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CD103$^+$ intraepithelial lymphocytes—a unique population in microsatellite unstable sporadic colorectal cancer

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Abstract

Colorectal cancers with microsatellite instability (MSI) typically show increased numbers of intraepithelial lymphocytes (IEL) in comparison to microsatellite stable (MSS) cancers. The aim of this study was to determine the phenotype of this unique lymphocyte population in MSI and MSS colorectal cancers. Twenty-four individuals with sporadic colorectal cancer (17 MSI, 7 MSS) were included in this study. Intraepithelial and stromal lymphocytes were detected using immunohistochemistry with anti-CD8 and anti-CD103 antibodies, and two observers independently quantified the numbers of lymphocytes. CD103$^+$ ($\alpha^E\beta_7^+$) IELs detected within tumour tissue co-expressed CD8$^+$ while the stromal lymphocytes were phenotypically heterogeneous, with respect to CD8$^+$ and CD103$^+$ expression. MSI colorectal cancers harboured increased numbers of CD8$^+$CD103$^+$ IELs, as well as CD8$^+$CD103$^-$ and CD8$^+$CD103$^+$ stromal lymphocytes, when compared with MSS colorectal cancers. CD103$^+$ IELs were found at 27-fold greater numbers in the tumour epithelium than in normal epithelium from the same patient ($P=0.001$, Wilcoxon matched pairs test). From our findings, we have proposed a mechanism for the homing of these $\alpha^E\beta_7^+$ lymphocytes to tumour tissue in MSI and MSS colorectal cancers.

Keywords: Lymphocytes; MSI; MSS; Colorectal cancer; Mechanism

1. Introduction

A genome wide instability in repetitive DNA sequences, referred to as 'microsatellite instability' (MSI) is observed in 10–15% of sporadic colorectal cancers [1] and in nearly all cases of Hereditary Non-polyposis Colorectal Cancer (HNPCC) [2]. Microsatellite instability is caused by loss of expression of a mismatch repair gene: *hMSH2, hMLH1, hPMS1, hPMS2, hMSH6/GTBP or hMSH3*, either as a result of mutations or epigenetic silencing [3–7].

Irrespective of their origin, MSI colorectal cancers have a distinctive pathological appearance. They are predominantly found in the right side of the colon, are large in size, and are typically poorly differentiated and mucinous [8–10]. MSI cancers also display three patterns of infiltrating lymphocytes, including a Crohn's-like immune response [11], a peritumoral reaction, and intraepithelial lymphocytes (IELs) [8].

In normal human intestinal tissue, IELs are characterised by their unique expression of the integrin $\alpha^E\beta_7$ [12–15]. These cells play an important role in mucosal defence since they are the first lymphoid cells to encounter dietary antigens and pathogens [16–18]. Additional evidence suggests that these IELs are comprised of phenotypically distinct subsets of CD8$^+$ T cells [19–22].

When found within the neoplastic epithelium of MSI colorectal cancers, IELs have been identified as CD8$^+$ cytotoxic T cells that are capable of inducing tumour cell apoptosis [23,24]. Not surprisingly, it has been speculated that the high numbers of IELs in MSI colorectal cancers may play an important role in inhibiting the progression of these tumours. If this was the case, then it may account for the improved prognosis of individuals with such tumours [25].

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Despite the possible importance of these $CD8^+$ IELs in colorectal cancer, their exact phenotype and their relationship to the IELs found in normal colonic mucosa remains unknown.

In the present study, we examined the phenotype of intraepithelial and stromal lymphocytes found in both normal mucosa and tumour tissue from individuals with either MSI or microsatellite stable (MSS) sporadic colorectal cancer, in order to determine if $\alpha^E\beta_7$ is expressed on the intraepithelial lymphocytes seen within these cancers.

