

Eotaxin-2 and Colorectal Cancer: A Potential Target for Immune Therapy

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Abstract **Purpose:** To study the production of chemokines by colorectal hepatic metastases. **Experimental Design:** Biopsies of resected colorectal hepatic metastases and nonneoplastic adjacent liver tissue were screened for chemokines using protein arrays and results were confirmed by ELISA and immunohistochemistry. **Results:** Two chemokines, eotaxin-2 and MCP-1, were found at elevated levels within the tumor biopsy compared with adjacent liver. The relative increase in expression from tumor was much higher for eotaxin-2 than MCP-1, with 10 of 25 donors having a >100-fold increase in expression compared with 0 of 24 donors for MCP-1. In a parallel analysis, eotaxin-2 was also found at elevated levels in the tumor region of primary colorectal cancer biopsies. Immunohistochemical staining indicated that carcinoembryonic antigen – positive tumor cells stained strongly for eotaxin-2, implicating these cells as the predominant source of the chemokine. *In vitro* studies confirmed that several colorectal tumor lines produce eotaxin-2 and that secretion of this chemokine could be depressed by IFN- γ and enhanced by the Th2-type cytokines interleukin-4 and interleukin-13. Jurkat T cells were engineered to express the receptor for eotaxin-2 (CCR3). These cells effectively migrated in response to eotaxin-2 protein, suggesting that immune cells gene modified to express a chemokine receptor may have improved abilities to home to tumor. **Conclusions:** Taken together, these observations confirm eotaxin-2 as a chemokine strongly associated with primary and metastatic tumors of colorectal origin. Furthermore, the importance of this result may be a useful tool in the development of targeted therapeutic approaches to colorectal tumors.

Chemokines are small molecules that mediate the migration of lymphocytes and other immune cells around the body through binding of appropriate G protein – coupled receptors (1). To date, at least 50 human chemokines and 18 chemokine receptors have been described and it is the complex interplay and degeneracy of these receptors and ligands that form a

network thought to control cellular migration patterns. It is becoming increasingly clear that the chemokine network plays an important role in cancer through its effect on the growth and metastasis of tumor cells as well as in manipulating host-tumor interactions (2). Examples include the chemokine receptor CXCR4, which has been identified on at least 23 different tumor cell types and has also been shown to play an important role in the migration of metastatic tumor cells *in vitro* (3–6). In colorectal cancer, strong CXCR4 expression is significantly associated with increased stage, lymph node metastasis, and reduced 3-year survival (7, 8). Furthermore, chemokines can also drive tumor cell proliferation as observed in glioblastoma cells, which expressed high levels of CXCR4 (9).

Colorectal cancer is the third most common cancer in the United States with 50% to 60% of patients progressing to develop hepatic metastases (10). Despite this prevalence of clinical cases, relatively little is known about the chemokine profile of hepatic colorectal metastasis. Two independent studies have shown an increased proportion of CCR5⁺ CXCR3⁺ CD3⁺ cells within tumor-infiltrating lymphocytes or within the invasive margin of hepatic colorectal metastases (11, 12), whereas a high frequency of CD3⁺ T cells and CD68⁺ monocyte/macrophages has been identified at the interphase between neoplastic and nonneoplastic tissue of hepatic tumor metastasis (13). Increased numbers of macrophages have also been found in hepatic colorectal metastases compared with liver tumor metastases of breast origin (14). Both interleukin

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(IL)-1 β and tumor necrosis factor- α were identified by real-time PCR in liver tissue surrounding the metastatic tumor (13), whereas the presence of certain CXC (IL-8) and CC (MIP-1 α , MIP-1 β , and MCP-1) chemokines has been identified in tumor and surrounding tissue by immunohistochemical methods (12). Taken together, these studies suggest that the chemokine network may be important in driving cellular infiltration into and surrounding colorectal hepatic metastases and as such may be important in the development and treatment of the disease. For example, tumor-specific chemokine signatures may allow the specific targeting of immune cells to tumor through the expression of the relevant chemokine receptor. This study used protein array technology to identify whether tumor-specific chemokines could be identified in biopsies of hepatic colorectal cancer metastasis after comparison with matched nonneoplastic tissue biopsies taken >5 cm from the tumor margin. These analyses identified several candidate chemokines, including eotaxin-2. Importantly, eotaxin-2 was found to be highly expressed in biopsies from primary colorectal cancer, suggesting that eotaxin-2 may be suitable as an immunotherapy target for primary and metastatic colorectal cancer.

Materials and Methods

General reagents and antibodies. AIM-V, DMEM Glutamax, and fetal bovine serum were obtained from Life Technologies (Invitrogen). Mouse IgG1 isotype control (Dako) and mouse anti-human carcinoembryonic antigen (CEA; Dako) were obtained from DakoCytomation. Mouse anti-human eotaxin-2 (MAB343) and matched antibody pairs MAB 343, 266, 279, and 208 and BAF 343, 266, 279, and 208 for eotaxin-2, IP-10, MCP-1, and IL-8 ELISAs were obtained from R&D Systems. The cell lines LS174T (colon adenocarcinoma; European Collection of Animal Cell Cultures), LOVO (colon adenocarcinoma; American Type Culture Collection), SW480 (colorectal adenocarcinoma; American Type Culture Collection), COLO 201 (colorectal adenocarcinoma; American Type Culture Collection), and COLO 205 (colorectal adenocarcinoma; American Type Culture Collection) were cultured in DMEM Glutamax plus 10% heat-inactivated FCS and passaged by addition of trypsin-EDTA (Sigma).

Tumor collection. All tumor samples were obtained with the approval of the North Manchester and South Manchester Local Research Ethics Committee and the written consent of the patients. Samples of colorectal hepatic metastases were obtained from patients undergoing hepatic resection at North Manchester General Hospital (Manchester, United Kingdom). All patients (4 females and 21 males) had been previously diagnosed with colorectal hepatic metastases and had a median age of 65 years (range, 49-74). Resected tumor and corresponding adjacent liver tissue 5 cm from the tumor margin (pathologically defined as normal liver) were transported to the laboratory in AIM-V medium on ice where a proportion of each sample was snap frozen and stored in liquid nitrogen or formalin fixed. Samples of hepatic metastases were also obtained from patients whose primary tumor was diagnosed as melanoma, renal cancer, breast cancer, neuroendocrine cancer, or rhabdomyosarcoma. Samples of primary colorectal tumors and corresponding bowel tissue >5 cm from the tumor margin (pathologically defined as normal bowel) were obtained from six patients undergoing surgery to have their tumor removed. Formalin-fixed, paraffin-embedded sections of normal liver from healthy donors free of liver disease were purchased from AMS Biotechnology (Europe) Ltd.

