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Inflammation and colorectal cancer: colitis-associated neoplasia

Sergei I. Grivennikov

Fox Chase Cancer Center, Cancer Prevention and Control Program, 333 Cottman Avenue
Philadelphia, PA, 19111-2497, USA

Abstract

Connection between inflammation and cancer is a rapidly developing field. Epidemiological data suggests that inflammation along with distinct arms of host immune system plays a very important role in development and progression of many different cancers. Inflammatory bowel diseases (IBD) is an important risk factor for the development of colon cancer, namely colitis-associated cancer (CAC). The molecular mechanisms by which inflammation promotes cancer development are still being uncovered and may differ between CAC and other forms of colorectal cancer. Recent work has shed light on the role of distinct immune cells, cytokines and other immune mediators in virtually all of the steps of colonic tumorigenesis, including tumor initiation and promotion as well as progression and metastasis. The close proximity of colonic tumors to the myriad of intestinal microbes, as well as instrumental role of microbiota in IBD, introduces microbes as new players capable of triggering inflammation and possibly promoting tumorigenesis. Various mechanisms of CAC tumorigenesis as well as new possible hints for the future approaches for prevention and therapy are discussed in this review.

Keywords

inflammation; colon cancer; inflammatory bowel disease; cytokines; bacteria

Introduction

Colorectal cancer (CRC) is the third common malignancy and one of the major causes of cancer-related death [1]. Since only about 20% of CRC cases can be genetically attributed to familiar history[2], the outstanding question is about the involvement and mechanisms by which overall environmental factors contribute to disease. Colitis associated cancer (CAC) is the type of colon cancer which is preceded by clinically detectable inflammatory bowel disease (IBD), such as Crohn's disease (CD) or Ulcerative colitis (UC) [3-6]. UC increases cumulative risk of CAC by up to 18-20%, while CD up to 8% after of 30 years of disease [5, 7, 8]. The overall exact increase in prevalence of CAC in IBD patients depends on disease severity and duration, patient groups analyzed, accessibility to preventive colonoscopies in normal cohorts and efficacy of anti-inflammatory therapies and IBD management [9-13]. In mouse models, only single injection of carcinogen azoxymethane (AOM) give rise to multiple colonic tumors, when coupled to the induction of chronic colitis [14, 15], while it takes multiple injection of carcinogen and longer time for tumors to form when inflammation is absent. These clinical and experimental observations clearly pinpoint CAC as classical inflammation-driven cancer and make mouse models of CAC extremely valuable for our understanding of general mechanisms which connect inflammation and cancer [16-18].

Tumor initiation in CAC, inflammation matters?

Since CRC tumors do not arise in the context of preceding inflammation, it is unlikely that inflammation plays a decisive role for CRC initiation. However, in the case of CAC clinically detectable IBD always precedes (sometimes by decades) tumor initiation and actually CAC risk directly correlates with the severity and longevity of active disease. How inflammation favor and what are the signaling nodes for the intersection of inflammatory and oncogenic pathways? Stepwise analysis of CAC and CRC development seems to be helpful in answering these questions. CRC tumorigenesis can be presented as a sequence of genetic alterations proposed by Fearon and Vogelstein as a ‘genetic pathway to colorectal cancer’ [19]. Wnt/ β -catenin signaling pathway is instrumental for the renewal of intestinal epithelium and is a critical regulator of normal and malignant cell proliferation. In sporadic CRC, mutations which lead to activation of Wnt/ β -catenin pathway happen early in over 90% of cancers, including the most often mutations in adenomatous polyposis coli (APC) tumor suppressor gene; GSK3 β a kinase, which controls APC and β -catenin stability and β -catenin itself [20-24]. Aberrant activation of Wnt/ β -catenin pathway not only ensues proliferation of cells, but also induces their “arrest” as they move towards the end of the crypt and prevents shedding of transformed cells [25-27]. Although in mouse models of CAC activatory β -catenin mutations can be found [28], current agreement is that mutations in APC and other activatory mutations happen rather late during the disease progression and follow earlier mutations in p53 and K-Ras, quite differently from what is observed during CRC development [6, 27, 29]. What is the explanation for the fact that despite its pivotal role in proliferation and tumor development, genetic alterations in Wnt/ β -catenin arm of signaling are observed late during CAC development? Several lines of evidence suggest that different inflammatory pathways can enhance β -catenin signaling in the absence of mutations. Mice deficient for IL-10 housed in conventional facilities, spontaneously develop colitis and later on, colorectal tumors, which typically do not display APC mutation but exhibit elevated nuclear activity of β -catenin [30, 31]. Several inflammatory pathways, including of NF- κ B, PI-3K and Akt pathways [32-34] can drive β -catenin nuclear accumulation even without any mutations in APC [35]. Cytokines, like TNF or soluble mediators such as prostaglandin E₂ (PGE₂), are overexpressed during inflammation and can be particularly responsible for activation of ERK, NF- κ B and PI3K-AKT pathways in epithelial cells, thereby increasing β -catenin signaling [36-38]. The initial step, which requires APC mutations to initiate CRC tumorigenesis, therefore, in some cases may be bypassed by inflammatory signals during initiation of CAC development. Since genetic alterations may be absent at this point, the assumption would be that inhibition of inflammation during CAC development sometimes can revert the growth and formation of intestinal tumors. Indeed, gut sterilization in IL-10^{-/-} mice decreases colitis and causes eradication of tumors (REF). While inflammation can temporarily bypass the mutation requirement for tumor initiation, next important hallmark of inflammation in cancer is actually its **ability to cause mutations**. Indeed, IBD and colitis induces robust genotoxic response [39], the fact also illustrated by decreased resistance of mice deficient in ATM (a molecule, critical for DNA damage response and repair) to dextrane sulfate sodium (DSS) colitis [40]. Inflammatory cells, such as activated macrophages and neutrophils, are potent sources of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), highly reactive and mutagenic entities which can DNA damage and mutations [41-44]. In genetic search of early oncogenic events in CAC, mutations in p53 gene have been identified, not only in dysplasia or carcinoma areas, but also in inflamed otherwise normal intestinal mucosa [45]. In humans, a chicken-and-egg problem nevertheless can be defined, whether the source of mutagens is primarily food-borne and inflammation just increases the accessibility of mutagens and intestinal stem cells, or whether noxious inflammatory products such as ROS, directly cause most of the DNA damage and tumor-initiating mutations. In mice kept in clean animal facilities on standard food, however, it is clear that

