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## Basic Study

## Cytotoxic CD8+ T cells and tissue resident memory cells in colorectal cancer based on microsatellite instability and BRAF status

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## Abstract

## BACKGROUND

Recent studies in non-colorectal malignancy have associated T resident memory ( $T_{RM}$ ) cells with improved patient survival. It is unknown if  $T_{RM}$  plays a role in colorectal cancer (CRC).

## AIM

To examine the potential role of  $T_{RM}$  cells in providing immunogenicity in CRC stratified by microsatellite instability (MSI) and BRAF status.

## METHODS

Patients with known MSI and BRAF mutation status were eligible for inclusion in this study. CRC tumour sections stained with haematoxylin and eosin were

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microscopically reviewed and the images scanned prior to assessment for location of invading edge and core of tumour. Sequential sections were prepared for quantitative multiplex immunohistochemistry (IHC) staining. Opal Multiplex IHC staining was performed with appropriate positive and negative controls and imaged using a standard fluorescent microscope fitted with a spectral scanning camera (Mantra) in conjunction with Mantra snap software. Images were unmixed and annotated in inForm 2.2.0. Statistical analysis was performed using Graphpad Prism Version 7 and Stata Version 15.

## RESULTS

Seventy-two patients with known MSI and BRAF status were included in the study. All patients were assessed for MSI by IHC and high resolution capillary electrophoresis testing and 44 of these patients successfully underwent quantitative multiplex IHC staining. Overall, there was a statistically significant increase in CD8+ T<sub>RM</sub> cells in the MSI (BRAF mutant and wild type) group over the microsatellite stable (MSS) group. There was a statistically significant difference in CD8+ T<sub>RM</sub> between high level MSI (MSI-H):BRAF mutant [22.57, 95% confidence interval (CI): 14.31-30.84] *vs* MSS [8.031 (95% CI: 4.698-11.36)], *P* = 0.0076 and MSI-H:BRAF wild type [16.18 (95% CI: 10.44-21.93)] *vs* MSS [8.031 (95% CI: 4.698-11.36)], *P* = 0.0279. There was no statistically significant difference in CD8 T cells (both CD8+CD103- and CD8+CD103+T<sub>RM</sub>) between MSI-H: BRAF mutant and wild type CRC.

## CONCLUSION

This study has shown that CD8+ T<sub>RM</sub> are found in greater abundance in MSI-H CRC, both BRAF mutant and MSI-H:BRAF wild type, when compared with their MSS counterpart. CD8+ T<sub>RM</sub> may play a role in the immunogenicity in MSI-H CRC (BRAF mutant and BRAF wild type). Further studies should focus on the potential immunogenic qualities of T<sub>RM</sub> cells and investigate potential immunotherapeutic approaches to improve treatment and survival associated with CRC.

**Key Words:** Tissue resident memory cells; Resident memory T cells; Colorectal cancer; Microsatellite instability; BRAF; DNA mismatch repair; Immunotherapy; Prognosis

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**Core Tip:** Prior to this study, whether T resident memory (T<sub>RM</sub>) cells exist in colorectal cancer (CRC) was poorly understood. This study has identified and characterize T<sub>RM</sub> cells within human CRC. CD8+ TRM cells are found in greater abundance in both high microsatellite instability (MSI-H) BRAF mutant and MSI-H:BRAF wild type CRC when compared with their microsatellite stable counterpart. CD8+ TRM may play a role in the immunogenicity in both BRAF mutant and BRAF wild type MSI-H CRC. Further studies should focus on the potential immunogenic qualities of TRM cells and investigate potential immunotherapeutic approaches to improve treatment and survival associated with CRC based on TRM.

