Immune-Dependent and Independent Antitumor Activity of GM-CSF Aberrantly Expressed by Mouse and Human Colorectal Tumors

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Abstract
Granulocyte-macrophage colony-stimulating factor (GM-CSF/CSF2) is a cytokine produced in the hematologic compartment that may enhance antitumor immune responses, mainly by activation of dendritic cells. Here, we show that more than one-third of human colorectal tumors exhibit aberrant DNA demethylation of the GM-CSF promoter and overexpress the cytokine. Mouse engraftment experiments with autologous and homologous colon tumors engineered to repress the ectopic secretion of GM-CSF revealed the tumor-secreted GM-CSF to have an immune-associated antitumor effect. Unexpectedly, an immune-independent antitumor effect was observed that depended on the ectopic expression of GM-CSF receptor subunits by tumors. Cancer cells expressing GM-CSF and its receptor did not develop into tumors when autografted into immunocompetent mice. Similarly, 100% of the patients with human colon tumors that overexpressed GM-CSF and its receptor subunits survived at least 5 years after diagnosis. These data suggest that expression of GM-CSF and its receptor subunits by colon tumors may be a useful marker for prognosis as well as for patient stratification in cancer immunotherapy. Cancer Res; 73(1); 395–405. ©2012 AACR.

Introduction
The immune system can identify and destroy nascent tumor cells, in a process termed cancer immuno surveillance, which plays an important role in the defense against cancer (1, 2). The ability of the immune system to fight tumor cells has been used in the development of cancer immunotherapy treatments based on enhancing host antitumor responses (3). The cytokine granulocyte-macrophage colony stimulating factor (GM-CSF, also known as CSF2) functions as a hematologic cell growth factor, stimulating blood stem cells to produce granulocytes and monocytes (4, 5). In addition, GM-CSF leads to protective immunity, mainly by stimulating the recruitment, maturation, and function of dendritic cells (6–8), the most potent antigen-presenting cell (9). This effect of the cytokine on dendritic cells leads to the activation of the immune system against specific antigens (10). Therefore, one approach to cancer immunotherapy is vaccination with tumor-irradiated cells engineered to secrete GM-CSF (6, 7, 11–13). In this context, the action of GM-CSF on dendritic cells allows the immune system to be activated against the specific antigens directly provided by the tumor cells (10). This strategy has, for instance, been used in vaccination with irradiated GM-CSF–secreting melanoma cells resulting in enhanced host responses through improved tumor antigen presentation by recruited dendritic cells and macrophages (6).

GM-CSF has been shown to be ectopically secreted by cell lines derived from a variety of solid tumors (14, 15) but the immune-independent effect of tumor-secreted GM-CSF is still poorly understood. Contradictory results from different groups have shown that GM-CSF could either exert an antiproliferative effect (16, 17) or promote tumor growth (18–20). Furthermore, no reproducible influence on tumor growth rate has been reported (21, 22). GM-CSF has also been reported to be secreted by some primary tumors (23–27) but, to the best of our knowledge, there are no reports to date showing the expression of GM-CSF by...
primary colon tumor tissues nor its possible effects on the proliferation of this tumor type. Furthermore, the molecular mechanisms involved in the ectopic expression of GM-CSF by tumor cells are still largely unknown. Here, we provide evidence for the possible molecular mechanism involved and describe the antitumor effect of the process in colorectal cancer.

Materials and Methods

qRT-PCR
Total RNA was extracted from mouse colon cell lines, human colon cancer cell lines, and human samples using TRIzol Reagent (Invitrogen) following the manufacturer's procedure. DNA was removed with DNase I treatment with DNA-free kit (Ambion/Applied Biosystems) and cDNA was prepared using SuperScript II Reverse Transcriptase kit (Invitrogen) following the manufacturer's recommendations. We ran PCR reactions in triplicate using Sybr Green Master Mix (Applied Biosystems) in a 7900HT Real-Time PCR System (Applied Biosystems). We conducted relative quantification of gene expression based on standard curve transformations of Ct values. Results from each target gene were normalized against their respective ubiquitous housekeeping gene. Primer sequences are listed in Supplementary Table S2.

