

# Increased tumor infiltration by mucosal-associated invariant T cells correlates with poor survival in colorectal cancer patients

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**Abstract** The infiltration of tumors by lymphocytes is a prognosis factor in colorectal cancer (CRC). The magnitude and quality of this infiltration have emerged as important component of the clinical outcome in these patients. Specifically, markers associated with functional cell-mediated immunity, i.e., a Th1 immune response, are independent markers of better prognosis, whereas Th17-associated components are deleterious and correlate with poorer survival. Mucosal-associated invariant T (MAIT) cells are a recently described T cell subset with tissue-homing properties. They display a restricted TCR repertoire specific for widely conserved microbial ligands, and display antibacterial properties upon release of Th1-like, Th17-like, and/or cytotoxic granules. MAIT-cell-specific transcripts have been found in kidney and brain cancer, but have not been studied in other sites. In this study, we retrospectively analyzed by confocal microscopy the presence of MAIT cells within colorectal tumors as compared with paired healthy tissues. We observed a significant although variable increase, both in density and in proportion of overall tumor-infiltrating T lymphocytes inside the tumors. Importantly, survival curves as well as multivariate analysis showed that

patients displaying a higher recruitment of MAIT cells in their tumor, as compared with the neighboring healthy tissue, showed a less favorable clinical outcome. This study suggests that including MAIT-cell-specific markers or transcripts in the analysis of tumor-infiltrating lymphocytes could be a benefit to the diagnosis and follow-up of CRC patients.

**Keywords** Colorectal cancer · Tumor-infiltrating lymphocytes · Mucosal-associated invariant T cells · MAIT cells · Prognosis · IL-17

## Abbreviations

CD	Crohn's disease
CNS	Central nervous system
CRC	Colorectal cancer
DFS	Disease-free survival
MAIT	Mucosal-associated invariant T cells
MR1	MHC-related 1
MS	Multiple sclerosis
ROR $\gamma$ t	RAR-related orphan receptor gamma t
OS	Overall survival
TIL	Tumor-infiltrating lymphocytes

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## Introduction

Solid tumors can be infiltrated by lymphocytes at variable levels. The presence of tumor-infiltrating lymphocytes (TIL), mostly T cells, has been historically interpreted as the demonstration of the immunogenicity of cancer cells and a subsequent anti-tumoral immune response. Major studies from many groups have then demonstrated the existence of tumor-associated antigens recognized by T cells, uncovering the mechanisms by which the adaptive

immune system can detect and destroy cancer cells. However, the presence of TIL is seldom associated with cancer regression. Numerous studies, mostly in the past decade, have addressed this apparent contradiction and have provided a highly complex and dynamic picture of the relationship between the immune system and tumors [1]. Schematically, the presence of lymphocytes by itself is not sufficient to drive tumor regression; the outcome depends upon the precise composition, as well as the location, of these lymphocytes inside tumors [2]. An effective anti-tumoral response requires a Th1-like differentiation of T cells and an infiltration by both CD4<sup>+</sup> and cytolytic CD8<sup>+</sup> T cells [3], however, TIL often include ineffective Th2 T cells, as well as regulatory T cells (Tregs), which may prevent the action of anti-tumoral T cells [4–9]. IL-17-producing cells apparently show a dual effect on tumor prognosis, depending on the cancer type; they are seemingly deleterious in lung or colorectal cancer (CRC), whereas they may promote or help cancer regression in other situations such as ovarian or esophageal cancers [10–13].

Seminal studies by Galon et al. in CRC have shown that it may be possible to use several immune system markers to improve prognosis of patients, an analysis called the immunoscore. They elegantly showed that an abundance of Th1/Tc1/cytolytic T cells, detected by markers such as CD8, CD45RO, IFN $\gamma$ , and granzyme B, is an independent factor of better prognosis in CRC, whereas numerous Th17-associated transcripts are associated with a poor outcome [2, 10, 14]. Therefore, it is of great importance to deepen our analysis of in situ immune responses when new T cell subsets, not yet studied in this context, are identified.

