Review

Cytokine Networks in Animal Models of Colitis-associated Cancer

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Abstract. *Background/Aim: It is well-known that inflammatory bowel disease (IBD) poses an increased, yet not definitely estimated, risk of colitis-associated colon cancer (CAC), which is considered a more aggressive and distinct in both genetic and molecular levels clinical entity compared to sporadic colorectal cancer (CRC). The present review discusses the cytokine networks involved in CAC-based translational findings from suitable animal models of the disease. Moreover, we summarize the most prominent data concerning the role of Th1, Th2, Th17 and anti-inflammatory cytokines in the pathogenesis of CAC. Last, we briefly address the controversies between basic science findings in IBD and CAC and suggest further directions regarding research on cytokines. This review should serve as a primer for clinicians and surgeons to understand the rapidly evolving field of cytokines in the context of CAC. Materials and Methods: The MEDLINE database was thoroughly searched using the keywords: cytokines, colitis-associated cancer, animal models, carcinogenesis. Additional articles were gathered and evaluated.*

An etiologic role of inflammation in cancer pathogenesis has been suggested as early as in 1863 when Rudolf Virchow noted the presence of leucocytes in neoplastic tissues and suggested a connection between inflammation and cancer (1). Furthermore, he suggested an etiologic role for inflammation in cancer initiation stating that the "lymphoreticular infiltrate" reflected the origin of cancer at sites of chronic inflammation. It is estimated that colitis-associated cancer (CAC) accounts only for 1-2% of all colorectal cancer (CRC) cases. However, data suggest that 10-15% of deaths in inflammatory bowel disease (IBD) patients are attributed to CRC (2). The exact risk that IBD poses to patients is not clear since different groups have published different values probably reflecting methodological variances but is thought to be increasing with disease duration and approaching 10.8 % at 30 years after the diagnosis (3).

Concerning tumor biology, evidence deriving from two recent large retrospective studies (4, 5) suggests that CAC patients have a significantly lower 5-year survival compared to patients suffering from sporadic CRC, which could reflect a different, more aggressive tumor biology. Basic science research utilizing proper animal models, like the AOM/DSS model, has provided robust evidence regarding important differences in terms of timing and frequency of gene mutations compared to sporadic CRC (6).

At the cellular level, there is a variety of interactions between cancer cells and stromal cells that are present in the tumor microenvironment of CAC (7). Among the soluble factors that mediate these interactions, and consequently contribute to the disease pathogenesis, cytokines and the networks that they form have been the focus of intense research during the last decade.

Cytokine Networks

Pro-inflammatory cytokines and CAC: Tumor necrosis factor alpha (TNF-α). TNF-α has a pleiotropic action as a result of binding to TNFR type-I (TNFR1) and type-II (TNFR2). Cell death, altered target gene transcription and cytokine production are mediated by TNFR1, while binding to TNFR2

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has an anti-apoptotic effect, acting through an nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. These receptor-mediated signaling pathways regulate inflammatory cell infiltration in the lamina propria, epithelial/mucosal damage and cytokine expression in colonic mucosa in many animal models of colitis and CAC. In an elegant study by Kanneganti *et al*., *TNFR1*-knockout (KO) mice developed a much milder form of colitis and a reduced incidence of CAC in the context of AOM/DSS animal model of CAC as compared to WT mice. Furthermore, the authors concluded that TNF-α initiates and perpetuates many inflammatory reactions and efficiently recruits activated inflammatory cells to the site of injury or inflammation. TNF-α also efficiently activates NF-κB, MAPK and cell-death signaling pathways (Table I) (8).

Furthermore, the interaction between TNFR1 and TNFR2 signaling pathways is thought to play a crucial role in regulation of cellular homeostasis. Recent studies suggest that the functional differences between TNFR1 and TNFR2 are not absolute and the effects produced by these receptors may depend on the cell types. *TCRa*-KO mice do not develop colitis when maintained in a germ-free environment. The proinflammatory cytokine network generated by chronic inflammatory conditions, caused by dysregulated immune response to luminal bacterial antigens, may lead to TNFR2 expression in circulating endothelial cells (CEC). Blockade of TNF/TNFRs inducing signaling has been shown to be an effective therapeutic strategy for Crohn Disease (CD), as well as rheumatoid arthritis. The study of the TNFR family is likely to reveal novel roles for these receptors in epithelial cell function in normal and diseased states including chronic colitis and colitis-associated cancer (9).