2. Patients and methods

2.1. Patient selection

Seventeen MSI and seven MSS colorectal cancer specimens were obtained from 24 individuals who underwent surgical resection for colorectal carcinoma at St Vincent's Hospital, Sydney. Paired normal colonic mucosa was also obtained from eighteen of these individuals (13 MSI cases and 5 MSS cases). Informed consent was obtained from all individuals. The above-described cohort of patients was recruited from a previous study where the MSI status of all tumours was determined [26]. MSI and MSS tumours were matched for age. The histological type and stage of all of the tumours was determined without knowledge of their MSI status, according to the American Joint Committee (AJCC) criteria for colorectal carcinoma [27]. Tumours in which less than 10% of cells formed glands were classified as poorly differentiated, while those that contained greater than 50% extracellular mucin were classified as mucinous [28].

2.2. Immunohistochemistry

Immunohistochemistry for the detection of the hMLH1 and hMSH2 proteins was performed on paraffin sections as previously described in Ref. [26]. Immunohistochemical analysis of CD8 and CD103 expression on lymphocytes was performed on 4-μm acetone-fixed, frozen tissue sections as described below. Tissue sections were first blocked for endogenous biotin activity using a DAKO Biotin Blocking Kit (DAKO, Dakopatts, Denmark), and then incubated with 0.3% (w/v) hydrogen peroxide for 5 min. They were then incubated with 100 μl of anti-CD8 (DAKO, 1:50) or anti-CD103 (DAKO, 1:50) antibody at room temperature for 1 h.

After washing, the bound antibody was detected with a prediluted solution of biotinylated anti-mouse immunoglobulin (DAKO, Dakopatts, Denmark) and a prediluted solution of streptavidin horseradish-peroxidase (DAKO, Dakopatts, Denmark). Chromogen substrate was added (0.05% (w/v) 3,3′-diaminobenzidine), and after colour development the sections were counterstained with haematoxylin, dehydrated and coverslipped.

The number of $CD8^+$ and $CD103^+$ positive lymphocytes was determined independently by two observers who were blinded to the MSI status of the tumours. Lymphocytes were classified as intraepithelial if they were located between or in direct contact with tumour cells. Those lymphocytes within the lamina propria were classified as stromal lymphocytes. Each observer counted all lymphocytes within 10 random high power fields (0.23 mm^2/field), and counts were corrected for the percentage of epithelium or stroma in each of the fields. Final lymphocyte counts were calculated as the average of the counts of the two observers, and expressed as lymphocytes per mm^2 of tumour epithelium, stromal tissue or normal colon epithelium as appropriate.

2.3. Statistical analysis

For non-parametric data, the difference between groups was analysed by the Mann–Whitney test. Categorical

Table 1
The clinicopathological characteristics of tumours according to microsatellite instability status

	All patients ($n=24$)	MSI ($n=17$)	MSS ($n=7$)	P value
Characteristic				
Age, mean years±S.D.	75.8±9.7	73.7±10.3	80.7±6.1	NS
Sex, no. (%)				
Females	16 (67)	11 (65)	5 (71)	
Males	8 (33)	6 (35)	2 (29)	NS
Site, no. (%)				
Proximal	21 (88)	16 (94)	5 (71)	
Distal	3 (13)	1 (6)	2 (29)	NS
Stage (UICC), no. (%)				
I	2 (8)	1 (6)	1 (14)	
II	17 (71)	13 (76)	4 (57)	
III	3 (13)	2 (12)	1 (14)	
IV	2 (8)	1 (6)	1 (14)	NS
Differentiation, no. (%)				
Well or Moderate	18 (75)	12 (71)	6 (86)	
Poor	6 (25)	5 (29)	1 (14)	NS
Histopathological type, no. (%)				
Mucinous	10 (42)	10 (59)	0	
Non-mucinous	14 (58)	7 (41)	7 (100)	<0.01
hMLH1 IHC, no. (%)				
Present	7 (29)	2 (12)	6 (86)	
Absent	17 (71)	15 (88)	1 (14)	<0.001
hMSH2 IHC, no. (%)				
Present	21 (88)	14 (82)	7 (101)	
Absent	3 (13)	3 (18)	0	NS

