Tumour-infiltrating CD68+ and CD57+ cells predict patient outcome in stage II–III colorectal cancer

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Background: The aim of our study was to evaluate the prognostic role of immunological microenvironnement in stage II–III CRC patients.

Methods: We constructed a tissue microarray from 196 consecutive patients with stage II–III CRC and compared CD3, CD4, CD8, CD57, CD68, CXCL9/MIG, CXCL13, and PPARγ immunoreactivity in tumour samples and their matched non-tumour tissue. We assessed their association with relapse-free survival (RFS; primary endpoint) and overall survival (OS) in multivariate Cox models.

Results: Low densities of CD57+ and CD68+ tumour-infiltrating cells (TIC) independently predicted worse outcomes. A prognostic score combining CD57 (+, > vs −, ⩽2 cells per spot) and CD68 (+, >0 vs −, =0 cells per spot) TIC density discriminated CRC patients at low (CD68+/CD57+), intermediate (CD68+/CD57−), or high (CD68−/CD57−) risk, with hazard ratios for the intermediate-risk and high-risk groups of 2.7 (95% confidence interval (CI): 1.3–5.8) and 9.0 (3.2–25.4) for RFS, and 2.5 (1.2–5.1) and 10.6 (3.8–29.2) for OS, respectively, as compared with the low-risk group. Corresponding 5-year survival rates (95% CI) in the low-, moderate- and high-risk groups were 84% (71–91), 65% (54–74), and 12% (2–47), respectively, for RFS, and 91% (80–96), 76% (66–84), and 25% (7–59), respectively, for OS.

Conclusion: Tumour CD57+ and CD68+ TIC density assessment independently predicts survival in patients with stage II–III CRC. If validated, our score based on a quick, inexpensive, and well-established method such as point counting on diagnostic tissue sections could be used routinely as a prognostic tool in CRC patients.

Despite advances in screening, diagnosis, and treatment, colorectal cancer (CRC) is the third leading cause of cancer-related mortality (Greenlee *et al*, 2000). Pathological staging is currently the most accurate predictor of CRC prognosis and is used in clinical practice to select patients for adjuvant chemotherapy—more specifically, those with stage II (node-negative) CRC and stage III (node-positive) CRC, patients with stage I disease not deriving benefit from adjuvant chemotherapy due to an excellent prognosis, and those with stage IV disease being already metastatic (AJCC, 2010).

However, 10–40% of patients with stage II (node-negative) CRC and 20–80% of those with stage III (node-positive) CRC develop recurrence after curative-intent (R0) surgical resection (AJCC, 2010). Several molecular markers have been shown to predict the likelihood of recurrence in stage II and III CRC, including microsatellite instability (deficient mismatch repair (dMMR)) and allelic imbalances (for example, loss of 8p and 18q chromosomal arms) (Halling *et al*, 1999; Gryfe *et al*, 2000; Zhou *et al*, 2002). However, only the dMMR tumour phenotype remained a significant

prognostic factor in early CRC both in a meta-analysis and prospective trials (Popat *et al*, 2005; Hutchins *et al*, 2011). Recently, Pages *et al* (2005) showed that the absence of an immune response within primary colonic cancer (CC) (stage I–IV disease) was associated with early metastatic invasion and worse survival, and that the density of CD3 + CD45RO + tumour-infiltrating lymphocytes (TIL) was a better predictor of outcome than pathological staging according to the Union Internationale Contre le Cancer (UICC) tumour–node–metastasis (TNM) system (Galon *et al*, 2006). By contrast, Laghi *et al* (2009) showed that CD3 + TIL density was prognostic in stage II, but not in stage III CRC. Furthermore, apart from T-cell markers, other tumour immune markers related to macrophages, NK cells, or chemokines have also been found to predict outcome in CRC. However, these putative immune prognostic markers have not been assessed simultaneously in the same patient cohort (Coca *et al*, 1997; Pages *et al*, 2005; Galon *et al*, 2006; Forssell *et al*, 2007; Hojo *et al*, 2007; Laghi *et al*, 2009; Ogino *et al*, 2009; Ferdinande *et al*, 2011; Mlecnik *et al*, 2011a). In the present study, we aimed to evaluate the prognostic value of immune tumour-infiltrating cells (TIC) and chemokines in stage II and III CRC patients. To assess simultaneously on the same patients the prognostic value of multiple immune parameters, we used a tissue microarray (TMA) analysis.

MATERIALS AND METHODS

Patients. Patients with CRC who underwent primary surgery at our institution between 1990 and 2000 were selected according to the following criteria: curative-intent resection (negative (R0) surgical margin status) for pathologically confirmed colorectal adenocarcinoma, UICC TNM stage II or III tumour (AJCC, 2010), no preoperative chemotherapy, no family history of Lynch syndrome or adenomatous polyposis, and postoperative follow-up of at least 2 years. As increased numbers of TIL represent one of the particular pathological features of CRC with dMMR phenotype (Jass *et al*, 1998; Laghi *et al*, 2009), patients with dMMR CRC as defined by molecular analysis or immunohistochemistry (IHC) (Suraweera *et al*, 2002; Hampel *et al*, 2005) were excluded.

