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Potential association between TLR4 and chitinase 3-like 1 (CHI3L1/YKL-40) signaling on colonic epithelial cells in inflammatory bowel disease and colitis-associated cancer

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Abstract

Inflammatory bowel disease (IBD) is a group of inflammatory disorders in the small and large intestines. Several studies have proved that persistent and disregulated host/microbial interactions are required for the development of IBD. It is well known that chronic IBD is strongly associated with an increased risk of developing colorectal cancer by 0.5–1% annually, 8–10 years after the initial diagnosis. To detect the tiny dysplasia or early stage of cancer in chronic IBD patients, a tremendous amount of effort is currently directed for improving colonoscopic technology and noninvasive serological marker development. However, there is only a limited amount of data available to understand the exact mechanism of how long term chronic colitis is connected to the development of colorectal tumors. Recently, our group has identified that significantly increased expression of chitinase 3-like 1 (CHI3L1) molecule in non-dysplastic mucosa from patients with IBD and remote dysplasia/cancer, compared to patients with IBD without dysplasia or healthy controls. CHI3L1 seems to contribute to the proliferation, migration, and neoplastic progression of colonic epithelial cells (CECs) under inflammatory conditions. Furthermore, the CHI3L1-mediated intracellular signaling cascade is likely to interact with TLR4 signaling in CECs. In this review article, we have concisely summarized the cellular and molecular mechanisms underlining the development of IBD and colitis-associated cancer, with particular focus on the CHI3L1-and TLR4-signaling pathways in CECs.

Keywords

mammalian chitinase; inflammation; microbiota; colitis-associated cancer; autoimmunity

Introduction

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a complex disorder characterized by chronic inflammation of the gastrointestinal

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tract[1]. Involvement of bacteria, in particular the presence of commensal bacteria is required for the development of IBD[2, 3]. Data from animal models of colitis suggests that the fundamental mechanism of IBD is much more complicated than previously predicted, and several factors including cell types, tissue specificity, and genetic/environmental factors are tightly involved in the pathogenesis of IBD[4, 5]. Immunological abnormalities are also key factors in this pathogenesis[6]. IBD is most common in developed countries, affecting the quality of life of 1.4 million individuals in the United States[7], and the affected patients will suffer from the chronic inflammation throughout their lives. IBD is characterized by excess immune responses to the intestinal microbiota but differs in the site and nature of the inflammatory pathology depending on epithelial defense, IL-23/Th17 axis, and immune regulation[8–10].

Toll like receptors (TLRs) are type I transmembrane glycoprotein receptors. So far, thirteen TLRs (TLR1-TLR13) have been identified in humans and mice[11]. TLR-mediated adapter proteins and kinases play crucial roles in the following signaling pathways, which is divided into two major pathways, the MyD88 (Myeloid differentiation primary response gene 88)-dependent and TRIF (TIR-domain-containing adapter-inducing interferon-β)-dependent pathways. MyD88 and MyD88-like adapter mediate an early response, while TRIF and TRIF-related adapter molecule leads to the rather delayed cascade[12]. On cell surface, TLR2 forms heterodimer with TLR1 to recognize triacylated lipoproteins or TLR6 to recognize diacylated lipoproteins from Gram-positive bacteria, mycobacteria, or mycoplasma[13]. In contrast, TLR4 homodimer is the main receptor for Gram-negative bacterial LPS[14], of which ligation is conjugated with 3 accessory molecules, including CD14, LPS-binding protein (LBP) and MD-2[15, 16]. TLR4/MD2 expression on intestinal epithelial cells is negatively regulated and is kept at low levels under normal conditions, but is significantly upregulated during the development of IBD[17]. Recently, Ferwerda et al identified two polymorphisms of TLR4 Asp299Gly (D299G) and Thr399Ile (T399I) in populations from Europe, Asia and Africa, which have been positively associated with susceptibility to Gram-negative bacterial infections and septic shock [18]. Furthermore, a recent report revealed that breast cancer patients harboring the TLR4- D299G mutation is related to an increased frequency of cancer metastasis after conventional chemotherapy[19]. The same group also found that the TLR4 mutation in mice reduces the efficacy of both radiation as well as chemotherapy, suggesting a crucial role of TLR4-mediated signaling not only in inflammatory responses but also in cancer development.

Chitinase 3-like 1 (CHI3L1, also known as YKL-40) is classified in the glycosyl hydrolase 18 family based on the structural similarity with other chitinases such as plant and bacterial chitinases[20]. CHI3L1 is produced by restricted types of cells, including osteosarcoma cells, chondrocytes, smooth muscle cells, macrophages, neutrophils and Colonic epithelial cells (CECs)[21]. This protein is frequently found in inflammatory environments; however the factors determining its production in these pathological conditions are particularly unknown[22]. Our group has reported previously that CHI3L1 is highly induced in CECs and macrophages with intestinal inflammation and enhances potentially pathogenic, but not non-pathogenic, bacterial adhesion and invasion on/into CECs[23]. A Recent report from our group also suggests that CHI3L1 plays a major role in inflammation-associated neoplastic changes in CECs, and CHI3L1 may effectively promotes tumor development by enhancing cell

proliferation, migration, angiogenesis, and cell survival in CECs and macrophages[24, 25]. In this review article, we will discuss the potential linkage between TLR4- and CHI3L1-signaling pathways on CECs in inflammatory bowel disease and the subsequent colitis-associated neoplastic processes in epithelial cells.

