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Toll-like Receptor 4 Variant D299G Induces Features of Neoplastic Progression in Caco-2 Intestinal Cells and Is Associated With Advanced Human Colon Cancer

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Abstract

Background & Aims—The Toll-like receptor (TLR) 4 mediates homeostasis of the intestinal epithelial cell (IEC) barrier. We investigated the effects of TLR4-D299G on IEC functions.

Methods—We engineered IECs (Caco-2) to stably overexpress hemagglutinin-tagged wild-type TLR4, TLR4-D299G, or TLR4-T399I. We performed gene expression profiling using DNA microarray analysis. Findings were confirmed by real-time, quantitative, reverse-transcriptase polymerase chain reaction, immunoblot, enzyme-linked immunosorbent assay, confocal immunofluorescence, and functional analyses. Tumorigenicity was tested using the CD1 *nu/nu* mice xenograft model. Human colon cancer specimens (N = 214) were genotyped and assessed for disease stage.

Results—Caco-2 cells that expressed TLR4-D299G underwent the epithelial-mesenchymal transition and morphologic changes associated with tumor progression, whereas cells that expressed wild-type TLR4 or TLR4-T399I did not. Caco-2 cells that expressed TLR4-D299G had significant increases in expression levels of genes and proteins associated with inflammation and/or tumorigenesis compared with cells that expressed other forms of TLR4. The invasive activity of TLR4-D299G Caco-2 cells required Wnt-dependent activation of STAT3. In mice, intestinal xenograft tumors grew from Caco-2 cells that expressed TLR4-D299G, but not cells that expressed other forms of TLR4; tumor growth was blocked by a specific inhibitor of STAT3. Human colon adenocarcinomas from patients with TLR4-D299G were more frequently of an advanced stage (International Union Against Cancer [UICC] ≥III, 70% vs 46%; $P = .0142$) with metastasis (UICC IV, 42% vs 19%; $P = .0065$) than those with wild-type TLR4. Expression of

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STAT3 messenger RNA was higher among colonic adenocarcinomas with TLR4-D299G than those with wild-type TLR4.

Conclusions—TLR4-D299G induces features of neoplastic progression in intestinal epithelial Caco-2 cells and associates with aggressive colon cancer in humans, implying a novel link between aberrant innate immunity and colonic cancerogenesis.

Keywords

Intestine; Signaling; Epithelium

Innate immunity represents the first line of host defense in the intestinal epithelial cell (IEC) barrier.¹ Commensals induce innate immune responses that participate in important physiologic functions, including IEC differentiation, proliferation, and maturation.² To maintain mucosal homeostasis in the intestine, commensal-induced host-modulatory effects require basal state of Toll-like receptor (TLR) function, thus ensuring rapid restitution and limited inflammatory responses of IECs. Changes in commensal composition during colitis may trigger aberrant innate immune signaling, leading to colon tumor formation.³

TLR4 is the major receptor for lipopolysaccharide activation in conjunction with 3 accessory molecules: CD14, LBP, and MD-2. Under normal conditions, TLR4/MD-2 expression is generally low in IECs but significantly up-regulated in inflammatory bowel diseases (IBDs).^{4,5} TLR4 exerts critical IEC barrier-protective mechanisms that enhance wound healing.^{6,7} Recently, 2 common missense mutations, A896G (D299G) and C1196T (T399I), have been identified at frequencies up to 18%.⁸ The single TLR4-D299G haplotype is more prevalent in African populations, while the cosegregated TLR4-D299G/T399I is usually found in European individuals. Both mutations are located within the extracellular domain,⁹ resulting in conformational changes,¹⁰ and depending functionally on MD-2.¹¹ TLR4-D299G and TLR4-T399I have been associated with increased disease risk for gram-negative infections with septic shock,¹² *Helicobacter pylori*-mediated noncardia gastric carcinoma,¹³ and IBDs.¹⁴ The human TLR4-D299G mutation (but not the T399I mutation) has been shown to interrupt TLR4-mediated lipopolysaccharide signaling in airway epithelial cells.¹⁵ However, the functional phenotypic consequences of the TLR4-D299G variant in the intestinal epithelium have not yet been delineated.

In this study, we show that the TLR4-D299G mutation induces features of neoplastic progression in the IEC line Caco-2 in vitro and in vivo that correlates with increases in several genes and proteins associated with inflammation and/or tumorigenesis and involves *STAT3* activation. Furthermore, our findings show that human patients with colon cancer carrying TLR4-D299G may develop more frequently aggressive disease with metastasis than patients carrying TLR4-WT. Thus, our data imply a novel mechanistic link between aberrant innate immune signaling and malignant tumor progression in colon cancer.

