REVIEWERS COMMENTS - ANSWERS

Thank you for your interest and review of our manuscript.

Reviewer #1:
Thank you for the privilege of reviewing your work. The authors report that T RM cells exist in MSS, MSI-H BRAF mut and MSI-H BRAF wt CRC. However, it is in greater abundance in MSI-H than MSS CRC. This manuscript is well written. While interesting, the manuscript has number of small shortcomings.

1. In abstract section, methods is long, you may describe in briefly.

The methods section in the abstract has been significantly shortened.

2. In Method section, what is eligible criteria?

The eligibility criteria for inclusion in the study was based on availability of both MSI status and BRAF mutation data for consecutive patients held in our database. The limitation on the number of patients included in the study was mainly due to availability of BRAF staining as this was not routinely performed at our institution and therefore only a percentage of patients held in the database had this data.

3. You described 72 patients were eligible. How many patients did you analyze? When did you analyze?

All 72 patients had blocks retrieved and representative tumour and normal blocks inspected for inclusion in the study. However, 28 of the slides did not have both invading edge and core regions which was a requirement for the analysis. Therefore only 44 patient tumour samples were analysed by Opal multiplex IHC staining. The IHC analysis was performed after the selection of the appropriate specimens that contained the required regions.

4. 44 patients were successfully underwent multiplex immunofluorescence staining. Why did 28 patients go wrong?

Please see response to point 3 above.

5. Were samples collect from primary or metastatic lesion?

All samples were collected from the primary tumour form patients that had undergone resection for colorectal cancer.

6. In Table1, I cannot understand Stage A, B, C and D.

Stage A, B, C, D comes from the Australian Clinicopathological Staging system. It is similar to Stage I, II, III, IV with minor differences and so that it is more consistent with international literature, we have changed it to Stage I, II, III, IV. The primary reference for the ACPS is…….

Table 1. Australian clinicopathological staging

Stage	Substage	Spread
A	A1	Not beyond mucosa
	A2	Into submucosa but not beyond
	A3	Into muscularis propria but not beyond
B	B1	Beyond muscularis propria; free mesothelial surface not invaded; no lymph node metastases; no tumour in lines of resection; no distant metastases
	B2	As for Substage B1 but with free mesothelial surface invasion
C	C1	Metastatic spread to local lymph nodes irrespective of depth of direct spread of tumour; no tumour in lines of resection; no distant metastases
	C2	Metastatic spread to an apical lymph node, irrespective of depth of direct spread of tumour; no tumour in lines of resection; no distant metastases
D (incurable)	D1	Tumour involving a line of resection (histological)
	D2	Distant metastases i.e. metastases not removed in continuity with the bowel resection specimen (clinical or histological)

Note: (1) As the distal portion of the rectum lacks a peritoneal covering, tumours in this region cannot be classified substage B2. (2) Substage A1 as defined in this table is classified as stage 0 using the ACPS system

7. Table1, I cannot understand low grade, average grade and high grade. Did all the samples collected from cancer? 8.

The grade of cancer (low, moderate and high grade) is part of synoptic reporting for colorectal cancer. We have changed the wording of average to moderate.

The frequency of MSI changes in unresectable advanced cancer or postoperative cancer. You should add unresectable advanced cancer or postoperative cancer in the patient demographics

The aim of this study was not to look at MSI in unresectable advanced cancer. This is a basic sciences study looking at the immune landscape in particular T cells in colorectal cancer based on MSI and BRAF status.

9. Statistically, I think that there is only one healthy control. I think we need more than 5 people

The healthy control was a baseline parameter for the technical calibration of the Opal multiplexing technique. This study primary and secondary objective was not to compare the three groups with healthy control, but rather to compare MSS vs. MSI-H BRAF +ve and MSI-H BRAF -ve subgroups.

Science editor:

(2) The "Author Contributions" section is missing. Please provide the author contributions;

The author contributions have been included at the end of the manuscript.

1. Author Contributions: J.T., A.F., U. P. conceptualised and designed the study experimental and analytical methods. J.T. wrote the manuscript. A.F. carried out the experiment. U.P. and K.S. supervised the project. J.T., A.F., U.P., K.S. and H. M. were involved in critical revisions of the manuscript.

(3) The authors did not provide original pictures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor;

The figures have been provided in powerpoint file type.

(4) PMID and DOI numbers are missing in the reference list. Please provide the PubMed numbers and DOI citation numbers to the reference list and list all authors of the references. Please revise throughout;

This has been done with thanks.

(5) The "Article Highlights" section is missing. Please add the "Article Highlights" section at the end of the main text;

An article highlights section has been added at the end of the main text.

ARTICLE HIGHLIGHTS

The presence of resident memory T (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, but there has been limited evidence on T_{RM} cells in the scientific literature in colorectal cancer (CRC). This is a landmark study on T_{RM} cells in CRC.

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

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

Fixed paraffin embedded (FFPE) tumour blocks were successfully stained on quantitative multiplex IHC staining. All IHC staining was performed using an OPAL Multiplex immunohistochemistry (IHC) assay kit (PerkinElmer, Waltham, Massachusetts, US) and optimised in-house.

This study has shown that CD8+ T_{RM} 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 MSS counterpart. CD8+ T_{RM} may play a role in the immunogenicity in both BRAF mutant and BRAF wild type MSI-H CRC. The abundance of T_{RM} cells may contribute to the favourable prognosis observed in MSI-H CRC when compared to microsatellite stable (MSS) CRC.

T_{RM} cells are found in greater abundance and contributes to the immunogenicity of MSI-H CRCs.

T_{RM} 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_{RM} cells, as well as therapeutic agents that may stimulate T_{RM} activity or adoptive cell transfer aimed at harvesting and utilising the patients' own immune cells such as specially altered T-cells to precisely and specifically target cancer cells.

(6) Authors should always cite references that are relevant to their study. Please check and remove any references that not relevant to this study.

This has been done with thanks.

Recommendation: Conditional acceptance.

Thank you. Additional language copy editing has been performed on the manuscript to improve any perceived deficiencies.