High Myeloperoxidase Positive Cell Infiltration in Colorectal Cancer Is an Independent Favorable Prognostic Factor

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Abstract

Background: Colorectal cancer (CRC) infiltration by adaptive immune system cells correlates with favorable prognosis. The role of the innate immune system is still debated. Here we addressed the prognostic impact of CRC infiltration by neutrophil granulocytes (NG).

Methods: A TMA including healthy mucosa and clinically annotated CRC specimens (n = 1491) was stained with MPO and CD15 specific antibodies. MPO+ and CD15+ positive immune cells were counted by three independent observers. Phenotypic profiles of CRC infiltrating MPO+ and CD15+ cells were validated by flow cytometry on cell suspensions derived from enzymatically digested surgical specimens. Survival analysis was performed by splitting randomized data in training and validation subsets.

Results: MPO+ and CD15+ cell infiltration were significantly correlated (p<0.0001; r = 0.76). However, only high density of MPO+ cell infiltration was associated with significantly improved survival in training ($P = 0.038$) and validation ($P = 0.002$) sets. In multivariate analysis including T and N stage, vascular invasion, tumor border configuration and microsatellite instability status, MPO+ cell infiltration proved an independent prognostic marker overall ($P = 0.004$; HR = 0.65; CI:±0.15) and in both training ($P = 0.048$) and validation ($P = 0.036$) sets. Flow-cytometry analysis of CRC cell suspensions derived from clinical specimens showed that while MPO+ cells were largely CD15+/CD66b+, sizeable percentages of CD15+ and CD66b+ cells were MPO−.

Conclusions: High density MPO+ cell infiltration is a novel independent favorable prognostic factor in CRC.

Introduction

Outgrowth and progression of human colorectal cancers (CRC) are driven by gene mutations and microsatellite instability tumor inherent characteristics [1,2], and by the interaction of cancer cells with microenvironmental stimuli provided by non-transformed cells [3,4]. In particular, cytokine and chemokine environment and infiltration by immunocompetent cells significantly influence CRC outcome [5–8].

Infiltration by activated CD8+ memory T cells and expression of IFN-γ gene within CRC were convincingly shown to be associated with favorable prognosis [5,7]. Furthermore, we and others have shown that FOXP3+ immune cell infiltration independently predicts improved survival in CRC [9,10].

The role of innate immune system cells was not studied in comparable detail and controversial data were reported regarding CRC infiltration by NK cells [11–14] and macrophages [15–17].

Granulocytes have largely been disregarded by tumor immunologists [18]. However, recent studies, mainly performed in

experimental models, suggest that neutrophil granulocytes might prevent metastatic cancer progression [19]. Furthermore, they were suggested to undergo cytokine driven differentiation into N1 and N2 cells endowed with anti- and pro-tumor properties, respectively [20,21]. These findings have led to a resurgent interest in granulocyte infiltration in cancer [22–24].

In previous work, we showed that CRC infiltration by CD33+/HLA-DR−/CD16+ myeloid cells is associated with improved patient survival [13]. Based on these phenotypic features, we hypothesized that CRC could be infiltrated by granulocytes with a favorable prognostic significance.

Myeloperoxidase (MPO) is a lysosomal enzyme produced in high amounts by neutrophilic granulocytes (NG) [25], especially during their early maturation phase. MPO catalyzes the production of hypochlorous acid from hydrogen peroxide and chloride anion and oxidizes tyrosine to tyrosyl radicals. Both hypochlorous acid and tyrosyl radicals are cytotoxic to a variety of microorganisms. Notably, MPO is also involved in the induction of granulocyte apoptosis following activation [26,27].

In a small series of CRC samples (n = 67), it has been shown that MPO+ cell infiltration is significantly higher in CRC than in normal colon mucosa [28]. However, prognostic relevance of CRC infiltration by MPO+ cells has not been addressed so far.

CD15, also known as Lewis X and stage-specific embryonic antigen 1, is a carbohydrate adhesion molecule expressed on mature neutrophils, mediating phagocytosis and chemotaxis [29]. Importantly, CD15 expression has been detected in tumor cells and found to correlate with poor prognosis in head and neck, gastric and lung cancers [30–32]. In CRC, expression of CD15 on tumor cells was shown to occur during progression to metastatic stages [33] and to be associated with high incidence of recurrences and poor survival [34,35]. However, the prognostic value of CRC infiltration by CD15+ immune cells has not been explored.

Here we show for the first time that a subgroup of CRC is characterized by a high infiltration by MPO+ and CD15+ positive cells. Most importantly, high MPO+ cell density in CRC is independently associated with favorable prognosis.

Materials and Methods

Ethics Statement

Written consent has been given from the patients for their information to be stored in the hospital database and used for research. The use of this clinically annotated TMA for research was approved by the corresponding Ethics Committee of the University Hospital of Basel (Ethikkommission beider Basel) and the ex vivo analyses were approved by the Institutional Review Board (63/07). For freshly excised clinical specimens included in this study written consent has been given from the patients undergoing surgical treatment at Basel University Hospital.

Tissue Microarray Construction

The TMA used in this work was constructed by using 1420 non-consecutive, primary CRCs, as previously described [36]. Briefly, formalin-fixed, paraffin-embedded CRC tissue blocks were obtained. Tissue cylinders with a diameter of 0.6 mm were punched from morphologically representative areas of each donor block and brought into one recipient paraffin block (30×25 mm), using a semiautomated tissue arrayer. Each punch was made from the center of the tumor so that each TMA spot consisted of at least a 50% of tumor cells. One core per case was used.

