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Intracellular colon cancer-associated *Escherichia coli* promote protumoral activities of human macrophages by inducing sustained COX-2 expression

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Intestinal dysbiosis has been reported in patients with colorectal cancer, and there is a high prevalence of *Escherichia coli* belonging to B2 phylogroup and producing a genotoxin, termed colibactin. Macrophages are one of the predominant tumor-infiltrating immune cells supporting key processes in tumor progression by producing protumoral factors such as cyclooxygenase-2 (COX-2). Here, we investigated whether B2 *E. coli* colonizing colon tumors could influence protumoral activities of macrophages. In contrast to commensal or nonpathogenic *E. coli* strains that were efficiently and rapidly degraded by macrophages at 24 h after infection, colon cancer-associated *E. coli* were able to resist killing by human THP-1 macrophages, to replicate intracellularly, and to persist inside host cells until at least 72 h after infection. Significant increases in COX-2 expression were observed in macrophages infected with colon cancer *E. coli* compared with macrophages infected with commensal and nonpathogenic *E. coli* strains or uninfected cells at 72 h after infection. Induction of COX-2 expression required live bacteria and was not due to colibactin production, as similar COX-2 levels were observed in macrophages infected with the wild-type colon cancer-associated *E. coli* 11G5 strain or a *clbQ* mutant unable to produce colibactin. Treatment of macrophages with ofloxacin, an antibiotic with intracellular tropism, efficiently decreased the number of intracellular bacteria and suppressed bacteria-induced COX-2 expression. This study provides new insights into the understanding of how tumor- infiltrating bacteria could influence cancer progression through their interaction with immune cells. Manipulation of microbes associated with tumors could have a deep influence on the secretion of protumoral molecules by infiltrating macrophages.

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Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies worldwide, making it the fourth most common cause of cancer deaths throughout the world.[1] CRC is a heterogeneous disease, including at least three major forms: hereditary, sporadic, and colitis-associated CRC. A large body of evidence indicates that genetic mutations, epigenetic changes, chronic inflammation, diet, and lifestyle deeply affect CRC onset.[2,3] In addition to these factors, gut microbiota dysbiosis has been reported in CRC patients.[4–9] Recent pyrosequencing analysis of CRC-associated bacterial microbiota has revealed dysbiosis with, in particular, overrepresentation of *Fusobacterium* and *Bacteroides*.[10–14] In addition, our group and others have shown that colonic adenomas, carcinomas, and the mucosa of CRC patients are abnormally colonized by *Escherichia coli* belonging to the B2 phylogroup, with a high prevalence of *E. coli* producing a genotoxin, termed colibactin, encoded by the *pks* genomic island.[15–20] It has been recently demonstrated that cells that survive infection with colibactin-producing *E. coli* display hallmarks of cellular senescence accompanied with production of reactive oxygen species and secretion of pro-inflammatory cytokines, chemokines, and proteases, inducing bystander genotoxic and oncogenic effects.[21,22]

Macrophages are one of the predominant components of murine and human tumor-infiltrating cells. By integrating signals present in the tumor microenvironment, macrophages acquire a new transcriptional program leading to secretion of the molecules displaying protumoral activities and hence supporting the key processes in tumor progression, including proliferation and survival of cancer cells,

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angiogenesis, immunosuppression, invasion, and metastasis.[23,24] Among these molecules, prostaglandin E2 (PGE2) is one of the major actors that orchestrate the protumoral response of macrophages.[25,26] Elevated PGE2 biosynthesis in tumor results from upregulation of cyclooxygenase-2 (COX-2). Role of COX-2 in CRC is particularly supported by its elevated expression found in ~50% of adenomas and 85% of adenocarcinomas,[27] its association with worse survival among CRC patients,[28] and the efficiency of non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors (COXIBs) to reduce the occurrence of sporadic CRC, and the number and size of adenomas in familial adenomatous polyposis patients and in *APC* knockout mice.[29] COX-2 expression is regulated at both transcriptional and posttranscriptional levels and mitogen-activated proteins kinase (MAPK) regulation has been reported to play a key role in regulation of COX-2 expression.[30–32]

In this study, we hypothesized that B2 *E. coli*-colonizing colon cancer tumors could influence protumoral activities of macrophages. We showed that colon cancer-associated *E. coli* survive, replicate, and persist within human macrophages and that macrophages infected with colon cancer *E. coli* expressed COX-2 at least until 72 h after infection. We identified the p38 MAPK signaling pathway as a target to control the number of intracellular bacteria within macrophages and to limit expression of the protumoral factor COX-2.

MATERIALS AND METHODS

Bacterial Strains

B2 *E. coli* strains used in this study were isolated from the tumors of colon cancer patients as previously described.[19,33] Bacteria were grown routinely in Luria Bertani (LB) broth or on LB agar plates overnight at 37 °C.

Gentamicin Treatment and Heat Killing of Bacteria

Bacteria were suspended at DO = 0.1 in PBS and were incubated for 30 min with gentamicin (Gm) at 5 mg/ml or incubated for 30 min at 65 °C before infection. Killing of bacteria was checked by plating on LB agar.

Construction of Isogenic Mutant

The 11G5Δ*clbQ* isogenic mutant was generated with a PCR product by using the method modified by Chaveroche *et al.*[22,34] The basic strategy was to replace a chromosomal sequence with a selectable antibiotic resistance gene, spectinomycin, generated by PCR.

Cell Culture

The human monocytic THP-1 cell line was maintained in an atmosphere containing 5% CO_2 at 37 °C in the culture medium recommended by ATCC. THP-1 monocytes were differentiated into macrophages by treatment with 20 ng/ml phorbol myristate acetate (PMA) for 18 h. Primary bone marrow-derived macrophages (BMDMs) were obtained from wild-type and TLR-4 knockout BALB/c mice, extracted from tibia and femur, and grown as previously described.[35]

Antibodies and Reagents

For western blot analysis, rabbit anti-GAPDH monoclonal antibody, rabbit anti-ERK1/2 polyclonal antibody, rabbit anti-phospho ERK1/2 monoclonal antibody, rabbit anti-p38 polyclonal antibody, rabbit anti-phospoho p38 monoclonal antibody, rabbit anti-SAPK/JNK monoclonal antibody, rabbit anti-phospho SPAK/JNK monoclonal antibody, rabbit anti-caspase-3 monoclonal antibody, and rabbit anti-PARP1 monoclonal antibody were purchased from Cell Signaling. Goat anti-COX-2 polyclonal antibody was purchased from Santa Cruz Biotechnology. Inhibitor for SAPK/JNK (SP600125) signaling pathway was purchased from Cell Signaling, and inhibitor for p38 signaling pathway (SB203580) was purchased from Calbiochem. For macrophage survival assay, ofloxacin and gentamicin were purchased from Euromedex and PMA was purchased from Sigma. For immunofluorescence analysis, bacteria were labeled with goat anti-*E. coli* polyclonal antibody (AbD Serotec) and Alexa 488-labeled anti-goat antibody (Invitrogen). For TLR-4 neutralization, macrophages were treated with goat anti-TLR-4 antibody (R&D Systems). Actin cytoskeleton was stained using TRITC-labeled phalloidin (Sigma). Hoechst 33342 was purchased from Sigma.

