

# Advanced Colorectal Cancer Is Associated with Impaired Interleukin 12 and Enhanced Interleukin 10 Production

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## ABSTRACT

Interleukin 12 (IL-12) is a heterodimeric cytokine that has been demonstrated to have a major role in stimulating a cell-mediated antitumor response. IL-10, a product of T helper 2 lymphocytes, is its most potent inhibitor. The aim of this study was to investigate whether patients with colorectal cancer had an imbalance in production of IL-12 and IL-10 preoperatively, and whether this was associated with advanced disease at surgery. Blood was obtained before surgery from 60 patients with colorectal cancer and from 30 controls. Peripheral blood mononuclear cells were incubated with *Staphylococcus aureus* Cowan's strain 1 *in vitro* for 24 h to assess IL-12 expression after stimulation, and serum was used for IL-10 measurement. IL-12 and IL-10 levels were assessed by ELISA. A single pathologist staged the tumors according to the tumor-node-metastasis (TNM) and Dukes' classifications. Patients with colorectal cancer had significantly lower levels of IL-12 ( $P < 0.001$ ) and higher levels of IL-10 ( $P = 0.004$ ) compared to controls. In addition, lower levels of IL-12 were detected in those patients who were node positive ( $P < 0.05$ ), had Dukes' C lesions ( $P \leq 0.001$ ), and T<sub>3</sub> or T<sub>4</sub> lesions ( $P < 0.033$ ) when compared to controls. Patients with Dukes' B and C lesions ( $P < 0.01$ ) and T<sub>3</sub> and T<sub>4</sub> lesions ( $P < 0.05$ ) also had higher levels of IL-10 compared to controls. This study is the first to demonstrate that patients with colorectal cancer have decreased IL-12 production and increased serum IL-10. This suggests an impaired T helper 1 cell-mediated antitumor response and provides some justification for exogenous IL-12 therapy or anti-IL-10 therapy in these patients.

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## INTRODUCTION

Colorectal cancer is the second most common cause of cancer death, with 28,000 new cases and 19,000 deaths per year in the United Kingdom. In recent years, there has been a minimal improvement in mortality, with an overall 5-year survival of less than 40% (1), which may be improved with appropriate adjuvant therapy (2).

Much interest has been concentrated on the potential use of cytokines, glycoproteins that play an important role in immunoregulation. Several cytokines have been demonstrated to exhibit a significant antitumor activity in man: (a) IFN- $\alpha$  in non-Hodgkin's lymphoma (3) and colorectal cancer (4, 5); (b) IL-4<sup>2</sup> in Hodgkin's disease (6); and (c) IL-2 in renal cell carcinoma and melanoma (7). However, response rates have been disappointing, with marked levels of toxicity, including the capillary leak syndrome after IL-2 administration (8).

IL-12 is a  $M_r$  70,000 heterodimeric cytokine composed of two *N*-glycosylated polypeptide chains of approximately  $M_r$  40,000 (p40) and  $M_r$  35,000 (p35) linked by a single disulfide bond (9, 10). It is produced by antigen-presenting cells, primarily those of the monocyte/macrophage system, and, to a lesser degree, by B lymphocytes.

Numerous *in vitro* and *in vivo* studies have demonstrated that IL-12 has a unique ability to skew the immune response in favor of a Th1 cytokine profile through the differentiation of naive Th cells (Th0) to the Th1 subset while inhibiting Th2 cell development and hence the characteristic cytokine expression of these latter cells (11-13). Th1 cells produce IFN- $\gamma$ , IL-2, IL-3, granulocyte macrophage colony-stimulating factor, TNF- $\alpha$ , and TNF- $\beta$ , whereas Th2 cells produce IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, granulocyte macrophage colony-stimulating factor, and TNF- $\alpha$ . The differences in cytokine secretion patterns lead to distinct effector functions. In general, Th1 cells preferentially induce cell-mediated immunity, whereas Th2 cells promote humoral immunity (14-16). IL-12 acts as a growth factor for activated NK and T cells (11), favors CTL generation (17-19), and induces the production of several cytokines, including TNF- $\alpha$ , IL-2, and, most importantly, IFN- $\gamma$  from these cells (9, 10, 14).

