Macrophage Infiltration in Tumor Stroma is Related to Tumor Cell Expression of CD163 in Colorectal Cancer

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Abstract The scavenger receptor, CD163, is a macrophage-specific marker. Recent studies have shown that CD163 expression in breast and rectal cancer cells is associated with poor prognosis. This study was conducted to evaluate the relationship between CD163 expression as a macrophage trait in cancer cells, and macrophage infiltration and its clinical significance in colorectal cancer. Immunostaining of CD163 and macrophage infiltration were evaluated in paraffin-embedded specimens, earlier analyzed for CD31, D2-40 and S-phase fraction, from primary tumors and normal colorectal mucosa of 75 patients with colorectal carcinoma. The

outcomes were analyzed in relation to clinical-pathological data. CD163 expression was positive in cancer cells in 20 % of colorectal cancer patients and was related to advanced tumor stages ($P=0.008$) and unfavorable prognosis ($p=0.001$). High macrophage infiltration was related to shorter survival and positive CD163 expression in tumor cells. The prognostic impact of macrophage infiltration was independent of tumor stage and CD163 expression in cancer cells ($p=0.034$). The expression of macrophage phenotype in colorectal cancer cells is associated with macrophage density in tumor stroma and lower survival rates. Macrophage infiltration has an independent prognostic impact on mortality in colorectal cancer. In accordance with previous experimental studies, these findings provide new insights into the role of macrophages in colorectal cancer.

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Introduction

Tumor microenvironment plays an important role in malignant disease and is influenced by several types of non-neoplastic cells, such as leukocytes and fibroblasts, in the tumor stroma. Monocytes are actively recruited to the tumor stroma, and tumor-associated macrophages (TAM) are common in the stromal compartment of several malignancies. Macrophages are derived from monocytes and show two polarization states in response to different micro-environmental signals [1]. M1 macrophages are pro-inflammatory and function as bactericidal and antigen-presenting cells. M2 macrophages have an immunosuppressive phenotype, stimulate Th2 cell differentiation and are activated by apoptotic cells and anti-inflammatory molecules such as IL-4, IL-13, and IL-10. TAMs represent the M2-type macrophages and play an

important role in tumor cell migration, invasion and metastasis. They also support angiogenesis and tissue repair [2, 3].

Experimental studies show that TAMs promote tumor progression, and high infiltration in many tumor types is correlated with lymph node involvement and distant metastasis [4].

The presence of macrophages directs tumours towards a histologically more malignant phenotype. Inhibition of macrophage infiltration in tumors may inhibit metastasis and progression of secondary tumors [5, 6]. The clinical significance of macrophage infiltration in tumor stroma, however, remains unclear. High infiltration of TAMs is correlated with poor prognosis in breast, prostatic, ovarian and cervical carcinoma [7]. In colorectal cancer, there are conflicting data on the clinical significance of macrophage infiltration, but several studies show that low macrophage density in tumor stroma is associated with an unfavorable prognosis [7–9].

Tumor growth results in local hypoxia, which in turn induces angiogenesis. Cancer cells release chemo-attractive factors, such as MCP-1 (monocyte chemoattractant protein-1) and RANTES (regulated upon activation, normal T-cell expressed and secreted), and actively recruit leukocytes to tumors [10, 11]. Once the bone marrow derived-cells (BMDC) arrive at the tumor site, they contribute to angiogenesis and tumor progression by expressing pro-angiogenic growth factors such as IL-1, VEGF, and IL-8 [7, 12]. Clinical studies have shown that increased infiltration of TAMs in solid tumors is associated with high micro-vessel density and poor prognosis. These data are particularly strong for hormone-dependent cancers, such as breast cancer. Macrophages may promote formation of lymphatic vascular networks in tumors, concomitant with neo-angiogenesis [13].

Cell fusion is a common and crucial biological process in mammalian development [14]. Heterotypic cell fusion between BMDCs and somatic cells, such as hepatocytes, cardiomyocytes and skeletal muscle cells, is reported in several studies [15, 16]. It has been proposed that cell fusion in cancer contributes to metastasis by producing hybrids with metastatic phenotypes when cancer cells spontaneously fuse with leukocytes that acquire genetic and phenotypic characteristics from both mother cells. From the cancer cells, they gain the functional ability to migrate from leukocytes and uncontrolled growth [17–21]. In an animal model with parabiosis, Powell and co-workers found evidence in vivo of fusion between macrophages and tumor epithelium during the course of tumorigenesis [22]. They could in later study provide evidence that transplanted hematopoietic cells incorporate into human intestinal epithelium through cell fusion [23]. Fusion events in human cancers, however, are difficult to detect in the clinical context due to the lack of safe tracing methods. Pawelek and co-workers reported evidence indicating fusion in two patients who developed renal-cell carcinoma after allogeneic bone-marrow transplantation from donors of the opposite gender [24, 25].