Materials and Methods

Antibodies and Reagents

Phospho-*STAT3* (Tyr705) XP, total *STAT3*, phospho-I κ B α , phospho- β -catenin, GAPDH, Alexa Fluor 488 phospho-histone H3, and Alexa Fluor 555 β -tubulin antibodies were from Cell Signaling (Danvers, MA). Bip/grp78 antibody was from BD Transduction (Franklin Lakes, NJ), DKK-1 antibody from R&D Systems (Wiesbaden, Germany), Cx43 antibody from Zymed (San Francisco, CA), and pan-cytokeratin antibody from Santa Cruz Biotechnology (Santa Cruz, CA). HA antibodies were from InvivoGen (San Diego, CA) and Cell Signaling. Alexa Fluor 647 phalloidin and Alexa Fluor 488 – conjugated goat anti-

mouse immunoglobulin G antibody were from Invitrogen (Carlsbad, CA). Horseradish peroxidase–conjugated anti-rabbit and anti-mouse antibodies were from GE Healthcare (Munich, Germany) and CY5- or fluorescein isothiocyanate–conjugated goat anti-rabbit immunoglobulin G antibodies from Jackson ImmunoResearch (West Grove, PA). STAT3 inhibitor VI (NSC74859), Wnt inhibitor (quercetin, dihydrate), and cyclooxygenase (COX)-2 inhibitor II (SC-791) were from Merck (Nottingham, United Kingdom) (please see Supplementary Materials and Methods). All other reagents were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise specified.

Cells, Plasmid Constructs, and Transfection

The IEC line Caco-2 (American Type Culture Collection #HTB-37; lot #1537739) tested negative for mycoplasma (MycoAlert assay; Lonza, Basel, Switzerland) was cultured as described previously.¹⁶ HA-tagged TLR4/wild-type plasmid (TLR4-WT) was obtained from InvivoGen. TLR4 mutants (D299G, T399I) were generated within the TLR4-WT expression construct and confirmed by sequencing (Trenzyme, Konstanz, Germany). Plasmids (EndoFree Maxi; Qiagen, Hilden, Germany) were transfected into Caco-2 cells on a 12-well plate (poly-D-lysine coated)¹⁶ using 1 μ g/well of TLR4-WT, TLR4-D299G, or TLR4-T399I (Lipofectamine LTX; Invitrogen). Stable IEC transfectant clones expressing TLR4-WT, TLR4-D299G, or TLR4-T399I were selected with 1 μ g/mL blasticidin (InvivoGen). Additional control cells were untransfected without any exogenous DNA (mock) or contained the empty vector (pUNO), as indicated. Transfection efficiency was evaluated by semiquantitative and real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis, as well as Western blotting and immunohistochemistry with anti-HA. Before analysis, cells (1.5×10^5 /mL) were cultured for 8 days in all experiments.

CD1 nu/nu Xenograft Model

Female CD1 *nu/nu* mice were purchased from Charles River (Sulzfeld, Germany) and housed under strict specific pathogen-free conditions at the Central Animal Facility, University Hospital of Essen (Essen, Germany). Protocols were in compliance with German law for use of live animals and approved by the Institutional Animal Care and Use Committee at the University Hospital of Essen and the responsible district government. Please see Supplementary Materials and Methods for details.

Human Colon Cancer Specimens

We performed a retrospective, single-center cohort study among the white patients with a diagnosis of colon cancer from March 2001 to November 2010 at the Division of Gastroenterology, Kliniken Essen-Süd (Essen, Germany). The protocol was approved by the Human Studies Committee at Kliniken Essen-Süd. The corresponding formaldehyde-fixed paraffin-embedded tissue collection samples were provided by the Institute of Pathology and Neuropathology, University Hospital of Essen. All tumors classified as adenocarcinoma were pathologically reviewed, and individual blocks were selected based on “maximum tumor cell extent” versus “negative oral/aboral surgical margin (R₀).” For the purposes of the present study, we excluded 17 patients from further analysis due to concomitant lymphoma, leukemia, or autoimmune disease, inadequate clinical staging at initial diagnosis, acute/chronic-active IBD, or polyposis coli. Genotyping of TLR4 (rs4986790: D299G, rs4986791: T399I) and TLR2 (rs5743708: R753Q) was performed by pyrosequencing (varionostic GmbH). Table 1 shows the clinicopathologic features of the remaining 214 cases.

Please see Supplementary Materials and Methods for details on RNA/DNA extraction, microarray analysis, quantitative RT-PCR analysis, protein analysis by immunoblotting,

enzyme-linked immunosorbent assay, Matrigel (BD, Franklin Lakes, NJ) invasion assay, and immunohistochemistry.

Statistical Analysis

The unpaired *t* test was used to calculate differences between means, and the Fisher exact test was used for contingency tables to compare patient proportions (GraphPad Prism version 4.03; GraphPad Software, La Jolla, CA). All tests were 2 tailed, and *P* values of < .05 were considered significant. All data are expressed as means $\pm$ SEM.