Bisulfite pyrosequencing
Genomic DNA isolation was conducted according to a standard phenol-chloroform extraction protocol. Bisulfite modification of DNA was conducted with the EZ DNA Methylation-Gold kit (Zymo Research) following the manufacturer's instructions. After PCR amplification of the region of interest with specific primers, pyrosequencing was conducted using PyroMark Q24 reagents, vacuum prep workstation, equipment, and software (Qiagen). Primer sequences for murine Gm-CSF and human GM-CSF are shown in Supplementary Table S2.

Human cell lines and human samples
Human colon cancer cell lines were cultured according to American Type Culture Collection recommendations. Cell lines were authenticated using short tandem repeat profiling of an extracted DNA sample using AmpF/STR Identifier for high resolution screening and intraspecies cross-contamination detection by Bio-Synthesis, Inc. Briefly, cell lines were grown at 37°C in 5% CO₂ in Dulbecco’s Modified Eagle’s Media supplemented with 10% FBS. Human cells were treated with the demethylating agent 5-aza-2′-deoxycytidine (AdC; Sigma) at various concentrations (2.5 and 5 μmol/L) for 72 hours. Healthy human- and primary tumor-colon samples were obtained from the Hospital Universitario Puerta de Hierro (Madrid, Spain) and the Asturias Tumor Bank of the Institute of Oncology (Asturias, Spain). Tissue collection and analyses were approved by the appropriate institutional review boards in accordance with national and EU guidelines.

Murine cell lines and shGm-CSf stable transfection
Mouse colon cancer cell lines were kindly provided by Dr. Ignacio Melero [Centro de Investigacion Medica Aplicada (CIMA), Navarra, Spain] and cultured at 37°C in 5% CO₂ in RPMI supplemented with 10% FBS and 50 μmol/L β-mercaptoethanol. MC38 and CT26 colon cancer cells were stably transfected with a HuSH 29-mer shRNA construct against Mus musculus Csf2 in pGFp-V-RS vector or a HuSH 29-mer Non-Effective Scrambled pGFp-V-RS vector (OriGene) following the manufacturer’s instructions. We selected different constructs of the 4 provided by the supplier to generate 2 independent clones from each cell line with Gm-CSf interference as well as a scramble.

In vitro growth experiments
Cell viability was determined by MTT assay on cells stably transfected with scramble and shGm-CSF vectors as described by Mosmann and colleagues (28). Ten replicates per condition and time point were assessed. Absorbance at 595 nm was measured with an automated microtitre plate reader Power Wave WS (BioTek). Cell proliferation rate was established by cell counting on consecutive days in scramble and shGm-CSF cells. Cells were seeded in triplicates in 24-well plates at a concentration of 1 × 10⁴ per well. Cells were collected daily for 5 days and viable cells, as assessed by trypan blue staining, were counted under a microscope in a hemocytometer. Colony formation assay was conducted as described in Franken and colleagues (29). Cells were then seeded at different densities (50, 100, and 200 cells) and after 2 weeks, stable colonies were fixed and stained with a mixture of 6.0% Glutaraldehyde and 0.5% Crystal Violet in water. After washing and being left to air dry, colonies were counted. The Plating Efficiency was calculated as a mean of the Plating Efficiency of the different cell dilutions.

Tumor transplantation and in vivo treatment studies
For tumor grafting, we subcutaneously (s.c.) injected 1 to 4 × 10⁵ tumor cells into each flank of recipient mice (Nude or NU/NU, C57BL/6 and BALB/c females, 6 to 8 week old, Charles River). Anti-PD1 was purchased from BioXCell and administered by intraperitoneal injection of 250 μg. Tumor volumes were measured twice or 3 times a week with calipers and calculated using the following formula: tumor volume = 0.4 × (length × width²). After sacrifice, tumors were excised and weighed.