prolonged chronic inflammation by itself can induce detectable DNA damage and intestinal tumors, in the absence of externally added mutagen [41], regardless whether colitis is caused by chemical injury or by colitogenic infection [41, 44, 46-49]. Inhibition of inducible NO-synthase, responsible for the production of RNI, or various approaches aimed at inhibition or scavenging ROS have been demonstrated to be effective to decrease DNA damage and delay intestinal tumorigenesis [42, 50-52]. It is worth to note that DNA damaging agents can be produced both by immune cells and also by epithelial and pre-malignant cells, and the latter can be primed to produce ROS and RNI by inflammatory cytokines, such as TNF and IL-1 [50, 52] (Figure 1). Further acceleration of CAC tumorigenesis by inflammation and inflammation-derived endogenous DNA damaging agents can be linked to oxidative inactivation of genes encoding DNA repair components, such as mismatch repair (MMR) [27, 42, 53]. On the other hand, it seems that ROS are required for the perpetuation of IBD, as inhibition of ROS by nanoparticles reduces IBD symptoms, may be via reducing the damage to epithelium and restoration of protective functions [54]. Further contribution of inflammation into direct mutagenesis within epithelial cells is illuminated by the ability of inflammatory cytokines to increase synthesis and activity of activation-induced cytidine deaminase (AID) enzyme, which is overexpressed in many cancers and causes mutations and genetic instability [55]. Indeed, sole targeting AID genetically or potentially pharmacologically, disrupts colorectal tumorigenesis [56].

Recently, it has become increasingly clear that somatically inheritable changes in cancer are not limited to mutations but also can include various *epigenetic changes*, which are further stably transmitted to oncogenic progenitors of tumor-initiating cells [27]. The importance of various inflammation driven epigenetic mechanisms in gene-silencing during IBD and CAC has been demonstrated. Methylation of histones and DNA in the loci with various tumor suppressor genes, such as APC and INK4a, and genes controlling DNA stability, such as MLH, has been reported [53, 57-61]. DNA methyl transferases Dnmt1 and Dnmt3 were shown to be activated by inflammatory signaling, particularly by cytokines such as IL-22 and IL-6, produced in UC and CAC [62-64]. These methylases have a broad impact on global methylation profile in normal and cancer cells [65] and required both for tumor initiation and tumor promotion in mouse models of CRC, as defined in *Apc^{min}* mice) [66].

Another mechanism of epigenetic silencing involves action of miRNAs, whose genes may be induced by inflammatory signaling. As a proof of principle, several genes known to be important in CAC and CRC development are subject to regulation by miRNA, including TGF β RII, MSH, K-Ras, Smad4, PTEN as well as APC [27, 67]. Given that over 500 miRNA's have been identified in mammalian genome, their expression can be influenced by many different mechanisms and that most of the miRNA's have quite a few targets [68], we should expect that the list of examples of miRNA controlling the expression of key genes in colitis and CAC will dramatically expand in the next few years. Particularly, several miRNA were identified, whose levels are upregulated during the transition of normal tissue to dysplasia, namely miR-122, miR-181a, miR-146b-5p, let-7e, miR-17, miR-143) [69]. However, these miRNA were downregulated as neoplastic tissue progressed to cancer [69] but at least two of them (let-7e and miR-17) were interfering with p53 pathway [69]. On the border between its role in tumor initiation (alterations, which lead to the emergence of pre-neoplastic cells and initial pre-cancerous lesions) versus its role in tumor promotion (proliferation and growth of initial lesion into a full-blown tumor) lies the role of inflammation in tissue injury and cell death. While that aspect has been reviewed elsewhere [70], some of the basic concepts ought to be mentioned. The first hint that tissue injury is important for tumor initiation and CAC tumorigenesis comes from epidemiological observation that CD confers smaller increase in CAC risk than UC [5, 7]. UC is often manifested with profound mucosal injury, including large ulcerations of mucosa, where entire crypts are subjected to cell death and are substituted with inflammatory infiltrates. As dying