MSI, microsatellite unstable; MSS, microsatellite stable; IHC, immunohistochemistry; NS, not statistically significant; S.D., standard deviation; UICC, International Union against Cancer. P values refer to differences between MSI and MSS cancers.

variables were compared with the use of the Pearson's Chi-square test, Spearman's Rank correlation test or Fisher's Exact test as appropriate. For paired data, the Wilcoxon matched pairs *t*-test was used to determine if one variable was significantly different between two matched groups. A two-sided probability of less than 0.05 was considered to be statistically significant.

When scatterplots were drawn, Pearson product moment correlation coefficients were calculated. A correlation coefficient (*r*) value of 1 was considered perfect. All data were analysed using the Statistical Package for the Social Sciences (SPSS) statistical software V9.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Phenotypic analysis of lymphocytes in MSI and MSS colorectal cancers

As shown in Table 1, the 24 tumour specimens in this study showed phenotypic features consistent with those previously described for MSI and MSS colorectal cancers. In particular, 17 MSI cancers showed a mucinous histology and reduced hMLH1 expression. Immunophenotypic analysis of the lymphocytes within the MSI and MSS tumours clearly demonstrated that the former harboured higher numbers of $CD8^+$ and $CD103^+$ lymphocytes (Fig. 1). Correlation of the $CD8^+$ and $CD103^+$ IEL counts indicated that approximately 90% of the $CD103^+$ IELs detected within tumour tissue also co-expressed $CD8^+$ (Fig. 2a). However, the wide distribution of the stromal lymphocyte counts (Fig. 2b) suggested that these lymphocytes were likely to be more phenotypically heterogeneous with respect to $CD8^+$ and $CD103^+$ expression.

3.2. Distribution of lymphocytes within MSI and MSS colorectal cancers

Analysis of all cell counts revealed that interobserver variation in lymphocyte counting was minimal ($P<0.01$, Pearsons correlation), and also confirmed the pattern of lymphocyte distribution seen by immunohistochemistry. MSI colorectal cancers had higher numbers of both $CD8^+$ and $CD103^+$ intraepithelial (Fig. 3a and b) and stromal lymphocytes (Fig. 3c and d) when compared with the MSS tumours.

