GM-CSF production by tumor cells is associated with improved survival in colorectal cancer

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GM-CSF is a powerful activator of myeloid cells. However, its role in cancer immunobiology is debated since it was shown to promote the generation of myeloid derived suppressor cells (MDSC). Here we report that GM-CSF induces in human macrophages the ability to inhibit the proliferation of colorectal cancer (CRC) cells “in vitro”. GM-CSF gene is expressed to significantly higher extents in CRC than in autologous healthy mucosa. By using a large (>1200) number of specimens, we demonstrate that in mismatch repair proficient (MMRp) cancers GM-CSF production by CRC cells is associated with improved survival in univariate and multivariate analyses. The favorable prognostic relevance of GM-CSF production by CRC cells is particularly evident in MMRp cancers where poor CD8+ T cell infiltration is detectable. These data underline specificities of CRC immunobiology and indicate that prognostic significance of defined tumor micro-environmental features critically depends on tumor types and related anatomic districts.
Abstract (249 words)

**Purpose:** Colorectal cancer (CRC) 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 co-cultured with CRC cells. Tumor cell proliferation was assessed by 3H-Thymidine incorporation. Expression of cytokine (CK) genes in CRC and autologous healthy mucosa (HM) was tested by quantitative, real-time PCR. A tumor microarray (TMA) including >1200 CRC specimens was stained with GM-CSF and M-CSF specific antibodies. Clinic-pathological features and overall survival were analyzed.

**Results:** GM-CSF induced CD16 expression in 66±8% of monocytes, as compared to 28±1% in cells stimulated by M-CSF (P=0.011). GM-CSF but not M-CSF stimulated macrophages significantly (P<0.02) inhibited CRC cell proliferation. GM-CSF gene was expressed to significantly (n=45, P<0.0001) higher extents in CRC than in HM whereas M-CSF gene expression was similar in HM and CRC. Accordingly, IL-1β and IL-23 genes, typically expressed by M1 macrophages, were expressed to significantly (P<0.001) higher extents in CRC than in HM. 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 (MMRp) CRC. Favorable prognostic effect of GM-CSF production by CRC cells was confirmed by multivariate analysis and was independent from CD16+ and CD8+ cell CRC infiltration. M-CSF expression had no significant prognostic relevance.

**Conclusions:** GM-CSF production by tumor cells is an independent favorable prognostic factor in CRC.
Introduction

Chronic inflammation is known to play a decisive role in cancer outgrowth and progression by powerfully shaping tumor microenvironment (1, 2). Tumor cells may produce factors promoting maturation and functional differentiation of resident pro-inflammatory cells. In turn, these cells may favor tumor angiogenesis and enhance cancer cell invasiveness. On the other hand, chemokine production within cancerous tissues may selectively chemoattract circulating cells expressing specific receptors, resulting in a peculiar composition of the cancer microenvironment, potentially affecting tumor progression and, ultimately, clinical prognosis (3-5). In particular, tumor infiltration by myeloid cells has frequently been associated with poor prognosis in different types of cancer, including, among others, breast, thyroid and renal cell carcinoma and melanoma (6).

Colorectal cancer (CRC) represents a major cause of cancer related death in different geographic areas. A variety of current experimental models of CRC 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 CRC incidence in humans (6, 7).

However, in sporadic CRC, 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 CRC, 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 CRC infiltration by T cells, and, in particular, by CD8+ lymphocytes, is associated with improved survival. These cells usually display a memory (8, 9) and activated (10) phenotype. CRC infiltration by FOXP3+ T cells has also been shown to be paradoxically associated with good prognosis (11, 12).

In contrast, the functional relevance of CRC infiltration by cells of the innate immune system is still unclear. NK cell infiltration is relatively rarely detectable and it is devoid of prognostic significance (13). Instead, at difference with a variety of cancers of diverse histological origin (14), CRC infiltration by macrophages has been...
shown to be associated with favorable prognosis (15). Therefore, in this context, CRC appears to represent an important exception.

