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Local and systemic Th17 immune response associated with advanced stage colon cancer

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Abstract

Background—Little is known about how the immunologic microenvironment changes during tumor progression and metastatic spread. Recently, murine models have shown the T-helper 17 (Th17) pathway to play an important role in promoting colorectal cancer (CRC). The purpose of this study was to compare cytokine profiles in the tumor microenvironment of CRC between local disease (stages I/II) and advanced disease (stages III/IV), and to determine whether these changes were manifest in the systemic circulation of patients with advanced disease.

Materials and methods—Serum and tissue cytokine profiles were assayed among patients with documented adenocarcinoma before surgical resection at a single institution from September 2014 to February 2015. Using the Bio-Plex Pro Human Th17 Cytokine Assay Kit (Bio-Rad Laboratories), the concentrations of multiple cytokines were determined. Multiple logistic regression analyses were used to evaluate the association between TNM staging and cytokine levels.

Results—A total of 33 patients with documented adenocarcinoma were included. None of the patients received neoadjuvant chemotherapy. American Joint Commission on Cancer TNM classification was used. Advanced disease was associated with elevated tumor levels of tumor necrosis factor- α , interleukin (IL)-4, IL-10, IL-17A, and IL-17F, and only stage IV showed elevated systemic levels of Th17-associated cytokines IL-17F, IL-23, and IL-25.

Conclusions—The Th17 pathway likely has important mechanistic implications in human CRC. Metastatic disease was associated with elevated Th17-associated cytokines both in colonic tissue and systemically. These changes in systemic expression of Th17-associated cytokines could establish novel pathways for CRC and warrant further investigation.

Keywords

Colorectal cancer; Inflammation; Th17; IL-17; Biomarkers

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Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths in the United States, and the prevalence of CRC is increasing worldwide.^{1–3} The etiology of sporadic CRC has been linked to several risk factors including: accumulation of genetic mutations, environmental factors such as smoking and obesity,^{4–6} as well as various inflammatory conditions.^{7,8} Complex interactions within the colonic microenvironment and gut microbiome are involved in the induction and progression of CRC.^{4,9} Disease progression has been linked to elevated levels of inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ)-induced protein 10, and interleukin (IL)-8.^{3,10}

T-helper 17 (Th17) associated cytokines have been shown to be involved in gastric carcinoma,^{11,12} ovarian cancer,¹³ non-small-cell lung cancer,¹⁴ and breast cancer,¹⁵ with the levels of IL-17 expression positively correlating with the aggressiveness of the malignancy. IL-17 is primarily produced by Th17 cells, which are known as a subset of T-helper cells that are different from the classic Th1 and Th2 cells.

Recent investigations using a murine model of CRC demonstrated upregulation of IL-17 signaling which promoted tumor growth and progression,¹⁶ with the production of the proinflammatory cytokines and chemokines increased during advanced stage CRC.¹⁷ Although the exact mechanisms are unclear, evidence has suggested that IL-17 may potentially act by inhibiting tumor cell apoptosis and antitumor responses, as well as by promoting tumor angiogenesis.¹⁸

There is a paucity of clinical data examining the relationship between the Th17 immune response in the tumor microenvironment and systemic cytokine levels during progression from local disease (stage I and II) to advanced disease (stages III and IV) in CRC. Based on the observations that inflammation is increased with advanced stage CRC, we hypothesized that Th17-associated cytokines in CRC would be increased in advanced disease compared with local disease. Furthermore, we hypothesized that these localized elevations of Th17-associated cytokines within the tumor could also be detected systemically in metastatic disease.

Materials and methods

Patients

The Internal Review Board and ethics committee of Albany Medical Center (Albany, NY) approved this study. Thirty-three consecutive patients with histologically confirmed sporadic colonic adenocarcinoma who underwent elective hemicolectomy between September 2014 and February 2015 at Albany Medical Center were enrolled in the study. Patients who had preoperative chemotherapy or prior history of colonic resection for colon cancer were excluded from the study. None of the patients had known hereditary cancer, ulcerative colitis, or Crohn's disease. Patients who could not provide informed consent were excluded. Written informed consents were obtained from all study participants before any research procedures being performed.