Clinicopathological Features

Clinicopathological data for the 1420 CRC patients included in the TMA were collected retrospectively in a non-stratified and non-matched manner. Annotation included patient age, tumor diameter, location, pT/pN stage, grade, histologic subtype, vascular invasion, border configuration, presence of peritumoral lymphocytic inflammation at the invasive tumor front and disease-specific survival (table 1). Tumor border configuration and peritumoral lymphocytic inflammation were evaluated according to Jass using the original H&E slides of the resection specimens corresponding to each tissue microarray punch [37]. The number of lymph nodes evaluated ranged between 1 and 61 with mean and median of 12 and 11, respectively. MMR status was evaluated by IHC according to MLH1, MSH2 and MSH6 expression [38]. Based on this analysis, the TMA included 1031 MMR-proficient tumors and 194 MMR-deficient tumors.

Overall survival was defined as primary endpoint. Follow-up data were available for 1379 patients with mean/median and IQR event-free follow-up time of 67.7/68 and 45–97 months.

Immunohistochemistry

Standard indirect immunoperoxidase procedures were used for immunohistochemistry (IHC; ABC-Elite, Vector Laboratories, Burlingame, CA). Briefly, slides were dewaxed and rehydrated in distilled water. Endogenous peroxidase activity was blocked using 0.5% H_2O_2. Sections were incubated with 10% normal goat serum (DakoCytomation, Carpinteria, CA) for 20 min and incubated with primary antibody at room temperature. Primary antibodies used were specific for MPO (clone 59A5 Novocastra, Newcastle, UK), CD15 (clone Carb-1, Leica Biosystems, Nussloch, Germany), CD16 (clone 2H7, Novocastra), CD68 (clone PG-M1, Dako, Glostrup, Denmark), FOXP3 (clone 236A/E7, Abcam, Cambridge, UK) and CD8 (clone C8/144B, DakoCytomation, Switzerland). Subsequently, sections were incubated with perox-idase-labelled secondary antibody (DakoCytomation) for 30 min at room temperature. For visualization of the antigen, sections were immersed in 3-amino-9-ethylcarbazole plus substrate-chro-mogen (DakoCytomation) for 30 min, and counterstained with Gill's hematoxylin.

Evaluation of Immunohistochemistry

MPO+ and CD15+ tumor infiltrating cells were counted for each punch (approximately one high power [20x] field) by a trained research fellow [R.D.]. Data were independently validated by two additional investigators [L.To. and C.H.] and a high Spearman correlation coefficient (= 0.82) and a highly significant (p<0.0001) correlation between measurements was observed. Evaluation of MLH1, MSH2, MSH6, CD16, CD68, CD8 and FOXP3 specific stainings in the CRC TMA under investigation was published previously [9,13,39].

Flow Cytometry Analyses

Following Institutional Review Board approval (63/07), tissues from surgically removed CRC and adjacent normal mucosa were minced, centrifuged, and resuspended in RPMI 1640 medium supplemented with 5% foetal calf serum, 2 mg/ml collagenase IV, 0.1 mg/ml hyaluronidase V, and 0.2 mg/ml DNAse I (Sigma Aldrich, Basel, Switzerland). Following a 1 hour digestion, cell suspensions were filtered and centrifuged. For phenotypic analysis of surface markers, cells were stained with mAbs for 15 minutes on ice in PBS, washed once with PBS 0.5% FCS, 0.5 M EDTA buffer and fixed in lysis buffer from BD Bioscience (1:10). Samples were then permeabilized in BD fixation/permeabilization buffer. For

Table 1. Characteristics of CRC patient cohort (n = 1420)*.

Characteristics	Number of cases or mean	Percentage or range
Age, years	71	(30–96)
Tumor size (mm)	75	(4–170)
Gender		
Female (%)	741	(52.2)
Male (%)	673	(47.4)
Anatomic site of the tumor		
Left-sided (%)	912	(64.2)
Right-sided (%)	488	(34.4)
T stage		
T1 (%)	62	(4.4)
T2 (%)	203	(14.3)
T3 (%)	899	(63.3)
T4 (%)	223	(15.7)
N stage		
N0 (%)	711	(50.1)
N1 (%)	358	(25.2)
N2 (%)	294	(20.7)
Tumor grade		
G1 (%)	31	(2.2)
G2 (%)	1177	(82.9)
G3 (%)	177	(12.5)
UICC		
Stage I (pN0 pT1 or 2) (%)	185	(13.6)
Stage IIA (pN0 pT3)+IIB-C (pN0 pT4) (%)	445+61	(37.2)
Stage III (pN>0) (%)	581	(42.7)
Stage IV metastasis (%)	88	(6.5)
Tumor border configuration		
Infiltrative (%)	871	(61.3)
Pushing (%)	513	(36.1)
Vascular invasion		
No (%)	1002	(70.6)
Yes (%)	383	(27)
Microsatellite Stability		
Proficient (%)	1031	(72.6)
Deficient (%)	194	(13.7)
Rectal cancers (%)	575	(40.5)
Overall survival time (months)	67.7	(0–152)
5-years survival % (95%CI)	56.4	(54–59)

*percentage may not add to 100% due to missing values of same variables; age and tumor size were evaluated using the Kruskal-Wallis test. Gender, anatomical site, T stage, N stage, grade, vascular invasion, and tumor border configuration were analyzed using the χ^2 test. Survival analysis was performed using the Kaplan-Meier method.
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intracellular staining, an anti-MPO reagent or an isotype matched negative control antibody were added for a 15 min incubation at room temperature. After a PBS wash, cells were suspended in wash buffer and analyzed by flow cytometry using a 2-laser BD FACSCalibur (Becton Dickinson, San Jose, CA). Results were analyzed by Cell Quest (Becton Dickinson, San Jose, CA) and Flow Jo (Tree Star, Ashland, OR) softwares.

