High Macrophage Infiltration along the Tumor Front Correlates with Improved Survival in Colon Cancer

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Abstract

Purpose: The role of macrophages in tumorigenesis is complex because they can both prevent and promote tumor development.

Experimental Design: Four hundred forty-six colorectal cancer specimens were stained with the pan-monocyte/macrophage marker CD68, and average infiltration along the tumor front was semiquantitatively evaluated using a four-grade scale. Each section was similarly scored for the presence of CD68 hotspots. Some aspects of macrophage-tumor cell interactions were also studied using *in vitro* coculture systems.

Results: Including all patients, regardless of surgical outcome and localization, survival increased incrementally with $CD68TF_{Mean}$ infiltration grade ($P = 0.0001$) but not in curatively resected colon cancers ($P = 0.28$). CD68 hotspot score ($CD68TF_{Hotspot}$) was divided into high and low. A high hotspot score conferred a highly significant survival advantage also in curatively resected colon cancer cases ($n = 199$, $P = 0.0002$) but not in rectal cancers. $CD68TF_{Hotspot}$ high turned out as an independent prognostic marker for colon cancer in multivariate analyses including gender, age, localization, grade, stage, tumor type, and lymphocytes at the tumor front, conferring a relative risk of 0.49 ($P = 0.007$). *In vitro* coculture experiments, using phorbol 12-myristate 13-acetate – activated U937 cells as macrophage model, revealed that a high ratio of macrophages to colon cancer cells inhibited cancer cell growth. This was partially dependent on cell-to-cell contact, whereas Boyden chamber cocultivation without cell-to-cell contact promoted cancer cell spread.

Conclusions: In conclusion, our data indicate that a dense macrophage infiltration at the tumor front positively influences prognosis in colon cancer and that the degree of cell-to-cell contact may influence the balance between protumorigenic and antitumorigenic properties of macrophages.

Solid tumors comprise not only malignant cells but also many other nonmalignant cell types such as fibroblasts, endothelial cells, and various infiltrating immune cells. Accumulating evidence from clinical and experimental studies has shown that immune cell infiltration can significantly affect the course of malignant transformation (1). Tumor-infiltrating macrophages often constitute a significant part of infiltrating immune cells (2), and as participants in the host response toward the tumor, macrophages can kill susceptible target cells through several different mechanisms, including secretion of tumor necrosis factor-α (3), nitric oxide (4), interleukin-1β (5, 6), and reactive oxygen intermediates (7, 8). However, although macrophages under certain conditions can kill tumor cells, several investigations have highlighted their potential role as tumor promoters. For example, macrophages can secrete a variety of factors that directly stimulate the growth and migration of tumor cells, such as platelet-derived growth factor, epidermal growth factor, and transforming growth factor-β (9), and angiogenesis-promoting factors like vascular endothelial growth factor and tumor necrosis factor-α (10, 11), as well as produce proteases (12, 13) that potentially could facilitate tumor invasion and metastasis. Studies done on clinical specimens have to some extent confirmed protumorigenic roles for macrophages. Thus, the presence of macrophages has been reported to be associated with poor prognosis in breast (14, 15), prostate (16), bladder (17), glioma (18), and cervical cancers (19, 20). On the other hand, in another study on prostate cancer, macrophages improved prognosis (21). There is also conflicting results about lung cancer (22, 23), whereas in stomach cancer, macrophages are associated with good prognosis (24). In a study encompassing 131 colorectal cancer cases, macrophages positively influenced prognosis, although not significant in multivariate analysis (25, 26). In addition, two studies on 26 and 30 patients, respectively, have reported

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that low infiltration of macrophages tended to occur with more advanced colorectal cancer (27, 28). Thus, results about macrophages and prognosis in cancer seem to be somewhat contradictory, and the fact that most previous clinical studies encompassed a relatively low number of cases may have contributed to this. Recently, it was shown in a larger clinical study that the overall inflammatory cell reaction at the tumor front was positively correlated with a favorable outcome (29).

Given the somewhat variable results in different cancer types, we considered it an urgent task to clarify how macrophages affect prognosis in colorectal cancers. To address this issue, we investigated macrophage infiltration along the tumor front in 446 unselected CRC specimens and correlated the results to various clinicopathologic variables in univariate as well as multivariate analyses. In addition, we analyzed some aspects of macrophage influence on colon cancer cell behavior *in vitro*.

Materials and Methods

Clinical samples. We studied tumor specimens from 488 consecutive patients diagnosed with colorectal cancer and tissues collected during primary tumor surgical resection over the period 1995-2003 at Department of Surgery, Umeå University Hospital, Sweden. Ten specimens were excluded due to lack of adequate tissue available (i.e., tumor front included in the specimen, or inadequate staining results for CD68). An additional 32 patients lacked follow-up data or died from perioperative complications, leaving 446 patients for survival analyses. Of these, 82 patients received preoperative radiotherapy (5 × 5 Gy), 39 received long-term radiotherapy (25 × 2 Gy), and 113 received postoperative adjuvant chemotherapy (mainly 5-fluorouracil/leucovorin).