mutated cells cannot give rise to tumor, it seems to be essential for CAC tumor initiation that these cells survive adverse conditions of chronic inflammation and injury. Inflammation results in production of cytokines and growth factors, which can act on epithelial and pre-malignant cells and protect them against apoptosis and other forms of cell death [17, 71]. Genetic ablation of NF- κ B activation or STAT3 expression in intestinal epithelial cells blocks the expression of several anti-apoptotic genes, including Bcl-xL, Bcl-2 and c-IAP, and renders cells susceptible to apoptosis caused by various stimuli, including chemically induced colitis [28, 72, 73]. The observed increased epithelial cell death and tissue injury in the absence of NF- κ B or STAT3 activation in epithelium results in more severe colitis, which typically would be expected to lead to enhanced tumorigenesis. Remarkably, however, a very pronounced decrease in tumor numbers is observed [28, 72, 73]. The likely explanation is that inflammation through NF- κ B and STAT3 enhances the resistance to cell death, and as more mutated pre-neoplastic cells are given a chance to survive, more tumors form. The second possible role of inflammation-induced injury and cell death in CAC tumor initiation originates from the requirement of pre-neoplastic cells to divide and expand to give rise to bulk of cell forming tumor. In mouse models, tumor development in response to carcinogen AOM or in genetic APC^{Min} model may be promoted by various colitogenic chemicals or infectious agents, which cause ulcerative colonic injury, such as DSS, oxazolone or enterotoxigenic bacteria *Bacteroides fragilis* [74-77]. Injury and ulceration induce wound healing-regeneration responses, which includes migration of stem cells, their enhanced proliferation and crypt fission and expansion to fill in for damaged mucosa. If these cells harbor oncogenic mutations, local repetitive injury and regeneration will instigate their proliferation and tumor formation [70]. The ability of inflammation to cause tissue injury potentially intersects with the ability of inflammation to cause oncogenic mutations described above. Although difficult to unequivocally demonstrate in experimental system, it is tempting to speculate that food borne mutagens are getting much better access to intestinal cells when tissue architecture is disrupted. Taken together, inflammation plays an important role in CAC tumor initiation, acting through several distinct mechanisms, including increasing activity of key oncogenic pathways, causing mutations and epigenetic changes in tumor initiating cells, promoting their survival and ensuring their inclusion into tissue regeneration program (summarize on Figure 1).

Inflammation as a stimulator of CAC growth- role of inflammation in tumor promotion

As tumor promotion in humans takes a lot of time (sometimes, decades) and precedes tumor progression and spread, targeting inflammation at the stage of tumor promotion will create an unique preventive and therapeutic window to extend the timeline when a tumor can be detected and removed before it spreads. Historically, also the ability to increase tumor growth and thereby to drive tumor promotion is the best defined feature of inflammation characterized by various genetic and molecular studies. During extensive genetic modeling of IBD and CAC in a last decade several interesting typical phenotypes were observed. First, molecules, whose inactivation leads to decrease in intestinal inflammation concomitant with reduction in CAC tumorigenicity. Second, molecules, whose inactivation results in exacerbation of both IBD and CAC. These two groups of molecules represent important positive and negative regulators of intestinal inflammation, and since CAC is promoted by inflammation, the mechanistic role of these molecules in IBD but not in CAC per se can be deduced from these experiments. Third interesting group of molecules actually regulates IBD in CAC in opposite directions, i.e. inactivation of such molecule aggravates IBD but despite increased local inflammation, CAC tumorigenicity is reduced. Such molecules can be deemed critical for the cross-talk between inflammation and oncogenesis.

First of all, the propensity of inflammation to drive tumor promotion directly stems from its ability to influence tumor initiation. Indeed, most of the effects of inflammation discussed above, such as the ability to cause mutation and epigenetic changes, as well as its causative involvement into tissue injury and regeneration continue to serve oncogenesis during tumor promotion stage. More genetic and epigenetic alterations, both cancer driving and passenger, accumulate as tumor grows and progresses and particularly K-Ras, B-Raf and p53 mutations are essential for tumor progression. Initial important observations in CRC revealed that consumption of non-steroid anti-inflammatory drugs (NSAIDs) such as aspirin and specific COX inhibitors reduces the incidence of CRC but also results in decrease in tumor growth [78]. Meanwhile, IBD, the underlying condition of CAC development, is inevitably associated with increased activation of transcription factor NF- κ B, which is a master-regulator of inflammatory responses [79, 80]. Notably, during IBD, NF- κ B is hyperactivated in both immune and epithelial cells as well as in many types of cancer, including CRC and CAC [28, 81, 82]. Inhibition of p65 expression in mice with anti-sense oligonucleotides impedes IBD development [83] and many current therapies of IBD, such as sulfasalazine, mesalamine and methotrexate are directly or indirectly targeting NF- κ B activation [27, 84]. A milestone discovery in the field came when NF- κ B activation was ablated specifically in epithelial cells, by virtue of conditional knockout of IKK β , a kinase involved in classical NF- κ B pathway activation [81]. In a mouse model of CAC, exposure to colonic irritant DSS caused increased apoptosis of epithelial cells lacking NF- κ B activity and therefore, not surprisingly, increased injury and aggravated chemically induced colitis [28]. What was initially surprising is that despite increased intestinal inflammation these mice developed much fewer CAC tumors, presumably because inflammation can stimulate tumorigenesis only as long as transformed cells retain their ability to survive and proliferate amid adverse conditions. Because of the NF- κ B inactivation, more epithelial cells underwent apoptosis and less transformed cells survived, in full agreement with the role of NF- κ B as transcriptional regulator of anti-apoptotic gene expression program [71, 85]. Quite different results were obtained when NF- κ B activity was ablated in myeloid cells, using LysMCre conditional deleter and IKK β floxed mice. Both CAC tumor size and tumor multiplicity were significantly reduced [28]. Since NF- κ B in myeloid cells controls the expression of multiple inflammatory cytokines and its inhibition indeed can ameliorate IBD development [28, 86], it has been suggested that NF- κ B dependent cytokines are critical mediators which signal from inflammatory cells to malignant cells in microenvironment [73]. Some of these tumor-promoting cytokines, including TNF and IL-1 [87, 88] have the ability to activate NF- κ B in malignant or epithelial cells, thereby completing the link between immune and epithelial NF- κ B activation. These results collectively implied NF- κ B driven inflammatory response into survival and proliferation of pre-malignant cells and served as first solid genetic proof for the connection between inflammation and cancer [28]. Since the levels of NF- κ B dependent and NF- κ B activating cytokines, such as TNF and IL-1, are elevated in IBD and CAC [88-90], and their inhibition is currently clinically used to treat IBD, these cytokines may represent potential attractive targets for CAC prevention and treatment.

