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## Characterization of an adaptive immune response in microsatellite-instable colorectal cancer

Florence Boissière-Michot, Gwendal Lazennec, H el ene Frugier, Marta Jarlier,  
Lise Roca, Jacqueline Duffour, Emilie Du Paty, Daniel Laune, France  
Blanchard, Florence Le Pessot, et al.

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1 **Characterization of an adaptive immune response in microsatellite-unstable colorectal**  
2 **cancer**

3

4

5 Florence Boissière-Michot,<sup>1#</sup> Gwendal Lazennec,<sup>2#</sup> H el ene Frugier,<sup>1</sup> Marta Jarlier,<sup>1</sup> Lise Roca,<sup>1</sup>

6 Jacqueline Duffour <sup>1</sup>, Emilie Du Paty,<sup>2</sup> Daniel Laune,<sup>2</sup> France Blanchard,<sup>3</sup> Florence Le Pessot,<sup>3</sup>

7 Jean-Christophe Sabourin,<sup>3</sup> Fr ed eric Bibeau<sup>1</sup>

8

9 Correspondence to: Fr ed eric Bibeau; Email: [Frederic.Bibeau@icm.unicancer.fr](mailto:Frederic.Bibeau@icm.unicancer.fr)

10

11 <sup>1</sup>Institut r egional du Cancer de Montpellier (ICM) - Val d'Aurelle, 34298, Montpellier, France

12 <sup>2</sup>UMR 3145 SYSDIAG CNRS, 34093, Montpellier, France

13 <sup>3</sup>University Hospital, 76038, Rouen, France

14

15 # These authors contributed equally to this work.

16

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20

21 **ABSTRACT**

22 Most sporadic or hereditary colorectal cancers (CRC) with microsatellite instability (MSI) are  
23 frequently characterized by an inflammatory infiltrate of lymphocytes and are associated with  
24 a better outcome than microsatellite stable (MSS) CRC, probably in relation to their more  
25 effective immune response. We investigated the inflammatory mechanisms in 48 MSI CRC and  
26 62 MSS CRC by analyzing i) the expression of 48 cytokines using Bio-plex multiplex cytokine  
27 assays and ii) the *in situ* immune response using immunohistochemistry with anti-CD3 (T  
28 lymphocytes), -CD8 (cytotoxic T lymphocytes), -CD45RO (memory T lymphocytes), -T-bet  
29 (Th1 CD4 cells) and -FoxP3 (regulatory T cells) antibodies. Statistically significant higher  
30 levels of RANTES (CCL5), IL-8 (CXCL8), MIG (CXCL9), IL-1 $\beta$ , IP-10 (CXCL10), IL-16,  
31 GRO $\alpha$  (CXCL1) and IL-1ra and lower levels of MIF were found in MSI CRC than in MSS  
32 CRC. Immunohistochemistry combined with image analysis indicated that the density of CD3<sup>+</sup>,  
33 CD8<sup>+</sup>, CD45RO<sup>+</sup> and T-bet<sup>+</sup> T lymphocytes was higher in MSI than in MSS CRC, whereas the  
34 number of regulatory T cells (FoxP3<sup>+</sup>) was not statistically different between groups. These  
35 results indicate that MSI CRC are associated with a specific cytokine expression profile that  
36 includes the cytokines RANTES, IP-10 and MIG, which are involved in the T Helper 1 response  
37 and in the recruitment of memory CD45RO<sup>+</sup> T cells. Our findings highlight the major role of  
38 adaptive immunity in MSI CRC, thus possibly explaining the better prognosis of this CRC  
39 subtype.

40

41 **Keywords:** colorectal cancer, microsatellite instability, inflammation, cytokines, chemokines.

42

43 **List of abbreviations and acronyms:**

44 CRC: colorectal carcinoma

45 MMR: mismatch repair

46 MSI: microsatellite instability

47 MSS: microsatellite stable

48 IHC: immunohistochemistry

49 Th1: T helper type-1 lymphocytes

50 UICC: [Union for International Cancer Control](#)

51 TMA: tissue microarray

52 *ct*: tumor center

53 *im*: invasive margin

54

## 55 INTRODUCTION

56 The immune contexture of human solid tumors has become an emerging hallmark of cancer  
57 and assessing its impact on the clinical outcome might lead to the identification of new  
58 prognostic markers.<sup>1, 2</sup> Indeed, colorectal carcinomas (CRC) that display a strong and  
59 coordinate adaptive immune response, as indicated by high density of CD45RO<sup>+</sup> memory and  
60 CD8<sup>+</sup> cytotoxic T lymphocytes, have been associated with a good prognosis.<sup>3-5</sup>  
61 CRC is considered to be a heterogeneous disease. About 85% of CRC occur in a context of  
62 chromosomal instability and 15% display a deficiency in the DNA mismatch repair (MMR)  
63 system<sup>6, 7</sup> linked to epigenetic or genetic mechanisms. Defects in the DNA MMR machinery  
64 leads to microsatellite instability (MSI), a condition in which repetitive DNA sequences named  
65 microsatellites accumulate mutations that can affect tumor suppressor genes and oncogenes.<sup>8</sup>  
66 Although not specific, pronounced peritumoral lymphoid reaction (Crohn's-like reaction) and  
67 dense infiltration of the tumor by lymphocytes are typically associated with MSI CRC<sup>9, 10</sup> and  
68 could contribute to their good prognosis.<sup>7, 11</sup> The immunogenicity of MSI CRC is attributed to  
69 the occurrence of microsatellite mutations generating immunogenic neo-antigens.<sup>12-14</sup>

70 One of the mechanisms involved in the recruitment of inflammatory cells at the tumor  
71 site is the expression of cytokines, chemokines and growth factors by the tumor and its  
72 microenvironment. However, beside its positive involvement in the identification and  
73 destruction of cancer cells, inflammation may also play an important role during cancer  
74 development and progression.<sup>15-17</sup> Initiation of carcinogenesis,<sup>18</sup> tumor progression,<sup>19</sup>  
75 angiogenesis<sup>20, 21</sup> and metastatic processes<sup>22, 23</sup> can be modulated by inflammation. Thus,  
76 inflammation appears as a key process, with dual functions, controlling the relationship  
77 between tumor cells and its microenvironment.<sup>24</sup> Some recent works have assessed the  
78 expression of various cytokines, chemokines and their receptors in CRC, but only focused on a  
79 limited number of factors and the tumor MMR status was rarely taken into consideration.<sup>3, 25-28</sup>  
80 Therefore, in this study we wanted to clearly determine the differential role of inflammatory

81 components and tumor microenvironment in CRC relative to their MMR status. To this aim,  
82 we characterized the immune cell infiltrate in 62 microsatellite-stable (MSS) and 48 MSI CRC  
83 by immunohistochemistry (IHC) and quantified their cytokine profile using multiplex-based  
84 assays.

