GM-CSF Production by Tumor Cells Is Associated with Improved Survival in Colorectal Cancer

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Abstract

Purpose: Colorectal cancer infiltration by CD16+ myeloid cells correlates with improved prognosis. We addressed mechanistic clues and gene and protein expression of cytokines potentially associated with macrophage polarization.

Experimental Design: GM-CSF or M-CSF–stimulated peripheral blood CD14+ cells from healthy donors were cocultured with colorectal cancer cells. Tumor cell proliferation was assessed by ³H-thymidine incorporation. Expression of cytokine genes in colorectal cancer and autologous healthy mucosa was tested by quantitative, real-time PCR. A tumor microarray (TMA) including >1,200 colorectal cancer specimens was stained with GM-CSF– and M-CSF–specific antibodies. Clinicopathological features and overall survival were analyzed.

Results: GM-CSF induced CD16 expression in 66% ± 8% of monocytes, as compared with 28% ± 1% in cells stimulated by M-CSF (P = 0.011). GM-CSF but not M-CSF–stimulated macrophages significantly (P < 0.02) inhibited colorectal cancer cell proliferation. GM-CSF gene was expressed to significantly (n = 45, P < 0.0001) higher extents in colorectal cancer than in healthy mucosa, whereas M-CSF gene expression was similar in healthy mucosa and colorectal cancer. Accordingly, IL1β and IL23 genes, typically expressed by M1 macrophages, were expressed to significantly (P < 0.001) higher extents in colorectal cancer than in healthy mucosa. TMA staining revealed that GM-CSF production by tumor cells is associated with lower T stage (P = 0.02), “pushing” growth pattern (P = 0.004) and significantly (P = 0.0002) longer survival in mismatch-repair proficient colorectal cancer. Favorable prognostic effect of GM-CSF production by colorectal cancer cells was confirmed by multivariate analysis and was independent from CD16+ and CD8+ cell colorectal cancer infiltration. M-CSF expression had no significant prognostic relevance.

Colorectal cancer represents a major cause of cancer-related death in different geographic areas. A variety of current experimental models of colorectal cancer induction do support the notion of an important causal role of inflammation (6, 7). Indeed, chronic inflammation, as observed in different types of inflammatory bowel diseases (IBD), is known to be associated with increased colorectal cancer incidence in humans (6, 7).

However, in sporadic colorectal cancer, accounting for a large majority of these tumors, evidence of a clinically significant inflammatory state, possibly associated with cancer outgrowth, is infrequently observed. While questioning the pertinence of several murine models to sporadic human colorectal cancer, these common clinical observations urge addressing the issue of the role of innate and adaptive immune responses in these cancers.

A number of studies have convincingly demonstrated that colorectal cancer infiltration by T cells and, in particular, by CD8+ lymphocytes, is associated with improved survival. These cells usually display a memory (8, 9) and it is devoid of prognostic significance (10). Instead, at different stages of colorectal cancer, CD8+ T-cell infiltration is particularly evident in MMRp cancers in which poor CD8+ T-cell infiltration is detectable. These data underline specificities of colorectal cancer immunobiology and indicate that prognostic significance of defined tumor microenvironmental features critically depends on tumor types and related anatomic districts.

In the same line, we have observed that infiltration by myeloid CD16+ cells represents a novel, independent, favorable prognostic factor in colorectal cancer (16).

In this study we have attempted to unravel mechanistic clues possibly underlying these effects, and to address the expression at the gene and protein level of cytokines and chemokines associated with chemotraction and functional polarization of macrophage subsets possibly endowed with antitumor potential.

Materials and Methods

Generation and phenotypic and functional characterization of polarized macrophages

Monocytes were isolated from peripheral blood mononuclear cells (PBMC) of healthy donors to a >98% purity by using anti-CD14-coated magnetic beads (Miltenyi). Purified cells were cultured for 6 to 7 days in the presence of recombinant GM-CSF (Laboratorio Pablo Cassará) or M-CSF (R&D Systems) at 50 to 5 ng/mL concentrations in RPMI 1640 medium supplemented with antibiotics, glutamine, nonessential aminoacids, sodium pyruvate, HEPES, β-mercaptoethanol and 10% fetal calf serum (FCS; all from Invitrogen Life Sciences), thereafter referred to as complete medium, according to previously published protocols (17).

Freshly isolated or cultured cells were stained with CD16-, CD163-, and CD204-specific fluorochrome-conjugated antibodies (Becton Dickinson), and analyzed by using a 2-laser FACSCalibur flow cytometer (Becton Dickinson). Propidium iodide (PI) positive cells were excluded from the analysis. Results were analyzed by Cell Quest (Becton Dickinson) and Flow Jo (Tree Star) computer softwares.