Another study from Popivanova *et al*. has revealed the crucial involvement of TNF-α in the initiation of chronic inflammation-mediated colon carcinogenesis. As a result, blocking of TNF-α reversed carcinoma progression even after colon carcinoma was established. Thus, drugs targeting TNF-α may be a useful weapon against malignancies, particularly those arising from chronic inflammation (10).

Interleukin 6 (IL6)

IL-6 plays an eminent role in the transition from acute inflammation to chronic colitis, as well as connecting innate immunity with acquired immunity (11). Macrophages that can recognize conserved pathogen-associated molecular patterns (PAMPs) produce and secrete IL-6 after being exposed to specific microbial molecules. IL-6 binds to a cell-surface receptor complex, which consists of an IL-6Rα chain (CD126) and the signal-transducing component gp130 (CD130). The ligand receptor interactions initiate signal transduction cascades through transcription factors, Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) (12). Also, it is well-known that IL-6 enhances the differentiation of Th17 cells under a combination with immunosuppressive cytokines (*e.g*., TGFβ) (13). IL-6 expression is significantly increased in IBD and in murine models of colitis and blocking of IL-6 signaling significantly inhibits the severity of colitis in murine models. In a study from Fenton *et al*., it was proved that IL-6 production is highly up-regulated in colorectal tumor-developing ApcMin mice and animal models of CAC (14, 15). IL-6 expression is mainly regulated by NF-κB activation and acts on both CECs and immune cells. Becker *et al*. showed that inhibition of IL-6 signaling significantly reduced tumor development in AOM-induced CAC model suggesting that IL-6 trans-signaling plays a pivotal role in the development of colon cancer (8, 11-16).

In a study conducted by Matsumoto *et al*., it was indicated that IL-6 trans-signaling plays an important role in the induction of colon carcinogenesis. However, the mechanisms underlying the regulation of IL-6 trans-signaling in the colonic mucosa remain largely unknown. It has been shown that the inflammatory macrophages in the colonic mucosa play essential roles in both the production of IL-6 and the shedding of soluble IL-6Ra (sIL-6Ra) from the cell membrane, thus inducing IL-6 trans-signaling in colonic epithelial cells during the development of inflammation-based colon carcinogenesis. Moreover, sgp130Fc, which is a competitive inhibitor of IL-6 trans-signaling, suppressed colon carcinogenesis. Therefore, the inhibition of IL-6 trans-signaling may prove to be a useful therapeutic system for the treatment of inflammation-based colon carcinogenesis (17).

Interleukin 11 (IL-11)

Putoczki *et al*. revealed that IL-11 has a stronger correlation with elevated STAT3 activation in human gastrointestinal cancers than IL-6. Using genetic mouse models, they revealed that IL-11 has a more prominent role compared to IL-6 during the progression of sporadic and inflammation-associated colon and gastric cancers. In these models, and in human tumor cell line xenograft models, pharmacological inhibition of IL-11 signaling alleviated STAT3 activation, suppressed tumor cell proliferation and reduced the invasive capacity and growth of tumors. These results identify IL-11 signaling as a potential therapeutic target for the treatment of gastrointestinal cancers.

Using mouse models of inflammation-associated and sporadic gastrointestinal cancers, it has been documented that the IL-11/STAT3 signaling axis is a more potent driver of tumor progression than IL-6. Pharmacological inhibition of IL-11/STAT3 in mouse models of gastrointestinal cancer and human tumor cell line xenografts inhibited the invasive capacity of neoplastic cells and reduced tumor growth. These data, according to this particular study, provide support for

the clinical development of IL-11signaling antagonists for the treatment of epithelial cancers (18).