Participant information

Preoperative patient demographics were collected and included age, gender, race, body mass index, smoking, and alcohol practices, tumor location, carcinoembryonic antigen level, and family history of colon cancer. Postoperative information was collected and included the total region of surgical resection, tumor histologic type and differentiation status, the presence of lymphovascular or perineural invasion, final American Joint Commission on Cancer (AJCC) TNM classification, and the presence or absence of metastases.

Serum samples

Serum samples were collected from all study participants at the time of the colectomy to be processed for systemic cytokine analysis. All participants had been fasting for 12 h before collection of the blood samples. Red-top tubes were used to collect the stored serum samples used for cytokine analysis. These samples were collected, processed, and stored at -80°C until they were analyzed for cytokine concentrations.

Tissue samples and culture

Resected specimens were dissected by a pathologist, and fresh samples were obtained from the tumor and uninvolved normal colon. The normal bowel samples were obtained from macroscopically normal appearing colon more than 10 cm from the tumor. The samples were transported on ice in phosphate-buffered saline directly to the laboratory for immediate processing. The tissue was gently washed three times in advanced DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with penicillin (100 IU/mL), and streptomycin (100 $\mu\text{g/mL}$). Each sample was then carefully blotted, weighed, and individually placed in a 24-well tissue culture plate (1 mL culture medium/well). Colonic tissue culture for cytokine analysis was performed according to previous protocols.^{19,20} Briefly, the culture medium consisted of RPMI-1640 (Thermo Fisher Scientific) supplemented by 10% heat inactivated fetal calf serum (Thermo Fisher Scientific), 2-mM L-glutamine (Thermo Fisher Scientific), penicillin (100 IU/mL), and streptomycin (100 $\mu\text{g/mL}$). After 18 h of incubation at 37°C in a humidified 95% air/5% CO_2 atmosphere, medium was removed, filtered, and stored at -80°C until required for cytokine assays.

Biochemical preparation and cytokine measurements

We hypothesized that advanced stage CRC would have higher levels of proinflammatory cytokines, thus we selected an assay kit that contained both proinflammatory and anti-inflammatory cytokines. The concentrations of IL-1 β , IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, CD40L, IFN- γ , and TNF- α were measured using the Bio-Plex Pro Human Th17 Cytokine Assay Kit (cat. no. 171AA001M, Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Briefly, cytokine assay plates were prewet with assay buffer and washed twice with wash buffer. Coupled magnetic beads were added to the 96-well plate, along with serial dilutions of the reconstituted standard and the samples. This mixture was left to incubate in the dark at room temperature on an orbital shaker for 60 min. Detection antibodies with antibody diluent were then added to the wells for an additional 30 min at room temperature, followed by washing and adding streptavidin-PE for 10 min at room temperature. The samples were washed, and

plates were run on the Luminex machine using Bio-Plex Manager v 6.0. Results of the cytokine concentrations in the supernatant of tissue cultures were converted to pg/mg of tissue weight/mL of culture medium based on the standards included on each plate.

Statistical analysis

Results of the quantification of TNF- α , IFN- γ , IL-4, IL-10, IL-17A, IL-17F, IL-23, IL-25, IL-31 expression are presented as the mean $\pm$ standard deviation. Student's two-tailed *t*-test was used. *P* values <0.05 were considered to reflect significance. All statistical analyses were performed using GraphPad PRISM software version 5 (GraphPad Software, San Diego, CA).

Results

Patient characteristics

Table summarizes the clinicopathologic characteristics of the 33 patients who underwent surgical resection for CRC. There were approximately equal numbers of each gender, 18 men (54.5%) and 15 women (45.5%). The median age was 65 y (range, 40–84 y). Tumor location was as follows: right-sided 36.4% (12/33), transverse colon 9.1% (3/33), left-sided 45.5% (15/33), and rectum 9.1% (3/33). None of the patients received neoadjuvant chemotherapy. AJCC TNM classification was used, with incidence as follows: stage I-18.2% (6/33), stage II-27.3% (9/33), stage III-45.5% (15/33), and stage IV with metastasis to liver-9.1% (3/33). The three patients with metastatic disease each presented to the emergency department with near-obstructing lesions with mild-to-moderate disease burden in the liver. The liver lesions were biopsied intra-operatively and shown to be adenocarcinoma by histology.

Tumor cytokine analysis

The anti-inflammatory cytokines IL-4 and IL-10 levels were significantly elevated in advanced disease (stage III and IV) compared to local disease (stage I and II; Fig. 1). TNF- α levels were also significantly elevated in advanced disease, while IFN- γ levels were not statistically different between local and advanced disease (Fig. 2). IL-17A and IL-17F levels were significantly elevated in advanced disease (Fig. 3).