Immunohistochemistry. Formalin-fixed tissue samples were embedded in paraffin wax and cut into 4- μ m sections. Antigen retrieval was done on hydrated sections by immersing in 1 L of pH 6 10 mmol/L

citric acid and microwaving at full power (800 W) for 25 min followed by 15-min cooling for sections to be stained for CEA. Antigen retrieval was done on hydrated sections by immersing in 250 mL of Target Retrieval solution pH 9 (Dako) and microwaving at full power (800 W) for 3 min followed by a further 15 min at 160 W (20%) for sections to be stained for eotaxin-2. Following the endogenous peroxidase block (0.03% hydrogen peroxide, 5 min) and a blocking step in 5% goat serum and 2% bovine serum albumin, colorectal hepatic metastases and adjacent liver sections were stained with 5 μ g/mL mouse anti-human CEA antibody, 6.6 μ g/mL mouse anti-human eotaxin-2, or mouse IgG1 isotype control at the corresponding concentration. Antibody binding was detected using the EnVision+ Detection System (Dako), and nuclei were counterstained with Gill 1 hematoxylin (Thermo Electron). Primary colorectal tumors and adjacent bowel tissue sections were stained by the same method except that anti-eotaxin-2 and relevant isotype control antibodies were used at 2.5 μ g/mL. Slides were analyzed on an Olympus BX51 microscope at \times 100 or \times 600 magnification.

Tumor cultures. Approximately 1 cm³ pieces of biopsy material were weighed and cultured in 24-well plates in 1 mL AIM-V medium for 24 h at 37°C, 5% CO₂. The supernatant was removed, and cellular debris was pelleted by centrifugation at 13,000 \times g for 5 min and stored at -80°C until required.

Protein arrays. Supernatants were analyzed for the expression of various chemokines and cytokines using the RayBiotech Cytokine Array V as per the manufacturer's protocol (RayBiotech, Inc.). Briefly, the array was blocked for 30 min at room temperature and then incubated with 1 mL of the tissue supernatants for 2 h at room temperature. The array was then probed for cytokine/chemokine expression using a cocktail of biotinylated antibodies followed by a 60-min incubation with streptavidin-horseradish peroxidase conjugate and signal was visualized by chemiluminescence detection using a Chemi-8000 digital imager (UVP) or by exposure to X-ray film. Chemokine spot density was analyzed using GeneTool software (Syngene) and calculated as the spot density / (density positive controls - density negative control) after background subtraction, and the fold increase of chemokine secretion from tumor over adjacent tissue was calculated.

Detection of chemokines by ELISAs. Tissue supernatants were thawed and analyzed for eotaxin-2, IP-10, MCP-1, and IL-8 by ELISA using matched antibody pairs MAB 343, 266, 279, and 208 and BAF 343, 266, 279, and 208 as described in the manufacturers' instructions (R&D Systems). Samples were added at a range of dilutions in AIM-V medium to ensure that they lay within the limit of sensitivity of the assay (31.25 pg/mL).

Real-time PCR. Snap-frozen biopsy samples (~0.5 cm³) were homogenized using a mortar and pestle and RNA was isolated using Trizol reagent (Invitrogen). Samples were then further purified using the RNeasy Cleanup Protocol (Qiagen) and up to 1 mg of total RNA was used to synthesize cDNA using the SuperScript II First-Strand cDNA Synthesis kit (Invitrogen). Real-time PCR for eotaxin-2 (forward primer, 5'-GCAGGAGCACATGCCTCAA-3'; reverse primer, 5'-GGCGTCCAGGTTCTTCATGTA-3') and β -2-microglobulin (forward primer, 5'-GGAATTGATTGGGAGAGCATC-3'; reverse primer, 5'-CAGGTCCTGGCTCTACAATTTACTAA-3') was done on an ABI 7700 Prism instrument using SYBR Green Mastermix (Applied Biosystems).

Production of eotaxin-2 by tumor cell lines. LOVO, LS174T, SW480, COLO 201, and COLO 205 (10⁶) were plated in 3 mL DMEM, 10% fetal bovine serum in six-well plates. After 24 h at 37°C, 1,000 IU/mL IFN- γ , 2 ng/mL transforming growth factor- β 1, and 10 ng/mL of IL-4, IL-10, IL-13, or IL-1 β (all from R&D Systems) were added in 0.5 mL and cells were cultured for a further 24 h. Supernatant was collected for analysis of eotaxin-2 levels by ELISA, and RNA was isolated by Trizol/RNeasy extraction. Real-time PCR for eotaxin-2 was done as detailed above.

Migration of CCR3.2A.MFEz Jurkats to eotaxin-2. Human CCR3 was isolated from human peripheral blood mononuclear cells by PCR