Statistical Analysis

Cut-off scores used to classify CRC with low or high MPO+ or CD15+ infiltration were obtained by regression tree analysis, evaluating sensitivity and false positive rate for the discrimination of survivors and non-survivors, on all tumor samples [40]. Specific scores were set at 60 cells/TMA-punch for MPO+ and at 46 cells/TMA-punch for CD15+ infiltration. Chi-Square or Fisher's Exact tests were used to determine the association of MPO+ and CD15+

infiltration and clinicopathological features. Univariate survival analysis was carried out by the Kaplan-Meier method and log rank test. The assumption of proportional hazards was verified for both markers by analyzing the correlation of Schoenfeld residuals and the ranks of individual failure times. Any missing clinicopathological information was assumed to be missing at random. Subsequently, MPO+ and CD15+ cell infiltration data were entered into multivariate Cox regression analysis and hazard ratios (HR) and 95% confidence intervals (CI) were used to determine associations with survival time. The multivariate Cox regression analysis was performed with 975 cases since missing values were excluded from the model.

Spearman's rank correlation was used to analyze the correlation between MPO+, CD15+, CD16+, CD68+, CD8+ and FOXP3+ cell infiltration. Statistical analyses were performed using R i386 Version 2.15.2 (http://www.R-project.org). Data reporting was performed according to the REMARK criteria [41].

Results

CRC Infiltration by MPO+ and CD15+ Cells: Detection and Association with Clinicopathological Features

MPO+ and CD15+ cells could be successfully identified in the TMA under investigation by specific mAbs. Figure 1A–D shows representative stainings of CRC with low and high MPO+ or CD15+ cell infiltration.

Out of 1420 CRC individual specimens, MPO expression could be evaluated in 1225 samples including 1031 MMR-proficient and 194 MMR-deficient CRC. CD15 expression was evaluable in 1191 CRC specimens, including 817 MMR proficient and 191 MMR deficient samples. Numbers of samples evaluated for each feature are indicated in absolute numbers and percentages in table 2. Dropouts were due to loss of punches during TMA staining preparation or missing information, and usually accounted for <15% of data (tables 1–2).

Normal colonic mucosa was indeed infiltrated by MPO+ and CD15+ cells (mean: 6.4, median: 2, range 0–59 cells/punch for MPO (n = 48) and mean: 4.3, median: 1, range 0–35 cells/punch for CD15 (n = 47), respectively). However, a significantly higher (P<0.001) infiltration by MPO+ and CD15+ cells was detectable in CRC samples (mean: 26.7, median: 23, range 0–150 cells/punch for MPO (n = 1225) and mean: 16.4, median: 7, range 0–125 cells/punch for CD15 (n = 1191), respectively; figure 1E–F).

