLR4 small molecule antagonist), PI-103 (PI3K inhibitor), or anti-β1 integrin blocking antibodies. Taken together, the results indicate that stimulation of the TLR4/MD2 complex by LPS activates PI3K/AKT signaling and promotes downstream β1 integrin function, thereby increasing the adhesiveness and metastatic capacity of CRC cells. Our findings suggest that inhibiting LPS-induced TLR4 signaling could improve therapeutic outcomes by preventing cancer metastasis during the perioperative period of CRC resection. *Cancer Res; 71(5); 1989–98. ©2011 AACR.*

Introduction

Colorectal cancer (CRC) is the fourth most common cancer and the second most common cause of cancer-related deaths in Canada (1) and the United States (2). As resection of the primary tumors is the treatment of choice, 30% of patients with stage III CRC develop local recurrence or distant metastasis within 5 years after curative resection (3). Despite lymph node negative status, 10% of patients with stage I/II diseases still develop recurrence within 5 years after curative resection (3). The basis for such high rate of recurrence is poorly understood.

Emerging data suggest surgical resection of CRC itself may promote local recurrence or distant metastasis (4). On the one hand, circulating tumor cells (CTC) can be detected in over 20% of the venous drainage blood collected from patients with lymph node negative CRC during curative resection (5). Quantity of these CTC can be significantly increased in the portal venous drainage during surgery suggesting manipulation of primary tumor may disrupt its structural integrity and increase hematogenous dissemination of tumor cells (6). On the other hand, surgical resection causes significant tissue trauma and systemic inflammation and bears risks of postoperative infectious complications. There are mounting evidences linking systemic inflammation and postoperative infection to recurrence of CRC (7–10). Surgical stress and postoperative infection trigger a cascade of inflammatory response mediated by various inflammatory cell types that can regulate tumor angiogenesis, secrete various chemokines and cytokines favoring tumor migration, survival and growth,

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and release various proteases facilitating extravasation of CTC to distant organs (11).

Lipopolysaccharide (LPS), a gram-negative bacterial antigen involved in the intraabdominal infectious complication from colorectal surgery, can cause significant systemic inflammation and severe sepsis. In addition, LPS-induced systemic inflammation was shown to increase hepatic recruitment of cancer cells in mice (12). LPS has been shown to bind directly to the TLR4/MD2 receptor complex (13, 14) that initiates the intracellular signaling cascade in a MyD88-dependant or MyD88-independent manner (15). LPS-induced TLR4 signaling leads to activation of various downstream Mitogen-Activated Protein Kinases (MAPK) that have been shown to play key roles in cell proliferation, apoptosis, and adhesion (15–19). Eritoran (E5564), second-generation lipid A analog, competes with LPS for the same hydrophobic binding pocket of MD2 and induces a different conformational change than LPS that reduces the stability of TLR4/MD2 complex and inhibits TLR4 signaling (13, 20).

As TLR4 expression has been found on several cancer cell types including human CRC cell lines (19, 21–24) and that its high expression is associated with liver metastasis and poor clinical prognosis in CRC patients (25), we aim to show the effects of LPS-induced TLR4 signaling on liver metastasis of CRC cells. Here we show LPS enhances liver metastasis of HT-29 cells in athymic nude mice after intrasplenic injection. These cells are more adherent to different ECM and HUVEC *in vitro* and are more likely to be attenuated within the hepatic sinusoids *in vivo* after LPS treatment. Although the phosphorylation of p38 and p42/44 (ERK1/2) MAPK remains unaltered, LPS increases serine-473 phosphorylation of AKT in HT-29 cells. The enhanced adherent effects can be blocked at 3 different levels using Eritoran (TLR4 antagonist), PI 103 (PI3K inhibitor) and anti-β1 integrin functional blocking antibodies. These data suggest LPS binding to TLR4/MD2 complex signals through PI3K/AKT pathway that activates β1 integrin increasing the adhesiveness and metastatic potential of CRC cells. Inhibition of LPS-induced TLR4 signaling may have a therapeutic value in the prevention of cancer metastasis during the perioperative period.

Materials and Methods

Antibodies and reagents

LPS derived from *E. coli* strain 055:B5, propridium iodide (PI), bovine serum albumin (BSA), and Poly-HEMA were purchased from Sigma-Aldrich. Kinase inhibitors (SB203580, PD184352, PI 103) were purchased from InvivoGen, US Biological, and Cayman Chemical, respectively. TLR4 antagonist (Eritoran) was a kind gift from Eisai Inc. Goat polyclonal anti-human TLR4 antibody was purchased from RnD Systems. Biotin-conjugated mouse anti-TLR4 antibody (clone HTA125), biotin-conjugated isotype control mouse IgG2a and Streptavidin–phycoerythrin were purchased from BD Pharmingen. Rabbit polyclonal anti-MD2 and anti-MyD88 antibodies were purchased from Abcam Inc. Rabbit monoclonal anti-CD14 antibody was purchased from Epitomics Inc. Mouse monoclonal anti-β1 integrin functional blocking antibody (4B4) and isotype control mouse IgG1 were purchased from Beckman Coulter. Rabbit polyclonal anti-AKT, anti-phospho-AKT (Ser473), anti-p44/42 and anti-phospho-p44/42 (Thr202/Tyr204) antibodies were purchased from Cell Signaling Technologies, Inc. Mouse monoclonal anti-p38α antibody and rabbit polyclonal anti-phospho-p38α (Thr180/Tyr182) antibody were purchased from Millipore, Inc. Collagen I, collagen IV, fibronectin and laminin were purchased from Roche Applied Science. Crystal violet was purchased from EM Science and carboxyfluorescein succinimidyl ester (CSFE) was purchased from Invitrogen, Inc.

