Published in final edited form as:

Inflamm Bowel Dis. 2009 July ; 15(7): 997–1006. doi:10.1002/ibd.20880.

Innate immune signaling by Toll-like receptor-4 (TLR4) shapes the inflammatory microenvironment in colitis-associated tumors

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Abstract

Patients with ulcerative colitis are at increased risk for developing colorectal cancer. We have shown that TLR4 is over-expressed in human colitis-associated cancer (CAC) and that mice deficient in TLR4 are markedly protected against colitis-associated neoplasia. We wished to elucidate the specific contributions of TLR4 signaling by myeloid cells and colonic epithelial cells (CEC) in colitis-associated tumorigenesis. TLR4-deficient mice or wild-type littermates (WT) were transplanted with bone marrow (BM) cells: TLR4^{-/-} BM→WT mice (TLR4-expressing CEC) and WT BM→TLR4^{-/-} mice (TLR4-expressing myeloid cells). Colitis-associated neoplasia was induced by azoxymethane (AOM 7.3mg/kg) injection and two cycles of dextran sodium sulfate (DSS) treatment. The number and size of dysplastic lesions were greater in TLR4^{-/-} BM→WT mice than in WT BM→TLR4^{-/-} mice ($P<0.005$). Histologically, TLR4^{-/-} BM→WT mice had greater numbers of mucosal neutrophils and macrophages compared to WT BM→TLR4^{-/-} mice. The chemokines KC and CCL2, important in recruitment of neutrophils and macrophages, respectively, were induced in mice expressing TLR4 in CEC rather than the myeloid compartment. The lamina propria infiltrate of mice expressing TLR4 in CEC was characterized by macrophages expressing Cox-2. Moreover, mice expressing TLR4 in CEC rather than the myeloid compartment had increased production of amphiregulin and EGFR activation. These findings indicate that TLR4 signaling on CEC is necessary for recruitment and activation of Cox-2 expressing macrophages and increasing the number and size of dysplastic lesions. Our results implicate innate immune signaling on CEC as a key regulator of a tumor-promoting microenvironment.

Keywords

Inflammation; colorectal cancer; mouse model; toll-like receptor; tumor microenvironment

Introduction

Inflammation and cancer are linked. This unfortunate connection is detrimental to patients with inflammatory bowel disease (IBD) who are at a substantially increased risk for

colorectal cancer. Even when cancer does not arise, the ominous presence of dysplasia often results in a total proctocolectomy since it is not currently possible to reliably predict who will progress to cancer or to halt the progression to cancer. Patients with ulcerative colitis have a lifetime relative risk of colorectal cancer that is five to eight times higher than the normal population 1-5. The exact mechanism by which chronic intestinal inflammation causes colorectal cancer remains uncertain 6-9.

We have recently demonstrated that TLR4 expression is upregulated in CAC lesions from patients with ulcerative colitis but not in the surrounding tissue 10. Moreover, investigators have examined pathologic specimens from patients with CAC and found that, compared to biopsies from ulcerative colitis, patients that did not develop cancer, colon biopsies from CAC patients had a higher degree of acute inflammation prior to the identification of cancer 8, 11. These results suggest that innate immune signaling in the intestine plays a critical role in the pathogenesis of CAC.

Animal models have provided a useful paradigm to study the contribution of intestinal epithelial cells or inflammatory cells in the pathogenesis of CAC. The combination of azoxymethane (AOM), a colonic genotoxic carcinogen, with dextran sulfate sodium (DSS), an inducer of colitis, has been frequently used as a mouse model of CAC 12, 13. The importance of epithelial and myeloid NF- κ B signaling in the development of CAC has been demonstrated using this model 14. More recently, a report has shown that TNF- α signaling via the p55 TNF receptor in myeloid cells is necessary for tumor development in the same mouse CAC model, whereas epithelial TNF receptor signaling does not affect tumor numbers 15. TNF- α potentially induces NF- κ B activation but can also be a NF- κ B-target gene 16, 17. These data suggest that inflammatory cytokines and NF- κ B activation may influence CAC development both at the level of the epithelial cell or the immune cell.