Macrophage Survival Assay

The numbers of intracellular bacteria within macrophages were determined by the gentamicin protection assay.[36] Briefly, THP-1 macrophages and BMDMs were infected for 20 min. Infected cells were then washed and incubated with culture medium containing gentamicin at 50 μg/ml for 40 min (1 h after infection) or 24 h (24 h after infection). When indicated, macrophages were treated for 1 h before infection with anti-TLR-4 antibody or an isotype antibody at 7.5 μg/ml, and infection was performed in the presence of the antibodies. When indicated, cells were treated with ofloxacin at 4 μg/ml in the gentamicin-containing medium at 6 h after infection. When indicated, the inhibitors of JNK (SP600125, 50 μM) and p38 (SB203580, 10 μM) signaling pathways were added in the gentamicin-containing medium at 1 h after infection.

Enzyme-Linked Immunosorbent Assays for PGE2 Quantification

The amount of PGE2 released in the culture supernatant was determined by enzyme-linked immunosorbent assay (Cayman Chemical). PGE2 concentration was assessed according to the manufacturer's instructions.

XTT Cell Viability Assay

Cells were plated at a concentration of 2×10^4 cells/well in 96-well plates in 100 μl of cell culture medium and infected at

a MOI of 10 as described above or incubated with staurosporin at 200 nM for 18 h. At 24, 48, or 72 h after infection, cell viability was evaluated using a XTT cell viability assay kit (Biotium) according to the manufacturer's instructions.

Immunoblot Analysis

Whole-cell protein extracts were prepared by using lysis buffer (25 mM Tris pH 7.5; 1 mM EDTA; 5 mM $MgCl_2$; 1% NP-40; 10% glycerol; 15 mM NaCl; 10 μl/ml sodium orthovanadate; and 1 mM PMSF). Proteins were separated on SDS/PAGE gels, transferred to nitrocellulose membrane, blocked for 2 h in Tris-buffered saline (TBS) solution containing 2% BSA, and probed overnight with primary antibodies and for 2 h with secondary HRP-coupled antibodies. GAPDH levels were used to normalize protein quantity. After membrane revelation using the ECL detection kit (Amersham), quantification was performed with ImageJ software.

Confocal Microscopy

Briefly, cells were fixed with 4% paraformaldehyde and immunostained overnight at 4 °C with the indicated specific primary antibodies. Cells were then incubated for 1 h with secondary antibodies. The slides were examined with a Zeiss LSM 510 Meta confocal microscope. Each confocal microscopy image is representative of at least three independent experiments.

Statistical Analysis

Data were expressed as means. Error bars represent s.e.m. Where appropriate, statistical analyses were done using ANOVA or Mann–Whitney using GraphPad Prism 6 software. A *P*-value of 0.05 was considered significant.

RESULTS

Colon Cancer-Associated *E. coli* Strains Survive, Replicate, and Persist within Human Macrophages

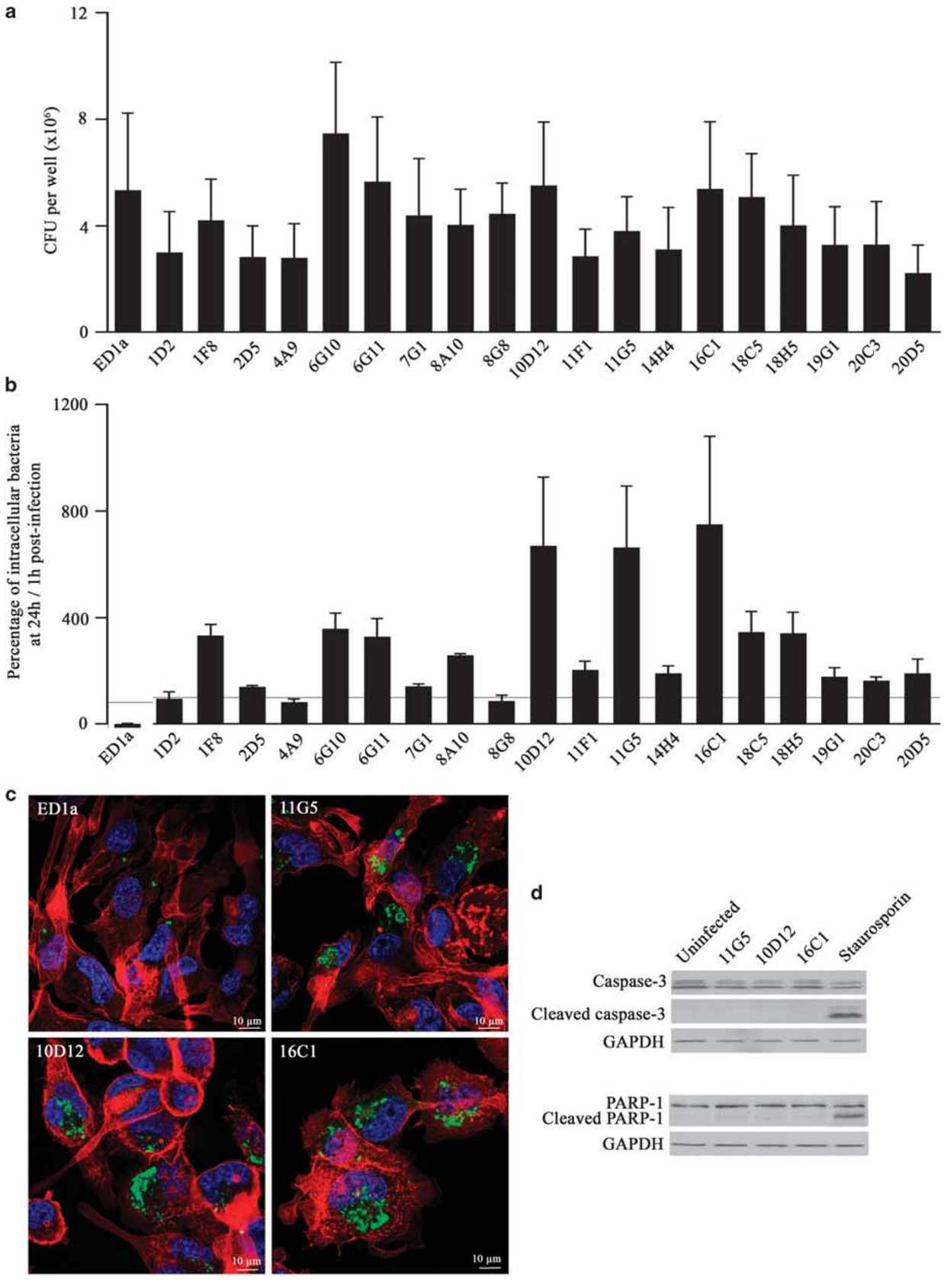
The ability of 19 strains of colon cancer-associated *E. coli* belonging to B2 phylogroup to resist killing by macrophage was assessed in human monocyte-derived THP-1 macrophages. The numbers of intracellular bacteria were determined at 1 and 24 h after infection (Figure 1a and b). No statistically significant difference was observed in the numbers of intracellular B2 *E. coli* at 1 h after infection, indicating similar internalization rates of bacteria within macrophages ($P = 0.97$; Figure 1a). In contrast to the commensal *E. coli* ED1a and Nissle 1917 strains or the nonpathogenic *E. coli* K-12 C600 strain, which were efficiently killed by macrophages, 16/19 (84%) colon cancer-associated *E. coli* strains were able to survive and/or replicate within macrophages with percentages of intracellular bacteria at 24 h compared with 1 h after infection, considered as 100%, ranging from 137.30 ± 7.67% to 746.90 ± 334.70% (Figures 1b and 3a). This result was confirmed by confocal microscopic examination of infected macrophages. At 24 h after infection, high numbers of intracellular bacteria were observed within macrophages infected with the *E. coli* 11G5, 10D12, and 16C1 strains, whereas only few bacteria were observed within *E. coli* ED1a strain-infected cells (Figure 1c). Analysis of *E. coli* 11G5, 10D12, and 16C1 strain-infected macrophages by transmission electron microscopy (TEM) revealed the existence of two types of bacteria-containing compartments, one containing only a few bacteria and another containing numerous bacteria (Figure 2). Survival of colon cancer-associated *E. coli* within THP-1 macrophages did not induce apoptosis as revealed by absence of cleaved Caspase-3 and PARP-1 in infected macrophages at 24 h after infection (Figure 1d). Cleaved Caspase-3 and PARP-1 were detected in macrophages incubated with staurosporin, an inducer of apoptosis (Figure 1d).