Results

TLR4-D299G Induces Aberrant Cytoskeletal Reorganization and Nuclear Atypia

To determine the effects of TLR4-D299G on cellular biology and function in the intestinal epithelium, we stably transfected the IEC line Caco-2 with HA-tagged mutant TLR4-D299G and, as controls, TLR4-WT or mutant TLR4-T399I. Endogenous messenger RNA (mRNA) expression of TLR4-WT was low in Caco-2 cells (regardless of confluency), consistent with previous results in primary human IECs in the healthy intestine.⁴ Equal transfection efficiency of receptor mRNA expression was confirmed in all clones (Supplementary Figures 1A and 4). TLR4-WT or mutants were constitutively glycosylated (Supplementary Figure 1B), implying functional integrity of the receptor.¹⁷ Level of glycosylation was slightly higher in both TLR4-T399I and TLR4-D299G than TLR4-WT, possibly due to posttranslational modifications. However, immunofluorescence with anti-HA showed comparable distribution of stable protein expression between the different clones (Supplementary Figure 1C). As shown in Figure 1A, Caco-2 cells overexpressing the TLR4-D299G mutant displayed significant morphologic changes when compared with the control clones. TLR4-D299G Caco-2 cells showed an elongated, flat shape, assuming a fibroblast-like appearance with actin cytoskeletal disorganization. Similar morphologic alterations were confirmed in 3 different TLR4-D299G stable Caco-2 clones (Figure 1B), in addition to the same clone (#7) at early passage. In contrast, the control clones maintained the typical cuboidal cellular shape with predominantly cortical actin architecture. We observed distinct mitotic abnormalities in enlarged TLR4-D299G Caco-2 cells, including multipolar spindles and misaligned chromosomes (Figure 1C and Supplementary Figure 2A), which were not apparent in the control clones. However, TLR4-D299G or TLR4-T399I Caco-2 cells were found to grow at comparable rates, while TLR4-WT showed decreased proliferation and lower metabolic activity (Figure 1D). Thus, stable expression of TLR4-D299G causes the IEC line Caco-2 to undergo morphologic changes suggestive of neoplastic progression.

TLR4-D299G Activates Genes Primarily Related to Inflammation and Tumorigenesis

To gain insight into the underlying mechanisms responsible for the altered cellular behavior of TLR4-D299G in the IEC line Caco-2, we next performed a broad gene expression profiling analysis to identify target genes specifically affected by the TLR4-D299G mutant. We compared basal mRNA expression between Caco-2-TLR4-WT, Caco-2-TLR4-T399I, Caco-2-TLR4-WT, and Caco-2 mock cells. Through hierarchical clustering of the microarray data sets (Figure 2A and Supplementary Table 1), we identified 281 genes significantly regulated with >2-fold differential expression (TLR4-D299G vs TLR4-WT). TLR4-D299G-dependent transcripts belonged primarily to canonical pathways associated with inflammation and/or metabolism (Supplementary Table 2). Fifty-two probes were identified as the major hit genes with expression that was most differentially changed in TLR4-D299G Caco-2 cells ($\log_2$ ratio cutoff, 2.0). Within this set, 39 genes were interconnected with tumorigenesis, acute phase response, and coagulation as main biologic functions (Figure 2B and Supplementary Figure 3). The Ingenuity (Redwood City, CA) knowledge base annotated 11 genes in this network (Figure 2B and Supplementary Figure 3).

specifically with the main biologic functions of “inflammation” and/or “inflammatory response” (A2M, ALCAM, ANXA1, C5, CD47, CHI3L1, HPX, IL2RG, TF, TFPI, SERPINC1). Real-time RT-PCR analysis of a selection of representative genes validated the gene expression changes observed by the array analysis. mRNA expression of acute phase response/inflammation markers linked to cancer (Figure 2C and Supplementary Table 3) was constitutively increased in TLR4-D299G compared with TLR4-WT or TLR4-T399I. In addition, TFF2 and TFF3 mRNA were significantly decreased in TLR4-D299G (Supplementary Tables 1 and 3), implying severe wound healing defects,^{18,19} yet only minor changes in gene expression levels of other TLRs were observed (Supplementary Figure 4). Enzyme-linked immunosorbent assay analysis of the supernatants (Figure 2D) confirmed baseline secretion of large protein amounts of proinflammatory coagulation and complement factors (A2M, TFPI, C3a, C5a) by TLR4-D299G, which were not produced by the control clones. Stable transfection did not appear to induce endoplasmic reticulum stress, secondarily leading to proinflammatory responses, because Bip/grp78 expression was not altered in any of the clones (Supplementary Figure 5A). These data imply that expression of TLR4-D299G in the IEC line Caco-2 induces enhanced production of distinct mediators of inflammation and cancer.