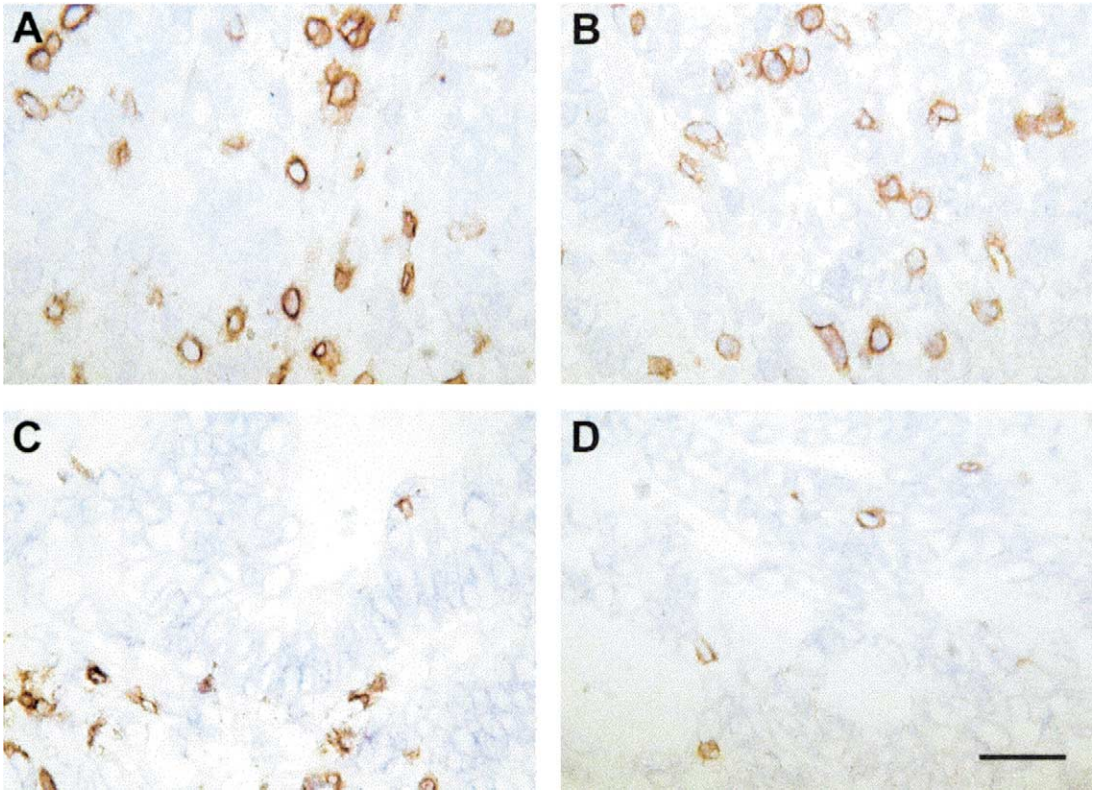

Fig. 1. Immunostaining of $CD8^+$ and $CD103^+$ lymphocytes in frozen tissue sections from microsatellite unstable (MSI) and microsatellite stable (MSS) colorectal cancers. The panels above show photomicrographs of representative fields from frozen sections of MSI (A and B) and MSS (C and D) colorectal cancer tissue stained with the anti-CD8 (A and B) and anti-CD103 (B and D) antibodies. $CD8^+$ (A) and $CD103^+$ (B) lymphocytes were more prominent within tumour epithelium of the MSI colorectal cancers compared with the MSS colorectal cancers. The MSS colorectal cancers harboured less $CD8^+$ (C) and $CD103^+$ (D) lymphocytes compared to the MSI colorectal cancers. Immunoperoxidase staining with 0.05% (w/v) 3,3′-diaminobenzidine and haematoxylin counterstain. All panels are of equal dimensions and the bar shown in panel d = 100 μm.

