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# CD103 and CD39 coexpression identifies neoantigen-specific cytotoxic T cells in colorectal cancers with low mutation burden

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## ABSTRACT

**Background** Expression of CD103 and CD39 has been found to pinpoint tumor-reactive CD8<sup>+</sup> T cells in a variety of solid cancers. We aimed to investigate whether these markers specifically identify neoantigen-specific T cells in colorectal cancers (CRCs) with low mutation burden.

**Experimental design** Whole-exome and RNA sequencing of 11 mismatch repair-proficient (MMR-proficient) CRCs and corresponding healthy tissues were performed to determine the presence of putative neoantigens. In parallel, tumor-infiltrating lymphocytes (TILs) were cultured from the tumor fragments and, in parallel, CD8<sup>+</sup> T cells were flow-sorted from their respective tumor digests based on single or combined expression of CD103 and CD39. Each subset was expanded and subsequently interrogated for neoantigen-directed reactivity with synthetic peptides. Neoantigen-directed reactivity was determined by flow cytometric analyses of T cell activation markers and ELISA-based detection of IFN- $\gamma$  and granzyme B release. Additionally, imaging mass cytometry was applied to investigate the localization of CD103<sup>+</sup>CD39<sup>+</sup> cytotoxic T cells in tumors.

**Results** Neoantigen-directed reactivity was only encountered in bulk TIL populations and CD103<sup>+</sup>CD39<sup>+</sup> (double positive, DP) CD8<sup>+</sup> T cell subsets but never in double-negative or single-positive subsets. Neoantigen-reactivity detected in bulk TIL but not in DP CD8<sup>+</sup> T cells could be attributed to CD4<sup>+</sup> T cells. CD8<sup>+</sup> T cells that were located in direct contact with cancer cells in tumor tissues were enriched for CD103 and CD39 expression.

**Conclusion** Coexpression of CD103 and CD39 is characteristic of neoantigen-specific CD8<sup>+</sup> T cells in MMR-proficient CRCs with low mutation burden. The exploitation of these subsets in the context of adoptive T cell transfer or engineered T cell receptor therapies is a promising avenue to extend the benefits of immunotherapy to an increasing number of CRC patients.

## BACKGROUND

Immune checkpoint blockade therapy is an effective treatment option for colorectal cancer (CRC) patients diagnosed with mismatch repair (MMR)-deficient tumors,

## WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Neoantigen-specific T cells infiltrate mismatch repair-proficient (MMR-proficient) colorectal cancers despite their low to moderate mutation burden. Their presence warrants the development of immunotherapeutic approaches that leverage their potential for the treatment of patients diagnosed with MMR-proficient colorectal cancers.

## WHAT THIS STUDY ADDS

⇒ Coexpression of CD103 and CD39 on CD8<sup>+</sup> T cells was found to be a feature of neoantigen-specific T cells and, therefore, codetection of these markers can be employed to enrich for neoantigen-specific cytotoxic T cells from bulk tumor-infiltrating T cell populations.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The discovery of cell surface markers that pinpoint neoantigen-specific CD8<sup>+</sup> T cells enables the development of adoptive T cell transfer products with increased anti-tumor activity and can support the discovery and exploitation of relevant T cell receptors for engineered T cell therapies.

while advanced MMR-proficient CRCs are generally refractory to immunotherapy.<sup>1–4</sup> These contrasting outcomes can, to a great extent, be attributed to the greater availability of somatically mutated antigens (neoantigens) in MMR-deficient CRCs.<sup>5</sup> The latter only comprise up to 5% of all advanced CRCs, thereby explaining the current limited applicability of immune checkpoint blockade in advanced CRC. Nevertheless, T cell responses against neoantigens have been extensively reported in patients diagnosed with MMR-proficient CRCs<sup>6–9</sup> and, importantly, a proportion of these cancers appears

sensitive to immune checkpoint blockade therapy in a neoadjuvant setting.<sup>3</sup>

Naturally occurring antitumor T cell responses have been identified in a plethora of cancer types including ones with low immunogenicity (eg, cholangiocarcinoma, ovarian, and breast cancer).<sup>8 10–14</sup> A major unaddressed question in the field is how to optimally leverage naturally occurring anti-tumor T cell responses to expand the benefit of immunotherapy to additional cancer patients. Adoptive T cell transfer (ACT) where patient's autologous tumor-reactive T cells are isolated and expanded in vitro to generate a therapeutic product is a straightforward approach to exploit tumor-reactive T cells.<sup>15–17</sup> Objective clinical responses have been observed in approximately 50% of melanoma patients on ACT treatment,<sup>17–19</sup> and encouraging outcomes have also been obtained in other solid cancers.<sup>14 20 21</sup> The generation of ACT products generally results from the uncontrolled expansion of polyclonal T cell populations where loss of tumor-reactive T cells can occur.<sup>22</sup> This undesired outcome may be favored when tumor-reactive T cells display dysfunctional phenotypes and are outnumbered by rapidly proliferating, non-tumor-reactive T cells. As previous works have shown, the enrichment of T cell populations with anti-tumor reactivity in ACT products is an attractive path to improve their efficacy.<sup>11 23</sup>

Several groups have proposed molecular surrogates that pinpoint T cells with anticancer reactivity, including PD-1, TIM-3, LAG-3, OX40, CD39, CD103 and CD137.<sup>24–33</sup> Previously, we reported that combined expression of CD103 and CD39 identifies tumor-reactive T cells and separates those from T cells with other specificities (eg, viral antigens).<sup>34</sup> CD103, also known as integrin  $\alpha E$ , can dimerise to for example, integrin  $\beta 7$  and orchestrate intraepithelial residency of T cells by binding to E-cadherin on epithelial cells.<sup>35</sup> CD39 is an ATP ectonucleotidase that is upregulated on chronically stimulated T cells and, together with CD73, produces adenosine which creates an immunosuppressive milieu. The expression of CD39 on T cells may

pinpoint chronic antigen stimulation which is likely to occur in the tumor microenvironment.<sup>27 36 37</sup> Combined, CD103 and CD39 might constitute ideal surrogates to pinpoint neoantigen-specific T cells.