Immunohistochemistry
Tissue microarray (TMA) blocks were sectioned at a thickness of 3 μm, dried for 1 hour at 65°C before the pretreatment procedure: deparaffinization, rehydration, and epitope retrieval in the Pre-Treatment Module, PT-LINK (DAKO) at 95°C for 20 minutes in ×50 Tris/EDTA buffer, pH 9. The antibody against CD3 (clone M-20, sc-1127, Santa Cruz Biotechnology) was used to stain the sections, after blocking endogenous peroxidase. Following incubation, the reaction was visualized with the EnVision Detection Kit (DAKO) using diaminobenzidine chromogen as a substrate. Sections were counterstained with hematoxylin. Appropriate negative controls were also tested. For immunofluorescence, TMA sections were pretreated with sodium-citrate buffer pH 6 (5 minutes, 100°C). Slides were blocked with 10% donkey serum (2 hours) and
incubated with the antibody against CSF2RB (LS-B6993, Life-
span Biosciences) and active-beta-catenin (05-665, Millipore)
onight, 4°C. After rinsing with Dulbecco’s phosphate-buff-
erred saline, slides were incubated with Alexa 594 (A11072, Life
Technologies) or Alexa 488-conjugated secondary antibody
(A11017, Life Technologies). DNA was stained with 4’6-
diamidino-2-phenylidole and tissue sections imaged using a dig-
ital camera connected to a Leica microscope DMRXA.

RNA in situ hybridization
The experiments of RNA in situ hybridization were con-
ducted with the QuantiGene ViewRNA ISH Tissue Assay Kit
(Affymetrix) and the QuantiGene ViewRNA TYPE1 # Probe Set
for HUMAN-CSF2-colony stimulating factor 2 (granulocyte-
macrophage; Affymetrix) following the manufacturer’s instruc-
tions. Images were obtained using a confocal microscope Leica
TCS-SP2-AOBS.

Cytotoxicity assays
After mouse grafting, peripheral lymphocytes were isolated
from blood using Lympholyte-M (Tebu-bio). The protocol for
the isolation of tumor-infiltrating lymphocytes was adapted
from Radoja and colleagues (30). Cytotoxicity assays were
conducted to determine cell-mediated cytotoxicity against cell
lines injected in the mice flanks with CytoTox 96 Non-Radio-
active Cytotoxicity Assay (Promega) following the manufac-
turer’s instructions.

ELISA
Culture medium aliquots were frozen in liquid nitrogen
immediately after extraction and kept at −80°C until analysis.
ELISA was conducted with LEGEND MAX Mouse GM-CSF
ELISA kit (BioLegend) for mouse Gm-CSf detection and
Human GM-CSF ELISAPRO kit (Mabtech) for human GM-CSF
detection, following the instructions of the manufacturers.

Statistical analyses
For statistical comparisons we used Student’s t test (paired
and unpaired). We analyzed colon tumor-free survival using
Kaplan–Meier log-rank test. A P value of less than 0.05 was
considered statistically significant.

Results
Frequent aberrant promoter demethylation and
overexpression of GM-CSF in human colorectal cancer
To characterize GM-CSF expression in colon cancer, we
analyzed GM-CSF mRNA levels in 124 paired samples of
primary colon tumors and their healthy counterparts, and
included peripheral blood mononuclear cells (PBMC) as a
positive control. We found that samples from healthy colon
epithelium showed very low, or absent, GM-CSF expression,
whereas many tumors expressed GM-CSF above PBMC
levels (Fig. 1A). GM-CSF overexpression by tumors (consid-
ered as more than 2-fold increase over the levels from their
corresponding healthy counterparts) was observed in 37.9%
(47 of 124) of patients. We also studied 10 colon cancer cell
lines (CCL) and found that 6 of them (Caco2, HCT15,
HCT116, HT29, RKO, and SW48) did not express GM-CSF,
whereas 4 (SW480, DLD1, COLO205, and Co115) showed
expression values similar to, or above, PBMC levels (Fig. 1A
and Supplementary Fig. S1A). Our results show that GM-CSF
is frequently overexpressed in primary colon tumors as well
as in colon cancer cell lines.