Authenticated, established human colorectal cancer cell lines Colo205 and HCT116 were purchased from the European Collection of Cell Cultures (ECACC) and cultured in complete medium. To evaluate their cytostatic capacity, 6 to 7 days cytokine-stimulated macrophages (see above) were cocultured in 96-well plates (Falcon) at different effector:target ratios with 3,000 tumor cells for 2 days. 3H-Thymidine (Amersham GE) was then added (1 μCi/well) for overnight incubation. Cultures were then harvested and tracer incorporation was measured by β-counting.

Gene expression analysis

Total cellular RNA was extracted from surgical specimens of colorectal cancer and autologous healthy mucosa sampled at distance from the tumor and reverse transcribed. Predeveloped Taqman assays (Applied Biosystems) were used to quantitatively evaluate the expression of a panel of cytokine and chemokine genes by using ABI Prism 7300 PCR system (Applied Biosystems). Data are reported as relative expression normalized to GAPDH house-keeping gene amplification. Expression of individual genes was analyzed by using the 2−ΔΔCt method (18).

Tumor microarray construction

The tumor microarray (TMA) utilized in this study has been described in detail in previous reports (19, 20).
Briefly, it includes 1,420 unselected, nonconsecutive, primary sporadic colorectal cancers, treated between 1987 and 1996, and 71 normal mucosa specimens. These samples were collected from the Tissue Biobank of the Institute of Pathology, University Hospital Basel, performing translational research with the approval of the Ethical Committee Beider Basel (EKBB), in compliance with ethical standards and patient confidentiality. Tissue cylinders with a 0.6-mm diameter from formalin-fixed, paraffin-embedded tissue blocks from resected colorectal cancer were punched from representative tissue areas and brought into 1 recipient paraffin block (30 × 25 mm), using a semiautomated tissue arrayer. Punches were made from the center of the tumor to guarantee that each TMA spot included at least 50% tumor cells.

Clinicopathological annotation included patient age, tumor diameter, location, pT/pN stage, grade, histologic subtype, vascular invasion, border configuration, presence of peritumoral lymphocytic inflammation at the invasive tumor front and disease-specific survival (Table 1). Tumor border configuration and peritumoral lymphocytic inflammation were evaluated by using the original hematoxylin and eosin (H&E) slides of the resection specimens corresponding to microarray punches, as previously described (20). Numbers of lymph nodes evaluated ranged between 1 and 61 with mean and median of 12 and 11, respectively. MMR status was evaluated by immunohistochemistry according to MLH1, MSH2, and MSH6 expression (20), as previously described. The TMA under evaluation included 1,031 MMR-proficient and 194 MMR-deficient tumors. Follow-up data were available for 1,379 patients with mean/median and interquartile range (IQR) event-free follow-up time of 67.7/68 and 45 to 97 months.

**Immunohistochemistry**

Indirect immunoperoxidase protocol was used for immunohistochemistry (ABC-Elite, Vector Laboratories). Following slide dewaxing and rehydration endogenous peroxidase activity was blocked using 0.5% H2O2. Epitope retrieval was achieved by incubation in Epitope Retrieval Reagent 2 (EDTA buffer, pH 9; Leica Biosystems) at 100°C for 30 minutes, as previously described (20), before staining. The sections were treated with 10% normal goat serum and incubated with primary antibodies for 12 hours. Staining was visualized using DAB (3,3’-diaminobenzidine) (Dako, Glostrup, Denmark), and sections were counterstained with hematoxylin. Sections were treated with 10% normal goat serum and incubated with primary antibodies for 12 hours. Staining was visualized using DAB (3,3’-diaminobenzidine) (Dako, Glostrup, Denmark), and sections were counterstained with hematoxylin.

