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

Florence Boissière-Michot1,2, Gwendal Lazennec2,3, Hélène Frugier1, Marta Jarlier3, Lise Roca3, Jacqueline Duffour4, Emilie Du Paty2, Daniel Laune2, France Blanchard3, Florence Le Pessot5, Jean-Christophe Sabourin6, and Frédéric Bibeau1,*

1Pathology Department, Institut régional du Cancer de Montpellier (ICM)—Val d’Aurelle; Montpellier, France; 2UMR 3145 SYSDIAG CNRS; Montpellier, France; 3Biostatistics Department, Institut régional du Cancer de Montpellier (ICM)—Val d’Aurelle; Montpellier, France; 4Oncoimmunology, Institut régional du Cancer de Montpellier (ICM)—Val d’Aurelle; Montpellier, France; 5University Hospital, Rouen, France

*These authors contributed equally to this work.

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Abbreviations: CRC, colorectal cancer; ct, tumor center; IHC, immunohistochemistry; im, invasive margin; MMR, mismatch repair; MSI, microsatellite instable; MSS, microsatellite stable; Th1, T helper type-1 lymphocyte; TMA, tissue microarray; UICC, Union for International Cancer Control; y, years

Introduction

The immune contexture of solid tumors in humans has become an emerging hallmark of cancer and assessing its impact on clinical outcome might lead to the identification of new prognostic markers.1,2 Indeed, colorectal cancers (CRCs) that display a strong and coordinated adaptive immune response, as indicated by a high density of CD45RO+ memory and CD8+ cytotoxic T lymphocytes, are typically associated with a good prognosis.3-5 CRC is considered to be a heterogeneous disease. Approximately 85% of CRCs occur in a context of chromosomal instability and 15% display a deficiency in the DNA mismatch repair (MMR) system6-7 linked to either epigenetic or genetic alterations. Defects in the DNA MMR machinery naturally give rise to microsatellite instability (MSI), a condition in which repetitive DNA sequences named microsatellites accumulate mutations that can affect tumor suppressor genes and oncogenes.8 Although not specific to this disease, pronounced peritumoral lymphoid reaction (Crohn-like reaction) and dense infiltration of the tumor by lymphocytes are typically associated with MSI CRC9,10 and could contribute to its good prognosis.7,11 The immunogenicity of MSI CRC is attributed to the occurrence of microsatellite mutations that generate immunogenic neo-antigens.12-14

One mechanism involved in the recruitment of inflammatory cells at the lesion is the expression of cytokines, chemokines, and growth factors by cancer cells and the tumor microenvironment. In addition to its positive involvement in the identification and destruction of malignant cells, inflammation may also play an important role during cancer development and progression.15-17 Initiation of carcinogenesis,18 tumor progression,19 angiogenesis,20,21 and metastatic processes22,23 can be modulated...
by inflammation. Thus, inflammation appears to be a key process with dual functions mediating the relationship between cancer cells and the tumor microenvironment.24 Recent studies have assessed the expression of various cytokines, chemokines, and their receptors in CRC, but these studies only focused on a limited number of factors and the tumor MMR status was rarely taken into consideration.3,25-28 Therefore, in this study we aimed to delineate the differential role of inflammatory components and the tumor microenvironment in CRCs relative to their MMR status. To this end, we characterized the immune cell infiltrate in 62 microsatellite-stable (MSS) CRCs and 48 MSI CRCs by immunohistochemistry (IHC) and quantified their cytokine profile using multiplex-based assays.

**Results**

**Clinicopathologic features**

At the time of surgery, the median age was 72.5 y (range 30–95) for patients with MSI CRC and 65.0 y (range 30–86) for patients with MSS CRC (P = 0.206) (Table 1). The stage distribution of tumors was statistically different between groups (P = 0.018): 52% of MSI CRCs were classified as Stage II vs. 44% of MSS CRCs, whereas 29% of MSS CRCs were Stage IV vs. 6% of MSI CRCs. As expected, MSI CRC was more commonly identified in the right colon (65% were proximal to the splenic flexure, P = 0.018) and was more likely to be poorly differentiated than MSS CRC (35% vs. 8%, P = 0.004). MSI CRC also displayed a significantly higher level of lymphocytic infiltration (58% vs. 37%, P = 0.034) and an expansile tumor border configuration (55% vs. 33%, P = 0.040) as assessed by morphologic evaluation.