Th1 cytokines (IL-12, IFN-gamma)

It has been shown that lymphocytes and IFN-gamma collaborate to protect against the development of carcinogen-induced sarcomas and spontaneous epithelial carcinomas and also select for tumor cells with reduced immunogenicity. Thus, the immune response functions as an effective extrinsic tumor-suppressor system. However, this process also leads to the immunoselection of tumor cells that are more capable of surviving in an immunocompetent host, which explains the apparent paradox of tumor formation in immunologically-intact individuals (19).

Another experiment conducted by Brunda *et al*. demonstrated that IL12 has potent antitumor and antimetastatic activity against a number of murine tumors of various histological types. Therapeutic intervention by systemic administration of IL-12 can be initiated even when tumors or metastases are well-established, resulting in an increase in survival time (20).

Neurath *et al*. demonstrated the pivotal role of IL- 12 and IFN-gamma in a murine Thl model of chronic intestinal inflammation induced by repeated administration of the hapten reagent TNBS. They demonstrated that inflammation was abrogated by anti-IL-12 treatment, likewise with their effect on CD, and suggested that anti-IL-12 antibodies may have potential therapeutic utility in patients with this disease.(21)

Berg *et al*. demonstrated that IFN-γ could mediate many deleterious effects, such as altering gut physiology and decreasing epithelial barrier function. In addition, the ability of IFN-γ to increase cytokine production by macrophages exposed to luminal bacterial products could further enhance the inflammatory response, particularly in the absence of negative regulation by IL-10 (22).

Th2 cytokines (IL-4, IL-5, IL-13)

Osawa *et al*. suggest that IL-4 may have a direct effect on promoting tumor formation. Many malignant tumors express the IL-4 receptor, which is able to bind to both IL-4 and IL-13 and also the high affinity decoy receptor of IL-13, IL-13 receptor. However, the function of IL-4 and IL- 13 in tumor cells, especially in colonic cancer, is still not well elucidated.

Early studies showed that IL-4 and IL-13 had antitumor activity in mice by growth inhibition through the IL-4 receptor. However, subversion of host antitumor defenses has also been demonstrated for IL-13. Recent studies using tumor cell lines demonstrated that STAT6, IL-4 and IL-13 were capable of inhibiting tumor rejection. Thus, Th2-type cytokines appear to antagonize tumor immunosurveillance. In this particular study, IL-$4^{2/2}$ mice and IFN-$\gamma^{2/2}$ mice showed distinct expression patterns for β-catenin, cell membrane and nuclear staining in IL-$4^{2/2}$ and IFN-$\gamma^{2/2}$ mice, respectively, while WT mice had tumors of both patterns. This again suggests that Th2-cytokine predominance directly influences the mutation of DNA in epithelial cells. In summary, the results show that a predominance of Th2-type cytokines in the inflamed colon, which mimics mucosal immunity in ulcerative colitis (UC), promotes aberrant b-catenin expression and tumor formation (23).

Table I. *Summary of the pleiotropic actions of cytokines*.

Action	Cytokines
Pro-inflammatory cytokines	TNF-α, IL6, IL-11
Th1 cytokines	IL-12, IFN-γ
Th2 cytokines	IL-4, IL-5, IL-13
Th17 cytokines	IL-17, IL-23
Anti-inflammatory cytokines	IL-10, TGF-β

Th17 cytokines (IL-17, IL-23)

IL-23 is a heterodimeric cytokine consisting of two subunits, p40 (which is shared with IL-12) and p19 (also called IL-23α subunit). IL-23 binds to a specific receptor, which is formed by IL-12RB1 and IL-23R. Both IL23 (p19/p40) and IL-12 (p35/p40) can activate the STAT4 transcription factor and subsequently stimulate the production of IFN-γ. In addition, IL-23, TGFβ and IL-6 conjugationally stimulate naıve $CD4^+$ T-cells to differentiate into Th17 cells, which produce the proinflammatory cytokine IL-17. IL-23 plays an important role in the inflammatory response against infection and its expression is increased in inflammatory bowel diseases (IBD) and in colon cancer. Shan *et al*. demonstrated the antitumor activity of IL-23 by injecting BALB/c mice either with a murine CRC cell lines or with a murine cell lines retrovirally transfected with *mIL-23* gene. Interestingly, while mice with the CRC cell lines finally died, those injected with the cell lines that was retrovirally transfected with *mIL-23* displayed complete tumor rejection and survived (24). Furthermore, IL-23 secreted by tumor cells efficiently suppressed the growth of tumor and the survival of mice by enhancing the production of IFN-γ, IL-12 and TNFα. IL-23 and Th17 cytokines act coordinately to maintain the balance between tolerance and immunity in the gastrointestinal tract (8, 24).