In the same line, we have observed that infiltration by myeloid CD16+ cells represents a novel, independent, favorable prognostic factor in CRC (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 chemoattraction and functional polarization of macrophage subsets possibly endowed with anti-tumor 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 6-7 days in the presence of recombinant GM-CSF (Laboratorio Pablo Cassarà) or M-CSF (R&D Systems) at 50-5 ng/ml concentrations in RPMI1640 medium supplemented with antibiotics, glutamine, non essential aminoacids, sodium pyruvate, HEPES, β-mercaptoethanol and 10% 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 CRC 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, six-seven days cytokine stimulated macrophages (see above) were co-cultured in 96 well plates (Falcon) at different effector:target ratios with 3000 tumor cells for two days. 3H-Thymidine (Amersham GE) was then added (1µCi/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 CRC and autologous healthy mucosa (HM) sampled at distance from the tumor and reverse transcribed. Pre-developed 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 $2^{-\Delta\Delta C_T}$ 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 1420 unselected, non-consecutive, primary sporadic CRCs, 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 to ethical standards and patient confidentiality. Tissue cylinders with a 0.6 mm diameter from formalin-fixed, paraffin-embedded tissue blocks from resected CRC were punched from representative tissue areas and brought into one recipient paraffin block (30 x 25 mm), using a semi-automated tissue arrayer. Punches were made from the center of the tumor to guarantee that each TMA spot included at least 50% tumor cells.

Clinic-pathological 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/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 1031 MMR-proficient and 194 MMR-deficient tumors. Follow-up data were available for 1379 patients with mean/median and interquartile range (IQR) event-free follow-up time of 67.7/68 and 45-97 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, Newcastle, UK) at 100°C for 30 minutes, as previously described (20), prior to staining. The sections were treated with 10% normal goat serum.
(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 three 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 non-parametric Wilcoxon test for paired samples.

For outcome assessment, cut-off values used to classify CRC with low or high parameters of interest were obtained by ROC curves based on histoscore analyses (see above), evaluating sensitivity and false positive rate for the discrimination of survivors and non-survivors, on all tumor samples. Threshold values thus obtained were compared with the expression levels in non-malignant and malignant colon tissues and final threshold values were set according to biological significance. Chi-Square or Fisher’s exact tests were used to determine the association of GM-CSF infiltration and clinic-pathological features. Survival curves were constructed according to the Kaplan-Meier method. Log-ranks were calculated to test for differences between survival curves. Multivariate regression analysis was performed according to Cox Proportional Hazard Models including CD16+ and CD8+ cell infiltration, age, gender, T and N stage, tumor grade, vascular invasion, invasive margin, and MMR status. Wald tests statistic was used to test the hypothesis that
GM-CSF provides significant information to the model. Subsequently, data obtained from multivariate Cox regression analysis were tabulated including hazard ratios (HR) and 95% confidence intervals (CI). Multivariate Cox regression analysis was performed by using 955 cases since missing values were excluded from the model. M-CSF and CX3CL1 were not integrated in the Cox hazard regression model since specific staining did not show significant prognostic relevance in univariate analysis.

Spearman’s rank correlation was used to analyze the association between GM-CSF, M-CSF, CX3CL1 and CD16+ and CD8+ cell infiltration. Two-tailed P-values <0.05 were considered significant for all analyses. Statistical analyses were performed using R i386 Version 2.15.2 (http://www.R-project.org).
Results

**Phenotypes of GM-CSF and M-CSF activated monocytes**

Human CD14+ peripheral blood monocytes were cultured in the presence of GM-CSF or M-CSF. Consistent with the M1/M2 polarization model (5, 17), we observed that following a 6-7 day culture in the presence of 25-6.25 ng/ml GM-CSF, a significantly higher percentage of cells expressed CD16, as 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, \( P = 0.011 \)) (Figure 1A-B). In contrast, percentages of cells expressing CD204 molecular scavenger were significantly increased in M-CSF, as compared to 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 six experiments with cells from different donors are reported in figure 1A-B.