**Inflammatory infiltrates in MSS and MSI CRC**

Immunohistochemical (IHC) analysis of tumor inflammatory cells showed a higher infiltration of CD3+ lymphocytes in the tumor center (ct) and invasive margin (im) areas of MSI CRC compared with MSS CRC (mean ± SD: 1335 ± 1320 vs. 777 ± 718 in the ct area, P = 0.046; 1574 ± 1017 vs. 1183 ± 1342 in the im area, P = 0.008). The number of CD8+ lymphocytes also was significantly increased in MSI CRC compared with MSS CRC (717 ± 724 vs. 262 ± 349 in the ct area, P < 0.001; 837 ± 629 vs. 539 ± 631 in the im area, P = 0.001), indicating efficient recruitment of cytotoxic cells (Fig. 1).

Because T helper type-1 (Th1) lymphocytes play a crucial role in activating cytotoxic T lymphocytes, we quantified the T-bet+ population, which is representative of the Th1 CD4+ T-cell subsets. The density of T-bet+ cells in MSI CRC was significantly higher than that in MSS CRC for both the tumor center and invasive margin (453 ± 492 vs. 115 ± 141 in the ct area, P < 0.001; 115 ± 93 vs. 64 ± 74 in the im area, P = 0.001). Similarly, the mean number of CD45RO effector T cells was higher in MSI than in MSS CRC samples (1461 ± 1031 vs. 798 ± 743 in the ct area, P < 0.001; 2716 ± 1620 vs. 2195 ± 2186 in the im area, P = 0.025). On the other hand, FoxP3+ cells, which are representative of the regulatory T cell (Treg) population, were recruited to a similar extent in MSI and MSS CRC (250 ± 183 vs. 305 ± 237 in the ct area, P = 0.276; 343 ± 303 vs. 356 ± 441 in the im area, P = 0.490).

To determine whether other specific inflammatory populations were recruited, macrophages and B-lymphocytes were quantified using anti-CD68 and anti-CD20 antibodies, respectively. MSI CRC displayed a significantly higher number of CD68+ macrophages (626 ± 364 vs. 339 ± 285 in the ct area, P < 0.001; 908 ± 579 vs. 683 ± 653 in the im area, P = 0.019), whereas the density of tumor-infiltrating B cells was similar in both groups (36 ± 93 vs. 44 ± 168 in the ct area, P = 0.629; 255 ± 556 vs. 406 ± 993 in the im area, P = 0.712).

**Cytokine expression in MSS and MSI CRC**

We next measured cytokine expression using multiplex assays that allow the measurement of 48 cytokines. Many cytokines could not be detected (median = 0; IL-2, IL-4, IL-5, IL-9, IL-10, IL-13, CCL3, G-CSF, TNFα, PDGFβb) or were barely detectable (median <1 pg/µg of total protein; IL-1α, IL-2-Rα, IL-6, IL-7, IL-12 (p70), IL-17, IL-18, LIF, CCL11, CCL27, IFNγ, CCL2, CCL7, CCL4, β-FGF, β-NGF, IFN-α2, GM-CSF, M-CSF, SCF, TNFβ, TRAIL) in both MSS and MSI CRC protein samples (Table 2).

Among cytokines that were expressed, MSI CRC displayed a specific cytokine profile compared with MSS CRC: CCL5, CXCL8, CXCL9, IL-1β, CXCL10, IL-16, GROα, and IL-1ra were significantly overexpressed in MSI CRC, whereas the level of MIF was decreased (Table 2). CCL5, CXCL10, CXCL8, CXCL9, and IL-1β showed the strongest upregulation (between 12.9- and 2.3-fold) in MSI CRC compared with MSS CRC.