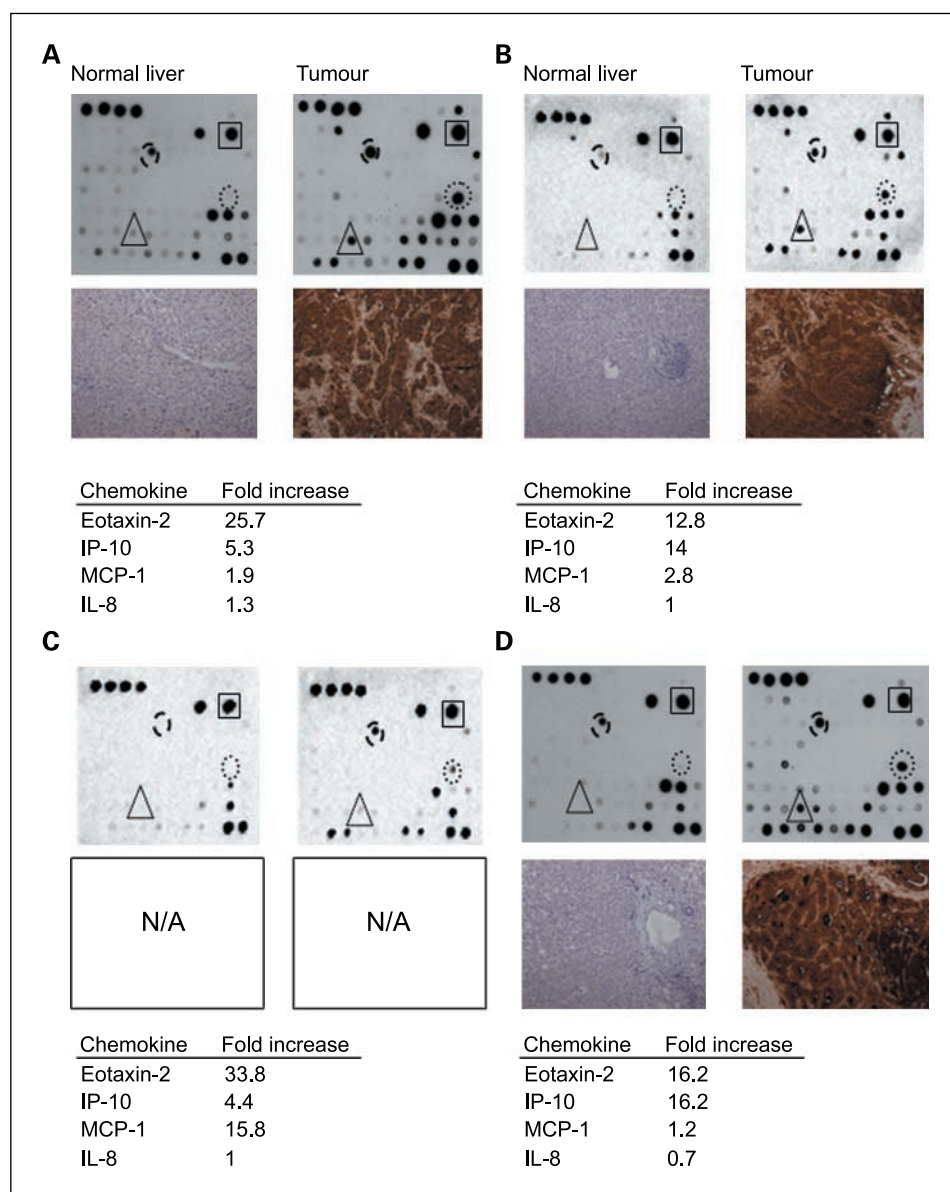
with *EcoRI* and *SalI* restriction sites introduced at the 5' and 3' end, respectively. CCR3 was cloned into the pMP71 retroviral vector upstream of a 2A cleavage sequence (15) and the CEA-targeting chimeric receptor MFEz consisting of an scFv linked to codon-optimized CD3 ζ (GenScript Corp.; ref. 16). Jurkat cells (American Type Culture Collection) were transduced with amphotropic retroviral particles generated by transient transfection of 293T cells (16) by centrifugation at $1,000 \times g$ for 3 h. Following a week of culture, transduced Jurkats were stained with anti-human CCR3-PE (R&D Systems), sorted on a FACSVantage (Becton Dickinson), and cultured in RPMI 1640 (BioWhittaker) plus 10% fetal bovine serum, 25 mmol/L HEPES, 50 nmol/L 2-mercaptoethanol, and 2 mmol/L l-glutamine. CCR3 expression by flow cytometry was analyzed using anti-CCR3 Alexa Fluor 647 (BD Biosciences). Migration of 10^5 Jurkats/transduced Jurkats across a fibronectin-coated 5- μ m Transwell insert (Corning) in the presence of 0 to 50 ng/mL of eotaxin-2 in the lower chamber in the presence of 500 ng/mL mouse IgG or anti-CCR3 (R&D Systems) was measured by counting the number of cells that had migrated to the lower chamber over a 4-h period by acquiring for 45 s on low flow on a FACSCalibur.

Results

Increased concentration of specific chemokines identified in culture supernatants generated from biopsies of primary colorectal hepatic metastases. Tissue biopsies and adjacent nonneoplastic tissue taken >5 cm from the tumor margin were initially collected from four patients undergoing surgical resection of hepatic metastases of colorectal origin and examined for CEA expression. Immunohistochemical staining confirmed that all tumor biopsies used in the study expressed CEA to high level, whereas the adjacent liver tissue was essentially devoid of this tumor-associated antigen (Fig. 1).

Approximately equal-sized (1 cm^3) biopsy samples were placed in culture wells and cultured for 24 h in serum-free culture medium, after which time the culture supernatants were analyzed for the presence of chemokines and cytokines leached from the tissue samples using a protein array. Across the four sample pairs, only three chemokines were found at consistently

Fig. 1. Analysis of chemokine secretion from colorectal hepatic metastases. Tissue samples (1 cm^3) taken from colorectal liver metastases (top right) or normal liver 5 cm from the tumor margin (top left) were cultured in 1 mL AIM-V medium for 24 h, and supernatants were analyzed for chemokine expression using the RayBiotech Chemokine Protein Array V. pI (A), pII (B), pIII (C), and pIV (D) were analyzed and the expression of eotaxin-2 (dotted circle), MCP-1 (hashed circle), IL-8 (square), and IP-10 (triangle) is highlighted. Spot density was quantified using GeneTool software, relative to positive and negative controls, and is expressed as the fold increase of spot intensity from hepatic metastases compared with normal liver (table). CEA expression on formalin-fixed paraffin sections of normal liver (bottom left) and colorectal liver metastases (bottom right) was detected by immunohistochemistry (EnVision).



increased levels in tumor compared with nontumor tissue. Eotaxin-2 was at the greatest differential (average increase tumor versus nontumor, 22.1 ± 9.5 -fold; range, 12.8-33.8), whereas two other chemokines (IP-10 and MCP-1) were also present at increased levels compared with matched adjacent tissue (10.0 ± 6.0 -fold; range, 4.4-16.2 and 5.4 ± 7.0 -fold; range, 1.2-15.8, respectively; Fig. 1). No other chemokines were found to be consistently increased in tumor versus adjacent tissue (Supplementary Table S1), including IL-8 (1.0 ± 0.3 -fold; range, 0.7-1.3), which was chosen as a control for further studies.

To confirm these initial results, tissue supernatants were generated from biopsies of matched, weighed tumor and adjacent liver tissue from a larger panel of donors (patient details provided in Supplementary Table S2) and the levels of eotaxin-2, IP-10 (both $n = 25$ donors), MCP-1 ($n = 24$ donors), and IL-8 ($n = 13$ donors) were quantified by ELISA (full details of analysis presented in Supplementary Table S3). This analysis supported the array results in that both eotaxin-2 and MCP-1 were found to be at significantly greater levels in tumor supernatants compared with the adjacent tissue control (Fig. 2). The average level of eotaxin-2 found across the range of donors was a surprisingly high 17.4 ng/g of tumor tissue (SEM, 11.2 ng/g of tissue; range, 0.1-77.9 ng/g of tissue) compared with an average of 0.2 ng/g (SEM, 0.15 ng/g; range, undetectable to 1.1 ng/g of tissue) found for adjacent liver tissue (eotaxin-2: tumor versus tumor-associated tissue, $P < 0.001$, Wilcoxon signed rank test; Fig. 2A). The absolute concentration of MCP-1 detected in tumor samples was lower than that of eotaxin-2 (7.7 ng/g of tissue; SEM, 5.1 ng/g; range, undetectable to 12.3 ng/g of tissue) but was also highly significantly increased when compared with that observed in matched adjacent liver tissue [0.4 ng/g of tissue; SEM, 0.1 ng/g; range, undetectable to 2.4 ng/g of tissue; MCP-1 (tumor) versus MCP-1 (adjacent liver tissue), $P \leq 0.001$, Wilcoxon signed rank test; Fig. 2B].