CD163 is a trans-membrane scavenger receptor for haptoglobin-hemoglobin complexes. It is specifically expressed by non-neoplastic monocytes/macrophages and by neoplasm's with monocytic/histiocytic differentiation, but not by other normal tissues [26]. CD163 is expressed in M2 macrophages [3]. Recent studies report that about 50 % of patients with breast cancer and 23 % with rectal cancer exhibit phenotypic macrophage traits by expressing CD163 antigen, which is associated with early distant recurrence and poor survival [27, 28]. It is suggested that macrophage traits in cancer cells might be explained by fusion between cancer cells and macrophages [29–33].

This is an exploratory study to primarily investigate TAM infiltration in relation to the presence of phenotypic macrophage traits, manifested by CD163 expression, in cancer cells and cancer cell proliferation in colorectal tumors. These factors are further analyzed in relation to variables related to angiogenesis and patient survival time.

Materials and Methods

Patient and Tissue Samples

Sections from formalin-fixed paraffin-embedded primary tumor tissue and adjacent normal mucosa were collected from 75 consecutive patients (46 colonic and 29 rectal cancer) operated during 1982–1986. All patients were diagnosed by conventional clinical methods and underwent surgical resection at the University Hospital in Linköping and Vrinnevi Hospital in Norrköping (Sweden). Ethical approval from the regional ethics committee in Linköping and patient consent to participate in research were obtained according to Swedish biobank law. The diagnosis of colorectal cancer was confirmed by routine histopathology. None of the patients received pre- or postoperative radiotherapy or chemotherapy since this was not a routine treatment during this period. The clinical-pathologic data were obtained from patient records.

Antibodies and Immune-Staining of Tissue Sections

Mouse anti-human monoclonal antibody (IgG1) against CD163 (clone 10D6 from Novocastra, England) was used as a macrophage marker. Lymphatic and blood vessels are examined by using mouse monoclonal D2-40 antibody (Abcam, Cambridge, UK) and mouse monoclonal anti-CD31 antibody (Dako, Denmark), respectively. These antibodies are well characterized and have been used in many previous studies [27, 34–39]. Serial sections of 5 µm were cut from formalin-fixed paraffin-embedded tissue blocks, deparaffinized in xylene and hydrated in a series of graded alcohols (100 %, 95 %, and 70 %). The sections were

stained with CD163, CD31 and D2-40 antibodies according to methods used in our previous studies [27, 40, 41, 28].

Evaluation of Histology and Immunostaining

Histology and immunostaining were evaluated independently by three of the authors (IS, RE and HO). Observer agreement was measured according to Cohen κ [42]. Positive staining of tissue macrophages was considered a positive internal control staining for CD163; as negative controls, the primary antibodies were replaced by an irrelevant isotype anti-mouse IgG1. The positive expression of CD163 was defined as granular cytoplasmic or cytoplasmic and membrane-staining pattern. The proportion of cancer cells staining for CD163 was scored in five grades: 0 %, 1–25 %, 26–50 %, 51–75 % and 56–100 %. TAMs and cancer cells could be distinguished on morphological grounds. TAMs nuclei were small and regular, whereas the cancer cells were atypical with pleomorphic and enlarged nuclei (Fig. 1). A tumor was evaluated as positive if it contained any positive cancer cells; otherwise it was considered negative. Inter-observer variation calculated as Cohen kappa index [42] was $\kappa=0.699$.

Microvessel density (MVD) was assessed by counting the vessels immunostained for D2-40 or CD31 antigen under light microscopy according to the first international consensus on the methodology, criteria of evaluation and quantification of angiogenesis in solid human tumors [43]. Briefly, the D2-40 or CD31 stained sections were initially scanned at $\times 2$ and $\times 10$ magnification and the areas with the highest number of vessels stained by CD31 or D2-40 (hot spots) were selected. Vessels were counted in three fields of the hot spots at $\times 40$ magnification, and the mean value of the three fields for each case was used. S-phase fraction was estimated according to our previous studies [44, 45]. For prognostic purposes, the median value of 8 % was used as a cut-off for further analysis.

S-phase Analysis

S-phase analysis was performed according to a protocol reported in previous study [45]. Briefly, 50 μm thick tumor section were deparaffinized with xylene and rehydrated stepwise with ethanol. The samples were treated with 0.4 % trypsin (Sigma) in a citrate buffer for 24 h before filtration through a nylon mesh and staining of the cell suspension with propidium iodide. The cell suspensions were analyzed with a FACScan flow cytometer (Becton-Dickinson, USA). Normal diploid cells from the same specimens were used as internal controls. Tumours were defined as diploid if a single G0/1 peak was obtained, and as non-diploid if more than one distinct G0/1 peak occurred. The number of S-phase cells was calculated by multiplying the number of channels between the G0/1 and G2/M peaks by the mean number of cells

Fig. 1 Expression of macrophage-specific antigen CD163 in colorectal cancer. Immunostaining shows (a) normal colon mucosa with no CD163 expression in epithelial cells. (b) Positive CD163 expression in cancer cells is manifested as cytoplasmic and membrane staining. (c) Tumor with negative CD163 expression in cancer cells. Non-neoplastic cells staining for CD163 demonstrate tumor-associated macrophages

per channel in S-phase interval. The mean value of S-phase was 8 % and was used as cut-off in later statistical analysis.

