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LPS receptor subunits have antagonistic roles in epithelial apoptosis and colonic carcinogenesis

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Colorectal carcinoma (CRC) is characterized by unlimited proliferation and suppression of apoptosis, selective advantages for tumor survival, and chemoresistance. Lipopolysaccharide (LPS) signaling is involved in both epithelial homeostasis and tumorigenesis, but the relative roles had by LPS receptor subunits CD14 and Toll-like receptor 4 (TLR4) are poorly understood. Our study showed that normal human colonocytes were $CD14^+TLR4^-$, whereas cancerous tissues were $CD14^+TLR4^+$, by immunofluorescent staining. Using a chemical-induced CRC model, increased epithelial apoptosis and decreased tumor multiplicity and sizes were observed in TLR4-mutant mice compared with wild-type (WT) mice with $CD14^+TLR4^+$ colonocytes. WT mice intracolonically administered a TLR4 antagonist displayed tumor reduction associated with enhanced apoptosis in cancerous tissues. Mucosa-associated LPS content was elevated in response to CRC induction. Epithelial apoptosis induced by LPS hypersensitivity in TLR4-mutant mice was prevented by intracolonic administration of neutralizing anti-CD14. Moreover, LPS-induced apoptosis was observed in primary colonic organoid cultures derived from TLR4 mutant but not WT murine crypts. Gene silencing of *TLR4* increased cell apoptosis in WT organoids, whereas knockdown of *CD14* ablated cell death in TLR4-mutant organoids. *In vitro* studies showed that LPS challenge caused apoptosis in Caco-2 cells ($CD14^+TLR4^-$) in a CD14-, phosphatidylcholine-specific phospholipase C-, sphingomyelinase-, and protein kinase C-ζ-dependent manner. Conversely, expression of functional but not mutant TLR4 (Asp299Gly, Thr399Ile, and Pro714His) rescued cells from LPS/CD14-induced apoptosis. In summary, CD14-mediated lipid signaling induced epithelial apoptosis, whereas TLR4 antagonistically promoted cell survival and cancer development. Our findings indicate that dysfunction in the CD14/TLR4 antagonism may contribute to normal epithelial transition to carcinogenesis, and provide novel strategies for intervention against colorectal cancer.
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Colorectal tumorigenesis proceeds via the accumulation of genetic and epigenetic alterations that promote unlimited cell proliferation, self-sufficient growth signaling, neovascularization, tissue invasion, and resistance to cell death.[1] The transformation of normal epithelium into colorectal carcinomas (CRC) is associated with the progressive inhibition of apoptosis; this confers a selective advantage for tumor cell survival and chemoresistance.[2,3] It is generally believed that sufficient epithelial apoptosis may hamper colon cancer formation in terms of incidence and growth rate.[4–6] Direct evidence for this was recently reported in mice deficient in pro-apoptotic molecules.[7,8] To date, the regulatory mechanisms of physiological apoptosis to eliminate premalignant cells in the gut remain incompletely understood.

Intestinal homeostasis is maintained by the dynamic, yet strictly regulated, turnover of epithelial cells. An imbalance in epithelial death *versus* survival/proliferative responses may lead to barrier dysfunction, chronic inflammation, and tumorigenesis.[9,10] Accumulating evidence indicates that gut microbiota and bacterial lipopolysaccharide (LPS) have critical roles in epithelial cell renewal under baseline conditions and on injury,[11,12] and are involved in the pathogenesis of colitis-associated CRC as well.[13–15] Given the juxtaposition of commensal bacteria and the gut mucosa, it has been assumed that normal epithelial cells are not equipped with LPS receptor complexes (CD14/TLR4/MD2) or express altered forms of receptors and signaling molecules to achieve immunotolerance.[15] Constitutive expression of CD14 was reported in the presence of negligible-to-low levels of Toll-like receptor 4 (TLR4) in normal human colonocytes,[16–18] whereas strong TLR4 immunoreactivity was detected in CRC.[18,19] Nevertheless, divergent cellular responses to LPS (death *versus* survival) have been reported among human CRC cell lines. Several laboratories, using Caco-2 cells, have

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Abbreviations: LPS, lipopolysaccharide; TLR, Toll-like receptor; CRC, colorectal carcinoma; WT, wild type; AOM/DSS, azoxymethane/dextran sulfate sodium; CO, commensal overgrowth; RT-PCR, reverse transcription-PCR; TUNEL, terminal deoxynucleotidyl transferase dUTP-biotin nick end labeling; DMEM, Dulbecco's modified Eagle's medium; PC-PLC, phosphatidylcholine-specific phospholipase C; SMase, sphingomyelinase; PKC, protein kinase C; MyD88, myeloid differentiation factor; MAPK, mitogen-activated protein kinase; IκB, inhibitor of κB; NFκB, nuclear factor-κB; IRF3, interferon regulatory factor 3; PMB, polymyxin B

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described increases in apoptotic cell death following apical LPS challenge,[20,21] whereas others have documented enhanced survival and proliferative responses of HT29 and SW480 cells to LPS.[22,23] Here we hypothesize that differing expression patterns of LPS receptor subunits on epithelial surfaces may have a determining role in cell death *versus* survival.

CD14, as the membrane-bound subunit of LPS receptor complex and lacking a cytoplasmic tail, has traditionally been regarded as merely a binding component for transferring LPS to TLR4. TLR4 subsequently activates downstream adaptors and signaling pathways, such as myeloid differentiation factor (MyD88), mitogen-activated protein kinases (MAPKs), inhibitor of κB (IκB)/nuclear factor-κB (NFκB), and interferon regulatory factor 3 (IRF3).[24,25] Recent findings in monocytes have indicated that LPS/CD14 binding triggers a cascade of lipid messenger signals before TLR4 trafficking to lipid rafts for complex formation. CD14-dependent lipid signaling includes the conversion of membranous phosphatidylcholine (PC) to diacylglcerol by PC-specific phospholipase C (PC-PLC) and the activation of sphingomyelinase (SMase) for sphingolipid metabolism and ceramide production. This process leads to the phosphorylation of protein kinase C (PKC) ζ, which recruits TLR4 to interact with CD14 (Cuschieri *et al*.[26] and Triantafilou *et al*.[27]). Lipid messengers, such as sphingolipids and ceramides, and their downstream PKCζ signals have been implicated in pro-apoptotic pathways and are considered tumor suppressors.[28–30] Decreased SMase activity and PKCζ levels have been observed in human colorectal tumors, correlated with poor prognosis.[31,32] In contrast, the TLR4/MyD88 and IκB/NFκB pathways are associated with anti-apoptotic and hyperproliferative responses.[5,33–35] Reduced colorectal tumor formation has been documented in TLR4(−/−), MyD88(−/−), and epithelial-specific IκB kinase β-deficient mice as compared with wild-type (WT) mice.[5,19,36] These findings led us to speculate that the expression of CD14 and TLR4 on epithelial cell surfaces may provide antagonistic signals to counteract apoptotic responses to LPS and to influence tumor progression.

The aims of this study were to (1) investigate the expression patterns of LPS receptor subunits in normal and cancerous colonic epithelia in human and murine tissues; (2) examine the individual roles of CD14 and TLR4 in epithelial apoptosis and tumor formation using a mouse model of colitis-associated CRC; (3) assess the involvement of CD14-mediated lipid messengers and/or TLR4-dependent signaling in the mechanism of LPS-induced apoptosis using human carcinoma cell lines; and (4) evaluate whether TLR4 has an opposing role against CD14-mediated apoptosis to promote tumor cell survival.

Results

Human normal colonocytes express CD14 constitutively in the absence of TLR4, whereas cancerous cells display increased CD14 and TLR4 proteins. Surgical specimens from CRC patients were examined for the expression of LPS receptor subunits. Immunoreactivity on epithelial layers of morphologically normal regions was found positive to CD14 but negative to TLR4 or MD2 (Figure 1a), indicating that CD14 was the only receptor subunit constitutively expressed in normal colonocytes. In cancerous tissues, increased epithelial expression levels of CD14 and TLR4 were observed without a change in MD2 levels (Figure 1a). The expression levels of CD14 and TLR4 were quantified in normal epithelium and tumorous regions (Figures 1b and c).

Augmentation of LPS-induced epithelial apoptosis correlates with a reduction of colonic tumorigenesis in TLR4-mutant mice. A colitis-associated CRC mouse model administered three cycles of azoxymethane/dextran sulfate sodium (AOM/DSS) was used to examine the relationship between epithelial cell death and tumor development, and the extent of LPS exposure to gut mucosa during disease progression. First, baseline levels of CD14/TLR4/MD2 were determined in mouse colonocytes isolated from untreated BALB/c mice. Mouse colonocytes showed transcripts of the three receptor subunits (*CD14*, *TLR4*, and *MD2*) constitutively, and expressed CD14 and TLR4 proteins on the apical membrane of the surface epithelia and MD2 on the crypt epithelia (Figures 2a and b). These results indicate that epithelial LPS receptor expression patterns differ between mice ($CD14^+TLR4^+$) and humans ($CD14^+TLR4^-$) at steady-state conditions. To mimic the absence of TLR4 in normal human colonocytes, genetically deficient mice and their WT counterparts were used for mutagen administration.

WT BALB/c mice, which are known high responders to AOM mutagen exposure,[37,38] and TLR4-deficient mice harboring a spontaneous point mutation in the *Tlr4* gene at Pro712His (TLR4-mut mice; C.C3-$Tlr4^{LPS\text{-}d}$/J mouse strain with BALB/c background) were administered AOM/DSS. Mice were killed before (day 0) and 11, 25, 39, 68, and 80 days after the start of the first AOM injection, to examine the progression of tumorigenesis (Figure 3a). The genetic alteration and signaling dysfunctions were verified in TLR4-mut mice (Supplementary Figure S1). Multiple colonic tumors were observed (mainly distal colons) of WT mice with an incidence of 100% at late stages (i.e., 63 and 80 days) (Figures 3b and c). Dysplastic features and high Ki-67 staining (a proliferation marker) were noted in the tumors of WT mice (Figures 3d and e). The tumors of WT mice on 80 days mostly exhibited high-grade dysplasia to carcinoma. Moreover, the expression levels of CD14, TLR4, and MD2 were higher in tumorous tissues than in adjacent non-tumor tissues or in untreated (baseline) tissues (Figure 2b).