Cell culture

Human colorectal carcinoma cell lines (HT-29, LS-174T, CaCO2, and SW1222) were kind gifts from Nicole Beauchemin's laboratory (McGill University, Montreal, Canada). Cells were maintained in a subconfluent state using α-modified Minimum Essential Media (αMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Human umbilical vein endothelial cells (HUVEC) were cultured using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% FBS, 1% penicillin and streptomycin, 10 mmol/LHEPES, 2 mmol/L L-glutamine, 1500 U/mL heparin, and 37.5ug/mL endothelial cell growth supplement (Biomedical Technologies). Human leukemic monocyte lymphoma cell line U937 was a kind gift from Sabah Hussain's laboratory (McGill University, Montreal, Canada) and grown in DMEM supplemented with 10% FBS. All cells were incubated at 37°C with 5% CO2. All cell culture reagents except endothelial cell growth supplement were purchased from Wisent, Inc. For LPS treatment, subconfluent cultures of HT-29 or CaCO2 cells were treated with 1 μg/mL LPS for 4 hours. For TLR4 inhibition, 100 nmol/L Eritoran was added to the cell culture at the same time as LPS. For p44/42, p38 and PI3K inhibition, PD184352 (10 μmol/L), SB203580 (5 μmol/L), and PI 103 (5 μmol/L), respectively, were added 1 hour prior to the addition of LPS. For β1 integrin blockade, β1 integrin functional blocking antibody (2.0 μg/mL) or isotype control IgG1 (2.0 μg/mL) were incubated with single cell suspensions for 30 minutes on ice after LPS treatment.

Flow cytometry

To detect TLR4 cell surface expression, single cell suspensions were washed with staining buffer (PBS containing 1% FBS). Cells were then incubated with biotin-conjugated mouse anti-human TLR4 antibody at a concentration of 20 μL/1 × 10^6 cells for 30 minutes on ice. After washing with staining buffer, the cells were mixed with Streptavidin-phycoerythrin and immediately analyzed with a flow cytometer FACScan and CellQuest Software. Background staining was determined by staining cells with biotin-conjugated isotype control mouse IgG2a followed by Streptavidin–phycoerythrin incubation.

Cell proliferation assay

Cells of 0.2 × 10^6 with 100 μL of medium were seeded into each well of 96-well flat bottom plates. Cell proliferation was assessed by MTT assay kit as described in the manufacturer's protocol (RnD Systems) at 24, 48, and 72 hours.

Anoikis assay

Cells of 0.75×10^6 were seeded onto poly-HEMA coated 48-well plates. At 12 and 24 hours, 0.1×10^6 cells were stained with propridium iodide (5 μg/mL) for 15 minutes at room temperature and membrane integrity was assessed by flow cytometry.

Extracellular matrix adhesion assay

Ninety-six–well plates were coated with 100 μL/well of 50 μg/mL collagen I, 50 μg/mL collagen IV, 10 μg/mL fibronectin or 20 μg/mL laminin overnight at 4°C and blocked by 1% BSA for 1 hour at 37°C before seeding cells. 5×10^4 cells resuspended in 100 μL media were seeded in each well and incubated at 37°C for 1 hour. Each well was washed gently with PBS twice and attached cells were stained with 100 μL 1% crystal violet dissolved in PBS for 1 hour. Wells were washed with distilled water and left to air-dry overnight. Dyes were solubilized in 100 μL of 2% SDS at room temperature for 1 hour. Concentration was determined by measuring absorbance at 570 nm using a spectrophotometric microplate reader (Biotek Inc.).

In vitro endothelial adhesion assay

Cells were stained with 25 μmol/L CSFE at room temperature for 15 minutes. Cells of 10×10^3 were resuspended in 300 μL of HUVEC media and seeded on top of each HUVEC monolayer in a 48-well plate at 37°C for 1 hour. After washing with media and fixed with 4% paraformaldehyde, the number of adhered CSFE-labeled HT-29 cells was counted using an inverted fluorescent microscope (Nikon TE300).

Intravital fluorescent microscopy

Cells were stained with 25-μmol/L CSFE at room temperature for 15 minutes. About 6–8 week-old C57BL6 male mice (Charles River Canada) were anesthetized using subcutaneous injections of ketamine and xylazine. The abdomen was opened with a midline incision and then subcostal incisions. The falciform ligament was dissected away from the gallbladder and anterior surface of liver. CSFE-labeled cells of 50×10^3 were injected intrasplenically into each animal. Animals were then placed in a left lateral position on a plexiglass stage and the left lobe of liver was gently positioned on a glass cover slip that was then positioned over a 20× microscope objective of an inverted fluorescent microscope (Nikon TE300). Blood flow to the liver sinusoids was assessed to eliminate artifacts owing to severe hypotension. CSFE-labeled cancer cells were visualized along the edges of the exposed liver using epifluorescence. Numbers of attenuated fluorescently labeled cancer cells in 10 random microscope fields were counted between 5–10 minutes postinjection for each animal. Observer was blinded regarding to the treatment groups.

Experimental hepatic metastasis assay

Athymic nude mice (Taconic Farms, Inc) were anesthetized using isoflurane. Spleens were exposed through a small abdominal incision. 1×10^6 cells resuspended in 100 μL PBS were injected intrasplenically and the mice were splenectomized 1 minute later. Mice were sacrificed 5 weeks later. Surface liver metastases were counted.

Immunoblot analysis

Cells were treated with 1 μg/mL LPS for different time points (10, 20, 30 60, and 240 minutes). Cells were then lysed in 30 mmol/L Tris-HCl (pH 8.0), 1% Triton X-100, 150-mmol/L NaCl, 1-mmol/L Na3VO4, 1-mmol/L NaF, 1-mmol/L iodoacetamide, 0.5% deoxycholic acid sodium salt, 1-mmol/L phenylmethylsulfonyl fluoride, and 10% of a mini EDTA-free Protease Inhibitor cocktail tablet (Roche Applied Science) for 30 minutes on ice and collected by scraping. For the analysis of TLR4, MD2, CD14, and MyD88 total protein expression, various cell lines were detached and lysed with 10% SDS lysis buffer. After removing insoluble debris by centrifugation, supernatants were collected and protein concentration was quantified by Bradford protein assay according to manufacturer's instruction (Bio-Rad Laboratories). For each sample, 20-μg proteins were separated by 12% SDS-polyacrylamide gel. After transfer onto PVDF membranes, nonspecific binding was blocked with 5% milk for 1 hour at room temperature before incubation of various primary antibodies in a 1:1000 dilution. After washing extensively with TBS-T buffer (10 mmol/L Tris-HCL (pH 7.4), 100 mmol/L NaCl, and 0.1% Tween-20), the membranes were incubated with the corresponding HRP-conjugated secondary antibodies in a 1:5000 dilution. After exposing the membrane with the Pierce ECL substrate (Fisher) for 5 minutes, chemiluminescence was detected by Fuji Medical X-Ray films.