Evaluation of Macrophage Infiltration

CD163 is a macrophage-specific antigen expressed mainly by M2 macrophages. Non-neoplastic cells stained with CD163 were estimated as TAMs. To avoid overestimation of the number of TAMs which could have been due to extended cytoplasmic ramifications, we counted only cells with a visible nucleus. The infiltration of TAM in tumor stroma was evaluated over the whole section. The level of macrophage infiltration was assessed in four grades: no/weak (grade I), moderate (grade II), strong (grade III) and massive (grade IV) (Fig. 2). For further analysis, TAM infiltration was scored as low (grad I and II) and high (grade III and IV).

Statistical Analysis

Statistical analyses were performed using SPSS statistics version 19 (IBM, USA). Pearson Chi-Square was used to evaluate the relationship between CD163 expression and macrophage infiltration in relation to patient clinical data and tumor characteristics. Survival rates were estimated using Kaplan-Meier method and based on disease (CRC) specific mortality. Cox regression analysis was used to estimate the prognostic value of CD163 expression and macrophage infiltration in relation to disease-specific survival. P value less than 5 % was considered as statistically significant.

Results

CD163 expression by tumor cells and macrophage infiltration were examined in surgical specimens, including primary tumor ($n=75$), adjacent normal mucosa ($n=75$) and distal normal mucosa ($n=15$) from a total of 75 patients with colorectal cancer. The majority (86 %) of the patients was 60 years or older, and 53 % were male. The cancer cells expressed CD163 in 20 % ($n=9/46$) of the cases with colon cancer and 17 % ($n=5/29$) with rectal cancer (Table 1). Epithelial cells in normal mucosa adjacent to tumor margin and distal mucosal specimens did not show any CD163 expression.

High macrophage infiltration was significantly more frequent in CD163-positive tumors than in negative tumors ($p=0.018$). Macrophage infiltration was high in 6 (43 %) of 14 tumors expressing CD163 in cancer cells. In contrast, macrophage infiltration was high in 8 (15 %) of 53 tumors with negative CD163 expression (Fig. 3).

CD163 expression occurred mainly in advanced stages of colorectal cancer ($p=0.008$). Of 14 patients with positive CD163 expression, 13 (93 %) had colorectal cancer in stage III–IV. Thirty-six patients (59 %) with negative CD163 tumors had tumor stage I–II. In 15 patients (20 %), the tumors exhibited high macrophage infiltration. The infiltration level of TAMs was not statistically correlated with the tumor stage. The proportions of patients with high and low macrophage density were nearly even in the respective tumor stages. Gender, age, tumor localization and growth pattern were associated with neither CD163 expression in cancer cells nor macrophage infiltration (Table 1).

Fig. 2 Infiltration of tumor-associated macrophages (TAM) in colorectal cancer. TAM are stained with macrophage-specific antigen CD163. The sections show immunostaining representing different grades of macrophage infiltration; (a) no/low, (b) moderate, (c) high and (d) massive

Table 1 CD163 expression and macrophage infiltration in relation to clinical and biologic data

	CD163 expression in colorectal cancer cells			Macrophage infiltration with CD163 as a macrophage marker		
	Negative No. of Patients (%)	Positive No. of Patients (%)	<i>P</i> value	Low No. of Patients (%)	High No. of Patients (%)	<i>P</i> value
Gender						
Male	32 (80)	8 (20)	0.80	31 (78)	9 (22)	0.56
Female	29 (83)	6 (17)		29 (83)	6 (17)	
Age (year)						
<=59	9 (82)	2 (18)	1.0	9 (82)	2 (18)	0.98
60-69	16 (89)	2 (11)		14 (78)	4 (22)	
70-79	23 (79)	6 (21)		23 (79)	6 (21)	
>=80	13 (77)	4 (23)		14 (82)	3 (18)	
Localisation						
Colon	37 (80)	9 (20)	0.80	36 (78)	10 (22)	0.90
Rectum	24 (79)	5 (21)		24 (83)	5 (17)	
Stage						
I	8 (100)	0 (0)	0.008	8 (100)	0 (0)	0.16
II	27 (96)	1 (4)		22 (79)	6 (21)	
III	18 (69)	8 (31)		22 (85)	4 (15)	
IV	8 (61)	5 (39)		8 (61)	5 (39)	
Growth pattern						
Expansive	24 (86)	4 (14)	0.40	24 (86)	4 (14)	0.30
Infiltrative	35 (78)	10 (22)		34 (76)	11 (24)	
S-phase						
<8 %	30 (94)	2 (6)	0.032	30 (94)	2 (6)	0.006
>=8 %	22 (73)	8 (27)		20 (67)	10 (33)	
CD31						
<median	21 (81)	5 (19)	0.209	18 (69)	8 (31)	0.377
>median	24 (92)	2 (8)		20 (77)	6 (23)	
D2-40						
<median	22 (85)	4 (15)	0.500	19 (73)	7 (27)	0.622
>median	23 (88)	3 (12)		19 (73)	7 (27)