In contrast, no difference in average IP-10 concentration between tumor (7.5 ng/g of tissue; SEM, 3.0 ng/g; range, undetectable to 48.7 ng/g of tissue) and adjacent liver tissue (5.5 ng/g of tissue; SEM, 3.2 ng/g; range, undetectable to 80.8 ng/g of tissue; $P = 0.231$, Wilcoxon signed rank test; Fig. 2C) was found. However, in agreement with the array analysis, no significant difference in the concentration of IL-8 between tumor and adjacent liver could be found (tumor $21.5 \pm$ SEM 5.6 ng/g of tissue; range, 1.8-83.3 ng/g of tissue; adjacent liver $13.7 \pm$ SEM 2.6 ng/g of tissue; range, 0.5-34.1 ng/g of tissue; $P = 0.087$, Wilcoxon signed rank test; Fig. 2D).

In summary, protein array and ELISA analysis confirmed that eotaxin-2 and MCP-1 are present in tumor-derived supernatants at levels significantly higher than that found in local adjacent tissues, whereas no difference was found for either IP-10 or IL-8. The magnitude of the increase in eotaxin-2 found in tumor supernatants is further exemplified when considering that >90% (23 of 25) of donors showed an increase of >10-fold and 40% (10 of 25) of donors showed an increase of >100-fold in eotaxin-2 concentration above that of the adjacent matched liver tissue (Fig. 3A). In the case of MCP-1, only 42% (10 of 24) of donors possessed a >10-fold and no donors exhibited a >100-fold increase in chemokine concentration over control. Although the comparison of the average values of IP-10 between tumor and adjacent tissue showed no statistically significant difference, a few donors were identified as having large increases in chemokine concentration in tumor over control (16% for 10-fold increase and 8% for >100-fold increase). This observation is reflected in the fact that the median concentration of IP-10 in tumor was also higher than that of adjacent liver tissue (2.0 versus 0.7 ng/g of tissue).

Eotaxin-2 mRNA is up-regulated in biopsies of hepatic colorectal metastases. Quantitative PCR was done on nine donor samples to explore whether the levels of eotaxin-2

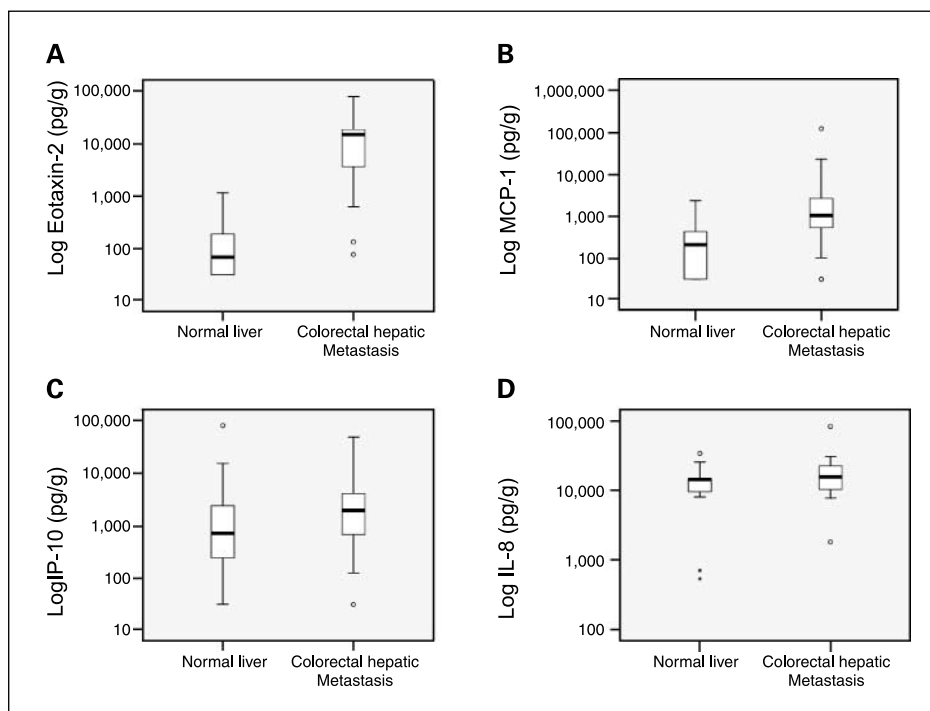


Fig. 2. Eotaxin-2 is highly expressed from colorectal hepatic metastases. Tissue samples (1 cm^3) taken from colorectal liver metastases or normal liver 5 cm from the tumor margin were cultured in 1 mL AIM-V medium for 24 h and supernatants were analyzed for eotaxin-2 (A), MCP-1 (B), IP-10 (C), and IL-8 (D) expression by ELISA. The limit of detection of the ELISA was 31.25 pg/mL. Values were corrected to pg/g tissue. Results are expressed as log box plots depicting the median (horizontal line), interquartile range (box), and the 95% confidence intervals (whiskers). Outliers are also shown (circles, asterisks).

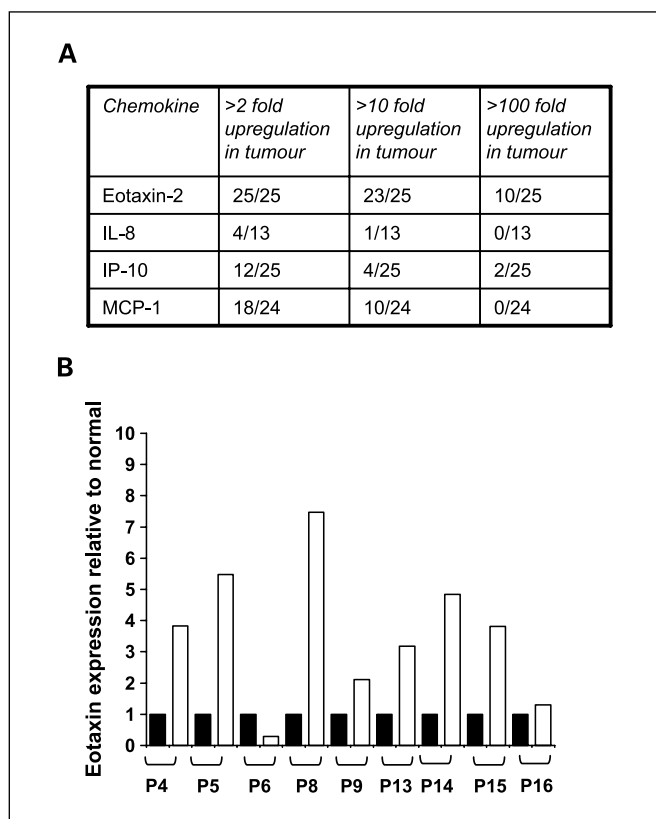


Fig. 3. A, relative fold increase in the level of the chemokines eotaxin-2, IP-10, MCP-1, and IL-8 from colorectal hepatic metastases compared with normal liver as quantified by ELISA. B, relative expression of eotaxin-2 mRNA compared with hypoxanthine phosphoribosyltransferase was determined by real-time PCR and data are expressed as the fold increase of eotaxin-2 mRNA from colorectal hepatic metastases (white columns) compared with normal liver (black columns).