### Table 1. Association of GM-CSF staining and clinicopathological features in colorectal cancer (n = 1,239)

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>Low Histoscorea N = 475 (38.3%)</th>
<th>High Histoscore N = 764 (61.7%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (n = 1,239), yr</td>
<td>Mean, range 69.4 (39–95)</td>
<td>69.8 (30–96)</td>
<td>0.537b</td>
</tr>
<tr>
<td>Tumor diameter (n = 1,235), mm</td>
<td>Median, mean, range 50, 49.7 (4–150)</td>
<td>45, 48.6 (5–160)</td>
<td>0.146c</td>
</tr>
<tr>
<td>Gender (n = 1,239)</td>
<td>Female 248 (52.2) 407 (53.3) 0.760c</td>
<td>Male 227 (47.8) 357 (46.7) 0.760c</td>
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<tr>
<td>Tumor location (n = 1,225)</td>
<td>Left-sided 297 (63.6) 502 (66.2) 0.380d</td>
<td>Right-sided 170 (35.4) 256 (33.8) 0.380d</td>
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<tr>
<td>pT stage (n = 1,213)</td>
<td>pT1-2 76 (16.5) 166 (22.1) 0.020d</td>
<td>pT3-4 386 (83.5) 585 (77.9) 0.020d</td>
<td></td>
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<tr>
<td>pN stage (n = 1,197)</td>
<td>pN0 230 (50.2) 406 (54.9) 0.126d</td>
<td>pN1-2 228 (49.8) 333 (45.1) 0.126d</td>
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<tr>
<td>Tumor grade (n = 1,212)</td>
<td>G1–G2 400 (87.5) 658 (87.2) 0.9195d</td>
<td>G3 57 (12.5) 97 (12.8) 0.9195d</td>
<td></td>
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<tr>
<td>Vascular invasion (n = 1,212)</td>
<td>Absent 324 (70.7) 559 (74.1) 0.222d</td>
<td>Present 134 (29.3) 195 (25.9) 0.222d</td>
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<tr>
<td>Tumor growth pattern (n = 1,212)</td>
<td>Pushing/expanding 150 (32.8) 310 (41.1) 0.004d</td>
<td>Infiltrating 308 (67.2) 444 (58.9) 0.004d</td>
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<tr>
<td>Peritumoral lymphocyte infiltration (n = 1,213)</td>
<td>Absent 357 (77.9) 598 (79.2) 0.655d</td>
<td>Present 101 (22.1) 157 (20.8) 0.655d</td>
<td></td>
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<tr>
<td>Local recurrence (n = 433)</td>
<td>Absent 69 (55.2) 185 (60.1) 0.410d</td>
<td>Present 56 (44.8) 123 (39.9) 0.410d</td>
<td></td>
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<tr>
<td>Distant metastasis (n = 440)</td>
<td>Absent 110 (85.3) 252 (81.0) 0.356d</td>
<td>Present 19 (14.7) 59 (19.0) 0.356d</td>
<td></td>
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<tr>
<td>Postoperative therapy (n = 437)</td>
<td>None 97 (75.7) 205 (60.9) 0.282d</td>
<td>Treated 31 (24.3) 39 (39.1) 0.282d</td>
<td></td>
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<tr>
<td>Overall survival (n = 1,206)</td>
<td>5-yr (95% CI) 51.3 (46.7–56.4) 0.0002e</td>
<td>62.1 (58.4–66) 0.0002e</td>
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aGM-CSF staining intensity (0–3) multiplied by frequency (%) of stained cells. Based on ROC curves analysis, a value of 115 was used to discriminate between samples with low or high histoscore. bTest was used for age analysis because of normal distribution; cWilcoxon (Mann–Whitney test) was used for tumor diameter analysis. dDiscrete/qualitative variables: χ² test; e-log-rank test was used to compare overall survival rates. Statistically significant P values are reported in boldface.

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(DakoCytomation) for 20 minutes and incubated for 60 minutes at room temperature with monoclonal antibodies recognizing M-CSF (110-57176; Novus Biologicals) or CX3CL1/fractalkine (89229; Abcam) or overnight at 4°C (21) with a GM-CSF–specific reagent (100-65022; Novus Biologicals). Slides were then incubated with peroxidase-labeled secondary antibody (DakoCytomation) for 30 minutes at room temperature, immersed in 3-amino-9-ethylcarbazole plus substrate-chromogen (DakoCytomation) for 30 minutes, and counterstained with Gill’s hematoxylin.

Evaluation of immunohistochemistry

Percentages of positive tumor cells and staining intensities in each punch were evaluated and samples were classified as negative (0), weakly positive (1), moderately positive (2), and highly positive (3). A histoscore was calculated by multiplying staining intensity (0–3) by percentages of positive cells, as previously described (22). Immunohistochemical slides were independently examined by 3 experienced pathologists (L. Terracciano, L. Tornillo, B. Angrisani) blinded to any prior information on clinicopathological features of the patients’ samples, with excellent correlation between measurements.

Statistical analysis

Gene expression data from different tissues were compared by using the nonparametric Wilcoxon test for paired samples.

For outcome assessment, cut-off values used to classify colorectal cancer with low or high parameters of interest were obtained by ROC curves based on histoscore analyses, evaluating sensitivity and false-positive rate for the discrimination of survivors and nonsurvivors, on all tumor samples. Threshold values thus obtained were compared with the expression levels in nonmalignant and malignant colon tissues and final threshold values were set according to the expression levels in nonmalignant and malignant colon tissues. Threshold values thus obtained were compared with cultures performed in the presence of the same concentrations of M-CSF (average ± SE: 66% ± 8.7% vs. 28% ± 11.4%, n = 6, P = 0.011; Fig. 1A and B). In contrast, percentages of cells expressing CD204 molecular scavenger were significantly increased in M-CSF cells, as compared with GM-CSF–stimulated cells (average ± SE: 85% ± 6.5% vs. 41% ± 10%, n = 6, P = 0.008). Percentages of cells expressing CD163 did not significantly differ in cells cultured in the presence of M-CSF or GM-CSF (average ± SE: 78% ± 6.5% vs. 65% ± 10.1%, n = 6, P = 0.27). Representative histograms and cumulative data derived from 6 experiments with cells from different donors are reported in Fig. 1A and B.