**Cytostatic activity of GM-CSF activated macrophages against CRC cells.**

We then tested the effects of GM-CSF and M-CSF stimulated macrophages on the proliferation of MMR proficient (MMRp) Colo 205 CRC cells. Following 6-7 days stimulation in the presence of 25-6.25 ng/ml GM-CSF, monocytes were able to significantly inhibit CRC cell proliferation (figure 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 (Figure 1C). Effects of GM-CSF stimulated monocytes were not mediated by soluble factors. Indeed, neither recombinant GM-CSF nor culture supernatants did inhibit CRC 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 10:1 E:T ratios were as effective as a 30\( \mu \)g/ml concentration of the pyrimidin analog 5FU in inhibiting CRC 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 CRC cells as targets (data not shown).
GM-CSF and M-CSF gene expression in CRC 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 genes in surgically excised paired specimens of CRC and autologous healthy mucosa (HM) sampled at distance from the cancerous tissue (23).

GM-CSF gene was expressed to significantly higher extents in CRC tissue, as compared with corresponding autologous HM (median, interquartile range (IQR): 6.167E-10, 2.7E-10-2.6E-10 vs. 4.03E-10, 0-1.91E-10, n=45, P<0.0001). In contrast, M-CSF gene expression was similar in healthy colon mucosa and in the corresponding CRC tissues (median, IQR: 1.8E-2, 5.6E-3-5.2E-2 vs. 3.8E-2, 2.3E-2-1E-1, n=46, P=0.25). Accordingly, GM-CSF/M-CSF gene expression ratio was significantly higher in tumor tissue than in the corresponding autologous mucosa (0.025 vs. 0.0014, P<0.0001) (Figure 2A).

Expression of genes predominantly associated with M1 and M2 macrophages in CRC and in corresponding, autologous healthy mucosa.

To obtain insights into specific gene signatures eventually detectable in clinical specimens, we assessed IL-23 and IL-1β gene expression in paired CRC and autologous healthy mucosa samples. We found that these genes were expressed to significantly higher extents in CRC than in matched HM (median, IQR: IL-23: 2E-3, 9E-4-5.1E-3 vs. 5E-4, 1.4E-4-1.1E-3, n=47, P<0.0001; IL-1β: 2E-2, 7.7E-3-5.2E-2 vs. 7.5E-3, 2.3E-3-1.6E-2, n=48, P=0.001; Figure 2B). However, expression of IL-12p35 gene, reportedly typically observed in M1 cells, was detectable to significantly higher extents in HM than in matched tumor tissues (median, IQR: 9.3E-4, 3E-4-2.2E-3 vs. 3.5E-4, 1.9E-4-8.5E-4, n=46, P=0.01, figure 2B).

M2 polarized macrophages are characterized by the ability to produce IL-10 (5). Indeed, we did not observe significant differences in IL-10 gene expression between HM and tumor tissue (median, IQR: 6.4E-4, 3.1E-4-1.2E-3 vs. 3.4E-4, 1E-4-1E-3, n=46, P=0.41).

Furthermore, expression of TNFα, IL-6 and IL-12p40 genes was also similarly detectable in HM and corresponding CRC tissue (median, IQR: TNF-α: 6.7E-4, 3.9E-4-1.3E-3 vs. 4.5E-4, 2.2E-4-9.2E-4, n=45, P=0.11).
1.7E10⁻⁴-1.6E10⁻³ vs. 8E10⁻⁴, 3.1E10⁻⁴-1.8E10⁻³, n=47, P=0.089; IL-6: 5.1E10⁻⁵, 8.6E10⁻⁸-6.1E10⁻⁴ vs. 2.7E10⁻⁴, 5.9E10⁻⁵-9.6E10⁻⁴, n=45, P=0.17; IL-12p40: 6.8E10⁻⁵, 3.19E10⁻⁵-2.7E10⁻⁴ vs. 7.5E10⁻⁵, 1.8E10⁻⁵-2.1E10⁻⁴, n=46, P=0.7, Figure 2B).

Thus, conventional patterns of polarized macrophage gene expression do not appear to fully fit gene signatures detectable in CRC (4, 5). However, consistent with gene expression profiles commonly attributed to polarized macrophages (5), M-CSF and IL-10 and GM-CSF and TNF-α gene expression in CRC tissues were highly significantly correlated (r=0.63, P<0.0001 and r=0.49, P<0.0001, respectively, Figure 2C).