Finally, variations in cytokine expression within the MSS CRC group were analyzed by comparing MSS CRC with strong lymphocytic infiltration and/or Crohn-like lymphocytic reaction (inflammatory MSS CRC, n = 29) to MSS CRC without these features (non-inflammatory MSS CRC, n = 33). The initial classification into these two subgroups by morphologic evaluation was validated by IHC, which confirmed that the density of intratumoral CD3+ lymphocytes was higher in inflammatory than non-inflammatory MSS CRC (882 ± 612 vs. 685 ± 798 cells/mm², P = 0.044; Table 3). Despite this significant difference, inflammatory and non-inflammatory MSS CRC had comparable cytokine contents. Conversely, although a similar density of CD3+ lymphocytes was observed in inflammatory MSS CRC and MSI CRC (882 ± 612 vs. 1335 ± 1320 CD3+ cells/mm², P = 0.391), the levels of CCL5, CXCL8, CXCL9, IL-1β, CXCL10, IL-16, and IL-1ra remained significantly higher in the MSI group (Table 3). These data suggest that the distinct cytokine expression profile observed in MSI CRC is linked to the MSI status as well as the unique inflammatory infiltrate observed in this CRC subgroup.

We also investigated the correlation between cytokine levels and specific immune cell densities in MSS and MSI groups. We identified significant correlations between different subsets of immune cells and chemokine expression, primarily in regards to CXCL9 and CXCL10 (Table S1). In MSI CRC, high levels of CXCL9 were associated with a significantly increased intratumoral density of CD3+, CD8+, and T-Bet+ T cells. Interestingly, the observed correlations between the
frequency of infiltrating-immune cells and CXCL9 expression appeared stronger in MSI CRC in which such occurrences also seemed to involve the CD45RO population, and were not limited to the center of the tumor. Taken together, these data showed that in situ immune cells are strongly associated with a specific chemokine profile indicating a distinct coordinated biological process.

| Parameter                              | All CRCs | MSS CRCs | MSI CRCs | P value |
|----------------------------------------|----------|----------|----------|---------|
| Total, n                               | 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-like reaction                    |          |          |          | 0.199   |
| Yes                                    | 31 (28)  | 14 (23)  | 17 (35)  |         |
| No                                     | 79 (72)  | 48 (77)  | 31 (65)  |         |
| Lymphocyte infiltration                |          |          |          | 0.034   |
| Yes                                    | 51 (46)  | 23 (37)  | 28 (58)  |         |
| No                                     | 59 (54)  | 39 (63)  | 20 (42)  |         |

Data were compared with the Fisher exact test or Mann–Whitney test as appropriate. *Assessed on H&E stained sections by a single pathologist blinded to the clinicopathologic data (No: no obvious tumor infiltrating lymphocytes; Yes: infiltrating lymphocytes present). Abbreviations: CRC, colorectal cancer; MSS, microsatellite stable; MSI, microsatellite unstable; NA, not available.