Tumour stage (TNM) and differentiation, vascular emboli, lymphatic invasion, and perineural invasion (VELIPI) (Pages *et al*, 2005) were determined from the histopathological reports obtained at the time of the resection.

Construction of TMAs. Paired, paraffin-embedded tumour (TT) and normal (NT) tissues from the whole patient population were used for the construction of TMA (product MTA1; Beecher Instruments, Sun Prairie, WI, USA). Three core biopsy samples measuring 0.6 mm in diameter (0.3 mm^2 per spot) from paraffin-embedded TT (centre of the tumour) and the NT counterpart blocks were arrayed into a recipient paraffin block (35 × 22 × 5 mm^3). Tissue microarray containing the tissue cores was then cut into 4-μm sections for hematoxylin and IHC staining.

Immunohistochemistry. The sections were affixed to Superfrost Plus slides (CML, Nemours, France), deparaffinised, and rehydrated. Antigen retrieval, consisting of microwave processing at 750 W and 150 W for 15 minutes each and pressure cooking in 0.01 M citrate buffer (pH 7.3), was applied. Dako Cytomation peroxidase blocking reagent (Dako, Les Ulis, France) was used to suppress non-specific staining due to endogenous peroxidase activity.

On the basis of previous published data on tumour immunity, immune cell recruitment and/or inflammation status of tumour environment, and the prognostic role of immune response in CRC, the following markers were selected for IHC analysis on TMA: CD3 (Pages *et al*, 2005; Galon *et al*, 2006; Laghi *et al*, 2009; Pages *et al*, 2009; Mlecnik *et al*, 2011b), CD68 (Ohtani *et al*, 1997; Funada

et al, 2003; Forssell *et al*, 2007; Algars *et al*, 2011; Zhou *et al*, 2011), CD57 (Coca *et al*, 1997; Jonges *et al*, 2001; Menon *et al*, 2004), CD8 (Pages *et al*, 2005, 2009), CD4 (Diederichsen *et al*, 2003; Hojo *et al*, 2007) in order to quantify macrophage and lymphocyte cell infiltration as effectors of IFN-γ production. In addition, the following chemokines known to be regulated by IFN-γ pathway were analysed: CXCL9/MIG (Mlecnik *et al*, 2011b), CXCL10/IP-10 (Mlecnik *et al*, 2011b), CXCL13/BCA1 (Lin *et al*, 2007; Agesen *et al*, 2012), Conversely, PPARγ (Ogino *et al*, 2009) and IDO (Brandacher *et al*, 2006; Ferdinande *et al*, 2011) previously identified as potential prognostic markers in CC and involved in inhibition of Th1 (IFN-γ producer T cells) and Tc1 (cytotoxic T cells) were also selected. The following monoclonal antibodies were used: anti-CXCL9 (clone 49106.11, R&D systems, Lille, France) at 1:100 dilution, anti-CXCL13 (clone 53610, R&D systems) at 1:20 dilution, anti-PPARγ (clone E-8, Santa Cruz Biotechnology, Heidelberg, Germany) at 1:10 dilution, anti-CD4 (clone 4B12, Novocastra, Nanterre, France) at 1:20 dilution, anti-CD8 (clone 4B11, Dako) at 1:25 dilution, anti-CD57 (clone NK1, Dako) at 1:100 dilution, anti-CD68 (clone PGM1, Dako) at 1:100 dilution, and polyclonal antibody anti-CD3 (Dako) at 1:100 dilution. All incubations were performed at room temperature. Immuno-peroxidase staining, using 3,3′-diaminobenzidine as a chromogen, was performed. Nuclei were counterstained with hematoxylin. The number of CD3-, CD4-, CD8- and CD57-positive (intra-epithelial and stromal) cells per spot was quantified by a single pathologist unaware of clinical data. Positivity for CD68 was scored quantitatively according to the number of positive stromal cells per spot: 0, no staining; 1, less or equal to 10-positive cells; 2, more than 10-positive cells. IHC staining of CXCL9, CXCL13/BCA1, and PPARγ was evaluated by semi-quantitative scores. For CXCL9, intact nuclear staining of the colonic crypts of the normal mucosa, lymphocytes, and endothelial cells was used as an internal positive control and was required for adequate evaluation. Normal immunoreactivity of the CXCL9 protein was defined as the presence of nuclear staining. The intensity of nuclear immunos-taining was evaluated by the following scoring system: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. Nuclear immunoreactivity for CXCL13/BCA1 was scored semi-quantitatively according to staining of stromal lymphocytes: 0, no staining; 1, few lymphocytes stained; and 2, numerous lymphocytes stained. Nuclear immunoreactivity for PPARγ was based on staining intensity and graded as 0, no staining; 1, mild-to-moderate staining; and 2, strong staining. The rounded average expression of the three core biopsy samples was used for each patient. No reliable IHC data could be obtained for CXCL10/IP-10 and IDO.

