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Tumor infiltration by FcγRIII (CD16)+ myeloid cells is associated with improved survival in patients with colorectal carcinoma

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Abstract

The prognostic significance of macrophage and natural killer (NK) cell infiltration in colorectal carcinoma (CRC) microenvironment is unclear. We investigated the CRC innate inflammatory infiltrate in over 1,600 CRC using two independent tissue microarrays and immunohistochemistry. Survival time was assessed using the Kaplan–Meier method and Cox proportional hazards regression analysis in a multivariable setting. Spearman's rank correlation tested the association between macrophage and lymphocyte infiltration. The Basel study included over 1,400 CRCs. The level of CD16+ cell infiltration correlated with that of CD3+ and CD8+ lymphocytes but not with NK cell infiltration. Patients with high CD16+ cell infiltration (score 2) survived longer than patients with low (score 1) infiltration ($p = 0.008$), while no survival difference between patients with score 1 or 2 for CD56+ ($p = 0.264$) or CD57+ cell ($p = 0.583$) infiltration was detected. CD16+ infiltrate was associated with improved survival even after adjusting for known prognostic factors including pT, pN, grade, vascular invasion, tumor growth and age [$(p = 0.001$: HR (95% CI) = 0.71 (0.6–0.9)]. These effects were independent from CD8+ lymphocyte infiltration [$(p = 0.036$: HR (95% CI) = 0.81 (0.7–0.9)] and presence of metastases [$(p = 0.002$: HR (95% CI) = 0.43 (0.3–0.7)]. Phenotypic studies identified CD16+ as CD45+CD33+CD11b+CD11c+ but CD64– HLA-DR–myeloid cells. Beneficial effects of CD16+ cell infiltration were independently validated by a study carried out at the University of Athens confirming that patients with CD16 score 2 survived longer than patients with score 1 CRCs ($p = 0.011$). Thus, CD16+ cell infiltration represents a novel favorable prognostic factor in CRC.

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Keywords

colorectal carcinoma; survival; CD16; tumor array; prognosis

Colorectal carcinoma (CRC) is the second leading cause of cancer-related death in industrialized countries. Surgery is routinely used to treat CRC patients at stage I–III while the use of adjuvant chemotherapy at different stages of the disease is debated.¹ Therapeutic monoclonal antibodies (mAbs) are also widely used, but indications for their administration still need to be fully clarified.² The identification of biological markers of potential prognostic significance, allowing correct stratification of CRC patients and helping in the selection of therapeutic options is urgently required.

The association between tumor infiltration by CD8+ T cells and long-term overall survival (OS) has been convincingly demonstrated in patients bearing CRC,^{3–5} but the role of innate immunity is still unclear.

Cancer cells frequently express the MICA/B antigens, the ligands of the NKG2D receptor,⁶ but presence and clinical significance of natural killer (NK) cells in tumor-microenvironment have not been thoroughly investigated in large series of cases.

Tumor-associated macrophages (TAMs) are frequently detectable within the tumor “milieu.” TAMs have been shown to display immunosuppressive and proangiogenic activity in a number of cancers, including breast and thyroid malignancies and melanoma⁷ while in others they might exert antitumor activities including direct and antibody dependent cellular cytotoxicity (ADCC) and antiproliferative effects.⁸ Notably, it has been recently suggested that the equation “TAMs equal tumor progression”⁹ may not apply to CRC.¹⁰

FcγRIII (CD16) is expressed by a subset of human monocytes accounting for ~10% of the total and likely representing precursors of tissue macrophages. CD16+ monocytes are proinflammatory cells,¹¹ often associated with sepsis¹² and HIV infection.¹³ The existence of a link between these cells and cancer is supported by evidence that numbers of CD16+ monocytes may be increased in patients with a variety of malignancies.¹⁴ However, their role in cancer immunobiology remains undefined.