**Discussion**

The tumor microenvironment encompasses the non-malignant tumor cells, including immune cells that could, under key circumstances, play an important role in constraining CRC progression.39,40 Here, we show that MSI CRC displays a specific in situ immune response and chemokine profile in comparison to that of MSS CRC. This particular MSI-specific inflammatory
Figure 1. For figure legend, see page 5.
microenvironment might explain the more favorable clinical course of this CRC subtype. Using image analysis that allows objective quantification of the positive cells and minimizes the observer’s bias, we found a significant increase in CD3+, CD8+, CD45RO+, and T-bet+ lymphocytes in MSI CRC relative to MSS CRC, whereas the density of FoxP3+ cells was similar in both groups. These results are in agreement with previous studies that also reported a relationship between MSI and density of CD3+, CD8+, and CD45RO+ cells in CRC. Several studies have provided compelling evidence that effector/cytotoxic (CD3+ and CD8+) and memory (CD45RO+) T cells play major roles in the antitumor immune response in CRC, and, that their high expression correlates with a good clinical outcome (reviewed by Fridman et al.2). CD8+ cytotoxic T lymphocytes can efficiently kill tumor cells and are mainly activated through the Th1 pathway. The Th1 pathway can be analyzed by assessing expression of the T-box transcription factor T-bet, which is crucial for the development of effector Th1 CD4 T cells59 and is currently the most specific marker for this cell subset. T-bet mRNA levels and T-bet in situ protein expression in CRC have previously been correlated with reduced tumor recurrence. Here, we show for the first time that T-bet+ lymphocytes are significantly upregulated in MSI CRC, highlighting an efficient Th1 response that could account for the good clinical outcome of this population. Similarly, the increased density of CD45RO+ cells in MSI CRC in comparison to MSS CRC might enhance the efficiency of the antitumor immune response. Indeed, CD45RO+ cells, which include both antigen-exposed CD4+ lymphocytes and CD8+ lymphocytes, respond faster and with increased intensity to antigenic stimulation than do naive T cells. Combined with the similar density of FoxP3+ cells found in MSI and MSS CRC, these results suggest that the lymphocyte balance is tipped in MSI CRC toward an effective host-mediated immune response rather than tolerance induction. Indeed, Tregs, which express the nuclear transcription factor FoxP3, dampen the antitumor immune response,53-37-43 and suppress the activity of cytotoxic T cells (reviewed in deLeeuw et al.44), thus maintaining immunologic tolerance. Few studies have evaluated the relationship between FoxP3+ and the MSI status, leading to controversial results in terms of prognosis.25,30,33,35,40,45,46

It has been proposed that the local immune response in MSI CRC could be related to the production of potentially immunogenic neopeptides resulting from frameshift mutations within microsatellite sequences. Accordingly, Tugeron et al. have described a significant association between CD3+ density and the overall number of frameshift mutations. The local inflammatory reaction evoked by cancer neoantigens preferentially arising in MSI CRC might thus promote cytokine production, which in turn, could expand the immune recruitment. On the other hand, the specific cytokine profile identified in this study involves mostly chemokines, specifically CXCL1, CXCL8, CXCL9, CXCL10, and CCL5. Based on the literature, these chemokines could, conceptually, be produced by immune cells. However, a number of colon cancer reports have shown these chemokines to be produced mainly by cancer cells and stromal cells, rather than by Th1, Th2, or Treg cells. Indeed, CXCL1 has been primarily detected in colon cancer cells and to a lesser extent in mesenchymal cells. Similar to CXCL10, CXCL8 has been shown to be primarily produced by carcinoma cells but has also been detected at weaker levels in macrophages, lymphocytes, and myofibroblasts. There are no publications describing the identity of the cells that produce CXCL9 in colon cancer, but the source could be neutrophils or M2 macrophages. Finally, one report has suggested that CCL5 is produced by lymphocytes in colon cancer, although CCL5 could be also produced by tumor-associated macrophages. The correlations that we observed between various cytokines and specific subsets of immune cells at particular locations in the MSS or MSI tumors suggest a fine-tuned regulation of the in situ inflammatory recruitment, but whether the cytokine profile is a cause or consequence of immune infiltration remains unclear. However, chemokines within the tumor stroma are generally thought to play a role in the recruitment of immune cells. Some of these chemokines are characterized by antitumor activity, whereas others are either pro-tumorigenic or have a controversial role. Specifically, besides its chemoattractant properties for T lymphocytes, monocytes, natural killer cells, and eosinophils, CXCL8 has been shown to be primarily produced by lymphocytes in colon cancer, although CCL5 has been shown to promote tumor growth and metastasis by inducing tumor cell proliferation, migration, angiogenesis, or expression of matrix metalloproteinases in various cancer types. Moreover, CCL5 can recruit Tregs within the tumor to kill cytotoxic CD8+ T cells, suggesting that its overexpression could promote an immunosuppressive tumor microenvironment that might enhance tumor progression. Similarly, CXCL8 possesses tumorigenic and proangiogenic properties in CRC both in vitro and in vivo. Nevertheless, in agreement with our study, Banerjea et al. described increased CXCL8 levels in MSI CRC. Therefore, the overexpression of cytokines such as CCL5 and CXCL8 in MSI CRCs that are considered to have a relatively good prognosis suggests the presence of regulatory pathways that counterbalance their protumorigenic effects. The Th1-type inflammatory mediators CXCL9 and CXCL10, two IFNγ-inducible CXCR3 ligands, act as angiostatic regulators and promote the infiltration and expansion of antitumor T lymphocytes, particularly CD8+ effector T cells and memory CD45RO+ T cells. We found that tumors displaying high levels of CXCL9 and CXCL10 also showed significantly higher densities of CD3+, CD8+, and T-Bet+ cells, with stronger in situ recruitment in MSI tumors. Our results are