Statistical analyses. The primary endpoint of the prognostic analysis was relapse-free survival (RFS), defined as the time from CRC diagnosis to locoregional relapse, distant relapse, or death related to CRC. Patients without any of these three events were censored at the time of last follow-up or at the death time in case of death from other cause. The secondary endpoint of the prognostic analysis was overall survival (OS), defined as the time from CRC diagnosis to death whatever the cause. Patients alive at the time of last follow-up were censored at that date. RFS and OS probabilities were estimating according to the Kaplan–Meier method. Follow-up duration was estimated by the reverse Kaplan–Meier method.

The prognostic impact of each clinicopathological factor was studied in univariate analysis using logrank test. Clinicopatholo-gical factors associated with RFS or OS with a *P*-value lower than 0.20 were tested in multivariate analysis using Cox model.

The prognostic impact of immune markers measured within the tumour was studied. CD3-, CD4-, and CD8-positive cells were dichotomised at their median value. Despite a median value of one per spot for CD57, we chose a threshold value of > 2 per spot that

seemed to the pathologist more reliable for the quantification. The number of CD68-positive cells per spot was studied in three categories: no staining; less or equal to 10-positive cells; and more than 10-positive cells. CXCL9/MIG was studied in three categories: no staining; weak staining; moderate to strong staining. PPARγ and CXCL13/BCA1 were studied in three categories as they were graded, that is, for PPARγ, no staining; mild-to-moderate staining; strong staining and for CXCL13/BCA1 no staining, few lymphocytes stained, numerous lymphocytes stained. The prognostic impact of each immune marker was studied using Cox model with adjustment for clinicopathological prognostic factors previously identified. All markers with a P-value lower then 0.20 in the Cox model were analysed all together to determine their independent prognostic value.

Immune markers staining were compared between TT and NT using paired t-test for quantitative variables and Bowker's test of symmetry for qualitative staining variables.

Comparisons of markers staining between patients who received preoperative radiotherapy (preop-RT) and other patients and associations between all markers staining in TT and in NT were analysed using χ^2-test or Fisher's exact test. As the number of tests realised for the latter analysis was high, Bonferroni correction was used ($P \leqslant 0.0018$ as the threshold of significance instead of 0.05). All statistical tests were two-sided. Statistical analyses were performed with the SAS System, version 9.1 (SAS, Grégy-sur-Yerres, France).

RESULTS

Patient characteristics. Characteristics of the 196 eligible patients are shown in Table 1. Adjuvant chemotherapy consisted of intravenous 5-fluorouracil and leucovorin in all the 115 (59%) patients who received adjuvant chemotherapy. The median duration of follow-up was 10 years (Interquartile range 6.8–13.3). Only 11 patients were followed less than 4 years. During follow-up, 73 recurrences and 72 deaths (cancer-related, 54) occurred. Five-year RFS and OS rates were 64% (s.e., 3.5) and 75% (s.e. 3.2), respectively. Prognostic analysis of clinicopathological factors (Cox model) identified tumour location (rectal vs colonic tumour), TNM stage (II vs III), and adjuvant chemotherapy (yes vs no) as independent predictors of RFS, and the same factors plus tumour grade for OS (Table 2).

Tissue microarray analysis. CD3, CD4, CD8, CD57, and CD68 cell density as for PPARγ, CXCL13/BCA1, and CXCL9/MIG staining intensity were significantly decreased in TT as compared with NT (each univariate, $P < 0.0001$, except for CD8, $P = 0.007$) (Figure 1).

Preop-RT impacted on all immune markers but PPARγ expression (Supplementary Table 1). The expression levels of CD57 and CD68 were significant higher in irradiated tumours ($n = 38$) than in non-irradiated tumours ($n = 158$) ($P = 0.002$ and $P < 0.0001$, respectively). The expression levels of CD57, CD68, and CXCL13/BCA1 were significant higher in normal tissue in patients who received preop-RT compared with non-irradiated patients ($P = 0.009$, $P = 0.004$, and $P = 0.010$, respectively), whereas those of CD3, CD4, CD8, and CXCL9/MIG were significant lower ($P = 0.018$, $P = 0.004$, $P < 0.0001$, and $P = 0.0017$ respectively). Therefore, we concluded that preop-RT significantly modified the tumour immune microenvironment, and decided to base further analyses on patient population who did not receive preop-RT.

