




Intrahepatic CD69⁺Vδ1 T cells re-circulate in the blood of patients with metastatic colorectal cancer and limit tumor progression

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ABSTRACT

Background More than 50% of all patients with colorectal cancer (CRC) develop liver metastases (CLM), a clinical condition characterized by poor prognosis and lack of reliable prognostic markers. Vδ1 cells are a subset of tissue-resident gamma delta (γδ) T lymphocytes endowed with a broad array of antitumor functions and showing a natural high tropism for the liver. However, little is known about their impact in the clinical outcomes of CLM.

Methods We isolated human γδ T cells from peripheral blood (PB) and peritumoral (PT) tissue of 93 patients undergone surgical procedures to remove CLM. The phenotype of freshly purified γδ T cells was assessed by multiparametric flow cytometry, the transcriptional profiles by single cell RNA-sequencing, the functional annotations by Gene Ontology enrichment analyses and the clonotype by γδ T cell receptor (TCR)-sequencing.

Results The microenvironment of CLM is characterized by a heterogeneous immune infiltrate comprising different subsets of γδ tumor-infiltrating lymphocytes (TILs) able to egress the liver and re-circulate in PB. Vδ1 T cells represent the largest population of γδ TILs within the PT compartment of CLM that is greatly enriched in Vδ1 T effector (T_{EF}) cells expressing constitutive high levels of CD69. These Vδ1 CD69⁺ TILs express a distinct phenotype and transcriptional signature, show high antitumor potential and correlate with better patient clinical outcomes in terms of lower numbers of liver metastatic lesions and longer overall survival (OS). Moreover, intrahepatic CD69⁺ Vδ1 TILs can egress CLM tissue to re-circulate in PB, where they retain a phenotype, transcriptional signature and TCR clonal repertoires resembling their liver origin. Importantly, even the increased frequencies of the CD69⁺ terminally differentiated (T_{EMRA}) Vδ1 cells in PB of patients with CLM significantly correlate with longer OS. The positive prognostic score of high frequencies of CD69⁺ T_{EMRA} Vδ1 cells in PB is independent from the neoadjuvant chemotherapy and immunotherapy regimens administered to patients with CLM prior surgery.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ The metastatic progression of colorectal cancer (CRC) to livers occurs in approximately 50% of patients and still represents an unmet clinical need lacking a cure.
- ⇒ The microenvironment of human liver is characterized by a heterogeneous distribution of distinct subsets of gamma delta (γδ) T lymphocytes with antitumor effector potential.

WHAT THIS STUDY ADDS

- ⇒ Intrahepatic CD69⁺ Vδ1 T cells in liver metastatic CRC (CLM) represent the predominant TIL subset with high antitumor effector functions that is also able to egress tumor and re-circulate in peripheral blood (PB).
- ⇒ Higher frequencies of both TI and PB CD69⁺ Vδ1 T cells are associated with a slower liver metastatic progression and a longer overall survival of patients with CLM.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ The enrichment of tissue-resident CD69⁺ Vδ1 T cells re-circulating at high frequencies in PB represents a new important clinical tool to help predicting either the natural history of CLM in 'liquid biopsies' or develop alternative therapeutic protocols of cellular therapies.

Conclusions The enrichment of tissue-resident CD69⁺ Vδ1 T_{EMRA} cells re-circulating at high frequencies in PB of patients with CLM limits tumor progression and represents a new important clinical tool to either predict the natural history of CLM or develop alternative therapeutic protocols of cellular therapies.

INTRODUCTION

The metastatic progression of colorectal cancer (CRC) to livers occurs in approximately

50% of patients and still represents an unmet clinical need lacking a cure. The surgical resection of colon liver metastases (CLM) is the only available treatment increasing patients' overall survival (OS), while definitive and shared clinical criteria that stratifies patients' eligibility to surgery and/or neoadjuvant conventional/biological chemotherapies (na-CHT) are still missing.¹

Targeting different aspects of antitumor immune responses in primary or metastatic tumors (MTs) is key for the development of new clinical tools to predict cancer progression and develop alternative therapeutic strategies. Indeed, since the diagnostic potential of resected/biopsied tumors is hampered by multiple methodological and ethical constraints, there is a growing interest in finding immune prognostic variables within the so-called 'liquid biopsies' from peripheral blood (PB). In this scenario, tumor-infiltrating (TI) lymphocytes (TILs) have been also found in the PB of patients affected by different cancers.^{2,3} The development of new single-cell technologies with an unprecedented degree of immunological resolution can now identify in 'liquid biopsies' T cells showing tumor specificities similar to those of their homologs in neoplastic lesions.^{4,5}