**Figure 1 (See previous page).** Distribution of immune cells in MSS and MSI colorectal cancers. (A–D) Comparison of the density of immune cells in human colorectal cancers with microsatellite stability (MSS; black bars) and microsatellite instability (MSI; gray bars). (A) The intratumoral (ct) and peritumoral (pm) densities of CD3+; CD8+; T-Bet; CD45RO+; FoxP3+; CD68+; and CD20+ cells were assessed by image analysis of tissue microarray spots. (B–D) Representative examples of staining are shown for CD8 (B), T-Bet (C), and FoxP3 (D); images show immunoperoxidase staining (×100) with the corresponding digital images (stained cells are represented in red). Statistical analyses were performed by non-parametric Mann-Whitney test; *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant
in line with previous reports of a correlation between high CXCL9 and CXCL10 mRNA expression in CRC and increased density of CD8+ and CD4+ cells and macrophages. Moreover, these chemokines have been associated with better outcome. Thus, together with the increased density of T-bet+ cells in MSI CRC, the higher expression levels of CXCL9 and CXCL10 suggest host protection via the generation of a Th1 immune response.

The role of CXCL1 in CRC progression is controversial. High CXCL1 expression has been associated with shorter recurrence-free survival in Stage III colorectal cancer patients and its downregulation results in a near-complete inhibition of tumor growth in nude mice. However, transcription is higher in less invasive tumors and in samples from patients aged <65 years. These results could be related to a stronger immune response in younger patients and to the fact that MSI CRCs are often early-stage tumors.

In summary, our data suggest a fine regulation of the immune contexture in MSI CRC, leading to efficient recruitment of inflammatory cells through the expression of specific chemokines. They also reveal a Th1-polarized immune response in MSI CRC through activation of the CXCL9/CXCL10 signaling axis. This translates into local recruitment or expansion of specific inflammatory populations that are involved in the anticancer response and potentiation of immunosurveillance, probably accounting for the favorable outcome of this particular subtype of colorectal cancer.

### Patients and Methods

**CRC samples and patients**

CRC resection specimens with documented MMR status and available frozen tissue samples containing at least 50% tumor cells were identified at the pathology departments of the Val d’Aurelle Cancer Centre and Rouen University Hospital. In total, 110 CRC samples, of which 48 had MSI, were selected for this study. Among the 48 MSI CRCs, 11 were from patients with Lynch Syndrome, as defined by the presence of a deleterious

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**Table 2. Differential expression of cytokines, chemokines, and growth factors in MSS and MSI colorectal cancers (continued)**

