Novel mediators of anti-tumor immunity: dissecting intratumoral immune responses at the single-cell level

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T cells with tumor-specific phenotypes largely originate from pericolic lymph nodes in colorectal cancer

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ABSTRACT

Background

Cancer immunotherapies aiming at reactivating anti-tumor T cell responses have yielded significant clinical benefits. To further improve the success rate of such T cell-based immunotherapies, it is important to characterize and understand the origin and dynamics of anti-tumor T cell responses. Previously, we showed that colorectal cancer (CRC) tissues contained specific populations of tissue-resident activated $CD8^+$ and $CD4^+$ T cells. Here, we aimed to identify their T cell receptor (TCR) repertoire, clonal frequencies, and clonal overlap with paired non-malignant tissues of the same patients.

Methods

Distinct $CD8^+$ and $CD4^+$ T cell populations, defined by the expression of markers of activation (CD38, PD-1) and tissue-residency (CD103), along with their non-activated and non-tissue-resident counterparts, were isolated from seven patients with CRC and processed for bulk TCRβ sequencing. The TCR repertoire in colorectal tumors was subsequently compared with that of matched samples from adjacent healthy mucosa, pericolic lymph nodes, and peripheral blood.

Results

We found clonal enrichment in tissue-resident, activated $CD8^+$ and $CD4^+$ T cells from colorectal tumors, with the most frequent T cell clone presenting frequencies ranging between 10-29% for $CD8^+$ T cells and between 8-39% for $CD4^+$ T cells in the different tumors. TCR clonotypes of tissue-resident, activated $CD8^+$ and $CD4^+$ T cells from colorectal tumors showed most overlap with matched pericolic lymph node samples, while their non-activated counterparts showed frequent TCRβ clonal overlap with T cells from adjacent healthy mucosa.

Conclusion

The TCRβ clonal overlap of tissue-resident, activated T cells with pericolic lymph node samples suggests that lymph nodes are an important source of tumor-reactive $CD8^+$ and $CD4^+$ T cells. These findings should be extended to a larger cohort of patients with CRC.

INTRODUCTION

T cell-mediated adaptive immune responses play an important role in cancer immunosurveillance and have predictive value with respect to natural disease progression[1,2] and response to immunotherapy[3-5]. Cancer cells express antigens presented by HLA class I molecules that may activate anti-tumor CD8+ T cell responses.[6] Intratumoral CD8+ T cells are particularly abundant in patients with DNA mismatch repair (MMR)-deficient cancers bearing a high mutational load and, therefore, a vast burden of antigens derived from somatic mutations (neoantigens).[7,8] Cancer immunotherapies targeting the T cell repertoire, such as T cell checkpoint blockade with antibodies against PD-1/PD-L1 and/or CTLA-4 are proving to be effective at restoring T cell-mediated immune responses.[5,9-11] However, durable clinical responses are observed in only a minority of patients, highlighting the need to better understand the origin and dynamics of anti-tumor T cell responses.

In colorectal cancer (CRC), the presence of intratumoral CD8+ T cells is correlated with improved prognosis.[12,13] We previously identified tumor tissue-specific CD8+ T cell clusters in colorectal tumors.[14] These T cell populations were characterized by the co-expression of tissue-residency (CD103) and activation markers (CD38, CD39, and PD-1), and were infrequent in colorectal healthy mucosa, pericolic lymph nodes, and peripheral blood samples. Interestingly, co-expression of CD39 and CD103 was reported to identify tumor-reactive CD8+ T cells in different types of cancer, including CRC.[15-17] Of note, tumor tissue-specific CD4+ T cells with a similar activated tissue-resident phenotype were found in colorectal tumor samples.[14] Understanding the clonal relationships between such distinct CD8+ and CD4+ T cell populations and their tissue of origin might shed light on the dynamics of anti-tumor T cell responses. In αβ T cells, most receptor diversity is contained within the third complementarity-determining region (CDR3) of the T cell receptor (TCR) alpha and beta chains. Targeted sequencing of the CDR3 region can identify T cell clones, clonal frequencies, and clonal expansion, a characteristic of antigen specific responses. Clonal enrichment of T cell populations has been shown to correlate with durable responses to PD-1 blockade.[3,18,19]

In this study, we performed DNA sequencing of the CDR3 region of the TCR beta-chain (TCRβ) to determine the TCR repertoires of tissue-resident, activated CD8+ and CD4+ T cells in colorectal tumors, and subsequently compare those with total CD8+ and CD4+ T cells from matched samples of adjacent healthy mucosa, pericolic lymph nodes, and peripheral blood from seven patients with CRC.

RESULTS

TCR repertoire analysis indicates clonal enrichment in tissue-resident, activated CD8+ and CD4+ T cells from colorectal tumors

TCR repertoire analysis was performed on DNA isolated from different FACS-sorted T cell populations from colorectal tumors defined as activated (CD38+ PD-1+) and non-activated

(CD38$^-$ PD-1$^-$), tissue-resident (CD103$^+$) and non-tissue-resident (CD103$^-$) (**Figure S1**). Those TCR sequences were compared to the CD8$^+$ and CD4$^+$ TCR repertoire of matched adjacent healthy mucosa, pericolic lymph nodes, and peripheral blood samples of the same patients (**Figure S1**). A total of 10,374 unique clonotypes were detected in the different T cell subsets isolated from colorectal tumors, 7,653 in adjacent healthy mucosa, 34,607 in pericolic lymph nodes, and 36,562 in peripheral blood samples (**Table 1**).

The majority of CD8$^+$ T cells in colorectal tumors contained a tissue-resident activated phenotype (CD103$^+$ CD38$^+$ PD-1$^+$), while in adjacent healthy mucosa non-activated cells were most abundant (**Figure 1A**). In pericolic lymph nodes and peripheral blood samples, almost all CD8$^+$ T cells consisted of CD103$^-$ CD38$^-$ PD-1$^-$ cells (**Figure 1A**). The Shannon diversity index was lower in intratumoral CD8$^+$ T cell populations as compared to CD8$^+$ T cells from adjacent healthy mucosa, pericolic lymph nodes, and peripheral blood samples of the patients, indicating a low CD8$^+$ TCR repertoire diversity in the tumors (**Figure 1B**). Within colorectal tumors, highest clonality was observed for tissue-resident activated (CD103$^+$ CD38$^+$ PD-1$^+$) CD8$^+$ T cell populations (**Figure 1C**; details are provided in Methods). Enrichment of clonotypes was detected in tissue-resident activated (CD103$^+$ CD38$^+$ PD-1$^+$) CD8$^+$ T cell populations from all patients, with the most frequent T cell clone presenting frequencies ranging between 10-29% in the different tumors (**Table 2**, **Table S1**).

Table 1. Overview of total numbers of cells, reads, and unique clonotypes detected in the clinical samples.