A large number of molecular alterations in cancer occur at
the epigenetic level (31). Cancer cells show aberrant gain or
loss of DNA methylation at the promoter region of many
genes (32); alterations that are directly involved in tumor-
igenesis as they can induce either repression of tumor
suppressor genes or activation of oncogenes (32, 33). To
study the possible aberrant epigenetic regulation of GM-CSF
in colon cancer, we determined the methylation status of

Figure 1. GM-CSF overexpression and DNA methylation levels at the GM-CSF promoter in human primary colon cancer. A, GM-CSF mRNA expression levels were determined by quantitative real-time PCR in 124 paired samples of primary colon tumors (Tumor) and their healthy counterparts (HC, healthy colon). Results from PBMCs were included as a positive control of GM-CSF expression. Human GM-CSF mRNA levels are expressed as a ratio in relation to glyceraldehyde-3-phosphate dehydrogenase mRNA levels. B, bisulfite pyrosequencing results from 74 colon primary tumors (Tumor) and their 74 paired HC tissues. A schematic representation of the studied region and the methylation values from 2 representative coupled samples are shown on the left. Black depicts methylated CpG and white indicates unmethylated CpG. On the right, a boxplot shows the methylation results of the 74 paired human samples. A significant difference in methylation values of tumor samples compared with their healthy counterparts was found (P < 0.001, paired t test).
GM-CSF promoter in 10 CCLs. This analysis showed that GM-CSF promoter was densely methylated in the 6 CCLs (Caco2, HCT15, HCT116, HT29, BKO, and SW48) that had very low levels of GM-CSF expression, whilst it was almost unmethylated in the 4 CCLs (SW480, DLD1, COLO205, and Co115) that presented high levels of GM-CSF expression (Supplementary Fig. S1A and S1B). With the aim of clarifying whether GM-CSF promoter demethylation is also a frequent in vivo event, we analyzed 78 primary colon adenocarcinomas and their 74 paired healthy colon tissues. Healthy tissues presented an average methylation level of 83.5%, whereas the mean methylation level of the tumors was 61.5% (P < 0.001; Fig. 1B). We detected hypomethylation at least a 20% change at the GM-CSF promoter in 38 of the 74 tumors (51.4%), thereby confirming this as a common event in vivo (Fig. 1B). To evaluate whether demethylation of the GM-CSF promoter is related to a genome-wide demethylation process, something frequently observed in tumor cells (34), we analyzed the methylation levels of the LINE-1 sequence by bisulfite pyrosequencing (35). A significant association (P < 0.001) between LINE-1 and GM-CSF hypomethylation was found (Supplementary Fig. S2), thereby consolidating the notion that DNA hypomethylation plays an important role in the aberrant regulation of GM-CSF in colon cancer.

Concurrent GM-CSF overexpression (Fig. 1A) and GM-CSF promoter DNA demethylation (Fig. 1B, right) suggested that DNA methylation plays an important role in the regulation of GM-CSF expression. To get a deeper insight into this issue, we analyzed the consequences of in vitro demethylation. Firstly, we measured GM-CSF promoter methylation and GM-CSF expression levels in the HCT116 cell line compared with a double DNMT1 and DNMT3B knock-out cell line (DKO) derived from HCT116. We observed genetically induced GM-CSF promoter hypomethylation and consistent GM-CSF overexpression in the DKO cell line (Supplementary Fig. S1C), supporting the existence of a relationship between GM-CSF promoter methylation and its expression. Furthermore, we analyzed GM-CSF mRNA levels in 4 colon cancer cell lines incubated with 2 different concentrations of the demethylating drug AdC. Treatment with this drug resulted in marked reactivation of GM-CSF expression in a dose-dependent manner in cell lines displaying high levels of GM-CSF promoter methylation (HCT15 and RKO), but not in those with low levels of methylation (Colo205 and Co115; Supplementary Fig. S1D). These data support the notion that aberrant GM-CSF promoter demethylation plays an important role in GM-CSF overexpression in colon cancer.