| Marker | MSS CRC (n = 62) | MSI CRC (n = 48) | Ratio (MSI/MSS) | P |
|--------|-----------------|-----------------|----------------|---|
|        | Mean | SD  | Median | Range | Mean | SD  | Median | Range |   |
| CCL5   | 0.40 | 0.80 | 0.13   | [0–4.29] | 5.16 | 18.13 | 1.37   | [0–125.92] | 12.9 | <0.001 |
| CXCL8  | 14.62 | 40.13 | 2.19   | [0–284.63] | 77.05 | 149.22 | 12.70  | [0.01–507.61] | 5.3  | <0.001 |
| CXCL9  | 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 |
| CXCL10 | 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 |
| CXCL1  | 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-1α  | 80.72 | 116.43 | 37.96  | [0.27–506.35] | 105.98 | 123.77 | 58.83  | [5.61–567.4] | 1.3  | 0.049 |
| CCL4   | 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   | [0–0.65]  | 0.05 | 0.07  | 0.01   | [0–0.33]   | -    | -    |
| CCL3   | 0.00 | 0.01  | 0.00   | [0–0.04]  | 0.02 | 0.05  | 0.00   | [0–0.24]   | -    | -    |
| IFN-γ  | 0.08 | 0.17  | 0.00   | [0–0.89]  | 0.25 | 0.44  | 0.02   | [0–1.97]   | -    | -    |
| IL-18  | 13.32 | 47.74 | 0.85   | [0.02–270.58]| 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   | [0–0.16]  | 0.01 | 0.03  | 0.00   | [0–0.18]   | -    | -    |
| 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   | [0–0.19]  | 0.03 | 0.04  | 0.00   | [0–0.15]   | -    | -    |
| IL-7   | 0.59 | 1.56  | 0.00   | [0–8.9]   | 0.83 | 1.72  | 0.01   | [0–9.39]   | -    | -    |
| CXCL12 | 2.17 | 3.05  | 1.54   | [0–16.09] | 1.55 | 1.93  | 0.43   | [0–7.67]   | 0.7  | 0.342 |
| CCL7   | 0.97 | 1.93  | 0.16   | [0–10.74] | 1.01 | 1.38  | 0.63   | [0–7.81]   | -    | -    |

The amount of each factor was quantified using two multiplex assays and expressed as pg/µg of total protein extracted from MSS or MSI tumor samples containing at least 50% tumor cells. Data were compared using a non-parametric Mann–Whitney test. Abbreviations: CRC, colorectal cancers; MSS, microsatellite stable; MSI, microsatellite instable.
### Table 2. Differential expression of cytokines, chemokines, and growth factors in MSS and MSI colorectal cancers (continued)

| Marker | MSS CRC (n = 62) | MSI CRC (n = 48) | Ratio (MSI/MSS) | P |
|--------|-----------------|-----------------|----------------|---|
|        | Mean  | SD | Median | Range        | Mean  | SD | Median | Range        |            | |
| CCL27  | 0.28  | 0.21 | 0.26 | [0–1.17]     | 0.23  | 0.22 | 0.22 | [0–0.84]     | -          | - |
| TNFβ   | 0.02  | 0.02 | 0.03 | [0–0.1]      | 0.02  | 0.03 | 0.00 | [0–0.08]     | -          | - |
| 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 | [0–0.53]     | -          | - |
| SCGFβ3 | 2.54  | 3.21 | 1.28 | [0–15.46]    | 2.57  | 3.70 | 1.13 | [0–18.79]    | 1.0        | 0.404 |
| CCL2   | 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 | [0–0.59]     | 0.05  | 0.08 | 0.00 | [0–0.38]     | -          | - |
| IL-10  | 0.00  | 0.01 | 0.00 | [0–0.03]     | 0.00  | 0.00 | 0.00 | [0–0.01]     | -          | - |
| IL-17  | 0.05  | 0.08 | 0.00 | [0–0.03]     | 0.08  | 0.15 | 0.00 | [0–0.55]     | -          | - |
| 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 |
| IFNα2  | 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 | [0–0.32]     | 0.06  | 0.27 | 0.00 | [0–1.74]     | -          | - |
| IL-2   | 0.01  | 0.02 | 0.00 | [0–0.12]     | 0.00  | 0.01 | 0.00 | [0–0.05]     | -          | - |
| 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 | [0.26–120.91] | 9.18  | 9.98 | 5.16 | [0.09–46.65] | 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 | [0–0.24]     | 0.05  | 0.13 | 0.00 | [0–0.7]      | -          | - |
| IL-9   | 0.01  | 0.02 | 0.00 | [0–0.11]     | 0.00  | 0.01 | 0.00 | [0–0.08]     | -          | - |
| 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]        | -          | - |

The amount of each factor was quantified using two multiplex assays and expressed as pg/µg of total protein extracted from MSS or MSI tumor samples containing at least 50% tumor cells. Data were compared using a non-parametric Mann–Whitney test. Abbreviations: CRC, colorectal cancers; MSS, microsatellite stable; MSI, microsatellite instable.