Systemic cytokine analysis

In the serum samples, the proinflammatory cytokine TNF- α levels were not significantly different between stage I-III *versus* stage IV, although the levels of the proinflammatory IFN- γ were significantly elevated in patients with metastatic disease (Fig. 4). Systemic levels of the Th17-associated cytokines IL-17F, IL-23, IL-25, and IL-31 were all significantly elevated in stage IV patients compared to stage I-III patients (Figs. 5 and 6). IL-17A was not detected in the serum of any of the patients.

Discussion

Understanding the mechanisms by which advanced and metastatic CRC has escaped immune surveillance remains a challenging and clinically significant problem. The tumor

microenvironment is extremely complex, as evidenced by complex pathways that include cytokines that are both anti-inflammatory and tumorigenic (IL-4 and IL-10) along with cytokines that are proinflammatory and tumorigenic (IL-17).^{21,22} Th17 cells and IL-17 have long been known to modulate different types of malignant tumors^{14,15,23–26} and often demonstrate prognostic value. In gastric cancer, for e.g., a higher percentage of IL-17 producing cells correlates with poor response to chemotherapy and decreased overall survival.^{27,28} Recently, murine models of CRC have shown that Th17 cells and IL-17 play a direct role in tumor-associated inflammation and cancer development.²⁹

The Th17-associated cytokine family is extremely complex and modulates multiple cellular responses including mucosal homeostasis.³⁰ IL-17 is primarily produced by Th17 cells and mediates autoimmunity and chronic infections through a variety of downstream cytokines, chemokines, and antimicrobial proteins.³¹ It has been shown to play a major role in enhancing chemotaxis in the innate immune response through proinflammatory mediators such as IL-6, TNF- α , C-reactive protein, matrix metalloproteases, and antimicrobial proteins.³²

Recent studies have revealed that IL-17 can promote tumor growth by multiple mechanisms. First, models of hepatocellular carcinoma and melanoma have shown enhanced tumor growth by inhibiting specialized immune cell infiltration.^{33,34} Other studies in IL-17-receptor (IL-17R) deficient mice have demonstrated that tumor growth is markedly reduced, and apoptosis is enhanced.^{35,36} Furthermore, experimental results from *in vivo* studies of human non-small-cell lung cancer in severe combined immunodeficiency mice have revealed that endogenous IL-17 upregulates the production of angiogenic CXC chemokines.³⁷ IL-17 can modulate the surrounding microenvironment and promote cancer cell invasion and metastasis via remodeling of the extracellular matrix.^{38–41} Finally, IL-17 is able to facilitate epithelial-mesenchymal transition, which plays an important role in tumor metastasis, by direct NF- κ B-mediated upregulation of ZEB1.⁴²

Although there are extensive data in animal models and *in vitro* highlighting the mechanisms by which IL-17 contributes to tumorigenesis, there are relatively few studies correlating Th17-associated cytokines with clinical outcomes, and even less data correlating IL-17 levels in the tumor microenvironment with systemic expression of Th17-associated cytokines. For instance, a recent 2015 meta-analysis by Zeng *et al.* of 28 studies evaluating the prognostic significance of IL-17 in all solid tumors included only four studies that evaluated peripheral IL-17 levels in their analysis (hepatocellular carcinoma, gastric cancer, and ovarian cancer).^{8,27,43,44} Our study is one of the first to correlate tumoral IL-17A with systemic IL-17 in CRC. We found that IL-17A is significantly increased in CRC patients with advanced disease ($P < 0.05$), along with significantly elevated systemic levels of Th17-associated cytokines IL-17F, IL-23, IL-25, and IL-31. IL-17F levels were significantly decreased in local disease CRC compared with advanced stage CRC. Interestingly, IL-17A was not detected in the serum of any of the patients. The reason for this is unclear, but a possible explanation is that IL-17A is selectively recruited and localized to the site of the tumor through an unknown mechanism.

There are several limitations of our study. Our results are applicable to sporadic, nonfamilial cases of CRC in patients who did not receive neoadjuvant chemotherapy and may not be generalizable to all patients with sporadic CRC. There were only three patients with stage IV disease which is a relatively small sample size to make comparisons to. Because of the relatively small sample size of our study, further validation of our findings by large prospective studies is warranted.