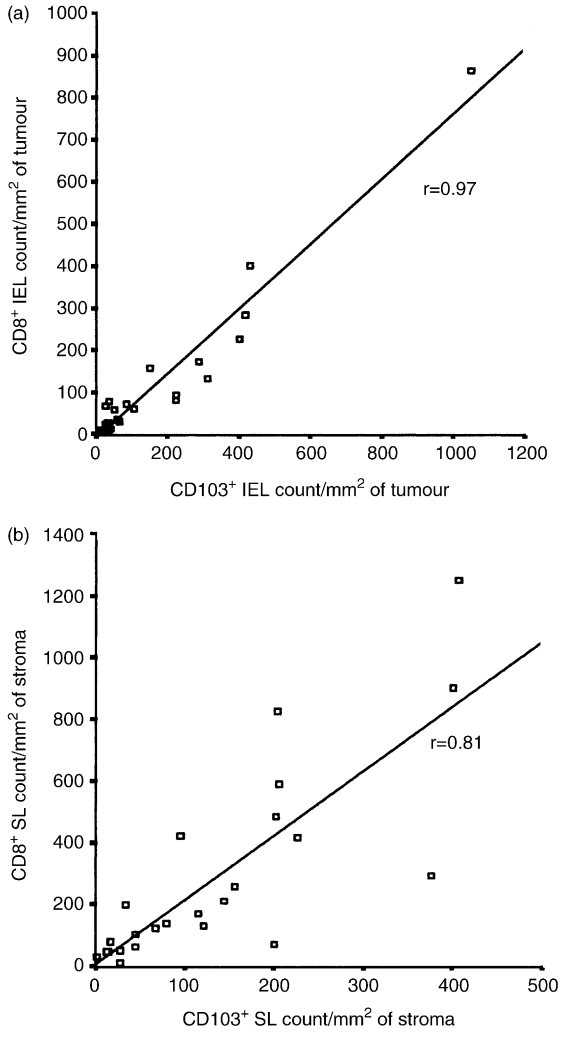

Fig. 2. Correlation between the $CD8^+$ and $CD103^+$ intraepithelial lymphocyte (IEL) and stromal lymphocyte (SL) counts in both microsatellite stable (MSS) and microsatellite unstable (MSI) colorectal cancers. To investigate the phenotypes of the intraepithelial (a) or stromal lymphocyte (b) population in the MSI and MSS colorectal cancers, mean $CD8^+$ and $CD103^+$ lymphocyte counts from the 24 tumours were plotted as shown above. There is a strong correlation between the $CD8^+$ and $CD103^+$ intraepithelial lymphocyte populations. In comparison, the distribution and correlation of the $CD8^+$ and $CD103^+$ stromal lymphocyte counts, suggest a more heterogeneous population of lymphocytes within the stroma of the tumours. r = Pearson product moment correlation coefficient.

Intraepithelial lymphocytes within the MSI cancers were most likely to be of the $CD8^+CD103^+$ phenotype. MSI colorectal cancers also contained large numbers of stromal lymphocytes expressing either the $CD8^+CD103^+$ or $CD8^+CD103^-$ phenotype. While the number of $CD8^+CD103^+$ intraepithelial lymphocytes within the MSS colorectal cancers was small in comparison to the MSI tumours, the MSS tumours also harboured a large population of $CD8^+CD103^-$ stromal lymphocytes.

No statistical difference was found in the number of $CD8^+$ and $CD103^+$ lymphocytes between the MSI and MSS tumours, largely reflecting the marked variation in the numbers of lymphocytes within the MSI tumours themselves. However, when classified as showing either low or high levels of $CD103^+$ IELs (with a cut-off count of 100 IELs/mm^2 of tumour), MSI tumours were associated with a high $CD103^+$ IEL count ($P = 0.019$, Fisher's Exact test) and a poorly differentiated histology ($P = 0.044$, Fisher's Exact test). No other significant correlation was found between these two tumour subgroups (high versus low $CD103^+$ IELs) and the other clinicopathological features examined in this study.

3.3. CD103 up-regulation occurs locally within MSI colorectal cancer

To determine if the up-regulation of the $CD8^+$ and $CD103^+$ IELs within MSI colorectal cancer tissue was a local effect due to the tumour, or a systemic effect of the tumour immune response, matched normal colon tissue was immunostained for these lymphocyte markers.

A reservoir of $CD8^+$ stromal lymphocytes was present in the normal colonic mucosa from both MSI (median: 49.7 lymphocytes/mm^2 of stroma, range: 14.2–123.3) and MSS (median: 42.1, range: 32.2–78.9) colorectal cancer patients. However, there were very low numbers of $CD103^+$ IELs in normal colonic mucosa from both MSI (median: 5.38 lymphocytes/mm^2 of normal epithelium, range: 0–19.16) and MSS patients (median: 6.1, range: 0–16.58).

The tumour tissue from MSI colorectal cancer patients contained a 27-fold greater number of $CD103^+$ IELs (median: 149.27 lymphocytes/mm^2 of tumour epithelium, range: 10.23–1050.87) compared with the normal colonic mucosa (median: 5.38 lymphocytes/mm^2 of normal epithelium, range: 0–19.16) from these patients ($P = 0.001$, Wilcoxon matched pairs test). These two observations indicated that the increased number of $CD103^+$ IELs seen within the MSI tumours was not a result of the systemic up-regulation of $\alpha^E\beta_7$ on lymphocytes, but that up-regulation was occurring locally within the tumour tissue.