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## INTRODUCTION

Colorectal cancer (CRC) may be divided into microsatellite stable (MSS) and high microsatellite unstable (MSI-H) CRC. Approximately 15% of CRC are MSI-H and are more likely to be immunogenic with abundant tumour infiltrating lymphocytes (TILs)<sup>[1-3]</sup>. On the other hand, MSS CRC are less likely to be immunogenic, with

sparse/limited TILs. Abundance of TILs has been associated improved survival<sup>[4-14]</sup> and is widely believed to provide MSI-H CRC with a favourable prognosis<sup>[15,16]</sup> despite the higher grade features also associated with MSI-H CRC, such as larger diameter, poorly differentiated, mucinous tumours.

However, while it is widely known that MSI-H CRC is associated with TILs, there is significant debate as to the composition of TILs within CRC and which component of TILs is most important. Various reports have suggested that within TILs, CD8+, CD4+, CD3+, FOXP3+ and CD45RO+ regulatory cells have important roles in immunosurveillance. There has been no consensus in recent studies. Noshio *et al*<sup>[17]</sup> reported on the benefits of CD45RO+ cells, Guidoboni *et al*<sup>[18]</sup> on cytotoxic CD3+ and CD8+ cells, Salama *et al*<sup>[19]</sup> reported that FOXP3+ cells were associated with favourable prognosis respectively. Potentially, the interaction of all of these cells may provide benefit as these cells have different profiles and roles within an intricate orchestrated immune response. It has been believed for decades that T cells are recruited in response to cancer, and constantly recirculate in the blood and lymphatic system. The benefit of these TILs in CRC has been reported and has been subject to recent reviews<sup>[20,21]</sup>.

More recently a new lineage of T cells has been discovered which is believed to provide immunity to solid tumours<sup>[22-24]</sup>. Tissue resident memory ( $T_{RM}$ ) cells, particularly CD8+  $T_{RM}$ , have been identified in non-colorectal malignancies and its presence has already been shown to be associated with better survival outcomes in lung cancer<sup>[25]</sup>, ovarian cancer<sup>[26]</sup>, breast cancer<sup>[27-29]</sup> and melanoma<sup>[24,30]</sup>, particularly CD8+  $T_{RM}$ <sup>[24-27,31-36]</sup>. The difference between CD8+  $T_{RM}$  and conventional cytotoxic CD8+ T cells is that  $T_{RM}$  cells, by definition, do not recirculate—rather they remain in peripheral tissue. This is because  $T_{RM}$  cells are not just a continuous circulation of T cells, but cells committed to their tissue of residence including tumour tissue, thereby providing immediate and long-term protection against invaders.  $T_{RM}$  cells are believed to produce interferon-gamma, granzyme B and perforin<sup>[30]</sup>.

$T_{RM}$  cells at epithelial surfaces develop from precursor cells through a process that relies on transcription factors and cytokines<sup>[37]</sup> and can be identified by the expression of CD103 (and CD69). Both CD103 and CD69 contribute to the retention of these cells within epithelia and prevent these cells from exiting their resident tissue. CD103 is the alpha chain of integrin, which binds to E-Cadherin which is an epithelial adhesion molecule. Furthermore,  $T_{RM}$  cells express checkpoint receptors such as programmed death 1 (PD-1), CTLA4 and Tim-3, potential targets for current immunotherapy strategies. If present in CRC,  $T_{RM}$  may represent an avenue for improving survival in CRC.

In this study, we have chosen CD103 status to identify  $T_{RM}$  cells. CD39, CD69 and CD103 have been associated with  $T_{RM}$ <sup>[36,37]</sup>, with CD103 reported to strongly correlate with a favourable prognosis in cancer patients<sup>[38]</sup>. While there are studies which have suggested the importance of CD4+  $T_{RM}$  cells<sup>[39]</sup>, the majority of studies have associated improved survival with CD8+  $T_{RM}$  cells<sup>[24-26,31-36]</sup> and for this reason the main outcome measure chosen for this study was the abundance of CD8+  $T_{RM}$  in the CRC tumour microenvironment. Secondary outcomes included assessing the presence of CD8+  $T_{RM}$  and cytotoxic CD8+ non- $T_{RM}$  cells. Results of the above outcome measures were pooled by microsatellite instability (MSI) and BRAF status.