Sample	Colorectal tumor (N=7)			Adjacent healthy mucosa (N=7)**			Lymph node (N=7)**			PBMC (N=7)**		
Population	**All**	**CD8**	**CD4**	**All**	**CD8**	**CD4**	**All**	**CD8**	**CD4**	**All**	**CD8**	**CD4**
Total cells	149,975	58,841	91,134	102,137	45,878	56,259	1,076,387	106,207	970,180	2,866,060	921,506	1,944,554
Total reads	1,7596,118	7,127,674	10,468,444	8,319,137	3,740,426	3,073,885	15,847,118	5,508,073	5,944,784	16,531,234	5,586,063	7,593,642
Total unique clonotypes*	10,374	2057	8334	7653	1243	3009	34,607	3069	22,137	36,562	4826	26,875

*Defined as distinct CDR3β amino acid sequence. **For two samples, cells were not subdivided into $CD8^+$ and $CD4^+$ T cell populations as DNA was isolated directly from fresh-frozen tissue or EDTA blood.*

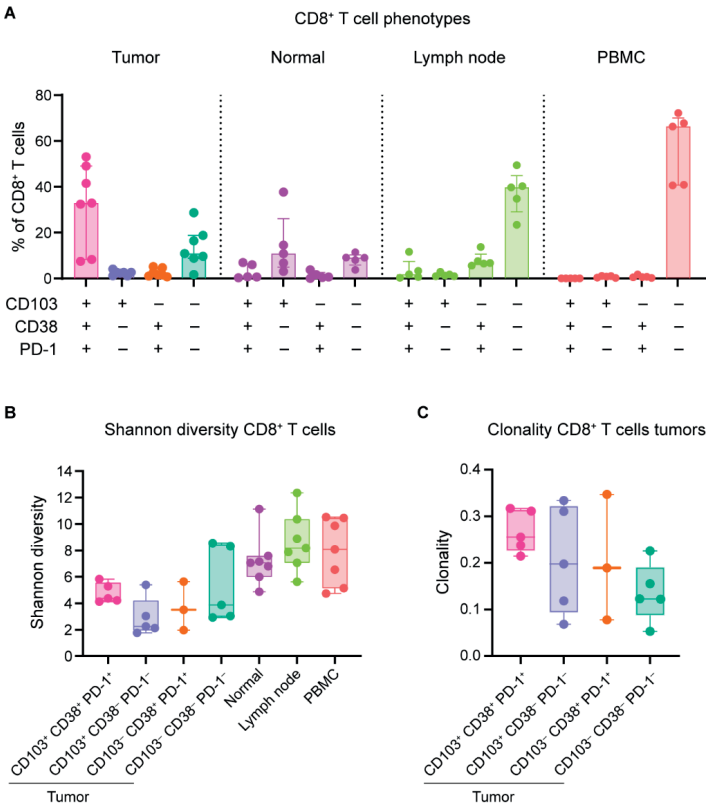

Figure 1. TCR repertoire analysis indicates clonal enrichment in tissue-resident, activated CD8⁺ T cells from colorectal tumors.

A. Frequencies of different CD8⁺ T cell populations among colorectal tumors, adjacent healthy mucosa ("normal"), pericolic lymph nodes, and peripheral blood samples from patients with CRC (N=7) as percentage of total CD8⁺ T cells by flow cytometry. Bars indicate median with IQR. Each dot represents an individual sample. **B**. Shannon diversity of different CD8⁺ T cell populations from colorectal tumors and CD8⁺ T cells from matched adjacent healthy mucosa ("normal"), pericolic lymph nodes, and peripheral blood samples from patients with CRC (N=7) by TCRβ sequencing. Box indicates median with IQR, whiskers min to max. Each dot represents an individual sample. **C**. Clonality of different CD8⁺ T cell populations from colorectal tumors from patients with CRC (N=7) by TCRβ sequencing. Details are provided in Methods. Box indicates median with IQR, whiskers min to max. Each dot represents an individual sample. Data from seven independent experiments.

Table 2. Top ten most frequent CDR3β sequences in tissue-resident, activated CD8⁺ T cells from colorectal tumors.

	CRC 164		CRC 181		CRC 220		CRC 102*		CRC 223*	
Rank	**CDR3β AA sequence**	**Frequency**	**CDR3β AA sequence**	**Frequency**	**CDR3β AA sequence**	**Frequency**	**CDR3β AA sequence**	**Frequency**	**CDR3β AA sequence**	**Frequency**
1	SARDPTSARQY	23.7%	ASSESTIGTQY	28.6%	ASSSRTSGGVGSYNEQF	26.9%	SARTSGGGETQY	10.4%	ASSLATGPNTEAF	13.8%
2	SAWDSPRGPRKLF	12.0%	ASSLREAGLAGGPFSYEQY	15.7%	ASRFLAGGHNEQF	14.5%	ASRKTLGGVRYGYT	7.6%	ASSLTVRYEQY	8.1%
3	ASSKGYGYT	8.4%	ASSATAGSNQPQH	10.5%	ASSWQGPNNEQF	5.5%	ASSLQSRDGYT	6.5%	ASSYTGSTYEQY	6.3%
4	ASRGQEAF	7.6%	ASSFLNEEEQY	6.0%	ASSQEDPYYEQY	3.1%	ASSRLAGRGEQF	6.4%	ASSLVGGPYEQY	6.1%
5	ASSTSGRGYEQY	6.1%	ASIITDSQETQY	3.3%	ASSSQGVEQY	3.0%	ASSYSGRGGDEQF	6.3%	ASSQDRGSYEQY	4.3%
6	ASSLIGSREQY	3.9%	ASIQQGNNQPQH	2.0%	SVGAGGTNEKLF	2.6%	ASSTGRSYGYT	5.4%	ASSFPSAAQY	4.2%
7	ASSLVSAHTNEQF	3.7%	ASSQEEGRQYGYT	2.0%	ASSMSGNYEQY	2.5%	SVKGGYEQY	4.3%	ASSLGWSYEQY	3.9%
8	ASSPTWYSGANVLT	3.5%	ASSQDRPLGAGTLPDTQY	2.0%	ASRGLAEKYNEQF	2.3%	ASSGGAYEQY	2.6%	ASSPDRDVYEQY	3.9%
9	ASSLTKRWGNNGETQY	3.1%	ASSNREGTEAF	1.6%	ASSPTTVGNQPQH	2.0%	ASQNGPGTEQF	2.5%	ASSQAGQPQH	3.3%
10	ASQPWASGAYEQY	2.5%	ASSPFNTEAF	1.6%	ASSMYADEQF	1.9%	ASSAGGVEQY	2.3%	ASSGGDLIANEQF	3.0%