Statistical analysis

The differences between experimental groups were analyzed using the Student's *t*-test. In all cases, a $P < 0.05$ was considered statistical significant.

Results

Expression of TLR4 signaling molecules in human CRC cell lines

Four different human CRC cell lines (HT-29, LS174T, CaCO2, and SW1222) have been tested for the expression of TLR4, which is essential for the downstream signaling cascade induced by LPS. Comparing with U937 cells, a monocytic cell line that is widely known to express TLR4 and responds to LPS, TLR4 expression was detected at a lower level in all CRC cell lines tested by immunoblot analyses (Fig. 1A). LS174T cells seemed to express relatively the lowest level of TLR4. Despite TLR4 expression detected with immunoblots in these CRC cell lines, cell surface expression of TLR4 could not be shown in CaCO2 cells by FACS analysis (Fig. 1B). Furthermore, CaCO2 cells were found to have the lowest level of MD2 and minimal MyD88 expression (Fig. 1A). These molecules have been shown to play an important role in LPS-induced TLR4 signaling pathway (15). In sum, HT-29 cells were found to have a more complete repertoire of TLR4 signaling molecules whereas CaCO2 cells were very limited.

LPS enhances experimental liver metastasis of HT-29 cells

Given that HT-29 cells express all the necessary components of LPS-induced TLR4 signaling, we aim to investigate if LPS can directly stimulate HT-29 cells and increase their metastatic

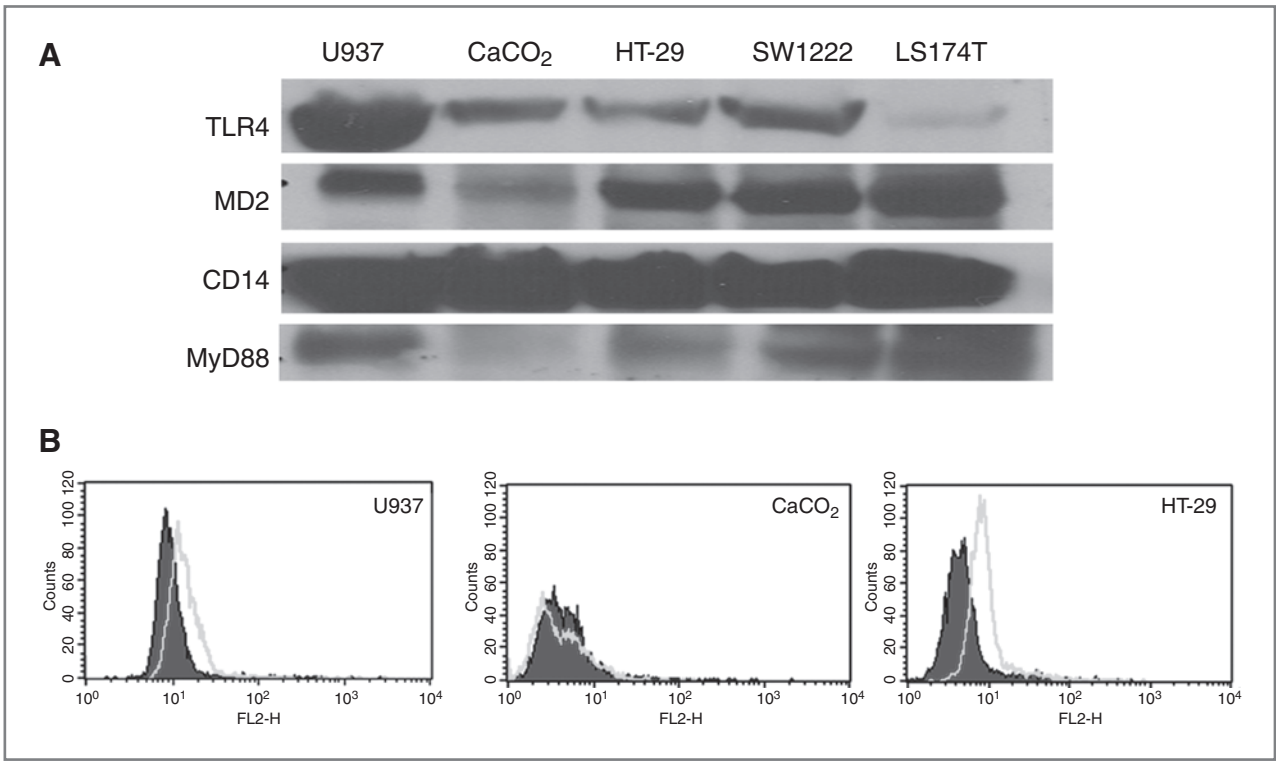

Figure 1. Expression of TLR4 signaling molecules in human CRC cell lines. A, immunoblot analysis using anti-TLR4, MD2, CD14 and MyD88 antibodies. B, cell surface expression of TLR4 detected by flow cytometry using anti-TLR4 antibody. Filled histograms denote background fluorescence; Line histograms denote TLR4 staining; Y-axis represents the number of cells; X-axis represents the level of fluorescence (FL-2) in a logarithmic scale [geometric mean: 14.4 (U937—positive control cell line), 12.1 (HT29) and 5.1 ($CaCO_2$)].

potential. CaCO2 cells lacking TLR4 surface expression were not used mainly because it is known as a nonmetastatic cell line. Subconfluent monolayer cultures of HT-29 cells were treated with or without LPS for 4 hours. LPS-treated or untreated HT-29 cells were injected intrasplenically into athymic nude mice. After 5 weeks of incubation, mice injected with LPS-treated HT-29 cells had a significantly higher number of liver surface metastatic nodules compared with those with untreated HT-29 cells ($P = 0.031$, Fig. 2). Coincubation of LPS and Eritoran (specific TLR4 antagonist) with HT-29 cells tended to attenuate the effect exerted by LPS (Fig. 2). These results indicated that LPS could increase the metastatic potential of HT-29 cells *in vivo* via activation of the TLR4 signaling pathway.