TLR4-D299G Induces Wnt Signaling and Associated Epithelial-to-Mesenchymal Transition

Wnt-mediated epithelial-to-mesenchymal transition (EMT) is a biologic process that has been implicated in the development and progression of colon cancer.²⁰ Our initial observations (Figure 1A) showed that Caco-2 cells expressing TLR4-D299G exhibit a fibroblast-like morphology suggestive of EMT, and therefore we assessed Wnt signaling and EMT-associated cellular changes in these cells. Expression of Wnt target genes (Cx43, DKK1; EMT markers: Snail2, VIM) was constitutively increased by TLR4-D299G (Figure 3A and Supplementary Table 3). Alterations in Cx43 have been linked to Wnt signaling²¹ and cancer progression²² as well as inflammation.¹⁶ Protein levels of Cx43 were decreased in TLR4-D299G (Supplementary Figure 5A), possibly due to proteasome-dependent degradation.¹⁶ Up-regulation of the Wnt antagonist DKK-1 (Supplementary Figure 5A) failed to inhibit Wnt signaling. As shown by confocal immunofluorescence (Figure 3B and Supplementary Figure 2B and C), TLR4-D299G significantly activated the Wnt/ β -catenin pathway, leading to intestinal epithelial dedifferentiation and a mesenchymal phenotype: (1) phosphorylated β -catenin translocated from the membrane to the cytoplasm and accumulated in the nucleus, (2) E-cadherin redistributed to the cytoplasm, which correlated with loss of cell polarity, and (3) the transcriptional factor Snail2, a central mediator of cell migration, anchored to the plasma membrane. These morphologic changes were not evident in the control cells.

TLR4-D299G Mediates Invasive Tumor Growth via Wnt-Dependent STAT3 Activation In Vitro

Concomitant reorganization of the actin cytoskeleton (Figure 1A) implied acquisition of a more motile intestinal epithelial phenotype by the TLR4-D299G gene variant. To test whether TLR4-D299G functionally induces invasive properties, we used an in vitro 3-dimensional Matrigel invasion assay. As shown in Figure 4A, Caco-2 cells expressing TLR4-D299G were highly invasive and displayed typical branching formations in Matrigel. In contrast, all the control clones that expressed other forms of TLR4 or mock did not show any invasive behavior. In parallel, levels of STAT3 mRNA were increased and nuclear STAT3 protein was constitutively phosphorylated in TLR4-D299G (Supplementary Table 3 and Figure 4B and C). Treatment with the specific STAT3 inhibitor NSC74859 repressed TLR4-D299G-dependent invasion (Figure 4D), implying that TLR4-D299G-mediated STAT3 activation is required for the invasive transformation. Furthermore, blockade of Wnt signaling by quercetin reduced the level of phospho-STAT3 (Figure 4C) and associated

invasion of TLR4-D299G Caco-2 cells (Figure 4D), suggesting that the TLR4-D299G-mediated Wnt pathway is important for persistent activation of STAT3 and the invasive phenotype. Of note, $\text{I}\kappa\text{B-}\alpha$ was phosphorylated in TLR4-T399I Caco-2 cells (Supplementary Figure 5A), but not in any of the other clones, suggesting that STAT3 in TLR4-D299G-induced cancer inflammation does not interconnect with the nuclear factor κB pathway. Although interleukin-6/Gp130 signaling has been linked to induction of STAT3 and development of cancer,²³ we did not detect any modulation of interleukin-6 mRNA in any clone (data not shown). In addition, we investigated whether other oncogenes may be involved, including COX-2 and c-Myc. Although COX-2 mRNA was increased in TLR4-D299G Caco-2 cells (Supplementary Table 3), levels of COX-2 protein were largely comparable between all clones (Supplementary Figure 5A) and administration of a specific COX-2 inhibitor did not influence TLR4-D299G-mediated cellular invasion (Supplementary Figure 5B). In contrast, mRNA and protein expression of c-Myc were comparable among the different clones (Supplementary Table 3 and Supplementary Figure 5A). These findings indicate that TLR4-D299G signaling activates the STAT3 pathway via Wnt, which drives invasive growth of the IEC line Caco-2.

TLR4-D299G Signaling Promotes Intestinal Xenograft Tumor Growth via STAT3 In Vivo

To investigate whether TLR4-D299G drives intestinal tumorigenicity in vivo, we used the CD-1 *nu/nu* mouse xenograft model.²⁴ As shown in Figure 5A and B, intestinal xenograft tumors from Caco-2 cells that expressed TLR4-D299G grew rapidly. Histopathology showed the morphology of highly cellular, undifferentiated adenocarcinoma in TLR4-D299G xenografts, with invasion of neighboring skeletal muscle (Figure 5C). In contrast, tumors from TLR4-WT, TLR-T399I, or mock xenografts failed to grow and began to shrink by day 8 after injection (Figure 5B), as seen previously in regular Caco-2 xenografts.²⁴ Cells were arranged in glandular formations lined by moderately well-differentiated columnar cells, without evidence of invasion (Figure 5C). Systemic treatment with the STAT3 inhibitor, but not control vehicle, resulted in inhibition of tumor growth, leading to degeneration of TLR4-D299G xenografts (Figure 5D). By day 11, sizes of TLR4-D299G xenografts were comparable to those observed of the other, untreated clones, with only few, scattered, and noninvasive tumor cell nests present. These data suggest that TLR4-D299G signaling promotes cancer growth and progression in human intestinal xenografts via STAT3 in vivo.