85

## 86 **RESULTS**

### 87 **Clinico-pathological features**

88 At the time of surgery, the median age was 72.5 years (range 30-95) for patients with MSI CRC  
89 and 65.0 years (range 30-86) for patients with MSS CRC ( $p=0.206$ ) (see Table 1). The stage  
90 distribution of tumors was statistically different between groups ( $p=0.018$ ): 52% of MSI CRC  
91 were classified as stage II (versus 44% of MSS CRC) and 29 % of MSS CRC as stage IV (versus  
92 6% of MSI CRC). As expected, MSI CRC were more commonly identified in the right colon  
93 (65% were proximal to the splenic flexure,  $p=0.018$ ) and were more often poorly differentiated  
94 than MSS CRC (35% versus 8%,  $p=0.004$ ). They also displayed a significantly higher level of  
95 lymphocyte infiltration (58% versus 37%,  $p=0.034$ ) and **expansile tumor border configuration**  
96 **(55% versus 33%,  $p=0.040$ )** as assessed by morphological evaluation.

97

### 98 **Inflammatory infiltrate in MSS and MSI CRC**

99 IHC analysis of tumor inflammatory cells showed a higher infiltration of CD3<sup>+</sup> lymphocytes in  
100 the *ct* and *im* areas of MSI CRC than MSS CRC (mean  $\pm$  SD: 1335  $\pm$  1320 *versus* 777  $\pm$  718 in  
101 the *ct* area,  $p=0.046$ ; 1574  $\pm$  1017 *versus* 1183  $\pm$  1342 in the *im* area,  $p=0.008$ ). The number of  
102 CD8<sup>+</sup> lymphocytes also was significantly more elevated in MSI CRC than MSS CRC (mean  $\pm$   
103 SD: 717  $\pm$  724 *versus* 262  $\pm$  349 in the *ct* area,  $p < 0.001$ ; 837  $\pm$  629 *versus* 539  $\pm$  631 in the *im*  
104 area,  $p=0.001$ ), indicating an efficient recruitment of cytotoxic cells (Figure 1).

105 As T helper type-1 lymphocytes (Th1) have a crucial role in activating cytotoxic T  
106 lymphocytes, the T-bet<sup>+</sup> population, which is representative of the Th1 CD4 T cell subset, was  
107 then quantified. The density of T-bet<sup>+</sup> cells in MSI CRC was significantly higher than in MSS  
108 CRC samples in both studied areas (mean  $\pm$  SD: 453  $\pm$  492 *versus* 115  $\pm$  141 in the *ct* area,  
109  $p < 0.001$ ; 115  $\pm$  93 *versus* 64  $\pm$  74 in the *im* area,  $p = 0.001$ ). Similarly, the number of CD45RO  
110 effector T cells was higher in MSI than in MSS CRC samples (mean  $\pm$  SD: 1461  $\pm$  1031 *versus*  
111 798  $\pm$  743 in the *ct* area,  $p < 0.001$ ; 2716  $\pm$  1620 *versus* 2195  $\pm$  2186 in the *im* area,  $p = 0.025$ ).  
112 On the other hand, FoxP3<sup>+</sup> cells, which are representative of the regulatory T cell (Tregs)  
113 population, were similarly recruited in MSI and MSS CRC (mean  $\pm$  SD: 250  $\pm$  183 *versus* 305  
114  $\pm$  237 in the *ct* area,  $p = 0.276$ ; 343  $\pm$  303 *versus* 356  $\pm$  441 in the *im* area,  $p = 0.490$ ).

115 To determine whether other specific inflammatory populations were recruited,  
116 macrophages and B-lymphocytes were quantified by using anti-CD68 and -CD20 antibodies,  
117 respectively. MSI CRC displayed a significant higher number of CD68<sup>+</sup> macrophages (mean  $\pm$   
118 SD: 626  $\pm$  364 *versus* 339  $\pm$  285 in the *ct* area,  $p < 0.001$ ; 908  $\pm$  579 *versus* 683  $\pm$  653 in the *im*  
119 area,  $p = 0.019$ ), whereas the density of tumor-infiltrating B cells was similar in both groups  
120 (mean  $\pm$  SD: 36  $\pm$  93 *versus* 44  $\pm$  168 in the *ct* area,  $p = 0.629$ ; 255  $\pm$  556 *versus* 406  $\pm$  993 in  
121 the *im* area,  $p = 0.712$ ).

122

### 123 **Cytokines expression in MSS and MSI CRC**

124 Using multiplex assays that allow the measurement of 48 cytokines, many cytokines could not  
125 be detected (median = 0; IL-2, IL-4, IL-5, IL-9, IL-10, IL-13, IL-15, MIP-1 $\alpha$ , G-CSF, TNF $\alpha$ ,  
126 PDGFbb) or were barely detectable (median  $< 1$ pg/ $\mu$ g of total proteins; IL-1 $\alpha$ , IL2-R $\alpha$ , IL-6,  
127 IL-7, IL-12(p70), IL-17, IL-18, LIF, Eotaxin, CTACK, IFN- $\gamma$ , MCP-1, MCP-3, MIP-1 $\beta$ ,  $\beta$ -  
128 FGF,  $\beta$ -NGF, IFN- $\alpha$ 2, GM-CSF, M-CSF, SCF, TNF $\beta$ , TRAIL) in both MSS and MSI CRC  
129 protein samples (Table 2).

130 MSI CRC displayed a specific cytokine profile compared to MSS CRC: RANTES, IL-  
131 8, MIG, IL-1 $\beta$ , IP-10, IL-16, GRO $\alpha$  and IL-1ra were significantly overexpressed, whereas the  
132 level of MIF was decreased (Table 2). RANTES, IP-10, IL-8, MIG and IL-1 $\beta$  showed the  
133 strongest increase (between 12.9 and 2.3-fold) in MSI CRC compared to MSS CRC samples.