MPO+ and CD15+ infiltration, as evaluated by absolute cell numbers, was significantly higher in MMR deficient than in MMR proficient CRC (median: 30 cells/punch in deficient vs. 21 cells/punch in proficient CRC P = 0.007 and 9 cells/punch in deficient
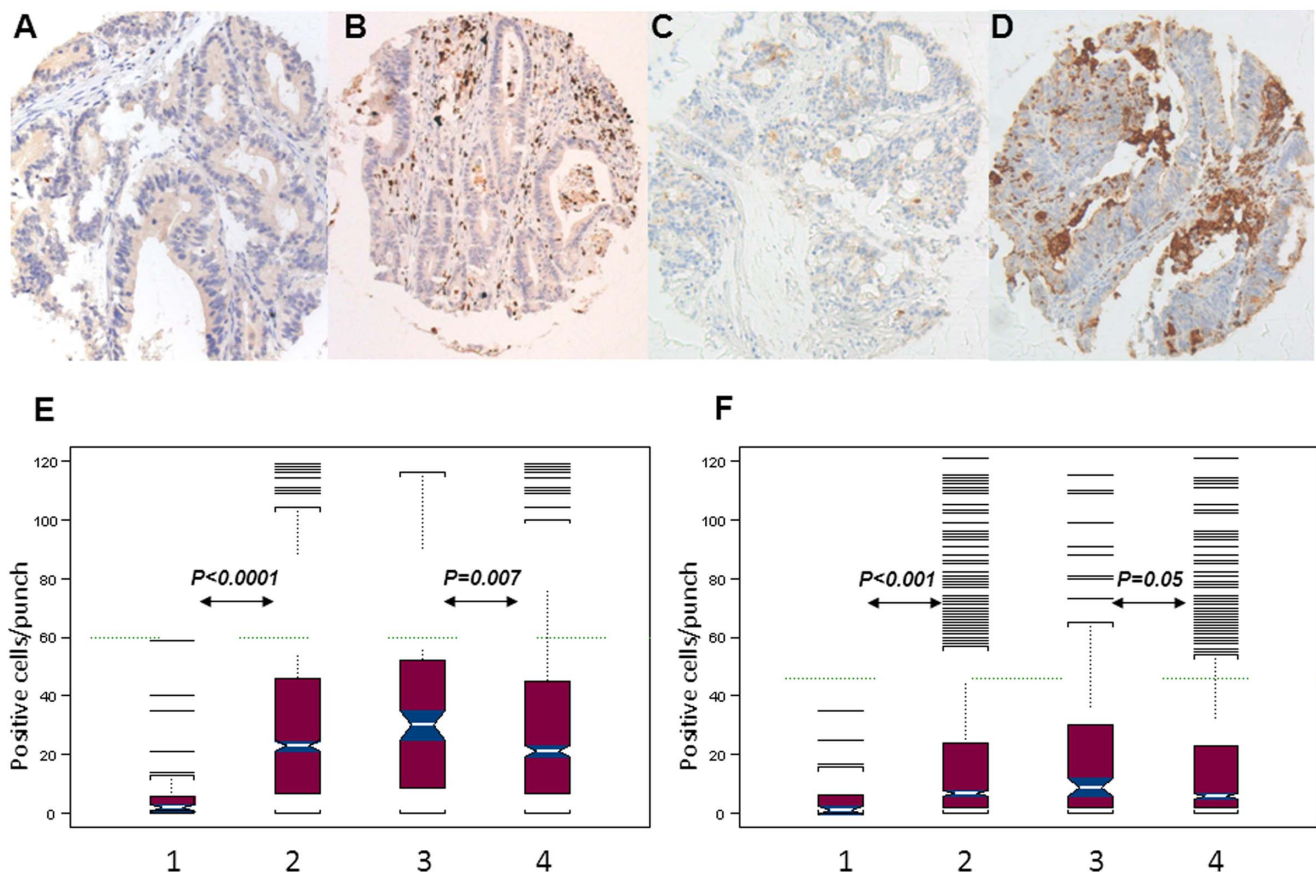
Figure 1. MPO and CD15 specific staining in CRC. CRC samples were stained with MPO and CD15 specific monoclonal antibodies (clone 59A5, Novocastra and clone Carb-1, Leica, respectively). Tumor punches are representative of low (panels A and C) and high (panels B and D) density of CRC infiltrating MPO+ (panels A–B) and CD15+ (panels C–D) cells, respectively. Magnification: 20x. Panel E reports the distribution of MPO+ cells in normal mucosa (E1), total CRC (E2), MMR-deficient CRC (E3) and MMR-proficient CRC (E4). The green line indicates the cut-off of 60 cells/punch as defined by regression tree analysis. Panel F reports the distribution of CD15+ cells in normal mucosa (F1), total CRC (F2), MMR-deficient CRC (F3) and MMR-proficient CRC (F4). The green line indicates the cut-off of 46 cells/punch as defined by regression tree analysis.
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Table 2. Association of MPO+ and CD15+ low and high immune cell density with clinicopathological features in CRC.

		MPO−		MPO+		p-value	CD15 −		CD15+		p-value
		N = 1047	(85.4%)	N = 178	(14.5%)		N = 1062	(89.2%)	N = 129	(10.8%)	
Age	years	71	(30–96)	73	(37–96)	**0.04**	71	(30–96)	71	(38–96)	0.45
Tumor diameter	mm	49	(8–120)	45	(4–170)	0.84	45	(4–160)	45	(8–170)	0.99
Gender	Female	546	(52.1)	90	(50.6)		558	(52)	70	(54.3)	0.64
	Male	501	(47.9)	88	(49.4)	0.75	515	(48)	59	(45.7)	
Tumor location	Left-sided	674	(64.4)	117	(65.7)	0.86	677	(63.1)	95	(73.6)	**0.018**
	Right-sided	361	(34.5)	60	(33.7)		385	(35.9)	32	(24.8)	
Histologic subtype	Mucinous	86	(8.2)	16	(9)	0.66	97	(9)	7	(5.4)	0.19
	Non-mucinous	955	(91.2)	158	(88.8)		965	(89.9)	122	(94.6)	
pT stage	pT1–2	182	(17.4)	48	(27)	**0.007**	190	(17.7)	41	(31.8)	**0.0004**
	pT3–4	833	(79.6)	129	(72.5)		857	(79.9)	87	(67.4)	
pN stage	pN0	527	(50.3)	101	(56.7)	0.16	536	(50)	72	(55.8)	0.35
	pN1-2	479	(45.7)	72	(40.4)		494	(46)	55	(42.6)	
Tumor grade	G1-2	894	(85.4)	146	(82)	0.224	907	(84.5)	110	(85.3)	0.68
	G3	129	(12.3)	28	(15.7)		138	(12.9)	18	(14)	
Vascular invasion	Absent	740	(70.7)	133	(74.7)	0.27	754	(70.3)	102	(79.1)	0.07
	Present	283	(27)	41	(23)		291	(27.1)	26	(20.2)	
Tumor border	Pushing	385	(36.8)	75	(42.1)	0.178	404	(37.7)	45	(34.9)	0.44
	Infiltrating	637	(60.8)	99	(55.6)		640	(59.6)	83	(64.3)	
PTL inflammation	Absent	819	(78.2)	132	(74.2)	0.224	835	(77.8)	97	(75.2)	0.3
	Present	205	(19.6)	42	(23.6)		211	(19.7)	31	(24)	
Local recurrence	Absent	212	(20.2)	54	(30.3)	**0.031**	241	(22.5)	26	(20.2)	**0.03**
	Present	162	(15.5)	23	(12.9)		180	(16.8)	8	(6.2)	
Distant metastasis	Absent	304	(29)	69	(38.8)	0.108	343	(32)	32	(24.8)	0.08
	Present	75	(7.2)	9	(5.1)		84	(7.8)	2	(1.6)	
Microsatellite stability	Deficient	159	(15.2)	35	(19.7)	0.161	167	(15.6)	24	(18.6)	0.45
	Proficient	888	(86.1)	143	(13.9)		729	(67.9)	88	(68.2)	
5-year survival rate	(95%CI)	55.6	(52.4–59)	68.5	(61.3–76.5)	**0.0009**	56.8	(53.6–60.2)	63.6	(55.1–73.4)	**0.033**