Among the 158 remaining patients, positive associations were found for TT expression between CD57 and CD68 ($P = 0.0006$), CXCL9/MIG and PPARγ ($P = 0.0015$), and CD8 and CXCL13/BCA1 ($P < 0.0001$), and a negative association for TT expression between CD8 and CXCL9/MIG ($P < 0.0001$). Positive associations

	n (%)
Age	
Mean ± s.d.	61.5 ± 11
Range	27–86
Sex	
Male	108 (55)
Female	88 (45)
Tumour site	
Colon, right-sided	51 (26)
Colon, left-sided	98 (50)
Rectum	47 (24)
Nodal status	
N0	99 (50)
N1	54 (28)
N2	31 (16)
N positive (not specified)	12 (6)
Tumour grade	
Well to moderately differentiated	167 (85)
Poorly differentiated	20 (10)
Missing	9 (5)
VELIPI	
0	67 (34)
1	122 (62)
Missing	7 (4)
Tumour stage	
II	99 (51)
III	97 (49)
Adjuvant chemotherapy	
5-Fluorouracil and folinic acid	115 (59)
No	81 (41)
Preoperative radiation therapy	
Yes	38 (19)
No	158 (81)

Table 1. Patient characteristics

Abbreviation: VELIPI = vascular emboli, lymphatic invasion and perineural invasion.

were also found for NT expression between CXCL9/MIG and both CD4 ($P < 0.0001$) and PPARγ ($P = 0.0001$), and CD8 and CXCL13/BCA1 ($P < 0.0001$), and a negative association between CD57 and CD4 ($P = 0.0005$) (Supplementary Table 2A and 2B).

Prognostic analyses. Five-year RFS and OS rates in the subset of patients who did not receive preop-RT ($n = 158$) were 69% (s.e. = 3.7) and 79% (s.e. = 3.4), respectively. The same prognostic clinicopathological factors as in the whole patient cohort were found in this patient subgroup (data not shown).

After adjustment for these factors (tumour location, stage, grade, and adjuvant chemotherapy), lower CD3 +, CD57 +, and CD68 + TIC densities were significantly associated with worse RFS and OS, and lower tumour expression of CXCL9/MIG with worse RFS (Table 3A, Figure 2). In multivariate analysis, only CD57 + and CD68 + TIC densities remained independently

Table 2. Five-year relapse-free and overall survival according to clinicopathological factors					
	Univariate analysis			**Multivariate analysis**	
Relapse-free survival	**n**	**5-Year rate**	**P-value[a]**	**HR (95% CI)**	**P-value[b]**
Sex					
Male	108	62			
Female	88	66	0.97		
Tumour site					
Colon	149	71		1.0	
Rectum	47	40	<0.0001	2.4 (1.5–3.9)	0.0004
Tumour grade					
Well-to-moderate differentiation	167	67			
Poor differentiation	20	42	0.012		
Tumour stage					
Stage II	99	73		1.0	
Stage III	97	54	0.0005	3.5 (2.0–6.1)	<0.0001
VELIPI[c]					
VELIPI no	67	70			
VELIPI yes	122	61	0.11		
Adjuvant chemotherapy					
Yes	115	68		1.0	
No	81	59	0.37	0.5 (0.3–0.9)	0.013
Preoperative radiation therapy					
Yes	38	42			
No	158	69	0.0008		
Overall survival					
Sex					
Male	108	74			
Female	88	76	0.88		
Tumour site					
Colon	149	80		1.0	
Rectum	47	58	0.0014	2.0 (1.2–3.3)	0.009
Tumour grade					
Well-to-moderate differentiation	167	81		1.0	
Poor differentiation	20	34	0.0001	2.3 (1.2–4.6)	
Unknown	9			1.5 (0.5–4.1)	0.045
Tumour stage					
Stage II	99	82		1.0	
Stage III	97	68	0.016	2.8 (1.6–5.0)	0.0004
VELIPI[c]					
VELIPI no	67	86			
VELIPI yes	122	69	0.30		
Adjuvant chemotherapy					
Yes	115	80		1.0	
No	81	68	0.068	0.5 (0.3–0.9)	0.02
Preoperative radiation therapy					
Yes	38	59			
No	158	79	0.0047		