MSI status was chosen because it is a biomarker widely considered to influence immunogenicity. BRAF status was also chosen because in CRC it has been shown that presence of BRAFV600E mutation nearly always excludes Lynch syndrome and absence of BRAF mutation (BRAF wild type) may be associated with Lynch syndrome (although 30%-40% of MSI-H:BRAF wild type may be sporadic)<sup>[40]</sup>. Thus this study examined the potential role of  $T_{RM}$  cells in providing immunogenicity in CRC stratified by MSI and BRAF status, with CRC divided into three groups: MSS, MSI-H:BRAF mutant (representing sporadic MSI-H, usually associated with epigenetic silencing of MLH-1) and MSI-H:BRAF wild type (approximately 60%-70% associated with Lynch syndrome)<sup>[40]</sup>.

## MATERIALS AND METHODS

### Patient

Seventy-two patients with known MSI and BRAF status confirmed by immunohistochemistry (IHC) and high resolution capillary electrophoresis were eligible for inclusion in this study. These patients also had detailed clinical, operative, pathological, adjuvant treatment and follow-up data from a cancer database which



was analyzed retrospectively. Institutional board approval was obtained, and patients had provided written consent for the use of their information for research (CH62/62011-136 HREC/11/CRGH206).

### Histopathology

Histopathology slides prepared with haematoxylin and eosin were reviewed using an Olympus BX53 microscope to find a representative slide which shows the tumour invading edge and core. These slides were then scanned electronically and the scanned images were reexamined by a pathologist to mark out the invading edge and core. From these blocks, representative slides were recut using a microtome in preparation for quantitative multiplex IHC staining.

Of the 72 original patient tumour specimens prepared on representative slides, 44 formalin fixed paraffin embedded (FFPE) tumour sections were successfully stained with quantitative multiplex IHC staining. All IHC staining was performed on 4- $\mu$ m-thick sections and stained using an Opal Multiplex IHC assay kit (PerkinElmer, Waltham, MA, United States) and optimised in-house. Briefly, FFPE tissue sections were deparaffinized and rehydrated prior to antigen retrieval by boiling in basic (10 mmol/L Tris base, 1 mmol/L EDTA, 0.05% Tween 20, pH 9.0) buffer. The sections were then incubated with 3% hydrogen peroxide for 20 min at room temperature before washing and blocking with Perkin Elmer Antibody diluent/Block buffer. Sections were then incubated with a single unconjugated primary antibody for 35 min, washed and then incubated with Opal Polymer HRP (Akoya Biosciences, United States) for 10 min. After washing, sections were incubated with Opal fluorochromes at a 1:100 dilution made up in tyramide signal amplification reagent (PerkinElmer, Waltham, MA, United States) for 10 min. The antigen retrieval step was repeated, and tissue was stained for subsequent antibodies as described above. Finally, sections were stained with DAPI (cell signalling technologies) for 5 min and then mounted using Prolong Diamond (Life Technologies). Slides were stored in the dark until imaging was complete. The following antibodies were used; CD8 (ab4055 clone; Abcam), CD103 [EPR4116(2) clone; Abcam] PD1 (NAT105 clone, Cell Marque). The Mantra imaging platform (PerkinElmer) was used for imaging in combination with Mantra snap software for data acquisition. For all quantitative analysis up to 10 randomly selected regions of interest per section were analyzed. InForm advanced image analysis software (PerkinElmer, MA, United States) (PerkinElmer) was used to process and analyze images, with each tissue manually segmented into tissue regions.