One of the unique aspects of the intestinal epithelium and the development of colorectal cancer is the role of intestinal bacteria. The intestinal epithelium is bathed in microbes and microbial products. Signaling through TLRs on either epithelial cells or lamina propria mononuclear cells activates NF- κ B and is necessary for intestinal homeostasis and repair 18-20. Germ-free rodents are protected from colitis-associated neoplasia supporting a role for bacterial recognition in CAC 21, 22. To delineate the role of TLR signaling in the development of CAC, we previously used TLR4 $^{-/-}$ mice and asked whether these animals were protected against colitis-associated neoplasia. We demonstrated that mice deficient in TLR4 are markedly protected against the development of neoplasia 10. By contrast, single immunoglobulin IL-1 receptor-related molecule (SIGIRR) deficient animals, which demonstrate increased TLR signaling, have increased intestinal inflammation and increased tumorigenesis following treatment with AOM-DSS 23, 24. Restoring SIGIRR expression in the epithelium reduces inflammation and tumorigenesis suggesting a role for epithelial TLR signaling in tumor development.

Based on our own work showing the importance of TLR4 in the development of colitis-associated neoplasia, we wished to determine whether TLR4 expression on the epithelium, myeloid cells, or both was required for the development of colitis-associated neoplasia. Traditionally, TLR signaling is thought to occur primarily in hematopoietic cells such as macrophages and dendritic cells. Using bone marrow chimeras, we demonstrate here that TLR4 expression by colonic epithelial cells (CEC) plays a greater role in the development of colitis-associated neoplasia than myeloid expression of TLR4. TLR4 expression by colonic epithelial cells (CEC) was able to direct recruitment of tumor-associated macrophages expressing Cox-2. Our data support targeted inhibition of TLR signaling for the prevention or treatment of CAC.

Materials and Methods

Allogeneic BM transplantation in mice

TLR4^{-/-} mice were purchased from Oriental Bio Service, Inc. (Kyoto, Japan). All knockout mice were backcrossed to C57BI/6J mice at least 8 generations. C57BL/6J mice were obtained from Jackson Laboratory as controls (Jackson Laboratory, Bar Harbor, Maine). Mice were kept in specific-pathogen free (SPF) conditions and fed by free access to a standard diet and water. All experiments were done according to Mount Sinai School of Medicine animal experimental ethics committee guidelines.

Six-week old C57BI/6J WT or TLR4^{-/-} recipient mice were irradiated with 10 Gy from a ¹³⁷Cs source delivered in two doses of 5 Gy each, 3h apart. BM cells were isolated from six to eight week old C57BI/6J or TLR4^{-/-} donor mice by flushing the bone shafts of the femurs and tibias with RPMI and $2 - 3 \times 10^6$ BM cells i.v. injected into the recipient mice 3h after last irradiation. WT mice were transplanted with TLR4^{-/-} BM (TLR4^{-/-} BM→WT) and TLR4^{-/-} mice were transplanted with WT BM (WT BM→TLR4^{-/-}). As controls, WT mice were transplanted with WT BM and TLR4^{-/-} mice transplanted with TLR4^{-/-} BM. Using C57BL/6-Tg (ACTB-EGFP) 10sb/J as donor mice, chimerism was confirmed by examining GFP⁺ cells in splenocytes eight weeks after transplantation by fluorescence activated cell sorter (FACS) analysis. Using this protocol, we have not seen any histological damage of intestinal mucosa induced by irradiation process at eight weeks of BM transplantation.

BM chimeric mice were injected with 7.4 mg/kg of AOM (Sigma, St. Louis, MO) intraperitoneally (i.p.) eight weeks after transplantation. After 14 days of AOM injection, mice were treated with 2.5% DSS (MW 36-50 kDa: ICN, Aurora, Ohio) in their drinking water for 7 days 10. This was followed by 14 days of normal water, another 7 days of 2.5% DSS treatment, and then normal water for an additional 14 days. During the DSS treatment and recovery phase, body weights, stool consistency, and stool occult blood were monitored, as described previously 10.

Mice were sacrificed on day 77 after AOM, 5 weeks after the second DSS cycle. Colons were removed and opened longitudinally. Cecum, proximal, and distal parts of the colon were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Histological assessment was performed by two independent gastrointestinal pathologists (R.X., H.C.) blinded to the mouse genotype and treatment. Severity of mucosal inflammation was graded using a standard scoring system described previously 10. To quantify the microscopic extent of dysplasia, paraffin-embedded colons were cut in 5 µm thick serial sections and every 20th section was analyzed for dysplasia. Number, size, and the percentage of the mucosal surface area containing dysplasia were determined microscopically. The size of the lesions was calculated using a scale micrometer on the microscope.