Gamma delta ($\gamma\delta$) T cells are a unique subset of T lymphocytes endowed with innate-like features due to their major histocompatibility complex (MHC)-unrestricted ability to activate and respond to stimuli.⁶ They represent the first cluster of cells appearing during T cell differentiation and play a crucial role in cancer immune-surveillance given their potent cytotoxicity and production of several antitumor cytokines.⁷ Indeed, the presence of $\gamma\delta$ T cells at high frequencies emerged as one of the most favorable and widest prognostic factors in cancer progressions (including CRC).⁸ Transcriptomic data and molecular analysis of human $\gamma\delta$ TCR repertoire revealed the existence of two major subsets of $\gamma\delta$ T cells, namely V δ 1 and V δ 2. The first one is highly enriched at tissue sites and owns features of local resident cells endowed with remarkable capacities in the clearance of malignant cells from different hematopoietic and solid cancers.^{9–13} V δ 2 T cells preferentially circulate in the bloodstream thus being targeted for developing protocols of *in vitro* expansion for adoptive cell transfer therapies.^{14,15} Moreover, a third population expressing V δ 3-TCR chain had been also described. These latter V δ 3 T cells exhibit a high liver tropism and are present at very low frequency in PB under homeostatic conditions, while expanding in bloodstream during viral infections and inflammatory diseases.^{16,17} However, a comparative cellular and molecular characterization of TI and PB $\gamma\delta$ T cells in human cancers is still lacking.

V δ 1 T cells represent the main subset of $\gamma\delta$ T lymphocytes in human liver. They include both tissue-resident and re-circulating cells playing a key role in hepatic immune homeostasis.^{18,19} Nothing is known about the clinical impact of hepatic V δ 1 T cells in limiting the progression of CLM. In this regard, we recently identified a specific subset of human intraepithelial gut NKp46⁺ V δ 1 T cells

able to limit the metastatic diffusion of primary CRC to liver.²⁰ Similar results have been also confirmed in mice.²¹ Moreover, increased frequencies of PB and intrahepatic $\gamma\delta$ T cells have been reported during chronic hepatitis and liver inflammation.^{22,23}

The present study provides an in-depth and integrated phenotypical and transcriptional characterization of TI and PB $\gamma\delta$ T cell subset in patients with CLM. In particular, we identified a subset of CD69⁺ V δ 1 TILs whose higher frequencies in tumor specimens are associated with lower numbers of metastatic lesions and longer OS of patients with CLM. Importantly, these latter immune cell subsets can egress the tumor and re-circulate in PB as CD69⁺ terminally differentiated (T_{EMRA}) V δ 1 cells. Taken together, these findings could remarkably improve our ability to predict the prognosis of patients with CLM and pave the ground for developing new strategies of adoptive cell transfer immunotherapies.

MATERIALS AND METHODS

Study design

The study characterized $\gamma\delta$ T cells in a real-life cohort of total 93 patients with CRC underwent hepatic resection of rectal metastasis mostly represented by older patients with mean age of 64 years, mainly synchronous clinical profile who received na-CHT treatment (table 1). We assessed phenotype, function, $\gamma\delta$ TCR clonotype and transcriptional profiles by single cell RNA-sequencing (scRNA-seq) of $\gamma\delta$ T cells isolated from PB, tumor-free liver parenchyma surrounding CLM and MT.

Cell preparation

Peripheral blood mononuclear cells obtained from the buffy coats of healthy donors (HDs) or from blood of patients with CLM were isolated by *Lympholyte* Cell Separation density gradient solution (Cederlane Laboratories, Burlington, Canada) according to the manufacturer's instructions. Liver specimens were dissociated by enzymatic digestion with 2 mg/mL of collagenase D (Roche Diagnostic; Indianapolis, Indiana, USA) in gentleMACS Dissociator (Miltenyi; Bergisch Gladbach, Germany) as previously described.²⁰ Lymphocytes were separated by 70%/30% discontinuous Percoll gradient (GE Healthcare; Chicago, Illinois, USA) and frozen in fetal bovine serum (Lonza, Verviers, Belgium) with 10% of dimethylsulfoxide (Lonza) before being stored in liquid nitrogen for further analysis.

Additional materials and methods are included in online supplemental materials.