LPS does not increase proliferation or survival of HT-29 cells

To decipher the possible mechanism that LPS enhances liver metastasis, we first examined the proliferative activity of HT-29 cells with or without LPS treatment. LPS-treated and untreated HT-29 cells were trypsinized and reseeded into 96-well plates at the same concentration. At various time points, using MTT assay, no significant change in cellular proliferation of HT-29 cells could be shown with LPS pretreatment (Fig. 3A). Similar result was obtained without trypsinization and reseeding of HT-29 cells (data not shown).

As proper anchorage to ECM is essential for normal cell survival, we then examined whether LPS-treated HT-29 cells were more resistant to anoikis, a form of apoptosis induced by lack of anchorage to ECM, so that they could survive longer in the circulation. LPS-treated and untreated HT-29 cells were kept on suspension by incubating on poly-HEMA coated plates at 37°C for 12 or 24 hours. Using propridium iodide staining for dying or dead cells that lack plasma membrane integrity, no significant difference in the percent cell death was detected between HT-29 cells pretreated with or without LPS (Fig. 3B). These results suggest that enhanced metastatic potential of LPS-treated HT-29 cells were not dependent on cellular proliferation or resistance to anoikis.

LPS enhances endothelial adhesion and hepatic recruitment of HT-29 cells

Without any evidence of proliferative and apoptotic changes after LPS treatment, we sought to determine if any alteration in the adhesive properties of CRC cells could explain the enhanced metastatic phenotype. To investigate the adhesive properties of HT-29 cells after LPS treatment, LPS-treated HT-29 cells were incubated on confluent monolayers of HUVEC at 37°C for 1 hour. A 50% increase in endothelial adhesion was observed in LPS-treated versus untreated HT-29 cells ($P < 0.05$; Fig. 4A). The latter effect was partially reversed by Eritoran indicating again the direct involvement of LPS-induced TLR4 signaling (Fig. 4A).

Using intravital fluorescent microscopy of the liver (12), recruitment of fluorescently labeled cancer cells can be

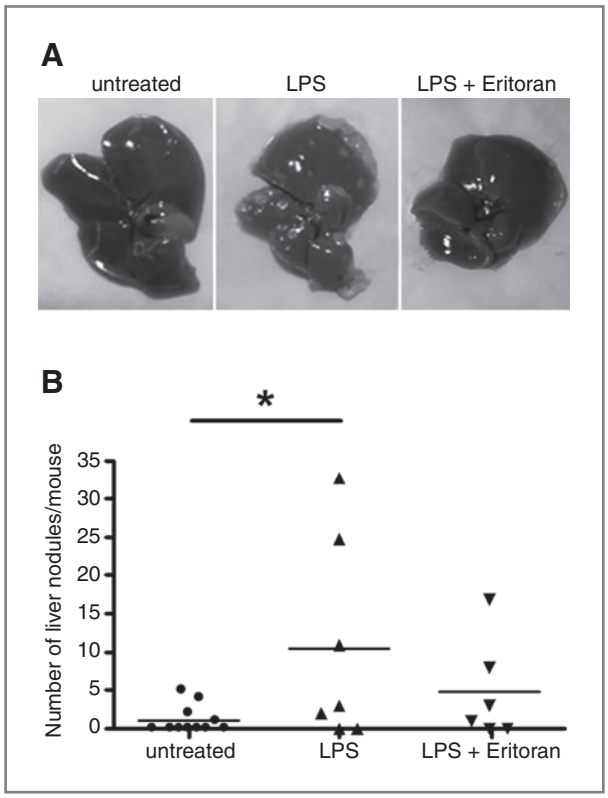

Figure 2. LPS increases liver metastasis of HT-29 cells *in vivo*. A, representative livers 5 weeks post–intrasplenic injection of 1×10^6 HT-29 cells in athymic nude mice [left, untreated cells; middle, cells pretreated with LPS (1 μg/mL); right, cells treated with LPS (1 μg/mL) + TLR4 small molecule inhibitor Eritoran (100 nmol/L)]. B, graphic representation of metastatic tumor load estimated by counting the number of metastatic nodules on the liver surface of individual mice from 3 different treatment groups shown in A. The horizontal bar represents the average number of metastatic nodules. *, $P = 0.031$ in the comparison between untreated and LPS-treated groups.