Real Time PCR

One µg of total RNA isolated with RNA Bee (Tel-Test, Inc., Friendwood, TX) was used as the template for single strand cDNA synthesis using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. Quantitative real-time PCR was performed for Cox-2, amphiregulin, and β-actin using TaqMan probes. The primers and probes used in this study are as follows (5' to 3' direction), for mouse Cox-2: sense primer, AAG GAA CTC AGC ACT GCA TCC, anti-sense primer, ACA GGG ATT GGA ACA GCA AGG A, and probe, ACC GCC ACC ACT ACT GCC ACC TCC; for mouse amphiregulin: sense primer, TGT CAC TAT CTT TGT CTC TGC CAT, anti-sense primer, AGC CTC CTT CTT TCT TCT GTT TCT, and probe, TCC TCG CAG CTA TTG GCA TCG GCA; for mouse β-actin: sense primer, ATG ACC CAG ATC

ATG TTT G, anti-sense primer, TAC GAC CAG AGG CAT ACA, and probe, CGT AGC CAT CCA GGC TGT GC. All TaqMan probes and primers were designed using Beacon Designer 3.0 software (Premier Biosoft International, Palo Alto, CA). The cDNA was amplified using TaqMan universal PCR Master Mix (Roche, Indianapolis, IN) on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA), programmed for 95°C for 10 minutes, then 40 cycles of: 95 °C for 15 seconds, 60 °C for 1 minute.

Real-time PCR for CCL2 and KC was performed using SYBR Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan) with the standard SYBR Green setting of 7900HT. The primers for mouse CCL2 are: sense primer, GCT GGA GCA TCC ACG TGT T, anti-sense primer, ATC TTG CTG GTG AAT GAG TAG CA; for mouse KC: sense primer, AAT GAG CTG CGC TGT CAG TG, anti-sense primer, TGA GGG CAA CAC CTT CAA GC. β -actin primers are the same primers used for TaqMan real-time PCR.

The amplification results were analyzed using SDS 2.2.1 software (Applied Biosystems, Foster City, CA) and the gene of interest was normalized to the corresponding β -actin results. Data were expressed as fold induction relative to the lowest gene product amplified.

Western Blot Analysis

Mouse colon samples were taken at the time of sacrifice and frozen at -80°C. Tissue lysates were prepared using a lysis buffer containing 50mM Tris HCl, 50mM NaF, 1% Triton $\times$ 100, 2mM EDTA, and 100mM NaCl, with a proteinase inhibitor cocktail (Calbiochem, San Diego, CA). Protein concentration was determined by the Bradford method using Bio-Rad Protein Assay Dye and SmartSpec™ 3000 (Bio-Rad Laboratories, Hercules, CA). Twenty-five μ g of the lysates were subjected to 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). The membrane was blocked in 5% skim milk and was immunoblotted with anti-phospho-EGFR (Tyr 1173, goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) antibody for 1 hour, followed by incubation with HRP-conjugated anti-goat IgG (Zymed Laboratories, South San Francisco, CA). After stripping with Restore™ Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL), the membrane was further blotted with anti-EGFR (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with HRP-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA). The membrane was exposed on an x-ray film using an enhanced chemiluminescent substrate SuperSignal West Pico Trial Kit (Pierce Biotechnology, Rockford, IL). Phospho-EGFR band intensity was calculated using NIH Image 1.62 by normalizing with the intensity of the corresponding EGFR band.

Immunofluorescent and immunohistochemical studies

Paraffin embedded sections were incubated in 10% normal goat serum for 1hr and stained with anti-murine Cox-2 antibody (1: 200, Cayman, Ann Arbor, MI) overnight at 4°C, followed by FITC-conjugated anti-rabbit IgG (1:200, Sigma, St. Louis, MO) for 1h at room temperature. The specificity of staining was confirmed using Cox-2 blocking peptide (Cayman, Ann Arbor, MI) according to the manufacturer's instructions or using rabbit isotype control antibody instead of the primary antibody (Zymed Laboratories, South San Francisco, CA).