*percentage may not add to 100% due to missing values of same variables; variables are indicated as absolute numbers, %, median or range; age and tumor size were evaluated using the Kruskal-Wallis test. Gender, anatomical site, T stage, N stage, grade, vascular invasion, and tumor border configuration were analyzed using the χ^2 test. Survival analysis was performed using the Kaplan-Meier method.
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vs 6 cells/punch in proficient CRC $P = 0.05$, for MPO and CD15 respectively; figures 2E–F).

Regression tree analysis defined cut-off scores for MPO+ and CD15+ CRC infiltrating cells detected in individual punch biopsies (n = 60 and n = 46, respectively) were used to assess clinicopathological correlations.

Univariate Cox regression analysis revealed that high density MPO+ cell infiltration (≥60 cells/punch), detectable in 14.5% of tumors, was significantly associated with older age of patients ($P = 0.04$). Most importantly, it was significantly associated with early (pT1–2) tumor stage ($P = 0.007$), absence of local recurrence ($P = 0.031$) and higher 5-year survival rate ($P = 0.0009$) (table 2).

High density of CD15+ infiltrating cells (≥46 cells/punch), detectable in 10.8% of tumors, was significantly associated with left sided location ($P = 0.018$), early (pT1–2) stage ($P = 0.0004$), absence of local recurrence ($P = 0.03$) and higher 5-year survival rate ($P = 0.033$) (table 2).

Correlation between MPO+, CD15+, CD16+ and CD68+ Tumor-infiltrating Myeloid Cells

To explore relationships between tumor infiltration by cells expressing MPO and other immune markers (CD15, CD16, CD68, CD8, FOXP3) expressed by immunocompetent cells infiltrating CRC, data from additional immune-histochemical stainings of the same TMA from previous studies were used [9,13,39]. The statistically strongest correlation was detectable between MPO+ and CD15+ cell infiltration (r = 0.75, P<0.0001), whereas correlations of lesser significance were detectable with CD16+ (r = 0.32), CD68+ (r = 0.35), CD8+ (r = 0.13) and FOXP3+ (r = 0.21) cell infiltration.

Ex vivo Characterization of MPO+ cells from Freshly Removed CRC

To assess in detail phenotypic characteristics of tissue infiltrating MPO+ cells, freshly excised CRC (n = 8) were enzymatically digested, and single cell suspensions were analyzed by flow cytometry. Examples of this phenotypic analysis are reported in figure 2a.
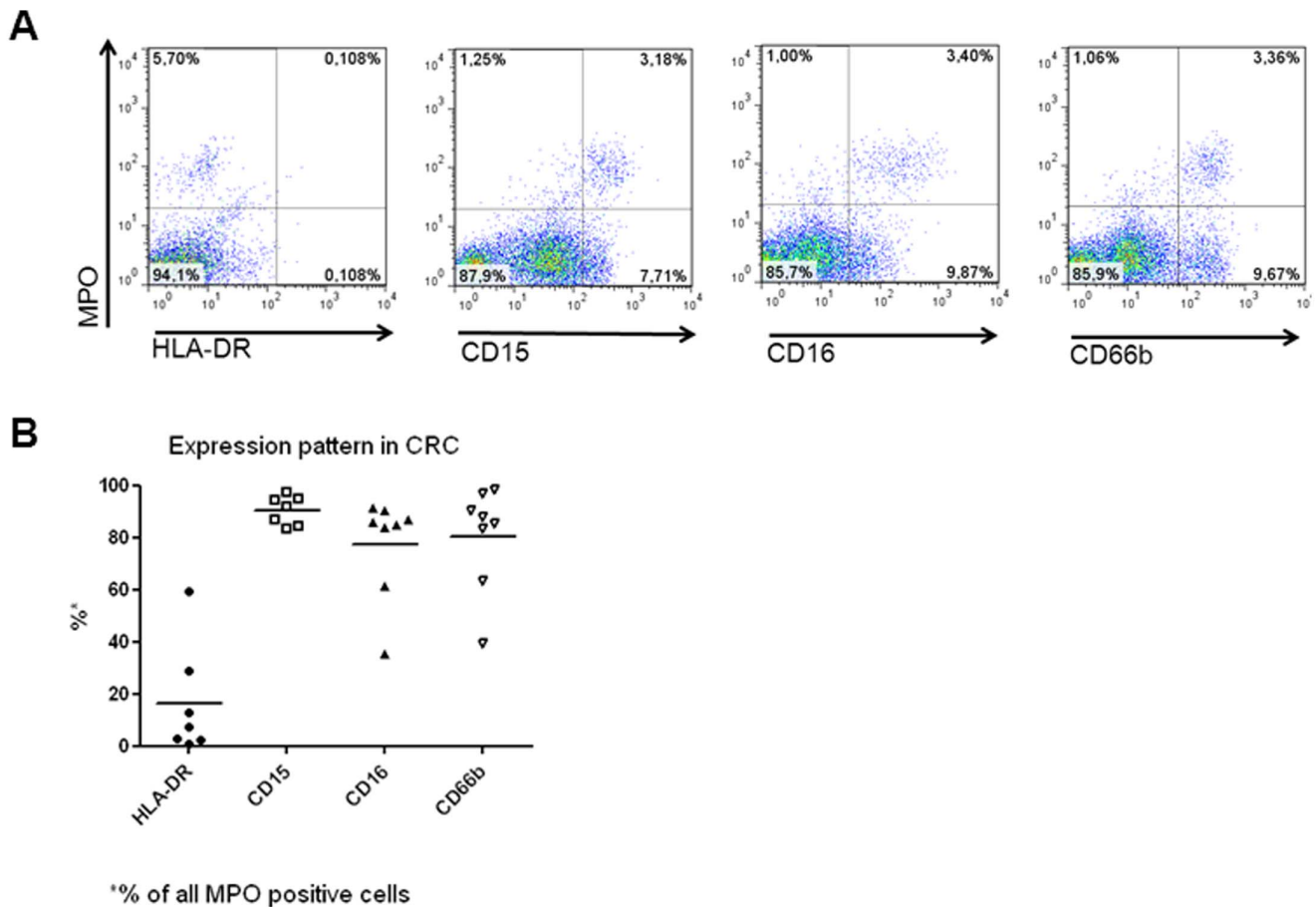
Figure 2. Phenotypic characterization of CRC infiltrating MPO+ cells. CRC surgical specimens were enzymatically digested and immediately stained with fluorochrome labeled mAbs recognizing MPO, HLA-DR, CD66b, CD15 and CD16, as indicated in ''materials and methods''. Panel A reports one representative staining, whereas panel B summarizes results from freshly excised specimens (n = 8) regarding the expression of the indicated markers in CRC infiltrating MPO+ cells.
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The large majority of CRC infiltrating, MPO+ cells were CD15+ (90.3%±5.6%), CD16+, (77.6±19.4%), and CD66b+ (80.6±19.9%; figure 2b). Interestingly, MPO+ cells detectable in autologous normal mucosa displayed a similar (P>0.05) marker expression pattern (data not shown). Notably however, sizeable percentages of MPO−/CD66b+ cells (47.3±35.7%) and, more expectedly, of MPO−/CD15+ cells (78.0±17.7%) of possibly cancerous nature were also detectable in CRC derived cell suspensions.