We previously demonstrated the existence of double-positive (DP), CD103<sup>+</sup> and CD39<sup>+</sup>, CD8<sup>+</sup> T cells in tumor digests of MMR-proficient CRCs, but the enrichment of neoantigen-specific CD8<sup>+</sup> T cells within this population has not yet been demonstrated.<sup>34</sup> Therefore, we investigated in this study whether neoantigen-reactivity is contained within DP CD8<sup>+</sup> T cell subsets in MMR-proficient CRCs and whether their specific isolation increases the capacity to detect neoantigen-specific T cells in comparison to reactivity assays performed on bulk tumor-infiltrating lymphocyte (TIL) populations.

## METHODS

### Tumor characterization

The original tumor location, clinical stage and Human Leucocyte Antigen (HLA) class I expression in the tumor cells are summarized in [table 1](#). Only one patient, NIC16, received neoadjuvant chemoradiotherapy to which no clinical response was observed. The HLA class I status of the tumors was determined through immunohistochemical detection of HLA class I molecules with the HCA2 (1:3200; Nordic MUBio, Susteren, The Netherlands) and HC10 (1:3200; Nordic MUBio) clones, and  $\beta 2$ -microglobulin (clone EPR21752-214; 1:4000; Abcam, Cambridge, UK), as described previously.<sup>38</sup> The MMR status of the tumors was determined in a diagnostic setting at the department of pathology of the LUMC.

### Collection and culturing of patient material

Patients' peripheral blood samples were obtained prior to surgery. Peripheral blood mononuclear cells (PBMC) were isolated from the heparinized venous blood by Ficoll-Amidotrizoate (provided by the LUMC pharmacy) gradient centrifugation. Tumor and corresponding

**Table 1** Tumor characteristics

Patient ID	Tumor location	TNM stage	HLA IHC	CMS class
NIC4	Colon ascendens	pT3N0M0	Positive	4
NIC5	Sigmoid	pT3N2M0	Positive	2
NIC7	Sigmoid	pT2N1M0	Weak	2
NIC16	Rectum	ypT2N0M0	Positive	2
NIC17	Sigmoid	pT1N1M0	Positive	2
NIC20	Splenic flexure	pT3N0M0	Positive	2
NIC22	Rectum	pT2N0M0	Positive	2
NIC25	Hepatic flexure	pT3N1bM0	Defect	4
NIC27	Rectum	pT2N0M0	Heterogeneous	2
NIC38	Colon ascendens	pT1N0M0	Positive	3
NIC39	Rectum	pT3N2bM1	Positive	3

CMS, consensus molecular subtype; IHC, immunohistochemistry; TNM, tumor node metastasis.

normal colorectal tissue samples were obtained following surgery under supervision of a pathologist. Part of the tumor materials was snap-frozen, the remaining was cut into small fragments. Some small fragments were digested to single cell suspensions using 1mg/mL collagenase D (Roche, Basel, Switzerland) and 50µg/mL DNase I (Roche) in IMDM medium (Lonza BioWhittaker, Breda, The Netherlands), supplemented with 2mM Glutamax (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 20% Fetal Bovine Serum (Sigma-Aldrich, Saint Louis, Missouri, USA), 1% penicillin/streptomycin (Thermo Fisher Scientific), 1% Fungizone (Thermo Fisher Scientific), 0.1% Ciprofloxacin (provided by the LUMC pharmacy), and 0.1% Gentamicin (Sigma-Aldrich). The fragments were incubated for 30 min at 37°C and mechanically dissociated on a gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) in gentleMACS C tubes (Miltenyi Biotec). The suspension was passed through a 70µm strainer (Miltenyi Biotec) and the flow through was cryopreserved.

In addition, 6–12 small tumor fragments were directly placed in culture in a 24-well plate with medium (IMDM (Lonza BioWhittaker), supplemented with 7.5% heat-inactivated pooled human serum (Sanquin, Amsterdam, The Netherlands), 1% penicillin/streptomycin (Thermo Fisher Scientific), and rIL-2 (1000 IU/mL; Aldesleukin, Novartis, Basel, Switzerland) for the outgrowth of TIL. After 14–21 days of culture, the T cells were counted and cryopreserved. To increase the number of T cells available for neoantigen-reactivity assays, TIL were expanded using a rapid expansion protocol in media containing rIL-2 (3000 IU/mL), OKT3 (Miltenyi Biotec, 30 ng/mL), and irradiated (40 Gy) feeder cells (100–200-fold excess) for 4–5 days, after which the culture was continued while refreshing medium with rIL-2 (3000 IU/mL), three times a week until a total culturing period of 2 weeks. The proportion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the final expansion product was assessed by flow cytometry (online supplemental table S1).

### Sorting and expansion of CD8<sup>+</sup> T cell subsets

CD8<sup>+</sup> T cell fractions were sorted on a BD FACSAria II (BD Biosciences, Franklin Lakes, New Jersey, USA) from single cell tumor digests and cultured as described previously.<sup>34</sup> In short, cells were selected for flow cytometric cell sorting based on the phenotypic markers CD45<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD45RA<sup>-</sup>, CCR7<sup>+/+</sup>, CD39<sup>+/+</sup> and CD103<sup>+/+</sup>. Additionally, the markers CD69, CD127 and PD-1 were assessed for phenotypic analyses. CD8<sup>+</sup> T cells were sorted based on the expression of CD39 and/or CD103 (double-negatives (DN), single-positives (SP), and DP) and each subset was cultured separately in RPMI-1640 (Gibco, Thermo Fisher Scientific), supplemented with 10 ng/mL IL-15 (BioLegend, San Diego, California, USA), 2 mM glutamine (Gibco, Thermo Fisher Scientific), 1% non-essential amino acids (Gibco, Thermo Fisher Scientific), 1% sodium pyruvate (Gibco, Thermo Fisher Scientific), penicillin (50 IU/mL) + streptomycin (50 µg/

mL; Gibco, Thermo Fisher Scientific) and 10% pooled human serum (in house). T cells were stimulated with 1 µg/mL PHA (Remel, Dartford, UK) in the presence of irradiated (50 Gy) allogeneic feeder cells (2\*10<sup>5</sup> cells/well) in a 96-well round-bottom plate. If necessary, cells were restimulated in order to yield enough cells for downstream analysis. Cells were cryopreserved for analyses at later stages, after a total culturing period of 2–3 weeks. The DP subset of NIC5 and NIC39 did not expand and could, therefore, not be inspected for their ability to recognize neoantigens.