Prognostic relevance of GM-CSF expression in CRC.

We then explored GM-CSF and M-CSF expression, at the protein level, by using a TMA including 50 HM tissues and 1239 different CRC specimens annotated with clinic-pathological data. Specific staining was evaluated by multiplying staining intensity (0-3) by percentages of positive cells (22).

In 60% of CRC 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 HM specimens, GM-CSF specific staining of tumor cells was weak or negative (Figure 3A-B). GM-CSF specific histoscore median values were 140, 170, 105 and 170 in HM, total CRC, and MMRd and MMRp CRC, respectively (supplementary figure 1). Thus, CRC MMRp expressed significantly more GM-CSF protein (P=0.0001) than MMRd CRC. In the latter cancers, histoscore values were even lower than in HM.

Based on this analysis, and on results of ROC curves and regression trees, we established GM-CSF threshold histoscore value for survival analyses at 115. Analysis of TMA data (table 1) indicates that CRC displaying high 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 appeared to be correlated with GM-CSF expression (p=0.0002 at five years, n=1206), as detectable at the protein level. In particular, this effect was specifically observed in MMRp CRC (n=1014; P<0.0001). In contrast, GM-CSF expression had no effect on overall survival of patients with MMRd CRC (n=192; P=0.927) (Figure 3E-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 HM and 743 CRC. M-CSF staining was usually diffuse with different intensity (Figure 3C-D). Absent or very low intensity (below the score of 115) was observed in 48.6% (19/37) of HM and in 82% (614/743) of the CRC (P=0.002). No differential M-CSF expression was detectable MMRp and MMRd tissues (P=0.6, supplementary figure 1). In the CRC patients 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 (Figure 3G-H).

Correlations between GM-CSF production and CRC infiltration by immunocompetent cells.

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

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

Combined Kaplan-Meier survival analysis (Figure 4A-B) indicates that patients with CD16+ cell infiltration of MMRp CRC 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 CRC, GM-CSF production did not appear to significantly influence overall survival. No effects were detectable in MMRd cancers.

GM-CSF staining was also unrelated with CRC infiltration by CD8+ cells (r Spearman: 0.09). However, most interestingly, in CRC 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 CRC (Figure 4C-D).

Expression of CX3CL1/fractalkine gene in CRC
CX3CL1/fractalkine has been shown to selectively attract CD16+ monocytes, which do express cognate CX3CR1 receptor (24) and CX3CL1/fractalkine gene expression has been suggested to associate with favorable prognosis in CRC (25).

We observed that CX3CL1/fractalkine gene is expressed to significantly higher extents in CRC than in corresponding HM (median, IQR: 1.8E10⁻², 9.3E10⁻³-8.5E10⁻² vs. 9.2E10⁻³, 4.6E10⁻³-4.7E10⁻², n=22, P=0.0028) and that the specific gene product is detectable by ELISA in supernatants from established CRC cell lines (Supplementary Figure 2A-B). Most interestingly, CX3CL1/fractalkine protein is also detectable in CRC (Supplementary Figure 2C-D). TMA analysis indicates that this protein is detectable to significantly higher extents in CRC than in HM (P=0.0045). However, its expression was devoid of prognostic significance and unrelated to CRC infiltration by CD16+ myeloid cells (data not shown).
Discussion

In previous work we showed that CRC 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 “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 chemoattraction of macrophage subsets possessing anti-tumor 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/anti-tumor M1 macrophages, respectively (5, 17). Here we show that upon GM-CSF but not M-CSF “in vitro” stimulation, peripheral blood monocytes from healthy donors become capable of exerting cytostatic effects on CRC cells. On the other hand, the analysis of >40 matched pairs of CRC and autologous healthy mucosa clearly indicates that malignant tissues are typically characterized by an increased expression of GM-CSF gene, as compared to autologous healthy mucosa. Accordingly, CRC tissues are characterized by a cytokine gene expression signature reminiscent, although not fully matching, of that observed in activated M1 cells, including high IL-1β and IL-23 gene expression (5).