Prognostic Significance of MPO+ and CD15+ Cell Infiltration in the CRC Microenvironment

Median survival time was 50 and 46 months for patients with high or low MPO+ cell density, respectively. High MPO+ cell infiltration was significantly (P = 0.0003) associated with better prognosis (0.59 HR, 95%CI: 0.45–0.74), as compared to tumors with low MPO+ cell infiltration in univariate Cox regression analysis. Upon splitting of the cohort in a test and a validation set, high score MPO+ cell infiltration was still associated with significantly improved survival (P = 0.038 and P = 0.002, respectively; figure 3A–B). Several randomizations of the overall cohort were tried and all results were found to be comparable.

In univariate analysis survival was also increased in case of high score CD15+ cell infiltration (P = 0.051, figure 3C). A combination

analysis however, showed that MPO is the dominant marker associated with improved prognosis, without relevant additive benefit provided by CD15 positivity (figure 3D).

Most importantly, in multivariate analysis, high score MPO+, but not CD15+, cell infiltration was independently associated with favorable prognosis after adjusting for several known prognostic factors such as age, sex, T stage, N stage, tumor grade, vascular invasion, tumor border configuration and microsatellite stability (P = 0.004; table 3). Also in the two stratified collectives the effect of MPO+ cell infiltration on survival of patients with CRC remained significant (P = 0.048 and P = 0.036 in the testing and validation set, respectively).

Discussion

To the best of our knowledge, this is the first study identifying MPO+ neutrophil granulocyte tumor infiltration as an independent favorable prognostic factor in CRC.