### Neoantigen detection and binding prediction

Genomic DNA was isolated from snap-frozen tumor and corresponding normal colorectal tissues in order to prepare sequencing libraries as described previously.<sup>6</sup> In brief, the NEBNext Ultra Directional RNA Library Prep kit for Illumina (New England Biolabs) and the NEBNext rRNA depletion kit (New England Biolabs) were employed to generate RNA sequencing libraries which were sequenced at Macrogen (Seoul, South-Korea) or Novogene (Cambridge, United Kingdom). The NEBNext Ultra II DNA Library Prep kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA) and the IDT xGEN Exome target kit (Integrated DNA Technologies, Leuven, Belgium) were used for preparation of the exome libraries. All kits were used according to the manufacturer's instructions.

RNA sequencing reads were first aligned to the human reference genome (build hg38) using STAR (V.2.7.3a).<sup>39</sup> For exome sequencing, the obtained 150 bp paired-end reads were mapped against the human reference genome (hg38) using the Burrows-Wheeler Aligner 3 algorithm (BWA-MEM V.0.7.17) BWA-MEM.<sup>40</sup> Picard Tools was used to remove duplicate reads<sup>41</sup> and the Genome Analysis Toolkit 7 (GATK V.3.8<sup>42</sup>) for base quality calibration. Subsequently, variant calling was done using a combination of three software tools, muTect 2, varScan and Strelka.<sup>43–46</sup> The resulting .vcf files were then combined into a single file using GATK CombineVariants.<sup>42</sup> RNAseq read counts for each variable allele was added to the identified variant chromosomal positions using the bam-readcount tool.<sup>47</sup>

Functional annotation of the variants was done using the Ensembl Variant Effect Predictor.<sup>48</sup> Variants annotated as protein-altering were further investigated if at least one read with the alternative allele was present in the RNAseq data. In order to exclude false positives, variants were visually inspected using Integrative Genomics Viewer (IGV, Broad Institute).<sup>49–51</sup> The 25-mer peptide sequences were generated for all the identified variants considered to be true. In case of frameshifts and stop loss mutations, several peptide sequences were generated which overlapped for at least half of the sequence (online supplemental table S2).

### T cell reactivity assay

For the T cell reactivity assays, Epstein-Barr virus-transformed lymphoblastoid B cell lines (EBV-LCL) were

used as antigen-presenting cells rather than monocyte-derived dendritic cells because of the limited availability of autologous PBMCs. Autologous PBMC were immortalized by incubation with supernatant of the marmoset B cell line containing infectious particles of EBV strain B95-8 for 1 hour at 37°C in culture medium (RPMI-1640 (Lonza BioWhittaker), supplemented with 5 µg/mL PHA (Thermo Fisher Scientific), 10% FCS, L-glutamine (4 mM), 1% penicillin/streptomycin (Thermo Fisher Scientific). The EBV-LCL were cultured for at least 3 weeks while refreshing the medium twice a week and cryopreserved for later use.

Neoantigen-directed reactivity of the T cells was investigated by a coculture assay as described previously.<sup>6</sup> In short, autologous EBV-LCL were irradiated (60 Gy) and cocultured overnight with 20 µg/mL synthetic long peptides (25 amino acids). For all the identified variants, 25-mer peptide sequences were synthesized (Cell and Chemical Biology department at the LUMC, or PepScan, Lelystad, The Netherlands). T cells were added to the EBV-LCL at a 1:2 ratio, that is, respectively 15,000 and 30,000 cells per well. As negative controls, unloaded EBV-LCL with or without DMSO corresponding to the peptide solution were used. *Staphylococcus aureus* enterotoxin B (SEB; 0.5 µg/mL; Sigma-Aldrich) and PMA/Ionomycin (P/I; 20 ng/mL and 1 µg/mL, respectively; Merck, Darmstadt, Germany) were used as positive controls.

T cell reactivity was determined by performing ELISA to detect IFN-γ and granzyme B (Mabtech, Stockholm, Sweden) in the co-culture supernatants. In addition, CD137 expression on CD8<sup>+</sup> T cells and CD40L and OX40 expression on CD4<sup>+</sup> T cells were measured by flow cytometry on an LSRFortessa (BD Biosciences) making use of an antibody panel including anti-CD3-Amcyan (SK7, 1:20, BD Biosciences), anti-CD4-PE-CF594 (RPA-T4, 1:50, BD Biosciences), anti-CD8-APC-Cy7 (SK1, 1:40, BD Biosciences), anti-CD45-PerCP-Cy5.5 (2D1, 1:20, BD Biosciences), anti-CD40L-PE (TRAP1, 1:10, BD Biosciences), anti-CD137-APC (4B4-1, 1:100, BD Biosciences) and anti-OX40-FITC (ACT35, 1:20, BioLegend) antibodies. If T cell reactivity was detected by two independent assays, the respective neoantigen was taken along for further validation using HPLC-purified wild-type and mutant peptide sequences.

In order to confirm whether reactivity derived from CD4<sup>+</sup> or CD8<sup>+</sup> T cells in TIL cultures, we analyzed intracellular IFN-γ expression following the reactivity assays. Expanded TIL were cocultured with the respective neoantigen-loaded EBV-LCLs, as described above, and 10 µg/mL brefeldin A was added to the medium after 1 hour of coculture. The next day, T cells were inspected for the presence of intracellular IFN-γ-BV421 (4S.B3, 1:20, Biolegend), in combination with CD4-PE-CF594 (RPA-T4, 1:50, BD Biosciences), CD8-FITC (SK1, 1:20, BD Biosciences) and an APC-Cy7 live-dead marker (1:20, Invitrogen, Carlsbad, California, USA). Six thousand cells per subset were measured on a LSRFortessa (BD Biosciences).

### CD8<sup>+</sup> T cell characterization by imaging mass cytometry

T cell infiltration was assessed on formalin-fixed paraffin-embedded tissue sections using a general immunophenotyping imaging mass cytometry panel as described previously.<sup>52</sup> For this study, we focused on the detection of CD3, CD4, CD8, CD39 and CD103, from a total of 40 markers on 18 MMR-proficient CRC patients (online supplemental table S3). H&E stains performed on consecutive tumor tissue slides were used to determine regions of interest. The 1000×1000 µm tissue areas were ablated and acquired by the Hyperion mass cytometry system (Fluidigm, San Francisco, California, USA). The generated MCD files were exported and visualized with the Fluidigm MCD viewer to set signal threshold per marker in order to better separate antibody signal from noise.