Abbreviations: CI = confidence interval; HR = hazard ratio; VELIPI = vascular emboli, lymphatic invasion and perineural invasion.
[a]log-rank P-value.
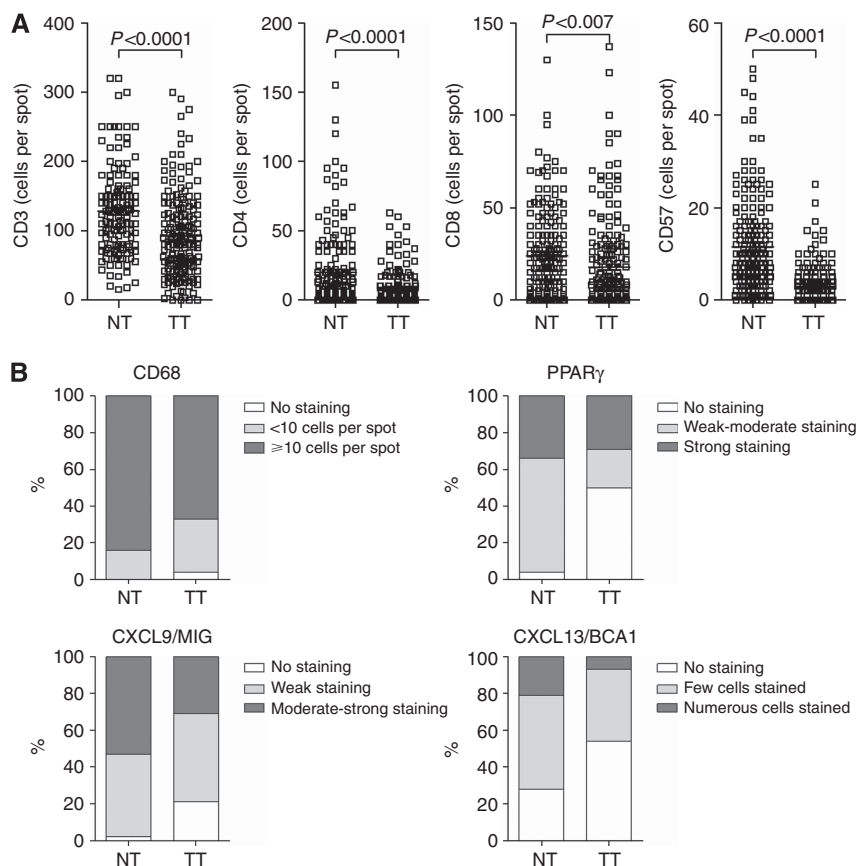[b]Cox-Wald χ^2-test P-value.
[c]Vascular/lymphatic/perineural invasion.

Figure 1. Immune environment differ between tumour tissue and matched normal tissue. (A) Enumeration of positive cells per spot in normal (NT) and tumour tissues (TT) was assessed for CD3, CD4, CD8, and CD57. (B) As in A but CD68-positive cells were studied in three semi-quantitative categories: no staining (white bars); 1 to 10 cells per spot (light grey bars); more than 10 cells per spot (dark grey bars) as for CXCL9/MIG, PPARγ but using three qualitative categories: no staining (white bars); weak to moderate staining (light grey bars); moderate to strong staining (dark grey bars) as indicated in the figure, and for CXCL13/BCA1 no staining (white bars); few cells stained (light grey bars); numerous cells stained (dark grey bars). Paired t-test for quantitative variables and Bowker's test of symmetry for qualitative staining variables, $P < 0.05$ was considered as significant.

associated with RFS and OS (Table 3B). The hazard ratio (HR) for the risk of relapse was 2.7 (95% confidence interval (CI): 1.2–5.7) in patients with ⩽2 CD57 + TIC per spot as compared with those with >2 CD57 + TIC per spot, and 3.5 (1.4–9.1) in patients with no CD68 + TIC as compared with those with >10 CD68 + TIC per spot. However, the risk of relapse was not significantly increased in patients with 1–10 CD68 + TIC per spot as compared with those with >10 CD68 + TIC per spot (HR: 1.2; 95% CI: 0.6–2.3). Similar results were observed regarding OS (Table 3B). Of note, the prognostic effect of CD57 + and CD68 + TIC densities on RFS and OS did not significantly differ according to the disease stage (that is, stage II vs stage III) (Supplementary Table 3).

Finally, we generated a prognostic score based on the combination of CD57 + (+, > vs −, ⩽2 cells per spot) and CD68 + (+, >0 vs −, =0 cells per spot) TIC densities. This three-category prognostic score discriminated CRC patients at low (CD68 +, CD57 +), intermediate (CD68 +, CD57 −), or high (CD68 −, CD57 −) risk, with HRs for the intermediate-risk and high-risk groups of 2.7 (95% CI: 1.3–5.8) and 9.0 (3.2–25.4) for RFS, and 2.5 (1.2–5.1) and 10.6 (3.8–29.2) for OS, respectively, as compared with the low-risk group. Corresponding 5-year survival rates in the low-, moderate-, and high-risk groups were 84% (71–91), 65% (54–74), and 12% (2–47), respectively, for RFS, and 91% (80–96), 76% (66–84), and 25% (7–59), respectively, for OS (Figure 3).

DISCUSSION

In the present study, we showed that tumour infiltration by CD57 + and CD68 + cells is an independent prognostic factor for RFS and OS in patients with pMMR CRC, regardless of disease stage (that is, stage II or III, Supplementary Table 3). Although CD3 + TIC density was also prognostic for RFS and OS in univariate analysis in accordance with other reports (Pages et al, 2005), it was correlated with CD57 + TIC density and did not remain an independent prognostic factor after taking into account CD57 + TIC density in multivariate analysis.