Cytostatic activity of GM-CSF–activated macrophages against colorectal cancer cells

We then tested the effects of GM-CSF– and M-CSF–stimulated macrophages on the proliferation of MMR proficient (MMRp) Colo 205 colorectal cancer cells. Following 6 to 7 days stimulation in the presence of 25 to 6.25 ng/mL GM-CSF, monocytes were able to significantly inhibit colorectal cancer cell proliferation (Fig. 1C). This effect was dependent on effector:target (E:T) ratios and on GM-CSF doses used in the initial stimulation phase. In sharp contrast, monocytes cultured in the presence of the same concentrations of M-CSF, were devoid of antiproliferative ability, irrespective of E:T ratios (Fig. 1C). Effects of GM-CSF–stimulated monocytes were not mediatred by soluble factors. Indeed, neither recombinant GM-CSF nor culture supernatants did inhibit colorectal cancer cell proliferation. Moreover, macrophages did not induce apoptosis of target cells, as indicated by lack of annexin V binding, but rather exerted cytostatic effects. Interestingly, GM-CSF–stimulated monocytes at 1:1 E:T ratios were as effective as a 30 μg/mL concentration of the pyrimidin analog 5FU in inhibiting colorectal cancer proliferation (data not shown).

Comparable results were observed upon culture in the presence of GM-CSF but not M-CSF–stimulated monocytes by using MMR-deficient (MMRd) HCT116 colorectal cancer cells as targets (data not shown).

GM-CSF and M-CSF gene expression in colorectal cancer and in corresponding, autologous healthy mucosa

To obtain an insight into local tumor microenvironment conditions, we then addressed the expression of GM-CSF and M-CSF.
and M-CSF genes in surgically excised paired specimens of colorectal cancer and autologous healthy mucosa sampled at distance from the cancerous tissue (23).

Figure 1. Phenotypic and functional differentiation of GM-CSF– and M-CSF–stimulated monocytes. Peripheral blood CD14⁺ monocytes from healthy donors were magnetically sorted and cultured in the presence of GM-CSF or M-CSF (12.5 ng/mL). Cells were then washed and stained with mAbs recognizing the indicated markers (A and B). Representative results referring to uncultured monocytes (gray lines), M-CSF treated (black lines), and GM-CSF treated (shaded profiles) are shown in A, whereas B reports cumulative data from 6 independent experiments. GM-CSF or M-CSF–stimulated (12.5 ng/mL) cells were then cocultured with Colo205 cells at the indicated E:T ratios in flat bottom 96-well plates in triplicates. Tumor cell proliferation was assessed by ³H-thymidine incorporation on day 3. Control data refer to Colo205 cells cultured in the absence of myeloid cells (C). Data refer to 1 representative experiment out of 4 performed with cells from different donors with similar results.

GM-CSF gene was expressed to significantly higher extents in colorectal cancer tissue, as compared with corresponding autologous healthy mucosa [median, IQR: 6.167E10⁻⁹, 100], [median, IQR: 6.167E10⁻⁹, 100].
Expression of genes predominantly associated with M1 and M2 macrophages in colorectal cancer and in corresponding, autologous healthy mucosa

To obtain insights into specific gene signatures eventually detectable in clinical specimens, we assessed IL23 and IL1β gene expression in paired colorectal cancer and autologous healthy mucosa samples. We found that these genes were expressed to significantly higher extents in colorectal cancer than in matched healthy mucosa (median, IQR: IL23: 2.1E10−3 vs. 6.4E10−5, n = 45, P < 0.0001; IL1β: 2.3E10−2 vs. 3.8E10−3, 47, P < 0.0001; IL10; 5.2E10−1, n = 48, P = 0.001; Fig. 2B). However, expression of IL12p35 gene, reportedly typically observed in M1 cells, was detectable to significantly higher extents in healthy mucosa than in matched tumor tissues (median, IQR: 2.2E10−3 vs. 3.5E10−4, 1.9E10−4−8.5E10−4, n = 46, P = 0.01; Fig. 2B).

M2 polarized macrophages are characterized by the ability to produce IL10 (5). Indeed, we did not observe significant differences in IL10 gene expression between healthy mucosa and tumor tissue (median, IQR: 9.3E10−4 vs. 3.5E10−4, 3.1E10−4−1.2E10−3 vs. 3.4E10−4, 1.9E10−4−1E10−3, n = 46, P = 0.41).

Furthermore, expression of TNFα, IL6, and IL12p40 genes was also similarly detectable in healthy mucosa and corresponding colorectal cancer tissue (median, IQR: TNFα: 6.7E10−5 vs. 1.7E10−4−1.6E10−3, 3.1E10−4−1.8E10−3, n = 47, P = 0.089; IL6: 5.1E10−5, 8.6E10−6−6.1E10−4 vs. 2.7E10−4, 5.9E10−5−9.6E10−4, n = 45, P = 0.17; IL12p40: 6.8E10−5−3.19E10−5 vs. 2.7E10−4−7.5E10−5, 1.8E10−5−2.1E10−4, n = 46, P = 0.7; Fig. 2B).