4. Discussion

This study further confirms the observation that MSI colorectal cancers harbour increased numbers of intraepithelial lymphocytes (IELs), in comparison to MSS colorectal cancers [9,23]. Immunohistochemical analysis of these IELs within colorectal cancer tissue has to date been performed using only anti-CD3 and anti-CD8 antibodies [23,25]. By using a monoclonal anti-CD103 antibody that is specific for the α^E subunit of the $\alpha^E\beta_7$

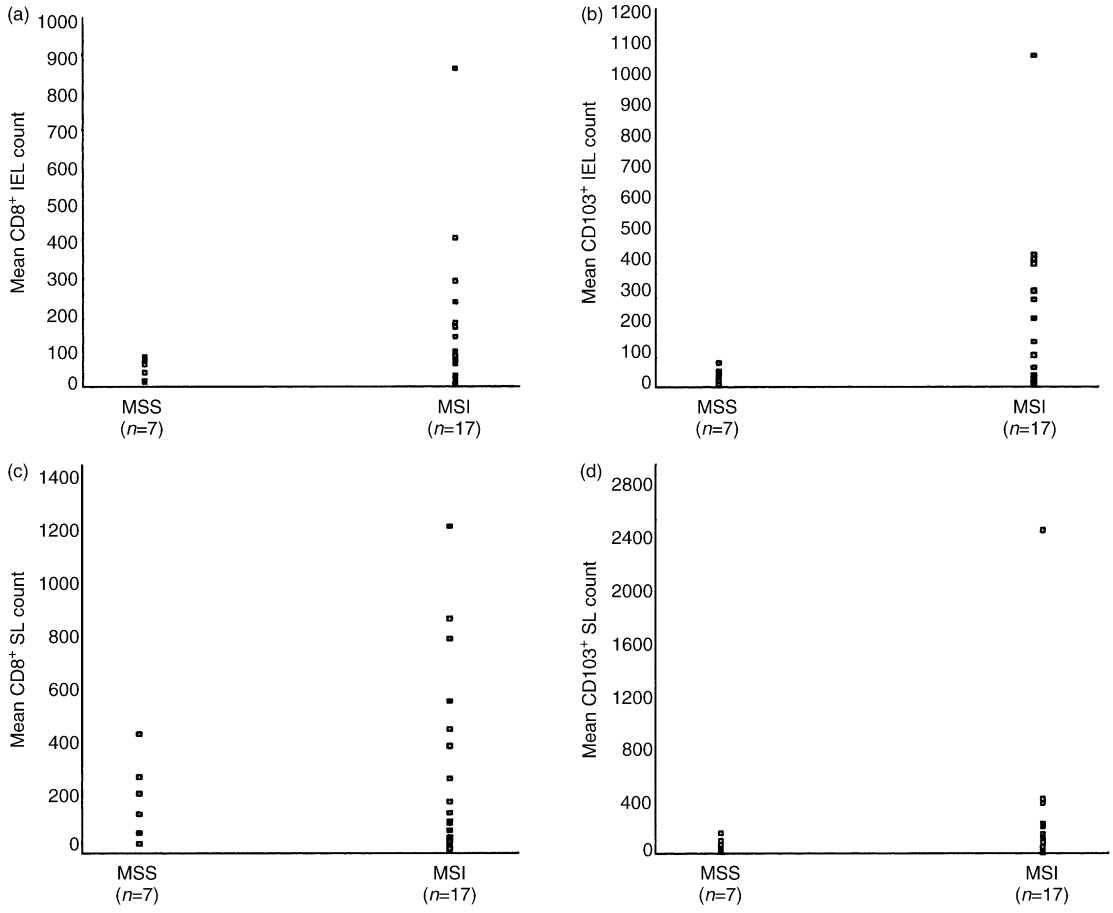

Fig. 3. Distribution of $CD8^+$ and $CD103^+$ intraepithelial lymphocyte (IEL) and stromal lymphocyte (SL) counts in microsatellite stable (MSS) and microsatellite unstable (MSI) colorectal cancers. Lymphocyte counts were expressed as per mm^2 of tumour or stroma where appropriate. MSI colorectal cancers had higher numbers of both $CD8^+$ IELs (median: 81.28 versus 36.26) and $CD103^+$ IELs (median: 149.27 versus 41.18) compared with the MSS tumours (a and b). MSI colorectal cancers also had higher numbers of $CD8^+$ (median: 170.5 versus 122.4) and $CD103^+$ (median: 144.48 versus 34.13) stromal lymphocytes (c and d) than their MSS counterparts.