MAIT cells are innate-like T cells with a restricted antigenic repertoire, specific for highly conserved microbial antigens presented by a monomorphic MHC class I-like molecule, MHC-related 1 (MR1) [15]. They display potent anti-bacterial and anti-fungal functions and probably represent an important first line of defense against microbial infections at mucosal surfaces [16, 17]. Indeed, they are abundant in the human blood but also in the liver and the gut lamina propria; furthermore, they may be rapidly recruited at sites of inflammation [18]. Activation and, in some cases, recruitment of MAIT cells have been shown in infectious diseases [19–21], but also in autoimmune/inflammatory pathologies, such as inflammatory bowel diseases, multiple sclerosis, and psoriasis [22–25]. Interestingly, one publication also reported the presence of MAIT-cell-specific transcripts in brain and kidney tumors [26]. MAIT cells display a mixed Tc1/Tc17 phenotype and function: They are CD8<sup>+</sup>, effector/memory T cells, and they mostly produce IFN $\gamma$  and TNF $\alpha$  upon stimulation; however, they can be cytolytic and secrete IL-17 after specific stimuli; furthermore, they express Th17-associated markers, such as RAR-related orphan receptor gamma t (ROR $\gamma$ t) and CD161 [18]. MAIT

cells have never been specifically studied in the context of CRC. Interestingly, at least in the blood of healthy subjects, they express both markers identified as good (CD8, granzyme, CD45RO) and bad prognosis (ROR $\gamma$ t) in CRC.

In this study, we retrospectively performed a descriptive analysis of MAIT cells inside a series of colorectal tumors and paired healthy intestinal tissue. We show that MAIT cells are specifically recruited inside tumors in CRC, but the level of infiltration is highly variable. Importantly, we found that an important infiltration by MAIT cells is correlated with a poorer outcome in patients, suggesting that they could represent an additional easy-to-use marker of prognosis in the context of CRC.

## Materials and methods

### Patients

This retrospective study included 35 patients undergoing surgery for CRC in the period 2005–2011. All patients gave prior informed consent. Relevant demographic and clinical data were collected and are listed in Table 1. The TNM stage was estimated using guidelines from the Union for International Cancer Control (UICC). Disease-free survival was defined as the time (in months) between surgery and any disease-related event, i.e., relapse or death. Overall survival was defined as the time between surgery and cancer-related death. We analyzed for each patients paired biopsies collected within the tumor and in the adjacent healthy tissue.

### Reagents

The detection of lymphocytes in biopsies was performed on 10- $\mu$ m formaldehyde-fixed frozen sections. The antibody panel included polyclonal rabbit anti-CD3 (Dako), polyclonal goat anti-CD26 (R&D Systems), and monoclonal anti-V $\alpha$ 7.2 (Biolegend) detected by the respective secondary antibodies: donkey anti-rabbit AlexaFluor 546, donkey anti-goat AlexaFluor 488, and donkey anti-mouse A647 (Invitrogen).

### Confocal microscopy

The analyses were performed by a trained technician without knowledge of the origin (tumoral or healthy) of the biopsies. Fields were chosen randomly but according to the T cells density, such that a minimum of 300 CD3<sup>+</sup> T cells/biopsy could be counted. Three-to-six fields were analyzed per biopsies. MAIT cells were identified and counted as CD3<sup>+</sup>CD26<sup>+</sup>V $\alpha$ 7.2<sup>+</sup> cells. The frequency of MAIT cells was calculated as follow: % MAIT cells = (number of

**Table 1** Demographic, clinical, and histological characteristics of the patients analyzed in the study

Total number	35
Sex ( <i>n</i> )	
Male	18
Female	17
Mean age at diagnosis	72
Min	51
Max	89
Histology ( <i>n</i> )	
Glandular	33
Mucinous	2
Tumor grade ( <i>n</i> )	
Well differentiated	21
Moderately differentiated	11
Poorly differentiated	3
Tumor localization ( <i>n</i> )	
Right	13
Left	12
Sigmoid	5
Unknown	5
Tumor infiltration ( <i>n</i> )	
T3	15
T4	20
TNM stage ( <i>n</i> )	
I	0
II	10
III	17
IV	8
DFS median (months)	24
OS median (months)	80

CD3<sup>+</sup>CD26<sup>+</sup>Vα7.2<sup>+</sup> cells/number of CD3<sup>+</sup>). The number of cells/mm<sup>2</sup> was extrapolated from the number of fields analyzed. Images were acquired at magnification 40×. Analyses were performed on the LSM 780 (Zeiss).