All routinely stained sections were reviewed by one observer (R.P.), who did all histopathologic classifications including stage, grade, tumor type (mucinous or nonmucinous), growth pattern (pushing or infiltrating), and lymphocytic reaction at the tumor front. Clinical data were obtained by reviewing the patient records (Å.Ö.) and survival data were collected during spring 2005. The study was approved by the local ethical committee of Umeå University.

Immunohistochemistry. Specimens were, according to routine procedures at the Department of Clinical Pathology, Umeå University Hospital, fixed in 4% formaldehyde and embedded in paraffin. One 4-μm section from each patient was cut, dried, dewaxed, and rehydrated before microwave treatment in citrate buffer (pH 6.0) for 3 × 5 min. A semiautomatic staining machine (Ventana ES, Ventana, Inc., Tucson, AZ) was used for the immunohistochemical procedures. Anti-CD68 monoclonal antibody (KP-1, DakoCytomation, Glostrup, Denmark) was used at a concentration of 1:4,000. The slides were counterstained with hematoxylin.

CD68 evaluation. CD68 immunostaining was evaluated along the tumor front over the whole section (7-10 view fields per section) and average infiltration ($CD68TF_{Mean}$) was semiquantitatively graded as no/weak (grade 1), moderate (grade 2), strong/robust (grade 3), and massive infiltration (grade 4). Tumors classified as 1 included totally negative specimens as well as specimens containing some scattered CD68-positive cells along the tumor margin. Tumors were classified as 2 when CD68 staining was continuous along the tumor margin but was not extended from the tumor front more than one cell layer on average. CD68 staining that, on average, extended two to three cell layers from the tumor margin over the whole section was classified as 3; whereas to be classified as 4, CD68 staining should extend several cell layers from the tumor margin in all fields. Examples of classified tumors are given in Fig. 1. The specimens were evaluated twice by the same observer without any knowledge about prognosis or clinicopathologic variables. Eighty-seven percent of the specimens were judged identically between evaluations. Disagreements were evaluated a third time followed by a conclusive judgment. CD68 hotspots ($CD68TF_{Hotspot}$) were defined as infiltration grade of the two highest view fields, evaluated as for

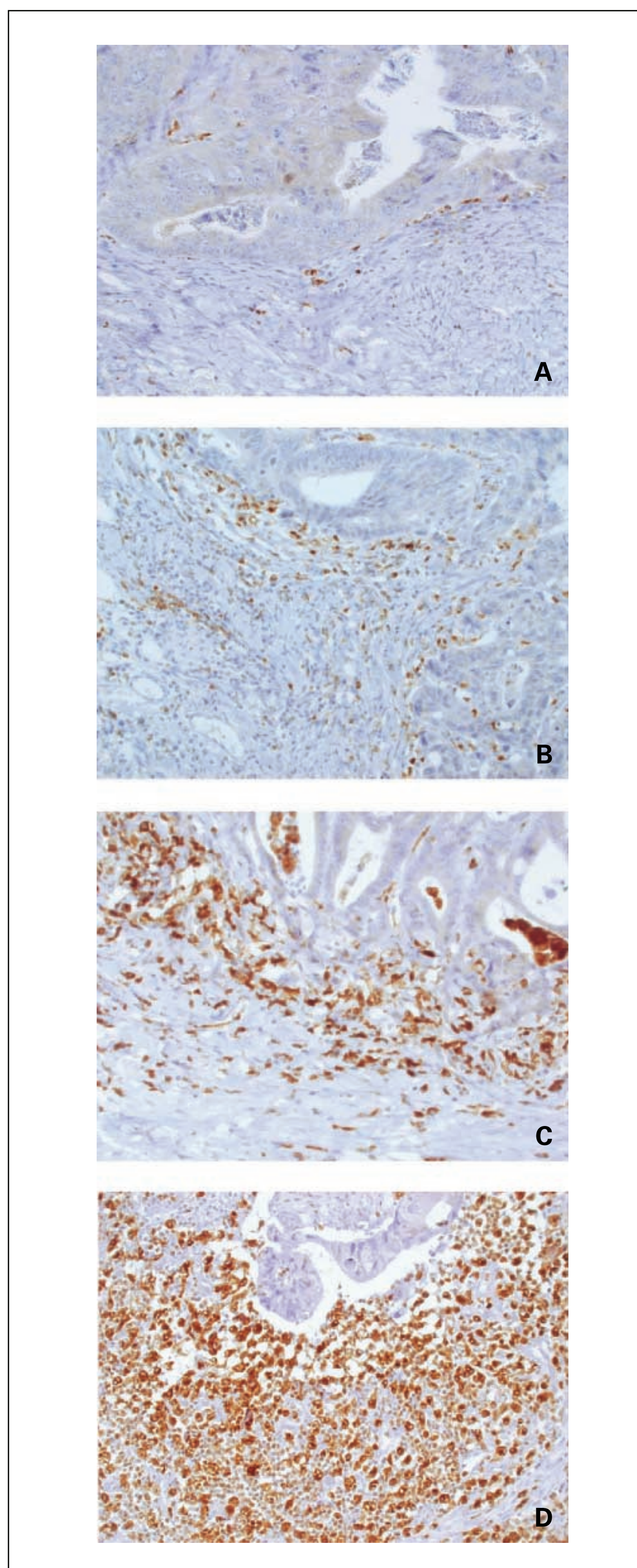

Fig. 1. Examples of stainings representing the different grades of macrophage infiltration; no/low (*A*), moderate (*B*), high (*C*), and massive (*D*).