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germline mutation of a DNA damage repair gene involved in the MMR system. All samples were procured from fresh biopsies that were taken in the vicinity of the tumor invasion front and flash-frozen in liquid nitrogen. Tumor samples were collected following French laws under the supervision of an investigator and declared to the French Ministry of Higher Education and Research (declaration number DC-2008–695). All patients were informed about the use of their tissue samples for biological research and a written informed consent was systematically obtained for analysis of germline MMR gene mutations. The study was approved by the local translational research committee and was performed in accordance with the Helsinki Declaration of 1975. All samples were anonymized and analyses were performed blinded to the clinicopathologic data. Hematoxylin and eosin slides were reviewed by a gastrointestinal surgical pathologist (FB) to identify morphologic features, including histologic differentiation, lymphocytic infiltration, Crohn-like reaction, and tumor border configuration. All tumors were staged according to the TMN classification system (7th edition) of the Union for International Cancer Control (UICC). The clinicopathologic features of the patients are reported in Table 1. MMR status assessment

MMR status was assessed by IHC analysis for the expression of the hMLH1, hMSH2, hMSH6, and PMS2 proteins and by PCR analysis of microsatellites as previously described.71

Protein extract preparation

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

Bio-Plex multiplex cytokine assays

Two Bio-Plex ProTM Human kits (BioRad, #171-A11127 and #171-A11171) were used to measure the amount of cytokines, chemokines, and growth factors in CRC samples according to the manufacturer’s instructions, as previously described.72 The first multiplex assay detected 27 proteins (27-plex assay: IL-1β, IL-1ra,
Table 3. Inflammatory cell populations and cytokine expression in inflammatory MSS, non-inflammatory MSS, and MSI colorectal cancers