mRNA in tumor and adjacent liver tissue supported the protein analysis. All nine donor samples showed up-regulation of eotaxin-2 in tumor samples by ELISA analysis but the fold increase varied widely (2.3- to 2,493-fold above matched adjacent liver tissue). Quantitative PCR analysis confirmed that eotaxin-2 mRNA was present at levels >2-fold increased in metastatic tissue than corresponding adjacent liver in seven of nine patients, with one further donor exhibiting a fold increase that was <2-fold in magnitude (Fig. 3B). In contrast to eotaxin-2, IL-8 mRNA levels were found to be an average of 40-fold higher (± 36) in six samples of tumor tissue compared with adjacent liver tissue, a result in contradiction to that observed in ELISA analysis of cultured biopsies. The inference is that the level of IL-8 in short-term culture of "normal" liver samples may be artificially high perhaps due to hypoxia within the biopsy sample, thereby reducing the difference observed between tumor and normal biopsy samples, a phenomenon reported elsewhere (17). However, correlations between mRNA and ELISA analysis were consistent within both experimental methods for eotaxin-2.

Taken together, these data support the view that eotaxin-2 is present at high levels in hepatic colorectal metastatic tumor tissue and these levels are significantly above that observed in adjacent tissue. To determine whether these high levels of eotaxin-2 were a feature specific to colorectal liver metastases or were common to all types of liver tumor metastases, several

biopsies of liver metastases derived from varying primary tumors were obtained. Although rare in number, donors with liver metastases originating from rhabdomyosarcoma, melanoma, breast, renal, and neuroendocrine tumors were identified, but of the five tumor samples, eotaxin-2 was only detected to any significant level in the renal cell carcinoma tumor metastasis but at a level less than that in matched adjacent liver (Supplementary Fig. S1). MCP-1 was found to be up-regulated in four of the five samples.

Eotaxin-2 is present at high level in primary colorectal tumor. Because the presence of eotaxin-2 seemed to be associated specifically with liver tumor metastasis of colorectal origin, an obvious question was whether primary colorectal tumors also produced significant levels of eotaxin-2 or whether the production of this chemokine was liver specific. Primary colorectal tumor biopsies or associated bowel tissue taken at least 5 cm from the tumor margin were collected at the time of surgery and cultured overnight in serum-free medium to release chemokines and cytokines. Six separate donor samples were analyzed and eotaxin-2 was found at statistically significant higher levels in the tumor samples as against the matched control (tumor $5.5 \pm \text{SEM } 2.8$ ng/g of tissue; range, 0.1-19.1 ng/g; adjacent bowel tissue $1.1 \pm \text{SEM } 0.4$ ng/g of tissue; range, undetectable to 2.7 ng/g; tumor versus adjacent bowel tissue, $P = 0.028$, Wilcoxon signed rank test; Fig. 4A). No significant differences between tumor and associated bowel tissue-derived MCP-1 ($n = 11$; $P = 0.176$), IP-10 ($n = 9$; $P = 0.929$), and IL-8 ($n = 6$; $P = 0.273$) were detected (Fig. 4B-D).

Cellular localization of eotaxin-2 in hepatic colorectal metastases and primary colorectal tumor biopsies. In an attempt to identify which cell type was predominantly responsible for the production of this chemokine, fixed serial sections were stained for eotaxin-2 (one representative donor is shown in Fig. 5A and B, three further donor samples are presented in Supplementary Fig. S2). H&E staining of adjacent liver tissue confirmed that these sections were histologically normal with intact liver parenchyma and associated hepatic arteries and bile duct (Fig. 5A). However, the tumor sections showed distinct tumor invasion with disrupted liver architecture and intense tumor-associated expression of CEA (Fig. 5B). CEA was also observed in stromal material both within and close to the tumor, consistent with the fact that CEA is shed from the tumor cell surface (18). Importantly, serial tumor sections also stained strongly for eotaxin-2 where staining was predominantly cytoplasmic in location (Fig. 5B). However, there was weak staining for eotaxin-2 both in the normal liver tissue situated close to the tumor margin and also observed in the adjacent liver tissue section (Fig. 5A and B). Interestingly, the bile duct visible within the portal triad of the adjacent liver tissue sections was very strongly positive for eotaxin-2 while remaining completely negative for CEA (Fig. 5A and B). In these sections, both tissue stroma and infiltrating cells seemed to be negative for eotaxin-2. Liver sections from two normal healthy donors were also analyzed and no eotaxin-2 was detected in the bile duct or the hepatocytes except at very low levels in the hepatocytes in donor 2 (Supplementary Fig. S3).

In addition, primary colorectal tumors and adjacent bowel tissue from two donors were also analyzed (one representative donor is shown (Fig. 5C and D), the remaining one donor is shown in Supplementary Fig. S2). Once again, H&E staining confirmed the normal histopathologic nature of the adjacent

bowel tissue (Fig. 5C) with the apical glandular cells weakly positive for CEA as documented for the normal gastrointestinal expression of this antigen (19). However, the tumor sections displayed intensely staining CEA-positive tumor tissue, including stromal cells and glandular crypts (Fig. 5D). The basal end of the adjacent bowel glands was strongly positive for eotaxin-2, whereas strong cytoplasmic eotaxin-2 staining was observed equally distributed within the glandular cells of the bowel tumor with stroma and infiltrating cells again negative for eotaxin-2.

Eotaxin-2 production by colorectal cancer cell lines is modulated by Th1/Th2 cytokines. Five colorectal cell lines were assessed for their ability to produce eotaxin-2. Three lines, SW480, COLO 201, and COLO 205, all secreted readily detectable levels of eotaxin-2 protein, whereas LOVO and LS174T cells did not (Fig. 6A). Culture with IFN- γ resulted in down-regulation of protein secreted in SW480 and COLO 201 cells (Fig. 6A). Quantitative PCR analysis indicated that eotaxin-2 mRNA levels were decreased in all five cell lines after IFN- γ treatment (Fig. 6B). Interestingly, IL-4 and IL-13 both up-regulated the expression of eotaxin-2 in LS174T and LOVO cells at the protein (Fig. 6C) and mRNA level (Fig. 6D), whereas transforming growth factor- β 1, IL-1 β , and IL-10 had no significant effect. However, this array of cytokines failed to up-regulate protein or mRNA levels in SW480 and COLO 205 cells, which spontaneously produce high levels of eotaxin-2. Overall, this *in vitro* analysis indicates that certain Th1- and Th2-type cytokines can influence eotaxin-2 expression, suggesting that the balance of cytokines present within the tumor may play a role in driving production of this chemokine from tumor cells.