Monocytes/macrophages may polarize as M1 or M2 cells. M1 macrophages, typically characterized by high Fcγ expression,¹⁵ may display a proinflammatory potential mediating antitumor activities⁸ while M2 anti-inflammatory cells promote cancer cell growth possibly by fuelling angiogenesis and immunosuppression.^{16,17}

Importantly, CD16 is also expressed on NK cells^{18,19} and NK cell infiltration could represent an important survival factor in CRC patients.²⁰ In addition, dendritic cells (DC) may also express CD16²¹ and they have been shown to infiltrate CRC,²² although their prognostic significance is debated.^{23,24}

We investigated phenotype and clinicopathological role of CD16+ cells infiltrating CRC using two independent CRC tissue microarrays (TMAs). We found that CD16+ myeloid cell infiltration represents a strong, novel and independent prognostic pro-survival factor in CRC patients.

Material and Methods

Antibodies

Anti-MICA/B mAbs have been previously described.^{25,26} Biotinylated anti-CD16, anti-CD56, anti-CD57, anti-CD68 and isotype-matched mouse mAbs were purchased from DAKO (Glostrup, Denmark) and Novocastra (Newcastle, UK). Fluorescein isothiocyanate (FITC)-conjugated anti-CD1a, anti-CD4, anti-CD11c, anti-CD14, anti-CD16, anti-CD45, anti-CD64, anti-CD83, anti-CD86, anti-HLA-DR, isotype-matched mAbs, phycoerythrin (PE)-conjugated anti-CD1a, anti-CD4, anti-CD8, anti-CD11c, anti-CD16, anti-CD56, anti-CD68, anti-CD83, anti-CD86, isotype-matched mAbs, allophycocyanin (APC)-conjugated anti-CD1a, anti-CD3, anti-CD8, anti-CD11c, anti-CD33, anti-CD56, anti-NKP46, anti-NKG2D, isotype-matched mAb, Cy5-conjugated anti-CD16 and iso-type-matched mAb were purchased from BD Bioscience (San Jose, CA).

TMA and immunohistochemistry

This study was performed by using two independent TMAs including over 1,600 CRC. The Basel TMA comprised 1,420 unselected, nonconsecutive cases while the Athens TMA included 220 mismatch repair (MMR) proficient CRC. Both TMAs were constructed as described.²⁷ Available clinicopathological data included pT, pN stage, tumor grade, vascular invasion, tumor budding, tumor location, CD8+ T-cell infiltration, adjuvant therapy and patients survival.

TMAs were immunostained with biotinylated mAbs. MICA/B was detected using the anti-MICA/B, WW6B7 mAb (see above) and a secondary biotinylated rabbit-anti-mouse IgG antibody.

Flow cytometry analysis of cell suspension from CRC surgical specimens

Following the Basel Institutional Review Board approval (63/07), tissues from surgically removed, CRCs were minced, centrifuged and resuspended in RPMI 1640 medium supplemented with 5% FCS, 2 mg/ml collagenase IV, 0.1 mg/ml hyaluronidase V and 0.2 mg/ml DNase I (Sigma Aldrich, Basel, Switzerland). After a 12-hr digestion, cell suspensions were filtered and centrifuged. Mononuclear cells were isolated by Ficoll-Hypaque gradient separation, stained with the appropriate fluorochrome-conjugated mAbs and analyzed by flow cytometry using a 2-laser BD FACSCalibur (Becton Dickinson, San Jose, CA). Propidium iodide (PI) positive cells were excluded from the analysis. Results were analyzed by Cell Quest (Becton Dickinson, San Jose, CA) and Flow Jo (Tree Star, Ashland OR) computer softwares.