RESULTS

V δ 1 T cells in CLM predict longer patients' overall survival

We first characterized by immunohistochemistry (IHC) the frequency and distribution of TILs within CLM. Among CD3⁺ T cells, $\gamma\delta$ TILs are significantly higher in numbers within peritumoral tissue (PT) compared with

Table 1 Cohort characterization of patients with CLM

Feature	Variable	Patients with CLM (total no=93)
Age (mean±SD)		64 (±11)
Sex, n (%)	Male	51 (54.8)
	Female	42 (45.2)
K-RAS status, n (%)	Wild type	38 (40.9)
	Mutated	38 (40.9)
	n/a	17 (19.2)
Liver metastases (n) (median=4; range: 1–30)		
Disease-free interval, n (%)	Synchronous	65 (69.9)
	Metachronous	28 (30.1)
Liver localization of metastases, n (%)	Unilobar	41 (44.0)
	Bilobar	52 (56.0)
T status of the primitive lesion, n (%)	T1-2	13 (14.0)
	T3-4	74 (80.0)
	n/a	6 (6.0)
N status of the primitive lesion, n (%)	N+	65 (70.0)
	N0	22 (23.7)
	n/a	6 (6.3)
Type of surgery, n (%)	Major hepatectomy	9 (9.7)
	Limited resection	84 (90.3)
na-CHT, n (%) (courses median=8; range: 1–34)	Total	72 (77.4)
	Monotherapy (FOLFOX/FOLFIRI/XELOX)	14 (19.4)
	Combination therapy with anti-VEGF (bevacizumab or aflibercept)	38 (52.8)
	Combination therapy with anti-EGFR (cetuximab or panitumumab)	20 (27.8)
Without na-CHT, n (%)		21 (22.6)
Immunotherapies, n (%)		0 (0.0)
Recurrence, n (%)	Total	61 (65.6)
	Only liver	32 (52.5)
	Only lung	7 (11.5)
	Liver and lung	11 (18.0)
	Others	11 (18.0)
Positive status of HBV/HCV infections, n (%)		0 (0.0)

Patients with CLM were stratified according to age, sex and major clinical manifestations. Synchronous or metachronous CLM status is considered to be those detected within or after 12 months after diagnosis of the primitive lesion, respectively. Liver localization. Unilobar localization: nodules are located in one hemiliver (right or left hemiliver). Bilobar localization: lesions are located both on the right and on the left hemiliver. T status: the size of the tumor and any spread of cancer into nearby tissue. T1: the cancer has grown through the inner lining of the bowel, or into the muscle wall, but no further. T2: the tumor has grown into the muscularis propria, a deeper, thick layer of muscle that contracts to force along the contents of the intestines. T3: the tumor has grown into the outer lining of the bowel wall but has not grown through it. T4: the tumor has grown into the outermost layers of the colon or rectum and through the visceral peritoneum (T4a) but has not reached nearby organs. N status: N+ patients with diffusion of the adenocarcinoma to any lymph nodes at any levels. N0: no infiltration of the lymph nodes. Type of surgery: major hepatectomies are defined according to the Brisbane Classification.

CLM, colon liver metastatic cancer; EGFR, epidermal growth factor receptor; FOLFIRI, 5-fluorouracil/irinotecan; FOLFOX, 5-fluorouracil/oxaliplatin; HBV/HCV, hepatitis B/C virus; K-RAS, Kirsten rat sarcoma virus; n/a, missing data; na-CHT, neoadjuvant conventional/biological chemotherapies; VEGF, vascular endothelial growth factor; XELOX, capecitabine/oxaliplatin.

the core of highly fibrotic MT (figure 1A,B and online supplemental figure 1A). Additionally, multiparametric flow cytometry data showed that Vδ1 T cells represent

the main subset of $\gamma\delta$ TILs in CLM, as their frequencies are significantly higher compared with Vδ2 T cells