### Statistical analysis

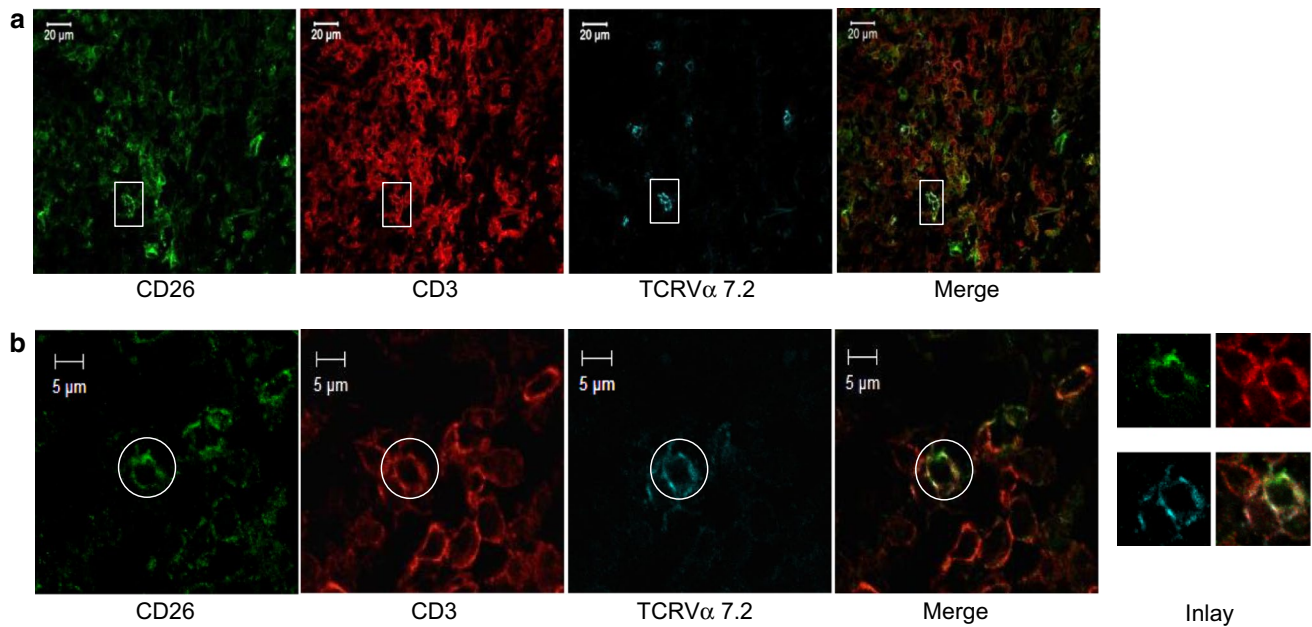
Statistical analyses for paired healthy and tumor samples were performed with a Wilcoxon test. Unpaired analyses according to clinical data used a Mann–Whitney test. In both cases, a value of  $p < 0.05$  was considered significant. The Pearson's coefficient ( $r$ ) enabled to analyze linear correlations. The Kaplan–Meier method was used for calculation of survival probabilities and the log-rank test for comparison of survival curves. Multivariate analysis was performed with a Cox proportional hazards model including the following parameters: age (4 groups), TNM stage (II, III or IV), tumor differentiation (poor, moderate, or well differentiated), CD3 infiltration index (calculated as

the ratio between the number of CD3<sup>+</sup> cells in the tumoral vs. healthy tissue), and the MAIT cell infiltration index (with a cutoff at 3). A value of  $p < 0.05$  was considered significant. Univariate analysis and survival probabilities were performed with GraphPad Prism; multivariate analysis was performed with Stata13.