For the double immunofluorescent staining of CD68 and Cox-2, sections were incubated with 0.1% Trypsin (Sigma, St. Louis, MO) CaCl₂ dissolved in 0.05M Tris-Hcl pH 7.6 for 15 min at 37 °C. Subsequently, sections were blocked in a 5% skim milk for 1h and then incubated with the rat anti-CD68 antibody (1:50, MCA1957S, Serotec Ltd., Raleigh, NC)

overnight at 4°C. After washing in PBS, sections were incubated with TRITC-conjugated rabbit anti-rat IgG (1:200, Sigma, St. Louis, MO) for 1h at room temperature. Then sections were re-incubated with 5% skim milk followed by Cox-2 staining as described above using FITC-conjugated goat anti-rabbit IgG (1:200, Sigma, St. Louis, MO). The number of Cox-2-positive cells plus CD68-positive cells infiltrating the tumor tissue or lamina propria in the surrounding mucosa was counted with double-stained slides at 400X magnification.

Assessment of proliferation

Colonic tissue sections were examined for cell proliferation by bromodeoxyuridine (BrdU) labeling. Mice were injected with 120 mg/kg of BrdU (Sigma, St. Louis, MO) i.p., 90 minutes prior to sacrificing, and colonic tissues were stained for BrdU using a BrdU staining kit (Zymed Laboratories Inc, South San Francisco, CA) according to the manufacturer's instructions. The number of BrdU-positive cells per well-oriented crypt was calculated every 3 crypts for each colon segment at high magnification under light microscopy.

Measurement of PGE₂

Production of PGE₂ in the tissue culture supernatant was determined using a monoclonal EIA kit (Cayman, Ann Arbor, MI) according to the manufacturer's instructions and as described previously 25, 26. Briefly, colonic samples from TLR4^{-/-} and WT mice were washed in cold PBS containing penicillin, streptomycin, and fungizone (100U/ml each). 100 mg tissue fragments from the distal part of the colon closest to the anus were cultured for 24 hours in 12 well flat bottom plates in serum free RPMI 1640. Culture supernatants were harvested for PGE₂ measurement.

Statistical analysis

Data were presented as mean ±SEM. The significance was analyzed by Kruskal-Wallis test followed by multiple comparisons between the groups using GraphPad Prism version 3.0 (San Diego, California). Fisher's exact probability test was used to compare tumor incidence. Student t-test was used to identify significant differences when comparing two samples with Microsoft Excel. *P* values were considered significant when < 0.05.

Results

Enhanced development of inflammation-induced colorectal tumors when mice express TLR4 in colonic epithelial cells

We have demonstrated that mice deficient in TLR4 are markedly protected against development of colitis-associated neoplasia in the AOM-DSS model 10. To examine the contribution of TLR4-expressing CEC versus myeloid cells in the development of colitis-associated neoplasia, we used bone marrow chimeras in the AOM-DSS model (Figure 1A, Table 1). The incidence of dysplastic lesions was significantly reduced when TLR4^{-/-} mice were used as recipients (TLR4 in myeloid compartment) compared to using WT mice (TLR4 in CEC and stroma) as recipients. The number of dysplastic lesions per colon was strikingly different between animals with or without TLR4 expression in CEC. There were no differences in the number of dysplastic lesions between WT mice receiving TLR4^{-/-} BM and WT mice receiving WT BM, suggesting a significant role of TLR4 signaling by CEC in the development of colitis-associated neoplasia. Moreover, the size and extent of dysplasia was also significantly greater when TLR4 was expressed by CEC rather than by myeloid cells (Table 1). TLR4^{-/-} mice receiving WT BM did, however, develop some dysplastic lesions supporting a contribution from myeloid TLR4 in dysplasia development whereas TLR4^{-/-} mice receiving TLR4^{-/-} BM did not. These results indicate that the TLR4 signal from myeloid cells may contribute to some extent to the development of colitis-associated

neoplasia but the TLR4 signal from CEC is sufficient to recapitulate the neoplasia regardless of the TLR4 signal from myeloid cells.

Consistent with our findings above, BrdU labeling demonstrated significantly higher epithelial proliferation in the mice with TLR4 in CEC than the mice with TLR4 in myeloid cells (Figure 1B). Epithelial proliferation was similar in WT BM→TLR4^{-/-} mice compared to TLR4^{-/-} BM→TLR4^{-/-} mice, suggesting myeloid expression of TLR4 has no effect on the epithelial proliferation when epithelial expression of TLR4 is absent. However, TLR4^{-/-} BM→WT mice continued to have less epithelial proliferation than WT BM→WT mice. These data support that epithelial expression of TLR4 drives proliferation in this model.