Another critical signaling hub in intestinal epithelial cells is represented by transcriptional factor STAT3. Activation of STAT3, particularly by cytokines, but also via cell intrinsic mechanisms, is important for regulation of anti-apoptotic genes such as Bcl-xL or Bcl-2, tissue-resistance factors, such as Hsp70, RegIII and S100A9, cell cycle regulators, like Cyclin D1 or c-Myc and angiogenic factors, such as bFGF and VEGF [71, 91]. Indeed, inactivation of STAT3 in intestinal epithelium affects both cell survival and cell proliferation during acute or chronic colitis [72, 73, 92] and in cancer model decreases CAC tumor multiplicity and diminishes tumor growth [72, 73]. Therefore, NF- κ B and STAT3 in epithelial and premalignant cells play distinct but somewhat overlapping roles, particularly due to their binding the same set of promoters [93] and the ability to regulate activity of each other [94, 95]. Importantly, many inflammatory cytokines can activate STAT3 in epithelial

cells, because their receptors coupled with JAK/STAT pathway are expressed not only by immune cells, but also by many non-hematopoietic cells, including stromal cells and normal and malignant epithelium. IL-6 is a multifunctional cytokine, which is produced by many cell types and is instrumental for the development of normal immune responses, tissue regeneration but also for pathological conditions including autoimmunity and various types of cancers [96-98]. IL-6 is a potent STAT3 activator, although it can also activate Ras/Erk and PI3K/Akt pathways [98]. IL-6 plays an important role in IBD development and disease maintenance, both in mouse models and in human patients, and its inhibition has been proven effective to ameliorate IBD [99-101]. This can be attributed to the ability to regulate the survival of colitogenic T cells, differentiation of T helper IL-17 producing (Th17) cells, suppression of T reg's, regulation of myeloid cell recruitment and other functions primarily within the cell of the immune system [99, 102-104]. On the other hand, IL-6 levels are elevated systemically and also locally during tumorigenesis, including in CRC and CAC tumors [73, 105, 106] and IL-6 can stimulate colon cancer cell proliferation and growth [105] as well as mediate protection of intestinal epithelium and wound healing during injury and colitis [73, 107]. Many of described effects of IL-6 in premalignant and cancer cells are likely to be mediated by STAT3 [71, 95]. IL-6 expression in T cells is controlled by NFATc2, transcription factor involved into CAC development [108], while in myeloid and many other cell types NF- κ B serves as a prime transcription factor for IL-6 induction [28, 82, 109]. Hence, the paradigm is created where activation of NF- κ B in immune cells results in production of inflammatory cytokines (such as IL-6), which drive activation of STAT3 in epithelial cells. Genetic ablation of IL-6 or its pharmacological inhibition decreases tumor cell proliferation and reduces CAC tumor multiplicity and growth [73, 106], mimicking more severe phenotype of epithelial cell specific deletion of STAT3 [73, 106]. Indeed, STAT3 is activated not only by IL-6 but also by other cytokines and factors in the microenvironment, including IL-11, IL-22, HGF and EGF family members, at least during colitis stage and potentially during CAC and CRC tumorigenesis [91, 92, 110]. Particularly, it has been shown that IL-6 signaling in epithelial and adenoma cells increases the expression of VEGFR2 on the surface of CAC adenoma cells and therefore allows tumor cells to benefit from high levels of VEGF in their surroundings [111]. Once VEGFR2 is overexpressed because of the inflammatory stimuli, it can continuously activate STAT3 and other oncogenic pathways, because its ligand VEGF is abundant in the tumors. In addition, cell intrinsic activation of STAT3 by protein kinases and oncogenes such as c-Met or Src is of great importance during tumor development [91], but it yet has to be determined whether that arm of STAT3 activation can be influenced by inflammation particularly during CAC tumorigenesis.

Other cytokines whose levels are elevated during IBD and CAC may serve as important regulators of inflammation and CAC tumorigenesis, albeit not necessarily acting on epithelial and pre-malignant cells directly. IL-23, a member of IL-12 cytokine family, recently emerged as a critical regulator of IBD in humans and in various mouse models [112-115] and its promoter polymorphisms are associated either with decrease or increase in IBD risk [116]. While normally detectable IL-23 expression is only limited to the terminal ileum and colon [117], its levels are dramatically upregulated in various cancers, including CRC [118, 119], and IL-23 –deficient mice do not develop tumors in two step inflammatory skin tumorigenesis model [118]. In recently developed CAC model, where inflammation driven by infection with enterotoxigenic *B. fragilis* enhances and facilitates colorectal tumor formation in APC^{Min} mice, pharmacological blockade of IL-23R with neutralizing antibodies reduced tumor-associated inflammation and tumor growth [77]. In a mouse model of spontaneous CRC [120], the lack of local protective barriers in tumors cause translocation of microbial products and increase in IL-23 production [121]. Inactivation of IL-23 and IL-23R in that model results in decreased tumor numbers and size, as well as in reduction in intra-tumoral levels of many pro-inflammatory cytokines, such as IL-6, IL-17A, IL-17F and IL-22 [121].