1. TLR4 expression on colonic epithelial cells (CECs)

TLRs, are a class of transmembrane, non-catalytic pattern recognition receptors whose role is to discern self from non-self by broadly conserved molecular patterns[26]. They are directly involved in the induction of pro/anti-inflammatory genes and the activation of immune responses[27]. TLRs comprise a total of 13 mammalian transmembrane proteins, 10 in humans and 12 in mice, which each contain multiple leucine rich repeat motifs in a large extracellular domain, and a highly conserved region in the intracellular tail named the toll-interleukin receptor (TIR) domain. This TIR domain contains homo and hetero-dimmer sub-units, which interact with receptor ligands, and recruit adapter proteins for downstream cell signaling[28, 29].

TLR4 is the major intracellular signaling complex for lipopolysaccharides (LPS), which are found in gram negative bacteria cell membranes and act as a direct ligand to the receptor[30]. TLR4 is also capable of binding to other exogenous stimuli such as fusion proteins from respiratory syncytial virus[31], envelope proteins from mouse mammary tumor virus[32] and bacterial HSP60[33]. TLR4 is found in a receptor complex requiring several accessory molecules: CD14, LPS-binding-protein (LBP), and MD-2[34]. LPS is transferred to cell surface CD14 by LBP, which then presents the ligand to TLR4 which is bound to MD2. Without interacting with MD2, TLR4 is nonfunctional and unable to induce the subsequent signaling cascade[35]. Recently, it was shown that CD14 is required for the microbe-induced endocytosis of TLR4 to the various cell organelles[36].

TLR4 induces a signaling cascade primarily dependent on MyD88, which is a universal adapter protein used by all TLRs except TLR3. Downstream, TLR4-mediated signaling leads to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein-1 (AP-1), mitogen-activated protein kinase (MAPK) and other transcription factors. However, it is also capable of signaling through MyD88 independent TRIF pathway[27]. Through both MyD88 dependent NF- κB and MAPK activation, and TRIF pathway activation, transcription of inflammatory cytokines are initiated[37].

Under normal conditions within the colonic epithelium, TLR4 expression in human colonic mucosa samples is barely detectable through immunohistochemical staining[38]. TLR4 is down regulated at the cell surface, and the mechanism of hyporesponsiveness in CECs to gut lumen LPS was found to be due to a down-regulation of MD2 and TLR4 expressions. However intracellular TLR4 remained functionally intact, perhaps as a potential defense against pathogenic stimulus, capable of rapidly upregulating TLR4 and MD2 without causing chronic over-activation[39]. Another group found that CECs do not express mRNA or protein for CD14 in the human CEC lines HT-29, Caco-2, and T-84, and mouse CEC line CMT93, and this has been proposed as another mechanism behind CEC hyporesponsiveness[40]. TLR inhibition is required to prevent inappropriate activation despite large amounts of LPS in the gut lumen. It has also been shown that the CECs transfer TLR4

from the cell surface to the cytoplasm to reduce lumenal sensing, as well as up-regulate the TLR downstream inhibitory molecule toll interacting protein (Tollip) to prevent over stimulation[41]. Other inhibitory controls include single immunoglobulin IL-1R -related molecule (SIGIRR) which inhibits TLR4's interaction with MyD88 through its TIR domain[42]. Triad domain-containing protein 3 variant A (TRIAD3A) is a E3 ubiquitin protein ligase which interacts with TIR-domain containing proteins such as toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), toll-like receptor adaptor molecule 1 (TRIF), and receptor interacting protein-1 (RIP-1)[43]. Knockdown of TRIAD3A upregulates NF-κB, while over-expression down regulates NF-κB[44]. Interleukin-1 receptor-associated kinase 1-M (IRAK-M) inhibits MyD88 associated signaling by preventing the dissociation of IRAKs to MyD88 and the formation of IRAK-TRAF6 (Tumor necrosis factor receptor associated factor 6) complexes[45]. A20 is a negative regulator of TLR signaling and a de-ubiquitinating enzyme, which removes K63-linked ubiquitin molecules from TRAF6 to abrogate downstream signaling to NF-κB and other pro-inflammatory cytokines[46]. A recent study has proved that A20 is an early response negative regulator of TLR5 signaling in intestinal epithelial cells during inflammation that regulates the innate immune system in the intestine[47]. Additional negative regulatory molecules include microRNA miR-21, which suppresses programmed cell death protein 4 (PDCD4), an activator of NF-κB, thus regulating the inflammatory response to LPS[48]. Vaccinia virus protein (VACV) A52R interferes with the association of IRAK2 and TRAF6 to prevent complex formation, with this protein being a viral mechanism to suppress host defense[49]. TRAF family member-associated NF-κB activator (TANK) contrary to its name, negatively regulates TLR4 signaling through binding to TRAF6 and preventing its ubiquitination, unlike A20, which removes ubiquintin molecules after they have been attached[50]. ST2825, a synthetic peptido-mimetic compound and a possible strategy for clinical treatment, prevents recruitment of IRAKs by MyD88[51]. In the MyD88 independent pathway, Deubiquitinating enzyme A (DUBA) cleaves the polyubiquitin chains on TRAF3, suppressing production of type I interferons[52]. We have summarized the regulatory mechanisms of TLR4 signaling in Figure 1.