In contrast, 0 and 90% of TLR4-mut mice had colorectal tumors after 63 and 80 days, respectively. The tumor multiplicity and mean tumor area were significantly lower in TLR4-mut mice compared with WT mice (Figure 3c). The tumors in TLR4-mut mice on 80 days displayed low- to high-grade dysplasia. Using a method of apoptotic terminal deoxynucleotidyl transferase dUTP-biotin nick end labeling (TUNEL), we observed higher numbers of TUNEL-positive cells per area of tumor in TLR4-mut mice than in WT mice (Figure 3f). At early time points (i.e., 0, 11, 25, and 39 days), increased mucosal cell apoptosis was observed in TLR4-mut mice but not in WT mice (Figures 3g and h). Mucosal cell proliferation levels, measured by immunoblotting for proliferating cell nuclear antigen, did not differ between WT and TLR4-mut mice at early times (Figure 3h). These results suggest that

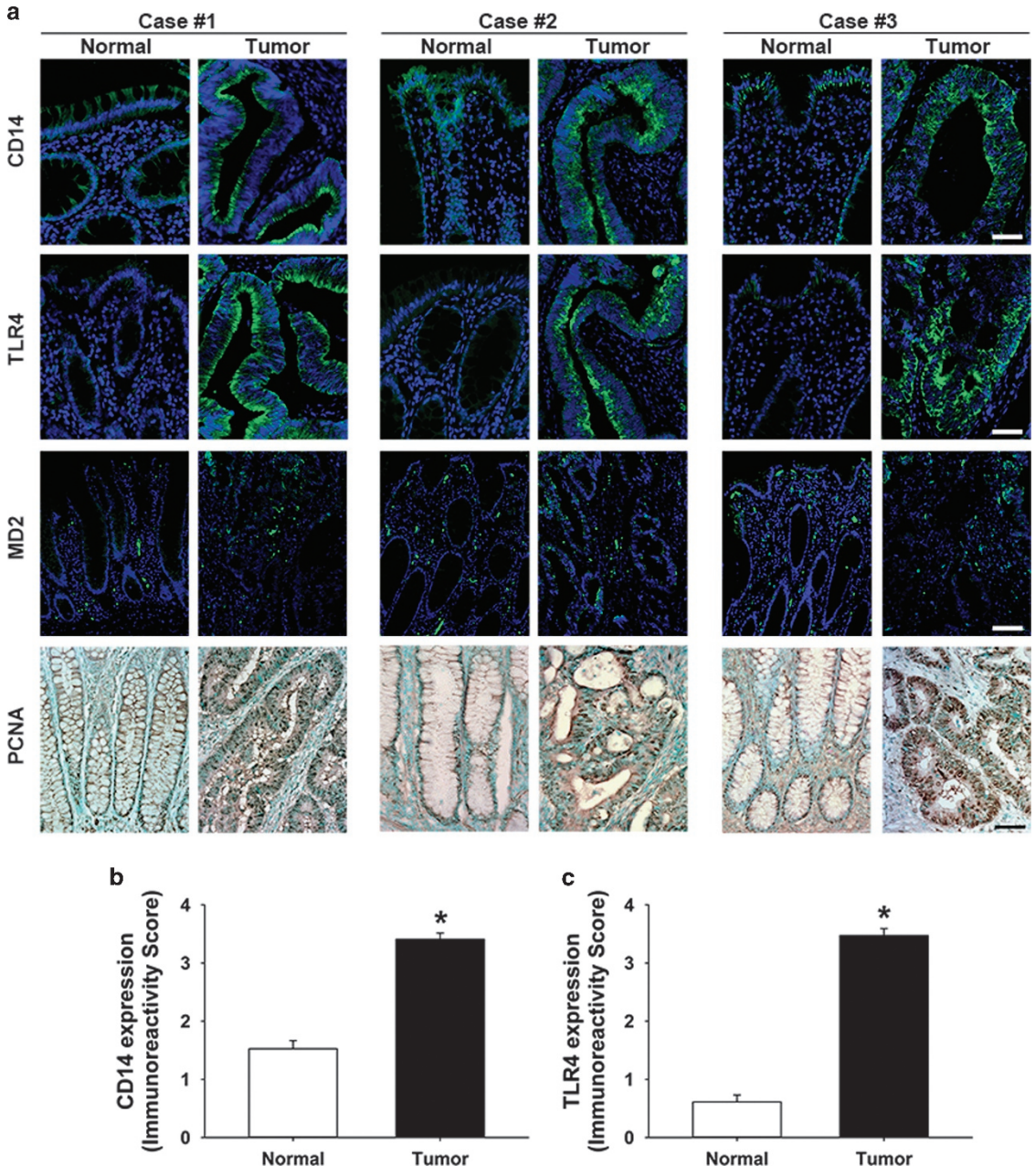


Figure 1 Human colonocytes in normal tissues express CD14 constitutively in the absence of TLR4, whereas cancerous tissues display enhanced levels of CD14 and TLR4. (**a**) Representative images of human surgical specimens of colonic adenocarcinoma from three clinical cases examined for the expression of LPS receptors. Fluorescent images indicate immunoreactivity to CD14, TLR4, or MD2 (green) superimposed with nuclear staining (blue). Apical expression of CD14 and the absence of TLR4 and MD2 were found on epithelial cells of morphologically normal tissues. Increased CD14 and TLR4 levels were observed in tumorous regions with no change in MD2 patterns. The levels of proliferating cell nuclear antigen (PCNA) in colonic tissues are in brown. Scale bar: 50 μm. (**b** and **c**) Immunoreactivity score of CD14 and TLR4 expression in normal epithelium and tumorous regions from thirty specimens. $^*P<0.05$ *versus* normal

colonocytes are prone to apoptosis and are less susceptible to tumorigenesis in the absence of TLR4 signaling.

Intracolonic blockade of TLR4 reduced tumor growth in WT mice. A TLR4 antagonist eritoran[39,40] was intracolonically administered to WT mice, to examine its effect on epithelial apoptosis and tumorigenesis. Mice were given eritoran or vehicle at different time points following CRC induction (Figure 4a). Significantly decreased tumor multiplicity and area were observed in mice with preventive and treatment regimen (Figures 4b and d). Tumor reduction correlated with enhanced apoptosis in cancerous tissues of mice administered eritoran, compared with those given vehicle (Figures 4c and e).

Increased sensitivity to bacterial LPS triggers colonic epithelial apoptosis in a CD14-dependent manner. Mucosa-associated LPS levels in colonic tissues were

elevated following AOM/DSS induction in WT and TLR4-mut mice, irrespective of mouse strain (Figure 3i). To examine whether DSS alone could increase mucosal LPS binding, mice were given DSS in drinking water without AOM injection. DSS challenge increased colonic mucosa-associated LPS content in both WT and TLR4-mut mice (Figure 3i), but only TLR4-mut mice displayed elevated mucosal apoptosis (Figure 3g). As low-dose (2–2.5%) DSS was not toxic to epithelial cells *in vitro*,[5,41] and DSS alone did not increase mucosal apoptosis in WT mice (Figures 3g and h), we reasoned that epithelial cell death observed in TLR4-mut mice could be caused by increased sensitivity to LPS secondary to DSS challenge.

To verify whether increased sensitivity to commensal microbes and bacterial LPS causes epithelial apoptosis, and to evaluate the involvement of CD14 in the apoptotic response, mouse colons were subjected to non-ischemic loop ligation for the induction of commensal overgrowth (CO) *in vivo* or were mounted on tissue baths for LPS challenge from the mucosal side *ex vivo*. In the *in vivo* model, elevated mucosal apoptosis was detected only in TLR4-mut, but not WT, mice after induction of CO (Figure 5a). Increased gut-associated bacterial counts and mucosa-associated LPS levels were confirmed in both WT and TLR4-mut mice following loop ligation (Figures 5b and c).

In the *ex vivo* setting, colonic tissues obtained from untreated mice were LPS-challenged from the mucosal side. Augmented epithelial apoptosis was observed in TLR4-mut, but not WT, tissues (Figures 5d and e). The LPS-induced epithelial apoptotic response was also seen in TLR4(−/−) mouse tissues, but not in CD14(−/−) mouse tissues or their WT counterparts (Supplementary Figure S2). To examine the involvement of epithelial CD14 in the apoptotic response, neutralizing anti-CD14 were administered intracolonically before LPS challenge in TLR4-mut mice. A significant reduction in the mucosal cell death level was observed in tissues pretreated with anti-CD14 compared with isotype antibody (Figure 5f). Anti-CD14 binding to epithelial surfaces was confirmed by immunostaining (Figure 5g).

LPS/CD14-induced epithelial apoptosis was prevented by TLR4 in primary colonic organoids derived from WT mouse crypts. Primary cultures of colonic organoids derived from mouse crypts were also developed for assessment of receptor expression and death response to LPS (Figure 6a). The presence of *CD14*, *TLR4*, and *MD2* transcripts were identified along with *Lgr5* (a marker of stem cells) in colonic organoids (Figure 6b). No sign of apoptosis was found after LPS challenge in WT colonic organoids; gene silencing of *TLR4* but not *CD14* increased the level of cell death (Figure 6c). In contrast, LPS-induced apoptosis was