As IL-23 is required for the stabilization and effective recruitment of Th17 cells, one potential mechanism of its action may be exerted through the regulation of IL-17 production. Indeed, inhibition of IL-23R in *B. fragilis* APC^{Min} model inhibits IL-17A production by T cells [77]. Furthermore, IL-17A recently has been proven as an important pro-tumorigenic cytokine in various CAC models, including AOM+DSS model [77, 122] and inflammatory APC^{Min} model [77]. Chae et al also found that IL-17A signaling is important for spontaneous small intestinal polyposis in APC^{Min} mice [123] and we confirmed these observations in mouse CRC model via ablation of IL-17RA, a receptor for both IL-17A and IL-17F [121]. IL-17A and IL-17F have very similar signaling patterns although different sources and kinetics and magnitude of production [124]. Although both IL-17A and IL-17F are required for spontaneous CRC tumorigenesis [123, 125], in chemically induced colitis and in CAC tumorigenesis they play quite opposing roles [122, 126][124]. IL-17A deficient mice develop less CAC tumors of smaller size, underscoring the role of IL-17A in regulation of intestinal inflammation [127, 128], intratumoral inflammation [129, 130], and tumor growth [122, 126, 130]. APC^{Min} mice with established *B. fragilis* infection and chronic colitis exhibit lower tumor burden when IL-17A is neutralized [77]. On the contrary, despite ablation of IL-17F decreases spontaneous small intestinal polyposis in APC^{Min} mouse model, it increases CAC tumorigenesis in AOM+DSS model [126]. Notably, there are examples of immunologically relevant molecules and cytokines, whose gene deletion suppresses or has no effect on spontaneous colon adenoma formation and growth, but dramatically increases intestinal inflammation and injury during chemically-induced colitis and therefore promotes CAC tumorigenesis because CAC development and growth directly correlates with the degree of inflammation. Among them are mutations in genes such as COX2, MyD88, IL-18 and various components of inflammasomes [78, 131-136]. While previously mentioned IL-6 and IL-17 have their receptors expressed by epithelial cells, and therefore may be implicated into direct immune cell-to-cancer cell signaling, IL-23 likely regulates immune cells in the microenvironment, but does not act on cancer cells directly. It is possible that IL-23 controls the expression of various pro-inflammatory and pro-tumorigenic cytokines, such as IL-6 and IL-17, and these cytokines transmit oncogenic effect of IL-23 in tumor microenvironment. Somewhat similar function has been assigned to IL-21, a primarily T cells derived cytokine, in CAC model [129, 130]. Inactivation of IL-21 signaling resulted in decreased DSS colitis and CAC tumorigenesis, while the levels of IL-6 and IL-17 were also reduced [129, 130]. Importantly, reduction in IL-6, IL-17 and tumorigenicity was also accompanied by increase in production of IFN γ , an important anti-tumorigenic cytokine, which activates various cytotoxic cells, myeloid cells and can have direct effect on intestinal epithelium and malignant cells [129, 130]. It remains to be determined to which extent increased levels of IFN γ in IL-21 deficient mice subjected to CAC stimulate protective tumor immunosurveillance or whether distinct portion of effect is mediated by reciprocal lack of IL-17 production and action of IFN γ on epithelial cells. While we studied several examples of how pro-inflammatory cytokine regulate CAC tumorigenicity, the assumption is that most of the anti-inflammatory cytokines will have a protective role both in IBD and in CAC. Indeed, as an extreme case, mice lacking components of TGF β signaling pathway, such as TGF β RII in T cells [137], develop lethal autoimmune phenotype which includes aggravated spontaneous IBD and *Il10*^{-/-} mice also can develop spontaneous colitis and exhibit enhanced CAC formation and growth when housed in dirtier conditions [31, 138]. Furthermore, IL-10 is important for CAC prevention in *Helicobacter*-infected alymphocytic *Rag*^{-/-} mice [47]. TGF β RII and Smad's are also protective in CAC, as their inactivation or partial blockade of their signaling in increased CAC tumorigenicity [106, 139, 140]. EGF signaling is important for epithelial survival and regeneration, so in the presence of EGF signaling intestinal epithelium can resist greater insults and as a result, supply of EGF ligands or activation of EGFR protects mice from DSS colitis, maintaining integrity of epithelial barriers. Since inflammation is essential for CAC formation and these mice do not develop robust DSS colitis, the presence

of EGF signaling actually makes these mice resistant to CAC [141], quite contrary to what is seen in spontaneous CRC without inflammation, where EGFR signaling is a prominent target for anti-cancer therapy [142]. Taking together, it can be concluded that inflammation plays a pivotal role in tumor promotion and growth, some of its features important during tumor initiation are also important during tumor promotion and that inflammatory cytokines serve as important mediators of continuous cross-talk between immune system and tumor cells. Most of the measures, which improve intestinal barrier and/or inhibit inflammatory response, including supply of anti-inflammatory mediators or pharmacological inhibition of inflammatory mediators will likely be effective for therapy and prevention of CRC.

Microbes, intestinal inflammation and CAC

When we think about the mechanisms which trigger and sustain IBD and CAC-associated inflammation, it is hard to overlook that mammalian intestine is a home to a huge number of microbes, fungi and viruses, whose variety goes probably well beyond 1000 species [143]. With the highest representation of microorganisms in the colon and cecum, their total amount may extend past 10^{13-14} cells and genetically constitute up to 90% of genes and DNA amount in a human or mouse body [143, 144]. Vast majority of intestinal bacteria are anaerobic and can be subdivided into four divisions: 60% of which belong to *Firmicutes* class (mainly composed of *Clostridium spp.*) and over 20% belonging to *Bacteroides* class (reviewed in [27]). Intestinal microbiome in general serves us well providing cues for immune system development and maturation, aid in food digestion, vitamin synthesis and production of regulatory substances such as butyrate [145-150]. Relevant for tumor development in CAC and CRC is that intestinal bacteria are important for metabolic activation of different carcinogens and mutagens, including model pro-carcinogen AOM, environmental polyamines, and alkylating agents [15, 27]. The absence of intestinal bacteria in germ-free mice dramatically decreases the frequency of oncogenic mutations and tumor formation in the AOM + DSS model of CAC and the *Apc^{min}* model of CRC [132, 151, 152]

Gut microorganisms also can play “active” role in IBD and CAC development or suppression, i.e. by their capability to induce pro-inflammatory responses, such as the induction of IL-17 or IFN γ pro-inflammatory responses [153-156] or specifically activate suppressive IL-10 cytokine response [157-159]. Alternatively, it seems likely that many commensals may have a “passive role” at IBD prevention by educating and maturing immune system and by establishing and occupying ecological niches in the gut, making them inaccessible for pathogenic microorganisms.