The role of Th17 cells in chronic intestinal inflammation has been under debate. While the potential relevance of Th17 cells has been highlighted by the finding that Th17 cells accumulate in the mesenteric lymph nodes and colonic lamina propria in a T-cell transfer model of chronic colitis, recent studies demonstrated that IL-17A production by T-cells, originally

identified as a transcript from a rodent T-cell hybridoma, is not essential to induce such chronic T-cell–dependent colitis. However, IL-23, a heterodimeric cytokine that drives Th17 cell activation, has been shown to play a pathogenic role in chronic intestinal inflammation. In that study, it was reported that *Ror*--deficient T-cells migrated to the colon but failed to induce mucosal IL-17 production and colitis development upon T-cell transfer in RAG1/mice. This finding was associated with diminished numbers of both dendritic cells and granulocytes at the site of inflammation in the absence of ROR T-cell signaling potentially due to regulation of chemokine expression by Th17 cells. In any case, these data demonstrate the importance of T-cell–ROR signaling in chronic colitis *in vivo*. In additional studies, it is observed that neither of the Th17 cell–derived cytokines IL-17A and IL-17F alone is required for the development of adoptive transfer colitis. Furthermore, although recombinant IL-22 has been recently shown to protect mice from a Th2 model of colitis in mice resembling human ulcerative colitis, it was shown that *IL-22*–deficient T-cells are fully capable to induce T-cell–mediated colitis in the adoptive transfer model, yet not excluding the physiological importance of this cytokine. The IL-17 cytokine family consists of several members out of which IL-17A and IL-17F are expressed by T-cells and display the most homology. Next, the potential functional redundancy of both cytokines in experimental colitis was assessed revealing that functional deficiency of both IL-17A and T cell–derived IL-17F, by neutralization of IL-17A after IL-17F/T-cell transfer significantly, reduces the severity of colitis. These findings suggested redundant effects of IL-17A and IL-17F *in vivo* (25).

In addition IL-17 plays a major role in stimulating granulopoiesis and mobilizing granulocytes into sites of inflammation, thus linking lymphoid and myeloid host defense mechanisms. Th17 cells, rather than Th1 cells, have been shown to be the major effector cells in experimental autoimmune encephalomyelitis, experimental arthritis and IL-10–deficient mice with colitis. Increased IL-23 and IL-17 expressions have been identified in the lesions of human inflammatory bowel disease and neutralizing antibody to IL-12p40 significantly reduces IL-17 expression in the lesions of Crohn's disease concomitant with a clinical response to this agent. The data show that commensal bacteria stimulate murine dendritic cells to produce IL-23, as well as IL-12, and that IL-23 supports colitogenic CD4 Th17 cells, a lineage distinct from colitogenic CD4 Th1 cells (26).

Anti-inflammatory cytokines and CAC: IL-10, TGF-β

IL-10 is a key anti-inflammatory cytokine produced primarily by monocytes, T-cells and B-cells. IL10 can block NF-κB activity and also regulates the JAK/STAT signaling pathway (27). IL-10 interacts with IL-10 receptor α subunit (IL-10RA) and inhibits the synthesis of pro-inflammatory cytokines, such as IL-6. As a result *IL-10*-KO mice develop spontaneous colitis, which is similar to human IBD, in particular CD. These mice also develop colitis-associated cancer, which is associated with the over-production of Th1 cytokines. AOM/DSS-induced CAC promotes inflammation-mediated colonic tumor growth in *IL-10* KO mice (8, 27).