Unlike in previous studies (Pages et al, 2005; Galon et al, 2006), we used stringent patient eligibility criteria and methods for this study. First, only patients with stage II or III disease were included. In fact, additional prognostic biomarkers are mainly needed for such patients, as the vast majority of stage I CRC patients will never relapse after curative surgery while most stage IV CRC patients will die from their disease. Second, we excluded patients with dMMR CRC, as an increased number of TIL is a well-known pattern in dMMR CRC (Jass et al, 1998; Laghi et al, 2009). Third, we excluded patients who received preop-RT. As previously suggested by others, we showed here that preop-RT strongly influenced immune microenvironment. Indeed, radiation therapy may enhance tumour-associated antigen and major

Table 3A. Prognostic value of CD3, CD8, CD4, CD57, CD68, CXCL9/MIG, PPARγ, and CXCL13/BCA1 in tumour tissue from patients without preop-RT after adjustment for anatomoclinical factors (tumour location, stage, grade, and adjuvant chemotherapy)							
		Relapse-free survival			Overall survival		
	n	HR	95% CI	*P*	HR	95% CI	*P*
CD3							
CD3 ≤80 cells per spot	76	2.1	1.1–4.0		2.0	1.1–3.8	
CD3 >80 cells per spot	73	1		0.02	1		0.025
Missing	9						
CD4							
CD4 ≤2 cells per spot	88	0.9	0.5–1.6		1.3	0.7–2.4	
CD4 >2 cells per spot	66	1		0.72	1		0.41
Missing	4						
CD8							
CD8 ≤8 cells per spot	80	1.4	0.8–2.6		1.5	0.8–2.7	
CD8 >8 cells per spot	74	1		0.25	1		0.22
Missing	4						
CD57							
CD57 ≤2 cells per spot	100	3.2	1.5–6.6		2.9	1.4–5.9	
CD57 >2 cells per spot	56	1		0.002	1		0.003
Missing	2						
CD68							
CD68 =0 cells per spot	8	5.4	2.1–13.4		7.6	3.0–18.8	
1≤ CD68 ≤10 cells per spot	55	1.4	0.8–2.7		1.9	1.0–3.5	
CD68 >10 cells per spot	94	1		0.0016	1		<0.0001
Missing	1						
CXCL9/MIG							
No staining	36	3.3	1.3–8.4		3.2	1.2–8.4	
Weak staining	80	2.3	1.0–5.3		2.6	1.0–6.3	
Moderate to strong	42	1		0.041	1		0.063
Missing	0						
PPARγ							
No staining	73	1.3	0.7–2.5		0.7	0.4–1.4	
Mild-to-moderate	33	0.7	0.3–1.8		0.7	0.3–1.7	
Strong staining	51	1		0.38	1		0.57
Missing	1						
CXCL13/BCA1							
No staining	85	1.0	0.3–3.3		3.0	0.4–22.2	
Few lymphocytes stained	57	1.0	0.3–3.3		3.3	0.4–24.5	
Numerous lymphocytes stained	12	1		0.98	1		0.51
Missing	4						
Abbreviations: CI = confidence interval; HR = hazard ratio.							

histocompatibility complex-I expressions, increase CD8+ and CD4+ tumour cell infiltration, and favour the antigen-capture by dendritic cells that may promote immunological recognition of tumour cells (Lugade *et al*, 2008; Teitz-Tennenbaum *et al*, 2008; Sharma *et al*, 2011). Recent works have highlighted the immuno-logical pathways involved in immunogenic cell death after irradiation of tumour in mice models. Irradiation may lead to calreticulin exposure at the cell surface of tumour cells, secretion of HMGB1 and ATP from tumour cells and IL-1β from antigen-presenting cells (APC) resulting in a better uptake of apoptotic bodies by APC and their maturation leading to an induction/amplification of specific tumour antigen IFNγ + CD8+ T cells at the tumour site (Apetoh *et al*, 2007; Obeid *et al*, 2007; Ghiringhelli *et al*, 2009). Therefore, we excluded patients who received preop-RT from the analysis. Fourth, we showed that the stainings of all the immune markers we studied were significantly decreased in the centre of the tumour. This suggests that tumour microenvironment hampers immune cells to infiltrate deeply within the tumour. Furthermore, we only focused the analysis of the immune infiltrate in the centre of the tumour as our TMA did not target the invasive margin of the tumour samples. Therefore, we did not assess the peritumoural nor the crohn-like lymphocytic reactions, which have been shown to be
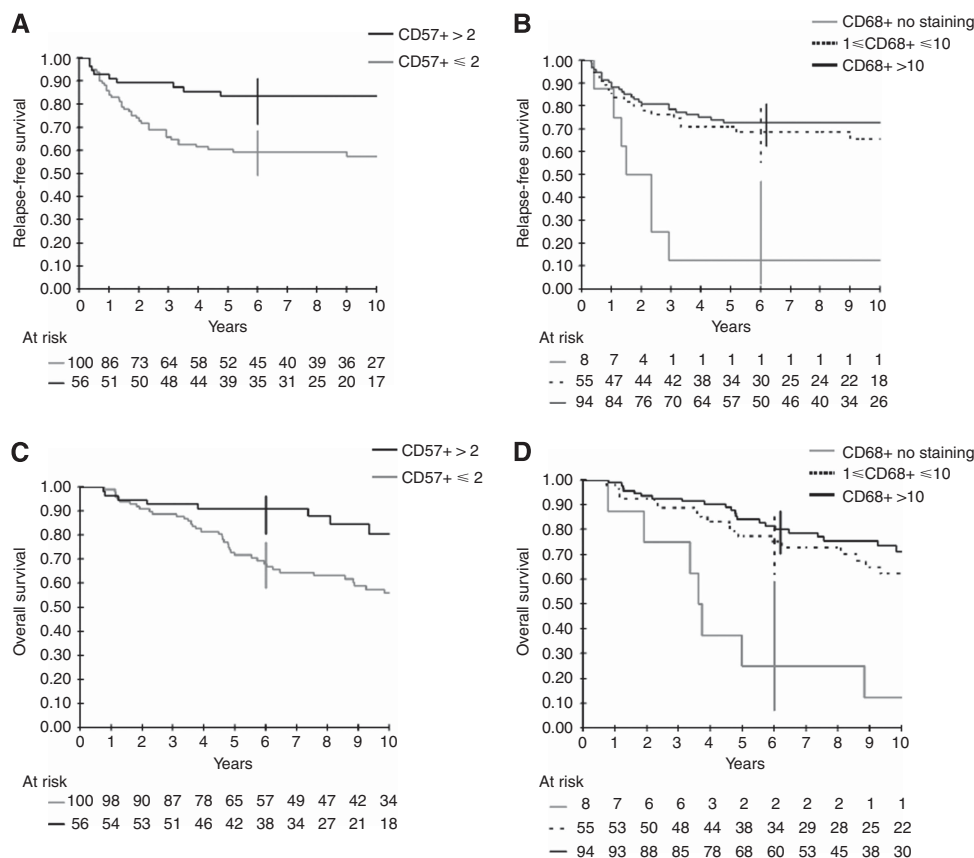
Figure 2. Relapse-free survival according to CD57 (**A**) and CD68 (**B**) tumour-infiltrating cells density and OS according to CD57 (**C**) and CD68 (**D**) tumour-infiltrating cells density.