Characterization of the tumor microenvironment in TLR4-dependent inflammatory colorectal cancer

Having established a system that permits us to study the contribution of epithelial versus myeloid TLR4 signaling in inflammatory colorectal cancer, we examined the role of TLR4 expression on the tumor microenvironment. Neutrophil infiltration of the mucosa in ulcerative colitis correlates with development of CAC 8· 11· 27. We compared the inflammatory infiltrate in our bone marrow chimeras treated with AOM-DSS. Histologically, WT mice transplanted with TLR4^{-/-} BM had a dense infiltrate of neutrophils not seen in TLR4^{-/-} mice receiving WT BM (45.4 ± 46.2 vs., 17.5 ± 19.3 in HPF, $P<0.05$) (Figure 2A). We also found a significant difference of neutrophil infiltrate between WT mice receiving WT BM (49.5 ± 26.7 in HPF) and TLR4^{-/-} mice receiving WT BM ($P<0.01$). These data demonstrate that CEC expression of TLR4 is necessary and sufficient for recruitment of neutrophils to the lamina propria.

In addition to neutrophils, tumor-associated macrophages (TAM)s play a central role in the establishment and growth of colorectal cancers 28· 29. We found that WT mice transplanted with TLR4^{-/-} BM have a higher number of tumor-associated macrophages compared to tumors in TLR4^{-/-} mice receiving WT BM (Figure 2B). There was no statistical difference of the number of tumor-associated macrophages between WT mice transplanted with TLR4^{-/-} BM and WT mice transplanted with WT BM (data not shown). The expression of TLR4 in CEC was also associated with higher numbers of lamina propria macrophages. Thus, in spite of the fact that TLR4 was not expressed on hematopoietic cells, epithelial expression of TLR4 could direct recruitment of neutrophils and macrophages. Differences in chemokine expression will be discussed below.

To determine whether these lamina propria macrophages could function to support tumor development, we examined expression of Cox-2 and production of PGE₂. Intestinal inflammation results in increased expression of Cox-2, which is thought to contribute to human CAC in a variety of ways 30· 31. We first asked whether Cox-2 mRNA expression was increased in our bone marrow chimera model. By real-time PCR, Cox-2 expression is significantly higher when TLR4 is expressed by CEC than by myeloid cells (Figure 3A). Although there was no statistical difference ($P=0.11$), there was an almost 50% decrease in Cox-2 expression when WT mice received TLR4^{-/-} BM compared to WT receiving WT BM, suggesting a contribution of myeloid TLR4 signaling in mucosal Cox-2 expression. PGE₂ production mirrored expression of Cox-2 but was also seen in TLR4^{-/-} mice receiving WT BM (myeloid TLR4) (Figure 3B). To determine the source of Cox-2, we used immunofluorescent staining of AOM-DSS-treated bone marrow chimeras. Cox-2 positive cells were found in the lamina propria infiltrate and coincided with CD68-positive cells (Figure 3C). The number of Cox-2 expressing macrophages was higher in the lamina propria and tumors of TLR4^{-/-} BM→WT mice compared with WT BM→TLR4^{-/-} mice. These results demonstrate that TLR4 expression by CEC recruits macrophages that express Cox-2 or induces local expression of Cox-2.

TLR4-dependent chemokine expression in inflammatory colorectal cancer

Given that TLR4 signaling by CEC is associated with an inflammatory infiltrate consisting of neutrophils and macrophages, we hypothesized that chemokines induced by CEC may be responsible for recruitment of these inflammatory cells. CCL2, a monocyte chemotactic protein-1 (MCP-1), has been implicated in recruitment of TAMs in colorectal cancer and CCL2 can be stimulated by TLR4 32-35. KC, a major neutrophil chemoattractant, is also induced by TLR4 and has been implicated in cancer cell growth 32. We examined mucosal expression of CCL2 and KC mRNA by real-time PCR. Mucosal expression of both CCL2 and KC mRNA was higher in TLR4^{-/-} BM→WT mice than in WT BM→TLR4^{-/-} mice (Figure 4A, B). Since WT BM→TLR4^{-/-} mice had similar expression levels of CCL2 and KC mRNA when compared to TLR4^{-/-} mice transplanted with TLR4^{-/-} BM, we conclude that the CEC-derived TLR4 signal, but not the myeloid-derived TLR4 signal, is responsible for chemokine expression.