**MMR-deficient colorectal tumors.*

In line with previous observations,[14] CD103$^+$ CD4$^+$ T cells were less prevalent in colorectal tumors as compared to their CD8 counterpart (**Figure 2A**). Tissue-resident activated (CD103$^+$ CD38$^+$ PD-1$^+$) CD4$^+$ T cells were exclusively found in colorectal tumor samples (**Figure 2A**). Within CD103$^-$ CD4$^+$ T cells, both activated (CD38$^+$ PD-1$^+$) and non-activated (CD38$^-$ PD-1$^-$) populations were abundant in CRC tissues (**Figure 2A**). In adjacent healthy mucosa, pericolic lymph nodes, and peripheral blood samples, CD4$^+$ T cells lacking CD103, CD38, and PD-1 were most prevalent (**Figure 2A**). Intratumoral CD4$^+$ T cells showed a low Shannon diversity as compared to matched samples from adjacent healthy mucosa, pericolic lymph nodes, and peripheral blood of the patients (**Figure 2B**). In line with the intratumoral CD8$^+$ T cells, tissue-resident activated (CD103$^+$ CD38$^+$ PD-1$^+$) CD4$^+$ T cells showed the highest clonality in colorectal tumors (**Figure 2C**; details are provided in Methods). Clonal enrichment was observed in tissue-resident activated (CD103$^+$ CD38$^+$ PD-1$^+$) CD4$^+$ T cell populations from all patients, with the most frequent T cell clone presenting frequencies ranging between 8-39% in the different tumors (**Table 3**, **Table S2**). Of note, CD103$^+$ CD38$^-$ PD-1$^-$ CD4$^+$ T cells were not detected in such frequencies to allow TCRβ sequencing.

We next examined whether homology could be observed within CDR3β sequences detected in the intratumoral T cells. For CD8$^+$ T cells, we found CDR3β sequences that were shared by two different colorectal tumors (**Table 4**). The first of those CDR3β amino acid sequences has been reported in dysfunctional CD8$^+$ T cells in hepatocellular carcinoma.[20] Interestingly, we detected a CDR3β sequence that was shared by CD4$^+$ T cells in three different colorectal tumors (**Table 5**). The CDR3β amino acid sequence had frequencies ranging from 0.11 – 2.26% in the tumors (**Table 5**). This specific sequence has been reported in effector (CD57$^+$CD28$^-$) CD4$^+$ T cells in fibrosing mediastinitis.[21]

Tissue-resident, activated CD8$^+$ and CD4$^+$ T cells in colorectal tumors show most overlapping TCR clonotypes with pericolic lymph nodes

The CDR3β sequences detected in intratumoral CD8$^+$ and CD4$^+$ T cell populations were subsequently compared to the CDR3β repertoires of CD8$^+$ and CD4$^+$ T cells from matched samples of adjacent healthy mucosa, pericolic lymph nodes, and peripheral blood to investigate TCRβ clonal overlap. An average of 19% of the T cell clones were shared between the intratumoral tissue-resident activated (CD103$^+$ CD38$^+$ PD-1$^+$) CD8$^+$ T cells and CD8$^+$ T cells from pericolic lymph node samples (**Figure 3A**, **Figure S2**). This indicates a strong clonal relationship between tissue-resident, activated CD8$^+$ T cells and CD8$^+$ T cells from the lymph nodes. In contrast, the non-activated counterpart (CD103$^+$ CD38$^-$ PD-1$^-$) showed frequent TCRβ clonal overlap with CD8$^+$ T cells from healthy mucosal tissue (**Figure 3A**, **Figure S2**), indicating that such T cells with intraepithelial phenotypes migrate from healthy mucosal tissue to the tumor tissue but do not become activated. For the non-tissue-resident, non-activated (CD103$^-$ CD38$^-$ PD-1$^-$) CD8$^+$ T cells, most TCRβ clonal overlap was found (shared) with CD8$^+$ T cells from blood samples (**Figure 3A**, **Figure S2**). Last, CD103$^-$ CD38$^+$ PD-1$^+$ CD8$^+$ T cells had an equivalent distribution of TCRβ clonotypes with CD8$^+$ T cells from adjacent mucosa, lymph node, and peripheral blood samples (**Figure 3A**, **Figure S2**).

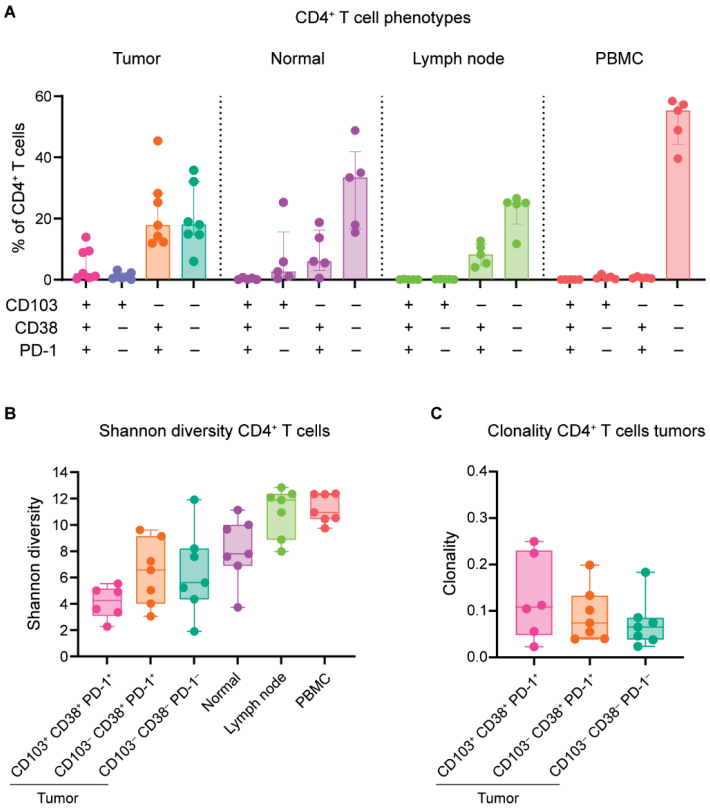

Figure 2. TCR repertoire analysis indicates clonal enrichment in tissue-resident, activated CD4⁺ T cells from colorectal tumors.

A. Frequencies of different CD4⁺ T cell populations among colorectal tumors, adjacent healthy mucosa ("normal"), pericolic lymph nodes, and peripheral blood samples from patients with CRC (N=7) as percentage of total CD4⁺ T cells by flow cytometry. Bars indicate median with IQR. Each dot represents an individual sample. **B**. Shannon diversity of different CD4⁺ T cell populations from colorectal tumors and CD4⁺ T cells from matched adjacent healthy mucosa ("normal"), pericolic lymph nodes, and peripheral blood samples from patients with CRC (N=7) by TCRβ sequencing. **C**. Clonality of different CD4⁺ T cell populations from colorectal tumors from patients with CRC (N=7) by TCRβ sequencing. Details are provided in **Methods**. **B,C**. Box indicates median with IQR, whiskers min to max. Each dot represents an individual sample. CD103⁺ CD38⁻ PD-1⁻ CD4⁺ T cells were not detected in such frequencies to allow TCRβ sequencing. Data from seven independent experiments.

Table 3. Top ten most frequent CDR3β sequences in tissue-resident, activated CD4⁺ T cells from colorectal tumors.