Fig. 3 Macrophage infiltration in colorectal tumor stroma in relation to CD163 expression in cancer cells. CD163 expression was significantly more frequent in tumors with high macrophage infiltration ($P=0.018$)

CD163 expression, macrophage infiltration and angiogenesis

MVD in tumor tissue was estimated by using CD31 as a blood vessel marker. D2-40 was used to stain lymphatic vessels and determine LVD. For CD31, mean MVD in all tumors was 124 microvessels per x200 field (range 13–372, $SD \pm 67$), whereas mean LVD was 17 lymphatic microvessels per x200 field (range 0–127, $SD \pm 18$). MVD and LVD were correlated neither with CD163 expression in colorectal cancer cells nor macrophage infiltration in tumor stroma (Table 1).

Macrophage Infiltration and CD163 Expression are Associated with Cancer Cell Proliferation

The proliferation activity in cancer cells was determined by S-phase fraction. S-phase fraction exceeded 8 % in 8 of 10 patients with positive CD163 tumors, which is significantly more frequent compared with the CD163 negative tumors

($p=0.032$). Out of 12 tumors with high macrophage density, 10 (83 %) exhibit an S-phase fraction exceeding 8 % ($P=0.006$) (Table 1).

CD163 Expression, Macrophage Infiltration and Survival Time

The mean survival time in all patients was 63 months (SD ± 45). The survival time was significantly shorter in the patients with tumors expressing CD163 in cancer cells (Fig. 4a). The mean survival time for the patients with positive

CD163 tumor was 34 months (SD ± 45) with 1- and 5-years survival rates of 70 % and 21 %, respectively. Patients with negative CD163 tumors had a mean survival time of 70 months (SD ± 39) and 1- and 5-years survival rates of 90 % and 64 %, respectively.

High macrophage infiltration was also associated with significantly shorter survival time (Fig. 4b). The mean survival time in patients with high macrophage infiltration was 43 months (SD ± 40) and with low macrophage infiltration, 69 months (SD ± 46). Survival rates of 1- and 5-years in patients with high macrophage infiltration were 81 % and 38 %, respectively. The corresponding rates in the patients with low macrophage infiltration were 88 % and 61 %.

In an attempt to predict the prognostic value of CD163 and macrophage infiltration, Cox regression analysis was performed in relation to tumor stage. Regardless of tumor stage and CD163 expression, high macrophage infiltration was significantly related to poor survival ($P=0.034$, Table 2).

Discussion

The relationships between expression of CD163 in colorectal cancer cells, macrophage infiltration in tumor stroma and clinical outcomes were evaluated in 75 patients with colorectal cancer. CD163 expression was associated with advanced tumor stage, poor survival and macrophage infiltration in tumor stroma. Both macrophage infiltration and CD163 expression were significantly correlated to higher proliferation rate of cancer cells.

Cells belonging to the myeloid lineage are the major component of leukocyte infiltration in solid tumors. TAMs have complex dual functions in their interaction with neoplastic cells and are part of an inflammatory process that promotes tumor progression [46, 47]. The primary tumor can regulate the function of TAMs, including their tumor cell

Fig. 4 Kaplan-Meier survival curves for 75 patients with colorectal cancer. Patients with positive CD163 expression (**a**) and high macrophage infiltration (**b**) have lower survival time. The survival analysis is based on disease (CRC) specific mortality, and overall comparison is estimated according to Log Rank (Mantel-Cox) analysis

Table 2 Multivariate analysis of mortality in colorectal cancer in relation to CD163 expression, macrophage infiltration and tumor stage estimated according to Cox proportional hazard analysis

	Colorectal cancer death Hazard ratio (95 % C.I.)	Test for significance
CD163 expression		
Negative	1.0	
Positive	1.6 (0.7-4.0)	$P=0.211$
Macrophage infiltration		
Low	1.0	
High	2.5 (1.0-6.0)	$P=0.034$
Tumor stage		
Stage 1-2	1.0	
Stage 3-4	3.5 (1.5-8)	$P=0.004$

cytotoxicity. The clinical and pathophysiological significance of macrophage density in colorectal cancer is a controversial issue. The contradictory outcomes in clinical studies may be due to the use of different methods to evaluate macrophage infiltration and the identification of TAMs with different and less macrophage specific markers. CD163 is a macrophage-specific marker and is highly expressed by TAMs [48]. In the present study, macrophage density in tumor stroma was statistically related to poor survival. However, the present study is retrospective and the number of patients ($n=75$) included is limited, why these data can not be generalized. Studies with larger numbers of patients and specific macrophage markers, like CD163, are necessary to address this issue.