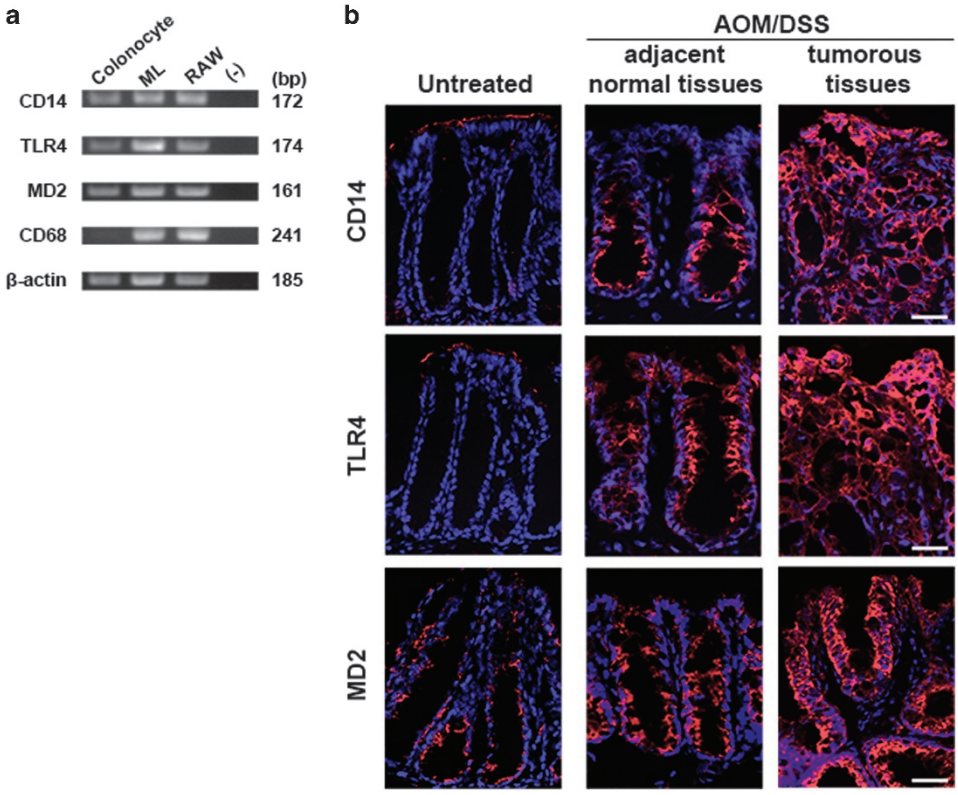
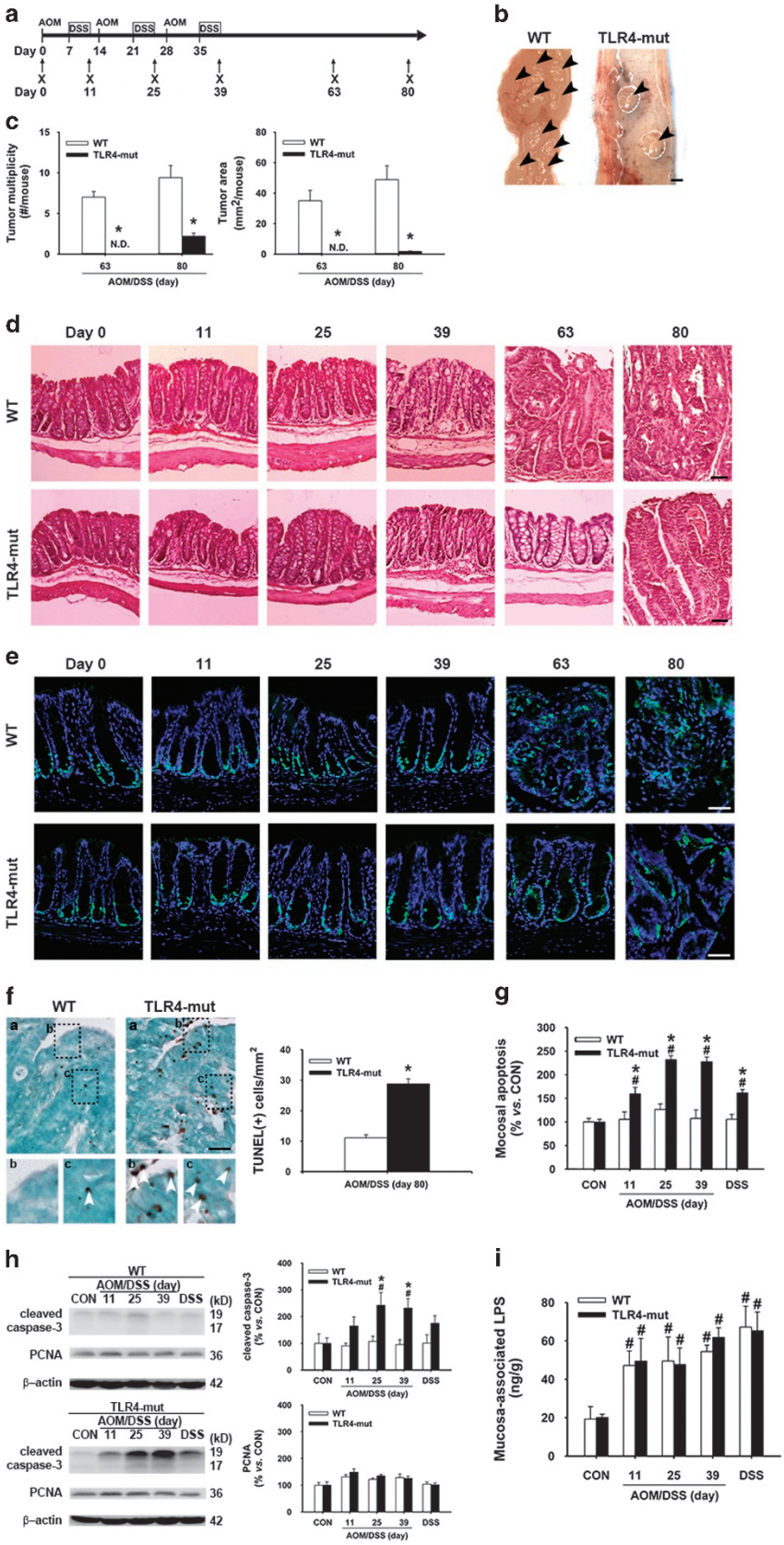


Figure 2 Baseline levels of CD14/TLR4/MD2 in mouse colonocytes and modification of expression levels in tumorous tissues. (**a**) Transcript levels of *CD14*, *TLR4*, and *MD2* in mouse colonocyte cell preparations, showing the absence of *CD68* transcripts (a marker of monocytes/macrophages). Mononuclear leukocytes (ML) and monocytic RAW264.7 (RAW) cells were used as positive controls for *CD68*. (**b**) Representative images of immunofluorescent staining of CD14, TLR4, or MD2 (red) in mouse colonic tissues, superimposed with nuclear staining (blue) for visual orientation. CD14 and TLR4 were localized to the apical membrane of the surface epithelium in untreated mouse colonic tissues, whereas MD2 was found on the crypt epithelium. Following three cycles of AOM/DSS, enhanced levels of CD14, TLR4, and MD2 were seen in tumorous tissues compared with adjacent normal tissues or untreated tissues. Scale bar: 50 μm. $n=10$/group


a
AOM
DSS
Day 0
7
14
21
28
35
11
25
39
63
80
b
WT
TLR4-mut
c
Tumor multiplicity (#/mouse)
Tumor area (mm²/mouse)
N.D.
AOM/DSS (day)
d
e
f
TUNEL(+) cells/mm²
AOM/DSS (day 80)
g
Mocosal apoptosis (% vs. CON)
CON
DSS
h
cleaved caspase-3
PCNA
β-actin
(kD)
i
Mucosa-associated LPS (ng/g)

observed in TLR4-mut colonic organoids, which was inhibited by *CD14* knockdown (Figure 6d). The knockdown efficiency in organoid cultures was confirmed by decreased transcript levels (Figures 6c and d).

Exposure to LPS increases apoptosis in Caco-2 cells ($CD14^+TLR4^-$) but not in HT29 cells ($CD14^+TLR4^+$). Human CRC cell lines, that is, Caco-2, T84, and HT29, were used to investigate the molecular mechanism underlying LPS-induced apoptosis. Constitutive levels of *CD14* transcripts and negligible amounts of *TLR4* and *MD2* were identified in Caco-2 cells (Figure 7A), whereas all three subunit transcripts were observed in T84 and HT29 cells (Figure 7A). Punctate apical CD14 staining was observed on Caco-2 and HT29 cells but not on T84 cells (Figure 7B). TLR4 immunoreactivity was observed on the apical membranes of T84 and HT29 cells but not on Caco-2 cells (Figure 7B). The mRNA and protein levels of CD14, TLR4, and MD2 were not altered after LPS challenge in any of the cell lines (Figures 7A and B). The expression patterns of Caco-2 cells ($CD14^+TLR4^-$) resemble those of normal colonocytes, whereas HT29 cells ($CD14^+TLR4^+$) resemble human cancerous tissues.

Death and survival responses to LPS challenge were assessed in the three cell lines. Caco-2 cells exposed apically to LPS (0, 5, 25, and 50 μg/ml) for 24 h showed increased DNA fragmentation and caspase-3 activation in a dose-dependent manner (Figures 7C and D). No indications of cell apoptosis were observed in LPS-challenged T84 or HT29 cells (Figures 7E and F). In Caco-2 cells, pretreatment with the caspase-3