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$CD68TF_{Mean}$, at a total magnification of ×200 and graded from 1 to 4. Intraobserver agreements for $CD68TF_{Hotspot}$ were 77%.

Statistics. Cross-tabulations were analyzed with Fisher's exact test. To test the linear association between two ordinal scale variables, the exact linear-by-linear association test was done. The Kaplan-Meier survival analysis was used to estimate the cancer-specific survival, and comparisons between groups were done with the log-rank test. Statistical analyses were done using SPSS statistical software version 12.0.1. $P < 0.05$ was considered statistically significant.

Cell cultures. HCT-116 and HT-29 cells were grown in 25-cm^3 culture flasks in 7 mL DMEM/10% FCS. Media were changed every third day and cells were harvested with trypsin-EDTA (PBS containing 0.53 mmol/L EDTA, 145 mmol/L NaCl, and 0.05% trypsin). U937 cells were activated for 48 h with 20 ng/mL phorbol 12-myristate 13-acetate (PMA) to promote macrophage differentiation (30, 31) and carefully decanted to remove nonadherent cells. Adherent cells were harvested by two subsequent incubations in PBS containing 25 mmol/L EDTA (no trypsin) for 10 min. PMA-activated U937 cells were washed twice in DMEM/10% FCS and subsequently added in different amounts directly to the culture plate containing preadhered cancer cells. HCT-116 or HT-29 cells (0.30×10^6) were seeded into a 25-cm^2 cell culture flask ($1.2 \times 10^4/cm^2$) and allowed to adhere and grow for 24 h; after which, PMA-activated U937 cells were added. After 2 days, nonadherent cells were gently washed away with PBS prewarmed to 37°C; after which, adherent cells were harvested with trypsin-EDTA and viable count was determined by trypan blue exclusion. Cells were stained with antihuman CD45 to detect remaining U937 cells, and only $CD45^-$ cells [65-95% of the recovered cells as determined by fluorescence-activated cell sorting (FACS)] were considered cancer cells.

For Boyden chamber cultures, 0.35×10^5 HCT-116 or HT-29 cells were seeded in 1 mL DMEM/10% FCS into each well of a 24-well plate. After 24 h, medium was replaced with 0.5-mL prewarmed fresh medium and the appropriate amount of PMA-activated U937 cells (in 250 μL DMEM) was added either directly into the well or into the Boyden chamber insert (0.4-μm pores; Millipore Corp., Bedford, MA). After 2 days, the adherent cancer cells were gently rinsed with 37°C PBS and harvested with trypsin-EDTA, counted, and stained for CD45.

Macrophage-conditioned medium. U937 cells were cultured at 0.5×10^6/mL in DMEM, 10% FCS containing 20 ng/mL PMA for 48 h. Nonadherent cells were discarded and remaining cells were washed twice in PBS prewarmed to 37°C and once in complete DMEM with 15-min incubation, and finally overlaid with DMEM containing 10% FCS (8 mL for a 75-cm^3 flask). After incubation for 24 h, the medium was filtered and used as macrophage-conditioned medium.

Migration assay. HCT-116 cells (50,000) or HT-29 cells (100,000) were placed in a 24-well cell culture insert (8 μm pore size; BD Biosciences, Franklin Lakes, NJ) in 400-μL DMEM containing 2% FCS and allowed to adhere for 2 to 3 h. Next, culture inserts were placed in 500-μL DMEM containing 10% FCS (control) or in 500-μL macrophage-conditioned DMEM, 10% FCS and incubated for 20 h. After washes in PBS, inserts were placed in ice-cold methanol for 1 min and washed again in PBS. Cells adhering to the inside of the insert were thoroughly scraped of with a cotton top and the insert placed in a solution of 0.5% crystal violet for 10 min. After washes in PBS, the filter was cut out and cells were counted at 100× in three randomly selected fields using a 10× ocular with a grid pattern.

Immunofluorescence. HCT-116 and HT-29 cells were seeded in 24-well culture plates over a cover glass. Twenty-four hours later, cells were overlaid with a culture insert containing PMA-activated U937 cells and incubated for 48 h. After fixation in 4% paraformaldehyde, cells were stained with a rabbit β-catenin antibody (Sigma, St. Louis, MO) and revealed with a Cy2-conjugated secondary goat anti-rabbit (Amersham, GE Healthcare, Little Chalfont, United Kingdom).

FACS. Cells were washed twice in FACS staining medium (PBS supplemented with 3% FCS) and incubated for 30 min on ice with FITC-conjugated antihuman CD45 (ImmunoTools GmbH, Friesoythe, Germany). Cells were washed twice, resuspended in FACS staining medium, and analyzed on a FACSCalibur (Becton Dickinson, Mountain View, CA).