134 Finally, variations in cytokine expression within the MSS CRC group were analyzed by  
135 comparing MSS CRC with strong lymphocytic infiltration and/or Crohn's-like lymphocytic  
136 reaction (inflammatory MSS CRC,  $n=29$ ) and MSS CRC without these features (non-  
137 inflammatory MSS CRC,  $n=33$ ). The initial classification in these two subgroups by  
138 morphological evaluation was validated by IHC: the density of intra-tumoral CD3<sup>+</sup>  
139 lymphocytes was higher in inflammatory than in non-inflammatory MSS CRC (mean  $\pm$  SD:  
140  $882 \pm 612$  versus  $685 \pm 798$  cells/mm<sup>2</sup>,  $p=0.044$ ; Table 3). Despite this significant difference,  
141 inflammatory and non-inflammatory MSS CRC had comparable cytokine content. Conversely,  
142 although a similar density of CD3<sup>+</sup> lymphocytes was observed in inflammatory MSS CRC and  
143 MSI CRC ( $882 \pm 612$  versus  $1335 \pm 1320$  CD3<sup>+</sup> cells/mm<sup>2</sup>,  $p=0.391$ ), the levels of RANTES,  
144 IL-8, MIG, IL-1 $\beta$ , IP-10, IL-16 and IL-1ra remained significantly higher in the MSI group  
145 (Table 3). These data suggest that the cytokine expression profile observed in MSI CRC is  
146 linked to the MSI status and to the distinct inflammatory infiltrate observed in this CRC  
147 subgroup.

148 We also investigated the correlations between cytokines levels and specific immune  
149 densities in MSS and MSI groups. We identified significant correlations between different  
150 subsets of immune cells and chemokine expression, mainly MIG and IP-10 (Supplementary  
151 Table 1). In MSS CRC high levels of MIG were associated with a significantly increased  
152 intratumor density of CD3, CD8 and T-Bet T cells. Interestingly, in MSI CRC, these  
153 correlations were strongest, also involving CD45RO population, and were not limited to the  
154 center of the tumor. Taken together, these data showed that *in situ* immune cells are strongly  
155 associated with specific chemokines pattern, indicating a specific coordinated process.



156

## 157 **DISCUSSION**

158 The tumor microenvironment, notably the immune response, may play an important role in  
159 CRC progression and control, in addition to the tumor morphological and molecular features.<sup>29,</sup>

160 <sup>30</sup> Here, we show that MSI CRC display a specific *in situ* immune response and chemokine  
161 profile compared to MSS CRC. This particular inflammatory microenvironment could explain  
162 the better clinical course of this CRC subtype.

163 By using image analysis that allows the objective quantification of the positive cells and  
164 minimizes the observer's bias, we found a significant increase of CD3<sup>+</sup>, CD8<sup>+</sup>, CD45RO<sup>+</sup> and  
165 T-bet<sup>+</sup> lymphocytes in MSI CRC compared to MSS CRC, whereas the density of FoxP3<sup>+</sup> cells  
166 was similar in both groups. These results are in agreement with previous studies that also  
167 reported a relationship between MSI and density of CD3<sup>+</sup>,<sup>31-33</sup> CD8<sup>+</sup>,<sup>26, 31, 33-35</sup> and CD45RO<sup>+</sup>  
168 in CRC.<sup>30, 35</sup> Several studies provide compelling evidence that effector/cytotoxic (CD3<sup>+</sup> and  
169 CD8<sup>+</sup>) and memory (CD45RO<sup>+</sup>) T-cells play major roles in the anti-tumor immune response in  
170 CRC and that their high expression correlate with a good clinical outcome (for a review, see  
171 Fridman et al.,<sup>2</sup>). CD8<sup>+</sup> cytotoxic T lymphocytes can kill efficiently tumor cells and are mainly  
172 activated through the Th1 pathway. One way to analyze the Th1 pathway is to assess the  
173 expression of the Tbox transcription factor T-bet, which is crucial for the development of  
174 effector Th1 CD4 T cells<sup>36</sup> and, to date, the most specific marker for this cell subset. T-bet  
175 mRNA levels and T-bet *in situ* expression in CRC have been previously correlated with reduced  
176 tumor recurrence.<sup>5, 26</sup> Here we show for the first time that T-bet<sup>+</sup> lymphocytes are significantly  
177 increased in MSI CRC, highlighting an efficient Th1 response that could account for the good  
178 clinical outcome of this population. Similarly, the increased density of CD45RO<sup>+</sup> cells in MSI  
179 CRC compared to MSS CRC might also strengthen the efficiency of the anti-tumor immune  
180 response. Indeed, CD45RO<sup>+</sup> cells, which include both antigen-exposed CD4<sup>+</sup> and CD8<sup>+</sup>  
181 lymphocytes, respond faster and with increased intensity to antigenic stimulation than naive T

182 cells.<sup>2</sup> Combined with the similar density of FoxP3<sup>+</sup> cells found in MSI and MSS CRC, these  
183 results suggest that in MSI CRC the balance is toward an effective host-mediated immune  
184 response rather than tolerance induction. Indeed, Tregs, which express the nuclear transcription  
185 factor FoxP3, modulate the anti-tumor immune response<sup>25, 33, 35, 37-43</sup> and suppress the activity  
186 of cytotoxic T cells (reviewed in deLeeuw *et al*<sup>44</sup>), thus maintaining immunologic tolerance.  
187 Few studies have evaluated the relationship between FoxP3<sup>+</sup> and the MSI status, leading to  
188 controversial results in terms of prognosis.<sup>25, 30, 33, 35, 45</sup> [Correale, 2010 #2278](#); [Lee, 2010 #2284](#)}