Statistical analysis

Cut-off scores for protein marker positivity were determined on the Test Group using receiver operating characteristic (ROC) curve analysis with the endpoint survival/death and the 0,1-criterion to select the most discriminating cut-off score from the ROC curve.²⁸ Cut-off scores were further validated by assessing the interobserver variability of positivity by a second (A.L.) and a third (D.C.) observer. Survival time differences were determined using the Kaplan–Meier method and the log-rank test. Multivariate analysis was performed by adjusting for well-established prognostic factors including pT and pN stage, tumor grade, vascular invasion, tumor border configuration and adjuvant therapy as well as CD8+ TILs and metastasis in a second analysis. Strength of correlations between different cell types was assessed using the Spearman Rank correlation coefficient.

NK cells were defined as CD16+CD56+CD57+ cells and CD56+CD16–CD57– cells,²⁹ whereas CD16+ TAMs were defined as CD16+CD56– cells.^{29,30} For the Basel CD16 study,

the level of innate inflammatory cell infiltration was scored as 1 and 2, when the CD16⁺ infiltrate consisted of ≤10 cells and >10 cells per punch, respectively. Accordingly, CD56⁺ and CD57⁺ cell infiltration was scored as 1 and 2, when the infiltrate consisted of ≤4 cells and >4 cells per punch, respectively, whereas for CD68⁺ cell infiltration scores 1 and 2 were set at ≤100 cells and >100 cells per punch, respectively. For the Athens CD16 study, similar to the Basel study, the cut-off was also set at 10 immunoreactive cells.

Results

Identification of CD16⁺ cells in the CRC microenvironment

To investigate the innate inflammatory infiltrate in CRC microenvironment, we used the Basel TMA including over 1,400 tumors with extensive clinicopathological information (Table 1). This TMA was stained for CD16 (1,150 evaluable CRCs: 269 score 1 and 881 score 2, see above), CD56 (1,203 evaluable CRCs: 743 score 1 and 460 score 2), CD57 (1,259 evaluable CRCs: 1,070 score 1 and 189 score 2) and CD68 (1,270 evaluable CRCs: 805 score 1 and 465 score 2) markers.

CD16⁺ infiltrate was found to be composed of large cells (40–50 μm) resembling macrophages (Fig. 1, upper left panels). Comparative analysis of CD16 and CD56 staining revealed a marked discrepancy between the two sets of data. Only 11.8% of CRC punches were infiltrated by ≤4 CD16⁺ cells whereas 61.8% of CRC punches were infiltrated by ≤4 CD56⁺ cells ($p = 2.2 \times 10^{-13}$) suggesting that CD16⁺ cell infiltrate was not associated with CD56⁺ cell infiltration. Figure 1 (lower left panel) shows examples of cell infiltration scores, namely score 1 CD16⁺ and CD56⁺ infiltrates (a, c) and score 2 CD16⁺ and CD56⁺ cell infiltrates (b, d). Notably, MICA/B, a ligand for NKG2D receptor expressed by NK cells was strongly expressed in >90% of the CRC specimens (data not shown).

We then evaluated the expression of CD57 and CD68. Only 21 of 1,270 evaluable tumors showed ≤4 CD68⁺ cells infiltration per punch. On the other hand, interestingly, 1,070 of 1,259 evaluable CRC lesions contained ≤4 CD57⁺ cells while 189 CRC only had >4 CD57⁺ cells. Taken together, these data suggested a myeloid nature of the CD16⁺ cell infiltrate.

Isolation of CD16⁺ cells from freshly removed CRC

The analysis of freshly excised surgical samples, following enzymatic digestion, showed that percentages of CD16⁺ cells observed were significantly higher than those of CD56⁺ cells (average ± SE = $1.83 \pm 0.31\%$ vs. $0.14 \pm 0.07\%$ of the whole CRC suspension, $n = 5$, $p = 0.005$, Table 2, upper panel). NKG2D and NKp46 markers were accordingly expressed in comparably ($p = 0.45$) low percentages of cells. Importantly, percentages of CD16⁺ infiltrating cells were significantly higher in suspensions from CRC tissues than in those from the corresponding autologous healthy mucosa (average ± SE = 1.66 ± 0.32 vs. 0.18 ± 0.054 , $n = 12$, $p = 0.0005$).