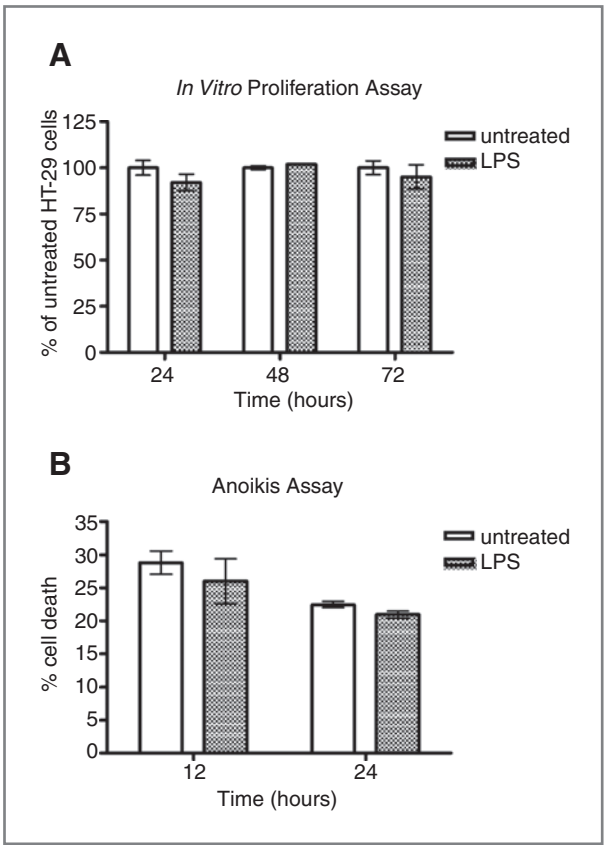

Figure 3. *In vitro* proliferation and anoikis assays. HT-29 cells were pretreated with or without LPS (1 μg/mL) for 4 hours and collected as single cell suspensions. A, cell proliferation measured by MMT assay at 24, 48, and 72 hours. The y-axis represents the relative absorbance at 570 nm compared with the untreated control at each time point. B, percent cell death detected by PI staining and flow cytometry after 12 and 24 hours on the poly-HEMA–coated 48-wells plates. No significant difference in proliferation (A) or anoikis (B) was detected. Error bar represents SEM of 3 independent samples.

directly observed in the hepatic sinusoidal circulation after intrasplenic injection. A 2-fold increase in hepatic recruitment was observed in LPS-treated HT-29 cells comparing with untreated HT-29 cells ($P < 0.05$; Fig. 4B). This effect was not observed in CaCO2 cells (Fig. 4B) that lacked cell surface expression of TLR4 suggesting the involvement of LPS/TLR4 signaling at the cell membrane level. The latter speculation was further reinforced by the partial reversal using Eritoran, a specific TLR4 antagonist (Fig. 4B). These results suggest LPS induces TLR4 signaling cascade leading to an increase in adhesive properties of HT-29 cells that likely have direct impact on endothelial adhesion, hepatic recruitment, and liver metastasis.

LPS-induced adhesion of HT-29 cells is β1 integrin dependent

To investigate the molecular changes responsible for the increased adhesion after LPS treatment, cell surface levels of various adhesion molecules were tested by FACS analysis. No change in the expression levels of ICAM1, NCAM, CEACAM, sialyl-Le^X, and various integrins (α2, α5, αV, and β1) could be detected in the HT-29 cells after 4 hours of LPS treatment by FACS analysis (data not shown).

Despite the lack of evidence of increased surface expression level of the adhesion molecules tested, increased binding to various ECM substrates, collagen in particular, were observed in HT-29 cells, but not CaCO2 cells, after LPS treatment (Fig. 5A and B). These effects were completely reversed by coincubation of LPS with Eritoran in HT-29 cells and not observed in $CaCO_2$ cells suggesting again a TLR4-dependent mechanism (Fig. 5A and B). As HT-29 cells bound mostly to collagen I/IV and much less to fibronectin and laminin, activation of β1 integrin, the major subunit for collagen binding (26), was most likely responsible for such phenotypic changes. The latter speculation was further supported by the complete phenotypic reversal through functional blockade of β1 integrin by specific antibody *in vitro* (Fig. 5C) and *in vivo* (Fig. 4C). These findings suggest increased adhesion of HT-29 cells induced by LPS was TLR4 and β1 integrin dependent.