To verify that GM-CSF promoter methylation and mRNA expression levels are related to protein levels and to determine whether ectopically overexpressed GM-CSF was secreted by cells, we measured GM-CSF protein levels in the culture media of 2 colon cancer cell lines (HCT116 and DLD1) by ELISA. We confirmed that GM-CSF protein levels are directly associated with mRNA levels and, inversely related to GM-CSF promoter methylation in both colon cancer cell lines (Supplementary Fig. S1E). This suggests that promoter demethylation-related GM-CSF overexpression leads to secretion of the cytokine by colon cancer cells.

Immune-dependent antitumor effect of GM-CSF ectopically secreted by colon tumors

Given the antitumor effect of GM-CSF when administered as a vaccine in an immunotherapy context (6, 11), we tested whether the overexpression of this cytokine by colon cancer cells could mimic this antitumor action. To evaluate a possible immune-dependent effect of GM-CSF in colon cancer, we knocked down Gm-CSF expression by stable RNA interference in the murine Gm-CSF–expressing colon cancer cell lines CT26 and MC38, which are poorly methylated at the Gm-CSF promoter (Supplementary Fig. S3A, S3B, S3C, and S4A). To discard a possible immune-independent effect of GM-CSF, we analyzed the expression of the Gm-CSF plasmatic membrane receptor subunits Csf2ra and Csf2rb in these same murine colon cancer cell lines. MC38-derived clones expressed Csf2ra, whereas CT26-derived clones did not. All clones expressed similar low levels of Csf2rb (Supplementary Fig. S3D and S4B). Then, we s.c. injected CT26 cells with knocked-down Gm-CSF expression (shGm-CSF) and an isogenic scramble-transfected clone (scramble) that overexpressed Gm-CSF into a cohort of autologous immune-competent BALB/c mice. As CT26 clones do not express Csf2ra (Supplementary Fig. S3D and S4B), this model allows the comparison of 2 colon cell lines, differing in their Gm-CSF expression, when they encounter an immune-competent in vivo environment, excluding immune-independent effects. Abolishing Gm-CSF expression in CT26 cells resulted in a marked increase in tumor formation in vivo (Fig. 2A and Supplementary Fig. S4C). At the time the mice were sacrificed, the Gm-CSF expressing CT26 tumors had, on average, half the volume and weight of their non-Gm-CSF–expressing counterparts (Fig. 2A and Supplementary Fig. S4C). This led us to investigate the possible role of the immune system reasoning that it could be responsible for the observed antitumor effect of Gm-CSF (7, 36, 37). We studied T lymphocyte infiltration conducting immunohistochemistry of tumors resected from BALB/c mice. On average, we found more T infiltration in Gm-CSF expressing tumors than in their silenced counterparts (Fig. 2B). In addition, we measured the specific lysis conducted by lymphocytes isolated from BALB/c mice blood and found that lymphocytes from mice carrying Gm-CSF expressing tumors provoked a higher percentage of cell lysis than those from mice with nonexpressing tumors (Fig. 2C). We further addressed this issue using tumor-infiltrating lymphocytes extracted from tumors generated by scramble or an additional shGm-CSF clone of CT26 cell line in BALB/c mice. As shown in Supplementary Fig. S4D, we obtained the minimal required amount of lymphocytes for cytotoxicity assays in only 4 cases. While 4 cases are not sufficient for statistical analysis, we observed a tendency toward higher cytotoxicity specific lysis from lymphocytes extracted from Gm-CSF–expressing tumors (Supplementary Fig. S4E): results that we consider to provide evidence for the role of Gm-CSF in activating the immune system to limit tumor growth. Thus, ectopic expression of this cytokine by tumors might have an antitumor effect mediated by the immune system.
Immune-independent antitumor effect of GM-CSF ectopically secreted by colon tumors