Eotaxin-2 acts as a target for gene-modified immune cells. Given the high tumor-specific level of eotaxin-2 within colorectal cancer, the next stage was to investigate whether this chemokine could be used as a target for immunotherapy. To

this end, Jurkat T cells were transduced with the receptor for eotaxin-2 (CCR3) coexpressed with a CEA-specific chimeric immune receptor (16) and sorted based on expression of the CCR3 receptor. Seventy-five percent of sorted cells expressed CCR3 as determined by flow cytometry (Fig. 6E), whereas 95% of cells expressed the CEA-specific chimeric immune receptor (data not shown). Encouragingly, these cells migrated toward recombinant eotaxin-2 protein in a Transwell assay (Fig. 6F) and also showed a typical "bell-shaped" response curve where no migration was observed at higher concentrations of eotaxin-2 (50 ng/mL), indicating likely receptor desensitization at high ligand concentrations. Control nontransduced Jurkat T cells failed to respond to eotaxin-2, whereas migration of the transduced T cells was blocked using an antibody specific for CCR3, thereby confirming this receptor as driving migration of the transduced cells (Fig. 6F).

Discussion

Somewhat surprisingly, the chemokine eotaxin-2 (CCL24) was found to have the greatest significance in this comparative analysis between hepatic colorectal metastasis tumor biopsy with matched adjacent pathologically normal tissue, an observation that to our knowledge has not previously been reported.

Eotaxin-2 seems to be a chemokine that is specifically expressed within colorectal cancer primary and hepatic metastatic tumor and to far lower degree within surrounding nonneoplastic tissue. Other chemokines, such as MCP-1 and IP-10, were also found within tumor tissue as documented in other studies (12). However, despite limited sample numbers, liver metastases originating from rhabdomyosarcoma, breast, renal, and neuroendocrine tumors also had elevated MCP-1 levels in biopsy supernatants but did not seem to express

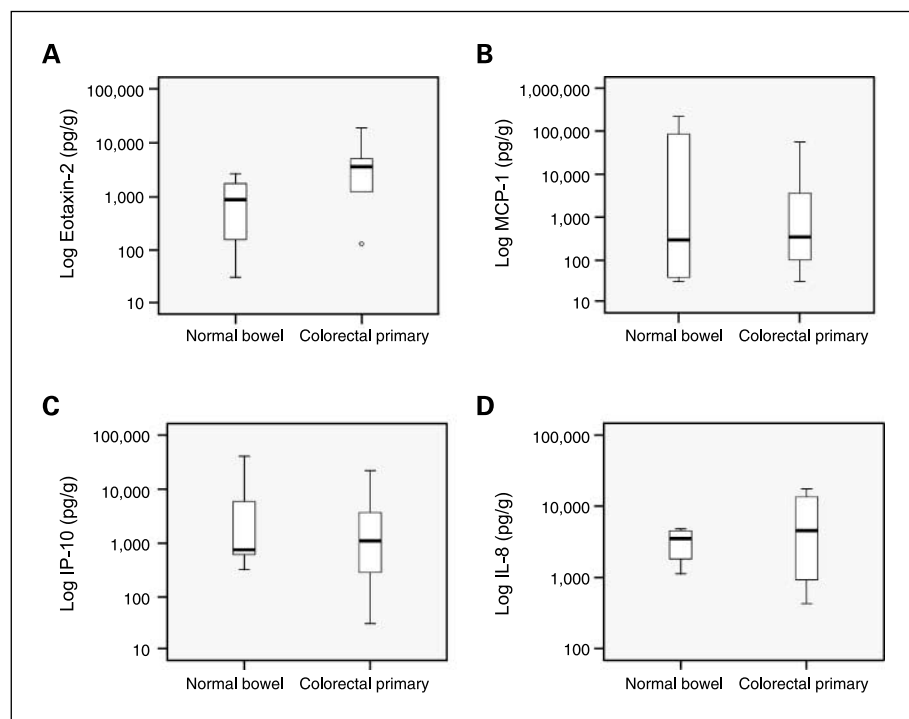


Fig. 4. Tissue samples (1 cm^3) taken from primary colorectal tumors or the normal bowel 5 cm from the tumor margin were cultured in 1 mL AIM-V medium for 24 h, and supernatants were analyzed for eotaxin-2 (A), MCP-1 (B), IP-10 (C), and IL-8 (D) expression by ELISA. Values below the limit of detection of the ELISA are not shown. Values were corrected to pg/g tissue. Results are expressed as log box plots depicting the median (horizontal line), interquartile range (box), 95% confidence intervals (whiskers), and outliers (circles). Data are indicative of 6, 11, 9, and 6 patients for eotaxin-2, IP-10, MCP-1, and IL-8, respectively.