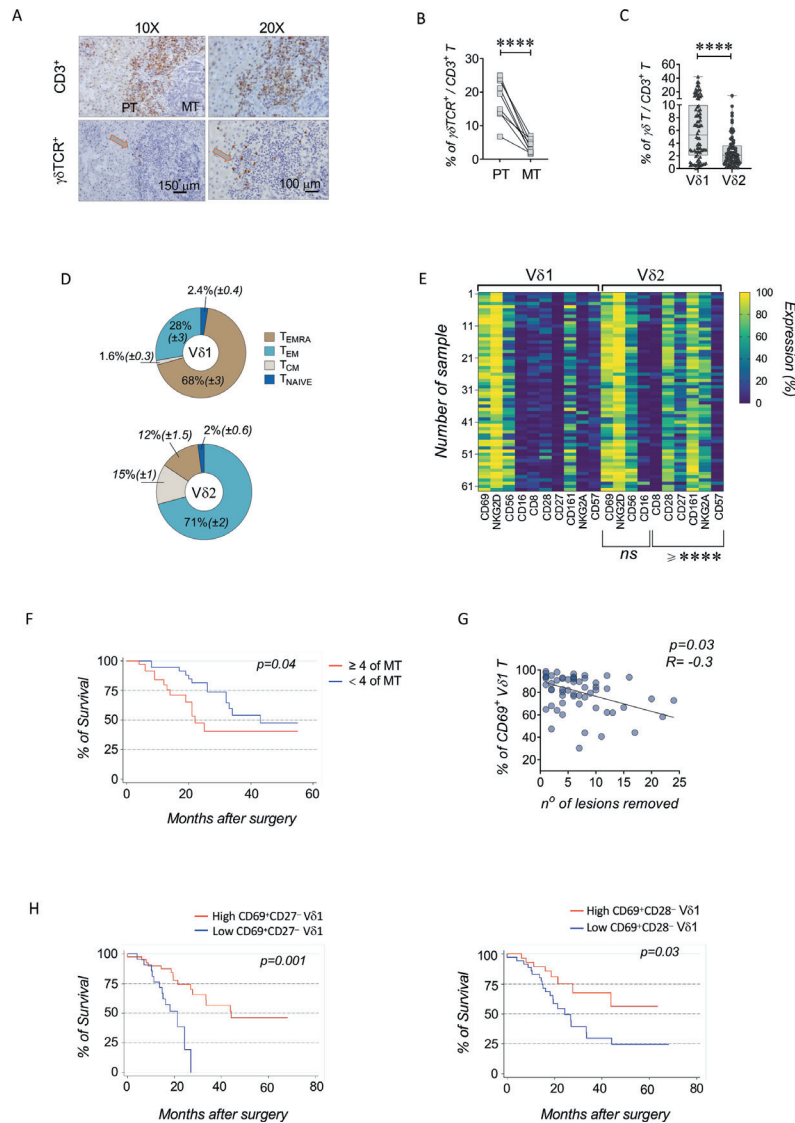


Figure 1 Cellular characterization and clinical impact of intrahepatic gamma delta ($\gamma\delta$) tumor-infiltrating lymphocytes (TILs) in colon liver metastatic cancer (CLM). (A) Representative immunohistochemistry (IHC) images showing $CD3^+$ (upper panels) and $\gamma\delta$ T cell receptor (TCR) $^+$ (lower panels) T cells within peritumoral tissue (PT) and metastatic tumor (MT) from one CLM liver section (out of 10) at 10 \times (left panel) and 20 \times (right panel) magnified views. Arrows indicate $\gamma\delta$ TCR $^+$ cells in lower panels. (B) Statistical graph from IHC data showing the mean frequency (%) of $\gamma\delta$ TCR $^+$ TILs among $CD3^+$ T cells in PT and MT areas (n=10). (C) Statistical graph from multiparametric flow cytometry data showing the mean (\pm SEM) frequency (%) of matched V δ 1 and V δ 2 T cells among $CD3^+$ T cells in PT (n=82). (D) Pie charts of multiparametric flow cytometry data showing the mean distribution (%) of naive T (T_{NAIVE}), T central memory (T_{CM}), T effector memory (T_{EM}) and terminally differentiated (T_{EMRA}) V δ 1 (upper chart) and V δ 2 (lower chart) T cells within PT (n=62). (E) Heatmaps of multiparametric flow cytometry data showing the expression of several surface markers between matched V δ 1 (left panel) and V δ 2 (right panel) TILs from PT (n=62). Group of markers which statistically differ between V δ 1 and V δ 2 TILs are annotated under the heatmap as ‘ \geq ****’ and indicates the range of significant p values; ‘ns’ indicates a group of markers not statistically significant. (F) Kaplan-Meier curve of postsurgical overall survival (OS; %) of patients with CLM based on the number of liver metastases (MT, cut-off \geq 4; n=60). (G) Spearman’s rank correlation between the frequency (%) of liver PT CD69 $^+$ V δ 1 T cells and the number of surgically removed liver metastasis (n=60). (H) Kaplan-Meier curves showing postsurgical OS (%) of patients with CLM based on the median frequencies (%) of CD69 $^+$ CD27 $^-$ (cut-off \leq 2%; left panel) and CD69 $^+$ CD28 $^-$ liver V δ 1 T cells in PT (cut-off \leq 5%; right panel) (n=63) Statistically significant p values are represented with the following number of asterisks (*): * $p \leq 0.05$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