Myeloid cell infiltration is known to promote tumor growth and to be associated with poor prognosis in a variety of human cancers [42]. In particular, tumor associated macrophages have been indicated as obligate partners for tumor progression and metastasis formation [43]. Granulocyte infiltration has also been found to be associated with poor prognosis in different tumors including lung cancers and renal and hepatocellular carcinoma [44–47]. In this
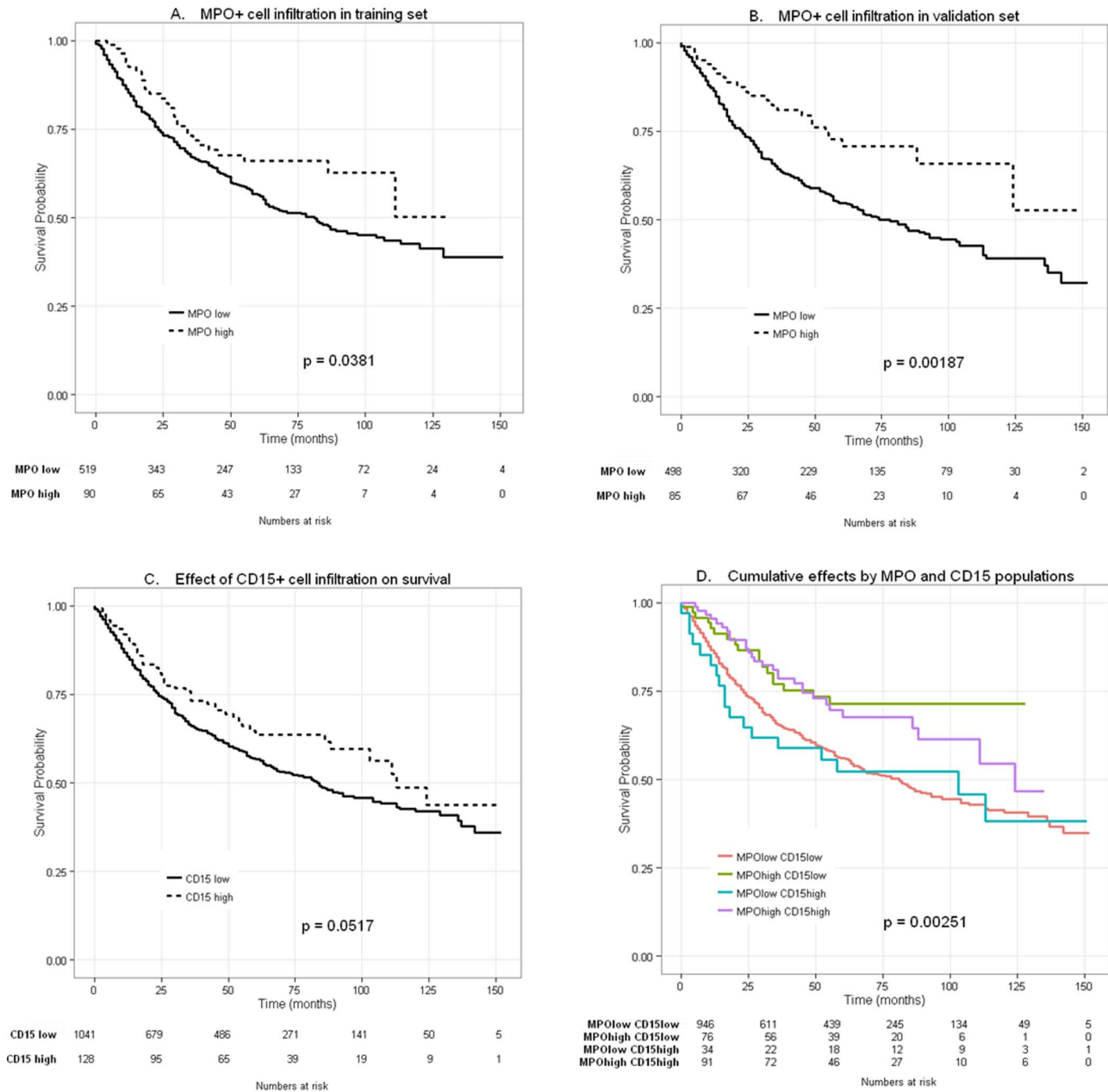
Figure 3. Effects of MPO+ and CD15+ tumor infiltration on overall survival in patients with CRC. Kaplan-Meier overall survival curves were designed according to MPO+ and CD15+ tumor infiltration in patients bearing CRC. In panels A–C, dotted lines refer to high density and black lines to low density infiltration according to cut-off values established by regression tree analysis (60 cells/punch for MPO+ and 46 cells/punch for CD15+ cell infiltration). Panels A and B report the effects of high MPO+ cell infiltration, as detected in the training (n = 609; 255 deaths observed in 519 patients with low CRC infiltration by MPO+ cells and 28 deaths observed in 90 patients with tumors with high MPO+ cell infiltration, P = 0.038) and in the validation set (n = 583; 234 deaths observed in 498 patients with low CRC infiltration by MPO+ cells and 23 deaths observed in 85 patients with tumors with high MPO+ cell infiltration, P = 0.002). Panel C reports the effect of high CD15+ CRC infiltration in the whole group of patients under investigation (n = 1169; 458 deaths observed in 1041 patients with low CRC infiltration by CD15+ cells and 47 deaths observed in 128 patients with tumors with high CD15+ cell infiltration, P = 0.051). In panel D cumulative effects of tumor infiltration by MPO+ and CD15+ cells were explored. Rosa line (430/946) refers to tumors with low MPO+/CD15+ cell infiltration. Light blue line (18/34) refers to tumors with low MPO+ and high CD15+ cell infiltration. Lila line (29/91) refers to tumors with high MPO+/CD15+ cell infiltration and green line (18/76) refers to CRC with high MPO+ and low CD15+ cell infiltration (n = 1147; 430 deaths observed in 946 patients with low CRC infiltration by MPO+ and CD15+ cells; 18 deaths observed in 34 patients with tumors with high CD15+ and low MPO+ cell infiltration; 29 deaths observed in 91 patients with high CRC infiltration by MPO+ and CD15+ cells; 18 deaths observed in 76 patients with tumors with high MPO+ and low CD15+ cell infiltration, P = 0.002).
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context, CRC might represent an interesting exception [48,49]. Indeed, controversial data have been reported on the prognostic significance of macrophage infiltration in CRC [50–54].

Neutrophil infiltration has been found to be increased in the transition from normal to dysplastic and cancerous mucosa [55].

Table 3. Multivariate Hazard Cox regression survival analysis.