	CRC 44*		CRC 179**		CRC 181		CRC 220		CRC 102***		CRC 223***	
Rank	CDR3β AA sequence	Frequency	CDR3β AA sequence	Frequency	CDR3β AA sequence	Frequency	CDR3β AA sequence	Frequency	CDR3β AA sequence	Frequency	CDR3β AA sequence	Frequency
1	ASSLRQGGDSPLH	12.1%	ASSAGTGDYGYT	24.7%	SASGLAGSLPHEQY	12.6%	ASSPHGQGARVGYT	8.4%	ASSYSAYSNQPQH	17.1%	ASSFRETNTEAF	38.8%
2	ATSSPSADTQY	11.5%	SARSREVTYEQY	22.0%	ASSPGQGRPEAF	7.8%	ASSLNQGEQY	7.6%	ASSLGGTGSTDTQY	10.2%	ATSRALREGLKTQY	7.9%
3	ATSASQGYTDTQY	10.9%	ASSLGKERGNQPQH	21.8%	SASGAGQPQH	6.2%	ATSETGYTDTQY	6.7%	SLRQDLEGYT	8.7%	ASRFGTSGIYEQY	6.9%
4	ASSFGLAGGPTGTDTQY	10.2%	SARAPGVAGSYEQY	21.4%	ASGRGVGQETQY	5.7%	ASSDVWTANEKLF	6.2%	ASSLDSNQPQH	8.0%	ASSLDGRYEQY	5.9%
5	ASRSGNWGYT	9.0%	ASTGETEAF	10.2%	SEGLGDNTQY	4.4%	ASQSFGTSYNSPLH	3.2%	SAKQGADQPQH	3.7%	ASGGYGYT	5.5%
6	ASFESGANVLT	8.4%			SARGLLAGGTPATDTQY	4.2%	ASSTGTSDGELF	3.0%	ASSSEAGWGNSPLH	3.4%	ASMQSSGANVLT	4.3%
7	ASSSGGYGYT	7.4%			ASSVYRENIQY	3.3%	SAYPEGQSYEQY	2.5%	ASSLDKYGYT	3.3%	ASSEAGGGAYTGELF	4.0%
8	ASSYSTSGGVPETQY	7.4%			ASSSGGISTKNIQY	3.0%	SARMLAAPTDTQY	2.1%	SARVQTGRSYGYT	2.9%	ASSTPSGGRGNTIY	3.8%
9	ASIPGQGLFSYNSPLH	7.1%			ASSYGTGTGRYGYT	2.5%	ASSRDSNQPQH	2.0%	ASSQGGDSPLH	2.3%	ASSYLGTGSHEQF	3.6%
10	ASSLGTADTQY	7.1%			SPGRPGTEAF	2.5%	ASSLPGTYPPDTQY	2.0%	ASSLGRSYNSPLH	2.2%	ASSLWSSTDTQY	2.8%

**For CRC 44, a total of 14 clonotypes were detected. **For CRC 179, a total of five clonotypes were detected. ***MMR-deficient colorectal tumors.*

Table 4. Shared CDR3β sequences by CD8⁺ T cells from different colorectal tumors.

	CRC 220			CRC 102*			CRC 223*		
CDR3β AA sequence	Frequency	CDR3β Vβ gene	CDR3β AA sequence	Frequency	CDR3β Vβ gene	CDR3β AA sequence	Frequency	CDR3β Vβ gene
ASSLGETQY	0.27%	TRBV7-7				ASSLGETQY	0.21%	TRBV5-6
ASSPGYEQY	0.25%	TRBV7-6	ASSPGYEQY	0.003%	TRBV12-3			

**MMR-deficient colorectal tumors.*

Table 5. Shared CDR3β sequences by CD4⁺ T cells from different colorectal tumors.

CRC 179			CRC 220			CRC 102*		
CDR3β AA sequence	**Frequency**	**CDR3β Vβ gene**	**CDR3β AA sequence**	**Frequency**	**CDR3β Vβ gene**	**CDR3β AA sequence**	**Frequency**	**CDR3β Vβ gene**
ASSRDSNQPQH	2.26%	TRBV6-1	ASSRDSNQPQH	1.95%	TRBV27	ASSRDSNQPQH	0.11%	TRBV7-2
			ASSLGGGTYNEQF	0.27%	TRBV28	ASSLGGGTYNEQF	0.01%	TRBV7-9
			ASSLGQGYTEAF	0.15%	TRBV4-3	ASSLGQGYTEAF	0.03%	TRBV11-3
			ASSRTASYEQY	0.43%	TRBV19	ASSRTASYEQY	0.10%	TRBV6-6
ASSRTGVYGYT	0.88%	TRBV6-1				ASSRTGVYGYT	0.03%	TRBV28
			ASSSWTGSYGYT	0.11%	TRBV5-1	ASSSWTGSYGYT	0.04%	TRBV27
SVSTNTGELF	1.20%	TRBV29-1				SVSTNTGELF	0.01%	TRBV29-1

**MMR-deficient colorectal tumor.*

Analysis of the CD4⁺ TCR repertoire of colorectal tumors *versus* paired tissues revealed that, in line with their CD8 counterparts, the tissue-resident activated (CD103⁺ CD38⁺ PD-1⁺) CD4⁺ T cells showed most TCRβ clonal overlap with pericolic lymph node samples (an average of 17%; **Figure 3B**, **Figure S3**). Within the CD103⁻ CD4⁺ T cells, minimal overlap in CDR3β repertoire was observed with CD4⁺ T cells from adjacent healthy mucosa, pericolic lymph nodes, or peripheral blood samples (**Figure 3B**, **Figure S3**). Activated (CD38⁺ PD-1⁺) populations shared little TCRβ clonal overlap with CD4⁺ T cells from lymph node and blood samples, and non-activated (CD38⁻ PD-1⁻) populations with CD4⁺ T cells from peripheral blood (**Figure 3B**, **Figure S3**). Of note, the large majority of TCRβ clonotypes detected in tissue-resident activated (CD103⁺ CD38⁺ PD-1⁺) CD8⁺ and CD4⁺ T cells in colorectal tumors did not share overlap with matched healthy mucosal tissue (**Figure 3A,B**).

Last, with the emerging data on the value of investigating co-expression of CD39 and CD103 as markers for tumor-reactive T cells,[15-17] we examined the expression levels of CD39 within the different T cell populations and found that CD39 was highly expressed by tissue-resident activated (CD103⁺ CD38⁺ PD-1⁺) CD8⁺ and CD4⁺ T cells (**Figure S4**). In addition, CD38 or PD-1 single-positive CD103⁺ CD8⁺ and CD4⁺ T cells showed expression of CD39. In contrast to CD8⁺ T cells, CD39 expression could also be observed on CD38⁺ PD-1⁺ CD103⁻ CD4⁺ T cells and CD38 or PD-1 single-positive CD103⁻ CD4⁺ T cells (**Figure S4**). These observations suggest that CD38 or PD-1 are better surrogates for tumor-reactivity than CD103 in the case of CD4⁺ T cells.