(figure 1C and online supplemental figure 1B). To assess the phenotype of $\gamma\delta$ TILs, we evaluated the expression of CD27 and CD45RA that are recognized markers of $\alpha\beta$ T cell differentiation. Moreover, the surface levels of both these markers are also indicative of the homeostatic status and effector functions of human $\gamma\delta$ T cells including

proliferative capacity, resistance to drug treatment, cytokine secretion and cytotoxic response.^{24–28} V δ 1 TILs with the CD27 $^-$ phenotype characterize two effector (T_{EF}) cell subpopulations that include T_{EMRA} (CD45RA $^+$ CD27 $^-$) and T effector memory (T_{EM} ; CD45RA $^-$ CD27 $^-$) cells. These cells represent the larger fractions of V δ 1 in CLM, while

phenotypes of naïve T (T_{NAIVE}) ($CD45RA^+CD27^+$) and T central memory (T_{CM} ; $CD45RA^-CD27^+$) V δ 1 T cells are present at very low frequencies. This is not the case of V δ 2 TILs, whose distribution in CLM is characterized by the predominant expansion of cells with the phenotype of T_{EM} together with lower frequencies of T_{CM} and T_{EMRA} (figure 1D). As expected,^{18,29} both V δ 1 and V δ 2 TILs express high levels of the tissue-residency and activating marker CD69. In accordance with more mature phenotype, V δ 1 TILs display low levels of CD27 and CD28, both markers of immature T cells (figure 1E and online supplemental figure 1C). Compared with the V δ 2 counterpart, V δ 1 TILs present higher frequencies of CD57, a T cell marker of proliferative-senescence found to be increased in V δ 2 T cells of patients with CLM.²⁴ Importantly, expression of CD57 in V δ 1 TILs inversely correlates with the expression of CD69 and several effector molecules such as NKG2D and CD56 (online supplemental figure 1C).

In addition, the surface levels of activating receptors NKG2D, CD56 and CD16 are similar between V δ 1 and V δ 2 TILs. On the other hand, V δ 2 TILs are characterized by the constitutive high expression of the inhibitory checkpoint NKG2A (figure 1E).^{25,30,31} We then assessed the prognostic values of V δ 1 TILs. In line with reported clinical data,³² we found that patients with CLM with less than four metastatic lesions had a significant longer OS compared with those individuals having four or more lesions (figure 1F). Importantly, higher frequencies of CD69⁺ V δ 1 TILs cells in CLM significantly correlate with lower numbers of metastatic lesions (figure 1G). We then combined flow cytometry data with the clinical outcome of patients with CLM stratified for high and low cut-offs of CD69⁺CD27⁻ and CD69⁺CD28⁻ V δ 1 effector T cells. Our results showed that high frequencies of both these CD69⁺ V δ 1 TIL subsets are associated with significantly longer OS, thus indicating their key role in limiting the metastatic progression of CRC to liver (figure 1H).

Transcriptional heterogeneity of $\gamma\delta$ TILs in CLM

To dissect the nature and functional relevance of $\gamma\delta$ TILs in the CLM microenvironment, we performed scRNA-seq analysis. To avoid sample selection bias, for scRNA-seq experiments we selected three representative patients (table 1) who underwent limited liver resection for synchronous CLM disease and were treated with standard combination na-CHT, prior surgery. PT and distal tumor-free (DT) $\gamma\delta$ TILs were performed starting from total CD3⁺ cells as described in online supplemental materials and methods. The unsupervised clustering obtained by performing dimensionality reduction based on uniform manifold approximation and projection (UMAP) identified eight clusters (c0–c7) among total $\gamma\delta$ T cells in PT and DT compartments (figure 2A). The analysis of TCR δ chain expression distinguishes three clusters of V δ 1 cells (c0, c3 and c4), while two cell clusters (c2 and c5) show a V δ 2-TCR repertoire. We also identified two additional clusters of cells (c1 and c6) expressing V δ 3 chain, thus confirming the presence of V δ 3 T cells in the human liver

(figure 2B).¹⁸ Cells lacking expression of *TRDV1-3* (c7) were excluded from further analysis. In line with our flow cytometry results (figure 1C,D), the relative enrichment analysis showed that V δ 1 TILs represent the dominant subset among all $\gamma\delta$ T cells in both PT and DT compartments (figure 2C). In particular, the cluster distribution in DT is characterized by the enrichment of clusters c0 and c4 (V δ 1 TILs) and c2 (V δ 2 TILs). On the other side, PT was mainly enriched in clusters belonging to V δ 1 (c0 and c3) and V δ 3 (c1) TILs (figure 2D). Regarding V γ chain recombination, V δ 1 TILs were mainly associated with V γ 3 and V γ 4, V δ 2 TILs with V γ 9 and V δ 3 TILs with V γ 5 chain (figure 2E).