### Results

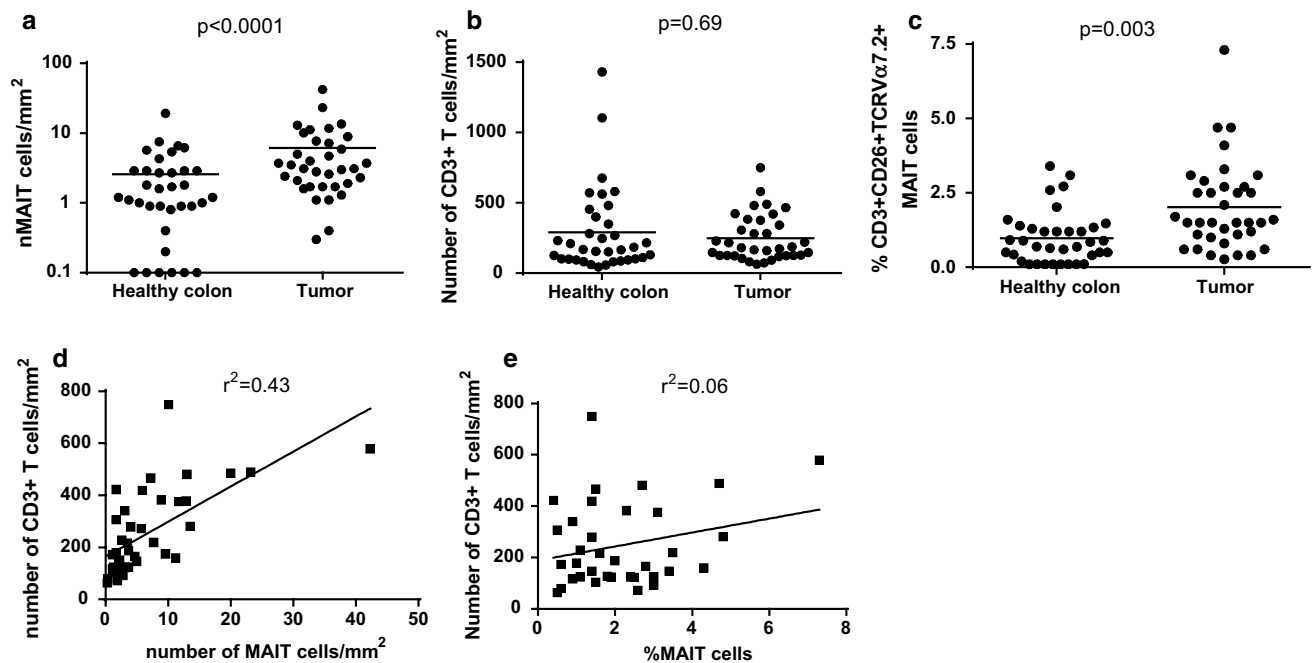
MAIT cells represent a significant proportion of CD3<sup>+</sup> T cells in the healthy lamina propria of the gut, but there is no data available regarding their frequency inside tumoral intestinal tissue. Therefore, we aimed at quantifying these cells inside colorectal tumors. Blood MAIT cells can be identified as CD3<sup>+</sup>TCRVα7.2<sup>+</sup> cells co-expressing high levels of CD161, IL-18Rα or CD26 [18]. Although CD161 may be the best marker for MAIT cell identification by flow cytometry, IL-18 Rα has been used with success for confocal microscopy analysis [18, 24]. In this study, we chose to identify MAIT cells as CD3<sup>+</sup>TCRVα7.2<sup>+</sup>CD26<sup>+</sup> cells by immunofluorescence microscopy, because we obtained better images with CD26 as compared with IL-18Rα or CD161. We first counted the number of triple-positive cells in a retrospective series of biopsies from colorectal tumors and paired healthy tissues (Fig. 1). We noted a significantly higher number of MAIT cells in the core of the tumors as compared to the healthy mucosa (6.1/mm<sup>2</sup> ±1.3 vs. 2.6/mm<sup>2</sup> ±0.6;  $p < 0.0001$ ; Fig. 2a). This increased number of MAIT cells could be the direct reflection of an increase in the number of total T cells inside tumors, as compared with healthy intestine. However, we found no such difference between paired tissues for CD3<sup>+</sup> T cells (247/mm<sup>2</sup> ±28 in tumors vs. 290/mm<sup>2</sup> ±51;  $p = 0.69$ ) (Fig. 2b). We therefore estimated the proportion of MAIT cells among total CD3<sup>+</sup> T cells (Fig. 2c) and observed a significant increased frequency of MAIT cells within the tumor (2 % ±0.25 vs. 0.98 % ±0.15;  $p = 0.003$ ). Although the number of MAIT cells per mm<sup>2</sup> somewhat paralleled the number of total CD3<sup>+</sup> T cells (Fig. 2d), their frequency was poorly correlated with the level of T cell infiltration (Fig. 2e). Therefore, the tumoral tissue harbors a higher number of MAIT cells than the healthy colon, resulting mostly from their increased frequency within infiltrating T cells.