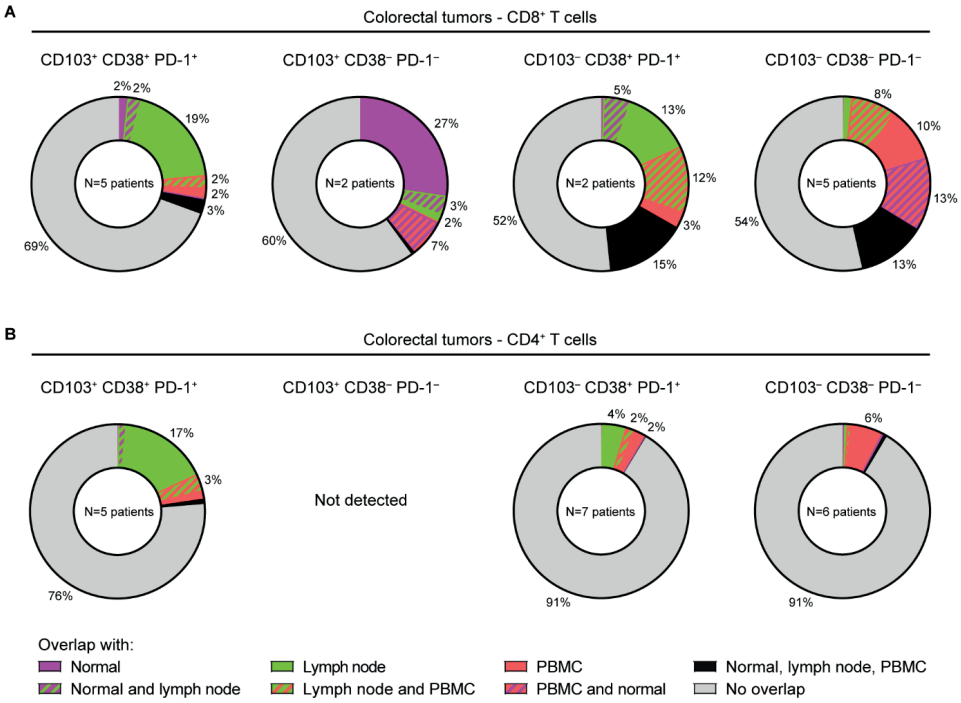

Figure 3. Tissue-resident, activated CD8⁺ and CD4⁺ T cells show most overlapping TCR clonotypes with pericolic lymph nodes.
A. Circular graphs showing the average frequency of overlapping TCR clonotypes of different CD8⁺ T cell populations from colorectal tumors with matched adjacent healthy mucosa ("normal"), pericolic lymph nodes, and peripheral blood samples from patients with CRC. **B**. Circular graphs showing the average frequency of overlapping TCR clonotypes of different CD4⁺ T cell populations from colorectal tumors with matched adjacent healthy mucosa ("normal"), pericolic lymph nodes, and peripheral blood samples from patients with CRC. CD103⁺ CD38⁻ PD-1⁻ CD4⁺ T cells were not detected in the tumor samples. **A,B**. Samples were only included if ≥ 10 clonotypes were detected. Data from seven independent experiments.

DISCUSSION

The field of cancer immunotherapy is rapidly developing and there is an emerging interest in (combinational) T cell checkpoint blockade immunotherapies designed to reactivate intratumoral T cells in cancers.[22,23] Unraveling the dynamic behavior of the intratumoral TCR repertoire and their capacity to recognize the tumor will be highly valuable for the understanding of anti-tumor T cell responses and ultimately for the development of novel T cell-mediated immunotherapeutic strategies. It is becoming increasingly evident that tissue-resident (CD103⁺) cells can be very abundant in CRC tissues, and have the capacity to recognize tumor cells.[24] However, it was shown that colorectal tumors can also contain so called 'bystander' T cells lacking tumor reactivity (with specificities for viruses such as EBV and CMV).[16] We and others previously identified tumor tissue-specific CD8⁺ and CD4⁺ T cell populations with an activated (CD38⁺, PD-1⁺, CD39⁺) tissue-resident (CD103⁺) phenotype in CRC.[14-16] These tissue-resident activated T cells were particularly enriched in MMR-deficient

CRCs.[14] Here, we specifically investigated the TCR repertoire, clonal frequencies, and clonal overlap with paired tissues of i) tissue-resident, activated (CD103$^+$ CD38$^+$ PD-1$^+$), ii) tissue-resident, non-activated (CD103$^+$ CD38$^-$ PD-1$^-$), iii) non-tissue-resident, activated (CD103$^-$ CD38$^+$ PD-1$^+$), and iv) non-tissue-resident, non-activated (CD103$^-$ CD38$^-$ PD-1$^-$) CD8$^+$ and CD4$^+$ T cells in colorectal tumors by bulk TCRβ sequencing. We showed that clonal enrichment was most frequently observed in the tissue-resident activated CD8$^+$ and CD4$^+$ T cell populations as compared to the other T cell populations, suggesting the existence of antigenic responses.

It has been proposed that the interaction of intratumoral T cells with tumor antigens in the tumor microenvironment can shape their phenotype toward an 'exhausted' cell state accompanied by the expression of different phenotypic markers depending on the cancer type.[25] Co-expression of CD39 and CD103 could function as markers for tumor-reactive CD8$^+$ T cells in different types of solid cancers including CRC.[15-17] In addition, expression of PD-1 and/or CD137 have been reported as markers for tumor-reactive T cells in melanoma and ovarian cancer.[25-29] However, the aforementioned markers not always separated tumor-reactive CD8$^+$ T cells from bystander T cells.[24] In our study, highest clonality was observed in the intratumoral CD103$^+$ CD38$^+$ PD-1$^+$ CD8$^+$ T cell populations, suggesting that tissue-residency and activation markers as CD103, CD38, PD-1 (and CD39) may serve as surrogates for tumor-reactive CD8$^+$ T cells. In contrast to CD8$^+$ T cells, phenotypic characterization of markers to identify tumor-reactive CD4$^+$ T cells are largely lacking. It was reported that CD39$^-$ CD4$^+$ T cells showed specificity for cancer-unrelated antigens (e.g. CMV epitopes).[30] However, no direct evidence has linked expression of CD39 to tumor-reactive CD4$^+$ T cells as of yet. In our data, clonal enrichment was mainly observed in the CD103$^+$ CD38$^+$ PD-1$^+$ CD4$^+$ T cell subset in colorectal tumors. This may suggest that tumor reactivity can be associated with expression of markers of tissue-residency (CD103) and activation/dysfunction (CD38, PD-1, CD39) for CD4$^+$ T cells in CRC. To test whether those CDR3β sequences of the clonally expanded CD103$^+$ CD38$^+$ PD-1$^+$ CD8$^+$ and CD4$^+$ T cells have the capacity to recognize the tumor, identified TCRs could be introduced into donor T cells and tested against the original tumor.