We then analyzed the different transcriptional profiles of the seven $\gamma\delta$ TIL clusters identified in CLM (figure 2F, figure 3). The tissue tropism of all these clusters was confirmed by expression of tissue and liver residency markers *CD69* and *CXCR6*, respectively. However, lower transcript levels of *CXCR6* and *CD69* were observed in c4 (ie, V δ 1 T cells), thus likely indicating their re-circulation from PB. Consistently with our flow cytometry data, V δ 1 TILs (c0, c3, c4) and V δ 3 TILs (c1, c6) express a *CD27^{low}/CD28^{low}* phenotype. On the other hand, V δ 2 TILs (c2, c5) express higher transcript levels of *CD27*, *CD28* and *IL7R*, thus showing an immature phenotype. Of note, *CD27^{low}* V δ 2 TILs in c5 are characterized by the specific signature of previously reported type 3 V δ 2 T cells (*CCR6*, *RORC*, *IL23R*, *DPP4*).³³

Besides some transcriptional factors (TFs) such as *ZNF331* and *JUNB* that are widely expressed in all $\gamma\delta$ TIL clusters, we detected specific TF signatures among different $\gamma\delta$ T cell types. Indeed, V δ 2 (c2 and c5) and V δ 3 (c1 and c6) T cells are characterized by high transcript levels of *ZBTB16* (*PLZF*) and *EOMES*, respectively. Conversely, the expression of *IKZF2* together with the concomitant lack of *EOMES* defines V δ 1 T cells (c0, c3, c4). The expression of genes associated with inhibitory signaling and tumor-promoting tolerance also revealed specific signatures among $\gamma\delta$ TILs in CLM. Indeed, high transcriptional levels of inhibitory Killer-cell immunoglobulin-like receptors (*iKIRs*) are present on V δ 1 (c0, c3, c4) and V δ 3 (c1, c6) TILs, while the expressions of *NKG2A* (*KLRC1*) and *KLRG1* distinguish V δ 2 TILs. Regarding immune-checkpoints expression, we found low/negative expression of *PDCD1* (programmed cell death protein 1) and *CTLA4*, across all $\gamma\delta$ TIL clusters with the only exception of *TIGIT* that resulted highly expressed mainly on V δ 3 T cells (c1, c6).

In general, all seven identified $\gamma\delta$ TIL clusters express high levels of several cytotoxic molecules (*GZMA*, *GZMB*, *GZMH*, *GZMK*, *GZMM*, *PRF1* and *CTSW*) and the activating receptor *NKG7*. Specifically, V δ 1 TILs (c0, c3, c4) can be distinguished for their high transcript levels of *KLRC3* (*NKG2E*) and *NCR3* (*NKp30*), while the expression of *KLRB1* (*CD161*) characterizes V δ 2 (c2, c5) and V δ 3 (c1, c6) TILs in CLM. Taken together, these transcriptional profiles demonstrate the overall high cytotoxic potentials of intrahepatic TILs in CLM and make it possible