Determination of intracellular CFU number in THP-1 macrophages and TEM examination of infected macrophages at longer time after infection revealed that the colon cancer-associated *E. coli* 11G5, 10D12, and 16C1 strains persist within macrophages until at least 72 h after infection (Figure 3a and b). Persistence within THP-1 macrophages at longer time after infection was also observed for the Crohn's disease-associated adherent-invasive *E. coli* (AIEC) reference strain LF82, which has been already described to persist in other macrophage cell lines.[37] No statistical difference in cell viability was observed between uninfected and infected macrophages by using XTT assay (Figure 3c), indicating that intracellular persistence of the bacteria does not induce host cell death.

Colon Cancer-Associated *E. coli* Bacteria Induce COX-2 Expression in Human Macrophages

We examined whether colon cancer-associated *E. coli* strains are able to modulate COX-2 expression and, as a conse-

Figure 1 Ability of colon cancer-associated *E. coli* to survive within macrophages. Human THP-1 macrophages were infected at a MOI of 100 with *E. coli* strains isolated from tumors of CRC patients or the nonpathogenic B2 *E. coli* ED1a strain. (**a**) The number of bacteria internalized within macrophages was determined at 1 h after infection. Results were expressed as numbers of colony-forming units (CFUs) per well. (**b**) The number of intracellular bacteria within macrophages was determined at 24 h after infection. Results were expressed as the number of intracellular bacteria at 24 h after infection relative to that obtained at 1 h after infection, taken as 100%. For all experiments, data are means ± s.e.m. of three independent experiments. (**c**) Confocal microscopic examination of cells infected with the nonpathogenic *E. coli* ED1a strain or the colon cancer-associated *E. coli* 11G5, 10D12, and 16C1 strains at 24 h after infection. The actin cytoskeleton and nucleus of cells were stained using TRITC-labeled phalloidin and Hoescht, respectively. (**d**) Cells were infected with the colon cancer-associated *E. coli* 11G5, 10D12, and 16C1 strains and the nonpathogenic *E. coli* ED1a strain. Caspase-3 and PARP-1 cleavage were analyzed by western blot at 24 h after infection. As a positive control for apoptosis, cells were treated for 3 h with staurosporin at 200 nM.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CFU per well (x10⁶)
12
8
4
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ED1a
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6G10
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8A10
8G8
10D12
11F1
11G5
14H4
16C1
18C5
18H5
19G1
20C3
20D5
b
Percentage of intracellular bacteria at 24h / 1h post-infection
1200
800
400
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ED1a
11G5
10D12
16C1
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11G5
10D12
16C1
Staurosporin
Caspase-3
Cleaved caspase-3
GAPDH
PARP-1
Cleaved PARP-1
GAPDH

quence, PGE2 secretion. For this, we focused on three colon cancer-associated *E. coli* strains displaying strong ability to survive within macrophages: 11G5, 10D12, and 16C1. As shown in Figure 4a and b, these strains induced COX-2 expression and PGE2 secretion in THP-1 macrophages. In addition, we showed that macrophages treated with gentamicin- and heat-killed colon cancer-associated *E. coli* 11G5, 10D12, and 16C1 strains produced significantly less amount of COX-2 than macrophages infected with live bacteria ($P<0.05$; Figure 4c), indicating that live bacteria are required to induce COX-2 expression.

The TLR4-dependent signaling pathways have been involved in induction of COX-2 expression by the uropathogenic *E. coli* J96 strain.[38] We investigated whether induced COX-2 expression by colon cancer-associated *E. coli* results from extracellular activation of TLR4 signaling and/or from stimulation by intracellular bacteria. Macrophages prepared from wild-type mice and TLR4 knockout mice and infected with the colon cancer-associated *E. coli* 11G5 strain expressed similar levels of COX-2 ($P=0.33$; Figure 4d). In addition, no significant modification of COX-2 expression was observed in 11G5-infected THP-1 macro-