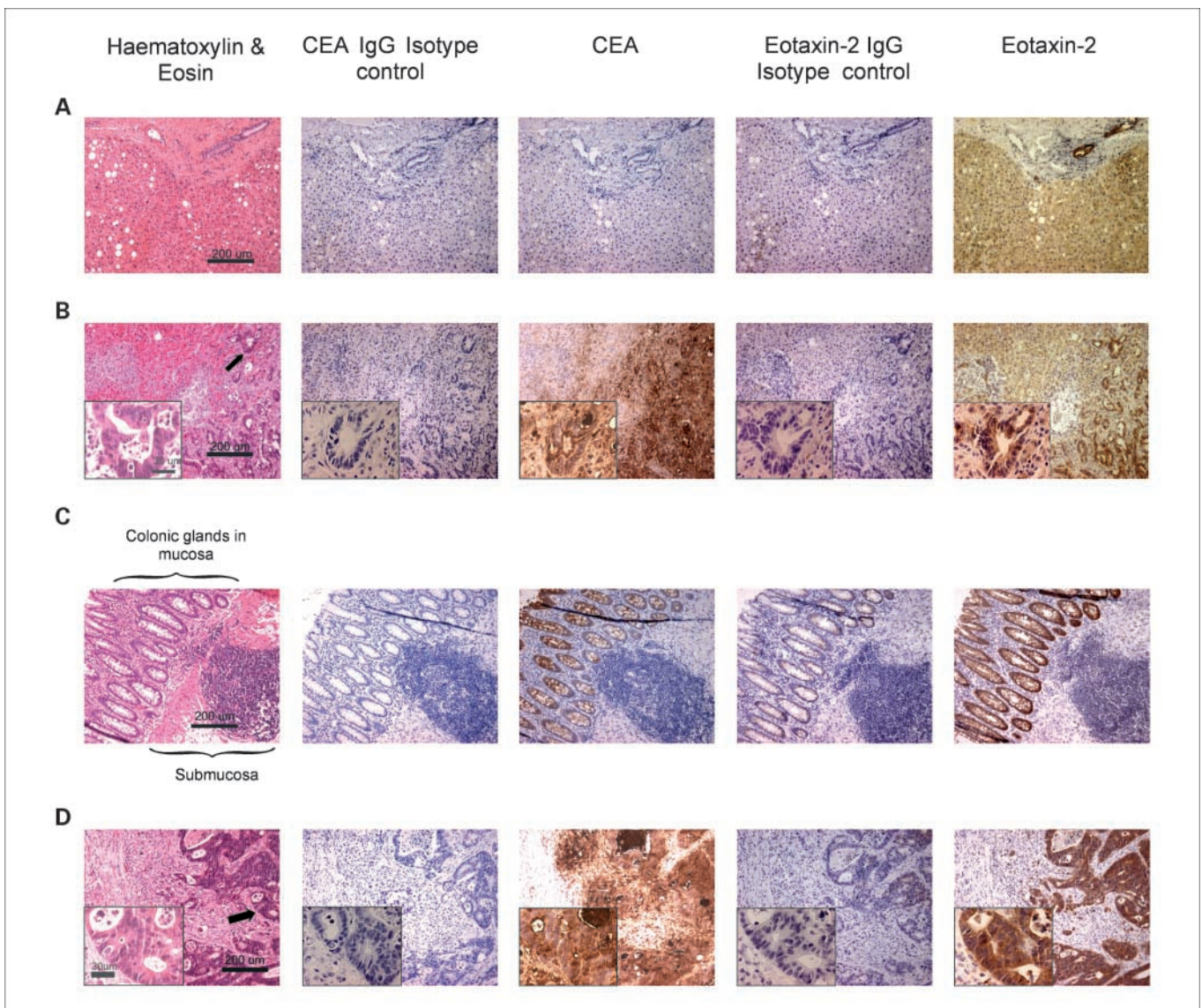


Fig. 5. Serial formalin-fixed, paraffin-embedded sections of normal liver (A), colorectal hepatic metastases (B), normal bowel (C), and primary colorectal tumors (D) were cut and antigen retrieval and immunohistochemistry were done as detailed in Materials and Methods. H&E, CEA, and eotaxin-2 staining is shown, along with the appropriate isotype control. Magnification, $\times 100$. B and D, insets, tumor cells at $\times 600$ magnification.

detectable levels of eotaxin-2. The observations that primary renal (20) and breast cancers (21) have also been shown to express MCP-1 further support our findings that MCP-1 expression is not specific to colorectal hepatic metastases. However, both primary colorectal and colorectal liver metastases produced significant levels of eotaxin-2, suggesting that the presence of this chemokine may be specific to this tumor type.

Eotaxin-2 belongs to the CC class of chemokines (β chemokines) and is one of three family members (including eotaxin-1/CCL11 and eotaxin-3/CCL26), which, despite relatively low levels of amino acid homology and distinct expression profiles, are characterized by being the major ligands of the chemokine receptor CCR3 (22). The cellular sources of eotaxin-2 include epithelial cells, such as bronchial epithelial cells (23), and can be induced in immune cells, including monocyte/macrophages (24). Gene expression is generally associated with allergic

responses with expression up-regulated in diseases such as asthma (25). Furthermore, gene expression of eotaxin family members is induced by the Th2-type cytokines IL-4 and IL-13 (26) and inhibited by the Th1-type cytokine IFN- γ (23).

However, relatively little is known about the role of these chemokines in relation to cancer. Eotaxin-1 has been identified in malignant oral squamous cell carcinoma cells, suggesting that epithelial cells were an important constitutive source of this chemokine and, furthermore, that tumor cells maintained this potential (27). In addition, eosinophil infiltration (possibly driven by eotaxin-1) has been correlated with an unfavorable prognosis in oral squamous cell carcinoma (28). However, the reverse has been documented in cervix, lung, and colon carcinomas (29–31) and it has been suggested that eosinophils may play a role in suppressing tumor growth (32). Eotaxin has also been shown to directly mediate angiogenesis of CCR3⁺ microvascular endothelial cells (33), and eotaxin-producing

melanomas have increased microvascular density and extensive thrombosis within the blood vessels of the tumor in a murine model system (34). It was hypothesized that eotaxin-1, although potentially responsible for recruiting eosinophils to the site of tumor and increasing blood vessel formation, may cause extensive thrombosis through degranulation of eosinophils and other cells (35), which may prevent effective infiltration of tumor by other components of the cellular immune system, such as cytotoxic T cells. Taken together with this study, the inference is that high levels of eotaxin-2 present within primary colorectal and liver metastatic tumors could be playing an important role in the conditioning of the tumor microenvironment.

Although the fold difference in levels of eotaxin-2 between tumor and adjacent liver tissue proved to be the greatest of those analyzed, it is the combinatorial effect of multiple chemokine families that provide the specific signature that may be playing the overall role in cellular recruitment toward tumor. In this context, our studies also identified and quantified the presence of MCP-1 and IP-10 in supernatants of colorectal hepatic metastases compared with adjacent tissue. IP-10 can be induced by IFN- γ in a variety of cell types, such as endothelial cells, monocytes, keratinocytes, and fibroblasts (36), whereas MCP-1 production can be induced in a wide number of cells, including macrophages, endothelial, and