Despite most intestinal bacteria in normal situation in humans and in mouse can be considered symbiotic or commensal, some of them can be in fact ‘conditionally pathogenic’. Experiments with gnotobiotic (‘germ free’) animals proved that even ‘normal’ microbiota in mice held in specific pathogen free (SPF) facilities plays an instrumental in initiation and induction of various forms of IBD. Indeed, germ-free mice do not develop colitis [143, 160-162]; and antibiotic treatment ameliorates colitis in animal models and sometimes in human patients, who, of course, harbor more complex potentially pathogenic microflora in comparison with mice housed under SPF conditions. In *Il10^{-/-}* mice, infection with different *Helicobacter spp.* results in CAC development, while eradication of bacteria by antibiotic treatment, ameliorated IBD and prevented CAC development [163]. On the other hand, transfer of microflora from SPF mice into germ-free mice (supposedly, only with commensal but not pathogenic organisms) reverted the inability of mice to develop IBD and, as a proof of concept, colonization of germ-free *Il10^{-/-}* mice with 2 species of otherwise commensal bacteria drives severe pancolitis [155]. Genetic predisposition and various forms of deregulation of immune tolerance against intestinal bacteria are believed to be major factors for IBD development. Since normally bacteria and immune system are separated

from each other by protective epithelial barrier, one would expect that effacing and translocating pathogens or combination between barrier breakdown and commensal flora translocation would be a pre-requisite for IBD development. Indeed enterotoxigenic *B. fragilis*, *Citrobacter rodentium* and adherent *E. coli*, pathogenic fungi, *Clostridium difficile* in humans or the use of DSS, which disrupts barrier and causes translocation of commensal microflora, effectively induce IBD and, in some cases, CAC [77, 161, 164-167]. The same can be observed in various murine strains with defects in immune defense or immune tolerance, infected with various bacteria, for example Rag deficient or *Il10*^{-/-} mice infected with *Helicobacter spp* develop spontaneous chronic colitis and later on colonic CAC adenocarcinomas [31, 46]. Furthermore, the importance of robust intestinal barrier is underscored by the fact that any interference with the quality of mucus layer or with tight junctions leads to increased susceptibility to intestinal inflammation [168, 169]. For example, inactivation of Muc2, a major component of mucus layer in the colon, causes spontaneous intestinal inflammation [170] that progresses to CAC even without introduction of additional mutations or carcinogens [171] and also accelerates adenoma growth when introduced into *Apc*^{Min} mice [172]. To some extent though, the disruption of the barrier can be compensated by the presence of T- and B-cell mediated adaptive immunity [173]. Prolonged DSS treatment, which promotes CAC development in WT or *Apc*^{Min} mice, also causes erosion of the intestinal epithelial barrier leading to microbial-dependent inflammation [41, 75]. Importantly, while current approaches in IBD treatment lead to the disease remission, they do not always result in complete mucosal healing [174], meaning that despite the absence of symptoms, local intestinal barrier are still defective and likely cycles of injury-regeneration are still ongoing. Those unhealed local regions likely will give rise to flares of IBD relapse [175, 176] and potentially may be “hot spots” of CAC emergence. However, it is important to note that increased intestinal bacteria accessibility does not always equal to IBD initiation, as in some cases disruption of intestinal homeostasis or protective immunity causes translocation of bacteria and systemic inflammation and various pathologies, like liver fibrosis, but nevertheless does not induce IBD [177-180]. Moreover, recent study nicely demonstrates that while different bacteria species can equally trigger IBD in mono-colonized germ-free *Il10*^{-/-} mice, some of the species, such as a specific adherent strain of *E. coli* expressing a specific pks pathogenicity island are much more effective at inducing CAC initiated by AOM injection [181].

Infection with a particular pathogen can cause acute colitis, but typically either results in lethality (rare) or in most cases, adequate immune response and pathogens elimination followed by mucosal healing. In most cases, it seems that IBD is induced and maintained by at least several microorganisms and often harbors signs of global dysbiosis, as referred to changes in the number, diversity and stability of microecosystems of commensal bacteria. Particularly various bacteria from the *Clostridia* group can be enriched in IBD and even in some CRC samples [182] and that representation of various constituents of gut microbiota likely affects CAC development [138, 160]. Other microorganisms are over-presented in samples from CD and UC patients, including species of *Chlamydia*, *Mycobacterium*, *Candida*, various adherent invasive *E. coli*, as well as *Proteus mirabilis*, *Klebsiella pneumoniae* and various *Proteobacteria*, including *Helicobacter* [183-185], but their real causative role in IBD remains to be determined. On the other hand, overall abundance of normal colonic constituents such as *Firmicutes* and *Bacteroidetes* decreases in IBD [185]. It is also worth mentioning that while increase in particular bacteria can change gut homeostasis and trigger IBD, noxious microenvironmental conditions in intestinal epithelium during IBD can affect microbial representation and further causing dysbiosis. IBD-causing or promoting dysbiosis can also be induced and perpetuated by the defects in various arms of host immunity, particularly in the systems of pathogen recognition. Mutations in *Tbet* (T-bet) [49, 186, 187], *Tlr5* (a receptor for flagellin) [188], defensins [189] and gene encoding components of inflammasomes, such as NLRP3, NLRC4

and NLRP6, as well as in NOD2 and NOD1 selects for microflora which predisposes otherwise SPF mice to spontaneous or easily inducible aggravated IBD [134-136, 190-194]. Inactivation of these molecules often increases CAC and CRC development in mice, and importantly the IBD, CAC and CRC susceptibility phenotype can be transferred with microbiota by co-housing gene-deficient animals with normal WT mice.