| Marker | Group 1 (n = 29) | Group 2 (n = 33) | Group 3 (n = 48) | Group 3 vs 1 |
|--------|------------------|------------------|------------------|-------------|
|        | Inflammatory MSS CRC<sup>(1)</sup> | Non-Inflammatory MSS CRC | MSI CRC | |
|        | Mean ± SEM | Median | Range | Mean ± SEM | Median | Range | Ratio | P | Mean ± SEM | Median | Range | Ratio | P |
| CD3 ct | 882 ± 61.2 | 710 | [65–2491] | 685 ± 79.8 | 451 | [53–3673] | 1.29 | 0.044 | 1335 ± 1320 | 905 | [13–5604] | 1.51 | 0.391 |
| CD45RO ct | 977 ± 787 | 705 | [64–2788] | 625 ± 667 | 521 | [0–3362] | 1.56 | 0.055 | 1462 ± 1031 | 1359 | [78–4595] | 1.49 | 0.031 |
| CD68 im | 779 ± 737 | 618 | [50–3678] | 601 ± 568 | 469 | [20–2669] | 1.29 | 0.241 | 908 ± 579 | 816 | [46–2022] | 1.17 | 0.188 |
| CD68 ct | 364 ± 276 | 328 | [14–1198] | 317 ± 296 | 249 | [20–1274] | 1.15 | 0.378 | 626 ± 364 | 596 | [43–1464] | 1.72 | 0.002 |
| FoxP3 ct | 254 ± 155 | 223 | [5–534] | 352 ± 285 | 255 | [54–1162] | 0.72 | 0.554 | 250 ± 183 | 206 | [15–820] | 0.98 | 0.701 |
| CD8 ct | 303 ± 420 | 155 | [5–1956] | 225 ± 272 | 148 | [7–1057] | 1.35 | 0.459 | 717 ± 724 | 505 | [31–2916] | 2.36 | 0.002 |
| CD45RO im | 2045 ± 1525 | 1715 | [139–5965] | 2326 ± 1525 | 1715 | [99–11293] | 0.88 | 0.553 | 2716 ± 1620 | 2614 | [125–6462] | 1.33 | 0.083 |
| T-bet ct | 105 ± 124 | 60 | [0–533] | 124 ± 159 | 53 | [9–664] | 0.85 | 0.241 | 453 ± 492 | 233 | [4–2053] | 1.17 | 0.188 |
| FoxP3 im | 326 ± 321 | 214 | [53–1508] | 383 ± 531 | 177 | [0–2568] | 0.85 | 0.378 | 250 ± 183 | 206 | [15–820] | 0.98 | 0.701 |
| CD8 im | 492 ± 460 | 345 | [40–1806] | 579 ± 751 | 268 | [18–3445] | 0.85 | 0.241 | 453 ± 492 | 233 | [4–2053] | 1.17 | 0.188 |
| CD3 im | 1041 ± 936 | 761 | [152–3805] | 1308 ± 1621 | 863 | [44–8474] | 0.79 | 0.554 | 837 ± 629 | 505 | [31–2916] | 2.36 | 0.002 |
| T-bet im | 61 ± 61 | 38 | [5–226] | 67 ± 67 | 36 | [0–388] | 0.85 | 0.554 | 115 ± 115 | 109 | [3–386] | 1.17 | 0.188 |
| IL-16 | 7.14 ± 3.84 | 6.48 | [1.75–14.05] | 11.63 ± 10.02 | 10.54 | [20.8–53.59] | 0.61 | 0.554 | 453 ± 149 | 123 | [4–2053] | 1.33 | 0.083 |
| IL-1ra | 56.38 ± 90.45 | 27.83 | [7–464.13] | 102.11 ± 132.94 | 44.21 | [27–506.35] | 0.55 | 0.554 | 105.98 ± 149 | 123 | [4–2053] | 1.33 | 0.083 |
| CCL5 | 0.29 ± 0.57 | 0.12 | [0–2.37] | 0.49 ± 0.96 | 0.15 | [0–4.29] | 0.59 | 0.554 | 5.31 ± 8.31 | 1.54 | [0–34.12] | 1.21 | 0.554 |
| CXCL8 | 12.27 ± 24.75 | 2.79 | [0–114.29] | 16.69 ± 50.25 | 1.51 | [0–284.63] | 0.74 | 0.554 | 77.05 ± 149 | 123 | [4–2053] | 1.33 | 0.083 |
| CXCL9 | 6.92 ± 8.76 | 2.66 | [0–16–2943] | 5.52 ± 8.86 | 3.73 | [0–49.90] | 1.25 | 0.554 | 28.51 ± 35.39 | 14.16 | [0–154.09] | 1.17 | 0.188 |
| IL-1β | 3.43 ± 12.71 | 0.09 | [0–68.39] | 1.2 ± 2.48 | 0.15 | [0–10.58] | 2.86 | 0.554 | 5.13 ± 8.31 | 1.54 | [0–34.12] | 1.21 | 0.554 |
| CXCL10 | 6.44 ± 9.98 | 1.85 | [0–44.67] | 5.31 ± 8.31 | 1.54 | [0–34.12] | 1.21 | 0.554 | 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 | 0.554 | 89.23 ± 60.19 | 76.16 | [0–350.63] | 1.01 | 0.554 |
| CXCL11 | 1.77 ± 2.51 | 0.89 | [0–12.89] | 1.99 ± 2.67 | 1.00 | [0–12.80] | 0.89 | 0.554 | 28.51 ± 35.39 | 14.16 | [0–154.09] | 1.17 | 0.188 |

1Based on the presence of a strong lymphocytic infiltration and/or Crohn’s-like lymphocytic reaction. The intratumoral (ct) and peritumoral (im) densities of CD3, CD8, T-Bet, CD45RO, FoxP3, and CD68 immunoreactive cells were assessed by image analysis of tissue microarray spots and expressed as number of cells/mm². The amount of each factor was quantified using two multiplex assays and expressed as pg/µg of total protein extracted from MSS or MSI tumor samples containing at least 50% tumor cells. Data were compared with a non-parametric Mann–Whitney test. Abbreviations: CRC, colorectal cancer; MSS, microsatellite stable; MSI, microsatellite instable.
CCL7 [MCP-3], M-CSF, MIF, CXCL9 [MIG], β [MIP-1]

and diaminobenzidine tetrahydrochloride–chromogen (DAB) as a chromogen.

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