Table 2 shows a phenotypic analysis of the inflammatory infiltrate obtained from 14 CRC lesions. It appeared to contain a heterogeneous cell population including T and myeloid CD16⁺ cells. CD16⁺ cells coexpressed to different extents CD45, CD11b, CD11c, CD33, CD68 and CD32 markers but lacked expression of CD64, HLA-DR, NK and mature DC markers including CD80 and CD86 (data not shown). Figure 1 (right, panel) shows flow cytometry profiles of a representative CD16⁺ cell infiltrate obtained from a CRC sample. These data indicate that the CD16⁺ infiltrate of the CRC is composed of cells of myeloid origin while the presence of NK cells is negligible.

Correlation between CD16+ cell infiltrate and CD3+ and CD8+ lymphocyte infiltration

In the Basel study, CD16+ cell infiltration was not associated with age, gender, histological subtype, location, TN stage and tumor grade (see below). The relationship between CD16+ and T-cell infiltration was specifically investigated. CRCs were divided in two groups. The first group was composed of 932 tumors with a proficient MMR system.³¹ This group was further randomized into two independent subgroups to provide additional statistical validation. A third group was composed of 187 CRC with a deficient MMR system. CRC infiltration by CD16+ cells did not correlate with CD56+ infiltration ($p > 0.05$) in any of the groups under investigation. In contrast, CD16+ cell infiltration was significantly correlated in all groups with CD3+ and CD8+ lymphocyte ($p < 0.001$) infiltration (Table 3).

Since CD8+ cell infiltrate has been associated with a favorable prognosis in CRC,^{3,5} these data suggested that CD16+ cell infiltration might also qualify as favorable prognostic factor.

Clinical significance of CD16+ cell infiltration in the CRC microenvironment

In univariate analysis of the evaluable CRCs of the Basel CD16 study, patients with score 2 CD16+ cell tumor infiltration showed a significantly higher 5-year survival rate than patients with score 1 infiltration (58.7% vs. 49.4%, $p = 0.008$, Fig. 2a). In contrast, Figures 2b and 2c show that no significant differences in 5-year survival were detectable between CRC with score 1 and score 2 CD56+ (51.7 vs. 56.5%, $p = 0.264$) or CD57+ cell infiltration (53.1 vs. 57.5%, $p = 0.583$).

To provide an external validation to these data, a CRC TMA, independently established in Athens and including over 200 MMR proficient specimens (Table 4), was also stained with anti-CD16 mAb. Of 199 evaluable CRCs, 55 showed score 1 and 144 score 2 staining. Figure 2d depicts results obtained in this study, indicating that, in agreement with the Basel TMA data, CRC patients with score 2 CD16+ cell infiltration also survived longer than patients with score 1 CD16+ cell infiltration (74.4 vs. 59.2%, $p = 0.011$).

These data indicate that a high CD16+ cell infiltrate is associated with long-term survival in patients with CRC while no prognostic role of NK cells could be observed.

Notably, CD68+ cell infiltration was devoid of prognostic significance (Fig. 3a). However, increased CD16+/CD68+ ratio significantly correlated with improved survival (Fig. 3b).

Multivariate analysis

In multivariate analysis, the beneficial effect of CD16+ cells on disease outcome was maintained after adjusting for other well-established prognostic factors including pT, pN, grade, vascular invasion, tumor growth pattern and age [$p = 0.001$: HR (95% CI) = 0.71 (0.6–0.9)]. CD16+ cell infiltration appeared to maintain its independent beneficial prognostic effect even in the presence of distant metastases [$p = 0.002$: HR (95% CI) = 0.43 (0.3–0.7)]. Remarkably, the prognostic value of CD16+ cell infiltration was independent from CD3+ T-cell infiltration [$p = 0.017$: HR (95% CI) = 0.78 (0.6–0.9)] and presence of CD8+TIL [$p = 0.036$: HR (95% CI) = 0.81 (0.7–0.9)].