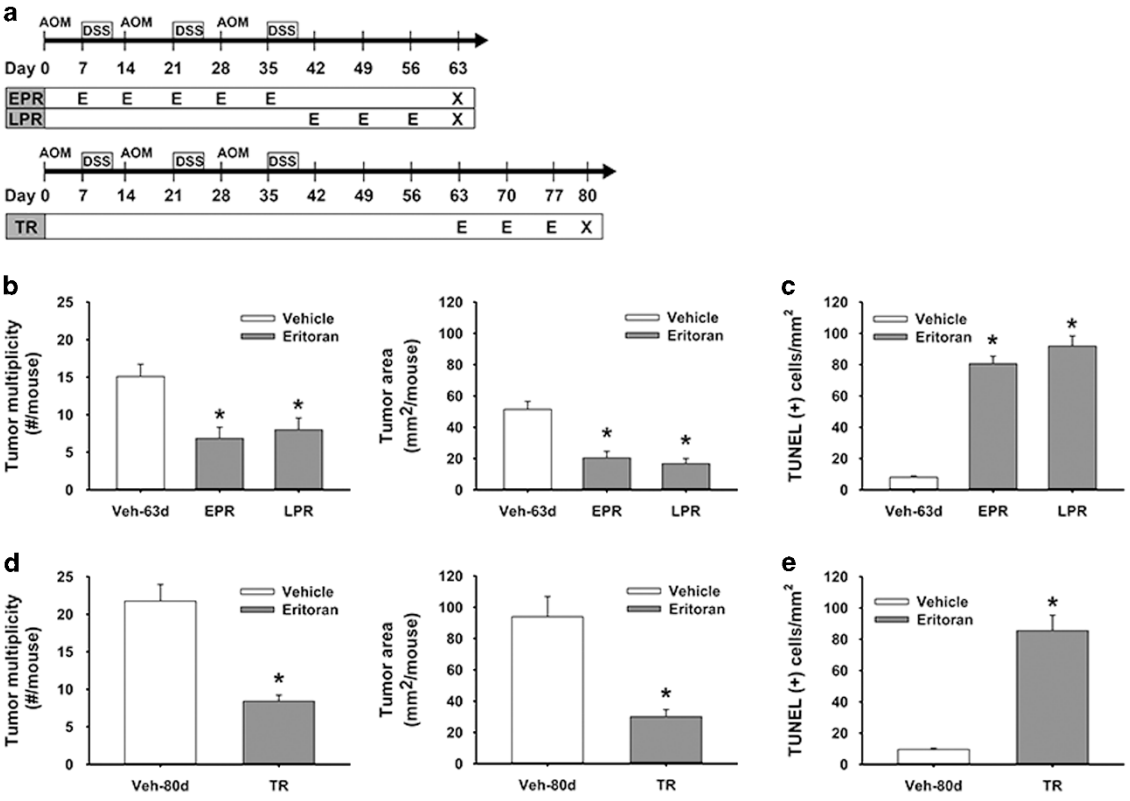


Figure 4 Decreased tumor growth was observed in WT mice intracolonically administered a TLR4 antagonist. Eritoran or vehicle (veh) was intracolonically given to WT mice at various time points, to evaluate its effect on CRC development. (**a**) Schema of early and late preventive regimen (EPR and LPR) and treatment regimen (TR). *E*, days administered eritoran; *X*, days for killing. (**b** and **c**) Tumor multiplicity and area per mouse, and number of apoptotic cells per mm^2 in tumors following preventive regimen. (**d** and **e**) Tumor multiplicity and area per mouse, and number of apoptotic cells per mm^2 in tumors following treatment regimen. $n = 8$–12/group. $^*P < 0.05$ *versus* veh

Figure 3 A reduction in tumor formation correlates with increase of epithelial apoptosis in TLR4-mutant mice after administration of AOM/DSS. (**a**) Timeline of experimental design. WT and TLR4-mut mice were subjected to three cycles of AOM/DSS for the induction of colitis-associated CRC. Mice were killed before (day 0) and after the first AOM injection on day 11, 25, 39, 63, and 80. (**b**) Representative images of distal colons on day 80. Arrowheads indicate tumors. Scale bar: 1 mm. (**c**) Tumor multiplicity and mean tumor area per mouse during late stages. N.D., non-detected. (**d** and **e**) Colonic histopathology and crypt cell proliferation levels were examined by hematoxylin and eosin staining, and by immunoreactivity to Ki-67 (green fluorescence) superimposed with Hoechst staining (blue fluorescence). Scale bar: 50 μm. (**f**) Number of apoptotic TUNEL-positive cells (arrowheads) per mm^2 in colonic tumors obtained from mice on day 80. Representative images of TUNEL staining (**a**) are shown at higher magnification (**b** and **c**). Scale bar: 50 μm. (**g**) Mucosal cell apoptosis was quantified in untreated controls (CON), after AOM/DSS, or after DSS alone. (**h**) Western blot analysis showing mucosal levels of cleaved caspase-3 and proliferating cell nuclear antigen (PCNA) in untreated CON, after AOM/DSS, or after DSS alone. (**i**) Mucosa-associated LPS amounts in untreated CON, after AOM/DSS, or after DSS alone. $n = 10$/group. $^*P < 0.05$ *versus* WT; $^{\#}P < 0.05$ *versus* CON

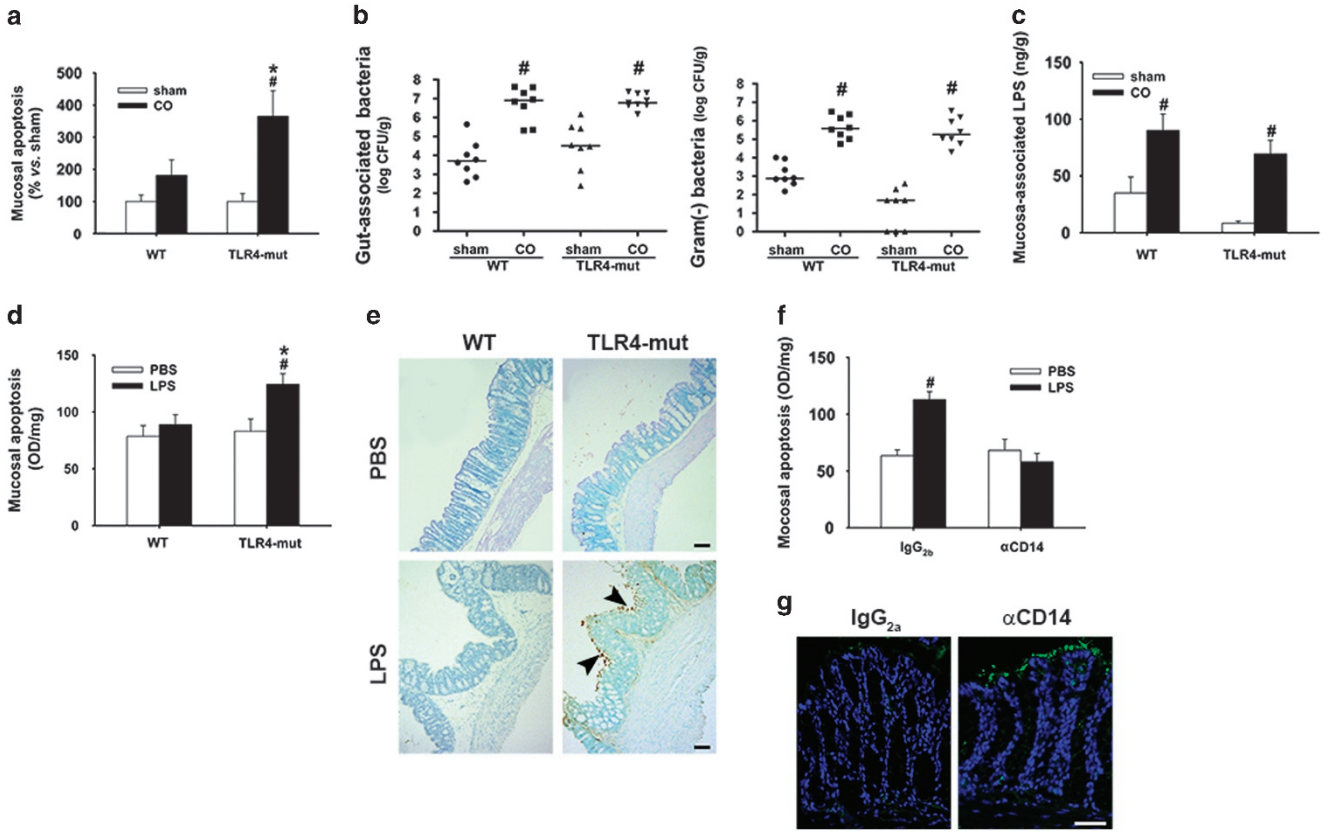


Figure 5 CD14-dependent epithelial apoptosis is augmented in colonic tissues of TLR4-mut mice after induction of CO or LPS challenge. WT and TLR4-mut mouse colons were subjected to non-ischemic loop ligation for induction of CO or were mounted on organ baths for LPS challenge from the mucosal side. (**a**) Augmented colonic mucosal apoptosis was detected in TLR4-mut but not WT mice after induction of CO. Sham-operated (sham) colons were without ligation. (**b**) Increased gut-associated and Gram-negative bacterial counts were confirmed after induction of CO in both WT and TLR4-mut mice. Each data point represents the bacterial count from one animal, and the median values are shown as bars. (**c**) Elevated mucosa-associated LPS levels were observed after CO in both mouse strains. (**d**) Colonic tissues obtained from untreated mice were challenged from the mucosal side with LPS or PBS, and increased mucosal apoptosis was seen in TLR4-mut mice compared with WT mice. (**e**) Apoptotic TUNEL-positive cells (arrowheads) were detected on the colonic epithelium after LPS challenge in TLR4-mut, but not WT, mice. Scale bar: 50 μm. (**f**) Intracolonic administration of neutralizing anti-CD14 antibody, but not its isotype IgG2b, decreased LPS-induced cell apoptosis. (**g**) Binding of anti-CD14 to epithelial surfaces was confirmed by immunofluorescent staining (green). Scale bar: 50 μm. $n = 6$–8/group. $^{*}P < 0.05$ *versus* WT; $^{\#}P < 0.05$ *versus* sham or PBS

inhibitor, z-DEVD, blocked LPS-induced cell apoptosis and tight junction disruption (Supplementary Figure S3A–D). Inhibition of apoptosis with polymyxin B (PMB) treatment confirmed the specificity of the lipid A moiety of LPS as the death trigger (Supplementary Figure S3A–D).

A mechanism of CD14-dependent lipid signaling without engaging TLR4 contributes to LPS-induced apoptosis. To identify the LPS receptor subunits involved in cellular apoptosis, Caco-2 cells were treated with neutralizing antibodies or siRNAs before LPS challenge. Pretreatment with neutralizing anti-CD14 significantly reduced the numbers of apoptotic cells following LPS challenge, whereas anti-TLR4 and isotype controls had no effect (Figures 8a and b). The LPS-mediated tight junction disruption was also attenuated by anti-CD14 but not by anti-TLR4 (Supplementary Figure S3E).

Gene silencing of *CD14* decreased the LPS-induced apoptosis levels in Caco-2 cells (Figure 8c). The knockdown efficiency and specificity of siCD14 was confirmed by immunoblotting (Figure 8c). Increased PKCζ phosphorylation following LPS challenge was attenuated by *CD14* knockdown (Figure 8d). Cells pretreated with inhibitors of PC-PLC, SMase, or PKCζ, and cells that were PKCζ gene silenced, displayed reduction in apoptosis (Figures 8c and e). Moreover, ceramide (C16) synthesis and PKCζ activation were confirmed in cells after LPS challenge (Supplementary Figure S4), whereas no changes in phosphorylated MAPKs, IκB, Akt, and IRF3 levels were seen (Supplementary Figure S5).