The activities of IL-12 are antagonized primarily by IL-10, which is produced mainly by Th1 cells and, to a lesser degree, by B cells, mast cells, and macrophages (20). IL-10 was first identified as a Th2 cell product that inhibited Th1 proliferation by down-regulating IFN- $\gamma$  and IL-2 production (21). IL-10 inhibits IFN- $\gamma$  production by suppressing the transcription of the IL-12 gene (20, 22).

<sup>2</sup> The abbreviations used are: IL, interleukin; Th, T helper; TNF, tumor necrosis factor; NK, natural killer; PBMC, peripheral blood mononuclear cell; SAC, *Staphylococcus aureus* Cowan's strain 1; mAb, monoclonal antibody; IQR, interquartile range.

Table 1 Patient details

Patients	No.	Mean age (yr)	Age range (yr)	Sex
Control	30	66	38–82	20 M:10 F
Colorectal cancer	60	69	32–92	29 M:31 F

Clinical and experimental data suggest that the host immune response may play a central role in patients with colorectal cancer. For example, lymphokine-activated killer cell activity has been shown to be impaired in patients with advanced gastrointestinal cancer (23), and lymphocytic infiltration within and around a tumor is one of the morphological features that has been associated with a better prognosis (24). A negative correlation between disease stage and IFN- $\gamma$  levels at the tumor site has been reported (25). IFN- $\gamma$  levels were also significantly lower in patients with distant metastases. Similarly, IL-2 production has been demonstrated to be impaired in patients with gastrointestinal cancer (26).

Because IL-12 is pivotal in inducing a cell-mediated immune response, including the production of IFN- $\gamma$  and IL-2, it has been postulated to have a role in inhibiting tumor growth and preventing tumor dissemination. The antimetastatic role of IL-12 in colon cancer has been demonstrated *in vivo* in a number of murine colon adenocarcinoma tumor models (27, 28) and *in vitro* on human colon cancer cell lines (29).

Phase I clinical trials of IL-12 for treatment of human malignancies have recently begun (30). However, it is not known whether patients with colorectal cancer have aberrant levels of IL-12. A study in patients with Sézary syndrome, an advanced form of cutaneous T-cell lymphoma, has shown significantly less IL-12 production by PBMCs when stimulated *in vitro* compared with that of controls. These patients also produced significantly less IFN- $\gamma$ , which was corrected by the addition of recombinant IL-12. The addition of an antihuman IL-10 mAb to the PBMC culture resulted in an increase in IL-12 and IFN- $\gamma$  production (31).

It was therefore our hypothesis that the previously demonstrated negative correlation between node metastases, IFN- $\gamma$  levels, and impaired IL-2 in patients with colorectal cancer was a result of impaired IL-12 production. A study was carried out to investigate (a) whether patients with colorectal cancer had impaired IL-12 production preoperatively, and (b) the relationship between IL-12, IL-10, and IFN- $\gamma$  levels and disease stage.

## PATIENTS AND METHODS

**Patients.** Consecutive patients undergoing surgery for primary colorectal cancer were recruited to the study. Age-matched patients undergoing elective hernia repair were used as controls. Any patients who had undergone preoperative adjuvant therapy, required blood transfusion, presented as emergencies with sepsis, or were taking steroids or other immunosuppressive therapy were excluded.

**Blood Samples.** Blood (30 ml) was taken preoperatively using a vacutainer (Becton Dickinson Vacutainer Systems, San Jose, CA). Twenty ml were required for isolation of PBMCs, and 2 ml were required for flow cytometry; 8 ml were spun down, and the serum was stored at  $-80^{\circ}\text{C}$ .

**Preparation of Human Lymphocytes.** Heparinized blood (20 ml) was diluted 1:1 with PBS, and then the PBMCs were isolated by the Ficoll-Hypaque (Histopaque-1077; Sigma-Aldrich Co., Ltd., Irvine, United Kingdom) density gradient purification technique (32). The number of PBMCs obtained was calculated using a hemocytometer, and cell viability was assessed by trypan blue exclusion.