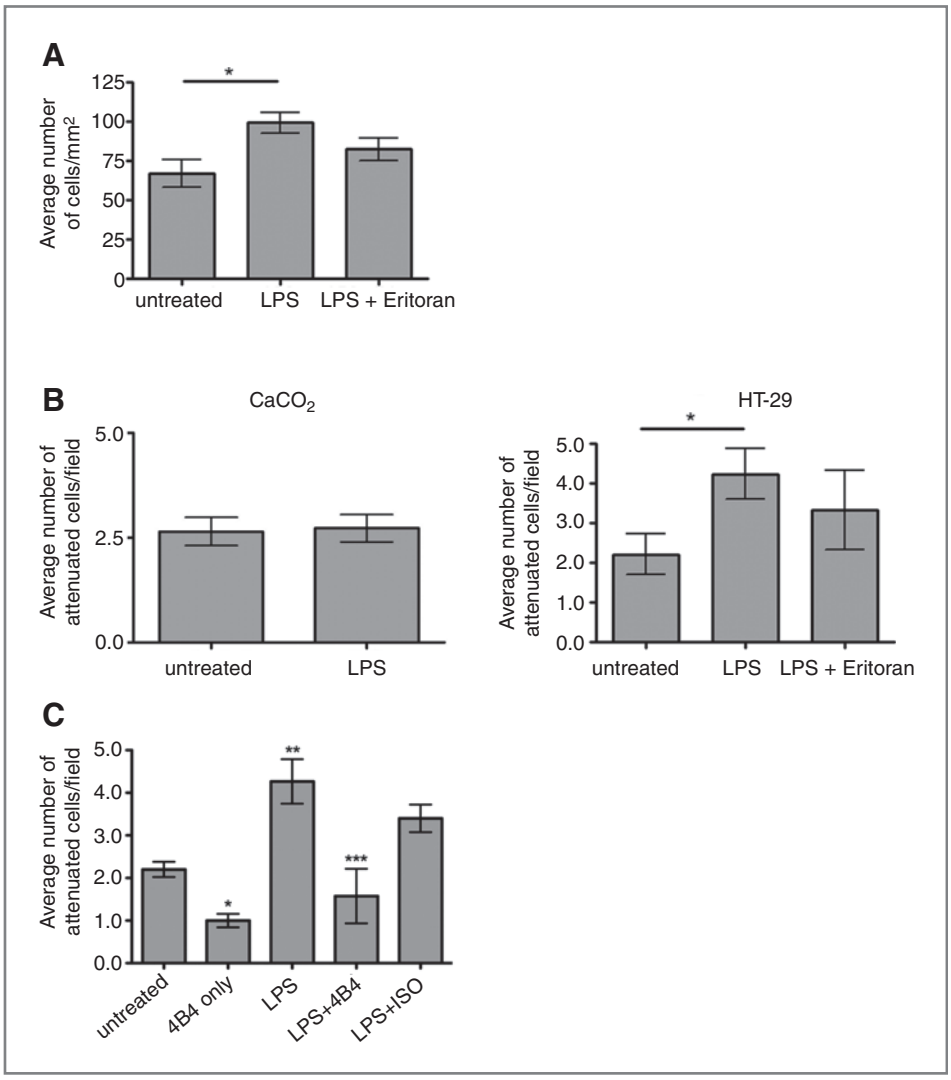

Figure 4. LPS increases *in vivo* hepatic recruitment and *in vitro* endothelial adhesion of HT-29 cells. HT-29 and $CaCO_2$ cells were pretreated with or without LPS (1μg/mL) and with or without Eritoran (100 nmol/L) for 4 hours, collected as single cell suspensions, and labeled with CSFE. A, average number of adhered HT-29 cells per mm^2 of confluent monolayer culture of HUVEC (8 wells/group). A 50% increase was observed between LPS-treated and untreated HT-29 cells (*, $P = 0.011$). Eritoran partially negated the observed LPS effect. Error bar represents SEM. B, average number of attenuated cells in hepatic sinusoids per microscopic view (20× objective) observed by intravital microscopy (8 mice/group). Although no difference was observed between LPS-treated or untreated $CaCO_2$ cells (left panel), a 2-fold increase can be detected in LPS-treated HT-29 cells versus untreated HT-29 cells (*, $P = 0.027$; right panel). In addition, Eritoran partially reversed the observed LPS effect. Error bar represents SEM. C, average number of attenuated cells in hepatic sinusoids per microscopic view (20X objective) observed by intravital microscopy (4 mice/group). Similar numerical difference was observed between LPS-treated HT-29 cells versus untreated HT-29 cells as in B (**, $P = 0.008$). β1 integrin functional blocking antibody (4B4) significantly decreased hepatic recruitment of both untreated (*, $P = 0.003$) and LPS-treated (***, $P = 0.03$) HT-29 cells to a similar level ($P = 0.417$ for comparison between untreated and LPS-treated HT-29 cells in the presence of 4B4 antibody). Note that there was no statistically significant difference between LPS-treated HT-29 cells in the presence or absence of isotype control (ISO) antibody.

PI3K/AKT is the central link between LPS/TLR4 signaling and β1 integrin-mediated adhesion

LPS-induced TLR4 signaling has been shown to activate a number of kinases including p38 MAPK, p42/44 (ERK1/2) MAPK and AKT in various cell types (15–19, 27). As $CaCO_2$ cells lacked cell surface expression of TLR4 and were unresponsive to LPS treatment, no change of phosphorylation in these kinases following LPS treatment was observed as expected (Fig. 6A). In contrast, the serine-473 residue of AKT was phosphorylated upon LPS treatment in HT-29 cells whereas no change in p38 and p42/44 (ERK1/2) MAPK phosphorylation was observed (Fig. 6B).

Upon LPS/TLR4 binding, PI3K has previously been shown to physically bind to MyD88, an immediate downstream signaling molecule of TLR4, and to mediate phosphorylation of its immediate downstream effector molecule AKT (27). To confirm the central role of PI3K/AKT in LPS-induced TLR4 signaling, phosphorylation of AKT and LPS-induced collagen binding were inhibited by Eritoran (TLR4 inhibitor) and PI 103 (a PI3K inhibitor), but not SB203580 (p38 inhibitor) or