### Statistical analysis

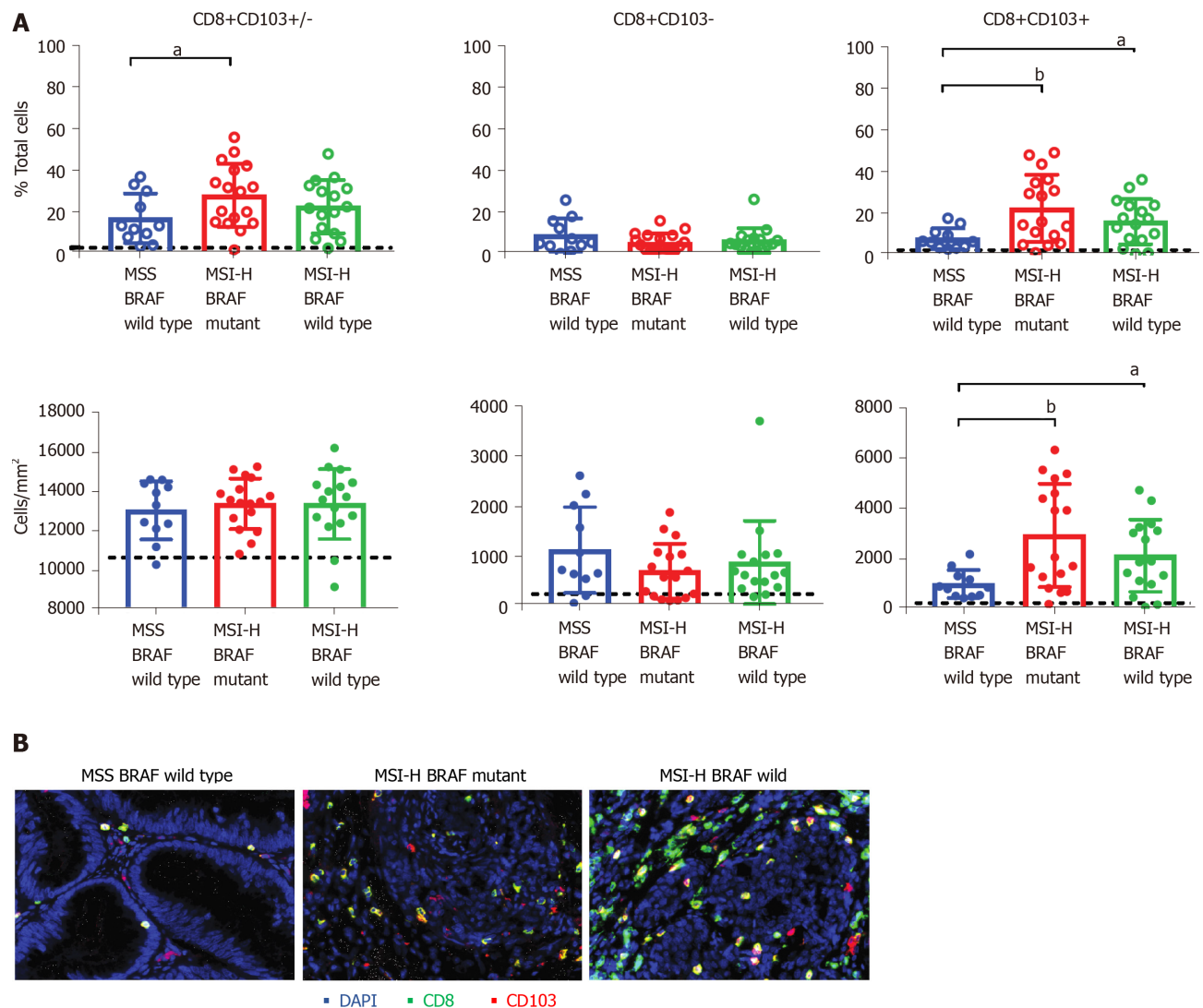
Statistical analysis was performed using Graphpad Prism Version 7 and Stata Version 15.