### Statistics

Paired samples Wilcoxon test was applied to test differential CD8<sup>+</sup> T cell infiltration in epithelial and stromal tissue compartments in tumors, as determined by imaging mass cytometry. One-way analysis of variance was applied to test differences in the relative frequency of cell surface markers on the different T cell subsets. Statistical testing and graphical representation were performed with Graphpad Prism V.9.3.1.

### Data availability

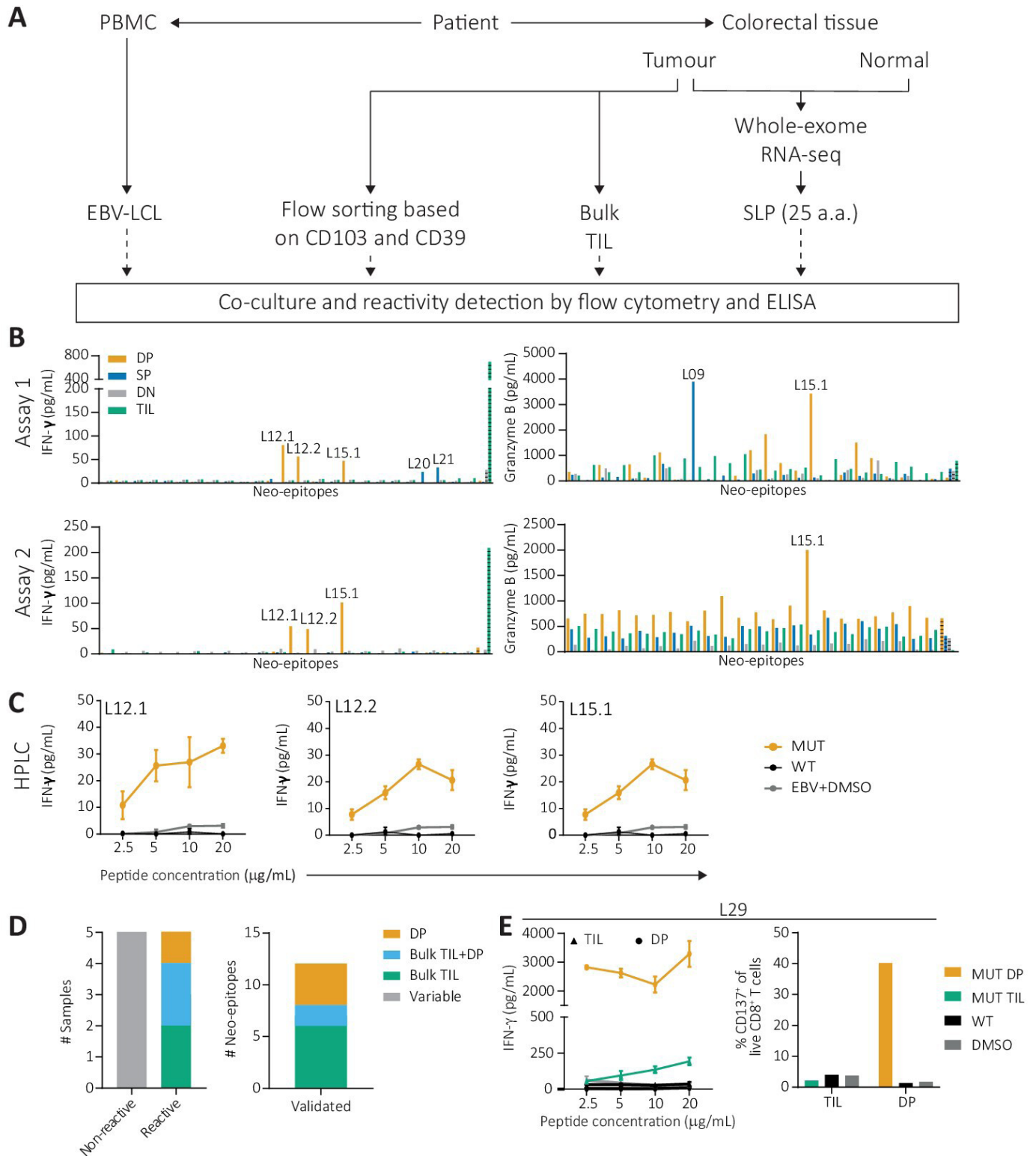
Additional data generated in this study are available on reasonable request to the corresponding author. RNAseq of the studied patients will be made available in the Sequence Read Archive of NCBI via PRJNA911749.

## RESULTS

### Neoantigen reactivity by cytotoxic T cells is contained within the CD103<sup>+</sup>CD39<sup>+</sup> subset

In order to evaluate whether neoantigen reactivity is associated with CD103<sup>+</sup> and CD39<sup>+</sup> expression on CD8<sup>+</sup> T cells, we evaluated T cell responses against synthetic peptides corresponding to neoantigens in bulk TIL and in CD103<sup>+</sup>CD39<sup>-</sup> (DN), CD103<sup>+</sup>CD39<sup>+</sup>/CD103<sup>-</sup>CD39<sup>+</sup> (SP), and CD103<sup>+</sup>CD39<sup>+</sup> (DP) CD8<sup>+</sup> T cell populations, isolated from single cell digests, derived from 11 MMR-proficient CRCs (figure 1A). All tumors retained β2-microglobulin expression and the majority of tumors were found to be proficient in HLA class I expression as determined by immunohistochemistry (table 1). Loss of HLA class I expression was observed in NIC25 while the tumor sample from NIC27 presented a heterogeneous pattern of HLA class I expression.