Interaction between neoplastic cells and the tumor microenvironment is crucial for tumorigenesis. In vitro co-culture of macrophages with tumor cells has been shown to result in accelerated tumor growth [49]. TAMs express several factors, such as EGF, MMP-9, TGF- β and CXCL-14, that stimulate tumor cell proliferation and survival [50, 51]. However, it is unclear whether macrophage density in tumor stroma is directly linked to tumor demography. In this study, macrophage infiltration was quantified in four degrees and shows that higher proliferation rates in cancer cells, estimated as S-phase fraction, are positively correlated ($p=0.006$) with increased macrophage density in the tumor stroma. These data are consistent with experimental in vitro studies suggesting macrophage promotion of tumor growth. To our knowledge this is the first report that shows correlation between macrophage density tumor stroma and cancer cell proliferation in human colorectal cancer samples.

It has previously been shown that macrophage traits, manifested as CD163 expression, in breast and rectal cancer cells are related to early distant recurrence and reduced patient survival [52, 27]. These observations are substantiated in the present study. Cell fusion as a biological process is consistent with the existing knowledge of macrophage functions. Macrophages fuse to become osteoclasts in bone tissue and giant cells in chronic inflammation. In vitro fusion between macrophages and malignant cells generates hybrids with enhanced metastatic ability [53, 29, 54, 55]. Heterotypic cell fusion between BMDCs and somatic cells has been demonstrated in several studies [56, 15, 57, 16] and might be a source of multiple tumor types [58]. In an experimental model of parabiosis, Powell and coworkers recently showed in vivo evidence of fusion between BMDCs and intestinal tumor cells. The most significant finding in this study is that in vivo-generated macrophage-tumor cell hybrids gain macrophage properties involved in tumor metastasis such as extravasation, migration, and immune evasion [59]. Silk et al. reported convincing data showing fusion between hematopoietic cells and epithelial cells in humans. Fusion between myeloid cells and tumor cells is a source for macrophage traits in cancer cells [32, 22]. In this study the macrophage-specific antigen, CD163, is expressed by cancer cells in about 20 % of patients

with colorectal cancer. CD163 expression is more common in advanced tumor stages, lower survival time and significantly associated with tumor cell proliferation with higher S-phase fraction ($p=0.032$). These results do not provide direct evidence for cell fusion theory, but is consistent with previous reports [60, 20, 55, 53, 61, 23], CD163 expression in cancer cells might be due macrophage-cancer cell fusion and contributes to tumor progression.

Conclusions

To our knowledge, this is the first study that shows macrophage traits in the colorectal cancer cells, manifested as CD163 expression, is correlated to high macrophage density in the tumor stroma. Both macrophage infiltration and CD163 expression in cancer cells are associated with higher cancer cell proliferation rates. This report is a hypothesis generating study that provide new insight on the role of macrophages in the tumor microenvironment and contributes to a better understanding of its pathophysiology in tumor progression.

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Competing of interest All authors have no conflicts of interest.

References

1. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A (2002) Macrophage Polarization: Tumor-Associated Macrophages as a Paradigm for Polarized M2 Mononuclear Phagocytes. *Trends Immunol* 23(11):549–555
2. Lin EY, Pollard JW (2007) Tumor-Associated Macrophages Press the Angiogenic Switch in Breast Cancer. *Cancer Res* 67(11):5064–5066
3. Sica A, Schioppa T, Mantovani A, Allavena P (2006) Tumour-Associated Macrophages are a Distinct M2 Polarised Population Promoting Tumour Progression: Potential Targets of Anti-Cancer Therapy. *Eur J Cancer* 42(6):717–727
4. Cecconi O, Calorini L, Mannini A, Mugnai G, Ruggieri S (1997) Enhancement of Lung-Colonizing Potential of Murine Tumor Cell Lines co-Cultivated With Activated Macrophages. *Clin Exp Metastasis* 15(2):94–101
5. Oosterling SJ, van der Bij GJ, Meijer GA, Tuk CW, van Garderen E, van Rooijen N, Meijer S, van der Sijp JR, Beelen RH, van Egmond M (2005) Macrophages Direct Tumour Histology and Clinical Outcome in a Colon Cancer Model. *J Pathol* 207(2):147–155. doi: 10.1002/path.1830
6. Miselis NR, Wu ZJ, Van Rooijen N, Kane AB (2008) Targeting Tumor-Associated Macrophages in an Orthotopic Murine Model of Diffuse Malignant Mesothelioma. *Mol Cancer Ther* 4:788–799. doi: 10.1158/1535-7163.MCT-07-0579