We observed in vitro that scramble and shGm-CSf clones from CT26 and MC38 cell lines showed variable growth patterns (Fig. 3A and Supplementary Fig. S5). Reduced Gm-CSF expression was associated with increased cell viability, cell growth, and colony formation ability in MC38 cells but its effects were not significant in CT26 cells (Fig. 3A and Supplementary Fig. S5). This observation led us to consider that, in addition to the immune-dependent effects described above, Gm-CSF expression may exert an immune-independent effect on tumor growth (16–20). This possibility was supported by the direct relationship between growth effects and the expression of Gm-CSF receptor subunits (38); only when both Gm-CSf receptor subunits were present (in MC38) could we detect a decrease in cell growth rate attributable to Gm-CSF (Fig. 3A and Supplementary Fig. S5). This immune-independent effect was confirmed in vivo by the s.c. injection of a scramble-transfected Gm-CSF-expressing MC38 clone and a Gm-CSF–silenced MC38 clone into immunodeficient nude mice, thereby allowing us to avoid interference of Gm-CSF effects on the immune system. Gm-CSF expression in scramble MC38 cells resulted in a significant reduction in tumor formation in vivo; at the time of sacrifice, Gm-CSF expressing MC38 tumors had, on average, 20% the volume and 20% the weight of those lacking Gm-CSf expression (Fig. 3B). Thus, our data suggest that the immune-independent antitumor action of tumoral Gm-CSF depends on the ectopic expression of Gm-CSF receptors by the tumor.
Synergic immune-dependent and immune-independent antitumor effect of tumoral GM-CSF

We studied the combinatory effect of Gm-CSf and its receptor subunits' expression using Gm-CSf–expressing and silenced MC38 clones injected into their autologous immune-competent C57BL/6 mice. In this model, where both immune-dependent and immune-independent effects coexist, we found that the MC38 scramble clone was detectable only for the first 10 days before disappearing completely (Fig. 4A and Supplementary Fig. S6). This effect did not occur on the wild-type MC38 clone (which does not express Cs2ra; Supplementary Fig. S7), which supports the notion that the immune-independent effect is crucial for the complete suppression of tumor growth in our mouse model, where the combination of immune-dependent and immune-independent effects of Gm-CSF leads to the complete loss of tumor formation capability.

To determine whether our findings in mice would also hold for human clinical samples, we analyzed GM-CSF, CSF2RA, and CSF2RB expression in a set of 124 colorectal cancer patients...
whom a long clinical follow-up was available. GM-CSF overexpression (more than 2-fold) was observed in 37.9% (47 of 124) of patients. CSF2RA and CSF2RB were overexpressed (more than 2-fold) in 22.6% (28 of 124) and 27.2% (34 of 124) of patients, respectively. Demographic and clinical characteristics of the patients included in our study are shown in Supplementary Table S1.

To test whether GM-CSF and its receptor were expressed by primary colon cancer cells, we conducted RNA in situ hybridization of GM-CSF as well as the immuno fluorescence detection of its receptor. As shown in Supplementary Fig. S8, we confirmed that cells from tumor colon epithelia aberrantly express these molecules. As expected, RNA in situ hybridization of GM-CSF was positively detected in tonsil and undetectable in muscle tissue (Supplementary Fig. S8A). GM-CSF receptor was located in the membrane of colon tumor epithelial cells, and colocalized with beta-catenin staining (which specifically marked epithelial cells, but not stroma cells or infiltrated lymphocytes; Supplementary Fig. S8B).

Concurrent overexpression of GM-CSF, CSF2RA, and CSF2RB, which was observed in 6.5% (8 of 124) of patients, was strongly associated with an increase in overall survival rates (Fig. 4B). All the patients with tumors overexpressing the 3 genes survived at least 5 years from diagnosis. This effect was not observed when comparing patients that overexpressed GM-CSF without concurrent changes in receptor subunit levels, or in patients that overexpressed both receptor subunits without changes in GM-CSF expression (Supplementary Fig. S9A and S9B), which sustains the notion that ectopic expression of GM-CSF and its receptor subunits by human colorectal tumors has a strong antitumor effect. Our data show that in 100% of cases where the 3 genes were overexpressed overall survival was dramatically increased. We would thus propose that GM-CSF in combination with its receptor subunits should be considered a valuable marker for diagnosis and prognosis in clinical studies that may prove helpful in the stratification of patients. Furthermore, demethylation-associated GM-CSF overexpression accompanied by CSF2RA and CSF2RB overexpression is a possible indicator of outcome in colorectal cancer patients.