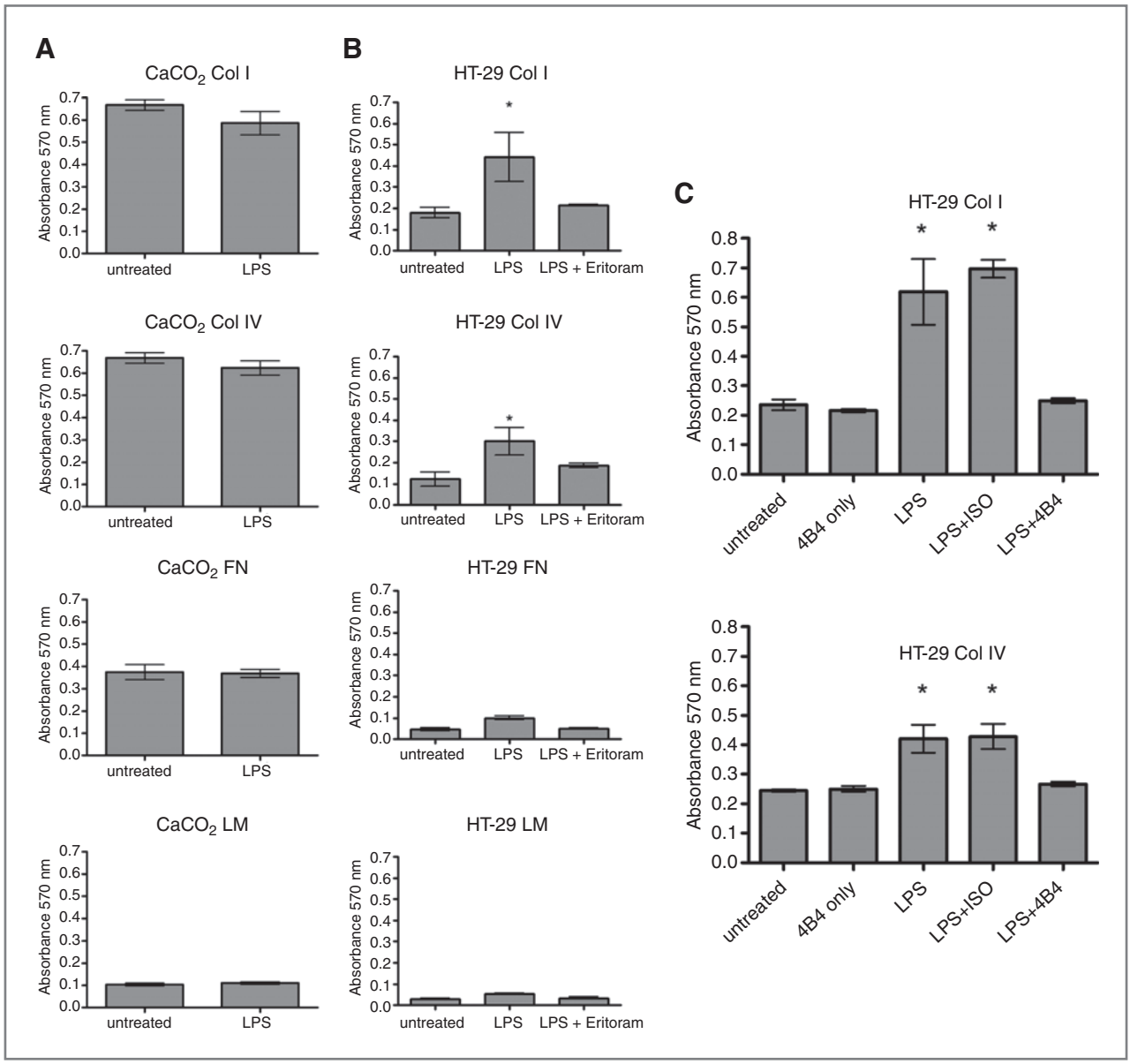

Figure 5. LPS enhances attachment of HT-29 cells to ECM. *A*, LPS treatment did not alter the binding ability of $CaCO_2$ cells for any of the 4 ECM substrates tested. B, LPS treatment significantly enhanced ECM binding ability of HT-29 cells. Coincubation with Eritoran completely reversed the LPS effects. C, although the β1 integrin functional blocking antibody (4B4) did not alter the baseline collagen binding of HT-29 cells, 4B4 completely abolished the LPS effect. The isotype control (ISO) had no impact on ECM binding as expected. Results are expressed as the mean ± SEM and are representative of 5 separate experiments. Each experiment was conducted in triplicate. Statistical significance was compared with the untreated cells. *, $P < 0.05$. ColI, Collagen I; ColIV, Collagen IV; FN, Fibronectin; LM, Laminin.

PD184352 (p42/44 inhibitor) (Fig. 6C and D). In sum, these results suggest LPS can induce PI3K/AKT pathway via TLR4 signaling that subsequently transduces an inside-out signaling cascade activating β1 integrin (28).

Discussion

Emerging evidences suggest systemic inflammation and postoperative infections lead to cancer recurrence (7–10). The mechanisms by which postoperative gram-negative bacterial infections promote cancer recurrence are poorly understood. Although LPS-induced systemic inflammation can increase cancer cell recruitment to the hepatic sinusoids (12) and liver metastasis *in vivo* (29), knowledge on the direct impact of LPS on the metastatic potentials of cancer cells *in vivo* is very limited. In this study, we have documented an increase in adhesion to endothelial cells and various ECM substrates *in vitro* and an enhancement in hepatic recruitment and liver metastasis *in vivo* by human colorectal cancer cells through LPS-induced TLR4 signaling.

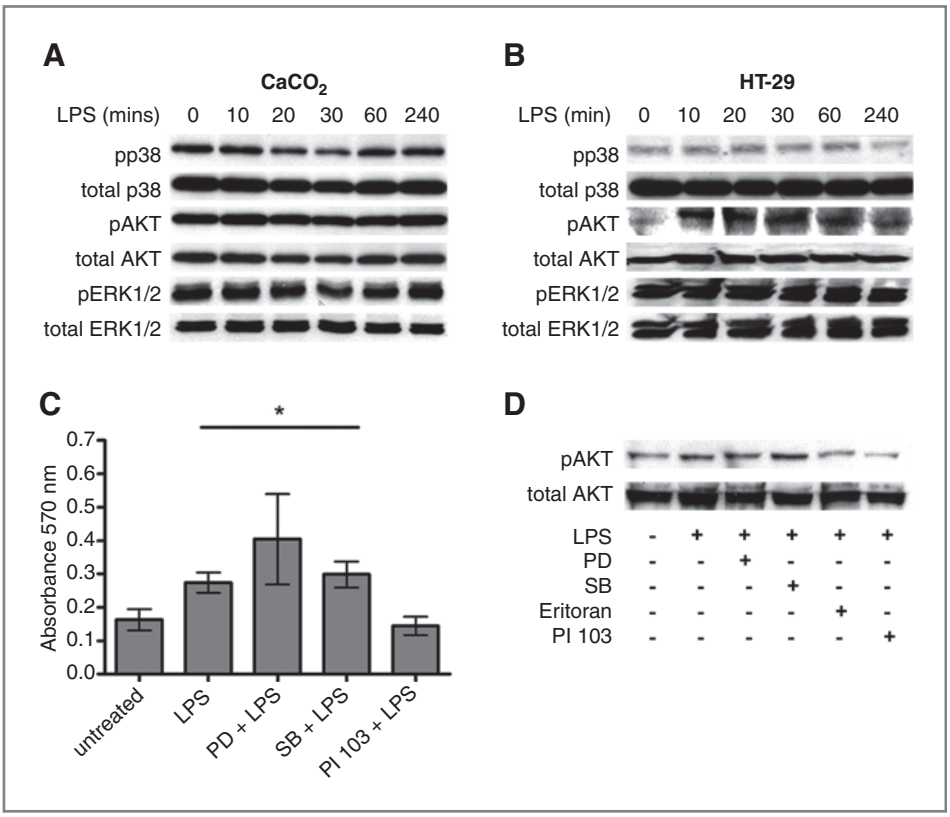

Figure 6. LPS induces PI3K/AKT-dependent adhesion. A, LPS treatment did not alter the expression level or phosphorylation of p38 MAPK, p44/42 MAPK and AKT in $CaCO_2$ cells. B, LPS treatment increased AKT phosphorylation at residue Ser473 after, but not those of p38 MAPK or p44/42 MAPK in HT-29. Expression levels of these kinases were not altered as well. C, PI 103 (PI3K inhibitor), but not SB203580 (SB; p38 MAP kinase inhibitor) and PD184352 (PD; p44/42 MAP kinase inhibitor), completely blocked the LPS effect on collagen binding of HT-29 cells. Error bars represent SEM. Statistical significance was compared with the untreated cells. *, $P < 0.05$. D, PI 103 (PI3K inhibitor) and Eritoran, but not SB203580 (SB) and PD184352 (PD), completely blocked the increased phosphorylation of AKT.