189 It has been proposed that the local immune response in MSI CRC could be related to  
190 the potentially immunogenic neopeptides produced by frameshift mutations in microsatellites  
191 sequences. Accordingly, Tougeron et al. have described a significant association between CD3<sup>+</sup>  
192 density and the overall number of frameshift mutations.<sup>46</sup> The local inflammatory reaction  
193 might thus promote cytokine production, which in turn, could expand the immune recruitment.  
194 On the other side, [the specific cytokine profile we identified in this study involves mostly](#)  
195 [chemokines, namely CXCL1 \(Gro- \$\alpha\$ \), CXCL8 \(IL-8\), CXCL9 \(MIG\), CXCL10 \(IP-10\) and](#)  
196 [CCL5 \(RANTES\). Based on the literature, these chemokines could be produced by immune](#)  
197 [cells, but a number of reports on colon cancer have shown that they are mainly produced by](#)  
198 [tumor cells and stromal cells rather than by Th1, Th2 or Treg cells. Indeed, CXCL1 is mainly](#)  
199 [detected in colon cancer cells and at a lesser extent in mesenchymal cells<sup>47, 48</sup>. As CXCL10<sup>49,</sup>](#)  
200 [<sup>50</sup>, CXCL8 is mainly produced by tumor cells<sup>51, 52</sup> but also detected at weaker levels in](#)  
201 [macrophages, lymphocytes and myofibroblasts<sup>52</sup>. There is not any paper describing the](#)  
202 [identification of the cells producing CXCL9 in colon cancer, but the source could be neutrophils](#)  
203 [<sup>53</sup> or M2 macrophages<sup>54</sup>. Finally, one report suggests that CCL5 is produced by lymphocytes](#)  
204 [in colon cancer<sup>50</sup>, even if in a general manner, CCL5 could be also produced by tumor](#)  
205 [associated macrophages<sup>55</sup>. The correlations we observed between various cytokines and](#)  
206 [specific subsets of immune cells to different locations in the MSS or MSI tumors, suggest a fine](#)  
207 [regulation of \*in situ\* inflammatory recruitment but whether the cytokine profile is a cause or](#)

208 consequence of immune infiltration remains to be fully understood. However, within the tumor  
209 stroma, chemokines are generally thought to play a role in the recruitment of immune cells.  
210 Some of these chemokines are characterized by anti-tumor activity, whereas others are either  
211 pro-tumorigenic or controversial. Specifically, besides its chemo-attractant properties for T  
212 lymphocytes, monocytes, natural killer cells and eosinophils,<sup>56</sup> RANTES promotes tumor  
213 growth and metastasis by inducing tumor cell proliferation, migration, angiogenesis, or  
214 expression of matrix metalloproteinases in various cancer types.<sup>57-60</sup> Moreover, it can recruit  
215 Tregs within the tumor to kill cytotoxic CD8<sup>+</sup> T cells,<sup>61</sup> suggesting that RANTES  
216 overexpression promotes an immunosuppressive tumor microenvironment, which might help  
217 tumor progression. Similarly, IL-8 possess tumorigenic and pro-angiogenic properties both in  
218 vitro and in vivo in CRC.<sup>62</sup> In agreement with our study, Banerjea et al. described increased  
219 IL8 levels in MSI CRC.<sup>63</sup> The overexpression of these chemokines in the MSI CRC, which are  
220 considered to have good prognosis, suggests the presence of regulatory pathways that  
221 counterbalance their pro-tumorigenic effects.

222 The Th1-type inflammatory mediators MIG (CXL9) and IP-10 (CXL10), two IFN- $\gamma$ -  
223 inducible CXCR3 ligands, act as angiostatic regulators<sup>64</sup> and promote the infiltration and  
224 expansion of anti-tumor T lymphocytes, particularly CD8<sup>+</sup> effector T cells<sup>65, 66</sup> and memory  
225 CD45RO<sup>+</sup> T cells.<sup>3</sup> In our hands, tumors displaying high levels of MIG and IP-10 were  
226 associated with significantly higher densities of CD3<sup>+</sup>, CD8<sup>+</sup>, T-Bet<sup>+</sup>, with stronger *in situ*  
227 recruitment in MSI tumors. Our results are in line with previous reports that showed a  
228 correlation between high *MIG* and *IP-10* mRNA expression levels in CRC and increased  
229 density of CD8<sup>+</sup>, CD4<sup>+</sup> cells and macrophages,<sup>3, 49, 50</sup> Moreover, these chemokines have been  
230 associated with better outcome.<sup>3</sup> Thus, together with the increased density of T-bet<sup>+</sup> cells in  
231 MSI CRC, the higher expression levels of MIG and IP-10 suggest a host protection via the  
232 generation of a Th1 immune response.

233 The role of GRO $\alpha$  in CRC progression is controversial. High GRO $\alpha$  expression has been  
234 associated with shorter recurrence-free survival in stage III patients<sup>27</sup> and its down-regulation  
235 resulted in a nearly complete inhibition of tumor growth in nude mice.<sup>67</sup> However, *GRO $\alpha$*   
236 transcription level is higher in the less invasive tumors and in samples from <65 years old  
237 patients.<sup>68</sup> These results could be related to the stronger immune response more frequently  
238 observed in younger patients and to the fact that MSI CRC are often early stage tumors.

239 In summary, our data suggest a fine regulation of the immune contexture in MSI CRC,  
240 leading to an efficient recruitment of inflammatory cells through the expression of specific  
241 chemokines. They also reveal a Th1-polarized immune response in MSI CRC through the  
242 activation of the IP-10/MIG axis. This translates in the local recruitment or expansion of  
243 specific inflammatory populations that are involved in the anti-tumor response and immune  
244 surveillance and probably accounts for the favorable outcome of this tumor subtype.

245

## 246 **PATIENTS AND METHODS**

### 247 **CRC samples and patients**

248 All CRC resection specimens with documented MMR status and available frozen tissue  
249 samples that included at least 50% of tumor cells were identified at the Pathology departments  
250 of the Val d'Aurelle Cancer Centre and Rouen University Hospital. [All samples have been](#)  
251 [homogeneously collected with fresh biopsies taken in the vicinity of the tumor invasion front](#)  
252 [before flash freezing in liquid nitrogen](#). Thus, 110 CRC samples of which 48 had MSI were  
253 selected for this study. Among the 48 MSI CRC, 11 were from patients with Lynch syndrome,  
254 as defined by the presence of a deleterious germline mutation of a gene of the MMR system.  
255 Tumor samples were collected following French laws under the supervision of an investigator  
256 and declared to the French Ministry of Higher Education and Research (declaration number  
257 DC-2008-695). All patients were informed about the use of their tissue samples for biological  
258 research and a written informed consent was systematically obtained for analysis of germline

259 mutations in the MMR system. The study was approved by the local translational research  
260 committee and was in accordance with the Helsinki Declaration of 1975. All samples were  
261 anonymized and analyses were performed blinded to the clinico-pathological data.  
262 Haematoxylin and eosin slides were reviewed by a gastrointestinal surgical pathologist (FB) to  
263 identify morphological features, including histologic differentiation, lymphocytic infiltration,  
264 Crohn's-like reaction and tumor border configuration.<sup>69</sup> All tumors were staged according to  
265 the TMN classification system (7<sup>th</sup> edition) of the Union for International Cancer Control  
266 (UICC). The patients' clinico-pathological features are reported in Table 1.