In agreement with the results emerging from the Basel TMA, the data obtained in the Athens study confirmed that infiltration of CD16+ cells was indeed an independent prognostic factor after adjusting for pT and pN stages and adjuvant therapy [$p = 0.033$: HR (95% CI) = 0.56 (0.3–0.9)]. Taken together, these data from two different studies clearly indicate that CD16+ cell infiltration represents a favorable prognostic factor in CRC.

Discussion

There is substantial evidence that adaptive immune response is able to counteract CRC progression.^{3,5} However, the role played by the innate immune system in this process is still controversial. Indeed, TAMs have widely been considered to promote cancer growth⁹ while the migration of NK cells into the tumor microenvironment has been associated with the inhibition of tumor development.²⁰

Several observations do indicate that CD34-derived myeloid macrophages may elicit antiproliferative effects on cancer cell lines *in vitro*.^{32,33} Furthermore, recently, it has been shown that CD68+ cell infiltration in the advancing margin of CRC is favorably associated with patients OS. However, the phenotype of the monocyte/macrophage subset involved was not characterized.¹⁰

Our data indicate that (i) CD16+ cells represent a major population of innate inflammatory cells infiltrating CRC lesions, (ii) there is no significant infiltration of NK cells in the CRC microenvironment and (iii) a considerably improved median OS of more than 10 years is associated with high CD16+ cell infiltration without apparent role of NK cells. Importantly, high levels of CD16+ cells infiltration in CRC are preferentially associated with CD3+, and CD8+ T-cell infiltration.

Since numerous tumor markers are available but only a few of them are useful,^{34–36} basic guidelines have been developed to characterize novel biomarkers.³⁷ We attempted to follow such indications with particular emphasis on data reproducibility. Thus, the results of the Basel study were independently reproduced by the Athens study confirming that CD16+ cell infiltration represents a favorable prognostic factor in CRC.

What is the origin of CD16+ cell infiltrate? Morphology and immunohistochemistry indicate that these cells are usually large (40–50 μ m), similarly to TAMs and phenotypically resembling immature DC (CD16+CD11b+CD11c+CD33+CD14–), although, in agreement with previous reports,^{24,38} they are HLA-DR–. Notably, the lack of NK cell infiltration appears to be frequent in epithelial cancer since similar results were observed in the microenvironment of renal,³⁹ breast and lung carcinoma as well.^{40,41}

Macrophages have been shown to polarize in response to environmental signals.¹⁵ In particular, M1 macrophages have been suggested to represent powerful proinflammatory effector cells, whereas M2 macrophages are involved in the fine tuning of the immune response and in the promotion of angiogenesis. Tumor-associated macrophages (TAM) have been suggested to exert predominantly protumoral functions consistent with a M2 polarization profile.^{9,16} However, “*in vitro*” studies⁴² suggest that M1 macrophages can be generated in the presence of GM-CSF, a cytokine typically detectable in CRC tissues and produced by CRC cells.^{22,43} Notably, M1 polarized macrophages are characterized by a high expression of CD16.¹⁵ Capitalizing on this background, our data may suggest that TAM infiltrating CRC might be M1 polarized. Therefore, they might be able to exert antitumor functions, possibly including direct antiproliferative effects or a persistently strong inflammatory activity, promoting the generation of an adaptive immune response. Alternatively, ADCC could also be involved. Indeed, the role of humoral immune response in the control of neoplastic growth has recently been re-evaluated since sera from patients with leukemias or solid tumors have been shown to contain antibodies directed against tumor antigens.^{44–46} It is tempting to speculate that CD16+ myeloid cells infiltrating CRC could represent effector cells of clinical relevance in the presence of an endogenous humoral antitumor responses.