LPS/CD14-mediated apoptosis can be overruled by functional TLR4 expression. We next examined whether functional TLR4 expression could serve as a pro-survival signal against apoptotic cell death caused by its coreceptor CD14. Caco-2 cells were transfected with either mock plasmids or a pMyc-CMV1-huTLR4 plasmid that encoded either WT or mutant TLR4 protein. Apical surface expression of TLR4 and Myc proteins was observed in pMyc-CMV1-huTLR4-transfected cells, whereas mock-transfected cells were negative for TLR4 staining (Figures 9a and b). CD14 and MD2 protein levels remained constant in mock- and TLR4-transfected cells (Figure 9b). A reduction in LPS-induced cell apoptosis was observed after transfection with WT TLR4, but not after transfection with TLR4

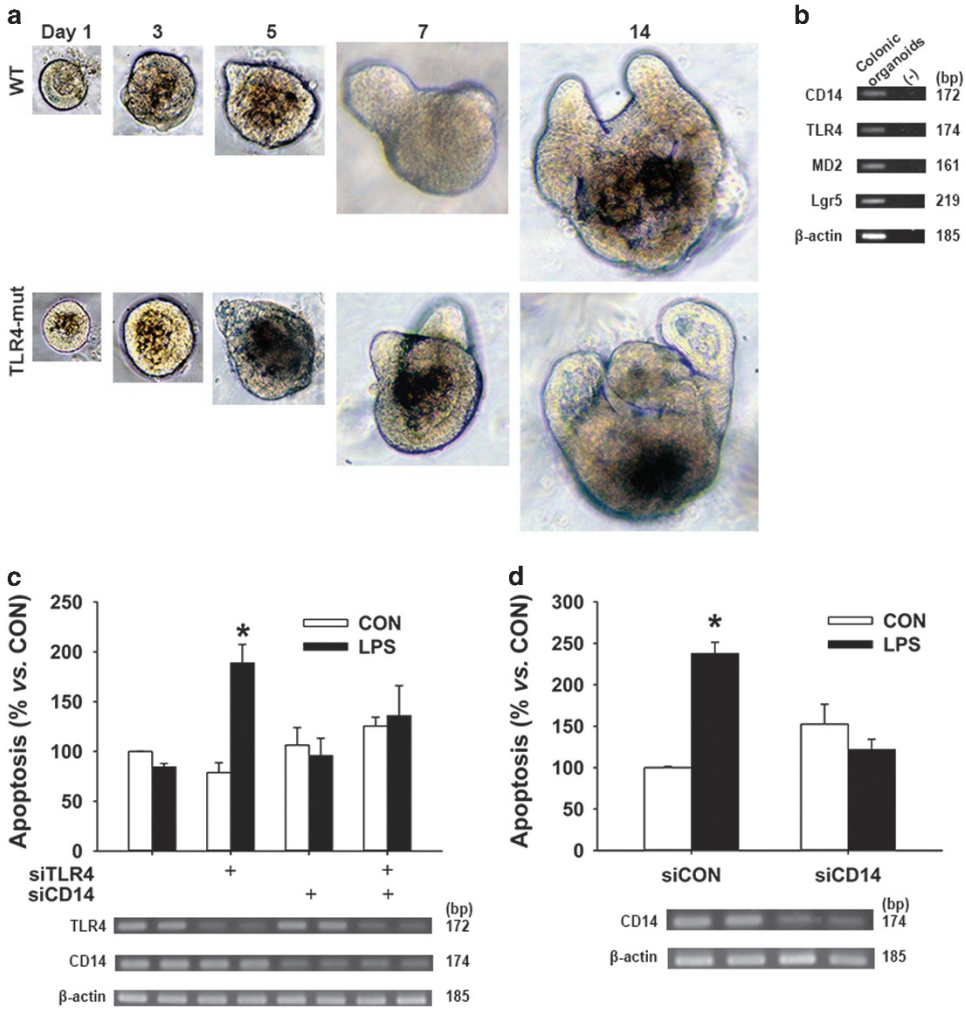


Figure 6 LPS/CD14-mediated epithelial apoptosis is prevented by TLR4 expression in primary mouse colonic organoids. (**a**) Representative images of colonic organoid cultures derived from colonic crypts of WT and TLR4-mut mice. Original magnification: ×200. (**b**) Transcript levels of LPS receptors (CD14, TLR4, and MD2) and Lgr5 in WT colonic organoids. (**c**) Apoptotic levels by LPS challenge in WT colonic organoids with gene silencing of *TLR4* and/or *CD14*. Organoids were either LPS challenged or untreated as controls (CON). (**d**) Apoptotic levels in TLR4-mut colonic organoids with gene silencing of *CD14*. $n=6$/group. $^*P<0.05$ *versus* respective CON

mutants (Asp299Gly, Thr399Ile, and Pro714His) (Figure 9c). Each TLR4 mutant group exhibited dysfunctional signaling after LPS challenge, compared with WT TLR4, as evidenced by the phosphorylation status of JNK and IκB (Figure 9d). Moreover, spheroid cultures of Caco-2 cells showed a twofold increase in apoptotic levels following LPS challenge, which was ablated by knockdown of *CD14* or overexpression of TLR4 (Figures 9e and f).

HT29 cells, which constitutively express TLR4 and CD14 proteins, were subjected to multiple gene knockdowns before LPS challenge for cell death responses. Immunoblottings indicated that single or double gene silencing approaches for TLR4, CD14, and/or PKCζ were specific and did not alter proteins other than the target molecule(s) (Figure 9g). Knockdown of TLR4 resulted in loss of survival and increased apoptosis following LPS challenge, compared with mock cells. In contrast, silencing of CD14 or PKCζ alone did not affect cell death levels (Figure 9h). Apoptotic levels of the double knockdowns, TLR4/CD14 or TLR4/PKCζ, were comparable to mock groups before and after exposure to LPS (Figure 9h).

Discussion

The present study aimed to dissect the individual roles of the LPS receptor subunits in the fundamental processes of cell death *versus* survival in the regulation of epithelial homeostasis and tumorigenesis. Our data showed that LPS/CD14-mediated colonocytic apoptosis, in the absence of TLR4 signaling, was negatively associated with cancer progression (i.e., delayed onset and reduced number and size of tumors). LPS-induced epithelial apoptosis was dependent on CD14-mediated lipid messengers and PKCζ phosphorylation, and the cell death process could be overruled by TLR4 expression. This study is the first to describe a reciprocal functional antagonism between CD14 and TLR4, which serves to balance epithelial cell demise and survival. Our findings provide a new perspective for the evolution of innate immunity for janus-faced microbial regulation of gut homeostasis.

Different expression patterns of LPS receptor subunits were identified on normal human colonocytes ($CD14^+TLR4^-$) and