267

### 268 **MMR status assessment**

269 The MMR status was assessed by IHC analysis of the hMLH1, hMSH2, hMSH6 and PMS2  
270 proteins and by PCR analysis of microsatellites as previously described.<sup>70</sup>

271

### 272 **Protein extract preparation**

273 Frozen CRC samples were sectioned into 15 µm-thin slices to obtain 25-100 mg of tissue that  
274 was collected in Lysing Matrix D tubes (MP Biomedicals, # 116913500). Samples were crushed  
275 in TEG (10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA and 10% glycerol) containing protease  
276 inhibitors (20 µg/ml aprotinin, 20 µg/ml leupeptin, 10 µg/ml pepstatin A and 0.40 µg/ml  
277 phenylmethylsulfonyl fluoride) using a MagNA lyser (Roche Diagnostics) and then centrifuged  
278 at 13,000g at 4°C for 20 minutes. Total protein concentration was measured in the supernatant  
279 using the Bradford assay.

280

### 281 **Bio-plex multiplex cytokine assays**

282 Two Bio-Plex Pro<sup>TM</sup> Human kits (BioRad, #171-A11127 and #171-A11171) were used to  
283 measure the amount of cytokines, chemokines and growth factors in CRC samples, following  
284 the manufacturer's instructions, as previously described.<sup>71</sup> The first multiplex assay detected 27

285 proteins (27 plex assay: IL-1 $\beta$ , IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10,  
286 IL-12 [p70], IL-13, IL-15, IL-17, Eotaxin (CCL11), b-FGF, G-CSF, GM-CSF, IFN- $\gamma$ , IP-10  
287 (CXCL10), MCP-1 (CCL2), MIP-1 $\alpha$  (CCL3), MIP-1 $\beta$  (CCL4), PDGFbb, RANTES (CCL5),  
288 TNF- $\alpha$ , VEGF) and the second one 21 additional factors (21 plex assay: IL-1 $\alpha$ , IL-2Ra, IL-3,  
289 IL-12 (p40), IL-16, IL-18, CTACK (CCL27), GRO- $\alpha$  (CXCL1), HGF, IFN- $\alpha$ 2, LIF, MCP-3  
290 (CCL7), M-CSF, MIF, MIG (CXCL9),  $\beta$ -NGF, SCF, SCGF- $\beta$ , SDF-1 $\alpha$  (CXCL12), TNF- $\beta$ ,  
291 TRAIL).

292 Coupled beads were incubated with 25  $\mu$ g of total protein samples in a final volume of  
293 50  $\mu$ l. Data on the antibody reactions were acquired using the Bio-Plex system, a dual-laser,  
294 flow-based microplate reader system (BioRad). The concentrations of each target protein  
295 (expressed as pg/ml for 25  $\mu$ g of total proteins) were matched to the clinico-pathological data.

296

### 297 **Tissue Microarrays**

298 After reviewing the archived tumor slides, tissue microarray (TMA) were prepared. Triplicate  
299 tissue cores (0.6 mm in diameter) were obtained from the tumor center (referred as *ct*) and from  
300 the invasive margin (referred as *im*), and arrayed using a manual arraying instrument (Beecher  
301 Instrument, MTA1).

302

### 303 **Evaluation of tumor-infiltrating inflammatory cells**

304 Tissue-microarray sections were incubated with monoclonal antibodies against CD3 (clone  
305 LN10, Menarini), CD8 (clone C8/144B, Dako), CD45RO (clone UCHL1, Dako), FoxP3 (clone  
306 236A/E7, AbCam), T-Bet (clone 4B10, SCB), CD20 (clone L26, Dako) and CD68 (clone KP1,  
307 Dako) on a Autostainer Link48 platform (Dako) using Flex<sup>®</sup> system for signal amplification  
308 and diaminobenzidine tetrahydrochloride–chromogen (DAB) as chromogen.

309 Immunoreactive cells were automatically quantified with the Spot Browser software  
310 (Excilone) as previously described.<sup>5</sup> Measurements were recorded as the number of positive

311 cells per mm<sup>2</sup> of tissue surface. Results were exported into an Excel file and data from triplicate  
312 cores were consolidated into a single score that was matched to the clinico-pathological data.

313

### 314 **Statistical analysis**

315 Continuous variables were described using mean, standard deviation, median and range. For  
316 categorical variables, frequencies and percentages were computed. Possible associations  
317 between the microsatellite status and the clinico-pathological parameters were investigated  
318 using the  $\chi^2$  test. The non-parametric Mann-Whitney test was used for continuous variables  
319 (quantification of cytokines and immunophenotypic markers). Differences were considered  
320 statistically significant when the p-value was < 0.05, except for the cytokine analyses for which  
321 the statistically significant threshold was [corrected with the Bonferroni method to account for](#)  
322 [multiple testing and set](#) at 0.001. All statistical analyses were performed using STATA 10.0  
323 (StataCorp).

324

325

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**Table 1. Characteristics of the study population**

<b>Parameter</b>	<b>All CRC <i>n</i> (%)</b>	<b>MSS CRC <i>n</i> (%)</b>	<b>MSI CRC <i>n</i> (%)</b>	<b>p- value<sup>1</sup></b>
Total. <i>n</i>	110	62	48	
Sex				1.000
Male	54 (49)	30 (48)	24 (50)	
Female	56 (51)	32 (52)	24 (50)	
Median age at surgery [range]	67.0 [30-95]	65.0 [30-86]	72.5 [30-95]	0.206
Stage				0.022
I	12 (11)	6 (10)	6 (13)	
II	52 (47)	27 (44)	25 (52)	
III	25 (23)	11 (17)	14 (29)	
IV	21 (19)	18 (29)	3 (6)	
Tumor location				0.018
Right-sided	57 (52)	26 (42)	31 (65)	
Other	53 (48)	36 (58)	17 (35)	
Histologic differentiation				0.004
Poorly differentiated	22 (20)	5 (8)	17 (35)	
Moderately differentiated	48 (44)	32 (52)	16 (33)	
Well differentiated	22 (20)	15 (24)	7 (15)	
Mucinous	18 (16)	10 (16)	8 (17)	
Tumor border configuration				0.040
Expansile	42 (42)	19 (33)	23 (55)	
Infiltrative	58 (58)	39 (67)	19 (45)	
NA	10	4	6	
Lymphovascular invasion				0.432
Yes	41 (37)	21 (34)	20 (42)	
No	69 (63)	41 (66)	28 (58)	
Perineural invasion				1.000
Yes	11 (10)	6 (10)	5 (10)	
No	99 (90)	56 (90)	43 (90)	
Signet ring cell carcinoma				1.000
Yes	4 (4)	2 (3)	2 (4)	
No	106 (96)	60 (97)	46 (96)	
Median number of lymph nodes examined [Range]	26 [3-84]	26 [3-84]	25 [4-71]	0.419
Crohn's-like reaction				0.199
Yes	31 (28)	14 (23)	17 (35)	
No	79 (72)	48 (77)	31 (65)	
Lymphocyte infiltration <sup>2</sup>				0.034
Yes	51 (46)	23 (37)	28 (58)	
No	59 (54)	39 (63)	20 (42)	