TLR4-D299G Is Associated With Aggressive Tumor Disease in Human Sporadic Colon Cancer

To assess the role of TLR4-D299G in the pathophysiology of human primary colon cancer, we examined sporadic human colon cancers (Table 1). Thirty-three of the 214 cases (15.4%) carried the D299G allele of the TLR4 gene, all in cosegregation with T399I (heterozygous, 30; homozygous, 3). Thus, the overall frequency of the TLR4-D299G mutation in our cohort was comparable to that previously reported in other European areas.⁸ Only one patient (0.5%) carried the single T399I allele of the TLR4 gene (Table 1), who was not further included in the following analysis. Primary human colon cancers carrying the TLR4-D299G variant showed a more progressive tumor stage distribution when compared with TLR4-WT at initial diagnosis (Table 1; $P < .005$). Advanced disease (International Union Against Cancer [UICC] $\geq$ III, 70% vs 46%; $P = .0142$) and distant organ metastasis (UICC IV, 42% vs 19%; $P = .0065$) at the time of diagnosis were significantly more frequently observed in patients with TLR4-D299G colon cancer than TLR4-WT (Figure 6A). Fourteen of the 214 patients (6.5%) carried the TLR2-R753Q variant (all TLR4-WT), but the overall tumor stage distribution of patients with TLR2-R753Q was comparable with TLR2-WT (Supplementary Table 4; $P > .05$).

No differences in mRNA expression of selected genes (STAT3, Cx43, TLR4, TFPI) were observed in normal, tumor-free (R₀) surgical margins between TLR4-WT and TLR4-D299G colonic tissues from representative patient groups (Figure 6B). However, TLR4 mRNA was significantly induced in sporadic human colon cancers, regardless of genotype. When compared with TLR4-WT, STAT3 mRNA was more increased in the group of TLR4-D299G cancers, but levels of STAT3 mRNA did not correlate directly with tumor stage (data not shown). In addition, significant elevation of Cx43 mRNA levels was confirmed in tumor areas carrying the TLR4-D299G variant, which was not evident in TLR4-WT. In contrast, mRNA levels of TFPI were equally induced in both cancer groups. These data indicate that primary human sporadic colon cancers with TLR4-D299G exhibit progressive tumor behavior associated with increases of STAT3 and Cx43 mRNA levels when compared with those with TLR4-WT.

Discussion

Chronic inflammation is an important component of tumor promotion and progression in the intestine.²⁵ Here, we identify a previously unappreciated role of the common human TLR4-D299G variant as a gain-of-function mutation with enhanced oncogenic potential in the intestinal epithelium. Our findings imply that TLR4-D299G drives malignant progression in human colon cancer.

In the present study, we used 2 different models. First, we stably transfected the IEC line Caco-2 with TLR4-WT, TLR4-D299G, or TLR4-T399I. We performed all experiments using the Caco-2 clones on day 8 after seeding. At that time point, Caco-2 cells are characteristic of an intermediate, noninvasive enterocyte/adenoma cell stage, exhibiting typical morphologic and functional properties of the mature enterocyte (including polarization),²⁶ and thus represent a suitable model to study molecular events involved in IEC differentiation and cancer progression steps. Although Caco-2 cells express a truncated form of APC, nuclear β -catenin does not accumulate and transcription of Wnt target genes is not inappropriately activated in polarized cells,²⁷ consistent with our results in the TLR4-WT, TLR4-T399I, or mock cells. Furthermore, Caco-2 cells harbor no oncogenic RAS mutations. Introduction of TLR4-D299G, but not TLR4-WT or TLR4-T399I, into Caco-2 cells induced dramatic phenotypic changes, resulting in cells that, by several criteria, modeled progression to undifferentiated, invasive carcinoma. Our data show that TLR4-D299G confers a gain-of-function phenotype, mediating significant increases in distinct tumorigenesis- and inflammation-related genes and proteins. Constitutive secretion of mediators of 3 major and interrelated proinflammatory pathways (acute phase, coagulation, and complement) was excessively induced by TLR4-D299G. TLR4-D299G caused up-regulation in expression levels of vimentin and Snail2, nuclear localization of β -catenin, and loss of cell polarity associated with redistribution of E-cadherin, all of which represent hallmarks of cancer-related EMT²⁰ In contrast, control Caco-2 clones did not show secretion of proinflammatory mediators or signs of EMT, which correlated with absence of tumor progression. Neoplastic progression was confirmed in 4 different TLR4-D299G Caco-2 clones, thus excluding effects of gene dosages and incidental or secondary gene mutations. Furthermore, using xenografts in mice confirmed TLR4-D299G-mediated acceleration of intestinal tumor growth with invasion in vivo.