The proportion of CD103 and/or CD39-positive CD8<sup>+</sup> T cell subsets was generally low among the total immune cell populations (CD45<sup>+</sup> cells) (online supplemental figure S1A). Furthermore, the relative frequencies of the flow sorted CD8<sup>+</sup> T cell populations (DP, SP and DN) were highly variable between patients (online supplemental figure S1B). In particular, SP CD8<sup>+</sup> T cells with CD39 expression were rare in this cohort which hampered



**Figure 1** Neoantigen-directed T cell reactivity assessment from bulk TIL and sorted CD8<sup>+</sup> T cell subsets according to CD39 and CD103 expression. (A) Schematic workflow of the experimental setup. (B) A representative example of the IFN- $\gamma$  and granzyme B ELISA measurements obtained in two independent assays performed in NIC16. Potential neoepitopes are depicted with the peptide number, for example, 'L12.1'. (C) Representative example of a validation experiment in NIC16. The differential IFN- $\gamma$  production upon coculture with the mutant peptide (yellow), the corresponding wild-type peptide (black) or a DMSO control (grey) was assessed in a peptide titration series ranging from 2.5 to 20  $\mu$ g/mL. (D) Summary of the number of patient samples in which no reactivity was detected (gray), or with T cell responses derived from the DP subset (yellow), bulk TIL (green) or both the bulk TIL and DP subset (light blue). (E) IFN- $\gamma$  production (left) and CD137 expression (right) on coculture of NIC4 bulk TIL (green) and DP subset (yellow) with the L29 epitope and controls. DP, double-positive; EBV-LCL, Epstein-Barr virus-transformed lymphoblastoid B cell lines; PBMC, peripheral blood mononuclear cell; TIL, tumor-infiltrating lymphocyte.

their specific expansion. NIC5 formed an exception as both CD103<sup>+</sup>CD39<sup>+</sup> and CD103<sup>+</sup>CD39<sup>-</sup> subsets could be separately expanded and taken along for neoantigen reactivity assays. The number of sorted cells varied from 18 to 31 000 cells per subset, with a median of 595 cells (online supplemental table S4). Most CD8<sup>+</sup> T cell subsets expanded more than 2000 times (median of 17 973 times) after culture, with the exception of two samples (NIC5 DP and NIC39 DP) which did not expand. Of note, the DN subset expanded at a higher rate than the subsets expressing CD39 and/or CD103 (online supplemental table S4). Also, preferential expansion of CD4<sup>+</sup> T cells over CD8<sup>+</sup> T cells was observed in the expanded bulk TIL samples (online supplemental table S1). Phenotypic analyses of the CD8<sup>+</sup> T cells subsets revealed that CD69 and PD-1 (traditional activation markers) expression were most frequent in the DP subset and less abundant in DN CD8<sup>+</sup> T cells, in contrast to CD127 (IL-7R) expression which was found to be enriched in DN CD8<sup>+</sup> T cells (online supplemental figure SIC-F).

Whole-exome and transcriptome sequencing were performed on cancer and healthy tissues for the identification of putative neoantigens. All CRCs presented a low number of non-synonymous mutations, with a median of 38 transcribed mutations (21–57, table 2). All expressed, non-synonymous mutations were considered potential neoantigens. T cell reactivity was assessed by measuring the capacity of T cells to secrete IFN- $\gamma$  and granzyme B (measured by ELISA) and to upregulate activation markers (measured by flow cytometry) after coculture with autologous EBV-LCL, loaded with mutated synthetic long peptides (25AA). The DN subset produced widespread, unspecific reactivity in the majority (n=6) of samples, which is likely explained by the presence of EBV-specific T cells in this population. Mutated peptides that consistently induced the secretion of IFN- $\gamma$  and/or granzyme B by T cells, in two independent replicate coculture assays, were considered as potential immunogenic neoantigens (figure 1B, online supplemental figure S2). For example, in patient NIC16, the peptides L12.1, L12.2 and L15.1 were identified as potential epitopes, while L09, L20 and L21 were not pursued further they only induced reactivity in one of the assays (figure 1B). Reactivities were confirmed by coculture with the mutant and the corresponding wild-type HPLC-purified peptides. Release of IFN- $\gamma$  or granzyme B, or CD137 upregulation on CD8<sup>+</sup> T cells with the mutant peptide and not the corresponding wild type was considered as a bona fide neoantigen-specific T cell response (figure 1C).

Neoantigen-specific T cell responses were validated in five patients (NIC4, NIC16, NIC20, NIC22, and NIC38), against 1–3 neoantigens per patient (figure 1D, table 2). The tumors of these patients were all found to have retained HLA class I expression on the surface of cancer cells, while two of the samples with no detectable neoantigen-reactivity displayed

abnormal HLA class I expression which could possibly interfere with the persistence of neoantigen-reactive CD8<sup>+</sup> T cells in the tissue. Interestingly, in these two patients the DP CD8<sup>+</sup> T cell subsets were also found to display suboptimal characteristics as the DP CD8<sup>+</sup> T cells of NIC25 were PD-1<sup>+</sup>CD39<sup>low</sup> and half of the DP CD8<sup>+</sup> T cells of NIC7 were PD-1<sup>+</sup>CD127<sup>+</sup>. These phenotypes suggest the absence (NIC25) or low abundance (NIC7) of properly activated CD8<sup>+</sup> T cells. Reactivity could only be detected in either the DP CD8<sup>+</sup> T cell subset or in the bulk TIL samples. None of the SP or DN subsets displayed neoantigen-specific T cell responses.

In figure 1C, representative assays are shown corresponding to the detection of neoantigen-specific reactivity in the DP CD8<sup>+</sup> T cell subset against two epitopes representing the *ENGASE* c.260T>G (p.C87W, peptide L12.1 and L12.2) and *SMPD4* c.646G>T (p.V216F, peptide L15.1) mutations. No reactivity for this patient could be detected in the bulk TIL. The DP CD8<sup>+</sup> T cell subset of NIC20 recognized two peptides corresponding to the *MYOBI* c.801A>C (p.E267D, peptide L16.2) and *KMT2C* c.8950dupA (p.S2984fs, peptide L27.2) mutations (online supplemental figure S3). Of note, cross-reactivity was also detected to the wild-type peptides of NIC20. The bulk TIL of NIC20 recognized the same peptide derived from the *MYOBI* mutation but not the one derived from *KMT2C*. In NIC4, reactivity against the mutation *PDP1* c.1024C>T (p.R342W, peptide L29) was detected in both DP CD8<sup>+</sup> and TIL samples.<sup>6</sup>

Neoantigen reactivity in bulk TIL samples—without concurrent reactivity of the DP CD8<sup>+</sup> T cell subset—was detected in NIC4, NIC22 and NIC38. For NIC22, bulk TIL recognized the peptides corresponding to the mutations *RRP15* c.187A>G (p.I63V, peptide L02) and *CHEK2* c.1418C>G (p.A473G, peptide L20; table 2). In NIC38, bulk TIL reactivity was detected to the epitopes from the following mutations: *RALGAPB* c.3410C>T (p.A1137V, peptide L36), *GNAS* c.557G>A (p.R186H, peptide L37) and *SHH* c.962G>A (p.R321H, peptide L48). In addition to the *PDP1* mutation that was recognized by both DP CD8<sup>+</sup> T cell and bulk TIL, two peptides showed reactivity only in the bulk TIL from NIC4 (*ACTR10* p.R213H, peptide L06; *RAE1* p.X369W, peptide L20).