In the mouse colon, TLR4 is located in the apical side of epithelial crypts and in lamina propria mononuclear cells to simplify the task of sensing gut contents[53]. In contrast, in the T84 human CEC line, TLR4 and/or MD-2 were found localized to the basolateral surface membrane. Potentially-pathogenic and pathogenic bacteria that transverse the colonic epithelium may efficiently encounter TLR4 and/or MD2 at this location. This is proposed as a physical mechanism for commensal hyporesponsiveness, while still maintaining the ability to activate pro-inflammatory genes in response to invading pathogens[54].

TLR4 expression is strongly upregulated and its presence detectable through immunohistochemistry in CECs of colonic biopsies obtained from patients with inflammatory bowel disease (IBD) including ulcerative colitis (UC) and Crohn's disease (CD)[17]. In both active and inactive regions of the terminal ileum of CD and UC patients, TLR4 expression was highly upregulated, which reflects a state of hyper-activation and maximization of responsiveness to the gut environment. Presumably, this abnormal expression of TLR4 on CECs may be associated with the exacerbation of chronic inflammation in IBD. Interestingly, differential TLR4-staining pattern was observed in the

basolateral and apical surfaces of the colon in UC and CD patients, respectively. Furthermore, TLR4-positive intestinal epithelial cells were observed in inactive UC and CD[14]. It has been suggested that host tolerance to luminal bacterial components (e.g. LPS) tends to be disregulated in IBD patients[55, 56]. This disregulation is likely to enhance the LPS recognition as a result of upregulated TLR expression on CECs under inflammatory conditions in the gut.

2. TLR4 polymorphism in inflammation and cancer

To date, at least 8 TLR4 receptor single nucleotide polymorphisms (SNP) have been identified[57]. Of these, the A896G (D299G) and C1196T (T399I) missense mutations are the most widely studied. They cause a conformation change in the extracelluar domain of TLR and a blunted response to LPS[58, 59]. These two SNPs have been implicated in several inflammatory disorders as well as cancer.

Both the D299G and T399I mutations have been associated with increased risk of gram negative bacterial infection with septic shock [18, 60], *Helicobacter pylori*-mediated gastric carcionoma[61], and head and neck squamous cell carcinomas in conjunction with increased chemotherapy resistance[62]. Interestingly, these mutations have been implicated in Malaria disease manifestation; with the D299G and T399I conferring, a 1.5- to-2.6-fold increased chance of severe malaria infection respectively. This has been hypothesized to be the result of reduced responsiveness to the *Plasmodium falciparum* glycosylphosphatidyl-inositol[63].

Specifically, The D299G mutation has been shown to inhibit LPS-mediated signaling in airway epithelial cells, and increase the risk of Crohn's disease but not ulcerative colitis[64] [Figure 2]. Recently it has been reported to induce neoplastic progression in intestinal epithelial cells, increase expression of pro-inflammatory genes and proteins, including alpha-2-macroglobulin (A2M), complement component 5 (CC5), CHI3L1, and tissue factor pathway inhibitor (TFPI); and was associated with more advanced and aggressive colon cancers in humans[65]. In breast cancer patients, the D299G mutation was associated with decreased binding of TLR4 to High-mobility group protein B1 (HMGB1); an “alarm” signal released by dying cancer cells that mediates the immune response against cancer, as well as quicker relapse after radiotherapy and chemotherapy[66]. Alzheimer's disease, in which inflammation plays a key role, the D299G mutation was associated with an increased risk of the disease, possibly due to increased production of inflammatory cytokines in the brain and decreased beta-amyloid clearance[67, 68]. An eight-fold increased risk of endometriosis was found in women carrying the D299G allele versus wild-type TLR4 due to increased peritoneal inflammation[69]. In the context of metabolic syndrome, D299G was associated with increased serum insulin levels, homeostatic model assessment of insulin resistance, and family history of type 2 diabetes[70].

Contact hypersensitivity to nickel containing jewelry, piercing, and coins has been shown to be mediated through TLR4 receptor binding, specifically to the Histidine 456 and 458 residues[71]. Mouse macrophages transfected with mutant Histidine 456 and 458 residues showed Ni^{2+} binding only occurs in human TLR4. This effect was independent of LPS signaling and as such, site-specific inhibition of TLR4 could be a potential treatment in patients with this allergy without negatively affecting the immune response.

Surprisingly, these mutations have been associated with a protective role in several conditions including myocardial infarction[72], increased survival after melanoma metastasis[57], and decreased levels of pro-inflammatory cytokines following cardiac surgery[73] and may even confer longevity by decreasing systemic inflammation[74]. Taken together, TLR4 mutations may have a variable role depending on the disease or condition.