Elevated levels of Th17-associated cytokines in advanced stage CRC are clinically significant and are associated with poor outcomes such as decreased overall survival and possible resistance to chemotherapeutic regimens. Our study is one of the first to show that elevated systemic Th17-associated cytokines in CRC is associated with metastatic CRC. In 2011 Liu *et al.*⁴⁵ reported a 34% decrease in 5-year overall survival stage III CRC that stained positive for IL-17. In a murine model of spontaneous CRC, Wang *et al.*²⁹ observed that administration of neutralizing IL-17A antibody reduced the growth of adenomas and enabled apoptosis and tumor shrinkage in response to 5-fluorouracil. The present study is important because it shows that the Th17 pathway is associated with higher tumor stage, and it sheds light on mechanistic changes that occur between stages III and IV, namely that these cytokines are released into the systemic circulation. Further studies investigating the therapeutic inhibition of IL-17A in advanced stage CRC is warranted and may provide new avenues for targeted tumor therapy.

Conclusions

In conclusion, the results of our study suggest that the Th17-associated cytokines, specifically IL-17, have important implications in human CRC. Our study is one of the first to correlate tumoral and systemic IL-17 with AJCC stage in CRC. Multiple Th17-associated cytokines were detected both in tumor tissue and systemically. These changes in the Th17 cytokine network within the tumor microenvironment which can be detected systemically in metastatic disease could yield new insight into mechanisms of tumor seeding and metastasis in CRC and warrant further investigation.

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Authors' contributions: S.P.S. contributed to conception and design, data collection, data analysis, writing and editing the article, as well as approving the final version of the manuscript. D.A. contributed to conception and design, data analysis, and editing the paper. S.C.S. contributed to critical revision of the article and approving the final version of the manuscript. E.C.L. contributed to conception and design, data collection, data analysis, critical revision of the article, as well as approving the final version of the manuscript.

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Fig. 1. Tumor IL-4 and IL-10 levels in local and advanced disease CRC. Each bar represents mean $\pm$ standard deviation (* $P < 0.05$, ** $P < 0.001$).

Fig. 2. Tumor TNF- α and IFN- γ levels in local and advanced disease CRC. Each bar represents mean $\pm$ standard deviation (* $P < 0.05$, n.s. = not significant).

Fig. 3. Tumor IL-17A and IL-17F levels in local and advanced disease CRC. Each bar represents mean $\pm$ standard deviation (* $P < 0.05$).

Fig. 4. Systemic TNF- α and IFN- γ levels in stage I–III *versus* stage IV disease. Each bar represents mean $\pm$ standard deviation (**** $P < 0.00001$, n.s. = not significant).

Fig. 5. Systemic IL-17F and IL-23 levels in stage I–III *versus* stage IV disease. Each bar represents mean $\pm$ standard deviation (* $P < 0.05$).

Fig. 6.

Systemic IL-25 and IL-31 levels in stage I-III *versus* stage IV disease. Each bar represents mean $\pm$ standard deviation (* $P < 0.05$, ** $P < 0.001$).

Table

Patient characteristics.

Patient characteristics	No. of patients	%
Total number of patients	33	
Gender		
Male	18	54.5
Female	15	45.5
Median age	65	
60	24	72.7
<60	9	27.3
Median CEA, range, ng/mL	14.35 (2.04–39.37)	
Tumor location		
Right colon	12	36.4
Transverse colon	3	9.1
Left colon	15	45.5
Rectum	3	9.1
Primary tumor		
T1	0	0.0
T2	9	27.3
T3	18	54.5
T4	6	18.2
Regional LN		
N0	12	
N1	18	54.5
N2	3	9.1
Histologic subtype		
Adenocarcinoma	21	63.6
Mucinous adenocarcinoma	12	36.4
Differentiation		
Well	15	45.5
Moderate	6	18.2
Poorly	12	36.4
Lymphovascular invasion		
No	15	45.5
Yes	18	54.5
Perineural invasion		
No	19	57.6
Yes	14	42.4
AJCC TNM stage		
I	6	18.2

Patient characteristics	No. of patients	%
2	9	27.3
3	15	45.5
4	3	9.1

AJCC = American Joint Commission on Cancer; CEA = carcinoembryonic antigen; LN = lymph node.