On the other hand, in a murine model, FcγR activation has been shown to regulate inflammation-associated squamous carcinogenesis of the skin.⁴⁷ These data may suggest that, depending on the type of oncogenic stimuli, and, eventually, on the anatomic district involved, these molecules might play different functional roles.

Recent advances have provided new tools for the treatment of CRC, including a series of mAbs with anticancer activity.^{48–50} However, incomplete information about underlying therapeutic mechanisms mediating anticancer effects has limited their clinical applications. Direct effects of mAbs on cancer cells might be complemented by immunological mechanisms related to mAb-mediated ADCC, particularly in the presence of high numbers of tumor infiltrating FcγR + cells.⁵¹

Therefore, CD16+ myeloid infiltration may prospectively be considered of potential relevance as a mechanism of action of therapeutic mAbs and it might contribute to the identification of subgroups of CRC patients potentially taking advantage of mAb-based treatments.

Although our data indicate that myeloid CD16+ cell infiltration represents an independent favorable prognostic marker in CRC, due to their antigen presenting skills, these cells may also be able to trigger T-cell responses specific for CRC tumor-associated antigens [45]. Indeed a high level of CD16+ cell infiltration correlates with CD8+ T-lymphocyte infiltration.

Altogether, this study, unprecedented for the size of its data base and supported by according evidence from an independent patient series, clearly indicates that, at difference with other cancers, TAM infiltration is associated with improved survival in CRC. These results set the stage for functional investigations addressing molecular mechanisms involved in the elicitations of these effects. Furthermore, they suggest that treatments taking advantage of M1 TAM and, possibly, helping to maintain a skewed functional profile, might be of potential relevance in CRC treatment.

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Abbreviations

ADCC	antibody dependent cellular cytotoxicity
APC	allophycocyanin
CI	confidence interval
CRC	colorectal cancer
Cy	cyanine
FITC	fluorescein isothiocyanate
HR	hazard ratio

mAb	monoclonal antibody
MMR	mismatch repair
NK	natural killer
OS	overall survival
PE	phycoerythrin
PI	propidium iodide
ROC	receiver operating characteristic
TAM	tumor-associated macrophages
TIL	tumor infiltrating lymphocytes
TMA	tumor microarrays

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Figure 1.

Phenotypic analysis of innate inflammatory infiltrate in the CRC microenvironment. Upper left panel documents CD16+ cell infiltration in CRC lesions. The four pictures show representative examples of different numbers of CD16+ cells infiltrating CRC microenvironment. Brown staining identifies labeled cells. The indicated marker highlights the size of CD16+ cells. Lower left panel documents infiltration by CD16+ and CD56+ cells in CRC. Panels (a) and (b) show two representative CRC lesions with 10 and >10 infiltrating CD16+ cells, corresponding to score 1 and 2 staining, respectively (see “Patients and Methods” section). Panels (c) and (d), show two representative CRC lesions with 4 and >4 infiltrating CD56+ cells, corresponding to score 1 and 2 staining, respectively. Right panel: Phenotypic analysis of CD16+ cells infiltrating a representative CRC specimen. Specific markers are indicated in individual histograms.

Figure 2.

CD16+ cell infiltration is associated with improved overall survival in CRC patients. Kaplan–Meier plots depict overall survival of patients bearing CRC showing score 1 (black lines) or 2 (purple lines) infiltration by cells expressing the indicated markers. Significance of differences eventually observed is also reported. Panels (a–c) refer to the Basel tumor array, whereas panel (d) reports data from the study independently performed in Athens.

Figure 3.

Effect of the combination of CD16+ and CD68+ cell infiltration on overall survival in CRC patients. Kaplan–Meier plot of panel (a) indicates that score of CD68+ cell infiltration of CRC is not associated with differential overall survival. In panel (b), CRC patients were subdivided in four subgroups according to CD16/CD68 specific ratio. Black line refers to a 0–0.18 CD16/CD68 positive cell infiltration ratio, whereas purple line, green line and blue line refer to 0.18–0.35, 0.35–0.52 and >0.52 ratios. Higher ratios are associated with significantly improved overall survival.