Patients with CRC are stratified using the TNM score, which takes into account the tumor infiltration inside the tissue (T), the number of infiltrated lymph nodes (N), and distant metastases (M). This score is a useful prognosis marker, as it strongly correlates with the overall survival. We asked whether the degree of MAIT cell infiltration and specific recruitment was correlated with the TNM score. In our cohort, no patients scored at stage I, whereas the great majority was at stage III ( $n = 17$ ), and a smaller fraction at



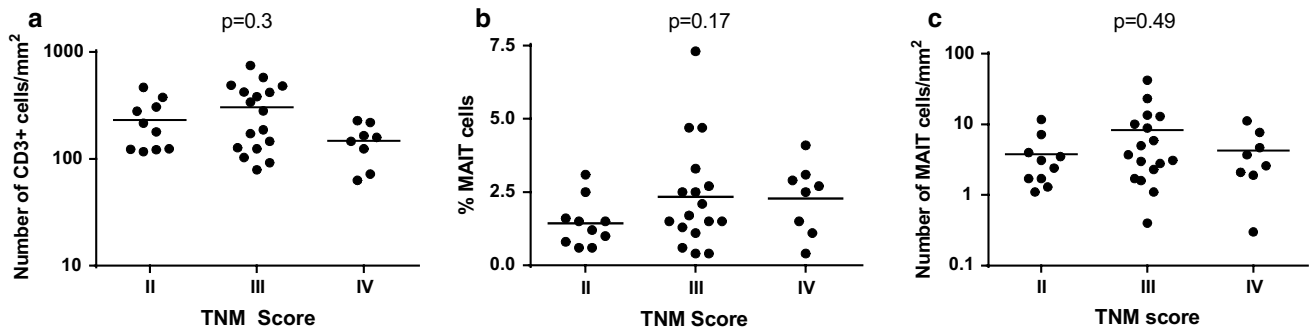
**Fig. 1** Immunofluorescent staining of tissue sections from a healthy colon with anti-CD3, anti-CD26, and anti-TCRV $\alpha$ 7.2. Cells staining positive for the three markers are counted as MAIT cells. Images are acquired on a LSM780 confocal microscope (Zeiss) at magnification 40 $\times$ . Two examples are shown. In (a), a panorama of a large field

was obtained by computerized combination of separate pictures of the whole area, by a process referred to as stitching. White squares identify triple-positive cells. **b** Example of one microscopy field, with one triple-positive MAIT cell marked by a white circle. The inlay on the right side of the panel shows a zoom on this triple-positive cell



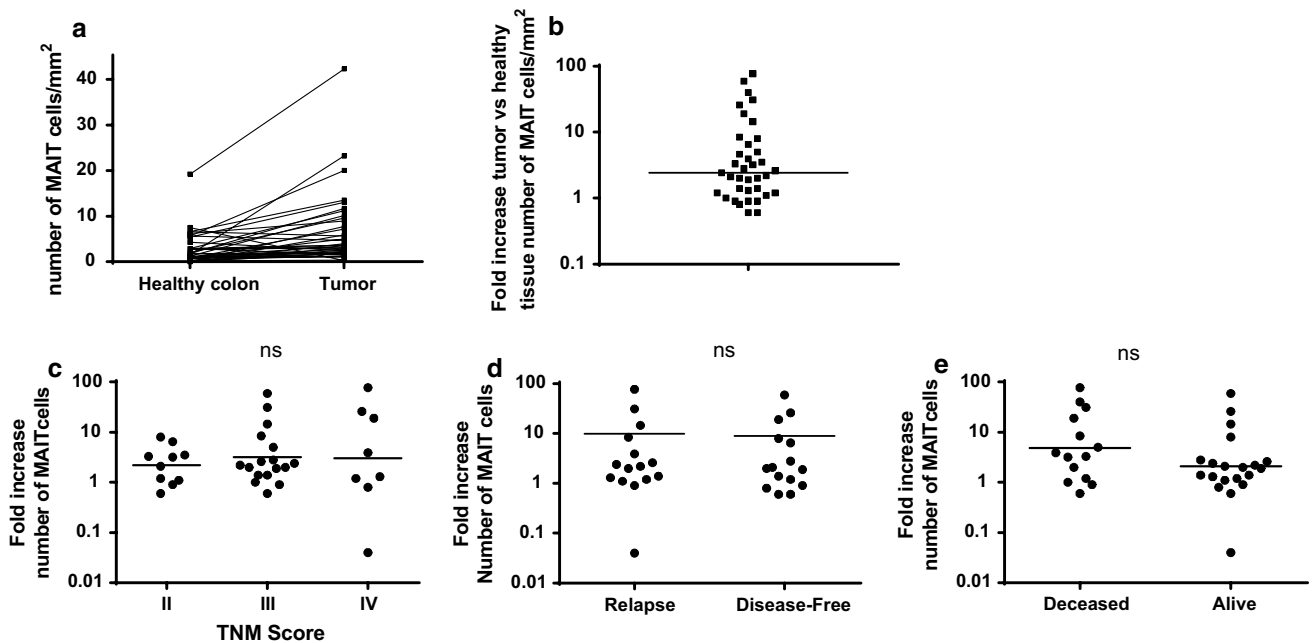
**Fig. 2** **a** Absolute values of MAIT cells per mm<sup>2</sup> in paired samples from healthy and tumoral tissue. **b** Absolute values of CD3<sup>+</sup> T cells per mm<sup>2</sup> in paired samples from healthy and tumoral tissue. **c** Frequency of MAIT cells among CD3<sup>+</sup> T cells in paired samples from healthy and tumoral tissue. **d** Correlation between the number of MAIT cells and the number of CD3<sup>+</sup> T cells in tumoral tissue. **e**