## RESULTS

### *T<sub>RM</sub> T cells are significantly increased in tumours with defective DNA mismatch repair*

Seventy-two patients with known MSI and BRAF status met the eligibility criteria for this study, and of these 44 patient tumour specimens were successfully stained by quantitative multiplex IHC staining (Figure 1). The percentage and densities of total CD8+ T cells, T<sub>RM</sub> T cells, and non-T<sub>RM</sub> CD8+ T cells were calculated for MSS, MSI-H:BRAF mutant and MSI-H:BRAF wild type subgroups. An unpaired student *t* test was used to compare the mean % T<sub>RM</sub>/non-T<sub>RM</sub> cells/total cells between the three groups for each outcome measure. Where there were significant outliers, the median was used instead of mean, and a Mann Whitney *U* test was performed to compare the median between the three groups. This showed that compared to healthy control all three groups had a statistically greater abundance of CD8+ T cells (Figure 1).

There was a significant difference in the proportion of CD8+ T cells between MSI-H:BRAF mutant and MSS subgroups with 17.24% [95% confidence interval (CI): 9.334-25.15] of nucleated cells in MSS compared to 28.14% (95%CI: 20.38-35.89) in MSI-H:BRAF mutant and 22.82 (95%CI: 16.02-29.62) in MSI-H:BRAF wild type subgroups. On Mann Whitney *U* analysis, there was a statistically significant difference between MSI-H:BRAF mutant and MSS subgroups (*P* = 0.0417). Although there was a greater percentage of CD8+ T cells in the MSI-H:BRAF wild type subgroup when compared to MSS, this did not reach statistical significance [22.82 (95%CI: 16.02-29.62) *vs* 17% (95%CI: 9.334-25.15), *P* = 0.2841].



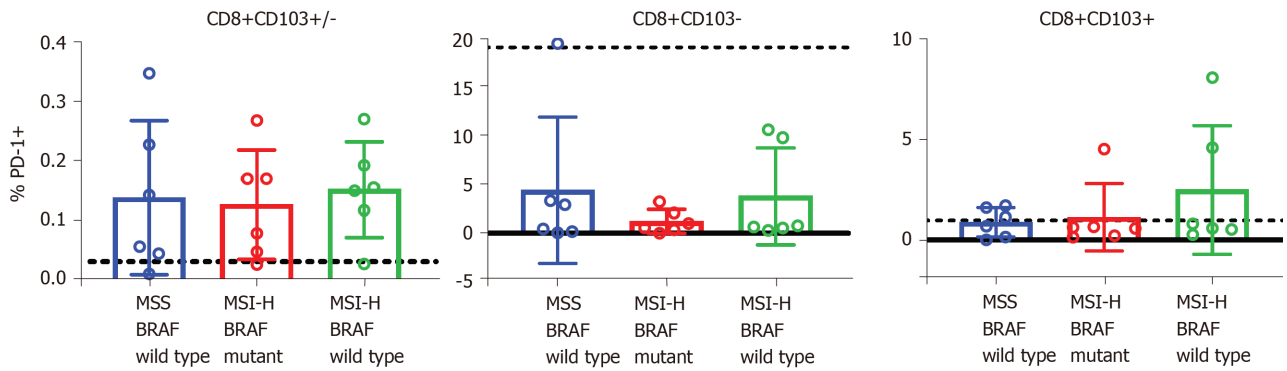
**Figure 1 Tissue resident memory T cells are significantly increased in microsatellite instability high tumours.** A: Quantitative immunohistochemistry staining of tumour specimen show the proportion (top panels) and densities (bottom panels) of total CD8+ T cells (left panels), CD103-CD8+ (non- $T_{RM}$ ) T cells (middle panels) and CD103+CD8+ resident memory ( $T_{RM}$ ) T cells (right panels) in CRC tumours with microsatellite stable BRAF wildtype and microsatellite instability (MSI) high BRAF mutants and MSI high BRAF wildtype groups. Figure shows the mean and SD of  $n = 11-17$  samples and the statistical differences were calculated using unpaired and non-parametric Mann-Whitney test ( $^aP < 0.05$ ;  $^bP < 0.001$ ). Black line shows the levels determined from normal healthy colorectal tissues ( $n = 1$ ); B: Representative tissue staining shows the staining for CD8 (green) and CD103 (red) and DAPI (blue) in the three groups of patients. MSS: Microsatellite stable; MSI-H: High microsatellite instability.