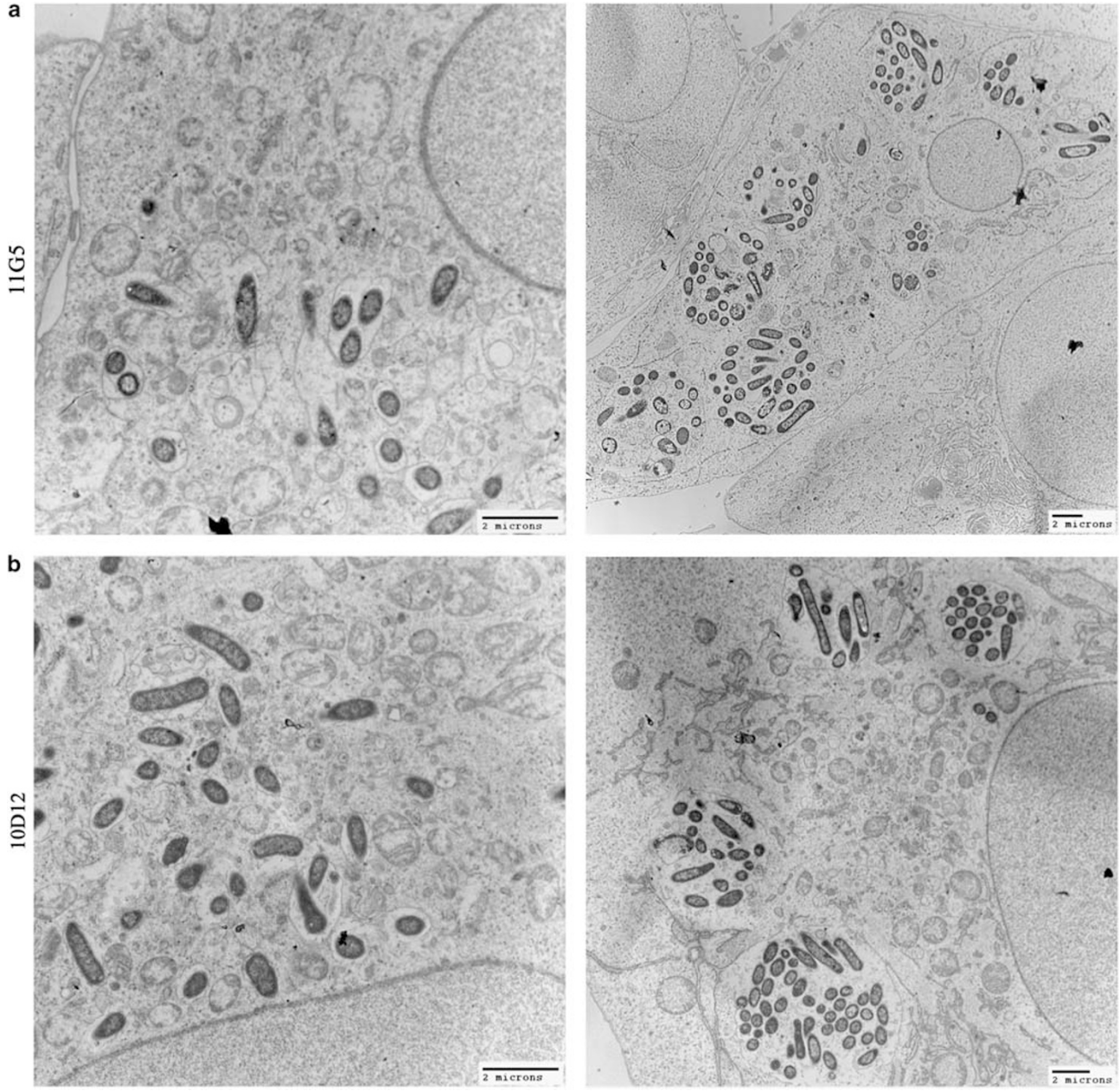


Figure 2 Transmission electronic microscopic examination of colon cancer-associated *E. coli*-containing vacuoles. THP-1 macrophages were infected at a MOI of 100 with the colon cancer-associated *E. coli* 11G5 (**a**), 10D12 (**b**), and 16C1 (**c**) strains, and infected macrophages were analyzed by TEM at 24 h after infection. TEM examination revealed two kinds of vacuoles, one containing a few bacteria (left panels) and another containing numerous bacteria (right panels). Bars = 2 μm.

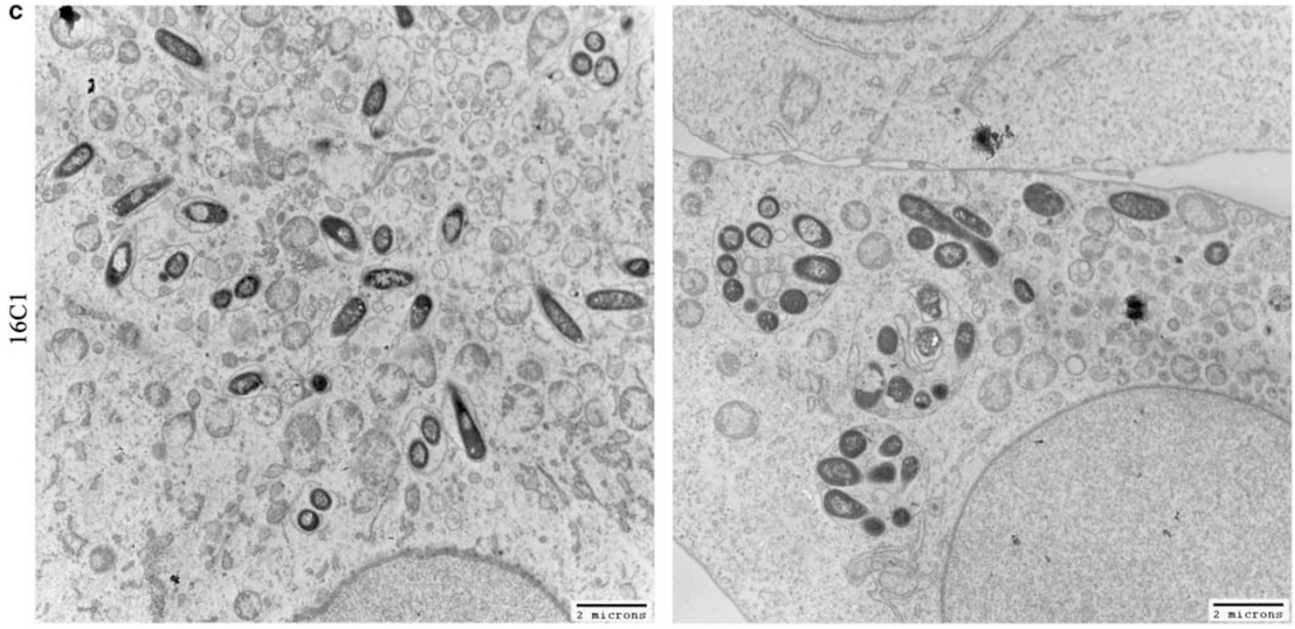


Figure 2 Continued.

phages treated with TLR4-neutralizing antibodies compared with those treated with isotype control antibodies (Figure 4e), indicating that the *E. coli* 11G5 strain induces COX-2 expression in a TLR4-independent manner.

Macrophages Infected with Colon Cancer-Associated *E. coli* Express COX-2 for Several Days

By infecting cells with the colon cancer-associated *E. coli* 11G5, 10D12, and 16C1 strains at different MOIs, we showed that the level of COX-2 expression was statistically correlated with the load of intracellular bacteria in THP-1 macrophages at 24 h after infection (11G5, $r = 0.9333$; 10D12, $r = 0.9167$; and 16C1, $r = 0.9833$), suggesting a link between intracellular bacteria and COX-2 expression by infected macrophages (Figure 5a–c). Interestingly, at 72 h after infection, we observed that macrophages infected with colon cancer-associated *E. coli* 11G5, 10D12, and 16C1 strains expressed significant increased COX-2 levels compared with uninfected cells and macrophages infected with the commensal *E. coli* ED1a and Nissle 1917 strains and the nonpathogenic *E. coli* K-12 C600 strain (Figure 5d). COX-2 levels in macrophages infected with commensal or laboratory *E. coli* strains, which are killed by macrophages as early as 24 h after infection, were similar to that of uninfected macrophages (Figure 5d). In order to investigate whether the ability of intracellular bacteria to induce COX-2 was unique to colon cancer-associated *E. coli*, we analyzed COX-2 expression in THP-1 macrophages infected with the AIEC reference strain LF82 that persist within macrophages at 72 h after infection (Figure 3a). Results showed that at 72 h after infection, THP-1 macrophages infected with LF82 bacteria expressed levels of COX-2 significantly increased compared with uninfected and macrophages infected with commensal and laboratory *E. coli* strains, and similar to those of macrophages infected with colon cancer-associated *E. coli* strains (Figure 5d).

We analyzed whether the induction of COX-2 expression by these strains could be suppressed by pharmacologically reducing the intracellular bacterial load in infected macrophages. Human THP-1 macrophages were treated at 6 h after infection with ofloxacin, a fluoroquinolone antibiotic able to penetrate eukaryotic cells and thus to target intracellular bacteria. Decrease in intracellular bacterial load by ofloxacin treatment was associated with significant reduction of COX-2 expression ($P = 0.05$; Figure 5e and f). Ofloxacin treatment did not modify COX-2 expression induced by LPS stimulation in THP-1 cells (Supplementary Figure S1). Altogether, these results demonstrated that THP-1 macrophages infected with colon cancer-associated *E. coli* express COX-2 for several days and that antibiotic treatment targeting intracellular bacteria could efficiently suppress COX-2 expression.