**Cell Culture.** The cells were suspended in RPMI 1640 (Life Technologies Inc., Ltd., Paisley, United Kingdom) supplemented with 1% FCS (10% for IFN- $\gamma$  culture), penicillin (100 units/ml), streptomycin (100  $\mu\text{g}/\text{ml}$ ), and glutamine (2 mM) to give a final concentration of  $1 \times 10^6$  cells/ml. Stimulation of IL-12 production was carried out with 0.0075% (w/v) fixed SAC (Pansorbin; Calbiochem-Novabiochem Corp., La Jolla, CA) added to the cells. Stimulation of IFN- $\gamma$  production was carried out with fixed phytohemagglutinin (Sigma-Aldrich Co., Ltd.). The cells were then incubated for 24 h at  $37^{\circ}\text{C}$  (33). After incubation, the supernatant was collected and stored at  $-80^{\circ}\text{C}$ .

**Measurement of Cytokine Levels.** IL-10 levels in serum and IFN- $\gamma$  and IL-12 levels in the cell culture supernatant (after stimulation) were measured in duplicate with the relevant solid-phase ELISA using the quantitative sandwich enzyme immunoassay technique (R&D Systems Europe, Abingdon, United Kingdom).

**Flow Cytometry Analysis.** Blood (1 ml) was added to the lysing solution (14 ml; 9 g of  $\text{NH}_4\text{Cl}$ , 1 g of  $\text{KHCO}_3$ , and 37 mg of tetrasodium EDTA dissolved in 1 liter of distilled water, adjusted to pH 7.3). The mixture was allowed to stand for 5 min and then centrifuged at 1300 rpm for 5 min. The supernatant was removed by aspiration, and the pellet of cells was washed with PBS, 2% FCS, and 0.01 M  $\text{NaN}_3$ . Centrifugation was repeated, the supernatant was discarded, and the pellet was resuspended in the wash buffer (1 ml).

FITC (10  $\mu\text{l}$ )- or phycoerythrin-conjugated mAbs specific for antigens CD56 (NK cells), CD3 (T lymphocytes), CD4 (helper T cells), CD8 (cytotoxic T cells), and CD14 (monocytes) were added to 50  $\mu\text{l}$  of the cell preparation [all mAbs were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA)]. After a 30-min incubation on ice in the dark, PBS, 2% FCS, and 0.01 M  $\text{NaN}_3$  (2 ml) were added to each sample. The cell suspensions were centrifuged at 1100 rpm for 5 min, the supernatant was aspirated, and PBS, 2% FCS, and 0.01 M  $\text{NaN}_3$  (0.5 ml) were added. Analysis was then carried out on a FACSCalibur flow cytometer (Becton Dickinson).

**Histological Assessment.** The tumors were staged by a single pathologist, who was blind to the results of the assays, according to the Dukes' (34) and tumor-node-metastasis (TNM; Ref. 35) classifications. The tumor volume was also calculated.

**Statistical Analysis.** Comparisons of samples to establish the statistical significance of difference were determined by Wilcoxon's rank-sum test and Spearman's correlation coefficient for nonparametric data. Results were considered to be statistically significant when  $P$  was  $\leq 0.05$ .

## RESULTS

A total of 90 patients was recruited for the study (60 patients with colorectal cancer and 30 controls). The clinical details are summarized in Table 1.

Table 2 Cytokine level and tumor volume versus node status

The IL-12 levels in supernatant after stimulation and IL-10 levels in serum for control and colorectal cancer patients subdivided into node-negative and node-positive groups are shown. The tumor volume is also shown. Results are expressed as the median level with the IQR.