“Per se”, these data might still be consistent with a pathogenic role of local inflammation in CRC, as suggested by a number of experimental models (6). However, by using a large number of surgical specimens (>1000) annotated with an exhaustive clinical database we report here that high GM-CSF expression at the protein level in CRC is associated with favorable prognosis, although only in MMRp cases. In contrast, M-CSF protein expression, as detectable in our TMA, does not appear to be significantly associated with clinic-pathological 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.

Due to 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 (DC) or recombinant viruses.

GM-CSF has also widely been used in combination with IL-4 or IFN type I (28) in the “in vitro” DC generation. A number of studies indicate that treatment of peripheral blood monocytes with GM-CSF leads to polarization towards 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) (29, 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 CRC cells do produce GM-CSF (21, 35). Interestingly, CRC cell lines producing GM-CSF have been suggested to be highly aggressive “in vivo” (36), possibly due to the activation of macrophages, promoting stromal reactivity. In addition, GM-CSF production by CRC 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 CRC 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 CRC 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 CRC microenvironment. First, we show here that GM-CSF is predominantly produced by MMRp CRC cells. Despite their higher genomic stability, these cancers are characterized by a more severe prognosis, as compared to MMRd CRC. Furthermore, we report that while recombinant GM-CSF is “per se” ineffective, CRC 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 CRC 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 into colonic tissues promoted by factors present in local microenvironment or from the selective chemoattraction 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 CRC. This chemokine has been found to be expressed in CRC 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 CRC (25, 39). Our data show that CX3CL1/fractalkine gene expression can indeed be observed to significantly higher extents in CRC than in matched HM. However, protein detection in CRC tissue sections is infrequent and devoid of clinical significance.

Taken together, these data suggest that CRC microenvironment contains factors promoting both local CD16+ myeloid cell differentiation and specific chemoattraction, such as GM-CSF and CX3CL1/fractalkine. However, while neither of these factors correlates significantly with CD16+ myeloid cell infiltration in CRC, GM-CSF detection is associated with favorable prognosis in a large CRC 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 alpha chain CD116, accompanied by hypo-responsiveness to cytokine stimulation, has recently been observed in peripheral blood monocytes and granulocytes from patients with IBD (41). On the other hand, 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 CRC cells, have been repeatedly tested in experimental models in the past. Dranoff et al., originally reported that irradiated, GM-CSF transduced, CT-26 are more effective than wild type cells in inducing anti-tumor immunity upon subcutaneous administration. However, live transduced CT-26 cells were not tested (42). Colombo et al 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.

While further research is warranted to clarify underlying molecular mechanisms, our data emphasize the prognostic significance of GM-CSF production by CRC cells. In this context, it is particularly interesting that GM-CSF appears to possess a major favorable prognostic significance in CRC which are not infiltrated by CD8+ T cells. Therefore, while adaptive immunity appears to play an important role in the control of CRC 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 CRC.

Cytokine and chemokine gene expression has been extensively investigated in CRC 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 appear to apply to CRC (46). For instance, at difference with a large number of cancer types, we and others have shown that CRC infiltration by FOXP3+ cells is associated to improved prognosis (11, 12). Accordingly, in keeping with the proposed CRC paradoxical scenario, we and others have previously observed that CRC infiltration by myeloid cells is also associated with relatively good prognosis (15, 16). Our data unravel a further important paradoxical CRC 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 anti-tumor roles. While underlining the complexity of CRC microenvironment, these findings suggest that the peculiar immunobiology of these cancers could provide important hints for the development of innovative treatments.

CRC treatment options, including curative or palliative surgical resection, neoadjuvant, adjuvant and palliative chemotherapy, are currently largely based on TNM staging. However, conventional staging appears to be relatively inefficient in daily clinical practice, frequently leading to over or under-treatment (47, 48). In this respect, analysis of CRC immunocontexture (49) appears 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 CRC 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.
Table 1: Association of GM-CSF staining and clinic-pathological features in colorectal cancer (n=1239).