Correlation between the frequency of MAIT cells and the number of CD3<sup>+</sup> T cells in tumoral tissue. Horizontal bars represent the mean values. Statistical analyses were performed with a matched Wilcoxon test. *p* values and Pearson's coefficient are indicated. *p* values <0.05 are considered significant



**Fig. 3** Number of CD3<sup>+</sup> T cells/mm<sup>2</sup> (a), the frequency of MAIT cells among CD3<sup>+</sup> T cells (b) and the number of MAIT cells/mm<sup>2</sup> (c) inside tumor samples were each analyzed according to the TNM

score for comparison. *Horizontal bars* represent the mean values. Statistical analyses were performed a multiple comparison ANOVA test. A value of  $p < 0.05$  was considered significant



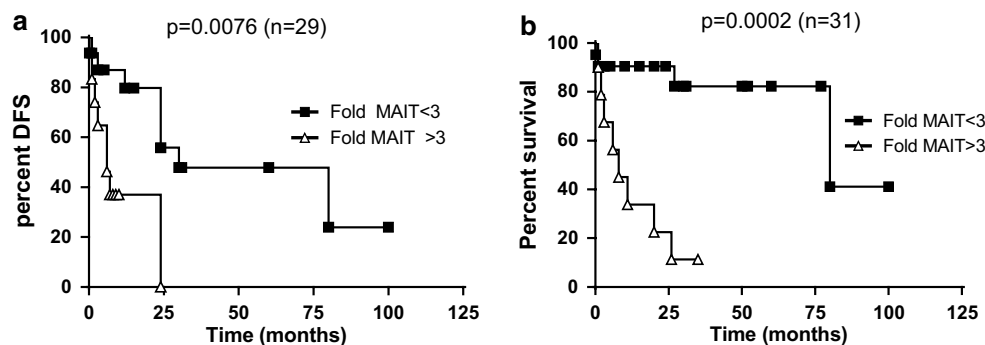
**Fig. 4** **a** Paired analysis of the absolute value of MAIT cells per mm<sup>2</sup> in paired samples from healthy and tumoral tissue (same data as in Fig. 2a). **b** Fold increase in the number of MAIT cells/mm<sup>2</sup> quantified in the tumor as compared to the paired healthy colon sample. *Each symbol* represents one patient. The *horizontal bar* delineates the geometric mean. **c** Fold increase in the number of tumor-infiltrat-

ing MAIT cells according to the TNM stage. Geometric means are shown. **d** Fold increase in the number of tumor-infiltrating MAIT cells in the disease-free group and the relapse group. Geometric means are shown. **e** Fold increase in the number of tumor-infiltrating MAIT cells according to the survival of the patients. Geometric means are shown

stage II ( $n = 10$ ) or stage IV ( $n = 8$ ). We first assessed the total number of T cells infiltrating the tumors according to the TNM score. As shown in Fig. 3a, similar numbers of T cells were found in stage II and III tumors, but this number dropped at stage IV ( $258.4/\text{mm}^2 \pm 34.4$ ,  $296.2/\text{mm}^2 \pm 46.1$  and  $145.3/\text{mm}^2 \pm 24.5$ , respectively); however, the difference did not reach statistical significance, probably because of the low number of stage IV tumors in our cohort. With respect to MAIT cells, we mostly observed a trend toward an increased infiltration from stage II to stage III ( $3.8/\text{mm}^2 \pm 1$  and  $8.3 \text{ mm}^2 \pm 2.6$ , respectively), but this difference did

not reach statistical significance (Fig. 3b, c). We conclude that MAIT cells infiltrate colorectal tumors at various levels, and this increased infiltration is mostly independent of the TNM score.