Node status	No.	IL-12 level (pg/ml)	IL-10 level (pg/ml)	Tumor volume (cm <sup>3</sup> )
		Median (IQR)	Median (IQR)	Median (IQR)
Control	30	31.9 (17.3–51.6)	2.56 (1.40–3.59)	
Node negative	44	13.6 (7.89–24.6) <sup>a</sup>	4.10 (2.44–6.59) <sup>a</sup>	6.00 (2.68–13.2)
Node positive	16	5.79 (0.24–21.0) <sup>b</sup>	4.15 (2.88–7.37) <sup>a</sup>	20.6 (13.8–37.5)

<sup>a</sup>  $P < 0.05$  when compared with controls.

<sup>b</sup>  $P < 0.05$  when compared with controls and node-negative tumor patients.

Table 3 Cytokine level and tumor volume versus Dukes' stage

The IL-12 levels in supernatant after stimulation and IL-10 levels in serum for control and colorectal cancer patients staged according to Dukes' classification are shown. The tumor volume is also shown. Results are expressed as the median level with the IQR.

Group	No.	IL-12 level (pg/ml)	IL-10 level (pg/ml)	Tumor volume (cm <sup>3</sup> )
		Median (IQR)	Median (IQR)	Median (IQR)
Control	30	31.9 (17.3–51.6)	2.56 (1.40–3.59)	
Dukes' stage				
A	15	8.21 (3.42–23.4)	3.50 (2.23–5.65)	2.25 (1.94–6.75)
B	29	16.0 (9.54–25.2)	5.33 (2.50–7.84) <sup>a</sup>	9.50 (4.75–15.8)
C	16	5.79 (0.24–21.0) <sup>a</sup>	4.15 (2.88–7.37) <sup>a</sup>	20.6 (13.8–37.5)

<sup>a</sup>  $P < 0.05$  when compared with controls.

Table 4 Cytokine level and tumor volume versus tumor stage

The IL-12 levels in supernatant after stimulation and IL-10 levels in serum for control and colorectal cancer patients staged according to the TNM classification system are shown. The tumor volume is also shown. Results are expressed as the median level with the IQR.

	No.	IL-12 level (pg/ml)	IL-10 level (pg/ml)	Tumor volume (cm <sup>3</sup> )
		Median (IQR)	Median (IQR)	Median (IQR)
Control	30	31.9 (17.3–51.6)	2.56 (1.40–3.59)	
Tumor stage				
T <sub>1</sub>	4	25.8 (7.85–38.3)	1.65 (0.89–3.66)	3.13 (1.31–5.50)
T <sub>2</sub>	15	10.1 (5.36–21.5) <sup>a</sup>	3.50 (2.49–5.65)	4.00 (2.25–7.00)
T <sub>3</sub>	35	13.8 (5.90–25.0) <sup>a</sup>	4.75 (2.45–6.59) <sup>a</sup>	14.0 (6.13–20.6)
T <sub>4</sub>	6	7.59 (2.72–13.9) <sup>a</sup>	7.95 (5.94–9.28) <sup>a</sup>	33.5 (16.4–72.4)

<sup>a</sup>  $P < 0.05$  when compared with controls.

Initial work demonstrated that the biologically active form of IL-12, IL-12p70, was not detectable in the serum using the currently available ELISA kits (data not shown). Therefore, it was necessary to stimulate isolated PBMCs with SAC, a non-specific cell stimulator. Stimulation of IL-12 production was carried out with fixed SAC for 24 h. The median level of IL-12 generated by patients with colorectal cancer, 12.5 pg/ml (IQR, 5.67–23.4 pg/ml), was significantly less than the levels produced by controls [31.9 pg/ml (IQR, 17.3–51.6 pg/ml);  $P < 0.001$ ]. When the patients with colorectal cancer were subdivided into node-negative and node-positive groups, the difference in IL-12 levels was statistically significant ( $P < 0.05$ ; Table 2). Patients with Dukes' C lesions at surgery also had lower levels of IL-12 production compared with those of patients with Dukes' B lesions ( $P = 0.027$ ) and with those of controls ( $P < 0.001$ ; Table 3). In addition, patients with T<sub>2</sub>, T<sub>3</sub>, and T<sub>4</sub> lesions had lower levels of IL-12 than did controls ( $P = 0.003$ ,  $P < 0.001$ , and  $P = 0.003$ , respectively; Table 4).