Colibactin Is Not Involved in the Induction of COX-2 Expression by Colon Cancer-Associated *E. coli*

As the *E. coli* 11G5, 10D12, and 16C1 strains harbor the *pks* genomic island encoding colibactin and as colibactin-producing *E. coli* have been shown to induce inflammation and oxidative stress as a consequence of senescence promotion,[21,22,33] we generated an *E. coli* 11G5 mutant strain unable to produce colibactin, by deleting *clbQ*, and analyzed its ability to survive within macrophages and to induce COX-2 expression. Similar levels of intracellular wild-type 11G5 and 11G5Δ*clbQ* bacteria were observed in THP-1 macrophages at 1 and 24 h after infection ($P = 0.75$; Figure 6a). In addition, deletion of *clbQ* did not significantly modify the level of

COX-2 expression induced by 11G5 bacteria ($P = 0.50$; Figure 6b). These results indicate that the ability of the *E. coli* 11G5 strain to resist killing by macrophage and to promote COX-2 expression is independent of colibactin production.

Intracellular Number of Colon Cancer-Associated *E. coli* and Bacteria-Induced COX-2 Expression Are Controlled by p38 MAPK

MAPK signaling pathways have been involved in transcriptional regulation of COX-2 expression.[39] The colon cancer-associated *E. coli* 11G5, 10D12, and 16C1 strains induced phosphorylation of JNK and p38 MAPKs, indicating activation of these signaling pathways (Figure 7a). No activation of ERK signaling pathway was observed (Figure 7a). To determine the contribution of JNK and p38 MAPK signaling pathway in the bacteria-induced COX-2 expression and in the intracellular persistence of colon cancer-associated *E. coli*, THP-1 macrophages were incubated with specific inhibitors of JNK (SP600125) and p38 (SB203580) after the infection period. These treatments had no effect on cell viability (Supplementary Figure S2). COX-2 expression was decreased in macrophages treated with specific inhibitors of p38 and JNK signaling pathways and infected with the 11G5, 10D12, and 16C1 strains (Figure 7b). Interestingly, significant decrease in the number of intracellular colon cancer-associated bacteria was observed at 24 h after infection only when p38, but not JNK, signaling pathway was inhibited (Figure 7c). Altogether, these results indicate that the p38 MAPK signaling pathway controls both numbers of intracellular bacteria and expression of the protumoral factor COX-2 in THP-1 macrophages infected with colon cancer-associated *E. coli*.

DISCUSSION

Macrophages are one of the most predominant tumor-infiltrating cell types and their role in cancer progression has been firmly established. By secreting a variety of factors including COX-2/PGE2, macrophages promote key steps of carcinogenesis. Acquisition of protumoral function by macrophages is directly influenced by tumor microenvironment. As several independent studies have reported high colonization by *E. coli* of adenomas and carcinomas in colon cancer patients,[15–19] we have analyzed the effect of such a microbial environmental factor on COX-2 expression in human macrophages.

Phagocytic uptake of pathogens by macrophages results in the formation of vacuoles that rapidly evolve into bactericidal

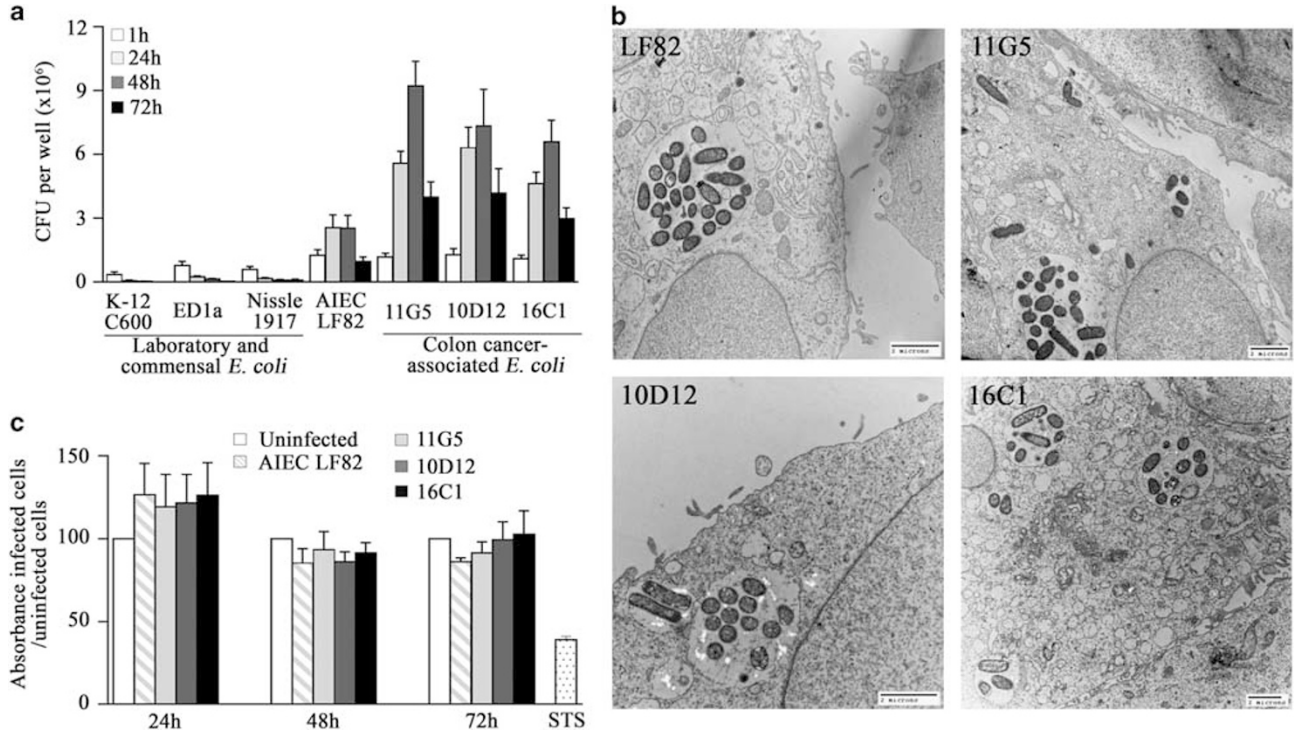
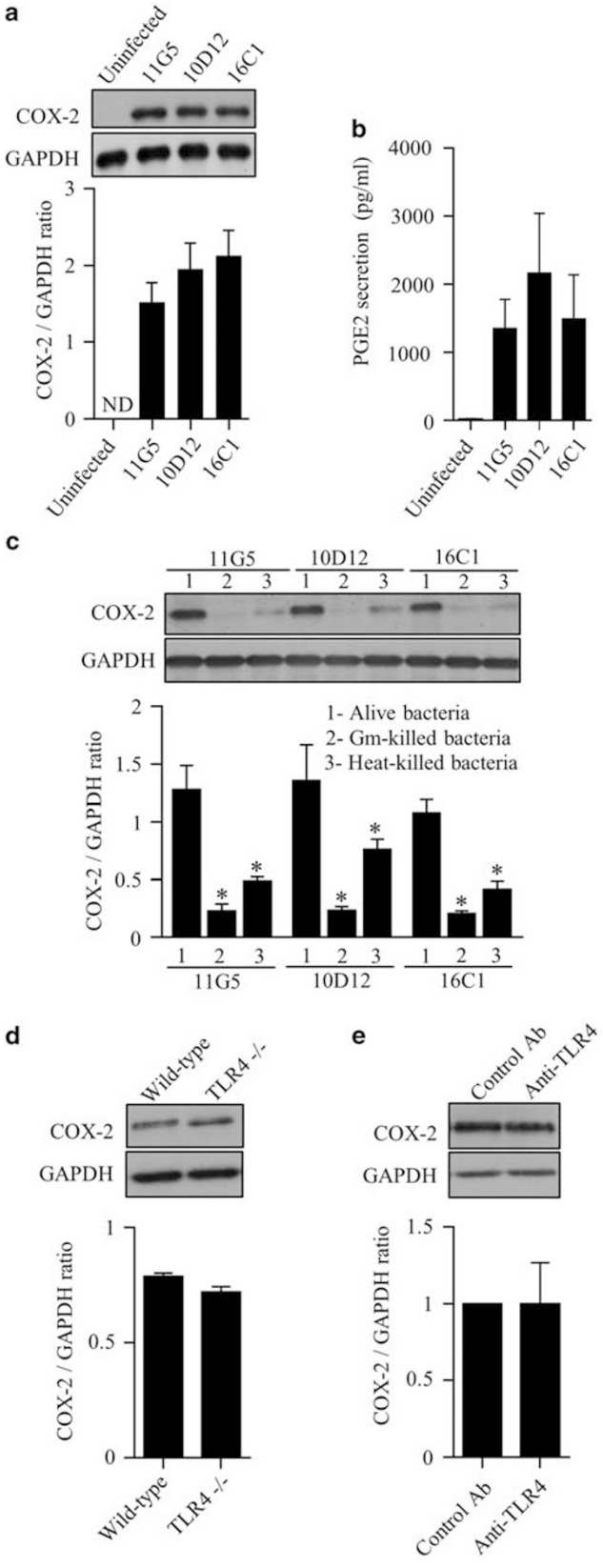