Although LPS may alter proliferation and apoptosis in cancer cells (30, 31), and potentially explain the increased metastasis with LPS incubation of cancer cells, we did not show any proliferative or apoptotic changes *in vitro* after 4 hours of LPS treatment. This may be owing to the duration and dose of LPS used. However, these findings do not exclude the possibility that these cells have a better capacity to survive and grow in the *in vivo* environment after hepatic recruitment. In fact, some studies have shown that LPS may induce secretion of immunosuppressive cytokines and proangiogenic chemokine (30–32). Nonetheless, the major phenotypic change observed in this study is the LPS-induced adhesion of human CRC cells. Despite the big debate on whether cell adhesion or sinusoidal size restriction play a more important role in hepatic recruitment of CTC, cell arrest in hepatic sinusoids has been recently shown to be reduced by anti-β1 or β4 integrin antibodies (33) and anti-sialyl-Le^X antibodies *in vivo* (12) suggesting cell adhesion plays a significant role in hepatic recruitment of cancer cells. Barthel et al. have recently shown that cell surface level of sialyl-Le^X moieties, selectin ligands contributing to the initial step of endothelial adhesion of circulating cells, can be upregulated by overexpressing α1,3-fucosyltransferases in prostate cancer cells (34). Although changes of cell surface level of sialyl-Le^X could not be detected 4 hours after LPS treatment in our present study, we have recently determined that sialyl-Le^X surface level on esophageal cancer cells can be increased after 48 hours of LPS treatment (Rousseau and Ferri, manuscript in preparartion). However, the ability of LPS to induce the expression of fucosyltransferases remains to be elucidated.

In contrast to the study published by Andrews et al. in which surface expression of β1 integrin was significantly increased in LS-174T cells, another metastatic human CRC cell line, as early as 1 hour after LPS treatment (35), cell surface expression level of β1 integrin did not change significantly in our HT-29 cells. Instead, we have seen an increase in β1 integrin-dependent adhesion, indicating a functional activation rather than an up-regulation or translocation onto the cell surface, which has been previously reported (36, 37). On the one hand, CTC-associated integrins can mediate cell adhesion by interacting directly with different ECM components within the hepatic sinusoids. Collagen has been shown to be present in the space of Disse where CTC can gain access through the fenestration on the hepatic sinusoidal endothelium (38, 39). On the other hand, integrins can bind directly to their binding partners on endothelial cells. For instance, VCAM1 has been shown to be the hepatic endothelial ligand of integrin α4β1 (40, 41).

Although LPS-mediated signaling in host cells is becoming clearer, the signaling pathways linking TLR4 binding and β1 integrin activation in cancer cells are largely unknown. In host cells, PI3K/AKT has previously been shown to be immediately downstream of MyD88-dependent LPS-induced TLR4 signaling (27). Upon LPS binding to TLR4, PI3K was shown to physically interact with MyD88 subsequently leading to the phosphorylation of AKT. Additionally, AKT was shown to activate β1 integrin as part of the inside-out signaling cascade (28). Through PI3K inhibition, we have shown here the essential role of PI3K/AKT in the crosstalk between LPS-induced

TLR4 downstream signaling cascade and integrin inside-out signaling pathway in CRC cells. On the contrary, p38 and p44/42 MAPK inhibitors failed to show independently any significant blockade of collagen binding, a β1 integrin-dependent adhesion (26). These results suggest that these MAP kinases may play a redundant role or may not be involved in this process.

This novel LPS-induced signaling pathway represents an interesting therapeutic target for patients undergoing curative resection of colorectal cancers. We have shown for the first time that LPS treatment on cancer cells can increase liver metastases *in vivo* and the cellular changes can be inhibited at three different levels using Eritoran (TLR4 antagonist), PI 103 (PI3K inhibitor) and anti-β1 integrin functional blocking antibody. TLR4 may be a better therapeutic target among the three candidates identified in this signaling pathway for the following reasons: (1) LPS binding to TLR4 is the triggering event in the cancer cells; (2) PI3K and β1 integrin are expressed in many cell types and involved in many normal cellular processes; nonspecific effects are anticipated with their inhibition. However, based on the conditions tested in this study, Eritoran can only partially reverse the LPS effect in our *in vivo* experiments. Further studies are required to optimize the pharmacodynamics and pharmacokinetics of Eritoran for the *in vivo* inhibition of TLR4 in the context of cancer metastasis.

Although these results may raise a significant interest in cancer therapeutics, especially on those patients who have infectious complications, the real question is when to treat these patients, that is, before or after anastomotic leak or abscess formation. Gram-negative bacterial infectious complications are frequent after colonic and rectal surgery, and the LPS/TLR4 signaling pathway could potentially be targeted in patients who are at high risk of developing these complications (e.g., obesity, malnutrition, prior-radiotherapy, peripheral vascular disease, and rectal anastomoses). Furthermore, there is evidence suggesting colon cancer cells may be exposed to low levels of systemic endogenous LPS within the tumors (42) in the absence of infections. Thus, targeting this pathway may be proven beneficial to patients with or without postoperative infectious complication.

In conclusion, we have shed light on the molecular and cellular mechanism of LPS-induced signaling pathway in CRC cells in this manuscript. LPS-induced TLR4 signaling in CTC contributes to their adhesiveness and metastatic capability and the blockage of this signaling pathway may prove to be beneficial to eradicate distal organ metastases. Further studies may include preclinical and clinical trials of the therapeutic agents used.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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