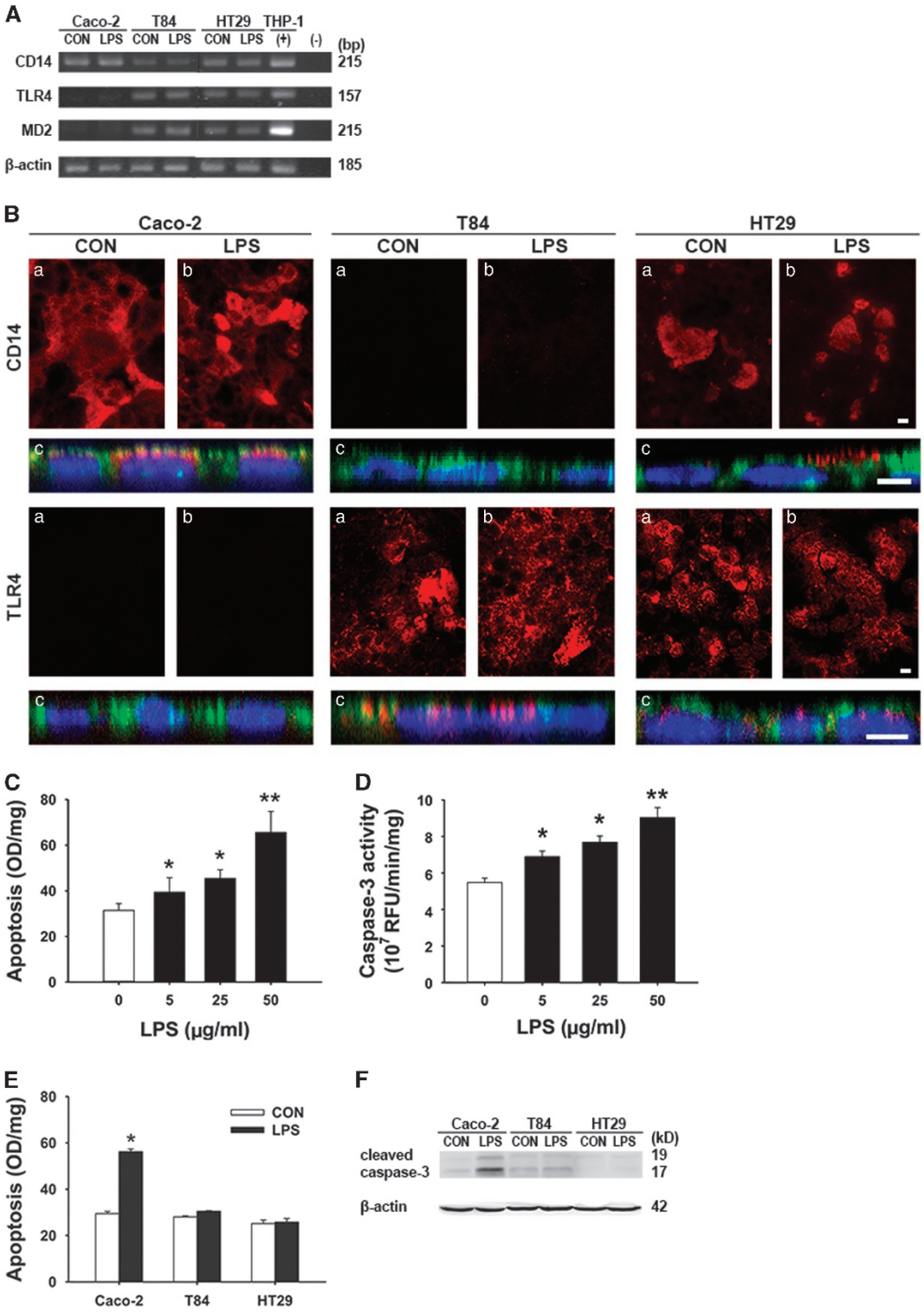


Figure 7 Apical exposure to LPS induces apoptosis in Caco-2 cells ($CD14^+TLR4^-$), but not in HT29 ($CD14^+TLR4^+$) or T84 ($CD14^-TLR4^+$) cells. The expression pattern of LPS receptors and death response to LPS were examined in three well-established human colorectal adenocarcinoma cell lines. (**A**) Levels of *CD14*, *TLR4*, and *MD2* transcripts in control (CON) and LPS-challenged Caco-2, T84, and HT29 cells. Promonocytic THP-1 cells were used as positive controls. (**B**) Representative immunostaining of CD14 and TLR4 proteins in control (**A** and **C**) or LPS-exposed (**B**) cells. Confocal microscopic imaging in **C** indicates apical staining of CD14 or TLR4 (red) superimposed with nuclei (blue) and phalloidin-labeled F-actin (green). Scale bar: 5 μm. (**C** and **D**) Caco-2 cells were challenged with various doses of LPS for 24 h and displayed increased DNA fragmentation and caspase-3 activation in a dose-dependent manner. $^*P<0.05$ and $^{**}P<0.005$ *versus* 0 μg/ml LPS. (**E**) LPS-induced apoptosis was observed in Caco-2 cells, but not in T84 or HT29 cells. $^*P<0.05$ *versus* CON. (**F**) Western blots showing increased caspase-3 cleavage after LPS challenge in Caco-2 cells, but not in T84 or HT29 cells. Experiments were repeated at least twice. Scale bar: 5 μm. $n=4$–6/group

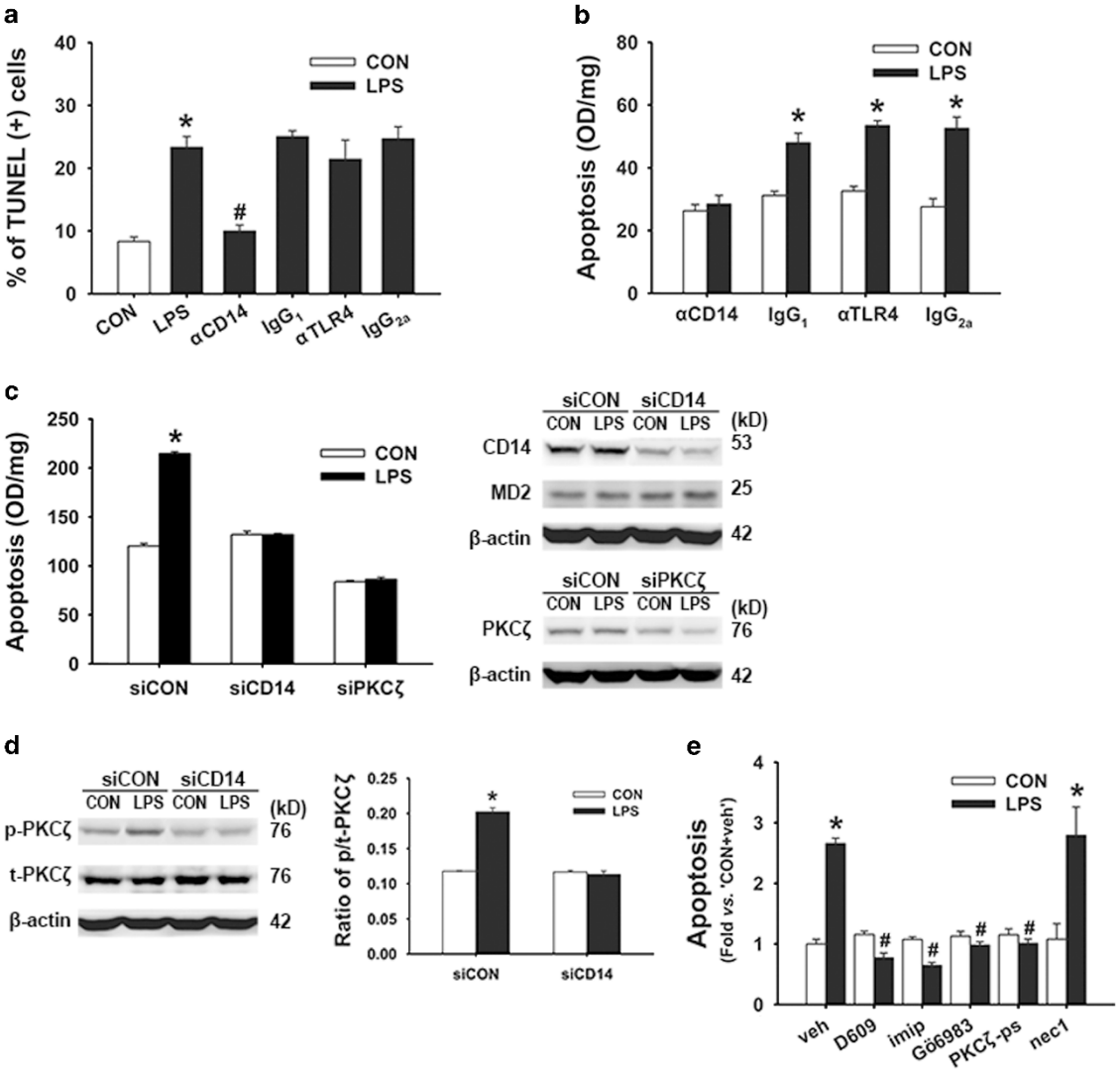


Figure 8 CD14-mediated lipid signaling is involved in LPS-induced apoptosis in Caco-2 cells. (**a**) Quantification of TUNEL-positive cells in control (CON) and LPS-challenged groups. The increase in the percentage of TUNEL-positive cells following LPS challenge could be inhibited with neutralizing anti-CD14 antibody, but not with anti-TLR4 or isotype antibodies. (**b**) LPS-induced apoptosis was inhibited by anti-CD14 but not anti-TLR4. (**c**) siRNA-mediated gene silencing of CD14 and PKCζ prevented LPS-induced cell apoptosis. Western blotting confirmed the knockdown efficiency of CD14 and PKCζ, and indicated that MD2 levels were not affected by siRNA treatment. (**d**) Hyperphosphorylation of PKCζ, caused by LPS exposure, was reduced in CD14 knockdowns. Representative blots of total (t)- and phospho (p)-PKCζ after CD14 knockdown. The p-PKCζ to t-PKCζ ratio was quantified by densitometric analysis. (**e**) Pretreatment with D609 (a PC-PLC inhibitor), imipramine (imip; a SMase inhibitor), Gö6983 (a PKC inhibitor), or PKCζ pseudosubstrate (ps; a specific inhibitor of PKCζ) suppressed LPS-induced cell apoptosis. No effects were observed when vehicle (veh) or necrostatin-1 (nec1; a necroptosis inhibitor) were used. Experiments were repeated at least twice. $^{*}P<0.05$ *versus* respective CON; $^{\#}P<0.05$ *versus* LPS or LPS+veh. $n=4$–6/group

cancerous tissues ($CD14^{+}TLR4^{+}$). *CD14* and *TLR4* gene polymorphisms also have been correlated with a higher risk of CRC development,[42–44] supporting a link between aberrant LPS recognition and cancer formation. Abundant studies demonstrated that TLR4/MyD88 signaling promotes colitis-associated CRC by causing cell hyperproliferation via cyclooxygenase-2, epidermal growth factor receptor, and β-catenin-dependent pathways.[19,35,36,45,46] Mutated TLR4 also contributes to the epithelial-to-mesenchymal transition.[43] Here we focused on the roles of LPS receptors on the dysregulation of epithelial death and survival responses for control of tumor progression. We demonstrated that in the absence of TLR4 signaling, colonocytes were prone to cell apoptosis and less susceptible to tumorigenesis, in keeping with previous findings of TLR4(−/−) and MyD88(−/−) mice.[19,36] Based on the data of primary organoid cultures, colonocytic apoptosis triggered by LPS hypersensitivity was dependent on CD14 and could be prevented by epithelial TLR4 expression. Moreover, intracolonic administration of a TLR4 antagonist eritoran significantly enhanced cell apoptosis in tumors and reduced CRC growth in WT mice, implicating therapeutic potential of the antagonist. Eritoran is a structural analog of the lipid A moiety of LPS that binds to the hydrophobic pocket of MD2 without causing its structural change necessary for dimerization of TLR4-MD2 complex and