In total, T cell responses were identified against 12 unique neoepitopes; 4 neoepitopes were recognized by the DP CD8<sup>+</sup> T cell subsets, 2 neoepitopes were detected by both the DP CD8<sup>+</sup> T cells and bulk TIL and, lastly, 6 neoepitopes were recognized only by the bulk TIL (figure 1D). Since a considerable number of neoepitopes were only recognized by the bulk TIL samples, we hypothesized that recognition of those epitopes could be mediated by CD4<sup>+</sup> T cells. Flow cytometry analyses revealed that all reactivity detected exclusively in bulk TIL samples was derived from CD4<sup>+</sup> T cells as determined by OX40 and/or intracellular IFN- $\gamma$  upregulation following coculture with peptides (figure 1

**Table 2** Patient's neo-epitopes to which T cell reactivity was detected

Patient ID	CMS	# Mut	# SLP	Genes	Mut cDNA	Mut aa.	Peptide	Peptide ID	Reactive T cell product	CD4/8
NIC4	4	30	39	ACTR10	c.638G>A	p.R213H	SVPEGVLEDIKAHTCFVSDLKRGLK	L06	TIL	CD4
				RAE1	c.1106A>G	p.X369W	WWLETLAQPELFLSTLPHLCTNLGP	L20	TIL	CD4
				PDP1	c.1024C>T	p.R342W	PKSEAKSVKQDWLLGLLMPFFRFG	L29	TIL, DP	CD8
NIC5	2	56	71	-	-	-	-	-	-	-
NIC7	2	33	44	-	-	-	-	-	-	-
NIC16	2	21	23	ENGASE	c.260T>G	p.C87W	PPLSSQRPRTLLWHDMMGGYLDDRF	L12.1	DP	CD8
				ENGASE	c.260T>G	p.C87W	PPLSSQRPRTLLWHDMMGGYLDDRD	L12.2	DP	CD8
				SMPD4	c.646G>T	p.V216F	LITQKPLPVSLHERTSDCAYFILVD	L15.1	DP	CD8
NIC17	2	43	47	-	-	-	-	-	-	-
NIC20	2	30	34	MYOB1	c.801A>C	p.E267D	RNAMQIVGFMHDHDAESVLAWAAVL	L16.2	TIL, DP	CD4/8
				KMT2C	c.8950dupA	p.S2984fs	NVTWSRVNHVFFESGCGAGKPRAHRSRSINS	L27.2	DP	CD8
NIC22	2	30	34	RRP15	c.187A>G	p.I63V	SEKDHFYSDDDAVEADSEGDAPCD	L02	TIL	CD4
				CHEK2	c.1418C>G	p.A473G	DLVKILLVDPKGRFTTEEALRHPW	L20	TIL	CD4
NIC25	4	38	47	-	-	-	-	-	-	-
NIC27	2	26	43	-	-	-	-	-	-	-
NIC31	3	54	75	RALGAPB	c.3410C>T	p.A1137V	EPANSRLPPLHLVLDSTIPGFFDDI	L36	TIL	CD4
				GNAS	c.557G>A	p.R186H	DYVPSDQDLRCHVLTSGIFETKFO	L37	TIL	CD4
				SHH	c.962G>A	p.R321H	VRPGQRVYVAEHDGDRRLPAAVH	L48	TIL	CD4
NIC39	3	57	66	-	-	-	-	-	-	-

aa, amino acid; CMS, consensus molecular subtype; DP, double-positive; Mut, mutation; SLP, synthetic long peptide.



(NIC16; online supplemental figure S3 (NIC38); online supplemental figure S4 (NIC4, NIC22)).

In sum, CD8<sup>+</sup> T cell reactivity in MMR-proficient CRC is largely contained within the DP subset and, importantly, the specific interrogation of this population allowed the discovery of neoantigen-specific reactivity that could not be detected in bulk TIL samples. In line with this, the higher levels of IFN- $\gamma$  production and CD137 expressing cells upon peptide stimulation reflects a higher frequency of neoantigen-specific cells in the DP CD8<sup>+</sup> subset than in the bulk TIL sample (figure 1E).

### CD103<sup>+</sup>CD39<sup>+</sup> CD8<sup>+</sup> T cell subsets are enriched in the epithelial compartment of CRC

To interrogate the distribution of the different CD8<sup>+</sup> T cell subsets in the tumor microenvironment of CRC, we applied imaging mass cytometry on 18 CRC tissues, including the 11 samples for which neoantigen reactivity was investigated (figure 2A,B). The relative frequency of the DP cells, in relation to the total number of CD8<sup>+</sup> T cells, was significantly higher (5.7x) in the epithelial compartment of tumor tissues than in the stromal areas (figure 2C, paired samples Wilcoxon test:  $p=0.002$ ). In the tumor stroma, CD8<sup>+</sup> T cells often lacked the coexpression of these markers (figure 2B) while the majority of intraepithelial CD8<sup>+</sup> T cells expressed CD103 and CD39 (figure 2A). This observation suggests a direct interaction between the DP CD8<sup>+</sup> T cells and cancer cells, therefore, supporting their important role in cancer immunity.