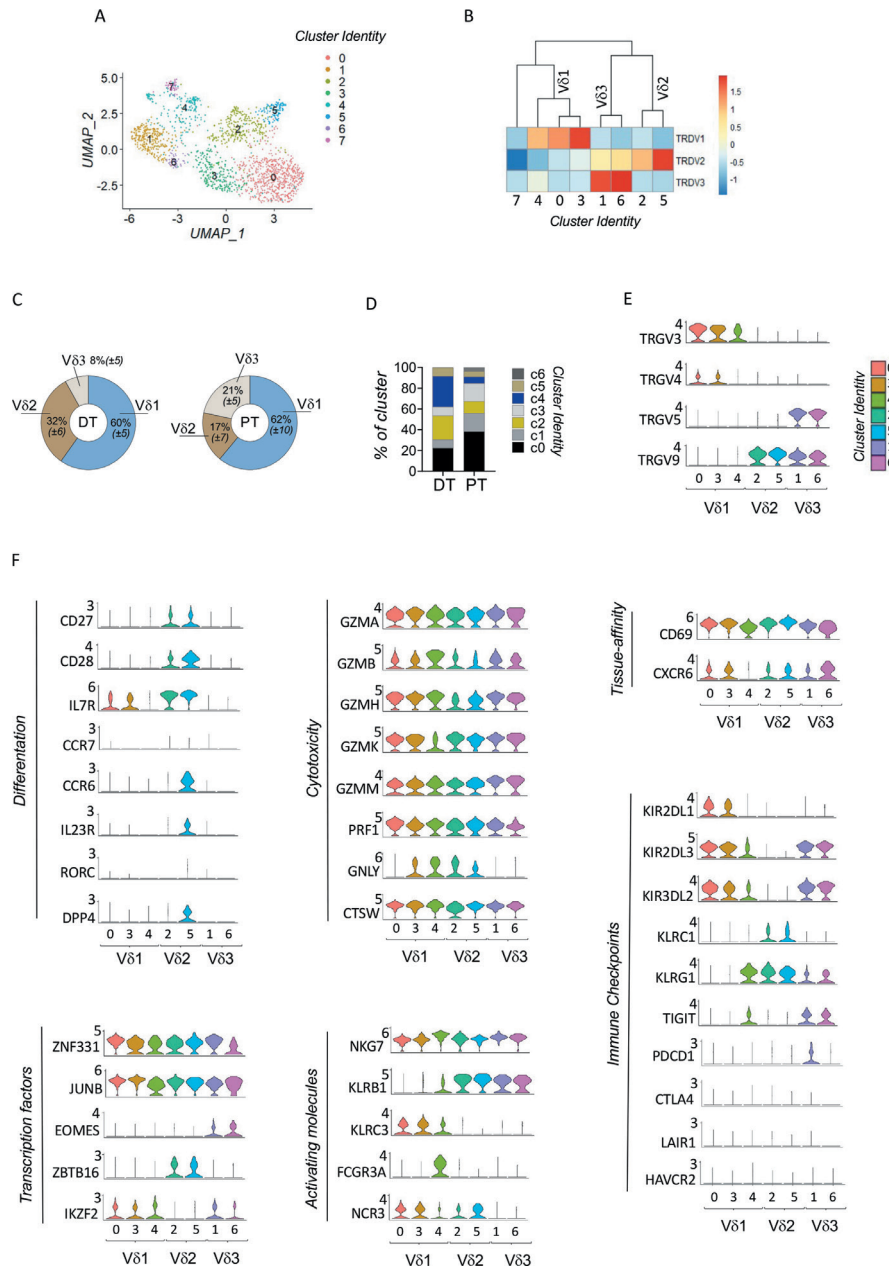


Figure 2 Single cell RNA-sequencing of gamma delta ($\gamma\delta$) tumor-infiltrating lymphocytes (TILs) in colon liver metastatic cancer (CLM). (A) Uniform manifold approximation and projection (UMAP) projection of $\gamma\delta$ T cells from peritumor (PT) ($n=1333$) and distal tumor-free (DT) ($n=424$) compartments of CLM from three patients underwent surgical liver resection. UMAP graph identifies eight specific clusters (left panel) and their distribution among DT (orange cells) and PT (light blue cells) compartments of CLM (right panel). (B) Heatmap showing the average of T cell receptor (TCR) δ chain expression within the eight $\gamma\delta$ T cell clusters identified by UMAP analysis. The expression values are zero-centered and scaled for each gene. (C) Pie charts showing the relative enrichment of V δ 1, V δ 2 and V δ 3 T cells (%) among DT (left panel) and PT (right panel) compartments of CLMs. (D) Bar plot graph showing the distribution (%) of $\gamma\delta$ T cell clusters among DT and PT compartments of CLM within the eight clusters identified from UMAP analysis. The proportion of $\gamma\delta$ T cells for each cluster across the DT and PT compartments were calculated as ratio between number of cells in each cluster and total number of cells in DT and PT, respectively. (E) Violin plot graph showing the expression of TRGV genes among the seven $\gamma\delta$ T cell clusters grouped according to their V δ 1, V δ 2 and V δ 3 T cell origin. (F) Violin plot graphs of selected genes among all $\gamma\delta$ T cell clusters grouped according to their V δ 1, V δ 2 and V δ 3 T cell origin. Each graph shows genes associated with cell differentiation status, transcription factors, tissue-affinity, cytotoxicity, inhibitory and activating molecules.

to define specific pathways and functional commitments among the three subsets of $\gamma\delta$ T cells infiltrating human liver during metastatic progression of CRC.