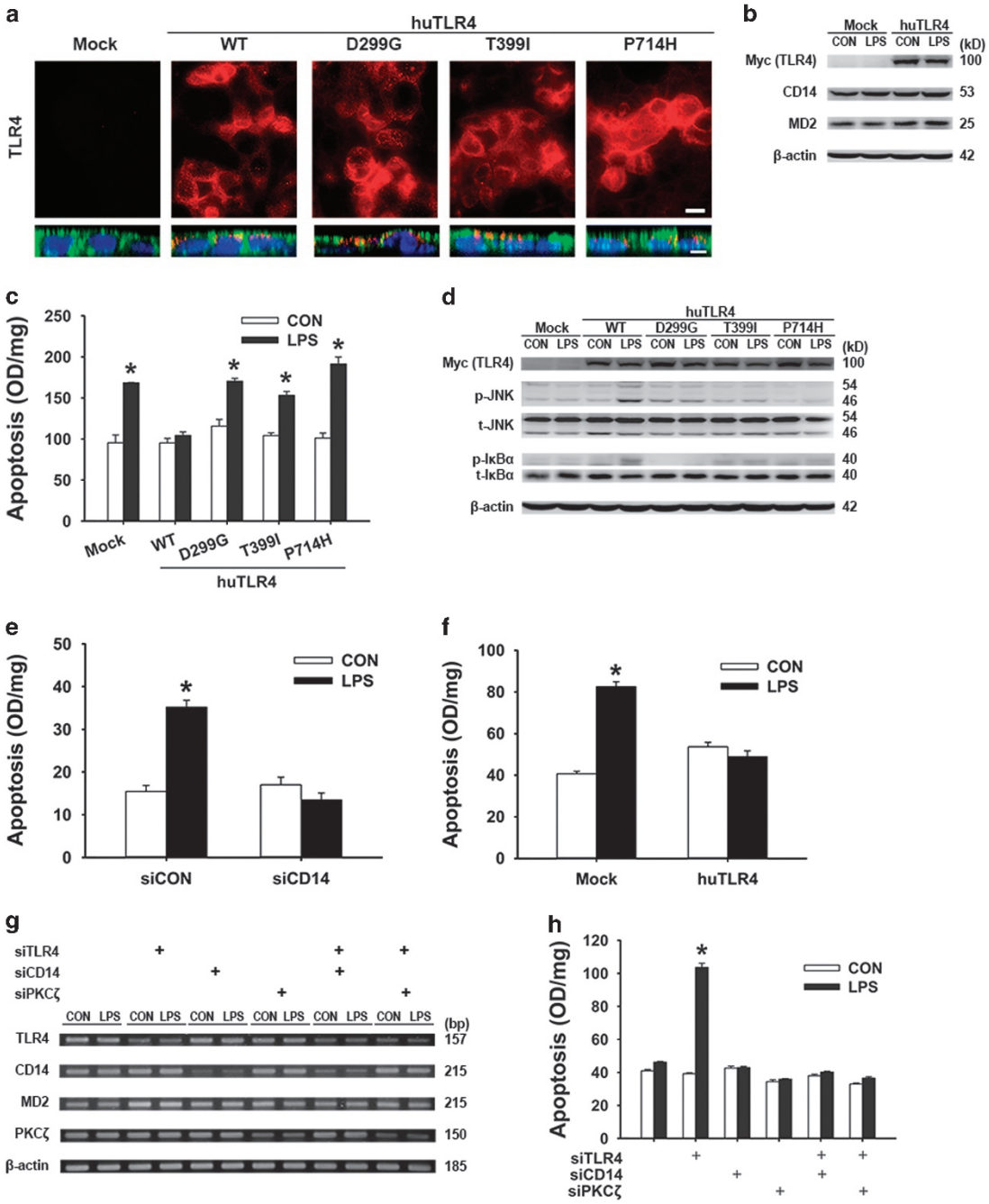


Figure 9 Expression of functional TLR4 inhibits LPS/CD14-induced apoptosis in Caco-2 cells. Caco-2 cells were transfected with mock plasmids or pMyc-huTLR4 expressing WT or mutant (Asp299Gly, Thr399Ile, or Pro714His) TLR4 before LPS challenge. (**a**) Apical expression of TLR4 proteins in Caco-2 cells was verified after transfection. Representative *en face* views of TLR4 staining (red) are shown in the upper panels, and superimposed confocal images of TLR4 (red), F-actin (green), and nuclei (blue) are shown in the lower panels. Scale bar: 10 μm. (**b**) Representative blots of TLR4, CD14, and MD2 in transfected cells. Expression of TLR4 did not affect CD14 or MD2 levels in control (CON) or LPS-challenged cells. (**c**) LPS-induced apoptosis was abolished in cells transfected with pMyc-huTLR4 harboring the WT TLR4 insert, but not with mock plasmids. Site-directed mutagenesis of TLR4 proteins eliminated the anti-apoptotic effects in Caco-2 cells. (**d**) Functional TLR4 signaling, defined as LPS-induced JNK and IκBα phosphorylation, was observed in Caco-2 cells transfected with WT TLR4, but not in cells harboring mutated TLR4. (**e** and **f**) Spheroid cultures of Caco-2 cells showed increase of apoptosis following LPS challenge, which was ablated by knockdown of *CD14* or overexpression of TLR4. *$P<0.05$ *versus* respective CON. (**g**) HT29 cells underwent single or double knockdown of *CD14*, *TLR4*, or *PKCζ*, as verified by PCR. (**h**) LPS challenge induced elevated apoptosis in HT29 cells with TLR4 knockdown, whereas no change in apoptosis was observed in CD14 or PKCζ knockdowns or in double knockdowns. All experiments were repeated twice. *$P<0.05$ *versus* respective CON. $n=4$/group

downstream signaling.[47] We suspect that tumor suppression by eritoran may be attributed to dual effects of TLR4 blocking and CD14 signaling. The opposing effects of CD14 and TLR4 on epithelial cell death and survival highlighted the indispensable roles of LPS receptor subunits in the regulation of tumorigenesis.

Oral DSS challenge may increase LPS exposure to gut mucosa, which could partially explain how low-dose DSS

induces mild colitis. High (5%), but not lower, concentrations of DSS are directly toxic to epithelial cells and may trigger severe inflammation.[41] Conversely, exposure to 1–3% DSS (non-cytotoxic doses) is associated with breakage of the epithelial barrier via unknown mechanisms. Our data and others have suggested that DSS, a polysaccharide, may modulate LPS binding on cell surfaces. Studies using monocytes and macrophages have reported that LPS binding through CD14 with the participation of scavenger receptor A (without engaging TLR4) is largely responsible for LPS internalization and detoxification.[25,48] The occupation of scavenger receptor A by DSS blocks LPS scavenging but enhances LPS signaling by increasing cell surface levels of CD14 and TLR4 (Czerkies *et al.*[48]). Taken together, increased adherence and receptor binding of LPS to gut mucosa may explain the colitogenic effects of low-dose DSS.

We further investigated the molecular mechanism of LPS/CD14-mediated colonocytic apoptosis in human epithelial cell cultures. In accordance with the expression patterns of LPS receptors, enhanced apoptotic death was observed in Caco-2 cells ($CD14^+TLR4^-$), but not in HT29 cells ($CD14^+TLR4^+$), following LPS challenge. Distinct protein and transcript expression patterns in CRC cell lines have been previously reported.[49–51] The doses of LPS (5–50 μg/ml) required to evoke epithelial apoptosis exceeded the basal level documented in the luminal contents of rodent intestines (~1.8 μg/ml)[52] and the blood concentration reported to stimulate monocytic cytokine secretion or septic responses (~10 ng/ml), suggesting that overgrowth or adherence of enteric bacteria may tip the balance from tolerance to cell death.

The apoptotic response required the activity of CD14-dependent lipid messengers (e.g., ceramide production and PKCζ phosphorylation) and the absence of TLR4 involvement. CD14-dependent TLR4-independent cell death also has been documented in terminally differentiated dendritic cells following exposure to high concentrations of LPS (1–10 μg/ml) as a means to regulate dendritic cell cycles.[53] However, this report found that macrophages did not die after stimulation with high-dose LPS and instead became refractory to further challenges via intracellular negative regulators to decrease TLR4 signaling.[53,54] Another study demonstrated that macrophages undergo apoptosis after anthrax bacterium challenge in the presence of p38 or proteasome inhibition via TLR4/IRF-3 pathways.[55] These findings suggest that LPS may trigger cell death response via different receptors on epithelial and immune cells that function as regulators of biological processes or as defensive and immune-evasive mechanisms.

Overexpression of functional TLR4 in epithelial cells overrules apoptotic responses triggered by its coreceptor, CD14. The pro-survival effects of TLR4 could be eliminated by site-directed mutagenesis at Asp299Gly, Thr399Ile, or Pro714His. These mutation sites correspond to the ligand-binding, coreceptor-binding, and signaling domains of TLR4, respectively.[56] This indicates that the anti-apoptosis responses were dependent on signaling pathways downstream of TLR4 rather than on their interaction with CD14. Our animal experiments involving spontaneous mutation of TLR4 at Pro712His supported this notion. A characterization of TLR-adaptor proteins and signaling pathways responsible for counteracting CD14/PKCζ-dependent cell apoptosis is in progress. The molecular cross-talk between CD14 and TLR4, and the role of MD2 in the antagonistic regulation of epithelial survival warrant further investigation.

In conclusion, we demonstrated that colonocytic apoptosis in response to bacterial LPS is mediated by CD14-dependent lipid messengers and may be counteracted by TLR4 signaling. Dysfunction in the CD14/TLR4 antagonism that regulates epithelial cell death and survival is correlated with tumor progression. The imbalances of CD14/TLR4 signaling may provide a working schema to explain the transition from normal cell turnover to carcinogenesis. Our data of tumor inhibition, partly via enhanced cell apoptosis, by intracolonic blockade of TLR4, highlighted a new direction for therapeutic intervention in human CRC.

Materials and Methods

Human colonic surgical specimens. Recruited subjects were patients referred for colonoscopy at National Taiwan University Hospital (NTUH). Thirty surgical specimens from the sigmoid colons of patients with CRC and respective normal mucosa were assessed. Written informed consent was obtained from all study subjects, and approval for this study was granted by the Research Ethics Committee of NTUH. Samples were fixed in 4% formaldehyde and paraffin-embedded sections were used for immunostaining of CD14, TLR4, and MD2. The percentage of CD14- or TLR4-positive epithelial/tumor cells were scored on a scale of 0–4 (0: no staining; 1: $\leq$10%; 2: 11–30%; 3: 31–50%; 4: $>$50%).[57]

Animals. WT mice (BALB/c and C57BL/6 strains), mice harboring a spontaneous mutation or knockout of the *TLR4* gene (C.C3-*Tlr4*$^{LPS\text{-}d}$/J strain and B6.B10ScN-Tlr4$^{LPS\text{-}del}$/J strain, respectively), and *CD14* gene knockout mice (B6.129S-Cd14^{tm1Frm}/J strain), aged 7–10 weeks, were used in this study. WT mice were purchased from the animal facility at National Taiwan University College of Medicine. C.C3-*Tlr4*$^{LPS\text{-}d}$/J mice were provided by Dr John T Kung. B6.B10ScN-Tlr4$^{LPS\text{-}del}$/J mice were provided by Dr Fuu Sheu. B6.129 S-Cd14^{tm1Frm}/J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All animals were raised in specific pathogen-free environments in temperature-controlled rooms (23 ± 2 °C) with 12 h light–dark cycles. Experimental procedures were approved by the Laboratory Animal Care Committee of National Taiwan University College of Medicine.