In our study, the large majority of TCRβ repertoires of intratumoral CD103$^+$ CD38$^+$ PD-1$^+$ CD8$^+$ and CD4$^+$ T cells did not share clonal overlap with T cells from matched healthy mucosal tissue. Instead, we found that CD103$^+$ CD38$^+$ PD-1$^+$ cells showed most overlapping TCR clonotypes with pericolic lymph nodes, for both CD8$^+$ and CD4$^+$ T cell populations, suggesting their potential origin from the lymph nodes. In the pericolic lymph nodes, T cells are primed by antigen presenting cells and subsequently migrate to the tumor where they recognize their cognate antigen and clonally expand. It has been proposed that upon TCR activation, intratumoral T cells may upregulate tissue-residency and dysfunctional markers that retain the cells within the tumor.[15,25] Our findings further support this hypothesis as CD103$^+$ CD38$^+$ PD-1$^+$ T cells were generally infrequent in the pericolic lymph node samples (**Figure 1**). We previously detected a high frequency of these cells in a tumor-positive lymph node sample.[14] TCRβ sequencing showed 86% overlap between TCR clonotypes of CD103$^+$ PD-1$^+$ CD4$^+$ T cells in the tumor sample with CD45RO$^+$ CD4$^+$ T cells in the pericolic lymph

node sample (data not shown). It would therefore be of interest to further study the TCR repertoires of tumor-positive lymph nodes to test for the presence of tumor-reactivity at those sites.

Due to the relatively small study size, it is important to extend our findings to a larger cohort of patients with CRC. Moreover, further studies could investigate whether the markers for tissue-residency and activation tested by us and others could be used to expand the Immunoscore[2,13], which may help to further segregate tumor-reactive T cells from bystander T cells, and add to its prognostic value for patients with CRC. Indeed, in a pilot study we observed that scoring $CD3^+CD103^+$ T cells further separated MMR-deficient from -proficient colorectal tumors as compared to scoring $CD3^+CD8^+$ T cells only (data not shown).

Altogether, our findings suggest that the addition of tissue-residency and activation markers as CD103, CD38, PD-1, and CD39 on intratumoral T cells may help in the search of finding enriched CDR3β sequences that correspond to tumor antigen-specific T cells. Immunotherapeutic strategies aiming at increasing the frequency of tumor antigen-specific TCRs (e.g. transduction of tumor antigen-specific TCRs on functional donor αβ T cells) may boost the clinical activity of T cell checkpoint blockade therapies for patients with CRC.

METHODS

Patient samples

Primary CRC tissues with matched adjacent healthy mucosa, pericolic lymph nodes, and peripheral blood samples were from patients with CRC (N=7) undergoing surgical resection of their tumor at the Leiden University Medical Center (LUMC, the Netherlands). All patients were treatment-naïve, and did not have a previous history of inflammatory bowel disease. Patients provided written informed consent, and the study was approved by the Medical Ethical Committee of the Leiden University Medical Center (protocol P15.282). All specimens were anonymized and handled according to the ethical guidelines described in the Code for Proper Secondary Use of Human Tissue in the Netherlands of the Dutch Federation of Medical Scientific Societies.

Processing of samples

Colorectal tumor tissues, adjacent healthy mucosa, and pericolic lymph nodes were processed as described previously.[14] Briefly, tissue specimens were minced into small fragments in a petri dish, followed by enzymatical digestion with 1 mg/mL collagenase D (Roche Diagnostics) and 50 µg/mL DNase I (Roche Diagnostics) in 5 mL of IMDM+Glutamax medium for 30 min at 37°C in gentleMACS C tubes (Miltenyi Biotec). Cell suspensions were mechanically dissociated during and after incubation with the gentleMACS Dissociator (Miltenyi Biotec). Thereafter, cell suspensions were filtered through a 70-µm cell strainer (Corning) and washed in IMDM+Glutamax medium. With regard to the peripheral blood samples, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (provided by apothecary LUMC) density-gradient centrifugation. Cell count and viability of the different samples were determined with the Muse Count & Viability Kit (Merck) on the Muse Cell Analyzer (Merck), and cells were cryopreserved in liquid nitrogen based on the number of viable cells until time of analysis.

FACS sorting

Cells from tumor digests, healthy mucosa, lymph nodes and PBMCs were thawed and rest at 37°C in IMDM+L-glutamine (Lonza) with 20% fetal calf serum (FCS) (Sigma-Aldrich) for 30min. After washing in FACS buffer (DPBS (Braun) with 1% FCS), cells were incubated with human Fc receptor block (BioLegend) and stained with the following cell surface antibodies: 1:100 anti-CD4-BV421 [clone RPA-T4, Sony], 1:200 anti-CD8a-BV650 [clone RPA-T8, BD Biosciences], 1:10 anti-CD103-FITC [clone Ber-ACT8, BD Biosciences], 1:160 anti-CD45-PerCP-Cy5.5 [clone 2D1, eBioscience], 1:30 anti-PD-1-PE [clone MIH4, eBioscience], 1:200 anti-CD38-PE-Cy7 [clone HIT2, eBioscience], and 1:60 anti-CD39-APC [clone A1, BioLegend] for 45 min at 4°C. To discriminate live from dead cells, a live/dead fixable near-infrared viability dye (1:1000, Life Technologies) was included in each staining. Thereafter, cells were washed three times in FACS buffer before cell sorting. Cells were resuspended in DPBS before sorting in 1.5 mL RNase-free Eppendorf tubes containing 80 mL of T1 buffer from the NucleoSpin Tissue XS kit. CompBeads (BD Biosciences) and ArC reactive beads (Life Technologies) were used for compensation controls. A gating strategy for single, live $CD45^+$ $CD8^+$ T cells as well as $CD4^+$ T cells was applied, from which the following T cell populations were sorted from colorectal tumor samples: i) $CD103^+$ $CD38^+$ $PD\text{-}1^+$, ii) $CD103^+$ $CD38^-$ $PD\text{-}1^-$, iii) $CD103^-$ $CD38^+$ $PD\text{-}1^+$, and iv) $CD103^-$ $CD38^-$ $PD\text{-}1^-$ (**Figure S1**). FMO controls for PE and PE-Cy7 were included on a PBMC samples used as positive control. In addition, $CD8^+$ and $CD4^+$ T cells were sorted from matched adjacent healthy mucosa, pericolic lymph nodes, and peripheral blood samples. Cells were sorted on a FACS Aria III 4L (BD Biosciences). Of note, for two patients (CRC44 and CRC102), no CD8/CD4 sorting was performed for healthy mucosa, lymph nodes, and peripheral blood samples, but instead fresh-frozen tissue slides (for healthy mucosa and lymph node samples) and EDTA blood (for peripheral blood samples) were used to isolate DNA.

DNA isolation

After sorting, cells were centrifuged for 5 min at 1,500 x *g* at 4°C and drop-wise resuspended in T1

buffer (80 mL for 10-100,000 cells and 200 mL for 100,000-10^7 cells). Samples were only included in downstream analysis if they contained at least 100 cells. Samples were transferred to -20°C until DNA isolation. DNA was isolated with the NucleoSpin® Tissue (XS) Kit (740952, Macherey-Nagel) according to the manufacturer's instructions. Upon thawing the samples, Proteinase K was added. Jurkat cells were included as control in a pilot experiment. DNA quality was assessed by running a qPCR on the *PPP2R1B* housekeeping gene on all samples before continuing with TCRβ sequencing.