Growth factor signaling in TLR4-dependent colitis-associated neoplasia

We have demonstrated above that TLR4 expression by CEC increases tumor incidence and size using a bone marrow chimera model. EGFR activation occurs in colorectal cancer and inhibition of this pathway is effective for treatment of cancer 36. Amphiregulin is an EGF-like molecule implicated in colorectal cancer 37. Both intestinal epithelial cells and myofibroblasts have been shown to produce AR 38, 39. Using our current system, we wished to address which TLR4-expressing cell type is responsible for expression of AR. Mucosal AR expression in TLR4^{-/-} BM→WT mice was significantly higher than in WT BM→TLR4^{-/-} mice and was similar to AR expression in WT mice transplanted with WT BM (Figure 5A). TLR4^{-/-} mice even when transplanted with TLR4 positive-myeloid cells, did not induce AR. EGFR activation as measured by Western blot mirrored the results of the AR experiments (Figure 5B). We conclude that TLR4 signaling by CEC is required for AR induction in a model of colorectal neoplasia and results in activation of the EGFR pathway.

Discussion

Although inflammation has long been recognized to engender cancer, we have not been able to harness a rational approach to its prevention or treatment. In the case of inflammatory bowel disease, less inflammation should mean less cancer and, by corollary, more effective anti-inflammatory treatments should mean less cancer. Studies have suggested a modest chemopreventive effect of 5-aminosalicylates 40. Part of the reason we may not see a better chemopreventive effect from treatment may be that previous episodes of severe inflammation cannot be undone. At the root of the problem may be coexistence with intestinal bacteria and persistent innate immune activation 41, 42. Our laboratory has tried to tease apart the contribution of innate immune signaling in colitis-associated cancer using a reductionist approach. Our previous data using human CAC tissue revealed that TLR4 is upregulated in cancer tissue compared with the surrounding mucosa. In an animal model, the complete absence of TLR4 protects the animals from developing inflammation-induced colorectal tumors 10. These studies could not ascertain which TLR4-expressing cell type was responsible for the oncogenic phenotype.

Our study is the first to show that CEC expression of TLR4 is more important for the development of inflammation-induced tumors than myeloid expression of TLR4. This is surprising given that epithelial expression of TLR4 is thought to be low under normal conditions 43, 44. During acute intestinal inflammation in mice and humans, TLR4 expression is increased—but these studies could not address whether function is increased 45, 46. In particular, previous studies have demonstrated increased TLR4 expression on

lamina propria macrophages in inflammatory bowel disease but could not address whether this is important for inflammation or inflammation-induced cancer 47.

We sought to determine the function of TLR4 in the intestinal mucosa and address an important issue: namely is TLR signaling on CEC or in the myeloid compartment necessary for inflammation-induced neoplasia? Like the intestine itself, the answer is multilayered. Previous studies by Karin et al. have shown that NF- κ B activation by epithelial cells increased tumor numbers but that myeloid NF- κ B activation was more important for tumor size 14. Although TLR4 activates NF- κ B, the Venn diagram of genes induced by NF- κ B and TLR4 signaling are distinct and merit their own careful investigation. Using bone marrow chimeras, investigators have found that MyD88-expressing myeloid cells such as macrophages are required for epithelial cell proliferation in DSS-induced mucosal damage 20-48. Again using bone marrow chimeras, Mizoguchi et al. has recently shown that TNF receptor 1 on myeloid cells protects the colonic epithelium from DSS-induced damage 49. Myeloid expression of the TNF receptor 1 is also important for tumorigenesis in the AOM-DSS model 15. These data point to an inter-relationship between the epithelium and the lamina propria myeloid compartment for CEC proliferation and ultimately neoplasia. In our work, the TLR4-responding cell type appears to be the CEC but the myeloid compartment, likely through TNF and other factors, promotes tumor growth.