Importantly, there were significant differences in the proportions of CD8+  $T_{RM}$  cells between the three subgroups of CRC. Compared to MSS, the MSI-H:BRAF mutant subgroup had a significantly higher proportion of CD8+  $T_{RM}$  cells (Figure 1) with 22.5% (95% CI: 14.31-30.84) in MSI-H:BRAF mutant subgroup and 8.0% in MSS subgroup (95% CI: 4.698-11.36,  $P = 0.0076$ ). Similarly, the MSI-H:BRAF wild type subgroup had significantly higher CD8+  $T_{RM}$  (16.18%, 95% CI: 10.44-21.93) when compared to MSS (8%, 95% CI: 4.698-11.36 and  $P = 0.0279$ ). The difference in abundance of  $T_{RM}$  between MSI-H: BRAF mutant and MSI-H:BRAF wild type subgroups was not significant (Figure 1). There was no statistically significant difference in non- $T_{RM}$  CD8+ cells between the three subgroups.

#### PD-1 expression on CD8+ T cells is not impacted by MSI status

We next sought to determine whether the expression of checkpoint receptor, PD-1 was impacted by MSI status or BRAF mutation. We enumerated the proportion of T cells expressing PD-1 in all three subgroups. PD-1 expression was present in both  $T_{RM}$  T cells and non- $T_{RM}$  T cell populations, although the proportion of non- $T_{RM}$  T cells expressing PD-1 was slightly higher than that of the  $T_{RM}$  T cell population (Figure 2). While there was a trend to increased PD-1+ expression in the  $T_{RM}$  T cell population in MSI-H:BRAF wild type compared to MSI-H:BRAF mutant and MSS subgroups, there





**Figure 2 Programmed cell death protein-1 expression on CD8+ T cells is not impacted by microsatellite instability or BRAF status.** Quantitative immunohistochemistry staining was used to determine the expression of programmed cell death protein-1 (PD-1) on CD8+ T cell populations. Figure shows the proportion of total CD8+ T cells (CD8+CD103+/-) (left panel), CD103-CD8+ (non- $T_{RM}$ ) T cells (middle panel) and CD103+CD8+  $T_{RM}$  cells (right panel) expressing PD-1. Figure shows the mean and SD for 5-6 samples/group. PD-1: Programmed death 1; MSS: Microsatellite stable; MSI-H: High microsatellite instability.

was, however, no statistically significant differences in the proportion of T cells expressing PD-1 between the three groups.

## DISCUSSION

Characteristics of TILs within CRC have been poorly defined to date, and there is a paucity of information on  $T_{RM}$  T cells in CRC. In humans, within the circulation, two types of memory T cells exist – central memory (TCM) and effector memory (TEM). For decades, it was thought that T lymphocyte populations in peripheral tissues including TILs were largely maintained by the continuous circulation of these memory T cells as well as other circulatory cells. Recent studies have challenged this concept and provided evidence for the existence of another subset of CD8+ T cells – tissue- $T_{RM}$  T cells. These cells are permanently resident (*i.e.*, do not recirculate) in both lymphoid and non-lymphoid tissues and represent a distinct subset to circulatory TEM or TCM.