TCRβ-chain sequencing

For bulk TCRβ repertoire profiling, TCRβ DNA libraries were constructed with the Ion AmpliSeq™ Library Kit Plus (4488990) and the Oncomine™ TCRβ-SR Assay (A39072) according to the user guide (MAN0017438). The individual libraries were quantified using the Ion Library TaqMan™ Quantitation Kit (4468802), and pooled to a total concentration of 25 pM. Final libraries were templated on ION 530™ Chips (A27764) using the Ion Chef™ System and sequenced on the Ion Genestudio™ S5 Series System with the Ion 510™ & Ion 520™ & Ion 530™ Kit - Chef (A34461) either in house or by GenomeScan BV (Leiden, the Netherlands) according to the user guide references (MAN0016854). All kits and equipment used, unless stated otherwise, are from ThermoFisher Scientific. The diversity analysis was performed with the IonReporter 5.14 software using the Oncomine TCRβ-SR - w1.2 - DNA - Single Sample (version 5.12) workflow with default settings.

Data analysis

Processed sequence data for each sample were analyzed with custom scripts in PostgreSQL (version 12.9). Number of clonotypes, CDR3β length, Shannon diversity, evenness, and the distribution and frequency of clones were processed for each sample. Clonality represented the distribution of clone sizes and was defined as 1 minus the normalized Shannon diversity of the TCRβ abundances (i.e., the evenness). Unique clonotypes were defined as TCRβ sequences with the same V-gene family, J-gene segment, and CDR3β amino acid and nucleotide sequences. For investigations of clonal relations between samples, the number of overlapping TCR clonotypes and their frequency were analyzed for the $CD8^+$ and $CD4^+$ TCR repertoires separately. Bars and boxes represent median with IQR.

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AUTHOR CONTRIBUTIONS

N.L. de Vries conceived the study. N.L. de Vries and J. Bras Gomes Nunes performed experiments. N.L. de Vries performed the computational analyses presented in this paper. N.L. de Vries, J. Bras Gomes Nunes, N.F. de Miranda, and F. Koning analyzed and interpreted the data. R. van der Breggen assisted with experiments. F. Koning and N.F. de Miranda supervised the study. The manuscript was written by N.L. de Vries, F. Koning, and N.F. de Miranda in collaboration with all co-authors.

COMPETING INTERESTS

The authors declare no competing interests exist.

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SUPPLEMENTAL FIGURES

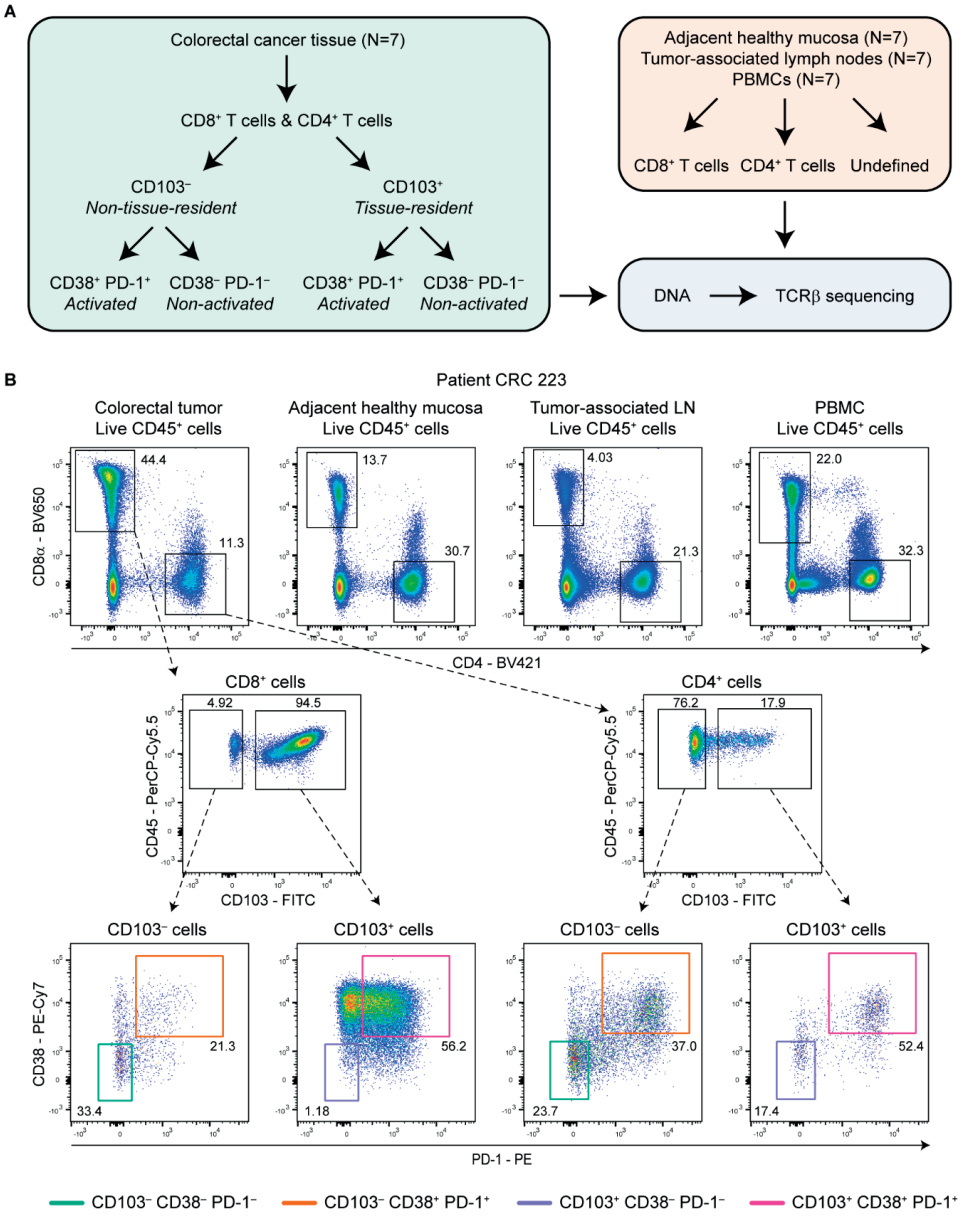

Figure S1. Gating strategy for the sorting of CD8⁺ and CD4⁺ T cell populations from colorectal tumors and matched non-malignant tissues.

A. Experimental design of TCRβ sequencing performed on DNA isolated from different T cell populations FACS-sorted from colorectal tumors and matched adjacent healthy mucosa, pericolic lymph nodes, and peripheral blood samples from patients with CRC. **B**. Flow cytometry gating strategy for the sorting of different CD8⁺ and CD4⁺ T cell populations from a representative MMR-deficient CRC sample with matched non-malignant tissues showing sequential gates with percentages.

Figure S2. Overlap in TCR clonotypes of different CD8+ T cell populations for each individual colorectal cancer patient.