3. Mammalian chitinases and chitinase-like proteins

Mammalian chitinases and chitinase-like proteins (CLPs) are members of the glycohydrolase family 18 enzymes[20,21]. Family 18 chitinases are characterized by an eight-fold alpha/beta barrel structure and are represented by bacteria, plants, fungi, insects, viruses, protozoan parasites, and mammals[75,76]. This family includes chitotriosidase and acidic mammalian chithnase (AMCase) which both possess chitinase enzymatic activity[77]. Chitinase activity appears to play an important role in various diseases such as malaria, parasitic and fungal infections[78]. CLPs include CHI3L1, which is also one of the members of glycohydrolase 18 family[79]. CHI3L1 selectively and strongly binds to chitin (a polymer of N-acetylglucosamine) but does not possess chitinase activity[80]. Mammalian chitinases and CLPs are produced by a small number of cell types including human synovial cells, osteosarcoma cells, chondrocytes, smooth muscle cells, macrophages, neutrophils, and CECs[81]. Chitinases are potent stimulators of the innate immune response. It has been shown that stimulation with LPS upregulates chitinase activity in human-monocytes and macrophages. Chitotriosidase is used as a marker for macrophage differentiation because it is expressed by the late stage of activated macrophages[82, 83]. AMCase is highly expressed in the glandular cells of the stomach and intestinal tissues under normal physiological conditions purportedly to aid in host defense and food processing[84]. CHI3L1 plays an important role in the processes of inflammation and it must be a crucial factor during the development of colitis under the controls of pro-inflammatory cytokines and chemokines on CECs[23, 85]. The chitin-binding motif (CBM) within CHI3L1 allows it to bind with chitin or chito-oligosaccharide. This CBM has been shown to be critical in activating the Akt pathway, which is closely associated with exacerbation and chronicity of IBD, as well as stimulating IL8 production, a pro-inflammatory cytokine, in SW480 cells[80]. CHI3L1 in serum has been used as a sensitive biomarker for early detection of several inflammatory disorders including IBD[86]. CHI3L1 mRNA level was found to be significantly upregluated after stimulating with pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNFα), IL-1β and IL-6 in CEC lines[23, 87, 88]. CHI3L1 is known to be a growth factor for connective tissue cells and adhesion molecule for endothelial cells[89]. Biological activities of CHI3L1 include regulation of cell proliferation, migration, and activation[24]. CHI3L1 has been upregulated in not only inflammatory conditions but also various solid tumors and is associated with the disease severity. Elevated level of CHI3L1 in serum was found in breast cancer, colorectal cancer, glioblastoma and malignant melanoma, extracellular myxoid-chondrosarcoma, and Hodgkin's lymphoma[90] and is positively associated with the poor prognosis of the cancers. CHI3L1-mediated signaling actively enhances the pro-inflammatory cytokine productions and cellular proliferation in CECs[91]. AMCase also possesses the ability to exacerbate local inflammation (e.g. allergic conjunctivitis) by facilitating the production of chemical mediators such as monocyte chemoattractant protein-1 and eotaxin-1[21, 92]. From the results, AMCase and CHI3L1 are likely to be used as

major and/or supplementary biomarkers for active inflammations as well as variety of cancers, including breast, ovarian, gastric, prostate, and colon cancer[93].

4. Potential role of CHI3L1 in colitis and colitis-associated cancer

CHI3L1 has been extensively studied in the context of rheumatoid arthritis[94] and asthma[95], two conditions characterized by excess inflammation conditions in joints and lung, respectively. However, its role in colitis and colitis-associated cancer is beginning to be elucidated. There are several types of colitis clinically, including autoimmune mediated- (Crohn's disease and ulcerative Colitis), idiopathic-, iatrogenic-, and infectious-types of colitis. Of note, patients with chronic IBD have a 0.5% to 1% increased risk of developing colitis-associated cancer after 10 years after the initial diagnosis of the colitis[96]. Colonic CHI3L1 mRNA expression is approximately 20-fold increased in patients with UC who harbored remote neoplastic lesions as compared to healthy individuals[24]. This result strongly suggests that CHI3L1 plays a major/direct role in inflammation-associated neoplastic changes in CECs.

CHI3L1 has been found to be increased in murine models of colitis including dextran sulfate sodium (DSS)-induced colitis, IL-10- or TCRα- deficiency-mediated chronic colitis, as well as in human patients with IBD. CHI3L1 was also found to be required for the adhesion and invasion of the pathogenic bacteria strains (e.g. *Salmonella typhimurium* and potentially pathogenic *Escherichia coli*) on/into CECs[23]. CHI3L1 was found to exacerbate the infectious colitis induced by *S. typhimurium* as well as DSS-induced acute colitis in C57Bl/6 wildtype mice. Blocking of CHI3L1 activity utilizing anti- CHI3L1 neutralizing antibodies resulted in improved recovery from the acute phase of DSS-induced-colitis as shown by improved clinical scores and percent body weight loss compared with the normal rabbit IgG-treated control group. Histologically, the antibody-treated group showed significantly less epithelial damage/proliferation and inflammatory cell infiltration in colonic lamina propria. Taken together, these data suggest that the neutralization of CHI3L1 suppresses the colonic inflammation in DSS-induced colitis by reducing the adhesion and internalization of luminal bacteria into the colonic mucosa and eventually suppresses their translocations into the mesenteric lymph nodes, spleen, and liver[23]. Interestingly, CHI3L1 has been associated with poor prognosis and decreased overall mortality in all types of cancer, in particular gastrointestinal neoplasia[92, 97]. CHI3L1 is increased in both IBD and colorectal cancer. Patients diagnosed with Crohn's disease have a 5.6 fold increased risk of colonic adenocarcinoma development[98], suggesting that CHI3L1 may play a pivotal role in the initiation and/or progression of IBD into colon cancer. CHI3L1 has been found to be over expressed in several types of solid cancer (breast-, colon-, lung-, kidney-, pancreas-, ovarian-, prostate-, and uterine carcinoma, osteosarcoma, oligodendroglioma, glioblastoma and germ cell tumors)[89]. Recently, CHI3L1 was found to be increased in the visceral fat biopsies of patients with diagnosed colon cancer. This result suggests that CHI3L1 is not only produced at the site of inflammation and released into serum, but is also secreted as an adipokine by visceral fat[99].