7. Bingle L, Brown NJ, Lewis CE (2002) The Role of Tumour-Associated Macrophages in Tumour Progression: Implications for new Anticancer Therapies. *J Pathol* 196(3):254–265. doi:10.1002/path.1027 [pii]
8. Tan SY, Fan Y, Luo HS, Shen ZX, Guo Y, Zhao LJ (2005) Prognostic Significance of Cell Infiltrations of Immunosurveillance in Colorectal Cancer. *World J Gastroenterol* 11(8):1210–1214
9. Lackner C, Jukic Z, Tsybrovskyy O, Jatzko G, Wette V, Hoefler G, Klimpfner M, Denk H, Zatloukal K (2004) Prognostic Relevance of Tumour-Associated Macrophages and von Willebrand Factor-Positive Microvessels in Colorectal Cancer. *Virchows Arch* 445(2): 160–167. doi:10.1007/s00428-004-1051-z
10. Dvorak HF (1986) Tumors: Wounds That do not heal Similarities Between Tumor Stroma Generation and Wound Healing. *N Engl J Med* 315(26):1650–1659
11. Murdoch C, Lewis CE (2005) Macrophage Migration and Gene Expression in Response to Tumor Hypoxia. *Int j of cancer J int du cancer* 117(5):701–708. doi:10.1002/ijc.21422
12. Chen JJ, Yao PL, Yuan A, Hong TM, Shun CT, Kuo ML, Lee YC, Yang PC (2003) Up-Regulation of Tumor Interleukin-8 Expression by Infiltrating Macrophages: Its Correlation With Tumor Angiogenesis and Patient Survival in non-Small Cell Lung Cancer. *Clin Cancer Res* 9(2):729–737
13. Sundar SS, Ganesan TS (2007) Role of Lymphangiogenesis in Cancer. *J Clin Oncol* 25(27):4298–4307. doi:10.1200/JCO.2006.07.1092
14. Vassilopoulos G, Russell DW (2003) Cell Fusion: An Alternative to Stem Cell Plasticity and its Therapeutic Implications. *Curr Opin Genet Dev* 13(5):480–485
15. Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A (2003) Fusion of Bone-Marrow-Derived Cells With Purkinje Neurons, Cardiomyocytes and Hepatocytes. *Nature* 425(6961):968–973
16. Nygren JM, Jovinge S, Breitbach M, Sawen P, Roll W, Hescheler J, Taneera J, Fleischmann BK, Jacobsen SE (2004) Bone Marrow-Derived Hematopoietic Cells Generate Cardiomyocytes at a low Frequency Through Cell Fusion, but not Transdifferentiation. *Nat Med* 10(5):494–501
17. Chakraborty AK, de Freitas SJ, Espreafico EM, Pawelek JM (2001) Human Monocyte x Mouse Melanoma Fusion Hybrids Express Human Gene. *Gene* 275(1):103–106
18. Chakraborty AK, Pawelek J (2007) Beta1,6-Branched Oligosaccharides Regulate Melanin Content and Motility in Macrophage-Melanoma Fusion Hybrids. *Melanoma Res* 17(1):9–16
19. Chakraborty AK, Sodi S, Rachkovsky M, Kolesnikova N, Platt JT, Bologna JL, Pawelek JM (2000) A Spontaneous Murine Melanoma Lung Metastasis Comprised of Host x Tumor Hybrids. *Cancer Res* 60(9):2512–2519
20. Pawelek JM (2008) Cancer-Cell Fusion With Migratory Bone-Marrow-Derived Cells as an Explanation for Metastasis: new Therapeutic Paradigms. *Future Oncol* 4(4):449–452
21. Pawelek JM, Chakraborty AK (2008) Fusion of Tumour Cells With Bone Marrow-Derived Cells: A Unifying Explanation for Metastasis. *Nat Rev Cancer* 8(5):377–386
22. Powell AE, Anderson EC, Davies PS, Silk AD, Pelz C, Impey S, Wong MH (2011) Fusion between Intestinal epithelial cells and macrophages in a cancer context results in nuclear reprogramming. *Cancer Res* 71 (4):1497–1505. doi:0008–5472.CAN-10-3223 [pii]10.1158/0008-5472.CAN-10-3223
23. Silk AD, Gast CE, Davies PS, Fakhari FD, Vanderbeek GE, Mori M, Wong MH (2013) Fusion Between Hematopoietic and Epithelial Cells in Adult Human Intestine. *PLoS One* 8(1):e55572. doi:10.1371/journal.pone.0055572
24. Chakraborty A, Lazova R, Davies S, Backvall H, Ponten F, Brash D, Pawelek J (2004) Donor DNA in a Renal Cell Carcinoma Metastasis from a Bone Marrow Transplant Recipient. *Bone Marrow Transplant* 34(2):183–186. doi:10.1038/sj.bmt.17045471704547 [pii]