Thus, conventional patterns of polarized macrophage gene expression do not seem to fully fit gene signatures detectable in colorectal cancer (4, 5). However, consistent with gene expression profiles commonly attributed to polarized macrophages (5), M-CSF and GM-CSF and TNFα gene expression in colorectal cancer tissues were highly significantly correlated (r = 0.63, P < 0.0001 and r = 0.49, P < 0.0001, respectively; Fig. 2C).

Prognostic relevance of GM-CSF expression in colorectal cancer

We then explored GM-CSF and M-CSF expression, at the protein level, by using a TMA including 50 healthy mucosa tissues and 1,239 different colorectal cancer specimens annotated with clinicopathological data. Specific staining was evaluated by multiplying staining intensity (0−3) by percentages of positive cells (22).

In 60% of colorectal cancer, a diffuse and strong GM-CSF–specific staining involving a large majority of tumor cells with a negligible contribution of interstitial cells could be observed. In the remaining 40% of cases, similarly to healthy mucosa specimens, GM-CSF–specific staining of tumor cells was weak or negative (Fig. 3A and B). GM-CSF–specific histoscore median values were 140, 170, 105, and 170 in healthy mucosa, total colorectal cancer, and MMRd and MMRp colorectal cancer, respectively (Supplementary Fig. S1). Thus, colorectal cancer MMRp expressed significantly more GM-CSF protein (P = 0.0001) than MMRd colorectal cancer. In the latter cancers, histoscore values were even lower than in healthy mucosa.

Based on this analysis, and on results of ROC curves and regression trees, we established GM-CSF threshold histoscore value for survival analyses at 11.5. Analysis of TMA data (Table 1) indicates that colorectal cancer displaying higher GM-CSF–specific staining are characterized by a significantly lower pT stage (P = 0.02), and a significantly (P = 0.004) more frequently detectable pushing/expanding, as opposed to infiltrating (20), growth pattern. Overall survival, as evaluated in the whole TMA seemed to be correlated with GM-CSF expression (P = 0.0002 at 5 years, n = 1206), as detectable at the protein level. In particular, this effect was specifically observed in MMRp colorectal cancer (n = 1014; P < 0.0001). In contrast, GM-CSF expression had no effect on overall survival of patients with MMRd colorectal cancer (n = 192; P = 0.927; Fig. 3E and F).

GM-CSF maintained its prognostic significance (P = 0.036) also in multivariate Cox regression analysis (Table 2), together with high CD16+ (P = 0.002) and CD8+ (P = 0.04) cell infiltration, age (P < 0.00001), gender (P < 0.0001), pT/N stage, vascular invasion, tumor border configuration, and microsatellite instability.

Detection of M-CSF could only be performed in a subset of the TMA including 37 healthy mucosa and 743 colorectal cancer. M-CSF staining was usually diffuse with different intensity (Fig. 3C and D). Absent or very low intensity (below the score of 115) was observed in 48.6% (19/37) of healthy mucosa and in 82% (614/743) of the colorectal cancer (P = 0.002). No differential M-CSF expression was detectable MMRp and MMRd tissues (P = 0.6; Supplementary Fig. S1). In the patients with colorectal cancer with higher M-CSF expression (129/743, 17%), we did not observe improved survival neither in MMRp (P = 0.124) nor in MMRd (P = 0.283) cases (Fig. 3G and H).

Correlations between GM-CSF production and colorectal cancer infiltration by immunocompetent cells

We explored the relationship eventually occurring between GM-CSF production by colorectal cancer cells and cancer infiltration by CD16+ or CD8+ cells, significantly associated with favorable prognosis (8–10, 16, 20).

Surprisingly, GM-CSF staining did not seem to be associated with CD16+ cell infiltration (P = 0.59).

Combined Kaplan–Meier survival analysis (Fig. 4A and B) indicates that patients with CD16+ cell infiltration of MMRp
Figure 2. Cytokine gene expression in freshly excised colorectal cancer and corresponding healthy mucosa. Total cellular RNA was purified from freshly excised colorectal cancer and autologous healthy mucosa specimens and reverse transcribed. Expression of GM-CSF and M-CSF genes was assessed by quantitative RT-PCR, by using GAPDH house-keeping gene, as reference. GM-CSF/M-CSF gene expression ratios were also calculated (A). The expression of additional cytokine genes was similarly evaluated (B), and the correlation between GM-CSF and TNFα, and M-CSF and IL10 gene expression was analyzed (C). n.s., nonsignificant.
Figure 3. Prognostic significance of GM-CSF and M-CSF protein expression in colorectal cancer. A colorectal cancer TMA was stained with GM-CSF (A and B) or M-CSF (C and D) specific reagents. Representative samples with low or high specific histoscores are shown in A and C and B and D, respectively (magnification: ×20). Based on ROC curves derived from histoscore data, the prognostic significance of GM-CSF (E and F) and M-CSF (G and H) could then be analyzed in MMRp (E and G) and MMRd (F and H) colorectal cancer. In both panels, red lines and black lines refer to cases with high and low cytokine expression, respectively. Number of events (= deaths) and total number of cases are also reported.
Table 2. Multivariate hazard Cox regression survival analysis