It has been reported that CHI3L1 plays a critical role in tumor angiogenesis by coordinating membrane-bound receptor syndecan-1 and integrin $\alpha_v \beta 3$ as well as stimulating focal

adhesion kinase (FAK) and MAPK, both which are involved in angiogenesis[100]. In human breast cancer, CHI3L1 was correlated with blood vessel density[101]. The molecule was also found to have an effect on extracellular tissue remodeling by binding specifically to collagen types I, II, and III[102], suggesting that CHI3L1 is involved in the processes of fibrillogenesis and tissue remodeling, both which are associated with tumor progression and fibrosis.

It has been previously demonstrated that intestinal epithelial cells express TLR4 and respond to LPS in a time-, dose-, and serum-dependent manner[103]. To examine whether CHI3L1 protein potentially enhances the TLR4 expression on CECs, we stimulated SW480 human colon cancer cells with LPS from E. coli O55:B5 (Enzo Life Sciences, Farmingdale, NY) or purified CHI3L1 (Quidel Corporation, San Diego, CA), which was purified from the culture supernatant of MG-63 cells in serum-free medium as previously described[104]. We selected SW480 CEC line for this experiment since it constitutively expresses TLR4 on the cell surface[105]. As shown in Figure 3, CHI3L1 protein does not stimulate the upregulated expression of TLR4 in SW480 cells after 30 minutes [Figure 3A] or 3 hours [Figure 3B] stimulation. In contrast, LPS significantly activates the expressions of TLR4 at the both time points [Figure 3]. Although CHI3L1 stimulation at the dose of 100 ng/ml does not alter the TLR4 expression, it has been found to activate NF-κB[24] and MAPK p42/p44[80] and phosphoinositide 3-kinase (PI3K)[106] mediated pathways in human synovial cells, fibroblasts, articular chondrocytes or CECs at the same dose. Both the MAPK and PI3K pathways are highly involved in cell growth, proliferation, survival, mitogenesis and apoptosis, as well as in the progress of cancer cellular transformation. It has been suggested that G-protein (guanine nucleotide-binding proteins), which are involved in transmitting chemical signals outside the cells and regulates MAPK-signaling networks, are involved in the action of most non-nuclear oncogenes and subsequent carcinogenesis[107]. These networks may enhance carcinogenic changes of epithelial cells with increased CHI3L1 expression under inflammatory conditions.

The canonical Wnt/β-catenin pathway is known to play a crucial role in UC-associated carcinogenic progression[108]. Our group has identified significantly increased expression and nuclear translocation of β-catenin in SW480 colonic cancer cell line after stimulating with a medium dose (50 ng/ml) of purified CHI3L1 protein[90]. This result suggests that CHI3L1 may play a direct role in inflammation based carcinogenesis by continuously activating the β-catenin pathway.

Recently, it was found that CHI3L1 mRNA was increased by 20-fold in non-dysplastic regions of human colonic biopsies of patients with IBD who had remote dysplasia and/or adenocarcinoma compared with healthy controls as detected by DNA-microarray analysis and RT-PCR[21]. The CHI3L1 message was also significantly increased, but by a lower extent, when compared to quiescent IBD patients without dysplasia. This suggests that CHI3L1 may be a useful and sensitive biomarker in detecting an early stage of colonic dysplasia in IBD patients. Specific expression of CHI3L1 was found in the specific types of CECs including Lgr5+ stem-like cells, Paneth cells and neuroendocrine-type cells in UC patients with dysplasia. In contrast, CHI3L1 expressing cells were not observed in UC patients without dysplasia. This finding strongly suggests that the increased CHI3L1 expression in specific cell types within the colonic crypts must be associated with the

neoplastic transformation of CECs in UC patients. One case in particular, a surgical resection from a patient with UC with mucinous adenocarcinoma and high-grade neuroendocrine carcinoma, had both tumors positively stained with anti-CHI3L1 antibody. In particular, as compared to the mucinous adenocarcinoma, the neuroendocrine tumor showed much more intense CHI3L1 staining in colon, which showed a large CHI3L1-positive metastatic niche formation to the liver. This finding strongly suggests that CHI3L1 may be associated with invasiveness and metastatic ability of malignant tumor cells.

The pro-inflammatory cytokine IL-6 has been shown to be a critical tumor promoter in the early stages of colitis-associated cancer, by enhancing proliferation and suppressing apoptosis[109]. These effects were found to be largely mediated by the activation of STAT3 transcription factor, which is also associated with cancer progression by increasing proliferation and inhibiting apoptosis. CHI3L1 production is enhanced by IL-6 stimulation[23], and as such, blocking IL-6-mediated CHI3L1 may be useful in preventing inflammation and subsequent inflammation-mediated carcinogenesis in epithelial cells[90].