<sup>1</sup> *p* value of Fisher's exact test or Mann-Whitney test as appropriate.<sup>2</sup> assessed on HES sections by a single pathologist blinded to the clinico-pathological data (No: no patent tumor infiltrating lymphocytes- Yes: infiltrating lymphocytes)

CRC, colorectal cancers; MSI, microsatellite instable; MSS, microsatellite stable.

542 **Table 2. Differential expression of cytokines, chemokines and growth factors in MSS and MSS**  
 543 **colorectal cancers (expressed as pg/ml for 25 µg of total proteins)**

Marker	MSS CRC (n=62)				MSI CRC (n=48)				Ratio (MSI/MSS)	Prb*
	Mean	SD	Median	Range	Mean	SD	Median	Range		
RANTES	0.40	0.80	0.13	[0-4.29]	5.16	18.13	1.37	[0-125.92]	12.9	<0.001
IL-8	14.62	40.13	2.19	[0-284.63]	77.05	149.22	12.70	[.01-507.61]	5.3	<0.001
MIG	6.17	8.77	3.20	[0-49.9]	28.51	35.39	14.16	[0-154.09]	4.6	<0.001
IL1-β	2.24	8.87	0.13	[0-68.39]	5.13	8.86	1.78	[0-41.93]	2.3	<0.001
IP-10	5.84	9.07	1.70	[0-44.67]	61.36	238.43	8.49	[0-1652.31]	10.5	<0.001
IL-16	9.53	8.03	7.91	[1.75-53.59]	14.90	14.89	12.10	[0-87.05]	1.6	0.011
GROα	1.89	2.58	0.94	[0-12.89]	2.84	3.58	1.40	[0-18.59]	1.5	0.033
MIF	106.66	53.64	92.40	[47.68-319.81]	89.23	60.19	76.16	[0-350.63]	0.8	0.039
IL-1ra	80.72	116.43	37.96	[.27-506.35]	105.98	123.77	58.83	[5.61-567.4]	1.3	0.049
MIP-1β	0.55	0.97	0.28	[0-5.92]	1.31	2.08	0.45	[0-10.25]	-	-
LIF	0.08	0.10	0.07	[0-.65]	0.05	0.07	0.01	[0-.33]	-	-
MIP-1α	0.00	0.01	0.00	[0-.04]	0.02	0.05	0.00	[0-.24]	-	-
IFNγ	0.08	0.17	0.00	[0-.89]	0.25	0.44	0.02	[0-1.97]	-	-
IL-18	13.32	47.74	0.85	[.02-270.58]	6.33	24.73	0.31	[0-160.42]	-	-
HGF	8.25	10.86	5.42	[1.55-63.03]	6.16	5.96	3.85	[0-23.19]	0.7	0.108
IL-13	0.02	0.04	0.00	[0-.16]	0.01	0.03	0.00	[0-.18]	-	-
IL-1α	0.26	0.49	0.15	[0-3.02]	0.38	0.62	0.16	[0-3]	-	-
βFGF	0.95	1.29	0.38	[0-4.6]	1.00	1.99	0.17	[0-9.14]	-	-
IL-12p40	2.14	2.72	1.24	[0-14.2]	1.81	2.69	0.69	[0-12.86]	0.8	0.258
CCL11	0.22	0.37	0.05	[0-1.71]	0.19	0.37	0.02	[0-1.73]	-	-
GM-CSF	0.03	0.05	0.00	[0-.19]	0.03	0.04	0.00	[0-.15]	-	-
IL-7	0.59	1.56	0.00	[0-8.9]	0.83	1.72	0.01	[0-9.39]	-	-
SDF1α	2.17	3.05	1.54	[0-16.09]	1.55	1.93	0.43	[0-7.67]	0.7	0.342
MCP-3	0.97	1.93	0.16	[0-10.74]	1.01	1.38	0.63	[0-7.81]	-	-
CCL27	0.28	0.21	0.26	[0-1.17]	0.23	0.22	0.22	[0-.84]	-	-
TNFβ	0.02	0.02	0.03	[0-.1]	0.02	0.03	0.00	[0-.08]	-	-
SCF	0.65	0.71	0.48	[0-3.29]	0.90	0.98	0.60	[0-3.47]	-	-
IL-12p70	0.18	0.31	0.04	[0-1.72]	0.10	0.14	0.02	[0-.53]	-	-
SCFGβ	2.54	3.21	1.28	[0-15.46]	2.57	3.70	1.13	[0-18.79]	1.0	0.404
MCP-1	0.16	0.25	0.08	[0-1.47]	0.16	0.31	0.01	[0-1.53]	-	-
βNGF	0.06	0.10	0.04	[0-.59]	0.05	0.08	0.00	[0-.38]	-	-
IL-10	0.00	0.01	0.00	[0-.03]	0.00	0.00	0.00	[0-.01]	-	-
IL-17	0.05	0.08	0.00	[0-.33]	0.08	0.15	0.00	[0-.55]	-	-
IL-6	0.35	0.76	0.01	[0-4.03]	0.74	1.72	0.02	[0-7.86]	-	-
IL-3	2.98	4.41	1.42	[0-20.35]	3.20	3.93	2.52	[0-18.77]	1.1	0.549
IFN-α2	0.45	0.30	0.43	[0-1.25]	0.42	0.32	0.44	[0-1.33]	-	-
PDGFbb	0.02	0.06	0.00	[0-.32]	0.06	0.27	0.00	[0-1.74]	-	-
IL-2	0.01	0.02	0.00	[0-.12]	0.00	0.01	0.00	[0-.05]	-	-
TRAIL	0.33	0.41	0.21	[0-1.89]	0.33	0.44	0.16	[0-2.24]	-	-
VEGF	12.94	20.97	5.10	[.26-120.91]	9.18	9.98	5.16	[.09-46.65]	0.7	0.786
IL-2Rα	0.32	0.37	0.23	[0-1.83]	0.34	0.38	0.21	[0-1.66]	-	-
TNFα	0.00	0.00	0.00	[0-0]	0.00	0.00	0.00	[0-0]	-	-
G-CSF	0.02	0.05	0.00	[0-.24]	0.05	0.13	0.00	[0-.7]	-	-
IL-9	0.01	0.02	0.00	[0-.11]	0.00	0.01	0.00	[0-.08]	-	-
M-CSF	0.43	0.51	0.33	[0-2.96]	0.36	0.30	0.35	[0-1.1]	-	-
IL-4	0.00	0.00	0.00	[0-0]	0.00	0.00	0.00	[0-0]	-	-
IL-15	0.00	0.00	0.00	[0-0]	0.00	0.00	0.00	[0-0]	-	-
IL-5	0.00	0.00	0.00	[0-0]	0.00	0.00	0.00	[0-0]	-	-