Mechanistically, the CEC TLR4 signal leads to chemokine expression, recruitment of neutrophils, and activation of macrophages, and EGFR activation. It is not necessary in our model for TLR4 to be expressed by the macrophages themselves in order for Cox-2 to be induced. On the other hand, mice with TLR4 expression only in the myeloid cells still develop tumors, but at significantly reduced numbers. These mice had levels of mucosal PGE₂ similar to mice expressing TLR4 in CEC. Thus, the myeloid TLR4 signal contributes to the development of neoplasia by inducing mucosal PGE₂. In addition, both Cox-1 and Cox-2 contribute to total levels of PGE₂ in the intestinal mucosa, which may explain why the PGE₂ production is not entirely reflected in differences in Cox-2 expression.

Although the primary role of TLRs in the periphery is to eradicate pathogens, the intestine has made adaptations to coexist with commensal bacteria. In fact, our work and others has highlighted the importance of TLR signaling in epithelial homeostasis 18-20. Proliferation and repair of the epithelium in response to bacterial invasion of the mucosa is intrinsic for the host to survive in the presence of potentially lethal amounts of intestinal bacteria. For patients with inflammatory bowel disease, the presence of luminal bacteria results in persistent innate immune activation. Innate immune signaling is thus a double-edged sword. Our work demonstrates that TLR4 signaling in discrete compartments in the intestine has distinct effects as they relate to inflammation-induced cancer. TLR4 signaling by the epithelium shapes the tumor microenvironment to optimally sustain the growth of a tumor. These observations can guide strategies directed at turning down the volume of TLR signaling in the epithelium to decrease the incidence of CAC.

Acknowledgments

This study was supported by NIH grants AI052266 (MTA), DK069594 (MTA), Career Development Award from CCFA (MF).

Abbreviations

TLR	toll-like receptor
WT	wild-type

DSS	dextran sodium sulfate
Cox	cyclooxygenase
EGFR	epidermal growth factor receptor
PGE₂	prostaglandin E ₂
AR	amphiregulin
BM	bone marrow
CAC	colitis-associated cancer
AOM	azoxymethane
TAMs	tumor-associated macrophages

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Figure 1. Incidence of dysplasia

A. The number of dysplastic lesions was counted per mouse. Data are gathered from five independent experiments. Bar represents mean (** $P < 0.001$, * $P < 0.01$, † $P < 0.05$).

B. Intestinal epithelial proliferation. BrdU labeling index was defined by counting BrdU positive epithelial cells per crypt. Data are represented as mean $\pm$ SEM. TLR4^{-/-}-BM→WT mice (n=10) had greater epithelial proliferation than WT BM→TLR4^{-/-} mice (n=16) (* $P < 0.001$), but less epithelial proliferation than WT BM→WT mice (n=9) (** $P < 0.0001$). Epithelial proliferation in WT BM→TLR4^{-/-} mice was similar to TLR4^{-/-}-BM→TLR4^{-/-} mice (n=8).

Figure 2. Characterization of the tumor microenvironment

A. Microscopic findings in TLR4^{-/-} BM→WT mice and WT BM→TLR4^{-/-} mice mucosa at day 77 of the AOM-DSS model (H & E staining). TLR4^{-/-} BM→WT mice (top panel) have a denser inflammatory infiltrate in the mucosa compared with WT BM→TLR4^{-/-} mice (bottom panel).

B. CD68 positive cells (macrophages) per HPF (400x) were counted in colon samples from AOM-DSS treated mice. Significantly greater numbers of macrophages were seen in TLR4^{-/-} BM→WT mice (n=10) compared to WT BM→TLR4^{-/-} mice (n=16) in both the tumors and surrounding lamina propria. Three fields per site (cecum, proximal, distal) were examined. Data are represented as mean ± SEM (**P* < 0.05).

Figure 3. Cox-2 expression from mucosal macrophages is dependent on TLR4 signaling by CEC
A. Cox-2 expression in the colon of AOM-DSS treated mice. Colonic samples were taken at day 77 in the AOM-DSS model. Cox-2 mRNA expression was analyzed by real time PCR. A significant difference is shown between TLR4^{-/-} BM→WT mice (n=10) and WT BM→TLR4^{-/-} mice (n=16). Cox-2 mRNA expression in WT BM→WT mice (n=9) was also significantly higher than TLR4^{-/-} BM→TLR4^{-/-} mice (n=8) or WT BM→TLR4^{-/-} mice (n=8). Data are represented as mean ±SEM of relative values of expression (**P* < 0.05).
B. PGE₂ production by colonic mucosa in the AOM-DSS model. There is a numerical difference but not significant difference between TLR4^{-/-} BM→WT mice (n=10) and WT BM→TLR4^{-/-} mice (n=16). Significant differences were found only between WT BM→WT mice (n=9) and TLR4^{-/-} BM→TLR4^{-/-} mice (n=8). Data are shown as mean ±SEM (**P* < 0.05).
C. Double immunostaining for Cox-2 (green - FITC) and the macrophage marker CD68 (red - TRITC) in TLR4^{-/-} BM→WT mice (top panel) and WT BM→TLR4^{-/-} mice (bottom panel) mucosa. The majority of Cox-2 expressing cells in the lamina propria are also positive for CD68.