	HR (95% CI)	P-Values
MPO (low vs high)	**0.65** (0.5–0.8)	**0.004**
Age (continuous)	**1.03** (1.02–1.03)	**<0.0001**
Gender (men vs women)	**1.21** (1.16–1.26)	**<0.0001**
pT stage (1,2,3,4)	**1.79** (1.71–1.87)	**<0.00001**
pN stage (0,1,2)	**1.93** (1.87–1.99)	**<0.0001**
Tumor Grade (1,2,3)	**1.23** (1.10–1.36)	**0.11**
Vascular invasion (0,1)*	**1.43** (1.33–1.53)	**0.0006**
Tumor border configuration (0,1)**	**1.52** (1.41–1.63)	**0.0003**
Microsatellite stability (deficient vs. proficient)	**1.70** (1.56–1.84)	**0.0002**

Multivariate analyses showing Hazard Ratios and p-value for all CRC (n = 975, due to missing values, see "materials and methods") conferred by high MPO density, age, sex, tumor size, nodal status, tumor grade, vascular invasion, tumor border configuration and microsatellite stability.
*0: absent, 1: present.
**0: pushing, 1: infiltrating.
doi:10.1371/journal.pone.0064814.t003

Furthermore, CRC infiltration by CD66b+ cells has recently been proposed to be associated with adverse prognosis [56].

In previous work we showed that CRC infiltration by CD16+ cells correlates with improved survival [13]. This marker is expressed in neutrophils, in a subset of macrophages and in NK cells [57,58]. Indeed, NK cell infiltration in CRC is negligible [13,14] and devoid of prognostic significance [59]. Since CRC infiltrating CD16+ cells are also CD11b+/CD33+/HLA-DR-, in this study we focused on the analysis of cells expressing MPO and CD15 granulocyte markers.

In univariate analysis high density CRC infiltration by cells expressing either marker was associated with improved survival. However, following adjustment for multiple comparisons carried out to compensate for the exploratory nature of this analysis this favorable prognostic relevance was maintained for MPO+ but not for CD15+ cell infiltration.

Importantly, in accord with a previously published report [55] we observed that MPO+ and CD15+ cells preferentially colonized CRC tissues while they poorly infiltrated normal colon mucosa, suggesting that they might be specifically recruited to the tumor microenvironment. Interestingly, MPO+ cell infiltration was higher in MMR-deficient than in MMR-proficient CRC as previously suggested by a study conducted with 67 samples from a limited number of cancers (n = 35) [28].

Flow-cytometric analysis of digested paired normal and cancerous tissues indicates that in both healthy mucosa and CRC infiltrating MPO+ cells are CD15+/CD16+/CD66b+/HLA-DR-, consistent with a granulocytic lineage. Most importantly however, we observed substantial percentages of CD66b+/MPO- cells infiltrating CRC. These data might explain the discrepancies between our findings and a previous report focusing on CD66b+ CRC infiltrating cells [56]. Notably, neutrophils with similar phenotypic characteristics have also been found to infiltrate head and neck cancers [60].

Similarly, the presence of CD15+/MPO− cells in healthy mucosa and cancerous tissues might explain the differential prognostic relevance of these markers. Notably however, MPO gene expression was undetectable in CRC or normal mucosa specimens (data not shown) consistent with a mature granulocyte nature of MPO+ infiltrating cells [61].

MPO activity has been suggested to contribute to the pathogenesis of degenerative diseases, including atherosclerosis, multiple sclerosis and Alzheimer disease [62]. Furthermore, high MPO activity or MPO+ cell infiltration have been detected in esophageal [63], and gynecological cancers [64,65] and in CRC [28,66,67], but their prognostic impact was not analyzed.

Despite early reports documenting their ability to mediate tumor cell cytotoxicity [68] granulocytes have largely been neglected by tumor immunologists [18]. However experimental models in the past have indicated that colorectal cancer cells transfected with G-CSF gene can be rejected by tumor infiltrating neutrophils upon interaction with IFN-γ producing T cells [69]. Granulocytes were also shown to express co-stimulatory molecules and to be able to present antigens [70,71], thus suggesting the possibility of a role in the initiation of antigen specific antitumor responses.

More recently, evidence of the ability of granulocytes to inhibit lung metastasis formation in an experimental breast cancer model was provided [19]. Furthermore, the capacity of granulocytes to undergo TGF-β dependent polarization into N1 and N2 functional profiles, characterized by anti- or pro-tumoral properties, respectively, similarly to macrophages, has also been documented [20].

The molecular background underlying CRC infiltration by MPO+ cells and its prognostic significance is unclear. These phenomena could reflect chemokine production by activated T cells, and therefore indirectly result from ongoing antitumoral adaptive responses. Alternatively, they might be related to the production of granulocyte attracting chemokines by tumor cells. At least CXCL8 (IL-8) is known to be produced by CRC cells. However, its production was suggested to be associated with increased angiogenesis and tumor dissemination [72]. On the other hand, we and others have previously shown that GM-CSF, promoting granulocyte maturation and survival, can also be produced by CRC cells [73,74].

Our results contribute to the characterization of the complex features inherent in gut microenvironment and with CRC-immune system interaction [75]. Further research is warranted to clarify molecular mechanisms underlying the independent prognostic impact of MPO+ cells in CRC. Importantly, here we show that CRC infiltrating MPO+ cells express CD16 Fcγ intermediate affinity receptor. The ability of granulocytes to mediate antibody dependent cellular cytotoxicity (ADCC) is debated. However, the availability of novel therapeutic mAb with glycoengineered Fc fragments characterized by increased affinity

for Fcγ receptors [76] might lead to a reevaluation of the effector significance of granulocytes.

Within this framework, it is tempting to speculate that neutrophil infiltration should be included in current prognostic models for CRC [77] and that it might represent an important novel stratification factor for randomization in specific clinical trials.

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