544 \* *p*-value of non-parametric Mann-Whitney test (only shown for biological markers with median ≥1 pg/ml)

545 CRC, colorectal cancers; MSI, microsatellite instable; MSS, microsatellite stable.

**Table 3. Inflammatory cell populations and cytokine expression in inflammatory MSS, non-inflammatory MSS and MSI colorectal cancers (expressed as number of cells/mm<sup>2</sup> and pg/ml for 25 µg of total proteins, respectively)**

Marker	Group 1 (n=29)				Group 2 (n=33)				Group 1 vs 2		Group 3 (n=48)				Group 3 vs 1	
	Inflammatory MSS CRC <sup>(1)</sup>				Non-Inflammatory MSS CRC				Ratio	Prb*	MSI CRC				Ratio	Prb*
	Mean	SEM	Median	Range	Mean	SEM	Median	Range			Mean	SEM	Median	Range		
CD3 <i>ct</i>	882	612	701	[65-2491]	685	798	451	[53-3673]	1.29	0.044	1335	1320	905	[13-5604]	1.51	0.391
CD45RO <i>ct</i>	977	787	705	[64-2788]	625	667	521	[0-3362]	1.56	0.055	1462	1031	1359	[78-4595]	1.49	0.031
CD68 <i>im</i>	779	737	618	[50-3678]	601	568	469	[20-2669]	1.29	0.241	908	579	816	[46-2022]	1.17	0.188
CD68 <i>ct</i>	364	276	328	[14-1198]	317	296	249	[20-1274]	1.15	0.378	626	364	596	[43-1464]	1.72	0.002
FoxP3 <i>ct</i>	254	155	223	[5-534]	352	285	255	[54-1162]	0.72	0.378	250	183	206	[15-820]	0.98	0.701
CD8 <i>ct</i>	303	420	155	[5-1956]	225	272	148	[7-1057]	1.35	0.459	717	724	505	[31-2916]	2.36	0.002
CD45RO <i>im</i>	2045	1525	1715	[139-5965]	2326	2651	1574	[99-11293]	0.88	0.553	2716	1620	2614	[125-6462]	1.33	0.083
T-bet <i>ct</i>	105	121	60	[0-533]	124	159	53	[9-664]	0.85	0.554	453	492	233	[4-2053]	4.30	<0.0001
FoxP3 <i>im</i>	326	321	214	[53-1508]	383	531	177	[0-2568]	0.85	0.603	343	303	259	[0-1159]	1.05	0.761
CD8 <i>im</i>	492	460	345	[40-1806]	579	751	268	[18-3445]	0.85	0.707	837	629	672	[57-3154]	1.70	0.006
CD3 <i>im</i>	1041	936	761	[152-3805]	1308	1621	863	[44-8474]	0.79	0.789	1573	1017	1363	[80-4059]	1.51	0.013
T-bet <i>im</i>	61	61	38	[5-226]	67	84	36	[0-388]	0.91	0.905	115	93	109	[3-386]	1.89	0.005
IL-16	7.14	3.84	6.48	[1.75-14.05]	11.63	10.02	10.54	[2.08-53.59]	0.61	0.059	14.90	14.89	12.10	[0-87.05]	2.09	0.002
IL-1ra	56.38	90.45	27.83	[7-464.13]	102.11	132.94	44.21	[.27-506.35]	0.55	0.080	105.98	123.77	58.83	[5.6-567.4]	1.88	0.007
RANTES	0.29	0.57	0.12	[0-2.37]	0.49	0.96	0.15	[0-4.29]	0.59	0.438	5.16	18.13	1.37	[0-125.92]	17.79	<0.0001
IL-8	12.27	24.75	2.79	[0-114.29]	16.69	50.25	1.51	[0-284.63]	0.74	0.494	77.05	149.22	12.70	[.01-507.6]	6.28	<0.0001
MIG	6.92	8.76	2.66	[0.16-29.43]	5.52	8.86	3.73	[0-49.90]	1.25	0.568	28.51	35.39	14.16	[0-154.09]	4.12	<0.0001
IL-1β	3.43	12.71	0.09	[0-68.39]	1.2	2.48	0.15	[0-10.58]	2.86	0.577	5.13	8.86	1.78	[0-41.93]	1.50	<0.0001
IP-10	6.44	9.98	1.85	[0-44.67]	5.31	8.31	1.54	[0-34.12]	1.21	0.843	61.36	238.43	8.50	[0-1652.31]	9.53	0.003
MIF	107.45	57.42	102.78	[50.73-319.81]	105.96	50.97	87.37	[47.68-226.69]	1.01	0.927	89.23	60.19	76.16	[0-350.63]	0.83	0.084
GROα	1.77	2.51	0.89	[0-12.89]	1.99	2.67	1.00	[0-12.80]	0.89	0.978	2.84	3.58	1.40	[0-18.59]	1.60	0.057

<sup>1)</sup> Based on the presence of a strong lymphocytic infiltration and/or Crohn's-like lymphocytic reaction.

\* *p*-value of non-parametric Mann-Whitney test

CRC, colorectal cancers; MSI, microsatellite instable; MSS, microsatellite stable.