25. Yilmaz Y, Lazova R, Qumsiyeh M, Cooper D, Pawelek J (2005) Donor Y Chromosome in Renal Carcinoma Cells of a Female BMT Recipient: Visualization of Putative BMT-Tumor Hybrids by FISH. *Bone Marrow Transplant* 35(10):1021–1024
26. Nguyen TT, Schwartz EJ, West RB, Warnke RA, Arber DA, Natkunam Y (2005) Expression of CD163 (Hemoglobin Scavenger Receptor) in Normal Tissues, Lymphomas, Carcinomas, and Sarcomas is Largely Restricted to the Monocyte/Macrophage Lineage. *Am J Surg Pathol* 29(5):617–624
27. Shabo I, Stal O, Olsson H, Dore S, Svanvik J (2008) Breast Cancer Expression of CD163, a Macrophage Scavenger Receptor, is Related to Early Distant Recurrence and Reduced Patient Survival. *Int j of cancer J int du cancer* 123(4):780–786
28. Shabo I, Olsson H, Sun XF, Svanvik J (2009) Expression of the Macrophage Antigen CD163 in Rectal Cancer Cells is Associated With Early Local Recurrence and Reduced Survival Time. *Int j of cancer J int du cancer*. doi:10.1002/ijc.24506
29. Larizza L, Schirmacher V, Pfluger E (1984) Acquisition of High Metastatic Capacity After in Vitro Fusion of a Nonmetastatic Tumor Line With a Bone Marrow-Derived Macrophage. *J Exp Med* 160(5): 1579–1584
30. Larsson LI, Bjerregaard B, Wulf-Andersen L, Talts JF (2007) Syncytin and Cancer Cell Fusions. *ScientificWorldJournal* 7:1193–1197. doi:10.1100/tsw.2007.212
31. Pawelek J, Chakraborty A, Lazova R, Yilmaz Y, Cooper D, Brash D, Handerson T (2006) Co-Opting Macrophage Traits in Cancer Progression: A Consequence of Tumor Cell Fusion? *Contrib Microbiol* 13:138–155
32. Pawelek JM (2005) Tumour-Cell Fusion as a Source of Myeloid Traits in Cancer. *Lancet Oncol* 6(12):988–993
33. Chakraborty AK, Pawelek J, Ikeda Y, Miyoshi E, Kolesnikova N, Funasaka Y, Ichihashi M, Taniguchi N (2001) Fusion Hybrids With Macrophage and Melanoma Cells up-Regulate N-Acetylglucosaminyltransferase V, beta1-6 Branching, and Metastasis. *Cell Growth Differ* 12(12):623–630
34. Droste A, Sorg C, Hogger P (1999) Shedding of CD163, a Novel Regulatory Mechanism for a Member of the Scavenger Receptor Cysteine-Rich Family. *Biochem Biophys Res Commun* 256(1): 110–113
35. Hogger P, Dreier J, Droste A, Buck F, Sorg C (1998) Identification of the Integral Membrane Protein RM3/1 on Human Monocytes as a Glucocorticoid-Inducible Member of the Scavenger Receptor Cysteine-Rich Family (CD163). *J Immunol* 161(4):1883–1890
36. Schluter C, Duchrow M, Wohlenberg C, Becker MH, Key G, Flad HD, Gerdes J (1993) The Cell Proliferation-Associated Antigen of Antibody Ki-67: A Very Large, Ubiquitous Nuclear Protein With Numerous Repeated Elements, Representing a new Kind of Cell Cycle-Maintaining Proteins. *J Cell Biol* 123(3): 513–522
37. Li SH, Li CF, Sung MT, Eng HL, Hsiung CY, Huang WW, Lin CN, Yu SC, Huang HY (2007) Skp2 is an Independent Prognosticator of Gallbladder Carcinoma Among p27 (Kip1)-Interacting Cell Cycle Regulators: An Immunohistochemical Study of 62 Cases by Tissue Microarray. *Mod Pathol* 20(4):497–507. doi:10.1038/modpathol.3800762
38. Roy S, Chu A, Trojanowski JQ, Zhang PJ (2005) D2-40, a Novel Monoclonal Antibody Against the M2A Antigen as a Marker to Distinguish Hemangioblastomas from Renal Cell Carcinomas. *Acta Neuropathol* 109(5):497–502. doi:10.1007/s00401-005-0999-3
39. Sharma S, Sharma MC, Sarkar C (2005) Morphology of Angiogenesis in Human Cancer: A Conceptual Overview, Histoprostic Perspective and Significance of Neoangiogenesis. *Histopathology* 46(5):481–489. doi:10.1111/j.1365-2559.2005.02142.x
40. Adell G, Zhang H, Jansson A, Sun XF, Stal O, Nordenskjöld B (2001) Decreased Tumor Cell Proliferation as an Indicator of the