**Anti-PD-1 adjuvant therapy improves the antitumor effects of tumoral GM-CSF**

Anti-PD-1 is an antibody raised against Programmed death 1 (PD-1; ref. 39), that enhances T cell activity in chronic pathologies such as cancer (7, 40, 41). The combination of PD-1 blockade with GM-CSF–secreting tumor cell immunotherapy has been recently shown to significantly improve antitumor
responses (41). To determine whether anti-PD-1 also potentiates the antitumor effect of Gm-CSF ectopically secreted by tumor cells, we injected Gm-CSF-expressing scramble and shGm-CSF CT26 cells into their autologous BALB/c mice and administered PBS or anti-PD-1 treatment. At the moment of sacrifice, we found a higher percentage of reduced tumor growth in mice with Gm-CSF–expressing tumors when they were treated with anti-PD-1 (Fig. 5). Furthermore, we found a 25% remission of tumors in mice treated with anti-PD-1 when the tumor did not express Gm-CSF (red bars), whereas there was a 50% remission, or growth arrest, in mice treated with anti-PD-1 that carried Gm-CSF–expressing scramble cells (blue bars). Photographs of 1 representative mouse from each analyzed group are also included.

Discussion

The cytokine GM-CSF was described because of its ability as a colony-stimulating factor to promote the formation of granulocytes and monocytes (4). However, GM-CSF also influences dendritic cells and this property is currently being used in various immunotherapy strategies to stimulate the antitumor action of the immune system (12). Importantly, the first US Food and Drug Administration approval in history for a therapeutic cancer vaccine was granted to Sipuleucel-T (Provenge; Dendreon, Inc.) for castration-resistant prostate cancer. Sipuleucel-T is an autologous dendritic cell-based vaccine in which cells are loaded with a fusion protein including GM-CSF. This vaccine is being studied in a phase III trial for patients with advanced melanoma because of the promising results from previous trials (43). In addition, the GVAX (Cell Genesys) approach in immunotherapy involves the administration of irradiated tumor cells that have been engineered to secrete GM-CSF and, in this manner, activate dendritic cells and the immune system with the antigens provided by the whole tumor cells. A significant body of preclinical data supported its antitumor efficacy, especially in combination with agents such as anti-CTLA-4, and anti-PD-1 (41, 44). However, phase III clinical trials of GVAX in patients with prostate cancer did not produce significant results (12). GM-CSF is a valuable tool in cancer immunotherapy but there are still several unanswered questions that need to be addressed before the complexity of the results from clinical trials can be explained.

Herein, we show that aberrant DNA demethylation of GM-CSF can lead to its ectopic secretion by colorectal tumors. However, the natural role of DNA methylation at the GM-CSF promoter is still largely unknown. According to previous DNA methylation data from Human Infinium Methylation Array 27k (Illumina; ref. 45), GM-CSF promoter is methylated in human stem cells and human healthy primary tissues but not in T CD4+ and T CD8+ lymphocytes, that is, it follows a tissue-specific pattern. Furthermore, comparing this methylation data with available gene expression data from Amazonia “Human body index” series (46), which comprises human transcriptome list annotations, we see that only the cells with GM-CSF promoter DNA demethylation (T CD4+ and CD8+ lymphocytes) are able to express this cytokine after activation (Supplementary Fig. S10). Therefore, GM-CSF expression could be under a double regulatory mechanism with a first level of regulation in which loss of DNA methylation would allow further gene expression after cell activation. Thus, GM-CSF
promoter demethylation might be important in priming this specific hematopoietic gene for activation/expression in response to external stimuli (45). This natural mechanism of regulation could suffer aberrant changes in colorectal cancers leading to its ectopic secretion by the tumor cells. Further studies are necessary to decipher the role of DNA methylation in the natural regulation of GM-CSF and the molecular mechanisms that lead to its loss in colon cancer cells.