Prior to this study the existence of  $T_{RM}$  cells in CRC was poorly understood and the aim of this study was to identify and characterize  $T_{RM}$  cells within human CRC. If present,  $T_{RM}$  cells may be important in the protection against recurrence, dissemination and metastasis. A favourable prognosis associated with the presence of  $T_{RM}$  cells has already been reported in studies of high-grade serous ovarian cancer patients, non-small cell lung carcinoma patients and breast cancer<sup>[41]</sup>. In lung cancer, although only a small proportion of CD8+ T cells were reported to be  $T_{RM}$ , the majority of  $T_{RM}$  cells were tumour-reactive. Studies in melanoma have also shown an association between  $T_{RM}$  and favourable prognosis<sup>[30,42]</sup>.

This present study showed that  $T_{RM}$  cells exist in MSS, MSI-H:BRAF mutant and MSI-H:BRAF wild type CRC. However, they are in greater abundance in MSI-H than MSS CRC. This is supported by the findings of a recent study by de Vries *et al.*<sup>[21]</sup> on 35 CRC tissues which demonstrated that CD103+  $T_{RM}$  cells were most abundant in MSI-H CRC. In that study 13 MSI-H CRC were included and BRAF status was not considered. Our study had 42 MSI-H CRC patients which were all subject to immunoscore, of which 33 went on to have multiplex IHC staining to look for presence of  $T_{RM}$  cells and of these 17 were BRAF mutant and 16 were BRAF wild type. Of the 30 MSS CRC patients, 11 went on to have multiplex IHC staining to look for presence of  $T_{RM}$  cells. Our study showed that both MSI-H:BRAF mutant and BRAF wild type subgroups had significantly greater abundance of  $T_{RM}$  cells when compared to the MSS subgroup. There was no statistically significant difference between MSI-H:BRAF mutant and BRAF wild type subgroups.

There was a trend to increased PD-1+ in the  $T_{RM}$  T cell population in MSI-H:BRAF wild type compared to MSI-H:BRAF mutant and MSS subgroups, but this was not statistically significant. This is an important finding as in advanced CRC this T cell subpopulation amongst other PD-1+ immune cells may be exhausted by PD-1/PD-1 ligand binding, a mechanism of escaping immunosurveillance by tumours. Reactivation of exhausted PD-1+  $T_{RM}$  cells may provide therapeutic opportunities that may be exploited in MSI-H:BRAF wild type CRC. PD-1 expression has been reported to be more prominent in cells with a  $T_{RM}$  phenotype<sup>[25,43]</sup>, and in the case of CRC it may

be their numbers that impacts the outcome.

The potential ways which CD8+ T<sub>RM</sub> cells can be harnessed to improve the success of immunotherapeutic targets has already been the focus of several general reviews<sup>[38,44-46]</sup> as well as in breast cancer<sup>[31]</sup> and melanoma<sup>[47,48]</sup>, with Edwards *et al*<sup>[42]</sup> reporting that CD8+ T<sub>RM</sub> cells being prognostic in metastatic melanoma and potentially initiating response to immunotherapy with anti-PD-1. Therefore, the role of T<sub>RM</sub> cells could have important implications for checkpoint-inhibition therapy in CRC<sup>[49]</sup>.

The mechanisms by which T<sub>RM</sub> cells confer immunity remains unclear. Recent studies by Park *et al*<sup>[24]</sup> have demonstrated that depletion of T<sub>RM</sub> cells in melanoma triggered tumour outgrowth of mice with occult melanoma, demonstrating that T<sub>RM</sub> cells have an important role in cancer surveillance and equilibrium and may keep cancer cells dormant<sup>[50]</sup>. This effect may be long term as T<sub>RM</sub> cell populations are able to persist in barrier tissues for a prolonged period of time<sup>[51]</sup>. Menares *et al*<sup>[52]</sup> suggest that the mechanism by which T<sub>RM</sub> cells provide immunity is *via* cross-talk with dendritic cells to trigger the spread of cytotoxic CD8+ T cells in response to tumour.