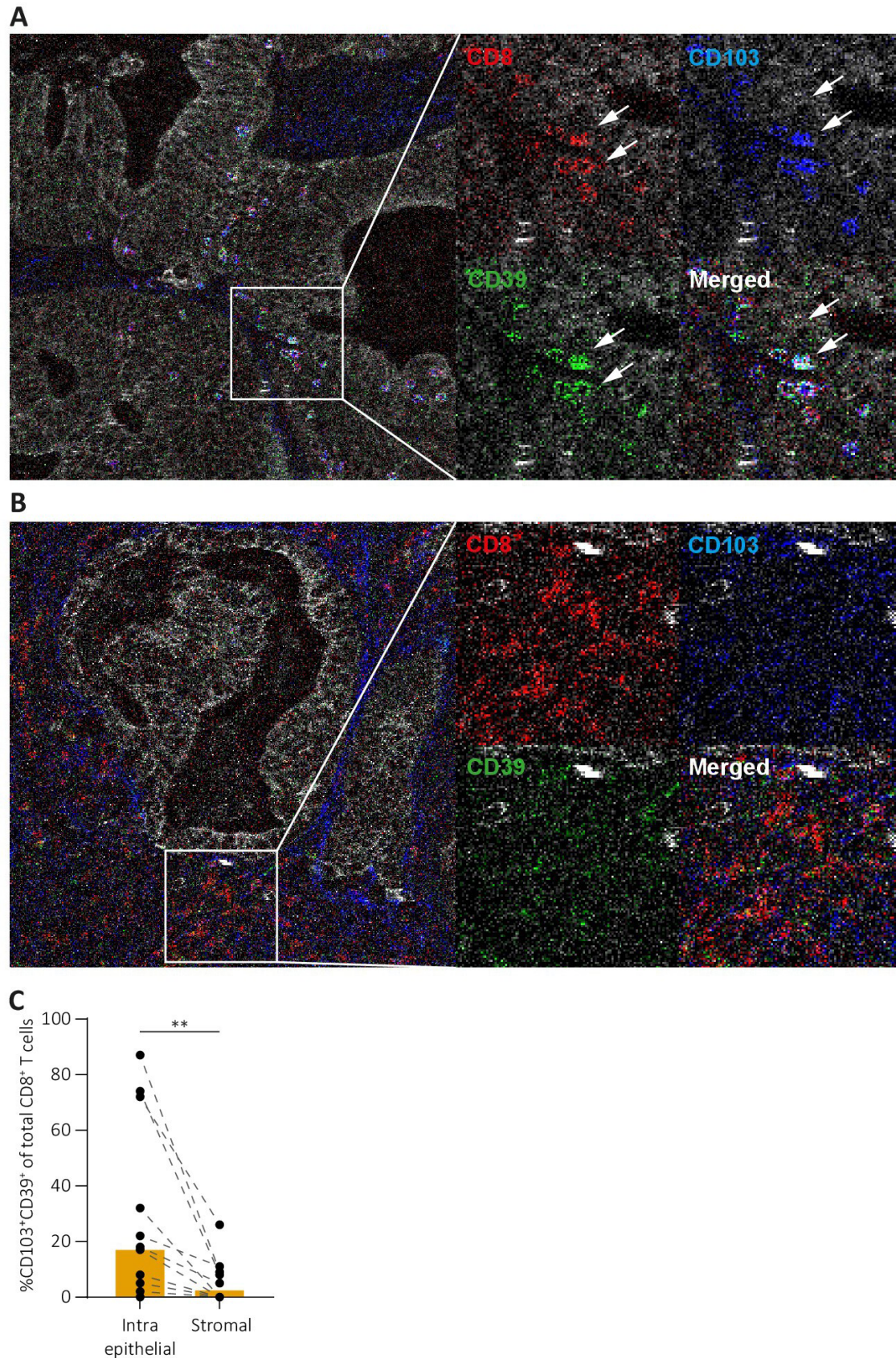
### DISCUSSION

Innovative treatment options are required for patients diagnosed with advanced CRC. Since we and others identified the presence of neoantigen-specific T cells in MMR-proficient CRC patients, there is a realistic expectation that T-cell based immunotherapy can also be successful in this patient group.<sup>6–9</sup> Furthermore, the majority of MMR-proficient colorectal tumors are found to retain HLA class I expression,<sup>38</sup> indicating that they remain susceptible to CD8<sup>+</sup> T cell-mediated tumor eradication. Their general refractoriness to immune checkpoint blockade treatment in the advanced setting might be explained by the low frequency of neoantigen-specific T cells in tissues but also a dominant immunosuppressive microenvironment like the one provided by TGF- $\beta$  activation in a substantial proportion of MMR-proficient CRC.<sup>6 53 54</sup>

An attractive path worth exploring for the treatment of cancers with low immunogenicity is the development of ACT protocols that specifically focus on neoantigen-specific T cells. To achieve optimal ACT treatments for those patients, the definition of cellular biomarkers that can be used as surrogates of neoantigen-specificity but also be targeted in cell sorting procedures is paramount.

Previously, CD103 expression on cytotoxic T cells was highlighted as a prognostic marker for breast and ovarian cancer patient survival,<sup>24 55 56</sup> and associated to positive treatment outcomes in lung and bladder cancer patients who received anti-PD-L1 therapy, intratumorally.<sup>57</sup> Also, loss of the CD103 ligand, E-cadherin, was found to reduce the response to checkpoint blockade therapy in murine melanoma.<sup>58</sup> Combined expression of CD103 and CD39 has been reported to pinpoint tumor-reactive CD8<sup>+</sup> T cells in melanoma and head and neck cancer.<sup>9 27 34</sup> DP CD8<sup>+</sup> T cells were more frequently positive for CD69 and negative for CD127 and expressed higher levels of PD-1. Together, this cell surface expression pattern supports a chronic activation phenotype in the DP CD8<sup>+</sup> T cell subset.<sup>36</sup> On the other hand, the expression of these markers did not provide enough specificity to define neoantigen-reactive T cells as compared with the codetection of CD103 and CD39.