As shown in Fig. 2, the number and proportion of MAIT cells, both in healthy tissue and in tumors, is highly variable between individuals. Furthermore, because they are a significant subset of T cells in the normal mucosa, it is difficult to estimate from the above data to what extent they are recruited inside the tumor. However, when analyzing paired samples for each patient, it became clear that



**Fig. 5** Kaplan–Meier analysis of survival probabilities between two groups of patients stratified according to the index of MAIT cell infiltration (<3 or >3). **a** Disease-free survival and **b** Overall survival.  $p$  values obtained with the log-rank test are indicated

**Table 2** Multivariate Cox proportional hazard analysis of the overall survival of the patients included in the study

	$p$	HR	95 % CI
Age (1)	0.35	1.96	0.47–8.11
TNM stage (2)	0.8	1.05	0.72–1.52
Differentiation (3)	0.65	0.77	0.24–2.41
CD3 index (4)	0.56	0.85	0.49–1.48
MAIT index (5)	0.008	8.82	1.77–43.98

(1) Patients were classified among 4 groups: <65 yo; >65 < 75 yo; >75 < 85 yo; >85 yo

(2) TNM stages: II, III or IV

(3) Poor, intermediate, well differentiated

(4) CD3 index was included as continuous variable

(5) Patients were classified among two groups: MAIT cell index <3 or >3

CI confidence interval, yo years old, HR hazard ratio

the level of specific recruitment inside tumors was highly variable and might be a better indicator of the behavior of MAIT cells (Fig. 4a). Indeed, when we calculated the specific recruitment of MAIT cells by the ratio between their number inside tumors and in healthy mucosa, there was a dramatic variation between individuals (Fig. 4b; range 0.6–77, median 2.4). We reasoned that this ratio might be a better marker to analyze differences between groups from a clinical viewpoint. However, using this index did not show any significant difference between tumors TNM stages (Fig. 4c). We then asked whether there was any link between this MAIT recruitment index and the patient's disease-free and overall survival. We found no difference between patients in remission and those who relapsed (Fig. 4d). Then, we compared this index between patients who died because of their disease and the group of patients who survived (Fig. 4e). Again, we observed no significant difference overall; however, it was clear that the bulk of patients alive had a smaller index (geometric mean 2.1,

25/75 percentile 1.1/2.7), with only four patients showing higher ratios. By contrast, patients who succumbed from cancer had a highly variable MAIT infiltration index, with several patients showing very high scores (geometric mean 4.9, 25/75 percentile 1.1/25). We turned then to a survival analysis of the CRC patients according to the MAIT recruitment index calculated as before. It turned out that the geometric mean of this ratio on the whole population studied was 3.4; therefore, we chose to set the cutoff to 3 and analyzed the impact of this index on the disease-free survival (DFS) and the overall survival (OS) by a Kaplan–Meier analysis. As shown in Fig. 5a, there was a significant trend toward better DFS in the patient group with a MAIT index <3 (median DFS 30 vs. 6 months,  $p = 0.0076$ ). More strikingly, the OS was clearly higher in the same group as compared patients with high index (median OS 80 vs. 8 months,  $p = 0.0002$ ; Fig. 5b). This result was further confirmed by multivariate analysis that included several relevant parameters, such as age, TNM score, differentiation stage of the tumor, as well as a CD3 infiltration index. A Cox proportional hazards model including all these covariates showed a highly significant impact of the MAIT cell infiltration index on OS, with  $p = 0.008$  and a hazard ratio of 8.8 (Table 2). We conclude from these data that patients with a high MAIT recruitment index, i.e., when the number of MAIT cells within the tumor is at least threefold higher than in the healthy mucosa, have shorter life time expectancy. Therefore, the infiltration of colorectal tumors by a high number of MAIT cells is a marker of bad prognosis.

## Discussion

MAIT cells are dedicated antimicrobial cells found in significant proportion in the healthy lamina propria of the intestine, where they are probably ideally located to promptly respond to bacterial and fungal infectious agents. They are also present in the peripheral blood, from where

they can be recruited to infected tissue, such as the lung in pulmonary tuberculosis. More generally, MAIT cells behave as inflammatory cells, capable of invading most inflammatory peripheral tissue. Indeed, they are found in the central nervous system (CNS) lesions of patients with multiple sclerosis (MS), in the psoriatic lesions of the skin, or in the inflamed gut mucosa of patients with Crohn's disease (CD) [22–24]. With respect to cancer, a previous study found an increased amount of transcripts for the MAIT cell-specific TCR $\alpha$  chain (V $\alpha$ 7.2-J $\alpha$ 33), in kidney and brain tumors, suggesting their potential to infiltrate tumoral tissues [26]. In this study, we show for the first time that MAIT cells constitute a substantial subset of TIL in CRC and that the importance of specific MAIT cell infiltration is a marker of bad prognosis in patients.