Table 3B. Multivariate analysis adjusted for anatomoclinical parameters (tumour location, stage, grade, and adjuvant chemotherapy) for patients without preop-RT (model based on 156 patients because of missing value for two patients)

	n	Relapse-free survival			Overall survival		
		HR	95% CI	P	HR	95% CI	P
CD57							
CD57 ⩽2 cells per spot	100	2.7	1.3–5.7		2.3	1.1–4.8	
CD57 >2 cells per spot	56	1		0.011	1		0.026
CD68							
CD68 = 0 cells per spot	8	3.6	1.4–9.1		5.3	2.1–13.6	
1 ⩽ CD68 ⩽10 cells per spot	55	1.2	0.6–2.3		1.6	0.9–3.0	
CD68 >10 cells per spot	93	1		0.03	1		0.002

Abbreviations: CI = confidence interval; HR = hazard ratio.

associated with longer survival in CRC (Ogino et al, 2009, 2010). However, the cell counting may be more reproducible in the centre of the tumour, the invasive/peritumoural margin being potentially difficult to delineate reliably and thus difficult to incorporate in the daily practice. In addition, Galon et al (2006) and others (Sinicrope et al, 2009; Ogino et al, 2010) showed that CD3 + TIL density within the tumour was also prognostic of disease-free survival (DFS), although less significantly than in the combined region

analysis (centre of the tumour and invasive margin) or than a score based on the overall lymphocytic reaction calculated as the sum of scores of Crohn's like lymphoid reaction, peritumoural lymphocytic reaction, and tumour-infiltrating lymphocytes (Ogino et al, 2009). In the study by Laghi et al (2009) in which patient selection was closer to ours (i.e., Stage II and III without preop-RT), CD3 + TIL density was found to be prognostic only in stage II, but not in stage III CRC. In this study CD3 expression was analysed as a continuous variable at the tumour invasive margin on whole slides and CD57 + tumour cell infiltrate was not studied (Laghi et al, 2009). Finally, Galon et al (2006) explored mainly CD3, CD8, and CD45RO and did not assessed CD57 + and CD68 + tumour cell infiltrate.

We found that the complete absence of CD68 + TIC is characteristic of a small subset of patients with a very poor prognosis. Several reports showed an association between lower CD68 + TIC densities and worse outcome in patients with CRC (Forssell et al, 2007; Algars et al, 2011; Zhou et al, 2011; Zlobec et al, 2011). However, whether CD68 staining corresponds to activated type 1 macrophages or regulatory/suppressive type 2 macrophages, and what are the exact roles of these distinct cell populations in the tumour microenvironment (e.g., induction of regulatory T cells by type 2 macrophages) remain to be elucidated.