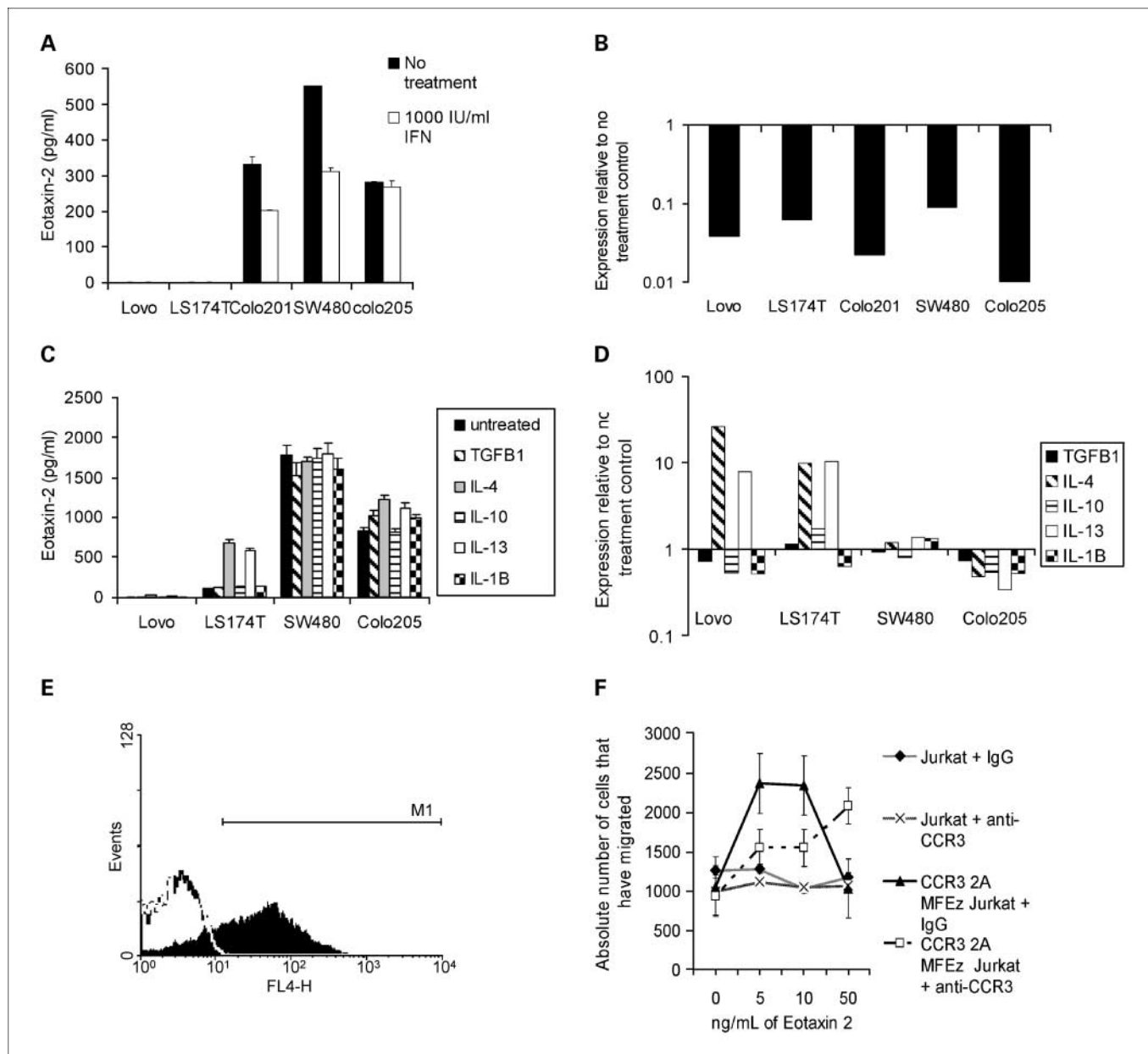


Fig. 6. Colorectal cancer cell lines (10^6) were cultured with IFN- γ , transforming growth factor- β , IL-4, IL-10, IL-13, or IL-1 β for 24 h. Eotaxin-2 production by ELISA (A and C) or quantitative PCR for eotaxin-2 transcript (B and D) is shown. Jurkats transduced with CEA-specific chimeric immune receptor and CCR3 were sorted and analyzed for expression of CCR3 by flow cytometry (E) Staining with anti-CCR3 Alexa Fluor 647 (filled histogram) and Alexa Fluor 647 IgG2b (open histogram) is shown. Migration of 10^5 control Jurkats/CCR3.2A, MFEz-transduced Jurkats across a 5- μ m fibronectin-coated Transwell over a 4-h period in response to 0 to 50 ng/mL of eotaxin-2 in the presence of 500 ng/mL. F, mouse IgG/ α CCR3 was measured by counting cells that had migrated to the lower chamber on a flow cytometer.

epithelial cells (37). Yoong et al. (12) described the presence of MCP-1 on tumor cells and IP-10 in infiltrating macrophages of colorectal hepatic metastases, which is consistent with the protein-based analysis done here. The same group analyzed tumor-infiltrating lymphocytes from colorectal cancer liver metastases and documented the presence of CXCR3⁺ tumor-infiltrating lymphocytes, which is consistent with the expression of the CXCR3 ligand IP-10, whereas only a low level of CCR3⁺ tumor-infiltrating lymphocytes was detected. On initial examination, this observation suggests that the high levels of eotaxin-2 are failing to induce the migration of CCR3⁺-expressing lymphocytes. However, *in vitro* studies have shown that CXCR3 ligands (including IP-10) can inhibit CCR3-mediated functional responses (including those driven by eotaxin-2), suggesting that IP-10 in colorectal cancer metastases may be regulating the functional activity of eotaxin-2 (38). Given the high levels of eotaxin-2 present within the tumor samples, there were surprisingly low numbers of eosinophils observed within tumor regions, suggesting that similar mechanisms inhibiting the influx of eosinophils may be at work here.

Alternatively, CD68⁺ macrophages/liver Kupffer macrophages are found around the tumor-normal tissue interface of liver colorectal cancer metastases at relatively high numbers (13) compared with liver metastasis of breast cancer origin (14). MCP-1 is a major chemotactic factor for macrophages (39); however, eotaxin-2 has also been shown to drive the recruitment of macrophages to sites of cutaneous injection in atopic and nonatopic volunteers (40) and as such may be playing a role here in the recruitment of macrophages to the site of tumor.

In reality, CCR3⁺ cells constitute a minority proportion of the T-cell population *in vivo*. In terms of immune therapy, transduction of immune effector cells with the CCR3 receptor may improve tumor targeting. Within this study, Jurkat T cells expressing the CCR3 receptor migrated in response to eotaxin-2 protein at levels corresponding to that found within the tumor biopsy samples (nanogram quantities). Similar approaches using chemokine targets have proven successful in other cancers

(17), suggesting that targeting of immune cells to sites of tumor may be possible through the expression of chemokine receptors. Further development of vectors will be required to optimize this approach. The vector used here used a bicistronic expression system encoding the CCR3 receptor with a tumor-targeting receptor. Such a system allows the potential of encoding multiple chemokine receptors, thereby providing a greater "fine tuning" of targeting. However, aside from the expression of chemokine receptors, understanding the combinatorial effects of the differing chemokines present within the tumor environment will be critical to ensure efficient targeting of gene-modified immune cells. For instance, this study suggests that the effects of MCP-1, IP-10, and IL-8 on immune cells expressing the CCR3 receptor would need examination. To this end, the development of suitable model systems will be central to the effective translation of this approach. As a final point, it is also clear that eotaxin-2 can be produced from normal tissues and particularly from tissues such as within the lung of asthmatic patients. Potential toxicities could arise from targeting of this tissue through CCR3-targeting approaches, implying that the use of multitargeting methods (such as the use of tumor-specific killing receptors) or control systems (e.g., suicide gene expression) should be used to reduce potential side effects.

In summary, the identification of high levels of eotaxin-2 chemokine, and the demonstration that T cells can be genetically engineered to migrate toward eotaxin-2, suggests that this chemokine may be of use as a target in the development of targeted immune cell therapies against colorectal carcinoma and, in particular, distant liver metastases of colorectal carcinoma origin.

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