Second, we used human colonic specimens to validate the association of TLR4-D299G with aggressive oncogenic behavior in the pathogenesis of primary sporadic colon cancer in a proof-of-concept study. Primary colon cancer endogenously carrying the TLR4-D299G mutant showed an advanced tumor stage distribution with frequent metastasis, while in TLR4-WT colon cancer, tumor stages were significantly lower at time of diagnosis. We confirmed significant elevation of the inflammation-/tumor-associated genes STAT3 and

Cx43 in association with TLR4-D299G. Regardless of genotype and disease stage, TLR4 mRNA and TFPI mRNA were both enhanced in primary human colon cancer. For this part of the study, we used formaldehyde-fixed paraffin-embedded tissue, which generally yields low amounts of RNA due to degradation and cross-linking. We decided against laser microdissection, because this could have further lowered RNA quantities and thus biased gene profiles, as previously shown.²⁸ Instead, all tumors were pathologically reviewed and those sections with maximal tumor cell extent and no surrounding healthy tissues were selected. We cannot exclude that remaining scattered stroma cells may have influenced tumor cell gene expression; however, in tumor-free margins of colonic specimens, we did not observe significant differences in mRNA levels between TLR4-D299G and TLR4-WT.

All of our patients were of European descent and had the cosegregated D299G/T399I-TLR4 haplotype. Due to evolutionary pressure during migration, distinct (single vs combined) TLR4 polymorphisms have unique distributions in different populations worldwide.⁸ Future studies will need to test the impact of the single TLR4-D299G mutation (which occurs mostly among African populations) in primary human colon cancer. It is possible that the single TLR4-D299G haplotype may have an even more profound phenotype than the combined TLR4-D299G/T399I haplotype in primary human colon cancer (eg, in black Americans who tend to have more advanced disease at diagnosis). In our in vitro studies, we tested the single polymorphisms individually and not the combined TLR4-D299G/T399I haplotype. It remains to be shown whether the single TLR4-T399I contributes to malignant progression in primary human colon cancer. Only one patient with cancer carried the single TLR4-T399I. In contrast to TLR4-D299G, insertion of TLR4-T399I alone in Caco-2 led only to elevation of some proinflammatory and tumorigenic genes, and these events did not mediate functionally cancer progression. It remains to be investigated whether selective activation of nuclear factor κ B by TLR4-T399I may have suppressed tumor growth. Collectively, our findings suggest that TLR4-D299G, and not TLR4-T399I, accelerates transition of colon cancer to invasion and metastasis in established tumorigenic cells.

Activation of the STAT3 pathway represents a central node for many converging cascades²⁹ associated with systemic acute phase³⁰ and cancer-promoting inflammatory responses.²³ STAT3 plays a pivotal role in the control of innate immunity in the intestine. Our findings imply that STAT3 is likely a principal target of TLR4-D299G, because STAT3 was constitutively tyrosine phosphorylated and translocated to the nucleus in TLR4-D299G, but not in the other IEC clones. In addition, a specific STAT3 inhibitor blocked the proliferative and invasive capacity of TLR4-D299G IEC in vitro (Matrigel invasion assay) and in vivo (mouse xenograft model of human colon cancer). Furthermore, STAT3 mRNA was more enhanced in primary human colon cancer expressing TLR4-D299G when compared with TLR4-WT. Wnt regulates normal embryonic development via STAT3.³¹ Our in vitro data suggest that STAT3 activation occurs through a Wnt/ β -catenin-dependent mechanism that drives tumor invasion, implying that TLR4-D299G-induced deregulated Wnt signaling may critically contribute to STAT3-mediated cancer progression of IECs. Downstream, STAT3 activation may lead to increased transcription of many different genes, including Cx43,³² which may drive inflammation and tumor development, thereby possibly establishing a feed-forward loop. Future studies will need to determine whether TLR4-D299G-induced Wnt signaling modules directly recruit STAT3 or other regulatory elements.

The TLR4-D299G variant has been associated with IBD susceptibility.¹⁴ Chronic recurrent intestinal inflammation in IBDs increases the risk of developing colon cancer.²⁵ Previous studies using AOM-induced chemical carcinogenesis in chronic dextran sulfate sodium-induced colitis showed that TLR4-deficient mice that fail to produce prostaglandin E₂ in COX-2-expressing macrophages are resistant to development of colon cancer,^{7,33} implying that complete loss of function in the murine TLR4 gene exerts antitumor effects. However,

our results show that COX-2 was not functionally involved in mediating the TLR4-D299G-induced effects. In contrast, others have shown that MyD88 signaling elicits protective effects via interleukin-18 against induction of colon cancer in the AOM/dextran sulfate sodium model³⁴ but drives intestinal tumorigenesis in *Apc*^{Min/+}.^{35,36} These varied reported effects of TLR4/MyD88 signaling in the development of colon cancer highlight the complexity and variability of experimental murine models, each depending on different immune, genetic, and environmental/commensal contexts.¹ Future studies will need to determine whether TLR4-D299G puts patients with IBD at increased risk for developing a more aggressive phenotype of colitis-associated neoplasia. It will be essential to dissect whether gene-gene and environmental-gene interactions in IBD and colitis-associated cancer may influence the functional outcome of the gain-of-function mutation TLR4-D299G in the intestinal epithelium (and other cell types).