<table>
<thead>
<tr>
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<th>HR (95% CI)</th>
<th>P</th>
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<tbody>
<tr>
<td>GM-CSF (low vs. high)</td>
<td>0.808</td>
<td>0.036</td>
</tr>
<tr>
<td>CD8 (low vs. high)</td>
<td>0.763</td>
<td>0.048</td>
</tr>
<tr>
<td>CD16 (low vs. high)</td>
<td>0.716</td>
<td>0.002</td>
</tr>
<tr>
<td>Age (continuous)</td>
<td>1.033</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>Gender (women vs. men)</td>
<td>0.656</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pT stage (1, 2, 3, 4)</td>
<td>1.900</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>pN stage (0, 1, 2)</td>
<td>1.882</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>Tumor grade (1, 2, 3)</td>
<td>1.259</td>
<td>0.11</td>
</tr>
<tr>
<td>Vascular invasion (0, 1)</td>
<td>1.413</td>
<td>0.002</td>
</tr>
<tr>
<td>Tumor border configuration (0, 1)</td>
<td>1.429</td>
<td>0.005</td>
</tr>
<tr>
<td>Microsatellite stability (deficient vs. proficient)</td>
<td>1.692</td>
<td>0.0009</td>
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NOTE: Multivariate analysis showing HRs and P values for all colorectal cancer (n = 975, because of missing values, see "Materials and Methods"), as conferred by high GM-CSF expression, CD8⁺ and CD16⁺ infiltrating cell density, age, gender, tumor size, nodal status, tumor grade, vascular invasion, tumor border configuration, and microsatellite stability.

a0: absent, 1: present.
b0: pushing, 1: infiltrating.

colorectal cancer and high GM-CSF production have a significantly better prognosis than those with low CD16⁺ cell infiltration and low GM-CSF production (P = 0.000193).

However, in CD16⁺ cell infiltrated colorectal cancer, GM-CSF production did not seem to significantly influence overall survival. No effects were detectable in MMRd cancers.
GM-CSF staining was also unrelated with colorectal cancer infiltration by CD8\(^{+}\) cells (r Spearman: 0.09). However, most interestingly, in colorectal cancer characterized by poor CD8\(^{+}\) T cell infiltration, a condition known to be associated with severe prognosis (8–10, 20), GM-CSF production by cancer cells was highly significantly correlated with improved overall survival in MMRp (P = 0.00004) but not in MMRd colorectal cancer (Fig. 4C and D).

Expression of CX3CL1/fractalkine gene in colorectal cancer

CX3CL1/fractalkine has been shown to be selectively attracted to CD16\(^{+}\) monocytes, which do express cognate CX3CR1 receptor (24) and CX3CL1/fractalkine gene expression has been suggested to associate with favorable prognosis in colorectal cancer (25).

We observed that CX3CL1/fractalkine gene is expressed to significantly higher extents in colorectal cancer than in corresponding healthy mucosa (median, IQR: 1.8E10–3–8.5E10–2 vs. 9.2E10–3–4.7E10–2, n = 22, P = 0.0028) and that the specific gene product is detectable by ELISA in supernatants from established colorectal cancer cell lines (Supplementary Fig. S2A and B). Most interestingly, CX3CL1/fractalkine protein is also detectable in colorectal cancer (Supplementary Fig. S2C and D). TMA analysis indicates that this protein is detectable to significantly higher extents in colorectal cancer than in healthy mucosa (P = 0.0045). However, its expression was devoid of prognostic significance and unrelated to colorectal cancer infiltration by CD16\(^{+}\) myeloid cells (data not shown).

Discussion

In previous work we showed that colorectal cancer infiltration by CD16\(^{+}\) myeloid cells is associated with improved prognosis (16). Here we have addressed mechanistic clues possibly underlying these effects, by analyzing the antitumor potential of \textit{in vitro} polarized macrophages. Furthermore, and most importantly, we have explored the expression at the gene and protein level of cytokines and chemokines associated with functional polarization and chemotraction of macrophage subsets possessing antitumor capacity and their prognostic significance.

M-CSF and GM-CSF are known to be involved in the polarization of anti-inflammatory/pro-angiogenic M2 and pro-inflammatory/antitumor M1 macrophages, respectively (5, 17). Here we show that upon GM-CSF but not M-CSF \textit{in vitro} stimulation, peripheral blood monocytes from healthy donors become capable of exerting cytostatic effects on colorectal cancer cells. However, the analysis of >40 matched pairs of colorectal cancer and autologous healthy mucosa clearly indicates that malignant tissues are typically characterized by an increased expression of GM-CSF gene, as compared with autologous healthy mucosa. Accordingly, colorectal cancer tissues are characterized by a cytokine gene expression signature reminiscent, although not fully matching, of that observed in activated M1 cells, including high \textit{IL1\(\beta\)} and \textit{IL23} gene expression (5).