- Effect of Preoperative Radiotherapy of Rectal Cancer. *Int J Radiat Oncol Biol Phys* 50(3):659–663
41. Gao J, Knutsen A, Arbmán G, Carstensen J, Franlund B, Sun XF (2009) Clinical and Biological Significance of Angiogenesis and Lymphangiogenesis in Colorectal Cancer. *Dig Liver Dis* 41(2):116–122. doi:10.1016/j.dld.2008.07.315
 42. Kundel HL, Polansky M (2003) Measurement of Observer Agreement. *Radiology* 228(2):303–308
 43. Vermeulen PB, Gasparini G, Fox SB, Toi M, Martin L, McCulloch P, Pezzella F, Viale G, Weidner N, Harris AL, Dirix LY (1996) Quantification of Angiogenesis in Solid Human Tumours: An International Consensus on the Methodology and Criteria of Evaluation. *Eur J Cancer* 32A(14):2474–2484
 44. Jansson A, Sun XF (1997) Ki-67 Expression in Relation to Clinicopathological Variables and Prognosis in Colorectal Adenocarcinomas. *APMIS* 105(9):730–734
 45. Sun XF, Carstensen JM, Stal O, Zhang H, Nilsson E, Sjødahl R, Nordenskjöld B (1993) Prognostic Significance of p53 Expression in Relation to DNA Ploidy in Colorectal Adenocarcinoma. *Virchows Arch A Pathol Anat Histopathol* 423(6):443–448
 46. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M (2013) Macrophage Plasticity and Polarization in Tissue Repair and Remodelling. *J Pathol* 229(2):176–185. doi:10.1002/path.4133
 47. Riboldi E, Porta C, Morlacchi S, Viola A, Mantovani A, Sica A (2013) Hypoxia-Mediated Regulation of Macrophage Functions in Pathophysiology. *Int Immunol* 25(2):67–75. doi:10.1093/intimm/dxs110
 48. Alexander P, Eccles SA, Gauci CL (1976) The Significance of Macrophages in Human and Experimental Tumors. *Ann N Y Acad Sci* 276:124–133
 49. Hagemann T, Robinson SC, Schulz M, Trumper L, Balkwill FR, Binder C (2004) Enhanced Invasiveness of Breast Cancer Cell Lines Upon co-Cultivation With Macrophages is due to TNF-Alpha Dependent up-Regulation of Matrix Metalloproteases. *Carcinogenesis* 25(8):1543–1549. doi:10.1093/carcin/bgh146bgh146 [pii]
 50. Leber TM, Balkwill FR (1998) Regulation of Monocyte MMP-9 Production by TNF-Alpha and a Tumour-Derived Soluble Factor (MMP-9). *Br J Cancer* 78(6):724–732
 51. Augsten M, Hagglof C, Olsson E, Stolz C, Tsagozis P, Levchenko T, Frederick MJ, Borg A, Micke P, Egevad L, Ostman A (2009) CXCL14 is an Autocrine Growth Factor for Fibroblasts and Acts as a Multi-Modal Stimulator of Prostate Tumor Growth. *Proc Natl Acad Sci U S A* 106(9):3414–3419. doi:10.1073/pnas.0813144106
 52. Shabo IOH, Sun XF, Svanvik J (2009) Expression of the Macrophage Antigen CD163 in Rectal Cancer Cells is Associated With Early Local Recurrence and Reduced Survival Time. *Int J Cancer* Accepted in Mars 2009
 53. Busund LT, Killie MK, Bartnes K, Seljelid R (2003) Spontaneously Formed Tumorigenic Hybrids of Meth A Sarcoma Cells and Macrophages in Vivo. *Int j of cancer J int du cancer* 106(2):153–159
 54. Rachkovsky M, Sodi S, Chakraborty A, Avissar Y, Bolognia J, McNiff JM, Platt J, Bermudes D, Pawelek J (1998) Melanoma x Macrophage Hybrids With Enhanced Metastatic Potential. *Clin Exp Metastasis* 16(4):299–312
 55. Busund LT, Killie MK, Bartnes K, Seljelid R (2002) Spontaneously Formed Tumorigenic Hybrids of Meth A Sarcoma and Macrophages Grow Faster and are Better Vascularized Than the Parental Tumor. *Int j of cancer J int du cancer* 100(4):407–413
 56. Nygren JM, Liuba K, Breitbach M, Stott S, Thoren L, Roell W, Geisen C, Sasse P, Kirik D, Bjorklund A, Nerlov C, Fleischmann BK, Jovinge S, Jacobsen SE (2008) Myeloid and Lymphoid Contribution to non-Haematopoietic Lineages Through Irradiation-Induced Heterotypic Cell Fusion. *Nat Cell Biol* 10(5):584–592
 57. Johansson CB, Youssef S, Kolekar K, Holbrook C, Doyonnas R, Corbel SY, Steinman L, Rossi FM, Blau HM (2008) Extensive Fusion of Haematopoietic Cells With Purkinje Neurons in Response to Chronic Inflammation. *Nat Cell Biol* 10(5):575–583. doi:10.1038/ncb1720
 58. Liu C, Chen Z, Zhang T, Lu Y (2006) Multiple Tumor Types may Originate from Bone Marrow-Derived Cells. *Neoplasia* 8(9):716–724
 59. Powell AE, Anderson EC, Davies PS, Silk AD, Pelz C, Impey S, Wong MH Fusion between Intestinal Epithelial Cells and Macrophages in a Cancer Context Results in Nuclear Reprogramming. *Cancer Res* 71(4):1497–1505. doi:10.1158/0008-5472.CAN-10-3223
 60. Pawelek JM, Chakraborty AK (2008) The Cancer Cell–Leukocyte Fusion Theory of Metastasis. *Adv Cancer Res* 101:397–444. doi:10.1016/S0065-230X(08)00410-7
 61. Houghton J, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H, Cai X, Fox JG, Goldenring JR, Wang TC (2004) Gastric Cancer Originating from Bone Marrow-Derived Cells. *Science* 306(5701):1568–1571. doi:10.1126/science.1099513