Figure 4. TLR4 signaling by CEC induces the expression of macrophage and neutrophil chemoattractant genes in the AOM-DSS model

A. CCL2 expression in the colonic mucosa measured by real time PCR. Samples were taken at day 77 in the AOM-DSS model. A significant difference is shown between TLR4^{-/-} BM→WT mice (n=10) and WT BM→TLR4^{-/-} mice (n=16). CCL2 mRNA expression in WT BM→WT mice (n=9) was also significantly higher than TLR4^{-/-} BM→TLR4^{-/-} mice (n=8) or WT BM→TLR4^{-/-} mice. Data are represented as mean ±SEM of relative values of expression (**P* < 0.05).

B. Real time PCR for KC mRNA expression in the colonic mucosa. A significant difference is shown between TLR4^{-/-} BM→WT mice (n=10) and WT BM→TLR4^{-/-} mice (n=16). KC mRNA expression in WT BM→WT mice (n=9) was also significantly higher than TLR4^{-/-} BM→TLR4^{-/-} mice (n=8) or WT BM→TLR4^{-/-} mice. Data are represented as mean ±SEM of relative values of expression (**P* < 0.05).

Figure 5. TLR4 signaling by CEC regulates mucosal amphiregulin expression and EGFR activation

A. AR expression in the colon of AOM-DSS model. AR mRNA expression was analyzed by real time PCR in the samples at day 77 in the AOM-DSS model. A significant difference is shown between TLR4^{-/-} BM→WT mice (n=10) and WT BM→TLR4^{-/-} mice (n=16). AR mRNA expression in WT BM→WT mice (n=9) was also significantly higher than TLR4^{-/-} BM→TLR4^{-/-} mice (n=8) or WT BM→TLR4^{-/-} mice. Data are represented as mean ± SEM of relative values of expression (**P* < 0.05).

B. Western blot analysis of phosphorylated EGFR and total EGFR in the colon. Results from representative samples obtained from two mice per condition are shown. 25 µg/lane of protein was loaded per lane. The membrane was sequentially probed for phospho-EGFR and total EGFR. Phospho-EGFR band intensity was quantified by normalizing with the corresponding EGFR band intensity.

Table 1

Incidence and size of polyps

	WT BM→WT (n=9)	TLR4 ^{-/-} BM →TLR4 ^{-/-} (n=8)	TLR4 ^{-/-} BM →WT (n=10)	WT BM→TLR4 ^{-/-} (n=16)	P value
Incidence (%)	100	0	80.0	43.8	0.26 ^a 0.006 ^b 0.08 ^c 0.03 ^d
Tumors/animal (range)	3.1 ± 0.4 (1 - 4)	0	2.8 ± 0.8 (1 - 7)	0.7 ± 0.2 (0 - 3)	0.37 ^a <0.001 ^b 0.002 ^c 0.07 ^d
Tumor size (mm) (range)	3.6 ± 0.3 (1 - 5)		2.8 ± 0.2 (2 - 5)	1.5 ± 0.2 (0.5 - 3)	0.06 ^a <0.001 ^b <0.001 ^c
Percentage of mucosal surface involved with tumor (range)	18.9% ± 4.2 (5 - 40)		8.3% ± 3.3 (5 - 20)	3.0% ± 0.8 (5 - 10)	0.05 ^a <0.001 ^b 0.016 ^c

^a WT BM→WT v.s. TLR4^{-/-} BM→WT

^b WT BM→WT v.s. WT BM→TLR4^{-/-}

^c TLR4^{-/-} BM→WT v.s. WT BM→TLR4^{-/-}

^d TLR4^{-/-} BM→TLR4^{-/-} v.s. WT BM→TLR4^{-/-}