Table 1

Clinicopathological features of colorectal cancer patients included in the Basel tissue microarray

Features	Frequency (%)
Gender (<i>n</i> = 1414)	
Female	741 (52.4)
Male	673 (47.6)
Patient age (years)	
Mean (range)	71.0 (30–96)
Histologic subtype (<i>n</i> = 1420)	
Nonmucinous	1301 (91.6)
Mucinous	119 (8.4)
Tumor grade (<i>n</i> = 1385)	
G1	31 (2.2)
G2	1177 (85.0)
G3	177 (12.8)
Tumor location (<i>n</i> = 1400)	
Left-sided	430 (30.7)
Rectum	482 (34.4)
Right-sided	488 (34.9)
MMR status (<i>n</i> = 1119)	
Proficient	932 (8.3)
Deficient	187 (16.7)
pT stage (<i>n</i> = 1387)	
pT1	62 (4.5)
pT2	203 (14.6)
pT3	899 (64.8)
pT4	223 (16.1)
pN stage (<i>n</i> = 1363)	
pN0	711 (52.1)
pN1	358 (26.3)
pN2	294 (21.6)
Distant metastasis (<i>n</i> = 452)	
Absent	370 (81.9)
Present	82 (18.1)
Vascular invasion (<i>n</i> = 1385)	
Absent	1002 (72.4)
Present	383 (27.7)
Peritumoral lymphocytic inflammation (<i>n</i> = 1386)	
Absent	1092 (78.8)
Present	294 (21.2)
Survival rate (%)	
5-year (95% CI)	56.4 (54–59)

Table 2

Phenotypic analysis of CRC inflammatory infiltrate

	P23	P25	P26	P28	P29	P30	P31	P33	P34	P35	P36	P37	P38	P39
% Positive cells in total tumor suspension														
CD16	0.90	2.99	1.37	1.81	2.11	10.5	3.1	20.82	11.1	2.67	1.90	2.2	2.8	7.6
CD56	0.16	0.46	0.03	0	0.07	-	-	-	-	-	-	-	-	-
NKp46	-	0.33	0.01	0.01	0.08	-	-	-	-	-	-	-	-	-
NKG2D	-	-	0.02	0.01	0.07	-	-	-	-	-	-	-	-	-
CD64	-	0.52	0.11	-	0.2	0.52	0.9	19.49	-	0.48	0.5	-	-	-
CD14	0.66	-	0.19	-	-	-	-	-	-	-	-	-	-	-
CD3	-	21.6	11.32	-	-	-	-	-	-	-	-	-	-	-
CD4	-	-	9.89	3.84	-	18.7	8.7	-	-	-	-	-	-	-
CD8	-	-	-	-	1.40	30.8	1.2	-	-	-	-	-	-	-
CD1a	-	-	0.08	0.05	0.09	-	-	-	-	-	-	-	-	-
CD45	-	37.3	-	11.5	10.42	-	-	-	-	-	-	-	-	-
CD11b	-	-	-	3.03	2.89	-	-	-	-	-	-	-	-	-
CD33	-	-	-	-	-	4.18	5.7	40.51	-	4.24	6.91	-	-	-
CD32	-	-	-	-	-	-	-	41.15	-	10.1	12.4	-	-	-
HLA-DR	-	-	1.62	1.93	0.94	-	-	-	-	13.78	11.12	-	-	-
% Positive CD16 ⁺ cells														
CD11b	-	-	-	95.6	98.3	-	-	-	-	-	-	-	-	-
CD11c	-	-	43.6	57.6	66.1	-	-	-	-	-	-	-	-	-
CD3	-	1.61	8.07	0	-	-	-	-	-	-	-	-	-	-
CD64	-	1.59	2.68	-	1.21	3.3	3.84	19	-	2.6	0.0	-	-	-
CD14	3.78	-	2.09	-	-	-	-	-	-	-	-	-	-	-
CD4	-	-	2	4	-	-	-	-	-	-	-	-	-	-
CD1a	-	-	1.12	0	0.83	-	-	-	-	-	-	-	-	-
CD56	2.94	1.59	4.49	0.37	2.47	-	-	-	-	-	-	-	-	-
CD83	-	-	5.03	0.08	3.10	-	-	-	-	-	-	-	-	-
NKG2D	-	-	5.83	4.84	7.63	-	-	-	-	-	-	-	-	-
NKp46	-	1.75	6.93	0.97	4.84	-	-	-	-	-	-	-	-	-
CD45	-	96.5	-	93.8	94.1	-	-	-	-	-	-	-	-	-