Results

CD68 expression. The majority of CD68-positive cells were located in the stroma and, in particular, along the tumor front. CD68-positive cells were mostly in apparent direct contact with or immediately adjacent to tumor cells lining the tumor front. Examples of representative staining fields of respective grade are given in Fig. 1. Although the majority of tumors displayed a fairly homogeneous CD68 infiltration pattern along the tumor front, there were also tumors containing small areas that showed CD68 infiltration considerably above average grade. This prompted us to evaluate $CD68TF_{Hotspot}$, similarly graded from 1 to 4. The frequencies of $CD68TF_{Hotspot}$ and $CD68TF_{Mean}$ are shown in a cross-tabulation (Table 1).

Correlation of macrophage infiltration to clinicopathologic parameters. Table 2 shows the correlation between $CD68TF_{Hotspot}$ and clinicopathologic variables. Similar correlations were obtained for $CD68TF_{Mean}$ (data not shown). When including all tumors, no correlation was seen to gender, age, preoperative radiotherapy, and whether the tumors showed an infiltrating or expanding growth pattern. On the other hand, there was a very strong correlation to tumor stage, grade, tumor localization, tumor type, as well as lymphocytic reaction at the tumor front (Table 2).

Survival analyses. Including all patients applicable to survival analyses ($n = 446$), regardless of surgical intent, outcome, and localization, survival increased incrementally with $CD68TF_{Mean}$ infiltration grade in a highly significant manner ($P < 0.0001$; Fig. 2A). Significance for $CD68TF_{Mean}$ was lost, however, when looking specifically on potentially curatively resected colon ($P = 0.28$) or rectal cancers ($P = 0.41$). $CD68TF_{Hotspot}$ was also highly significantly correlated to survival ($P < 0.0001$). The $CD68TF_{Hotspot}$ survival curves essentially clustered into two groups (i.e., grade 1 and 2 versus 3 and 4). Therefore, cases were regrouped into $CD68TF_{Hotspot}$ high (3 and 4) versus $CD68TF_{Hotspot}$ low (1 and 2) macrophage infiltration, with a significant survival advantage for the $CD68TF_{Hotspot}$ high group ($P < 0.0001$). This was also the case for potentially curatively resected colon cancers ($n = 199$,

Table 1. Cross-tabulation between CD68 count as mean value and CD68 hotspot value in 478 colorectal cancers

CD68 hotspot at invasive front		CD68 mean value at invasive front				P
		−/+	+	++	+++	
		1	2	3	4	
−/+	1	24	0	0	0	<0.001/<0.001*
+	2	27	62	0	0	
++	3	10	160	32	0	
+++	4	0	42	103	18	

*Exact linear-by-linear association test.

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Table 2. CD68 expression in hotspots at the tumor invasive front in relation to clinicopathologic characteristics in colorectal cancers

Variable	CD68 front hotspot				P
	−/+	+	++	+++	
	1	2	3	4	
Gender					
Male	15	47	104	95	0.49
Female	9	42	98	68	
Age, y					
≤59	2	17	30	41	0.37*
60-69	8	14	54	34	
70-79	12	43	67	44	
≥80	2	14	47	37	
Localization					
Right colon	11	41	62	39	0.012
Left colon	7	19	62	53	
Rectum	5	29	76	69	
Stage					
I	4	10	23	31	<0.001/<0.0001*
II	9	22	82	73	
III	2	21	46	28	
IV	8	35	44	23	
Grade					
Well	2	5	0	3	<0.001/<0.001*
Well-moderate	8	28	91	94	
Moderate-poor	9	39	90	55	
Poor	5	17	17	6	
Tumor type					
Mucinous	8	24	30	12	<0.001
Nonmucinous	16	64	168	149	
Growth pattern					
Pushing	9	20	69	57	0.17
Infiltrating	15	67	129	103	
Lymphocytic reaction tumor front					
Low	21	65	119	56	<0.001
High	3	23	79	105	
Preoperative radiotherapy					
No	22	74	170	127	0.29
Yes	2	15	30	34	

*Exact linear-by-linear association test.

$P = 0.002$; Fig. 2B) but not for rectal cancers ($n = 125$, $P = 0.37$; Fig. 2C).

Multivariate survival analysis. Next, a multivariate analysis was adopted to investigate the importance of CD68 expression in comparison with other prognostic parameters. Multivariate analysis was done including gender, age, localization, grade, stage, tumor type, and lymphocytes at the tumor front. In colon cancers, prolonged survival for $CD68TF_{Mean}$ was observed mainly in the group with grade 4 infiltration. Because all cases in this group survived, this parameter was not applicable in multivariate analysis (infinite risk estimation). However, $CD68TF_{Hotspot}$, which compares high (merged 3 and 4) versus low (1 and 2), turned out as an independent prognostic marker for colon cancer, conferring a relative risk of 0.49 ($P = 0.007$; Table 3). Notably, lymphocytic infiltration detected by H&E staining was significantly associated with prognosis in multivariate analysis (relative risk, 0.55; $P = 0.007$) if $CD68TF_{Hotspot}$ was excluded from the model, but lost significance when $CD68TF_{Hotspot}$ was included (Table 3). Again, there was no significant survival advantage conferred by high $CD68TF_{Hotspot}$ scores for rectal cancers (relative risk, 0.95; $P = 0.89$).