Circular graphs showing the frequency of overlapping TCR clonotypes of different CD8+ T cell populations from colorectal tumors with matched adjacent healthy mucosa ("normal"), pericolic lymph nodes, and peripheral blood samples for each patient with CRC. Samples with < 10 clonotypes are colored in red. Data from seven independent experiments.

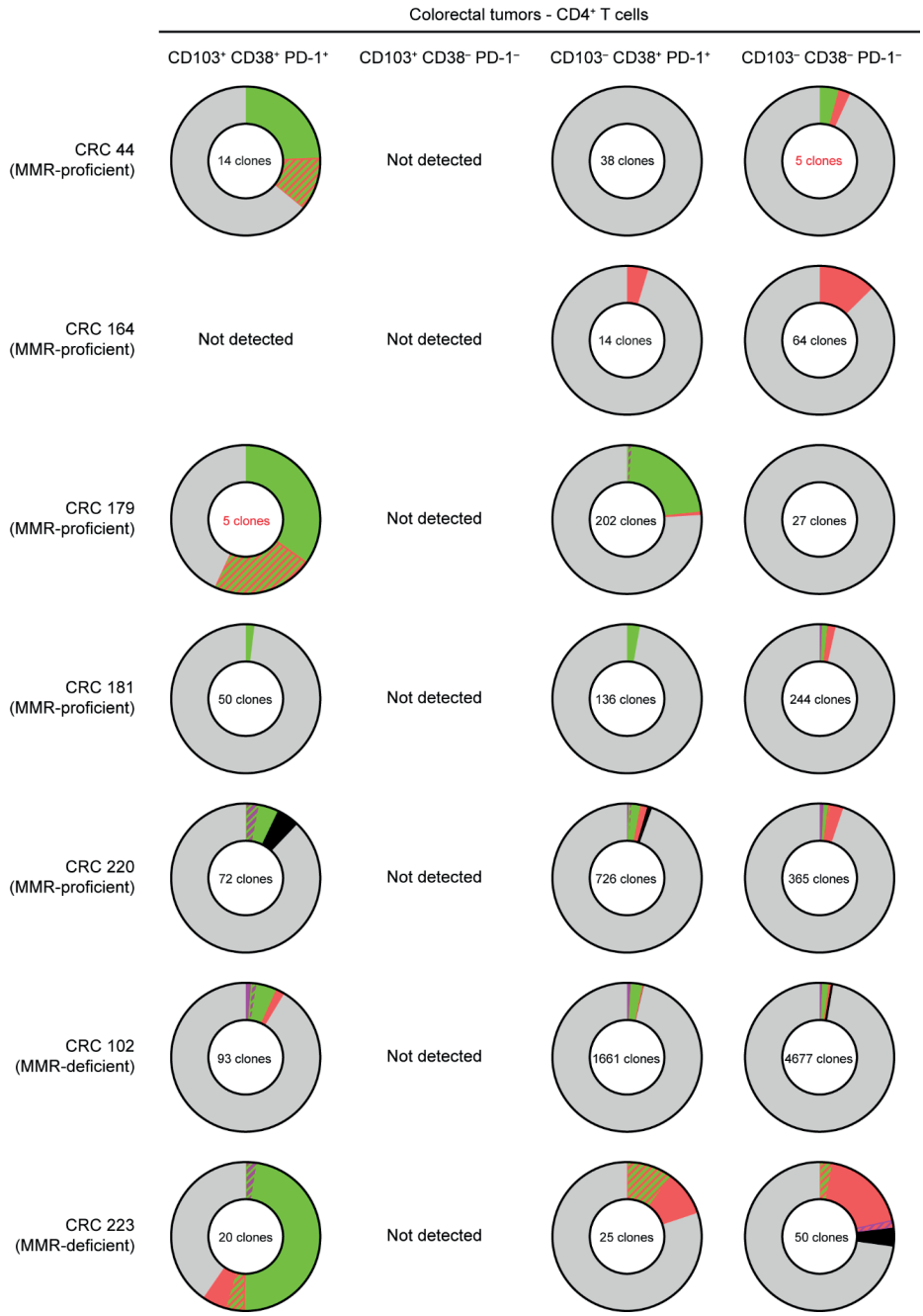

Figure S3. Overlap in TCR clonotypes of different CD4⁺ T cell populations for each individual colorectal cancer patient.

Circular graphs showing the frequency of overlapping TCR clonotypes of different CD4⁺ T cell populations from colorectal tumors with matched adjacent healthy mucosa ("normal"), pericolic lymph nodes, and peripheral blood samples for each patient with CRC. CD103⁺ CD38⁻ PD-1⁻ CD4⁺ T cells were not detected in the tumor samples. Samples with < 10 clonotypes are colored in red. Data from seven independent experiments.

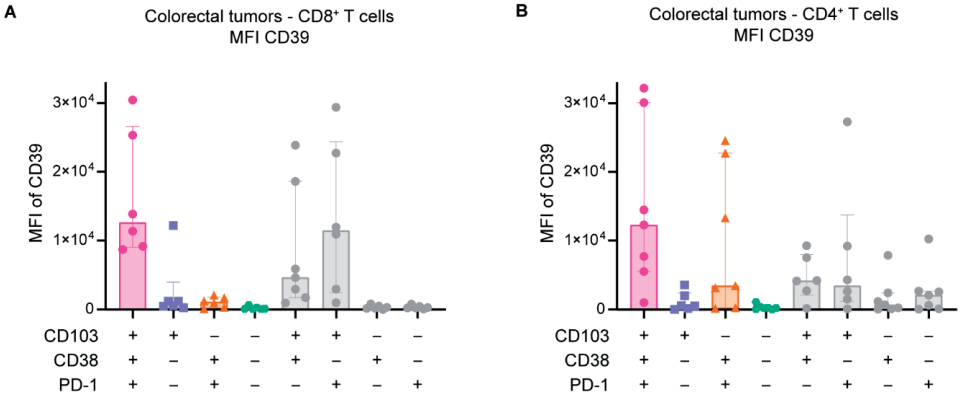

Figure S4. Tissue-resident, activated T cell populations show high expression levels of CD39.
A. MFI of CD39 in the different CD8⁺ T cell populations from colorectal tumors (N=6-7) analyzed by flow cytometry. Background MFI of CD39 of each unstained sample was subtracted. **B**. MFI of CD39 in the different CD4⁺ T cell populations from colorectal tumors (N=6-7) analyzed by flow cytometry. Background MFI of CD39 of each unstained sample was subtracted. **A,B**. Bars represent median with IQR. Samples were only included if ≥100 cells were present. Data from seven independent experiments.

SUPPLEMENTAL TABLES

The supplementary tables are available in the appendix to this thesis at the repository of Leiden University (https://hdl.handle.net/1887/3439882) and can be requested from the author.

Table S1: Overview of number of cells, number of clones, Shannon diversity, and clonality for $CD8^+$ T cells from all samples.
Table S2: Overview of number of cells, number of clones, Shannon diversity, and clonality for $CD4^+$ T cells from all samples.