Figure 3 Intramacrophagic persistence of *E. coli* strains isolated from colon cancer patients. THP-1 macrophages were infected at a MOI of 10 with the colon cancer-associated *E. coli* 11G5, 10D12, or 16C1 strains, the Crohn's disease-associated *E. coli* reference strain LF82, the commensal *E. coli* ED1a and Nissle 1917 strains, or the laboratory *E. coli* K-12 C600 strain. (**a**) The numbers of bacteria internalized within macrophages were determined at 1, 24, 48, and 72 h after infection. Results were expressed as numbers of colony-forming units (CFUs) per well. (**b**) Infected macrophages were analyzed by TEM at 72 h after infection. Bars = 2 μm. (**c**) Viability of uninfected and infected cells was assessed at 24, 48, and 72 h after infection using a XTT cell viability assay kit. As a positive control for cell death, cells were treated for 18 h with staurosporin (STS) at 200 nM.



organelles termed phagolysosomes through interactions with vesicles of the endosomal system. Pathogens often use phagocytic cells, in particular macrophages, to gain access to an intracellular niche where they can survive and replicate.[40–42] Shortly after internalization, many intracellular pathogens hijack the intracellular defense mechanisms to either subvert or delay the maturation process (eg, *Mycobacterium tuberculosis*, *Salmonella enterica* Typhimurium, *Brucella abortus*, and *Legionella pneumophila*), or to escape from their vacuoles (eg, *Shigella flexneri* and *Listeria monocytogenes*) and induce macrophage apoptosis.[41,43] In this study, we showed that the great majority of B2 *E. coli* strains isolated from colon cancer patients are able to survive, replicate, and persist within human macrophages for several days and do not induce host cell death. Interestingly, we demonstrated that the p38 MAPK signaling pathway is involved in the control of intracellular bacteria. Indeed, specific inhibition of p38 signaling pathway, but not that of JNK, induced a decrease in the number of intracellular colon cancer-associated *E. coli*. It could be speculated that colon cancer-associated *E. coli* hijack the p38 MAPK signaling pathway to create an intracellular niche where they can survive and replicate. MAPKs are involved in membrane trafficking, and thus control key steps of phagolysosome biogenesis[44–46] and regulate autophagy pathway.[47,48] Some intracellular pathogens hijack MAPK signaling pathways in favor of their survival. For example, intracellular replication of *Mycobacterium avium* is enhanced by p38 activation, whereas ERK activation has the opposite effect.[49] Activation of p38 plays a role in EEA1 exclusion, an early endosomal protein, from *M. tuberculosis* phagosomes and in inhibiting the maturation of mycobacterial phagosomes.[44] Dependence of the survival of *Brucella melitensis* on p38 and JNK activation has also been reported.[50]

Figure 4 COX-2 expression and PGE2 secretion by colon cancer-associated *E. coli*-infected macrophages. THP-1 macrophages were infected with the colon cancer-associated *E. coli* 11G5, 10D12, and 16C1 strains. (**a**) COX-2 expression was analyzed by western blot at 24 h after infection. Quantification of COX-2 expression relative to GAPDH was displayed below the representative immunoblot. (**b**) Levels of PGE2 secreted in cell supernatants were quantified by ELISA. (**c**) COX-2 expression was analyzed at 24 h after infection in macrophages infected with living or dead (gentamicin (Gm) or heat killed) bacteria by western blot. Quantification of COX-2 expression relative to GAPDH was displayed below the representative immunoblot. (**d**) Bone marrow-derived macrophages were prepared from wild-type and TLR-4 knockout mice and infected with the colon cancer-associated *E. coli* 11G5 strain. COX-2 expression was analyzed by western blot at 24 h after infection. Quantification of COX-2 expression relative to GAPDH was displayed below the representative immunoblot. (**e**) Human THP-1 macrophages were treated before infection with anti-TLR4 antibody or with an isotype control antibody. Infection with the *E. coli* 11G5 strain was performed in the presence of the antibodies. COX-2 expression was analyzed by western blot at 24 h after infection. Quantification of COX-2 expression relative to GAPDH was displayed below the representative immunoblot. For all experiments, data are means ± s.e.m. of three independent experiments.

In macrophages infected with colon cancer-associated *E. coli*, two kinds of vacuoles were observed, one containing a few bacteria and another containing numerous bacteria. Heterogeneity of intracellular bacterial population has already been reported for *Salmonella*.[35,51] Indeed, upon entry into macrophages, some *Salmonella* do not replicate and enter a dormant-like state. It is speculated that these living nonreplicating bacteria could represent a reservoir of persistent bacteria. Besides, some intracellular pathogens have adapted to resist the harsh conditions in the phagolysosomes (eg, Crohn's disease-associated *E. coli*),[52] and may require lysosomal or autophagosomal interactions to facilitate the delivery of nutrients ensuring their intracellular survival and growth (eg, *Brucella abortus* and *Yersinia*).[53,54]