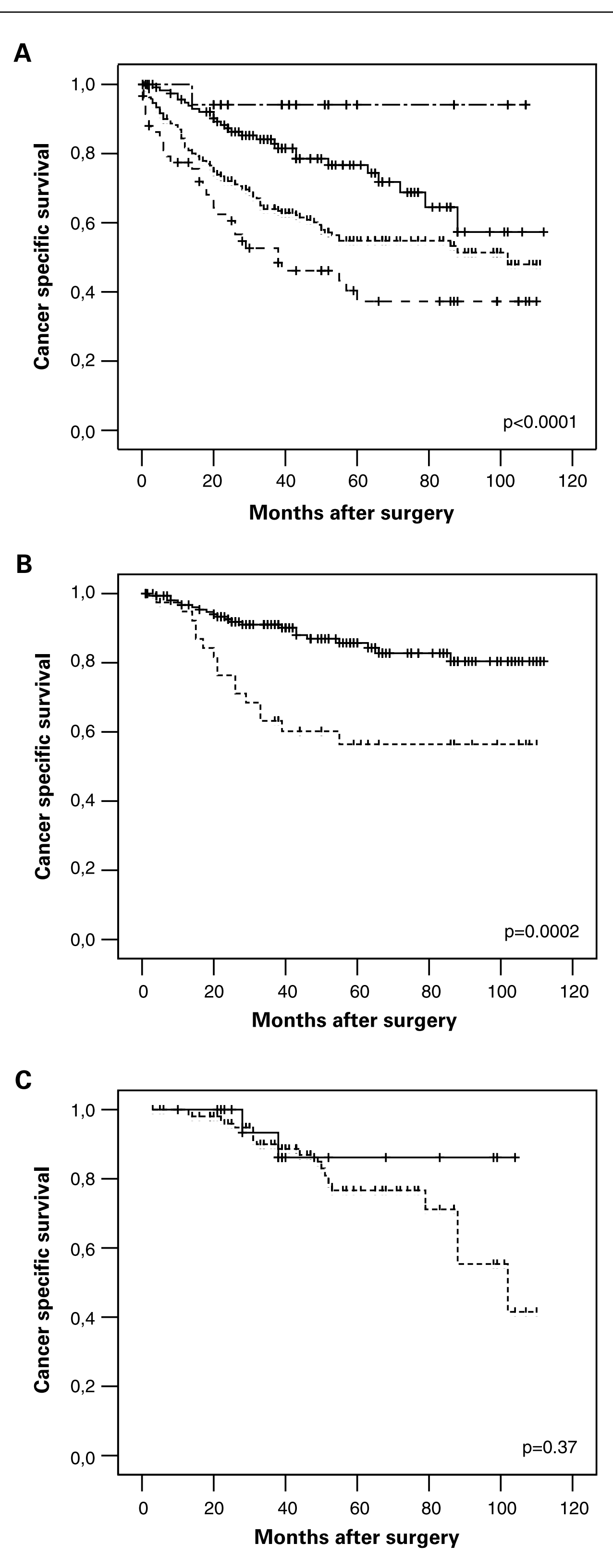

Fig. 2. Kaplan-Meier plots of all cancer specimens with follow-up data ($n = 446$) scored for $CD68TF_{Mean}$ (– – –, 1; −−−, 2; —, 3; −−·−−, 4) (*A*). Subgroup analysis of $CD68TF_{Hotspot}$ for curatively resected colon cancers ($n = 199$; —, high; – – –, low; *B*) and for curatively resected rectal cancers ($n = 125$; —, high; – – –, low; *C*).

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Table 3. Results of the Cox proportional hazard model in 270 colon carcinomas

Variable	Relative risk, e^{β} (95% confidence interval)	P
Age* (y)	1.009 (0.99-1.03)	0.43
Gender		
Male	1.00	
Female	1.22 (0.81-1.86)	0.34
Localization		
Right colon	1.00	
Left colon	0.84 (0.54-1.33)	0.46
Grade		
Well	1.00	
Well-moderate	0.76 (0.21-2.67)	0.67
Moderate-poor	0.83 (0.24-2.90)	0.77
Poor	1.08 (0.31-3.78)	0.91
Stage		
I	1.00	
II	0.85 (0.28-2.56)	0.78
III	1.90 (2.26-13.7)	0.27
IV	16.79 (5.56-50.7)	<0.0001
Tumor type		
Mucinous	1.00	
Nonmucinous	4.01 (2.02-7.95)	<0.0001
Lymphocytic reaction tumor front		
Low	1.00	
High	0.67 (0.42-1.07)	0.095
CD68 front hotspot		
Low	1.00	
High	0.49 (0.29-0.82)	0.007

*Continuous variable.