### Limitations

The use of a single marker (CD103) to define T<sub>RM</sub> cells is a limitation. However, many studies have consistently shown that CD103+CD8+ T cells have the hallmark of T<sub>RM</sub> T cells<sup>[24,53]</sup>. Further, the study sample size was small ( $n = 72$ ) and we were only able to successfully perform quantitative multiplex IHC staining and imaging on 44 samples. Nonetheless, this is to date the largest study on T<sub>RM</sub> cells in CRC.

## CONCLUSION

This study has shown that in CRC a significant fraction of TILs is made up of T<sub>RM</sub> cells and are found in greater abundance in MSI-H: BRAF mutant and MSI-H: BRAF wild type compared with MSS CRC tumours. Acknowledging that there is a complex interaction of factors at play in influencing the prognosis in CRC, the abundance of T<sub>RM</sub> cells may contribute to the favourable prognosis observed in MSI-H CRC when compared to MSS<sup>[15,16]</sup>. This is because MSS CRC has significantly less T<sub>RM</sub> cells when compared to MSI-H CRC. The abundance of T<sub>RM</sub> cells in MSI-H CRC may also explain the reduced likelihood of metastases in patients with MSI-H CRC<sup>[54]</sup> and also the difference in response to immunotherapy between MSI-H CRC and MSS CRC<sup>[20]</sup>. It is important to understand that while T<sub>RM</sub> cells can provide immunity in solid organ tumours, the immunity conferred is by no means absolute and cancers still arise and continue to disseminate despite the presence of T<sub>RM</sub> cells. Further studies should focus on the potential immunogenic qualities of T<sub>RM</sub> cells and investigate potential immunotherapeutic approaches to improve treatment and survival associated with CRC.

## ARTICLE HIGHLIGHTS

### Research background

The presence of resident memory T (T<sub>RM</sub>) cells has already been reported in studies of high-grade serous ovarian cancer patients, non-small cell lung carcinoma patients and breast cancer, but there has been limited evidence on T<sub>RM</sub> cells in the scientific literature in colorectal cancer (CRC). This is a landmark study on T<sub>RM</sub> cells in CRC.

### Research motivation

With T<sub>RM</sub> cells showing promise in several solid organ tumour studies, the premise of this study was to evaluate if T<sub>RM</sub> cells are present in CRC and thus potentially be a target for immunotherapy/novel target therapy in the future.

### Research objectives

The objective of this study was to examine the potential role of T<sub>RM</sub> cells in providing immunogenicity in CRC stratified by microsatellite instability (MSI) and BRAF status.

### Research methods

Formalin fixed paraffin embedded tumour sections were successfully stained using quantitative multiplex IHC staining. All IHC staining was performed using an Opal Multiplex immunohistochemistry (IHC) assay kit (PerkinElmer, Waltham, MA, United

States) and optimised in-house.

### Research results

This study has shown that CD8+ T<sub>RM</sub> are found in greater abundance in both high level MSI (MSI-H):BRAF mutant and MSI-H:BRAF wild type CRC when compared with their microsatellite stable (MSS) counterpart. CD8+ T<sub>RM</sub> may play a role in the immuno-genicity in both BRAF mutant and BRAF wild type MSI-H CRC. The abundance of T<sub>RM</sub> cells may contribute to the favourable prognosis observed in MSI-H CRC when compared to MSS CRC.

### Research conclusions

T<sub>RM</sub> cells are found in greater abundance and contributes to the immunogenicity of MSI-H CRCs.

### Research perspectives

T<sub>RM</sub> cells may be a target for immunotherapy/novel target therapy in MSI-H CRCs, and future research should focus on the potential value of T<sub>RM</sub> cells, as well as therapeutic agents that may stimulate T<sub>RM</sub> activity or adoptive cell transfer aimed at harvesting and utilizing the patients' own immune cells such as specially altered T-cells to precisely and specifically target cancer cells.

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