IL-10 is also a crucial anti-inflammatory cytokine produced by many cell types involved in the generation of the Tr1 subset of regulatory T-cells (Treg). The IL-$10^{-/-}$ mice are characterized by a mild disease progression involving spontaneous $CD4^+$ Th1-driven enterocolitis, dependent on IFN-γ for onset and IL-12 for sustaining disease and progression to adenocarcinoma, occurring from 5-6 months of age. Similar to the IL-$10^{-/-}$ mice, deficiency of CRF2-4, a subunit of the IL-10 receptor, results in development of splenomegaly and chronic colitis. Whereas the *IL-10R2/TGFβR2* double-knockout mouse develops rapid fulminant ulcerative colitis within weeks after birth, other double–knockouts, including *IL-10/Leptin* and *IL-10/iNOS*, develop similar mild colitis compared to IL-$10^{-/-}$ mice; their genetic status does not confer protection or rescue the phenotype of the IL-10 deficiency.(28)

Alterations of TGF-β signaling have been described in colorectal cancer, although the molecular consequences are largely known. By using transgenic mice over-expressing TGF-β or a dominant–negative TGF-βRII, Becker *et al*. demonstrated that TGF-β signaling in tumor infiltrating T lymphocytes controls the growth of dysplastic epithelial cells in experimental colorectal cancer, as determined by histology and a novel system for high resolution chromoendoscopy. At the molecular level, TGF-β signaling in T-cells regulated STAT-3 activation in tumor cells *via* IL6. IL6 signaling required tumor cell-derived soluble IL6-R rather than membrane-bound IL6-R and suppression of such TGF-β-dependent IL6 trans-signaling prevented tumor progression *in vivo*. Taken together, these data provide novel insights into TGF-β signaling in colorectal cancer and suggest novel therapeutic approaches for colorectal cancer based on inhibition of TGF-β-dependent IL6 trans-signaling (16).

Discussion

Basic research findings concerning cytokines in the context of CAC has followed the unraveling of the mechanisms and the cytokine patterns that contribute to IBD pathogenesis. Indeed, the impressive findings in IBD have greatly enhanced our understanding and fuelled the development of rational therapies with antibodies targeting specific cytokines with the prominent example of anti-TNF monoclonal antibodies, which have entered the clinical practice successfully. Findings and lessons from the one clinical

entity can be extrapolated and translated to the other. For example TNF-α and IL-6, that are prominent pro-inflammatory cytokines in IBD, are found to be key players also in CAC.

Critical Appraisal

However, we would like to underline that the molecular pathways of the two diseases should not be viewed as merely consecutive steps of the same continuum since there have been observed some discrepancies. In IBD for example, treatment with antibodies that neutralize both soluble TNF and membrane-bound TNF (such as infliximab and adalimumab) was highly effective and shown to induce T-cell apoptosis *in vivo*, in marked contrast with etanercept, an agent that preferentially blocks soluble TNF, and showed no therapeutic effect (29).

On the other hand, in the context of an established animal model of CAC (AOM/DSS), it has been shown that mice treated with etanercept, a soluble TNF receptor, carcinoma progression was reversed even after colon carcinoma was established (10).

Moreover, it is increasingly recognized that in many malignancies, with the predominant example of PDAC, tumor stroma, which consists of various cell sub-populations like endothelial cells, immune cells, neurons and fibroblasts, exerts a bidirectional feedback with the cancer cells initiating and promoting the malignancy. Interestingly, recently, it has been proposed that CAC follows this pattern of cancer progression and stroma plays a significant role interacting with the cancer cells (30).

Future Directions

Thus, we should think beyond the classic view of the degree and extent of inflammation as the sole etiological factors impacting on CAC development and study more extensively the role of cell populations like stroma fibroblasts, which preferentially get activated when inflammation subsides, since their physiological role encompasses the healing procedure. This view potentially explains why Tlr4–/– mice are more susceptible to experimental colitis but, at the same time, are also protected from colitis-associated tumor development (31). Furthermore, a recent elegant study showed that fibroblast–derived epiregulin promotes growth of colitis-associated neoplasms, whereas Ereg-deficient mice develop more severe acute experimental colitis, excluding in that way the possibility that Ereg controls tumor growth by directly regulating inflammation activity (30).

Thus, more vivid research is needed on the cytokine networks that participate to the healing process between the flares of the inflammation and their connection with stroma populations other than the immune cells, such as the heterogenous sub-populations of fibroblasts.

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