In conclusion, TLR4-D299G-mediated enhanced activation of the STAT3 pathway may contribute to poor prognosis and may serve as a target in colon cancer therapy in this subgroup of patients who are at high risk for developing advanced disease. Further studies in other cohorts are now needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

COX	cyclooxygenase
EMT	epithelial-mesenchymal transition
IEC	intestinal epithelial cell
RT-PCR	reverse-transcriptase polymerase chain reaction
TLR	Toll-like receptor
UICC	International Union Against Cancer
WT	wild-type

Figure 1.

(A) Cell shape and actin cytoskeletal architecture are disturbed in Caco-2-TLR4-D299G (clone #7) but not in the other clones, as visualized by bright field (*upper panel*; objective 10 $\times$; bar = 100 μm) and direct Alexa Fluor 647-phalloidin (*white*) immunofluorescence (*lower panel*; objective 20 $\times$ /0.75; zoom 1.0; bar = 100 μm), respectively. (B) Comparable changes in cytoskeletal reorganization were illustrated by Alexa Fluor 647-phalloidin (*white*) immunofluorescence staining (objective 40 $\times$ /1.3; zoom 1.0; bar = 50 μm) of 4 different TLR4-D299G transfected Caco-2 cell clones (clone #7 shown at earlier passage). (C) Mitotic bodies within multinucleated cells are formed in Caco-2-TLR4-D299G but not in the other clones, as visualized by confocal immunofluorescence (objective 63 $\times$ /1.4, zoom

1.0; *bar* = 20 μm) with staining for β -tubulin (Alexa Fluor 555: *red*) and phospho-histone-H3 (Alexa Fluor 488: *green*). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (*insets*). (A–C) Representative images from a single experiment that was repeated at least twice with identical results. *White arrow* indicates exemplary (A) enlarged cell with (C) aberrant mitosis. (D) Growth curve (*left*) and metabolic activity (*right*) of the Caco-2-TLR4-WT, -TLR4-D299G, -TLR4-T399I, and mock cell clones at 19, 43, and 67 hours, determined by bromodeoxyuridine incorporation and MTS assay, respectively. Data are presented as means $\pm$ SEM for 2 independent experiments ($n = 6$). * $P < .05$; ** $P < .01$; *** $P < .001$.

Figure 2.

(A) Hierarchical clustering analysis of selected gene regulation by TLR4-WT, TLR4-T399I, and TLR4-D299G in Caco-2 cells. Genes (281) that were differentially expressed at levels of mean average >2-fold within the 99% confidence interval in comparison between the data sets TLR4-D299G and TLR4-WT were included. Each row corresponds to a single gene. The column “mock” represents the mean of 3 independent samples and was specified as baseline experiment for all scaling. The columns “TLR4-WT,” “TLR4-T399I,” and “TLR4-D299G” contain the 3 individual samples (#1–#3), which are each compared with baseline. The color scale at the top right corner of the figure uses only the range of relative expression level values from the data displayed in the heat map (log₂ scale). Per-row color

scaling was applied for expression levels (*blue*, low; *yellow*, intermediate; *red*, high). A detailed list of these selected genes is provided in Supplementary Table 1. (B) Functional linkage of top-hit gene expression patterns by Ingenuity pathway analysis. A log₂ ratio cutoff of 2.0 was set to focus only on genes with expression that was most differentially regulated in the microarray data set TLR4-D299G vs TLR4-WT. Of the 52 hits identified in this data set, 39 genes were associated with their main biological functions and/or diseases in the Ingenuity knowledge base. Based on their connectivity, “tumorigenesis,” “acute phase response,” and “coagulation system” were identified. Thirteen gene hits did not fit into these Ingenuity pathway analysis algorithms and were therefore not considered here (*strong red*, highly up-regulated; *strong green*, highly down-regulated). (C) Increase of A2M, C5, CHI3L1, and TFPI mRNA expression in Caco-2-TLR4-D299G, as determined by real-time RT-PCR analysis. Results are shown in relation to expression of Gapdh mRNA. (D) Induction of protein secretion of coagulation (A2M, TFPI) and complement (C3a and C5a) factors in Caco-2-TLR4-D299G, as assayed by enzyme-linked immunosorbent assay. Data are presented as means ± SEM (n = 3). **P* < .05; ***P* < .01; ****P* < .001.

Figure 3.