Inhibition of colon cancer cell growth by direct contact with PMA-activated U937 cells but not by cocultivation without cell-to-cell contact. U937 is a cell line of monocytic origin that can be induced to differentiate into macrophage like cells by treatment with PMA (30). We cultured HCT-116 colon cancer cells with nonactivated and PMA-differentiated U937 cells for 2 days whereupon viable counts of adherent cells were determined. To avoid detecting cocultured U937 cells as cancer cells, recovered cells were stained with antihuman CD45 and analyzed by FACS. U937 cells readily expressed CD45, whereas it was not detected on cancer cells (Fig. 3A). Whereas PMA-differentiated U937 cells inhibited HCT-116 growth, negligible cancer cell growth inhibition was seen by coculture with untreated U937 cells (Fig. 3B). This growth inhibition may be a general feature of colon cancer cells because HT-29 and SW-480 cells were also found to be inhibited by coculture with macrophages, and preliminary results indicate that this applies to LoVo cells as well (data not shown). To determine if this effect was dependent on the density of PMA-activated U937 cells to cancer cells and/or required cell-to-cell contact, we cultured HCT-116 and HT-29 cells with increasing amounts of U937 cells added directly to the monolayer or in a Boyden chamber insert (Fig. 3C and D). Interestingly, when allowing communication but not direct contact between cancer and U937 cells, inhibition of cancer cell growth was no longer observed at a 4:1 ratio (Fig. 3C and D). The fact that some inhibition was observed at 10:1 also in Boyden chamber cocultures indicates that part of the growth inhibitory effect occurs through soluble mediators (Fig. 3C and D). Another more striking feature of the colon cancer cells communicating with PMA-activated U937 cells was a morphologic switch from epithelial sheets to more loosely associated or even single cells with a migratory phenotype (data not shown). To confirm that these cells indeed displayed enhanced migration, HCT-116 and HT-29 cells that adhered to the upper chamber of a culture insert were placed over either macrophage-conditioned DMEM or DMEM, and migration to the apical side of the insert filter was scored 20 h later. As depicted in Fig. 4A and B, these experiments revealed that migration of both cell lines was stimulated by the macrophage-conditioned medium. Finally, we also tested whether any biochemical changes occurred in the colon cancer cells communicating (but not in contact) with PMA-activated U937 cells. Evidence for loss of E-cadherin expression at adherens junctions between cells was obtained for HT-29 cells (Fig. 4C). For HCT-116 cells, little E-cadherin expression was detected at adherens junctions in either condition, probably due to the much lower expression in HCT-116 cells (data not shown). Interestingly, both cell lines, particularly evident for HCT-116, also showed a nuclear translocation of β-catenin (Fig. 4D). Thus, although the present results from the *in vitro* culture system cannot be automatically extrapolated to the situation in patients, they nevertheless indicate that the likelihood for protumorigenic actions increases when macrophages are not able to make contact with the colon cancer cells. Conversely, a high macrophage to tumor cell ratio favors colon cancer growth inhibition, which is consistent with the prolonged survival for tumor specimens with high macrophage infiltration observed in the clinical material.

Discussion

There are now a large body of evidence implicating macrophages as important players in the process of tumorigenesis. In the present study, we could in a relatively large clinical material subdivide the degree of macrophage infiltration at the tumor front, allowing for subgroup analyses. Our data suggested that an incremental increase in mean macrophage infiltration also incrementally improved survival, with a prominent apparent advantage in cases showing the most extreme CD68 mean infiltration. In addition, high CD68 hotspot score significantly improved survival and was shown to be an independent prognostic marker in multivariate analysis for colon cancer but not rectal cancer. The lack of prognostic significance in rectal cancers is most likely due to the preoperative radiotherapy given to about two thirds of these patients.

Our results were somewhat unexpected, given the dominating protumorigenic role in some other malignancies (2) and the fact that macrophages can secrete factors promoting angiogenesis and tumor growth (9). However, as illustrated by our *in vitro* Boyden chamber coculture experiments, protumorigenic properties could be unmasked when macrophage-to-tumor cell contact was denied. In these experiments, tumor cell migration, accompanied by nuclear translocation of β-catenin, was instead stimulated by the presence of macrophages. It has previously been shown that murine macrophages can kill glioma cells transfected with the membrane but not the secreted, isoform of macrophage colony-stimulating factor in a phagocytosis-dependent process (32). Thus, the degree to which the antitumorigenic abilities manifest may partly depend on the abilities of macrophages to come in direct contact with

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tumor cells, as well as to achieve a high macrophage to cancer cell ratio.

Although colon cancer cells are not immune to protumorigenic properties of macrophages *in vitro*, antitumorigenic properties seem to dominate in the more complex situation *in vivo*, altogether resulting in a favorable prognosis in our clinical material. These results are consistent with some previous studies that have linked decreasing amounts of macrophages with more advanced-stage tumors in colorectal cancer patients (27, 28, 33). In an attempt to link macrophage infiltration to prognosis using material from 131 patients, CD68 infiltration alone did not correlate with prognosis (25). Somewhat surprisingly, however, the presence of both CD68 and vascular endothelial growth factor in tumor-infiltrating macrophages/stroma significantly improved prognosis (25). Our study established an association of $CD68^+$ macrophages with improved prognosis. However, the question remains whether this reflects a predominant cytotoxic action of macrophages directly causing tumor regression, or whether it is more likely to reflect a gradual breakdown of immune responses eventually leading to tumor progression following a reduction in macrophage numbers, as proposed by Hakansson et al. (27). To that end, we have shown that under certain circumstances, PMA-activated U937 cells can inhibit the growth of some colon cancer cell lines. More significantly, it was recently reported that macrophage depletion in rats bearing colon cancer xenografts promoted enhanced cancer cell growth and impaired survival (34). Taken together, these results point to an important and direct role for macrophages in antitumor defense in colon cancer. Based on data presented herein, survival benefits require a high macrophage to cancer cell ratio, either along the whole tumor front or locally in "hotspots."