Given the significance of dysbiosis association with IBD and CAC, multiple attempts are being undertaken to deal with altered microbiota (reviewed in [162, 184]). Since generation of germ free patients is not an option and the overuse of antibiotics can lead to the selection of antibiotic resistant strains not only in humans (as in case of *C. difficile* infection) but also in overall clean mice [195], another approach is to correct altered microbiota composition by use of various probiotics, formulated from distinct and tested microbial species and/or changes in dietary habits which also have impact on quantitative and qualitative composition of microbiota [162, 184]. Preparations of *Lactobacillus* particularly were shown to inhibit intestinal inflammation and CAC in animal models [196-198]. As a proof of concept indeed a particular preparation of probiotic bacteria VSL#3 was shown to inhibit IBD and delay transition from inflammation to dysplasia in a rat model of CAC [199, 200]. Another approach at curing IBD and subsequently reducing CAC risk is fecal microbiota transplantation, which seems to be effective in otherwise untreatable forms of IBD and has the advantage that transferred microbiota bypasses the stomach and small intestine, where many important bacteria can be killed due to adverse conditions but the disadvantage of using 'bulk population' of microbes would be a concern about large area of uncertainty with regard to exact microbial entities being transferred and possible side effects [162, 201-204]. Again, these normal and symbiotic microbes may act through different mechanisms at IBD suppression, i.e. by outcompeting potentially pathogenic microflora and creating unfavorable conditions for its growth, but also by direct stimulation of tolerogenic and immunosuppressive arms of intestinal immunity.

An outstanding question about the role of microbes in connection between IBD and CAC remains whether microbes are only required for IBD, and then microbial driven inflammation accelerates CAC tumorigenesis as a dangerous side-effect, or there are special microbes which are increased during IBD and which actually specifically promote CAC tumorigenesis in situ? Enterotoxigenic *B. fragilis* seems to be a good candidate for the latter scenario, as that bacteria not only triggers colitis but also cleaves E-cadherin and enhances β -catenin signaling, clearly contributing to oncogenic signaling [77, 205]. Other bacteria though, including otherwise harmless commensals, upon injury to intestinal tumor would be able to translocate and trigger cytokine production by immune cells [27, 206]. Yet another related question worthwhile investigating is whether any microbe capable of triggering IBD will automatically promote CAC and CRC? Recent studies identified several microorganism linked to IBD but also enriched in tumors, particularly in CRC tumors. For example, *Fusobacterium* species are implicated into IBD development and also are enriched in CRC samples [207-209]. For a long time, connection between *Streptococcus bovis* and CRC has been noticed [150, 210] and patients which develop *S. bovis*- induced myocarditis are recommended to undergo colonoscopy check for colon cancer. Likely, CRC tumors serve as a point of entry for *S. bovis* and cause its dissemination. In addition, it is quite possible that immune response subverted by tumors is no longer sufficiently protective to keep *S. bovis* at bay. Another previously mentioned bacteria associated with CRC is enterotoxigenic strain of *B. fragilis* [211]. It is not clear yet how this bacteria may act to promote CRC in cases it does not cause colitis, but it is tempting to speculate that its ability to activate β -catenin signaling, disrupt E-cadherin and epithelial barriers and stimulate localized inflammatory response may be involved. As technology platforms for high throughput sequencing of bacterial 16S rRNA and bioinformatics support are getting much better and accessible to many research labs, one can expect that other species of commensal or pathogenic microbiota will be found

associated with CRC or CAC tumors. Important question is whether microbes specifically found to be enriched in tumors always play some role in tumor promotion or sometime are just innocent bystanders, which prefer a niche created by tumor but not normal environment. Former scenario may upgrade these microbes from just potentially valuable screening targets to valuable therapeutic and preventive targets. In summary, microbes play important and not entirely understood roles in IBD and CAC (Figure 2) and likely hold a lot of promises for better diagnostic, preventive and therapeutic approaches for both IBD, CAC and potentially for more common sporadic CRC as well as for many other cancers which develop at epithelial interfaces with a large exposure to microorganisms.