We found that CD57 TIC density tended to be associated with that of CD3 ($P = 0.003$, Table 2). Thus, these cells may represent a subpopulation of CD3 + T cells. However, the lack of correlation with CD4 + TIC density and the weak association with CD8 + TIC density ($P = 0.02$, Table 2) suggest that these cells may not belong to the conventional T-cell family. Okada et al (1995) found abundant CD57 + TIC in CRC patients, the majority of which being CD4 −, half CD8 +, and one third $\gamma\delta$TCR +. Other investigators have reported that CD57 + cells were one
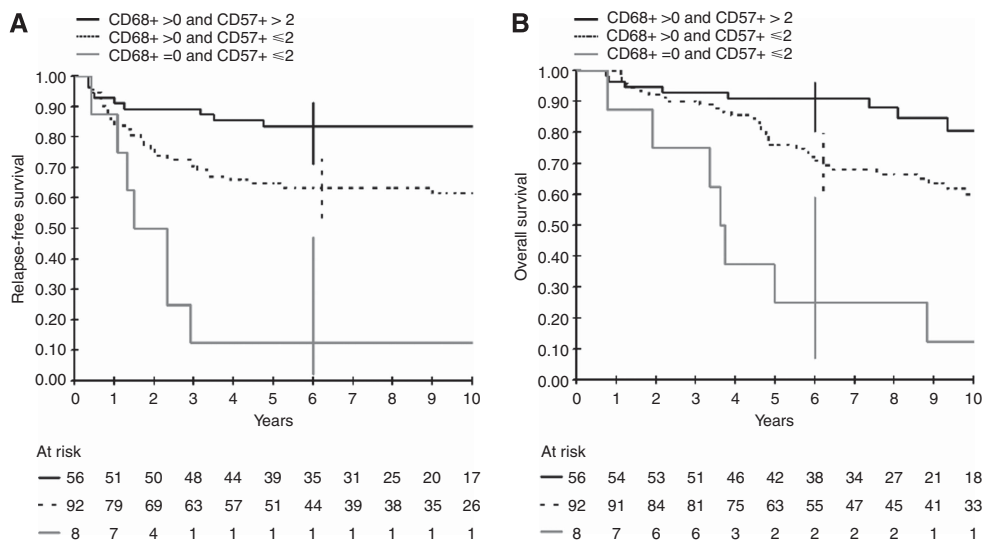
Figure 3. Relapse-free survival (**A**) and OS (**B**) according to the prognostic score combining CD57 and CD68 tumour-infiltrating cells density.

of the major TIL, especially in patients with gastric carcinoma (Karimine *et al*, 1994; Arinaga *et al*, 1995). However, they did not examine whether or not such CD57 + cells express CD3 (or TCR). In other words, they considered these cells as NK cells rather than CD3 + T cells (Karimine *et al*, 1994; Arinaga *et al*, 1995). More recently, CD8 + and CD57 + T cells with cytotoxic functions were found in patients with multiple myeloma (Sze *et al*, 2001). Higher numbers of CD57 + cells (mostly CD8 + T cells and NK cells) were associated with longer progression-free survival in patients with multiple myeloma treated with thalidomide (Mileshkin *et al*, 2007). These CD57 + T cells are often associated with chronic antigenic stimulation and denominated as 'highly antigen experienced CD8 + T cells'; in patients with cancer, chronic stimulation by tumour antigens may be responsible for the differentiation of these T cells within the tumour (Strioga *et al*, 2011). Of note, marked infiltration by CD57 + TIC at the advancing tumour margin was independently associated with longer DFS in one previous series of 93 CRC patients (Menon *et al*, 2004).

We found a strong association between CD57 + and CD68 + TIC densities. We built a strongly prognostic score based on these two immune markers, which allowed us to discriminate three groups of patients with stage II–III CRC harbouring very different risks of relapse and death. This suggests that CD57 + and CD68 + TIC may interact for their recruitment, reciprocal activation, and survival at the tumour site, favouring tumour clearance. However, our hypothesis warrants further investigations.

Our study has several limitations: its retrospective design, the relative small numbers of CRC patients included before the use of modern chemotherapy, that is, FOLFOX regimen that became the standard of care for patients with stage III colon cancer (Andre *et al*, 2004) as well as the unavailability of some established prognostic parameters in CRC patients (e.g., the refined TN substage and the number of examined nodes (AJCC, 2010)) that could not be included in our analyse. Considering the adjuvant treatment, we cannot exclude a potential interaction with such treatment and the immune system. However, there is no preclinical/clinical data supporting an immunogenic cell death induced by 5-FU. Conversely, oxaliplatin may influence anti-tumour immune responses as we showed previously (Tesniere *et al*, 2009). Therefore, such interactions warrant further investigation in future prospective trials. Furthermore, and as discussed earlier, TMA is by itself less indicative than whole slides and can not account for potential heterogeneity in TIC density within the tumour.

In conclusion, CD68 + and CD57 + TIC densities are independent prognostic biomarkers in stage II and III CRC, adding to the growing body of evidence for the influence of the host immune response on CRC outcome. If confirmed in other studies, our results may support the routine use of IHC assessment of both CD68 + and CD57 + TIC densities as a prognostic tool in patients with stage II–III CRC. Whether these markers may also predict the benefit of adjuvant treatment after primary surgery deserves to be assessed. Finally, our results argue for the potential therapeutic interest of immune modulators in CRC patients.

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