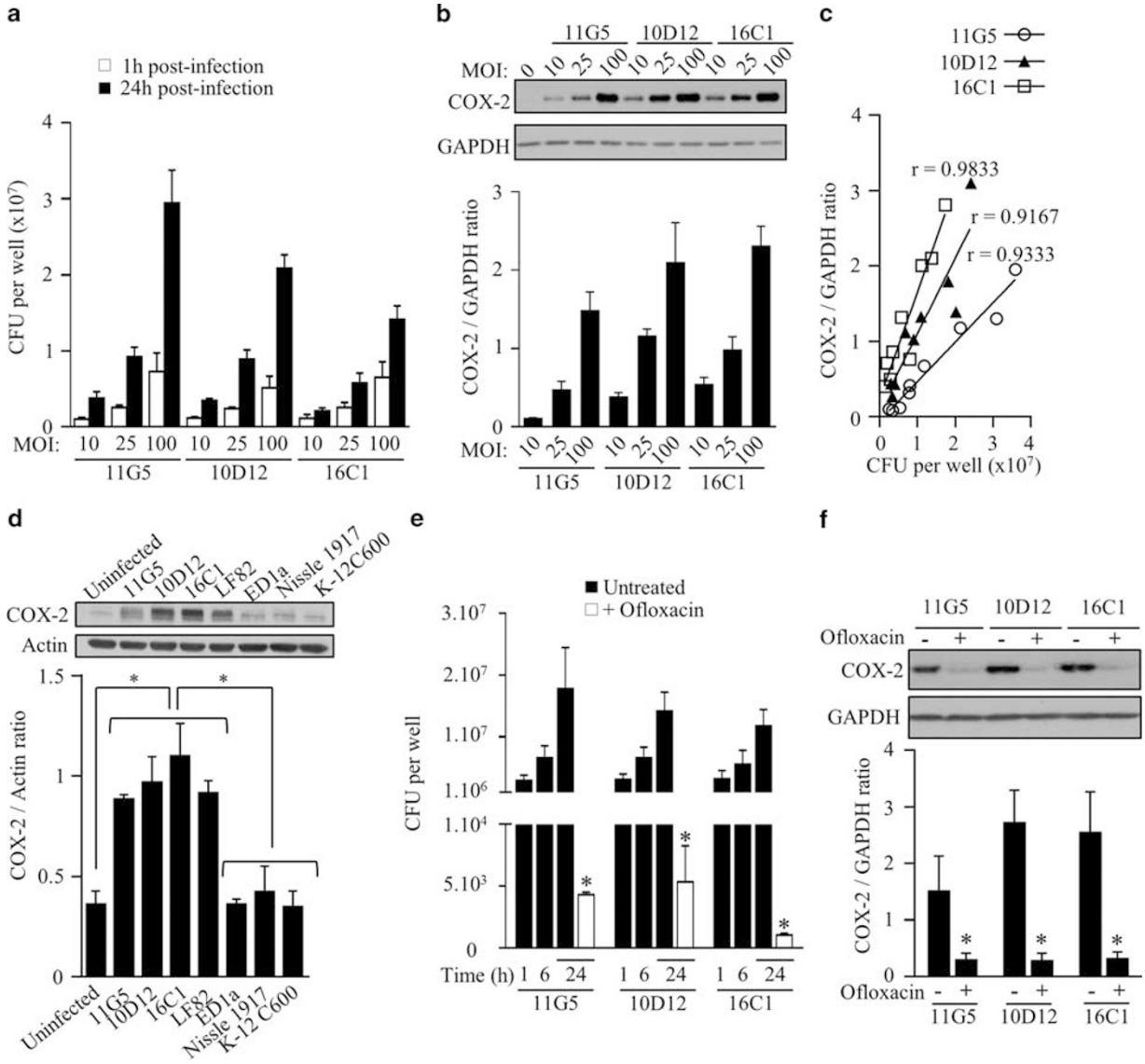


Figure 5 COX-2 expression in infected macrophages is correlated with the presence of intracellular bacteria. THP-1 macrophages were infected with the colon cancer-associated *E. coli* 11G5, 10D12, or 16C1 strains, the Crohn's disease-associated *E. coli* reference strain LF82, the commensal *E. coli* ED1a and Nissle 1917 strains, or the laboratory *E. coli* K-12 C600 strain. (**a**, **b**) THP-1 macrophages were infected at MOIs of 10, 25, or 100. (**a**) The number of intracellular bacteria within macrophages was determined at 24 h after infection. (**b**) COX-2 expression was analyzed at 24 h after infection, and quantification of COX-2 expression relative to GAPDH was displayed below the representative immunoblot. (**c**) Correlation between the number of intracellular bacteria and the ratio of COX2/GAPDH levels determined by Spearman's test. (**d**) THP-1 macrophages were infected at a MOI of 10. COX-2 expression was analyzed at 72 h after infection, and quantification of COX-2 expression relative to GAPDH was displayed below the representative immunoblot. (**e**, **f**) Infected macrophages were treated with ofloxacin at 6 h after infection and until 24 h after infection. (**e**) The numbers of intracellular bacteria were determined at 1, 6, and 24 h after infection. Results were expressed as CFUs per well. (**f**) COX-2 expression was analyzed at 24 h after infection, and quantification of COX-2 expression relative to GAPDH was displayed below the representative immunoblot. For all experiments, data are means ± s.e.m. of three independent experiments.

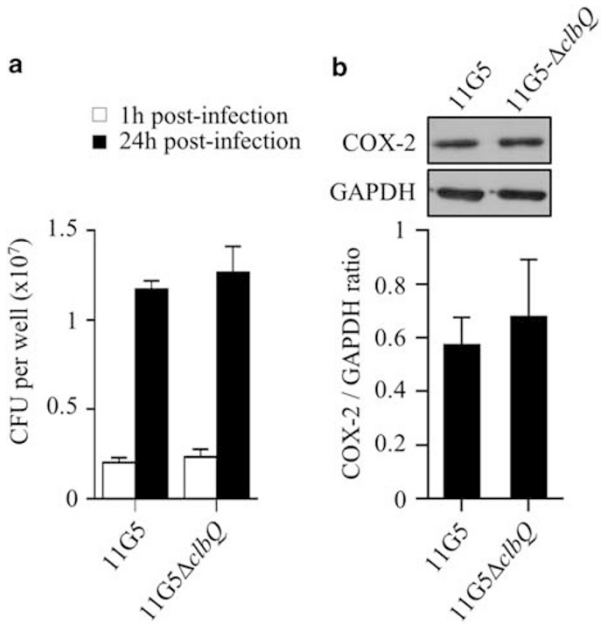


Figure 6 Colibactin is not involved in colon cancer-associated *E. coli*-induced COX-2 expression. THP-1 macrophages were infected with the *E. coli* 11G5 and 11G5Δ*clbQ* strains. (**a**) The numbers of bacteria internalized within macrophages were determined at 1 and 24 h after infection. Results were expressed as CFUs per well. (**b**) COX-2 expression was analyzed at 24 h after infection, and quantification of COX-2 expression relative to GAPDH was displayed below the representative immunoblot. For all experiments, data are means ± s.e.m. of three independent experiments.