<table>
<thead>
<tr>
<th>Clinic-pathological features</th>
<th>Histoscore</th>
<th>Histoscore</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=475 (38.3%)</td>
<td>N=764 (61.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age (n=1239) yrs</strong></td>
<td>Mean, range</td>
<td>69.4 (39-95)</td>
<td>69.8 (30-96)</td>
</tr>
<tr>
<td><strong>Tumor diameter (n=1235) mm</strong></td>
<td>Median, Mean, range</td>
<td>50, 49.7 (4-150)</td>
<td>45, 48.6 (5-160)</td>
</tr>
<tr>
<td><strong>Gender (n=1239)</strong></td>
<td>Female</td>
<td>248 (52.2)</td>
<td>407 (53.3)</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td>227 (47.8)</td>
<td>357 (46.7)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor location (n=1225)</strong></td>
<td>Left-sided</td>
<td>297 (63.6)</td>
<td>502 (66.2)</td>
</tr>
<tr>
<td><strong>Right-sided</strong></td>
<td>170 (35.4)</td>
<td>256 (33.8)</td>
<td></td>
</tr>
<tr>
<td><strong>pT stage (n=1213)</strong></td>
<td>pT1-2</td>
<td>76 (16.5)</td>
<td>166 (22.1)</td>
</tr>
<tr>
<td><strong>pT3-4</strong></td>
<td>386 (83.5)</td>
<td>585 (77.9)</td>
<td></td>
</tr>
<tr>
<td><strong>pN stage (n=1197)</strong></td>
<td>pN0</td>
<td>230 (50.2)</td>
<td>406 (54.9)</td>
</tr>
<tr>
<td><strong>pN1-2</strong></td>
<td>228 (49.8)</td>
<td>333 (45.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor grade (n=1212)</strong></td>
<td>G1-2</td>
<td>400 (87.5)</td>
<td>658 (87.2)</td>
</tr>
<tr>
<td><strong>G3</strong></td>
<td>57 (12.5)</td>
<td>97 (12.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Vascular invasion (n=1212)</strong></td>
<td>Absent</td>
<td>324 (70.7)</td>
<td>559 (74.1)</td>
</tr>
<tr>
<td><strong>Present</strong></td>
<td>134 (29.3)</td>
<td>195 (25.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor growth pattern (n=1212)</strong></td>
<td>Pushing/Expanding</td>
<td>150 (32.8)</td>
<td>310 (41.1)</td>
</tr>
<tr>
<td><strong>Infiltrating</strong></td>
<td>308 (67.2)</td>
<td>444 (58.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Peritumoral lymphocyte infiltration (n=1213)</strong></td>
<td>Absent</td>
<td>357 (77.9)</td>
<td>598 (79.2)</td>
</tr>
<tr>
<td><strong>Present</strong></td>
<td>101 (22.1)</td>
<td>157 (20.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Local recurrence (n=433)</strong></td>
<td>Absent</td>
<td>69 (55.2)</td>
<td>185 (60.1)</td>
</tr>
<tr>
<td><strong>Present</strong></td>
<td>56 (44.8)</td>
<td>123 (39.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Distant metastasis (n=440)</strong></td>
<td>Absent</td>
<td>110 (85.3)</td>
<td>252 (81.0)</td>
</tr>
<tr>
<td><strong>Present</strong></td>
<td>19 (14.7)</td>
<td>59 (19.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Postoperative therapy (n=437)</strong></td>
<td>None</td>
<td>97 (75.7)</td>
<td>250 (80.9)</td>
</tr>
<tr>
<td><strong>Treated</strong></td>
<td>31 (24.3)</td>
<td>59 (19.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Overall survival (n=1206)</strong></td>
<td>5-year (95%CI)</td>
<td>51.3 (46.7-56.4)</td>
<td>62.1 (58.4-66)</td>
</tr>
</tbody>
</table>
a) GM-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. T-test * was used for age analysis due to normal distribution; Wilcoxon (Mann-Whitney test) ** was used for tumor diameter analysis. Discrete/qualitative variables: Chi square test ***; Log-rank test**** was used to compare overall survival rates.
Table 2: Multivariate Hazard Cox regression survival analysis