The median level of IL-10 in the serum of patients with colorectal cancer, 4.11 pg/ml (IQR, 2.46–6.64 pg/ml), was

significantly higher than that of controls [2.56 pg/ml (IQR, 1.40–3.59 pg/ml);  $P = 0.004$ ]. When the effect of node status on IL-10 levels was assessed, the difference in IL-10 levels between node-positive and node-negative tumors was not significant ( $P = 0.920$ ; Table 2). However, patients with Dukes' B and C lesions at surgery had higher levels of IL-10 compared with those of controls ( $P = 0.008$  and  $P = 0.009$ , respectively; Table 3). In addition, patients with T<sub>3</sub> lesions had significantly higher IL-10 levels than did controls ( $P = 0.006$ ). Patients with a tumor stage of T<sub>4</sub> also had higher IL-10 levels than did patients with T<sub>1</sub>, T<sub>2</sub>, and T<sub>3</sub> lesions ( $P = 0.019$ ,  $P = 0.020$ , and  $P = 0.031$ , respectively; controls,  $P = 0.001$ ; Table 4). Analysis of the correlation between IL-10 and IL-12 levels by coefficient demonstrated a negative correlation in patients with colorectal cancer ( $P = 0.009$ ).

Analysis of the ability of PBMCs to produce IFN- $\gamma$  after stimulation demonstrated no significant difference between colorectal cancer patients and controls, whichever disease classification was used (Table 5).

Analysis of tumor bulk (Tables 2–4) demonstrated an

Table 5 IFN- $\gamma$  level versus node status

IFN- $\gamma$  levels after stimulation for control and colorectal cancer patients are shown. Colorectal cancer patients have also been divided into node-negative and node-positive groups. Results are expressed as the median level with the IQR.

Node status	No.	IFN- $\gamma$ (pg/ml)
		Median (IQR)
Control	16	105 (ND <sup>a</sup> -347)
Tumor present	24	ND (ND-150)
Node negative	20	ND (ND-106)
Node positive	4	137 (28.8-258)

<sup>a</sup> ND, not detectable.

increase in size with advancing stage, but there was no significant association between tumor bulk, IL-12, and IL-10.

Analysis of the relative numbers of NK cells, monocytes, and CD4<sup>+</sup> and CD8<sup>+</sup> T cells as assessed by flow cytometry demonstrated no significant difference between patients with colorectal cancer and controls (data not shown). A comparison of cell numbers with respect to cytokine levels showed an increased proportion of CD8<sup>+</sup> cells with increasing IL-12 levels; however, these differences were not statistically significant (Table 6). The flow cytometry results may well be explained by the action of IL-12 on cellular proliferation in a paracrine fashion at the tumor site, rather than systemically.

## DISCUSSION

IL-12 has been demonstrated to have an antitumor and antimetastatic role *in vivo* in a number of murine colon adenocarcinoma tumor models when given systemically (i.p.), intratumorally (27, 28), or by paracrine secretion using gene therapy techniques (36). Work *in vitro* on human colon cancer cell lines has also indicated that IL-12 exhibits an antimetastatic role. This is mediated through its inhibitory effect on colon cancer cell attachment, motility, and invasion, due in part to an increase in E-cadherin levels (29). The antitumor activity of IL-12 is mediated by its effects on NK and T cells, with tumor regression being correlated with an increased number of NK and CD8<sup>+</sup> T cells at the tumor site (37). In addition to activating immune effector cells, IL-12 induces high levels of IFN- $\gamma$  from these cells (38).

Results from the first Phase I clinical trial treating human malignancies with IL-12 have recently been reported (30). Of 40 patients, including 5 with advanced colorectal cancer, 1 partial response (renal cell cancer) and 1 transient complete response (melanoma) have been observed. Although clinical trials have begun, no work has been published investigating the levels of IL-12 in patients with colorectal cancer.