Along the tumor front, well-bordered, noninvasive tumor areas alternate with sites showing a more infiltrative growth pattern. Macrophage hotspots were often found at sites of infiltrative growth. Hauptmann et al. (35) have previously reported that 27E10-positive inflammatory macrophages were dominating at invasive areas and hypothesized that these macrophages could favor invasion. However, Hauptmann et al. did not assess prognosis in a clinical material. Given our results, an alternative possibility could be that a vigorous macrophage response (hotspot) at sites of ongoing invasion is an important feature of the protective action of macrophages that may delay or, in some cases, even prevent further tumor spread. It should also be noted that it was particularly at invasive sites, around small tumor nests, that a high macrophage to tumor cell ratio with efficient cell-to-cell contact was observed. The positive effect of massive infiltration was also reflected by the *in vitro* experiments allowing cell-to-cell contact, where colon cancer cell survival was reduced with increasing macrophage to cancer

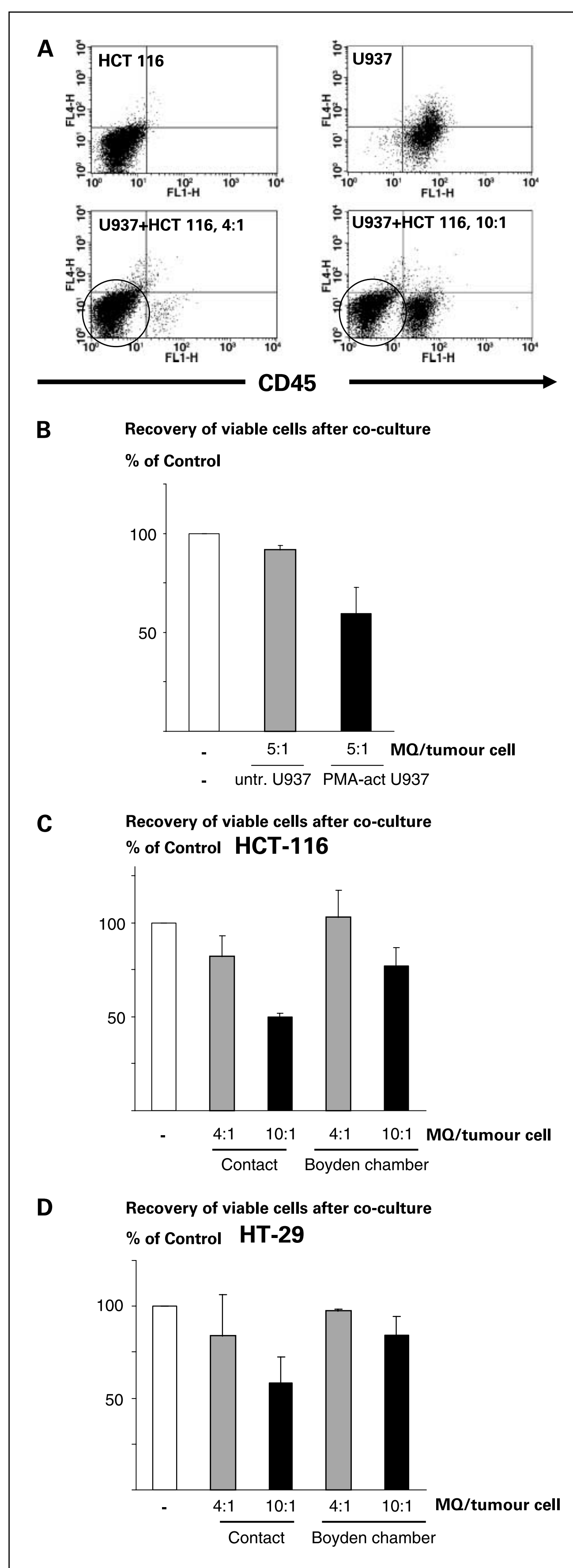

Fig. 3. PMA-activated U937 cells exert a cell-to-cell contact dependent growth inhibition on colon cancer cells *in vitro*. *A,* PMA-activated U937 cells were discriminated from tumor cells based on their expression of CD45. *B,* the number of viable cells recovered from wells with HCT-116 cells growing without U937 cells, overlaid with untreated U937 cells (5:1 macrophage to tumor cell ratio; *hatched column*), and overlaid with PMA-activated U937 cells (5:1 ratio; *black column*). The cell recovery is normalized to the control cells grown without macrophages (*white column*; set to 100%). Columns, mean calculated from two independent experiments; bars, SE. HCT-116 (*C*) or HT-29 (*D*) cells grown in 24-well plates without or with PMA-activated U937 cells in direct contact, or in Boyden chamber inserts, at 4:1 (*hatched columns*) and 10:1 (*black columns*) ratios. Columns, mean calculated from three independent experiments; bars, SE.