(A) Up-regulation of mRNA transcription of selective Wnt and/or EMT-associated target genes in Caco-2-TLR4-D299G, determined by real time RT-PCR analysis. Results are shown in relation to expression of Gapdh mRNA. Data are presented as means $\pm$ SEM ($n = 3$). * $P < .05$; ** $P < .01$; *** $P < .001$. (B) Shown (fluorescein iso-thiocyanate: green) are (i) nuclear accumulation of phosphorylated β -catenin, (ii) loss of cell polarity and redistribution of E-cadherin to the cytoplasm, and (iii) translocation and anchorage of the transcriptional factor slug to the plasma membrane in Caco-2-TLR4-D299G, in comparison to the other clones (i and iii: objective 63 $\times$ /1.4 oil; ii: objective 40 $\times$ /1.3 oil; all: zoom 1.0; i and iii: bar = 20 μ m; ii: bar = 51 μ m [3-dimensional distance]), as assessed by confocal immunofluorescence. White arrows indicate exemplary regions of interests. Note variations in cell size of Caco-2-TLR4-D299G. Representative stack scanning (52 stacks; Z-stack depth, 26 μ m) is shown in ii. Red and green lines indicate location of XZ/YZ stacks in XY

stack, and *blue lines* indicate location of XY stack in XZ/YZ stacks, respectively (ii). Nuclei were counter stained with 4',6-diamidino-2-phenylindole (*insets*).

Figure 4.

(A) Caco-2-TLR4-D299G are invasive, as determined by Matrigel invasion assays. Shown is reverse side of the culture insert stained with crystal violet (objective 20×/0.75; *bar* = 50 μ m). (B) STAT3 phosphorylation (Tyr705) is significantly induced in Caco-2-TLR4-D299G, as shown by confocal immunofluorescence (objective 63×/1.4 oil; zoom 1.0; *bar* = 20 μ m). (C) Increased phosphorylation status of STAT3 (Tyr705) in Caco-2-TLR4-D299G cell lysates by Western blotting (in the presence or absence of quercetin or vehicle [dimethyl sulfoxide]). Blot is reprobed with anti-STAT3 (total) and anti-Gapdh. (D) Quercetin (15 μ mol/L) or NSC74859 (100 μ mol/L) blocks invasive growth of Caco-2-TLR4-D299G in Matrigel invasion assays (objective 20×/0.75; *bar* = 50 μ m).

Figure 5.

Caco-2-TLR4-D299G xenografts exhibit substantially enhanced tumor growth in vivo, which is blocked by treatment with a specific STAT3 inhibitor. (A) Gross appearance of Caco-2-TLR4-WT versus Caco-2-TLR4-D299G xenografts (black arrows) at day 22 is shown. Each index in the ruler represents 1 mm. (B) Measurement of tumor volume of the Caco-2-TLR4-WT, Caco-2-TLR4-D299G, Caco-2-TLR4-T399I, or Caco-2 mock xenografts up to day 22. Assessment of (C) histopathology with representative tumor cross sections on day 22 (upper panel: H&E, objective 40×/0.95; bar = 50 μ m; lower panel: confocal immunofluorescence staining with anti-pan-cytokeratin (objective 20×/0.75; zoom 1.0; bar = 100 μ m). White arrows indicate invasion. (D) Measurement of tumor volume of TLR4-D299G xenografts with intraperitoneal treatment of STAT3 inhibitor or negative control (vehicle: dimethyl sulfoxide) up to day 11 (H&E, objective 10×/0.45; bar = 100 μ m). Data are presented as means $\pm$ SEM. ⁺ P > .05; * P < .05; ** P < .01; *** P < .001

Figure 6.

Advanced disease is more often diagnosed in patients with TLR4-D299G colon cancer than TLR4-WT (see Table 1). (A) The stacked bar graphs show the proportion of patients with colon cancer in each subgroup (TLR4-WT vs TLR4-D299G) with or without high-stage (UICC $\geq$ III) involvement (left) and distant or gan metastasis (right) at the time of diagnosis. (B) Expression levels of mRNA transcription of selected genes in formaldehyde-fixed paraffin-embedded human colon cancer specimens (R_0 margin [$n \geq 9$ /group] versus tumor area [$n \geq 20$ /group]) from representative patients with TLR4-WT or TLR4-D299G, as determined by real-time quantitative RT-PCR analysis. Results are shown in relation to

mRNA expression for the housekeeping gene Gapdh. Data are presented as means $\pm$ SEM.
* $P < .05$; ** $P < .01$; *** $P < .001$.

Table 1
Clinicopathologic Patient Characteristics (N = 214)

	TLR4-WT	TLR4-D299G/TLR4-T399I	TLR4-T399I
Total no. of patients with colon cancer	180 (84.1)	33 (15.4)	1 (0.5)
Sex			
Male	77 (43)	17 (52)	1
Female	103 (57)	16 (48)	0
Age (y), median (range) Stage (UICC) ^a	73 (45–92)	72 (39–99)	62
I (T1 or T2, N0, M0)	32 (18)	3 (9)	0
II (T3 or T4, N0, M0)	65 (36)	7 (21)	1
III (any T, any N, M0)	48 (27)	9 (27)	0
IV (any T, any N, M1)	35 (19)	14 (42)	0
Differentiation			
Well-moderate (G1–G2)	141 (78)	25 (76)	1
Poor (G3)	39 (22)	8 (24)	0
Location			
Proximal	86 (48)	15 (45)	0
Distal	94 (52)	18 (55)	1

NOTE. All values are expressed as n (%) unless otherwise indicated.

^a $P < .005$ (TLR4-WT vs TLR4-D299G/TLR4-T399I).