\textit{Per se}, these data might still be consistent with a pathogenic role of local inflammation in colorectal cancer, as suggested by a number of experimental models (6). However, by using a large number of surgical specimens (>1,000) annotated with an exhaustive clinical database, we report here that high GM-CSF expression at the protein level in colorectal cancer is associated with favorable prognosis, although only in MMRp cases. In contrast, M-CSF protein expression, as detectable in our TMA, does not seem to be significantly associated with clinicopathological features or overall survival. Importantly, TMA analysis reveals that GM-CSF is predominantly produced by tumor cells.

GM-CSF plays a key role in the differentiation and functional maturation of different myeloid populations. Because of its ability to activate antigen-presenting cells, this cytokine has been widely used in cancer immunotherapy (26, 27). GM-CSF–transfected primary tumor cells and established tumor cell lines have been used for vaccination purposes (27). Moreover, recombinant GM-CSF has been utilized as supportive cytokine to supplement immunization targeting tumor-associated antigens (TAA) implemented through administration of peptides, antigen-pulsed dendritic cells or recombinant viruses.

GM-CSF has also widely been used in combination with IL4 or IFN type I (28) in the \textit{in vitro} dendritic cell geneation. A number of studies indicate that treatment of peripheral blood monocytes with GM-CSF leads to polarization toward a M1 pro-inflammatory phenotypic and functional profile, whereas M-CSF promotes the differentiation of alternatively activated M2 macrophages possessing pro-angiogenic and anti-inflammatory properties (17).

However, GM-CSF has also been shown to promote the generation of myeloid-derived suppressor cell (MDSC; refs. 29 and 30), characterized by a powerful ability to inhibit T-cell proliferation and to promote the expansion of CD4\(^{+}\)/FOXP3\(^{+}\) regulatory T cells. Notably, increased numbers of myeloid cells with phenotypic and functional profiles closely overlapping those of MDSC have been detected in peripheral blood of patients bearing cancers following treatment with GM-CSF (31).

Myeloid cell colony-stimulating factors have been found to be produced by different types of carcinoma cells. In particular, GM-CSF production by tumor cells has been shown to be associated with increased recurrence rate and metastasis formation in head and neck cancers (32). Furthermore, GM-CSF production by breast cancer cells was suggested to enhance tumor growth and to promote the formation of bone metastases, possibly by stimulating resident macrophages or by inducing osteoclast differentiation and activation (33). Lung cancer cells have also been shown to produce GM-CSF and their proliferation may be enhanced by exogenous GM-CSF (34).

We and others have previously shown that colorectal cancer cells do produce GM-CSF (21, 35). Interestingly, colorectal cancer cell lines producing GM-CSF have been suggested to be highly aggressive \textit{in vivo} (36), possibly because of the activation of macrophages, promoting stromal reactivity. In addition, GM-CSF production by colorectal...
cancer cells from liver metastases has been suggested to promote tumor growth by a paracrine loop implying heparin-binding EGF production by activated tumor-infiltrating macrophages (37).

Most recently however, immune-dependent and immune-independent antitumor activities of GM-CSF in human colorectal cancer have been suggested (38). In a group of 124 patients, association with favorable prognosis was detectable in 8 patients bearing tumors concomitantly expressing genes encoding GM-CSF and both receptor subunits (38). However, MMR status of colorectal cancer was not analyzed, GM-CSF protein expression was not investigated and the association with macrophage and T-cell infiltration or with the expression of additional cytokines promoting their polarization was not explored.

Within this frame our data provide important novel information on the role of GM-CSF in colorectal cancer microenvironment. First, we show here that GM-CSF is predominantly produced by MMRp colorectal cancer cells. Despite their higher genomic stability, these cancers are characterized by a more severe prognosis, as compared with MMRd colorectal cancer. Furthermore, we report that although recombinant GM-CSF is per se ineffective, colorectal cancer cell lines are sensitive to the cell–cell contact-dependent cytostatic effects of GM-CSF–activated macrophages. However, although previously published data from our groups indicate that colorectal cancer infiltration by cells expressing CD16 is associated with improved prognosis (16), we did not observe any significant correlation between GM-CSF–specific staining and CD16+ cell infiltration in the TMA under investigation.

We reasoned that tumor infiltration by CD16+ myeloid cells might result from the functional maturation/differentiation of cells residing in colonic tissues promoted by factors present in local microenvironment or from the selective chemotraction of circulating cells endowed with specific phenotypic and functional features (24).