Activation of NF-κB is associated with cell survival, inflammation, and inflammation-associated carcinogenesis[110]. Stimulated SW480 cells with 80 ng/ml of CHI3L1 cells showed activation in select genes including IRAK1, IκB, NF-κB p65, MyD88, and increased NF- κB pathway activation in a dose dependent manner. Phosphorylation of IkBα was also found to be dose-dependent with highest expression at 80 ng/ml by Western blot analysis. Enhancement of the pro-inflammatory cytokine TNFα and chemokine IL-8 were also found to be dose-dependent at the levels of the previous stimulations. CHI3L1 has also shown potent epithelial growth stimulating effects in vitro. After treatment with CHI3L1 Colo205 and SW480 cell lines showed increased proliferation evaluated by BrdU (bromodeoxyuridine)-incorporation index[21]. This growth stimulating effect was found to be similar to IGF-1, a well-characterized growth factor for CECs. CHI3L1 was also found to increase cellular migration of SW480 cells in a Boyden chamber assay by six-fold. This effect was significantly diminished after incubating the cells in a rabbit anti-CHI3L1 polyclonal antibody, suggesting a CHI3L1-mediated cell migratory effect.

Taken together, it appears CHI3L1 plays a pivotal role in enhancing the adhesion and invasion of potentially pathogenic bacteria on/into CECs and in initiating/perpetuating colitis. CHI3L1 has also been discovered to be a valuable biomarker for cancer progression and prognosis. The recent works by our group have bridged this information in the context of colitis-associated cancer. CHI3L1 induces the production of inflammatory mediators, which are crucial in colitis-associated neoplasia formation as a result of the activation of MAPK- and NF- κB-signaling pathways[24, 25]. CHI3L1 highly contributes to cellular proliferation, survival, migration, and angiogenesis, which all play a critical role in maintaining the tumor-microenvironment.

5. Differential linkage between TLR4 and CHI3L1

The link between CHI3L1 and TLR4 has not been fully understood. Treatment of SW480 cells with TLR4 ligand LPS for 2 and 6 hours both exhibited a down-regulation of CHI3L1 expression [E. Mizoguchi, unpublished observation]. The 299th and 399th amino acids mutations in TLR4 protein has been described as a “loss-of-function” mutation which leads

to a hypo-responsive effect with blunted activation of NF- κB and decreased secretions of pro-inflammatory cytokine IL-1α after stimulation of airway epithelial cells with LPS[59]. It has been shown that Caco2 cells transfected with the D299G or T399I mutation both had significant increases in CHI3L1 mRNA[65]. This demonstrates a direct link between these TLR4 mutations and enhanced production of CHI3L1. In fact, we have identified the D299G/T399I mutations in SW480 CEC cells, and this could explain the downregulated responsiveness against LPS and upregulated endogenous expression of CHI3L1 in this cell line[23].

The D299G mutation in TLR4 was found to constitutively increase the expressions of Wnt target genes Connexin 43 and Dickkopf-related protein 1[62]. Similarly, the nucleic translocation of β-catenin after stimulating with CHI3L1 in SW480 cells was increased[90]. This suggests there is an indirect or perhaps direct, co-stimulatory effect of Wnt and/or PI3K/Akt signaling pathway activation[111]. In addition, Stat3 phosphorylation was found to be induced in Caco-2 cells[65], which have the TLR4 D299G mutation. As our group previously reported, CHI3L1 is induced by the proinflammatory cytokine IL-6, one of the potent signal transducers of STAT3[23]. It appears the D299G mutation in TLR4 and the upregulated CHI3L1 expression synergistically activate the β-catenin signaling pathway, which will be associated with the neoplastic change of CECs, subsequently. The two may work indirectly but additively to activate many of the proinflammatory pathways or other pathways that result in the disregulated function of epithelial cells. It can be speculated that colon cancer patients with elevated CHI3L1 that are also carriers of the D299G/T399I mutations in TLR4 will have a worse cancer prognosis and increased metastasis versus patients with elevated CHI3L1 and wild-type TLR4.

Another important and critical proinflammatory cytokine, TNFα, was found to increase the CHI3L1 expression in SW480 and T84 CEC lines[23]. Recent reports have shown that the T399I mutation in TLR4 enhances the expression of TNFα message, which is associated with the risk of nasopharyngeal carcinoma development[112]. This represents a potentially more direct link between the T399I mutation in TLR4 and excessive CHI3L1 expression in nasopharyngeal epithelial cells via TNFα.

In order to further elucidate the linkage between CHI3L1 expression and TLR4 mutation, our group has recently examined the presence of the TLR4 D299G and T399I mutations in various CEC lines by sequencing the PCR products, which were amplified with D299G and T399I specific primers as previously published[113]. As summarized in Table 1, we examined four separate human CEC lines, since these SNPs are associated with neoplastic changes, and thus cancerous cells would more likely exhibit these mutations. This information is also pertinent for IBD patients who may be placed at an increased risk for colorectal cancer. Both D299G and T399I mutations were identified in Caco2 and SW480 cells, which express the message of CHI3L1 endogenously[20]. In contrast, HT-29 cells do not have D299G mutation and lack the endogenous expression of CHI3L1. Therefore, we hypothesized that the presence of TLR4 mutations may directly or indirectly modulate the endogenous expression of CHI3L1. Presumably TLR4 D299G and T399I mutations keep the endogenous CHI3L1 levels aberrantly high, which may prevent the negative regulation of CHI3L1 expression after LPS/TLR4 ligation on CECs.