Our data indicate that tumor-secreted GM-CSF can induce immune-dependent antitumor responses but that it also has a strong direct antitumor action when the tumors express GM-CSF receptors. Although the immune-dependent antitumor effect of GM-CSF has been known for some time, hence its use in immunotherapy (12, 47), this is the first time that GM-CSF ectopically secreted by tumor cells is shown to have immune-dependent antitumor effects. This observation might be important for the clinical use of GM-CSF vaccines, and further studies should determine whether the effects of these vaccines are affected by tumor-secreted GM-CSF. On the other hand, the immune-independent effect of tumor-secreted GM-CSF is still poorly understood. Some studies have found it to exert an antiproliferative effect (16, 17), whereas others have shown it to promote growth (18–20) and yet others found it to have no reproducible influence on growth rate (21, 22). Two main points may explain, to a certain extent, these contradictory results: firstly, the dose of GM-CSF used may well be relevant to the evaluation of its effects at a physiologically relevant level and, secondly, the presence of GM-CSF receptor should also be ascertained to ensure that the studied cells do in fact have the means to signal GM-CSF presence. Our study goes further in the evaluation of immune-independent effects of GM-CSF as we investigate the effects in vivo and find that tumors with concurrent expression of Gm-CSF and its receptor do not grow when injected in their autologous immunocompetent mice. Furthermore, human colorectal tumors with concurrent over-expression lead to a very good prognosis and a 100% 5-year overall survival rate. These findings support the notion of the strong role of the GM-CSF receptor in the immune-independent action of GM-CSF on tumor growth and indicate the importance of verifying the expression of this receptor by tumor cells when selecting a strategy for the treatment of colorectal cancer. On the other hand, a previous report on skin cancer (48) showed the tumorigenic role of GM-CSF when this cytokine is combined with G-CSF. Together, these results indicate that the role of GM-CSF in cancer is complex and its tumorigenic effect might depend on the tumor context.

Unexpectedly, our data show that promoter demethylation-associated expression of GM-CSF in colon cancer cells impairs tumorigenesis suggesting that not all the epigenetic alterations occurring in a tumor necessarily favor tumorigenesis. This possibility is in line with reports showing that, in many gene promoters, aberrant de novo hypermethylation is not responsible for gene repression as these genes are already repressed in healthy tissues (49). Collectively, these data imply that a number of DNA methylation alterations in cancer are not subject to growth selection (49), which is in consonance with the differentiation between "driver" and "passenger" modifications (previously referred to as mutations, but nowadays acknowledged to also include epigenetic alterations) in the genome of cancer cells during the tumorigenic process (50, 51). Even though driver modifications are considered causative in the development of a tumor, other changes that could be considered passenger modifications, that is, those not directly related with the tumor development, may become crucial for tumor progression and be considered driver changes as long as they give tumor advantage against antitumor therapies.

Our results on human primary tumors may have clinical implications, as they suggest that expression of GM-CSF and its receptor subunits are indicators of good prognosis in colorectal cancer. In addition, tumoral expression of the GM-CSF receptor could also be useful for patient stratification in cancer immunotherapy (i.e., tumors expressing GM-CSF receptor may be better candidates to receive GM-CSF vaccination). The strong immune-independent antitumor activity of GM-CSF reported in this study suggests that future clinical trials with GM-CSF–secreting vaccines should also take into account the expression of the GM-CSF receptor by tumors. Moreover, the results obtained in previous Phase III clinical trials with these vaccines (37, 52) should be reevaluated taking into account the tumor expression of GM-CSF receptor subunits. In addition, our results indicate that anti-PD-1 treatment may enhance the antitumor effect of Gm-CSF ectopically secreted by tumors. Further studies are necessary to determine whether patients with tumors expressing GM-CSF are better candidates to receive anti-PD-1 or other enhancers of the clinical activity of GM-CSF (53).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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