CD103<sup>+</sup>CD39<sup>+</sup> CD8<sup>+</sup> T cells were previously found to be clonally expanded in tumor tissues and to present enhanced granzyme B expression in comparison to other CD8<sup>+</sup> T cell subsets.<sup>27 34</sup> In line with these findings, we show that CD8<sup>+</sup> T cell-derived neoantigen reactivity is limited to populations expressing CD103 and CD39. The relevance of DP CD8<sup>+</sup> T cells in the antitumor response is highlighted by the fact that their frequency within the tumor epithelium was significantly higher than in the stromal compartment, supporting the occurrence of physical interactions between this subset and cancer cells. In our study, 1–3 neoepitopes eliciting T cell reactivity were identified, per patient, which translates to a neoantigen detection rate of 2.6% in relation to the total number of expressed mutations. Importantly, the interrogation of neoantigen reactivity in the DP CD8<sup>+</sup> T cell subsets led to the identification of additional epitopes recognized by CD8<sup>+</sup> T cells as compared with bulk TIL. Furthermore, we observed that DN CD8<sup>+</sup> T cells expanded at a higher rate than SP and DP CD8<sup>+</sup> T cells, suggesting that the latter subsets may be under-represented in expanded bulk TIL populations. Furthermore, we also observed that bulk TIL products were, in general, enriched for CD4<sup>+</sup> T cells, again, affecting the probability that neoantigen-reactive CD8<sup>+</sup> T cells are contained within unselected T cell products. Altogether, our observations highlight the possibility of undertaking immunotherapeutic strategies in MMR-proficient CRC patients through the enrichment of CD103<sup>+</sup>CD39<sup>+</sup> neoantigen-specific CD8<sup>+</sup> T cells for the development of therapeutic T cell products. In parallel, it would be important to investigate whether clinical responses to immune checkpoint blockade therapies in MMR-proficient CRC<sup>3</sup> are related to the frequency of DP CD8<sup>+</sup> T cells. Interestingly, PD-1 expression on its own did not appear to be an ideal biomarker to pinpoint neoantigen-specific T cells in this cohort (online supplemental figure S1).



**Figure 2** CD103 and CD39 detection on tumor-infiltrating T cells using imaging mass cytometry. (A, B) Representative tissue sections illustrating T cell infiltration (CD8 in red, CD103 in blue, CD39 in green) in relation to cancer cells (keratin, white). The arrows highlight DP CD8<sup>+</sup> T cells. (C) Quantification of infiltration by DP populations as percentage of the total CD8<sup>+</sup> T cell infiltrate. The number of cells was measured for the epithelium and stroma separately, and compared between both compartments using a paired samples Wilcoxon test. \*\*, P ≤ 0.01. DP, double-positive.

Whether CD39 expression translates to an ‘exhausted’ or ‘dysfunctional’ T cell state that would compromise the exploitation of DP CD8<sup>+</sup> T cells for ACT, remains a point of discussion.<sup>34 37 59</sup> In the first days after activation of naïve T cells, transient CD39 expression takes place but, CD39—together with other coinhibitory molecules—can also be expressed by T cells after chronic antigen stimulation in the tumor microenvironment.<sup>60</sup> Nevertheless, we were able to expand DP CD8<sup>+</sup> T cells in vitro (median=7419 times) and their successful application in neoantigen reactivity assays demonstrates that this subset retains functional activity. Further studies should be performed to examine whether DP CD8<sup>+</sup> T cells still harbor functional cytotoxic capacity in vivo. Alternatively, the isolation of DP CD8<sup>+</sup> T cells could instead be employed for the identification of neoantigen-specific T cell receptors (TCRs) that can then be engineered into donor T cells with optimal functionality. Such an approach has recently been reported for the treatment of a patient with metastatic pancreatic cancer harboring the KRAS<sup>G12D</sup> mutation and expressing HLA-C\*08:02.<sup>61</sup> Specifically, the authors genetically engineered the patient’s T cells to express two TCRs, previously identified in a metastatic CRC patient, with that specific restriction. The infused ACT product led to regression of the metastases, which was still ongoing 6 months postinfusion. This case study illustrates the safety, feasibility and wide applicability of using previously identified TCRs in ACT treatments.

In addition to CD8<sup>+</sup> T cell-mediated responses, we observed CD4<sup>+</sup> T cell reactivity among the bulk TIL in several patients. This observation is in line with previous reports on MMR-proficient CRC where approximately half of the T cell reactivity was attributed to CD4<sup>+</sup> T cells.<sup>6–8</sup> Importantly, ACT approaches making use of neoantigen-specific CD4<sup>+</sup> T cells were shown to be successful, supporting the relevance of including CD4<sup>+</sup> T cells in ACT products for optimal tumor eradication.<sup>11 17</sup> However, little is yet known about cell surface markers that specifically pinpoint tumor-reactive T cell subsets among CD4<sup>+</sup> T cells. A recent study proposed that CD39 can guide the enrichment of tumor-reactive CD4<sup>+</sup> T cells.<sup>30 62</sup> Single cell transcriptomic data from head and neck, cervical and ovarian cancer samples revealed similar transcriptional programs between PD-1<sup>hi</sup>CD39<sup>+</sup> CD4<sup>+</sup> T cells and neoantigen-specific CD8<sup>+</sup> T cell subsets,<sup>32 63</sup> for example, *TOX* and *CXCL13* expression. However, as these markers are not expressed at the cell surface they cannot be employed for the specific isolation of neoantigen-specific CD4<sup>+</sup> T cells. Alternatively, combined expression of PD-1 and ICOS has been proposed to identify populations of neoantigen and tumor-associated antigen-specific CD4<sup>+</sup> T cells.<sup>64</sup> The limited availability of patient material in this cohort did not allow us to explore this question in more detail leaving this topic open for future investigations.

In conclusion, we report here that in MMR-proficient CRC patients neoantigen-directed CD8<sup>+</sup> T cell reactivity is mainly contained in the CD103<sup>+</sup>CD39<sup>+</sup> subset. Their isolation can be exploited to enrich ACT products for

tumor-reactive T cells and, thereby, improve the efficacy of the current ACT strategies. Furthermore, the specific focus on this CD8<sup>+</sup> T cell population can expedite the identification of therapeutically relevant TCRs. These strategies are highly promising to complement the current applicability of checkpoint blockade therapies.

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**Contributors** JvdB cultured the bulk TIL, performed the in vitro assays, interpretation of all experiments and drafted the manuscript. MvdP provided experimental support. MEI ran the imaging mass cytometry assays and analysis. DR processed and analyzed the genomic data. RvdB collected patient material and prepared the genomic libraries. KCMJP was responsible for the patient recruitment and clinical supervision. AFS performed pathologic inspection of the tumors. TD was responsible for the isolation and expansion of CD8<sup>+</sup> T cell subsets. EMEV and SHVdB have supervised T cell reactivity experiments. NFCCdM conceptualized this study, received funding, revised the manuscript and acts as guarantor.

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**Data availability statement** Data are available in a public, open access repository. All data relevant to the study are included in the article or uploaded as online supplemental information. Patient’s RNA sequencing data can be retrieved from the Sequence Read Archive of NCBI via PRJNA911749.

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