6. Future prospective

The D299G and T399I mutations in TLR4 seem to constitutively increase the expressions of CHI3L1 in CECs. It has been demonstrated that these mutations are associated with the carcinogenic changes of CECs[62]. However, their role in conjunction with CHI3L1 has yet to be completely unrevealed. It is likely that the coexistence of one or more TLR4 mutations and elevated CHI3L1 work synergistically to further promote carcinogenic changes of CECs under inflammatory conditions. Since SW480 cells show a blunted down regulation of CHI3L1 after stimulation with LPS, and patients with the D299G mutation were found with more advanced tumor grade and metastasis, perhaps these factors play together in a positive feedback loop increasing CHI3L1 expression in CECs, which potentially promotes the carcinogenic change of CECs in IBD. Further *in vitro* studies will be required to solidify this connection. Subsequent *in vivo* studies could be employed to determine the systemic effects of this mutation with CHI3L1-associated carcinogenic change of CECs. The presence of both TLR4 mutation and elevated CHI3L1 expression may represent co-morbidities, and could have important prognostic consequences in the diagnosis/treatment for patients with inflammatory disorders including IBD and colitis-associated cancer.

Conclusion

TLR4 represents a critical component of innate immune responses, of which its main function is sensing the existence of gram-negative bacteria in the gut. It is known that TLR4 expression is barely detectable under normal physiological conditions, but is strongly upregulated under inflammatory conditions including IBD. Two common missense mutations, D299G and T399I, alter the function of TLR4. In particular, D299G mutation leads to a hypo-responsive effect to LPS and both are associated with increased inflammation and risk of infection in several disorders. However, these mutations may also be protective in preventing systemic inflammation in several other disorders including myocardial infarction. D299G and T399I mutations have been associated with radio-resistance of head and neck squamous carcinoma cells as well as promoting the epithelial-mesenchymal transition, increased tumor grade, and metastasis in colon cancer. We here hypothesize that one of the potential mechanisms by which these mutations may increase the rate and severity of carcinogenesis is by increasing the expression of CHI3L1, which is involved in tissue remodeling, angiogenesis, and tumor progression. CHI3L1 is upregulated in many inflammatory disorders and cancers including IBD and colorectal cancer. The D299G mutation and cellular CHI3L1 stimulation were both found to independently activate the β-catenin trans-nucleic localization, which is associated with tumor initiation and progression.

Further research is required to determine and clarify the complete relationship between the TLR4 mutations and the CHI3L1 overexpression and their role in inflammatory disorders and cancer formation. We strongly hope that this mini-review would provide us some clues in developing the diagnostic as well as therapeutic strategies for patients with IBD and colitis-associated cancer in the near future.

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Abbreviations used

AMCase	acidic mammalian chitinase
CECs	colonic epithelial cells
CLPs	chitinase-like proteins
CD	Crohn's disease
IBD	inflammatory bowel disease
IECs	intestinal epithelial cells
TLRs	toll-like receptors
UC	ulcerative colitis
WT	wild-type
LPS	lipopolysaccharide
CHI3L1	Chitinase 3-like-1
TIR	toll-interleukin receptor
LPD	Lipopolysaccharide binding protein
MYD88	myeloid differentiation primary response gene 88
NF- κB	nuclear factor kappa-light-chain-enhancer of activated B cells
AP-1	activator protein-1
MAPK	mitogen-activated protein kinase
TRIF	TIR-domain-containing adaptor-inducing interferon-γ
Tollip	toll-interacting protein
SIGIRR	single immunoglobulin IL-1R -related molecule
TRIAD3	triad domain-containing protein 3 variant A
TIRAP	toll-interleukin 1 receptor domain containing adaptor protein
RIP-1	receptor interacting protein-1
IRAK	Interleukin-1 receptor-associated kinase
TRAF6	Tumor necrosis factor receptor associated factor 6
SNP	single nucleotide polymorphism
A2M	alpha-2-macroglobulin

CC5	complement component 5
TFPI	tissue factor pathway inhibitor
HMGB1	High-mobility group protein B1
TNF-α	tumor necrosis factor alpha
DSS	dextran sulfate sodium
TCRα	T-cell receptor alpha
Brd-U	Bromodeoxyuridine
FAK	focal adhesion kinase
PI3K	phosphoinositide 3-kinase
STAT3	Signal transducer and activator of transcription 3
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
PCR	polymerase chain reaction

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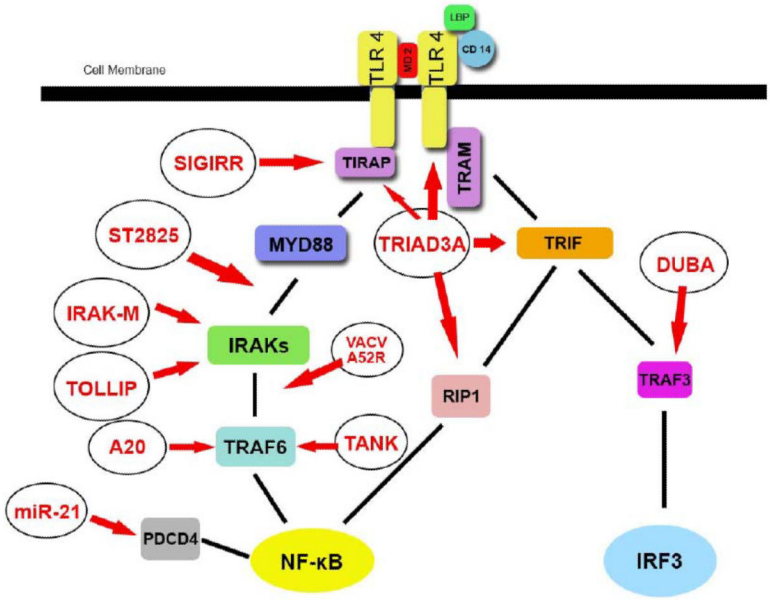
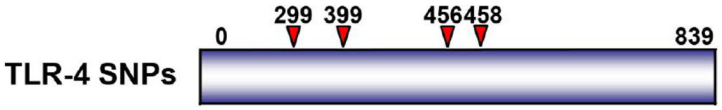