Models of colitis-associated CRC. Mouse models of colitis-associated CRC were prepared according to previously established methods, with some modifications.[5,19] Briefly, mice were injected intraperitoneally with AOM (10 mg/kg body weight; Sigma, St Louis, MO, USA) at the beginning of the experiment (day 0). After 7 days, 2% DSS (MP Biomedicals, Santa Ana, CA, USA) was administered in the drinking water for 4 days, followed by 3 days of regular water. This cycle of AOM/DSS was performed three times. Body weight was measured every week and the animals were killed at the indicated time intervals for macroscopic inspection, histological analysis, and intestinal sample collection. Tumor numbers were determined macroscopically under an anatomic microscope and the tumor area measured using imaging software. Tissues were fixed in 4% paraformaldehyde (PFA) and paraffin-embedded for histological assessment by a gastrointestinal pathologist blinded to mouse genotype and treatment group. In a different setting, mice were given 2.5% DSS in drinking water for 7 days and were killed for sample collection.

Tumor grading was classified as low-grade dysplasia, high-grade dysplasia, and carcinoma. Low-grade dysplasia indicates disorganized crypt structures associated with monolayered, hyperchromatic epithelial cells. High-grade dysplasia indicates disorganized crypt structures lined by stratified epithelial cells, back-to-back glands, high nuclear to cytoplasmic ratio, and loss of cellular polarity. Carcinoma was defined as loss of crypt architecture and penetration of the dysplastic region through basement membrane.[58]

Intracolonic administration of a TLR4 antagonist. A TLR4 antagonist eritoran tetrasodium (Eisai Inc., Andover, MA, USA)[39,40] was administered intracolonically at various time points to WT mice following induction of CRC. Eritoran is a synthetic analog of lipid A structure of LPS.[47] The dose of eritoran

administered was 10 μg dissolved in 150 μl PBS per bolus per mouse. Mice were killed at either 63 or 80 days after starting the AOM/DSS procedure for examination of tumor growth.

Measurement of cell apoptosis. Scraped colonic mucosa were homogenized and sonicated in lysis buffer, and the supernatant was used for cell apoptosis assays. A cell death detection ELISA kit (Roche, Basel, Switzerland) that specifically measures the histone region of mono- and oligo-nucleosomes was used as an indicator of DNA fragmentation.[20] In addition, a caspase-3 activity assay (Roche) was performed according to the manufacturer's instructions. Alternatively, tissues were fixed in 4% PFA for TUNEL staining by using a DNA fragmentation detection kit (Merck, Darmstadt, Germany). The percentage of TUNEL-positive cells was calculated, normalized, and expressed per 1 mm^2 from 10 tumor sections from each mouse group or per 100 epithelial cells in culture.

CO by intestinal loop ligation. Non-fasting mice underwent surgery for non-ischemic intestinal loop ligation to induce CO. Briefly, mouse colons were mechanically obstructed with 4–0 nylon at both ends, to produce loop ligation, starting distal to the cecocolonic junction and ending proximal to the rectum.[59] Sham-operated mice received laparotomy and mock manipulation of the gut without ligation. Animals were killed 24 h after surgery, and colonic tissues were collected for the measurement of gut-associated bacterial counts, mucosal LPS levels, and cell apoptosis.[60]

LPS challenge to mouse colonic tissues in *ex vivo* tissue baths. A 1-cm segment of colon was excised and immediately placed in warmed Krebs buffer. Two pieces of colonic tissues from each mouse were cut longitudinally into flat sheets and mounted on Ussing chambers, exposing the luminal and serosal sides of the intestine (an area of 0.7 cm^2) to 5 ml of circulating oxygenated Krebs buffer at 37 °C. The serosal buffer contained 10 mM of glucose as an energy source osmotically balanced with 10 mM of mannitol in the luminal buffer. Based on the LPS concentration optimized in our previous studies,[20,61] tissues were luminally challenged with LPS (50 μg/ml) or PBS for 2 h, and then were removed for measurement of cell apoptosis. Bacterial LPS was derived from *E. coli* O26:B6 and was purchased from Sigma.

In another experimental setting, colonic tissues were pretreated with neutralizing antibody to CD14 (25 μg/ml, BD Biosciences, San Jose, CA, USA) or with IgG2b isotype controls before LPS challenge. Briefly, mice were fasted but allowed to drink water *ad libitum*, to ensure no luminal contents were left in the proximal colonic segment. After anesthetizing, mice underwent aseptic laparotomy and the proximal colon was ligated into a 3-cm loop into which antibodies in 0.25 ml PBS were carefully administered using a 26-gauge needle with polyethylene tubing. After 30 min, colonic tissues were excised and mounted in tissue baths for mucosal LPS challenge.

Intestinal organoid cultures. Mouse colonic crypt cells were cultured as organoids following previously described methods,[62,63] with some modification. Briefly, cell clusters of the crypt fractions were isolated by using an EDTA-detachment method. After counting, around 1500 cell clusters were plated in 300 μl ice-cold Matrigel (BD Biosciences, 356235) (3 : 1 ratio with crypt culture medium) per well of 24-well plates. After the polymerization of Matrigel, 300 μl of crypt culture medium was overlaid. The crypt culture media is composed of advanced Dulbecco's modified Eagle medium (DMEM)/F12 (Life Technologies, Carlsbad, CA, USA) supplemented with 100 ng/ml mouse recombinant Wnt3A (R&D Systems, Minneapolis, MN, USA), 50 ng/ml epidermal growth factor (Life Technologies), 100 ng/ml Noggin (PeproTech, Rocky Hill, NJ, USA), 500 ng/ml R-spondin-1 (Peprotech), 1 μM Jag-1 (AnaSpec, Fremont, CA, USA), 10 μM Y-27632 (Sigma), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml Streptomycin (Life Technologies), and 2% heat-inactivated fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel). The overlaying medium was refreshed every 2–3 days. The organoids were grown for up to 14 days, and images were captured under a light microscope. Organoids were collected by dissolving Matrigel with recovery solution (BD Biosciences). The transcript levels of *CD14*, *TLR4*, *MD2*, and *Lgr5* were examined by reverse transcription-PCR (RT-PCR). Organoids grown for 5 days were challenged by adding LPS to the overlaying medium at a final concentration of 50 μg/ml for 24 h, and collected for measurement of cell death.

In another setting, gene silencing by siRNA was conducted 48 h before LPS challenge in organoids. Organoids were transfected with 50 nM of siRNA oligonucleotides from a pool of four target-specific 20–25 nt siRNAs that target CD14, TLR4, or scrambled controls (Santa Cruz, Dallas, TX, USA) using Lipofectamine RNAiMAX Reagent (Life Technologies) in serum-free basal DMEM/F12 medium for 6 h. The basal medium was switched back to serum-containing complete medium for 48 h, and organoids were then exposed to LPS for 24 h. Knockdown efficiencies in the transfected cells were confirmed by RT-PCR.

LPS challenge in cell cultures. Human CRC Caco-2 and HT29 cells were grown in DMEM (Life Technologies).[20,61,64] T84 cells were grown in DMEM/Nutrient Mixture F-12 Ham (DMEM/F-12) (Sigma). Cells were apically exposed to 50 μg/ml bacterial LPS (from *E. coli* O26:B6; Sigma) for 24 h, unless stated otherwise, and were examined for apoptotic levels.

In some experiments, cells were pretreated with inhibitors or blocking antibodies 30 min before LPS challenge. These included 120 μM Z-DEVD-FMK (z-DEVD, a caspase-3 inhibitor; Merck), 10 μg/ml PMB (a LPS antagonist; Sigma), 50 μM D609 (a PC-PLC inhibitor; Sigma), 5 μM imipramine (a SMase inhibitor; Sigma), 60 nM Gö6983 (a broad spectrum PKC inhibitor; Merck), 20 μM inhibitory PKCζ pseudosubstrate (Merck), 100 μM necrostatin-1 (a necroptosis inhibitor; Sigma), 10 μg/ml neutralizing mouse anti-human CD14 (R&D systems), 20 μg/ml neutralizing mouse anti-human TLR4 (eBioscience, San Diego, CA, USA), or their respective IgG1and IgG2a isotype controls. The topoisomerase-I inhibitor, camptothecin (50 μM; Sigma), was used as a positive control for apoptosis after 24 h of incubation.

siRNA-mediated knockdown. Cells were transfected with 50 nM of siRNA oligonucleotides targeting CD14, TLR4, or PKCζ (Santa Cruz) using Lipofectamine RNAiMAX Reagent (Life Technologies) in serum-free basal medium. After 24 h, cells were trypsinized and reseeded in culture plates at 0.5–1 $\times 10^6$ cells/well for 48 h with complete medium before LPS challenge. Our pilot study indicated tight junctional formation 48 h after reseeding, as measured by transepithelial electrical resistance.

Plasmid constructs and point mutation. The expression vector pMyc-CMV1-huTLR4 was kindly provided by Dr Douglas T Golenbock (University of Massachusetts Medical School, Worcester, MA, USA). Point mutations for Asp299Gly, Thr399Ile, or Pro714His were created by site-directed mutagenesis using KAPA HiFi PCR kits (Kapa Biosystems, Woburn, MA, USA), according to the manufacturer's instructions. Cells were transfected with pMyc-CMV1-huTLR4 plasmids (2.5 μg/well each) before LPS challenge.

Statistical analysis. All data were expressed as mean $\pm$ S.E.M., except that bacterial colony-forming units were presented as median values. When the normality assumption was appropriate, data were statistically analyzed by one-way ANOVA followed by the Student–Newman–Keuls *post-hoc* test for multiple comparisons. When comparing the differences between two groups of data that failed to satisfy the normality assumption, a non-parametric Mann–Whitney *U*-test was used. $P < 0.05$ was considered statistically significant.

Detailed information regarding isolation of mouse intestinal epithelial cells and blood mononuclear leukocytes, histopathological examination, RT-PCR, histopathological examination, immunofluorescence staining and confocal microscopy, western blotting, enzyme-linked immunosorbent assay (ELISA), quantification of mucosa-associated LPS levels, cell lines, lipid extraction and ultra-performance liquid chromatography–tandem mass spectrometry, and plasmid constructs and point mutation are provided in the Supplementary Text.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

Guarantor of integrity of the entire study, study concepts and design: LCY; data acquisition: WTK, TCL, HYY, CYC, YCA, YZL, LLW, YC, and YHT; data analysis/interpretation: WTK, TCL, HYY, CYC, TCL, SCW, YHN, and LWL; statistical analysis:

WTK and HYY; material and technical support: BRL, YC, YHT, JTK, and FS; obtained funding: LCY, SCW, and YHN; drafted manuscript or revised for important intellectual content, literature research, manuscript editing, and manuscript final version approval: all authors.

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