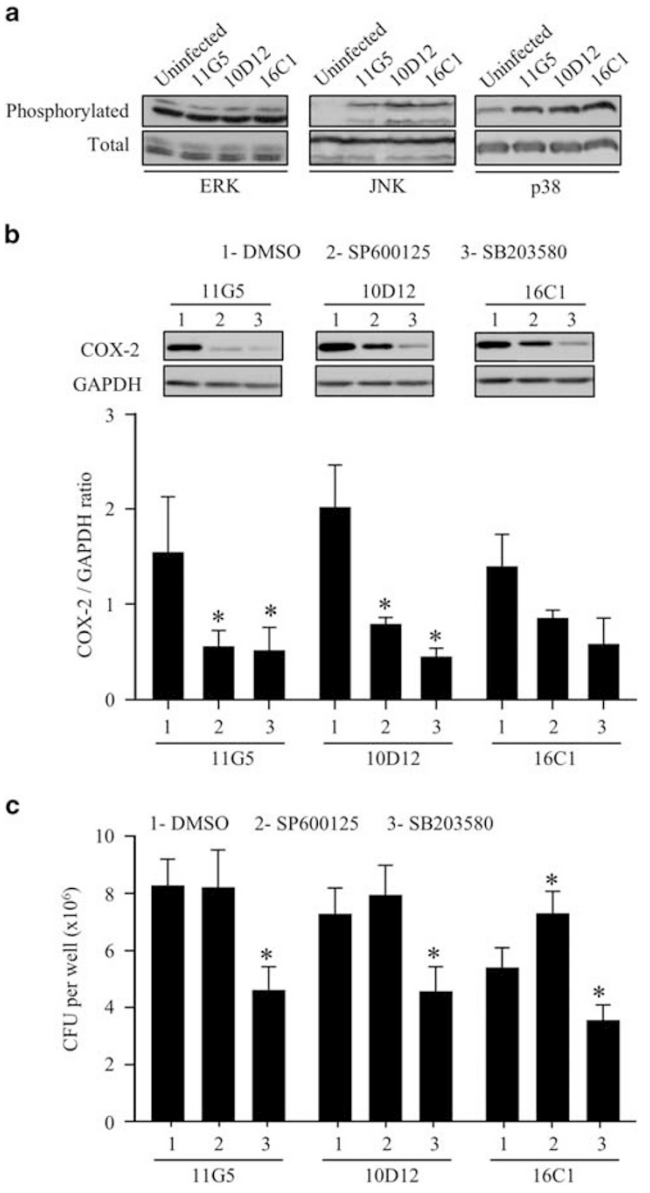


Figure 7 Role of MAPKs in the control of number of intracellular colon cancer-associated *E. coli* and in COX-2 induction in infected macrophages. THP-1 macrophages were infected with the colon cancer-associated *E. coli* 11G5, 10D12, or 16C1 strains. (**a**) Activation of JNK and p38 MAPK signaling pathways was analyzed by western blot using specific antibodies directed against phosphorylated and total proteins. (**b**, **c**) Infected macrophages were treated with specific inhibitors of JNK (SP600125) and p38 (SB203580) signaling pathways at 1 h after infection. (**b**) COX-2 expression was analyzed at 24 h after infection, and quantification of COX-2 expression relative to GAPDH was displayed below the representative immunoblot. (**c**) The numbers of intracellular bacteria were determined at 24 h after infection. Results are expressed as CFUs per well. For all experiments, data are means ± s.e.m. of three independent experiments.

COX-2 expression is low and undetectable in normal cells and is induced in response to various stimuli.[39] Elevated COX-2 expression is found in most colon cancer tissues and is associated with worse survival among CRC patients.[29] We reported here that infection of human macrophages with colon cancer-associated *E. coli* strains induces COX-2 expression for several days and that targeting intracellular bacteria by using antibiotic allows to reduce COX-2 level. This suggests that intracellular colon cancer-associated *E. coli* sustain COX-2 expression. However, induction of COX-2 expression by intracellular bacteria is not a unique feature of colon cancer-associated *E. coli* but rather a property shared by pathogenic *E. coli* able to persist intracellularly, as we observed that AIEC LF82 persistence in THP-1 macrophages was also associated with COX-2 expression.

Colon cancer-associated *E. coli* need to be alive to induce COX-2 expression, and the induced COX-2 expression level was correlated with the intracellular bacterial number. Induction of COX-2 expression by other pathogens associated with gastrointestinal cancer has been reported. For example, *Helicobacter pylori*, a persistent colonizer of the human stomach classified as carcinogen class I, and *Bacteroides fragilis*, a human anaerobic enterotoxinogen bacteria with oncogenic capabilities, induce COX-2 expression through production of toxins.[55,56] In addition, tumors of $Apc^{Min/+}$ mice infected with *Fusobacterium nucleatum* and human fusobacteria-positive colorectal carcinomas highly expressed COX-2.[12] Induction of COX-2 expression by pathogenic *E. coli* has also been reported. Uropathogenic *E. coli* stimulate COX-2

expression through activation of the pathogen recognition receptor TLR4 by type 1 pili expressed by bacteria.[38] We reported here that induction of COX-2 expression by colon cancer-associated *E. coli* does not require TLR4 as similar COX-2 levels were observed in TLR4-/- and wild-type infected macrophages. Cytotoxic necrotizing factor 1 (CNF), a toxin produced by some isolates of *E. coli* that cause extraintestinal infections, stimulates COX-2 expression via a RhoA-dependent signaling pathway.[57] PCR analysis of the colon cancer-associated *E. coli* 11G5, 10D12, and 16C1 strains revealed that they do not possess genes encoding CNF. However, these strains harbor the *pks* genomic island encoding colibactin, a genotoxin that has been associated with protumoral properties of *E. coli* strains isolated from patients with colon cancer.[18] Colibactin triggers senescence in host cells, and this is notably accompanied by production of reactive oxygen species and secretion of proinflammatory cytokines and chemokines.[21,22] Deletion of *clbQ* gene, which impaired colibactin synthesis, had no effect on induction of COX-2 expression by the colon cancer-associated *E. coli* 11G5, 10D12, and 16C1 strains in macrophages, indicating that B2 *E. coli* strains associated with colon cancer can promote protumoral response independently of colibactin production.

Increased levels of COX-2 in macrophages have been reported in human CRC and mouse models of CRC.[39,58–61] COX-2 expression is regulated at both the transcriptional and posttranscriptional levels, and the signaling pathways involved in COX-2 transcriptional regulation include NF-κB, PI3K/AKT, GSK/β-catenin, and MAPKs.[29] High expression of active MAPKs has been observed in the stroma of human colonic adenomatous polyps.[61] Involvement of MAPK signaling pathways in induction of COX-2 expression by colon cancer-associated *E. coli* was revealed by decrease in COX-2 level upon selective inhibition of these signaling pathways. Involvement of pro-inflammatory signaling pathways in COX-2 transcriptional induction by pathogens has been previously reported. Stimulation of COX-2 expression by *H. pylori* involves activation of p38 MAPK and PKC/c-Src/NF-κB signaling pathways in human gastric cancer cells.[56,62] *B. fragilis* enterotoxin increases COX-2 expression in a NF-κB-dependent manner in human intestinal epithelial cells.[55] In addition, ERK, JNK, and p38 MAPKs are critical for uropathogenic *E. coli*-induced COX-2 expression in bladder epithelial cells.[38]

In conclusion, colon cancer-associated *E. coli* strains belonging to B2 phylogroup are able to resist killing by macrophages and to persist several days after infection, and the presence of intracellular bacteria stimulates COX-2 expression. We identified the p38 MAPK signaling pathway as a target to control the number of intracellular colon cancer-associated *E. coli* within macrophages and to limit expression of COX-2 induced by these bacteria. Antibiotic treatment, allowing intracellular bacterial clearance, suppressed COX2 expression, suggesting that manipulation of microbes associated with tumors could have a deep influence on the secretion of protumoral molecules by infiltrated macrophages.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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