Figure 1. Schematic of TLR4 negative regulators

Arrows indicate sites of inhibition. Sigirr inhibits the initiation of this pathway by preventing TLR4's interaction with MyD88. IRAK-M and TOLLIP both prevent the dissociation of IRAKs from MyD88. TRIAD3A has several mechanisms of inhibition by interacting within the Toll-interleukin-1 receptor domains of TIRAP, TRIF, and RIP1. Overexpression of TRIAD3 also degrades TLR4. A20 is a de-ubiquitinating enzyme which removes ubiquitin moieties from TRAF6 to prevent downstream signaling. miR-21 is a microRNA targeting the protein PDCD4 and preventing activation of NF-κB. VACV A52R is a viral protein that prevents the formation of IRAK TRAF6 complexes. TANK prevents the ubiquitination of TRAF6 suppressing further downstream signaling. ST2825 a synthetic compound, prevents MyD88's association with IRAKs. DUBA cleaves polyubiquitin chains from TRAF3 to suppress type I interferons.



Mutations	Resulting conditions	References
Asp299Gly mutation	IBD	64
	Colon cancer	64
	Breast Cancer	66
	HNSCC	62
	Endometriosis	69
	Alzheimer's Disease	67,68
	Chemotherapy/Radiotherapy resistance	66
	Insulin Resistance	70
Thr399Ill mutation	IBD	64
	HNSCC	62
	Gastric Cancer	61
Histidine 456, 458	Ni^{2+}contact hypersensitivity	71

Figure 2. Amino acid locations of TLR4 SNPs and Histidine residues involved in nickel hypersensitivity within the TLR4 peptide sequence

Below is a list of conditions associated with the TLR4 SNPs and histidine residues. Abbreviations: IBD, inflammatory bowel disease; HNSCC, head and neck squamous cell carcinomas.

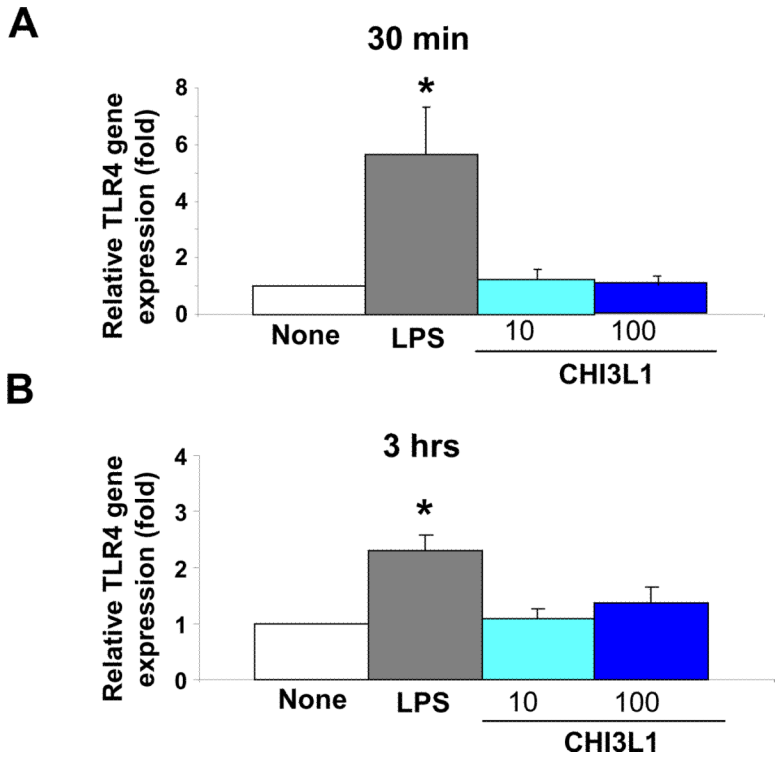


Figure 3. No enhanced activation of TLR4 in colonic epithelial cells by the stimulation with purified Chitinase 3-like 1 (CHI3L1) protein

SW480 human colon cancer cells at 95 % confluency were cultured without (none) or with low (10 ng/ml) or high (100 ng/ml) concentrations of purified CHI3L1 protein for 30 minutes (A) or 3 hours (B). As an internal control, cells were stimulated with LPS O55:B5 at high dose (≥10 μg/ml). Quantitative RT-PCR analysis for human TLR4 (Forward: 5'-AGACCTGTCCCTGAACCCTAT; Reverse: 5'- CGATGGACTTCTAAACCAGCCA) mRNA was performed in each group (n=6). CT values were normalized to the housekeeping gene (GAPDH) and shown as average ± standard error. **P*<0.05.

Table 1

CHI3L1 expression and TLR4 mutations in CEC lines

Cell Line	CHI3L1 Expression	D299G Mutation	T399I Mutation
SW-480	(+)*	(+)	(+)
Caco-2	(+)	(+)	(+)
Colo-205	(-)**	(+)	(+)
HT-29	(-)	(-)	(+)

* Presence of CHI3L1 expression or TLR4 D299G/T399I mutations.

** absence of CHI3L1 expression or TLR4 D299G/T399I mutations.