Therefore, we explored the potential prognostic role of CX3CL1/fractalkine, a chemokine selectively attracting CD16+ peripheral monocytes (24) in colorectal cancer. This chemokine has been found to be expressed in colorectal cancer cells and, based on the analysis of a small (n = 80) number of specimens, it has been suggested to be associated with favorable prognosis in colorectal cancer (25, 39). Our data show that CX3CL1/fractalkine gene expression can indeed be observed to significantly higher extents in colorectal cancer than in matched healthy mucosa. However, protein detection in colorectal cancer tissue sections is infrequent and devoid of clinical significance.

Taken together, these data suggest that colorectal cancer microenvironment contains factors promoting both local CD16+ myeloid cell differentiation and specific chemotraction, such as GM-CSF and CX3CL1/fractalkine. However, although neither of these factors correlates significantly with CD16+ myeloid cell infiltration in colorectal cancer, GM-CSF detection is associated with favorable prognosis in a large colorectal cancer subset.

Most obviously, other CD16- cell types possibly favoring tumor progression might be responsive to GM-CSF (40). Indeed, their activities might eventually "mask" or modulate the favorable effects of this cytokine promoting the expansion of CD16+ myeloid cells at the tumor site. Alternatively, in defined subgroups of patients, myeloid cells might be hypo-responsive to GM-CSF. Interestingly, a decreased expression of GM-CSF receptor α chain CD116, accompanied by hypo-responsiveness to cytokine stimulation, has recently been observed in peripheral blood monocytes and granulocytes from patients with IBD (41). However, recruitment, differentiation, and elicitation of antitumoral effects of CD16+ myeloid cells might require other factors in addition to GM-CSF. It is tempting to speculate that bacterial products possibly deriving from gut lumen might be of relevance in this context, possibly through TLRs triggering.

Indeed, GM-CSF–transduced murine CT-26 colorectal cancer cells have been repeatedly tested in experimental models in the past. Dranoff and colleagues originally reported that irradiated, GM-CSF–transduced, CT-26 are more effective than wild-type cells in inducing antitumor immunity upon subcutaneous administration. However, live transduced CT-26 cells were not tested (42). Colombo and colleagues have reported (43) that subcutaneous injection of live GM-CSF–transduced cells resulted in rapid tumor growth, similarly to wild-type cells. In both series of studies, cells were injected subcutaneously. Therefore, the role of mucosal immune response and gut microbiome could not be addressed. This aspect might represent a major difference between the above-cited experimental models and clinical reality. Furthermore, importantly, paradoxical effects of GM-CSF used as adjuvant for tumor-specific vaccination were more recently reviewed (44). These data suggest that low doses injected locally might be helpful, whereas systemic administration of high doses could be ineffective or detrimental.

Although further research is warranted to clarify underlying molecular mechanisms, our data emphasize the prognostic significance of GM-CSF production by colorectal cancer cells. In this context, it is particularly interesting that GM-CSF seems to possess a major favorable prognostic significance in colorectal cancer, which are not infiltrated by CD8+ T cells. Therefore, although adaptive immunity seems to play an important role in the control of colorectal cancer progression, other mechanisms, possibly related to innate immune system activation, might still be significantly active in its absence. Thus, GM-CSF might bona fide be included in the hierarchy of cell subsets and soluble factors of relevance in shaping the clinical course of colorectal cancer.

Cytokine and chemokine gene expression has been extensively investigated in colorectal cancer tissues (9, 45). However, to the best of our knowledge, this is one of the first studies addressing the prognostic significance of cytokine and chemokine expression at the protein level in a large number of patients.

It has been highlighted that a number of conventional assumptions related to cancer-immune system interaction...
do not seem to apply to colorectal cancer (46). For instance, at difference with a large number of cancer types, we and others have shown that colorectal cancer infiltration by FOXP3+ cells is associated to improved prognosis (11, 12). Accordingly, in keeping with the proposed colorectal cancer paradoxical scenario, we and others have previously observed that colorectal cancer infiltration by myeloid cells is also associated with relatively good prognosis (15, 16). Our data unravel a further important paradoxical colorectal cancer feature, represented by the favorable prognostic role of GM-CSF.

Most interestingly, our data reveal that CD8+ and CD16+ cell infiltration and GM-CSF production by tumor cells play independent antitumor roles. While underlining the complexity of colorectal cancer microenvironment, these findings suggest that the peculiar immunobiology of these cancers could provide important hints for the development of innovative treatments.

Colorectal cancer treatment options, including curative or palliative surgical resection, neoadjuvant, adjuvant, and palliative chemotherapy are currently largely based on tumor–node–metastasis (TNM) staging. However, conventional staging seems to be relatively inefficient in daily clinical practice, frequently leading to overtreatment or undertreatment (47, 48). In this respect, analysis of colorectal cancer immunoncontexture (49) seems to identify a set of markers largely independent from TNM staging but also associated with a high prognostic relevance, as detectable in large cohorts of patients. It is tempting to speculate that, in a next future, relatively limited constellations of markers, possibly including GM-CSF production by colorectal cancer cells, might be integrated into novel staging procedures, helping to identify subsets of patients eligible for effective therapies while sparing them unnecessary treatments and improving their quality of life.

References


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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