	P23	P25	P26	P28	P29	P30	P31	P33	P34	P35	P36	P37	P38	P39
CD68 int	-	-	62.4	43.7	-	55.2	-	-	33.7	16.7	59.1	-	62.7	84
CD33	-	-	-	-	-	30.9	51.9	74.5	-	83.1	68.9	-	-	-
CD32	-	-	-	-	-	-	48.5	-	53.7	54.7	-	-	-	-
HLA-DR	-	-	4.06	3.35	1.44	-	-	-	-	3.4	5.7	-	-	-

P: CRC patient; CD: cluster of differentiation; int.: intracellular; bold characters: phenotypic features of CD16+ cell infiltrate; -not done.

Table 3

Analysis of the correlation of colorectal cancer infiltration by CD16+ cells and by defined lymphoid cell populations according to MMR-proficient or MMR-deficient status

		CD56 ⁺	CD3 ⁺	CD8 ⁺
MMR proficient-1 (<i>n</i> = 466)	<i>r</i>	0.037	0.220	0.294
	<i>p</i>	0.435	<0.0001	<0.0001
MMR-proficient-2 (<i>n</i> = 466)	<i>r</i>	0.079	0.159	0.214
	<i>p</i>	0.096	<0.001	<0.0001
MMR-deficient (<i>n</i> = 187)	<i>r</i>	0.111	0.364	0.375
	<i>p</i>	0.183	<0.0001	<0.0001

Table 4

Clinicopathological features of MMR proficient colorectal cancer patients included in the Athens validation cohort

Features	Frequency (%)
Gender (<i>n</i> = 199)	
Male	99 (49.8)
Female	100 (50.2)
Tumour diameter (<i>n</i> = 198) (cm)	
Mean (range)	4.6 (1–12)
Patient age (<i>n</i> = 194) (years)	
Mean (range)	67.8 (35–93)
Histologic subtype (<i>n</i> = 199)	
Nonmucinous	174 (87.4)
Mucinous	25 (12.6)
Tumour grade (<i>n</i> = 199)	
G1	3 (1.5)
G2	125 (62.8)
G3	71 (35.7)
Tumour location (<i>n</i> = 199)	
Left-sided	124 (62.3)
Rectum	28 (14.1)
Right-sided	47 (23.6)
pT stage (<i>n</i> = 199)	
pT1	12 (6.0)
pT2	37 (18.6)
pT3	116 (58.3)
pT4	34 (17.1)
pN stage (<i>n</i> = 199)	
pN0	100 (50.3)
pN1	47 (23.6)
pN2	52 (26.1)
pM stage (<i>n</i> = 200)	
pM0	182 (91.0)
pM1	18 (9.0)
Vascular invasion (<i>n</i> = 203)	
Present	37 (18.2)
Absent	166 (81.8)
Lymphatic invasion (<i>n</i> = 203)	
Present	83 (40.9)
Absent	120 (59.1)
Adjuvant therapy (<i>n</i> = 209)	
None	71 (34.0)

Features	Frequency (%)
Treated	138 (66.0)
Survival rate (%)	
5-year (95% CI)	60.3 (50–69)