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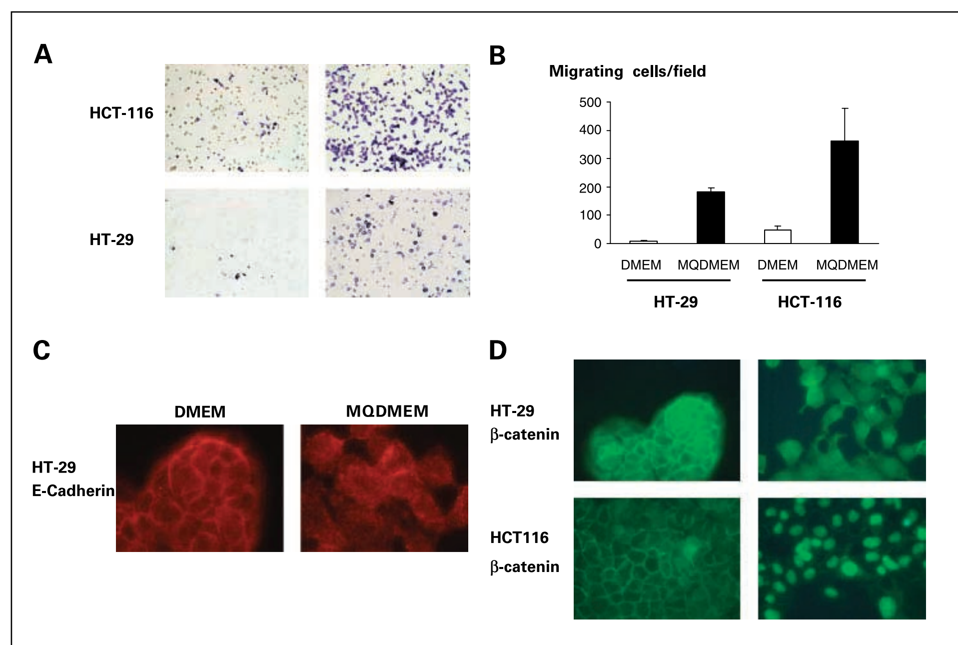

Fig. 4. Macrophage-conditioned medium promotes enhanced migration of colon cancer cells. *A,* colon cancer cells that adhered to the inside of a culture insert were allowed to migrate toward control medium (*left*) or macrophage-conditioned medium (*right*) for 20 h. *B,* average of migrating cells calculated from three fields per experiment. Columns, mean of two independent experiments; bars, SE. *C,* distribution of E-cadherin in HT-29 cells untreated or treated for 48 h with macrophages communicating through a 0.4-μm culture insert. *D,* nuclear translocation of β-catenin in HT-29 and HCT-116 cells treated for 48 h with macrophages communicating through a 0.4-μm culture insert.

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cell ratios. The highest cell ratios used in the coculture experiments corresponded roughly to those observed in hotspot grade 4. Thus, high macrophage infiltration is associated with improved prognosis in our clinical material, as well as in an animal model (34), and a high macrophage to tumor cell ratio inhibits cancer cell growth *in vitro*. Similarly, massive macrophage/monocyte infiltration was previously reported to be associated with tumor destruction, whereas a moderate infiltration resulted in growth of melanoma tumor xenografts (36).

Considering that we obtained a very strong correlation between lymphocytic and macrophage infiltration, lymphocytes seem to play an important role in the macrophage-promoted antitumorigenic defense. When staining a submaterial of 22 colorectal tumors, we found that $CD20^+$ cells (B cells), when present, mainly showed a follicular staining pattern distant from the tumor front, whereas $CD8^+$ cells, like $CD68^+$ cells, were localized along the tumor front.[4] High infiltration of $CD8^+$ T cells tended to follow high infiltration CD68.[4] Interestingly, whereas lymphocyte infiltration, as determined by routine H&E staining, was a strong prognostic marker in multivariate analysis when $CD68TF_{Hotspot}$ was not included as a parameter, its prognostic value was lost if $CD68TF_{Hotspot}$ was included. Combined with the finding that immune cell infiltrate was essentially absent in macrophage depleted colon tumor bearing rats (34), these results indicate that macrophages are essential for efficient recruitment of lymphocytes and that the interplay between macrophages and lymphocytes is of profound importance for an effective antitumor defense in colorectal cancer. The presence of high infiltration of both $CD8^+$ and $CD68^+$ cells has also been reported to positively influence prognosis in colon cancer patients (26), as well as the overall inflammatory cell reaction at the tumor front as evaluated in H&E-stained sections (29).

In conclusion, it is clear that monocytes/macrophages have protumorigenic as well as antitumorigenic properties in colon cancer. Assistance from T cells and direct macrophage-to-tumor cell contact may be required to manifest the antitumorigenic, or, alternatively, to counterbalance, the protumorigenic properties of macrophages in this cancer type. In addition, a high macrophage to cancer cell ratio increases the likelihood that the balance is shifted toward predominantly antitumorigenic properties, resulting in improved prognosis for colon cancer patients.

[4] Our unpublished observations.

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