Progression, invasion and metastasis- another call for inflammation

The role of immune cells and inflammation in CAC adenoma-to-carcinoma transition, invasiveness and metastasis is an interesting and largely unexplored topic because of the scarce availability of relevant in vivo models and occupancy of the field with the studies primarily addressing metastasis in CRC (reviewed in [27]). As mentioned before, p53 mutations in CRC are essential for the acquisition of malignant phenotype, and one important feature of CAC is that these mutation often happen early during the course of tumorigenesis, sometimes even within the inflamed, but otherwise normal (not yet transformed) epithelium. Early emergence of p53 mutations during the course of tumorigenesis provides a potential for CAC tumors, at least in human patients, to be aggressive and dangerous from the time of their initial emergence. Various molecular and cellular pathways participating during various stages of CAC and CRC metastasis, have been reviewed elsewhere [27, 95, 212], we will briefly summarize some of the findings. In general, chronic inflammation present during CAC development and progression can influence all proposed stages of tumor progression and metastatic dissemination [27, 213]. Acquisition of mesenchymal phenotype by the tumor epithelial cells, known as epithelial-mesenchymal transition (EMT) [214], apart from being largely under the control of TGF β signaling, can also be influenced by NF- κ B and/or STAT3 signaling [87, 212] and be regulated by various inflammatory cytokines, previously discussed in the context of tumor initiation and promotion. For example, TNF and IL-6, as well as IL-1 have been implicated into EMT-like processes and enhancement of metastatic behavior in colon cancer cell lines [215-217]. TNF signaling in particular was reported to stabilize key transcription factor Snail, which is pivotal for EMT [216] and on the other hand was shown to promote survival of single cancer cells in circulation and their successful extravasation and growth in the lungs [218]. A paradigm, where local inflammatory signal activates EMT, migration and metastasis of cancer cells deserves attention. Previous studies concentrated primarily on search for stable genetic (mutations) and, recently, epigenetic (stable chromatin marks) alterations in cancer cells, which predispose them to metastatic process. Meanwhile, most of the distant metastases exhibit epithelial phenotype, indicating that after EMT and successful metastasis, a reverse process called mesenchymal-to-epithelial transition (MET) likely takes place and that successful MET is required for metastasis, as for the first time demonstrated in experimental settings by Jing Yang and co-workers. This inevitable suggest the importance of EMT-reversibility, which is hard to explain if major driving force of EMT are genetic and epigenetic changes. Chronic inflammatory signals, however trigger EMT, but once metastatic seed leaves the site of local chronic inflammation, the inflammatory signal fades out and by the time that cell reaches the target organ, EMT promoting signal is gone and cell reverts to epithelial phenotype through MET. Another quest for metastatic cells is the creation of relevant chemokine gradient for the establishment of their motility and directed migration [219]. Chronic inflammation present during CAC likely create a 'rich chemokine environment', potentially much more prominent than in regular CRC. Recent work suggest crucial importance of CCL2/CCR2 signaling axis in cancer cells, endothelium and monocytes to promote lung metastasis of colon cancer cells [220]. Many different

chemokines in CAC are likely to mediate cancer cell migration and survival towards the circulation and to specific preferred target organs [219]. Normal inflammatory response typically aids our fight with infection and involves also activation of pathways, responsible for tissue remodeling. Inflammation therefore can regulate various proteases, whose activity is implicated into remodeling of extracellular matrix and in metastatic process [27, 221]. Overexpression of various metalloproteinases (MMP), such as MMP7 and MMP9 is a hallmark of many cancers, including colon cancer [91, 221]. Inflammation further can inhibit various inhibitors of tissue remodeling, such as TIMP3, various serpins or maspin [222, 223]. Taken together, inflammation plays a pivotal role in tumor progression and metastasis and we can expect more interesting discoveries in that area once appropriate in vivo models will be further advanced. From a clinical prospective indeed, it is the most important to learn how to target tumor progression and metastasis, as over 90% cancer related deaths are because of metastasis and not because of the primary tumor growth. Delaying tumor progression by various preventive (for example, anti-inflammatory agents and cytokine blockers) measures along with increase in early detection and tumor surveillance in CAC, will provide us with wider window of opportunity to detect and surgically remove primary tumor before its too aggressive and already had metastasized, because indeed anti-inflammatory agents are likely to influence tumor progression and malignization.

Taken together, inflammation plays a pivotal role at the creation of favorable environment for CAC initiation and emergence. Further, it promotes CAC tumor growth and progression and influences metastatic process. Inhibition of inflammation with either biological therapies or selective small molecular inhibitors therefore should be developed and tested for CAC prevention and treatment, with conjunction with currently used approaches such as surgery, chemo- and radiation therapy.

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Figure 1. The role of chronic inflammation in tumor initiation

Genotoxic compounds produced by inflammatory cells can damage DNA. In addition, signaling by distinct inflammatory cytokines upregulates ROS and RNI levels inside the target epithelial cells. Chronic intestinal inflammation is also intimately associated with a breakdown of protective intestinal barriers, which causes increased accessibility of inflamed epithelium to food borne and other types of mutagens. Altogether these processes contribute to enhanced mutagenesis required for tumor initiation. Furthermore, inflammatory signaling (particularly through cytokines) upregulates chromatin modifiers, miRNA and other epigenetic changes. Inflammatory signals also provide additional activation of pro-survival and proliferative pathways such as Akt, STAT3 and NF- κ B, as well as increased Wnt/ β -catenin signaling pivotal for adenoma formation and growth.

Figure 2. Interplay between microbiota, chronic inflammation and CAC tumorigenesis
Under normal conditions “good” or commensal microbes prevail, mediate immune system maturation, tolerance and also inhibit growth of ‘bad’ microbes probably via competition for ecological niches. Infection with colitogenic pathogens or shift in microflora causes increased growth of ‘bad’ microbes, which can cause aberrant immune responses, barrier disruption, production of inflammatory and pro-tumorigenic cytokines and metabolic activation of various carcinogens cells are also pro-tumorigenic. Intestinal inflammation in CAC can in turn further shape ‘bad’ microflora, selecting for the overrepresentation of particular species. Tumor may be enriched in distinct microflora, either indicating that these microbes may serve tumor-promoting role or the fact that for some reasons due to the tissue alterations tumor now is the best site for colonization by some particular microbes. Whether identity of ‘good’ microbes is much different from ‘bad’ ones remains an open question, because even truly commensal bacteria can (upon disruption of the barriers) can trigger inflammatory response to aid CAC development.