The difficulty in studying the recruitment of MAIT cells in cancer cells is related to their relative abundance in the normal mucosa. In this study, we found that an average of about 1 % of CD3<sup>+</sup> T cells were MAIT cells as defined as triple-positive CD3<sup>+</sup>CD26<sup>+</sup>TCR $\alpha$ V $\alpha$ 7.2<sup>+</sup> cells. This frequency is a bit lower but still similar to our previous observations on patients with CD, in which we observed an average of  $1.5 \pm 0.3$  % MAIT cells among intestinal T cells [24]. It is also close but a bit lower than estimates obtained from another study [27], where mononuclear cells from the intestinal lamina propria were isolated and MAIT cells enumerated by flow cytometry. The minor differences obtained could be related to the greater mean age of the patients studied here, as the proportion of MAIT cells may be impacted by immune senescence [28]. Furthermore, it is possible that the various strategies in use, in terms of markers (CD161 vs. IL18R $\alpha$  vs. CD26) as well as technology (in situ confocal microscopy vs. flow cytometry after cell extraction) somewhat impact on the precise results. Nevertheless, the comparison of those different data show that our strategy to enumerate MAIT cells in situ is valid and that they are found in higher numbers and proportion within the colorectal tumors. The paired analysis of samples in each patient enabled us to calculate a recruitment index of MAIT cells which demonstrated a prognostic value, as patients with a higher recruitment have a reduced OS.

In CRC, seminal studies by Galon et al. have demonstrated that the composition of TIL is variable and complex, and deeply influences prognosis. Indeed, the presence of high numbers of effector/memory cytotoxic T cells and Th1 CD4<sup>+</sup> T cells is an independent factor of better outcome in these patients [10, 14]. Interestingly, an important infiltration by IL-17-producing cells is a marker of bad prognosis in CRC, whereas it has a positive impact on other cancer types [12]. In our study, we found that an increased infiltration by MAIT cells identifies patients with a lower DFS and OS. Our current knowledge on MAIT cells functions show they have a mixed phenotype and cytokine secretion pattern between Tc1 and Tc17 cells [18]. On the one hand,

they express granzymes and perforin, which are cytolytic and secrete mostly IFN $\gamma$  and TNF $\alpha$  upon in vitro stimulation. On the other hand, they express most Tc17 markers, such as the transcription factor ROR $\gamma$ t and the surface molecule CD161 [18]. The parameters driving the functional differentiation of MAIT cells toward a Tc1, Tc17, or mixed Tc1/Tc17 phenotype are not known. Based on the results shown here, we hypothesize that in CRC, strong infiltration by MAIT cells may be accompanied by a differentiation of these cells toward a Tc17 phenotype. This hypothesis would be concordant with the aforementioned studies, where strong Th17 markers identified in patients with a poor prognosis would include MAIT cells. In addition, MAIT cells infiltrate several organs and tissues in chronic inflammatory diseases, and some studies suggest this is accompanied by a functional Tc17 differentiation [23, 24]. These results, together with this study, would be consistent with the idea that IL-17 production is maximal within tissues, as compared with blood cells. IL-17 production may be pro-oncogenic and therefore deleterious in the context of chronic inflammatory diseases [29, 30]. This further suggests that in diseases such as inflammatory bowel diseases, which are associated with the development of colorectal cancer [31], sustained infiltration by MAIT cells would increase the risk of cancer development. Future studies are required to confirm this hypothesis and to unravel the mechanisms involved in MAIT cells infiltration and differentiation in CRC.

The small number of patients included in our cohort does not permit to draw any definitive conclusion about the use of the MAIT cells infiltration index as a ready-to-use tool for prognosis assessment. However, our study suggests that it would be of great interest to include MAIT-cell-specific markers in the so-called tumor immunoscore, together with other already analyzed useful transcripts. Even if MAIT cells infiltration always comes together with a Th17-like differentiation of intra-tumoral T cells, and therefore does not represent an independent factor of outcome, they are easy to monitor and therefore may represent a useful tool as a marker of prognosis in CRC.

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#### Compliance with ethical standards

**Conflict of interests** The authors declare no conflicts of interests.

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