In the present study, we report that after stimulation of isolated PBMCs, IL-12 production was significantly impaired in patients with colorectal cancer, compared with that of controls. The decrease in IL-12 expression was most clearly seen in advanced disease at the time of surgery; node-positive/Dukes' C lesions, and T<sub>3</sub> or T<sub>4</sub> lesions. In contrast to a previous report by Numata *et al.* (25), no significant difference was observed in IFN- $\gamma$  production by PBMCs after phytohemagglutinin stimulation in colorectal cancer patients *versus* controls.

Table 6 IL-12 level versus fluorescence-activated cell-sorting analysis of lymphocyte populations

Percentage of NK cells and CD8<sup>+</sup> T cells as a proportion of lymphocytes assessed by flow cytometry compared with IL-12 levels after stimulation is shown. Results are expressed as the mean percentage of cells with the SE of the mean.

IL-12 level (pg/ml)	No.	NK cells	CD8 <sup>+</sup> cells
		Mean % $\pm$ SE	Mean % $\pm$ SE
<2.0	6	18.8 $\pm$ 5.38	18.7 $\pm$ 3.14
2.0-10	11	19.5 $\pm$ 2.71	21.9 $\pm$ 3.41
$\geq$ 10	16	18.8 $\pm$ 2.44	24.6 $\pm$ 3.33

Our results show that PBMCs from node-positive tumors produce significantly lower amounts of IL-12; hence, it may be possible to use IL-12 production as a prognostic marker to determine nodal spread. Obviously, these findings will need verification in a larger study.

Previous studies have demonstrated that the activities of IL-12 are primarily antagonized by IL-10, mainly at the level of cytokine gene transcription (20). Statistical analysis of IL-10 in the sera demonstrated that patients with colorectal cancer had significantly higher levels than did controls. IL-10 levels were also higher in patients with Dukes' B, Dukes' C, T<sub>3</sub>, and T<sub>4</sub> lesions at surgery compared with those in controls.

Although the results of impaired production of IL-12 are shown in an *in vitro* system using a nonspecific cell stimulator (SAC), we hypothesize that patients with colorectal cancer have an impaired IL-12-mediated antitumor response. As has been shown previously (14-16), a decrease in IL-12 expression is associated with a shift toward a Th2 response. Our theory is supported by the observation that the circulating serum IL-10 level was significantly increased. The imbalance in IL-12 and IL-10 expression would also explain the previously demonstrated locally impaired production of IFN- $\gamma$  and IL-2 in patients with colorectal cancer (25, 26). From this study, it is not possible to elucidate definitively whether a decrease in IL-12 production or an increase in IL-10 expression was the initiating event. The results suggest that a defect in IL-12 production occurs first, because there is a significant decrease in IL-12 production when comparing T<sub>2</sub> tumors with controls, but a significant increase in IL-10 levels is not seen until tumors reach T<sub>3</sub>.

In this particular study, it was impossible to assess whether cytokine levels in patients returned to those seen in controls after surgery, because all patients with advanced disease went on to chemotherapy regimes. This also means that no conclusions can be drawn as to whether the observed imbalances in cytokine expression are the cause or simply the effect of the disease. Intratumoral analysis of the local effects of IL-12 and lymphocyte subsets are currently being examined using immunohistochemical techniques.

Analysis of tumor bulk demonstrated the expected increase in size with advancing stage, although no direct correlation between tumor bulk and IL-12 or IL-10 levels was demonstrated when all of the colorectal cancer patients were studied. Previous studies have demonstrated that IL-10 is produced by certain colon cell lines (39). Whether the observed increase in IL-10

production seen with some larger tumors is due solely to the production of this cytokine by the tumor is currently being investigated.

Our study suggests that there is a group of patients with advanced colorectal cancer disease who have an impaired IL-12 response and enhanced IL-10 production that may well benefit from exogenous IL-12 therapy or anti-IL-10 therapy. Also, it has been shown that patients with impaired IL-12 and enhanced IL-10 production have increased tumor bulk and a more advanced stage at surgery. These measurements before surgery may indicate a group of patients who would warrant preoperative neoadjuvant therapy.

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