integrin, we were able to confirm that the IELs seen in both MSI and MSS colorectal cancers express the $\alpha^E\beta_7$ integrin [29]. Therefore, it is the accumulation of these $CD103^+$ lymphocytes, which is responsible for the prominence of IELs typical of the MSI colorectal cancer phenotype.

From this study of IELs, a model of lymphocyte homing in colorectal cancer tissue can be postulated. Firstly, as part of perhaps a non-specific tumour immune response, both MSI and MSS tumours appear to systemically recruit cytotoxic lymphocytes via a $\alpha^4\beta_7$: MAdCAM-1 interaction on intestinal endothelial cells [30,31]. We found that both MSI and MSS tumour tissues contain a large reservoir of $CD8^+$ stromal lymphocytes, which were most likely to be $CD103^-$ in the case of MSS tumours and either $CD103^+$ or $CD103^-$ in the case of MSI tumours.

Secondly, to move from the stroma to the epithelium, these lymphocytes must express the $\alpha^E\beta_7$ integrin. IELs within tumour tissue from both MSI and MSS colorectal cancer patients displayed the $CD8^+CD103^+$ phenotype. This finding is in agreement with studies that have shown that IELs are $CD8^+$ T cells [13,19,20].

In the case of MSI tumours, we found that while stromal lymphocytes were a heterogeneous population ($CD8^+CD103^-$ and $CD8^-CD103^+$), IELs were homogeneously $CD8^+CD103^+$. This suggests that once these stromal lymphocytes express the $\alpha^E\beta_7$ integrin, they migrate rapidly to the tumour epithelium, and is consistent with the known homing capacity of $\alpha^E\beta_7^+$ lymphocytes to epithelial cells [32].

Finally, the data from this study support the concept that local up-regulation of $\alpha^E\beta_7$ is a particular feature of MSI colorectal cancer, and is responsible for the increased number of IELs seen within these cancers. The mechanism for this up-regulation in MSI colorectal cancer remains unknown. However, it is known that $\alpha^E\beta_7$ expression is upregulated after exposure to Transforming Growth Factor-β1 (TGF-β1) in the normal intestinal mucosa [13,30,32–35]. Therefore, it would be reasonable to conclude that up-regulation of the $\alpha^E\beta_7$ integrin by TGF-β1 may also be important in the setting of MSI colorectal cancer.

In order to confirm this hypothesis, it would be necessary to quantify the levels of TGF-β1 in tumour tissue. This is technically difficult, since the TGF-β1 protein undergoes cleavage before binding to its receptors [36–38], and assays must therefore be able to discriminate between the active and latent forms of TGF-β1.

Following up-regulation of $\alpha^E\beta_7$ expression on lymphocytes, the expression of E-cadherin on tumour epithelium must play a significant role in lymphocyte retention, since this is the only known ligand for $\alpha^E\beta_7$ [31,39–41].

However, it is not yet clear how the $\alpha^E\beta_7$: E-cadherin interaction on IELs may be involved in the cytotoxic response to tumour cells [42–44].

In conclusion, it is IELs of the $CD8^+CD103^+$ phenotype that are more prominent in MSI colorectal cancer. It is likely that after trafficking of $CD8^+$ lymphocytes to MSI cancers, marked local up-regulation of $\alpha^E\beta_7$ expression occurs, and subsequent binding to E-cadherin is responsible for the accumulation of IELs that characterise these tumours.

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