<table>
<thead>
<tr>
<th></th>
<th>HR (95% CI)</th>
<th>P-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM CSF (low vs. high)</td>
<td>0.808 (0.706-0.909)</td>
<td>0.036</td>
</tr>
<tr>
<td>CD8 (low vs. high)</td>
<td>0.763 (0.626-0.899)</td>
<td>0.048</td>
</tr>
<tr>
<td>CD16 (low vs. high)</td>
<td>0.716 (0.608-0.824)</td>
<td>0.002</td>
</tr>
<tr>
<td>Age (continuous)</td>
<td>1.033 (1.028-1.038)</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Gender (women vs. men)</td>
<td>0.656 (0.554-0.757)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pT stage (1,2,3,4)</td>
<td>1.900 (1.807-1.993)</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>pN stage (0,1,2)</td>
<td>1.882 (1.809-1.954)</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Tumor Grade (1,2,3)</td>
<td>1.259 (1.114-1.403)</td>
<td>0.11</td>
</tr>
<tr>
<td>Vascular invasion (0,1)*</td>
<td>1.413 (1.300-1.525)</td>
<td>0.002</td>
</tr>
<tr>
<td>Tumor border configuration (0,1)**</td>
<td>1.429 (1.302-1.556)</td>
<td>0.005</td>
</tr>
<tr>
<td>Microsatellite stability (deficient vs. proficient)</td>
<td>1.692 (1.534-1.849)</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

Multivariate analysis showing Hazard Ratios and P-values for all CRC (n = 975, due to 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.

*0: absent, 1: present
**0: pushing, 1: infiltrating
Reference List


Legends to the figures

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 (panels A-B). Representative results referring to uncultured monocytes (gray lines), M-CSF treated (black lines) and GM-CSF treated (shaded profiles) are shown in panel A, whereas panel B reports cumulative data from six independent experiments.

GM-CSF or M-CSF (12.5 ng/ml) stimulated cells were then co-cultured 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 (panel C). Data refer to one representative experiment out of four performed with cells from different donors with similar results.

Figure 2. Cytokine gene expression in freshly excised CRC and corresponding healthy mucosa.

Total cellular RNA was purified from freshly excised CRC 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 (panel A). The expression of additional cytokine genes was similarly evaluated (panel B) and the correlation between GM-CSF and TNFα, and M-CSF and IL-10 gene expression was analyzed (panel C). n. s. : non significant.

Figure 3. Prognostic significance of GM-CSF and M-CSF protein expression in CRC.

A CRC TMA was stained with GM-CSF (panels A-B) or M-CSF (panels C-D) specific reagents. Representative samples with low or high specific histoscores are shown in panels A and C and B and D, respectively (magnification: x20). Based on ROC curves derived from histoscore data, the prognostic significance of GM-CSF (panels E-F) and M-CSF (panels G-H) could then be analyzed in MMRp (panels E
and G) and MMRd (panels F and H) CRC. 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.

**Figure 4. Prognostic significance of GM-CSF in CRC as related to levels of infiltration by immunocompetent cells.**

The prognostic significance of GM-CSF production was analyzed in tumors stratified according to their high or poor infiltration by CD8+ or CD16+ cells (10, 15, 20) in MMRp and MMRd CRC. Kaplan Meier curves in panels A and B display the combined effects of GM-CSF expression (score threshold at 115) and CD16+ cell infiltration (16) in MMRp and MMRd CRC, respectively. Black lines: both markers low; blue lines: both markers elevated; red lines: high CD16+ cell infiltration and low GM-CSF expression; green lines: low CD16+ cell infiltration and high GM-CSF expression. Number of events (=deaths)/total number of cases are also reported.

Similarly, Kaplan Meier curves in panels C and D display the combined effects of GM-CSF expression and CD8+ infiltration (10, 20) in MMRp and MMRd CRC, respectively. Black lines: both markers low; blue lines: both markers elevated; red lines: high CD8+ cell infiltration and low GM-CSF expression; green lines: low CD8+ cell infiltration and high GM-CSF expression. Number of events (=deaths)/total number of cases are also reported.
GM-CSF production by tumor cells is associated with improved survival in colorectal cancer

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