**Supplementary Table 1. Immune Cell Densities According to Chemokine Expression Levels**

		IL-8 low	IL-8 high	Prb*	MIG low	MIG high	Prb*	IL1- $\beta$ low	IL1- $\beta$ high	Prb*	IP-10 low	IP-10 high	Prb*	IL-16 low	IL-16 high	Prb*	MIF low	MIF high	Prb*
MSI	CD3 <i>ct</i>	1018.6	751.0	0.145	403.6	1577.4	<0.0001	1007.1	715.1	0.307	639.0	1200.5	0.056	751.0	1073.7	0.069	840.8	956.1	0.874
	CD3 <i>im</i>	1746.3	1249.3	0.114	909.5	1591.8	0.032	1478.9	1350.1	0.526	1611.7	1336.0	0.248	1611.7	1302.8	0.741	1609.4	1323.5	0.934
	CD8 <i>ct</i>	524.8	473.6	0.351	147.2	951.9	<0.0001	612.7	293.8	0.166	276.0	881.9	0.010	311.5	759.5	0.010	473.6	524.8	0.803
	CD8 <i>im</i>	764.2	632.4	0.409	480.1	999.8	0.004	724.5	598.3	0.313	598.3	894.3	0.020	617.3	787.4	0.465	661.2	680.2	0.953
	CD45RO <i>ct</i>	1362.4	1356.2	0.519	778.8	1709.8	0.001	1394.1	1227.9	0.318	980.8	1618.0	0.039	1059.3	1564.7	0.045	1520.7	1172.0	0.182
	CD45RO <i>im</i>	2474.1	2753.6	0.962	1516.7	3279.8	0.053	3244.9	2072.7	0.139	2007.3	3017.7	0.509	2353.7	3244.9	0.777	3302.8	2449.2	0.318
	T-Bet <i>ct</i>	319.1	231.2	0.509	147.5	410.7	0.000	276.9	214.0	0.540	153.8	324.9	0.038	199.0	321.3	0.097	231.2	279.0	0.892
	T-Bet <i>im</i>	94.0	110.3	0.556	48.5	147.8	0.001	111.5	90.1	0.589	103.4	119.4	0.212	91.3	119.7	0.126	103.7	114.4	0.916
	FoxP3 <i>ct</i>	206.5	210.8	0.604	194.2	239.1	0.358	197.6	230.4	0.404	197.4	223.7	0.850	159.3	246.1	0.124	189.9	223.7	0.690
	FoxP3 <i>im</i>	259.4	271.7	0.697	221.6	261.9	0.981	207.8	348.0	0.109	301.8	208.5	0.129	221.6	261.9	0.788	221.6	324.5	0.733
	CD68 <i>ct</i>	626.6	506.1	0.570	400.8	690.8	0.084	704.8	408.1	0.188	531.8	650.3	0.363	415.3	690.8	0.139	676.8	500.5	0.633
	CD68 <i>im</i>	921.0	609.3	0.509	601.9	942.4	0.164	1070.3	550.2	0.023	771.0	921.0	0.777	847.9	778.4	0.962	857.6	774.9	0.664
	CD20 <i>ct</i>	8.4	4.4	0.539	3.1	11.5	0.127	9.9	5.9	0.555	8.3	7.1	0.909	6.8	11.5	0.065	9.9	3.1	0.089
	CD20 <i>im</i>	42.1	66.8	0.443	18.4	87.6	0.031	69.1	33.7	0.725	64.6	43.5	0.751	18.4	82.3	0.126	44.9	82.3	0.679
MSS	CD3 <i>ct</i>	510.0	636.9	0.281	499.1	698.2	0.051	540.4	588.4	0.451	540.4	698.2	0.060	555.6	674.3	0.418	416.5	674.3	0.050
	CD3 <i>im</i>	741.3	827.6	0.482	611.6	845.4	0.206	751.0	808.1	0.994	758.5	808.1	0.589	623.5	897.6	0.246	746.2	817.8	0.416
	CD8 <i>ct</i>	108.0	155.2	0.379	82.3	173.7	0.014	141.9	155.2	0.314	108.0	162.6	0.058	126.6	158.7	0.342	82.3	189.3	0.051
	CD8 <i>im</i>	314.4	327.7	0.954	253.6	368.7	0.445	329.0	293.9	0.594	271.1	333.7	0.718	253.6	353.2	0.535	218.7	381.5	0.042
	CD45RO <i>ct</i>	529.3	663.9	0.207	553.3	623.3	0.380	529.3	663.9	0.388	578.7	493.8	0.513	520.5	563.4	0.786	389.4	721.7	0.044
	CD45RO <i>im</i>	1228.2	1722.6	0.149	1597.8	1862.2	0.133	1144.2	1721.6	0.348	1597.8	1721.6	0.348	1144.2	1721.6	0.264	1609.8	1807.7	0.554
	T-Bet <i>ct</i>	49.9	57.7	0.897	43.8	143.0	0.001	61.9	52.7	0.762	43.8	137.9	0.001	49.5	84.7	0.106	41.4	114.0	0.116
	T-Bet <i>im</i>	33.9	44.5	0.693	33.9	52.6	0.229	31.4	52.6	0.447	31.4	58.8	0.028	42.8	36.2	0.569	31.8	55.9	0.149
	FoxP3 <i>ct</i>	255.0	232.8	0.588	229.5	255.0	0.379	255.0	232.8	0.598	161.4	290.8	0.035	199.5	318.9	0.015	275.9	232.8	0.961
	FoxP3 <i>im</i>	239.2	185.8	0.851	184.1	203.4	0.708	317.9	156.9	0.226	210.2	187.2	0.507	165.6	236.8	0.480	218.3	151.6	0.614
	CD68 <i>ct</i>	211.9	317.6	0.356	195.2	353.4	0.038	243.2	282.7	0.468	211.9	353.4	0.188	208.5	282.7	0.364	217.6	402.1	0.098
	CD68 <i>im</i>	507.0	531.3	0.420	440.5	708.0	0.099	519.4	500.3	0.842	500.5	531.3	0.773	531.3	500.3	0.706	475.0	632.7	0.399
	CD20 <i>ct</i>	6.2	6.5	0.349	3.6	21.3	0.018	5.8	15.2	0.257	5.8	15.2	0.213	4.7	10.9	0.223	4.2	11.4	0.058
	CD20 <i>im</i>	49.5	50.3	0.448	61.2	47.1	0.638	38.7	54.3	0.255	61.2	44.6	0.565	45.8	64.1	0.844	49.3	54.3	0.413

\* *p*-value of non-parametric Mann-Whitney test; Only chemokines displaying significant differences for a lymphocytic population and/or localization were shown in this table.