Functional commitments of $\gamma\delta$ TILs in CLM

The functional annotations of $\gamma\delta$ TILs were performed by Gene Ontology (GO) enrichment analysis on sc-RNA seq data from CLM specimens. We first observed that

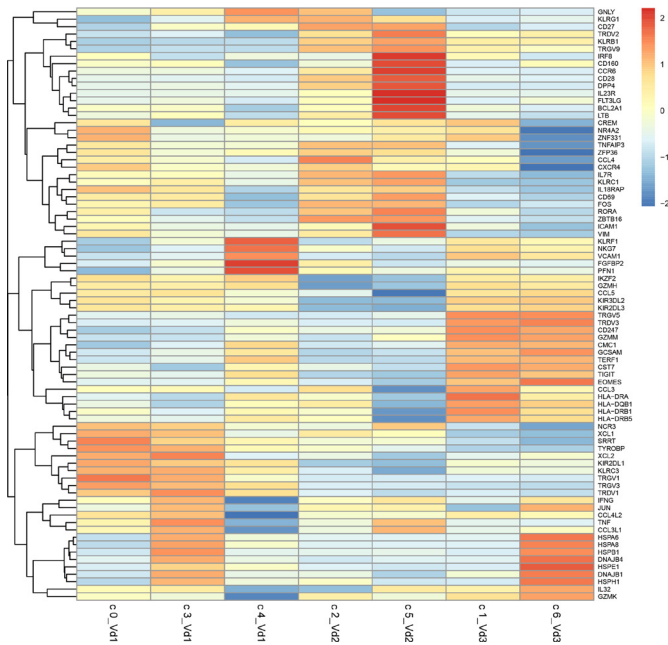


Figure 3 Differentially expressed genes among gamma delta ($\gamma\delta$) tumor-infiltrating lymphocyte (TIL) clusters in colon liver metastatic cancer (CLM). Heatmap showing the values of 238 differentially expressed genes (DEGs) (adjusted $p < 0.05$) coming from pairwise comparison between cluster 0 (c0) against each identified $\gamma\delta$ T cluster. Expression values are zero-centered and scaled for each gene.

all $\gamma\delta$ TILs share functional pathways highly associated with T cell activation, immune effector responses, leukocytes proliferation and regulation of cell-to-cell adhesion (figure 4A). The different ranges of functional annotations across specific $\gamma\delta$ TIL subsets are due to the diverse degrees of gene transcripts and their response/adaptation to tumor microenvironment. In this context, V δ 2 TILs can be distinguished for the enrichment in pathways associated with T cell differentiation and B cell activation. As expected,³³ type 3 V δ 2 TILs in c5 are specifically linked to the functional pathway of T helper 17 (Th-17)-IL-17-producing cells. However, we did not observe any IL-17A production. Instead, we found higher transcriptional level of *tumor necrosis factor* (*TNF*) among type 3 V δ 2 TILs (figures 3 and 4C).

On the other side, V δ 3 TILs are characterized by the high transcript levels of *HLA-DR* coded molecules associated with antigen processing and presentation. Moreover, V δ 3 TILs show high responsiveness to interferon-gamma (IFN- γ) in association with increased transcriptions of heat shock proteins (HSPs) that are, in turn, linked to biological pathways of protein stability and folding. These latter functional features mainly characterize c6 of V δ 3 TILs.

The GO pathways enriched at the highest levels among V δ 1 TILs encounter innate immune response, cytokine production and cell killing. In particular, V δ 1 TILs show great pro-inflammatory cytokine-secretion signature (*Cy-SI*) characterized by high expression of *IFN- γ* , *TNF* and *X-C motif chemokine ligand 2* (*XCL2*) (figure 4A–C and online

supplemental figure 2). Considering the positive prognostic impact of high frequencies of CD69⁺ V δ 1 TILs in the clinical outcomes of CRC and CLM (figure 1F–H),²⁰ we evaluated whether the functional annotations of this specific *Cy-SI* among V δ 1 TILs played any role in limiting the clinical progression of metastatic CRC. To do so, we searched for patients with CRC in The Cancer Genome Atlas (<https://cancergenome.nih.gov>) datasets by using the Gene Expression Profiling Interactive Analysis 2 (GEPIA2) web server. In the absence of available meta-data on a specific cohort of patients with CLM, we selected individuals affected by CRC at highest risk of liver metastases. These patients are characterized by low-frequency microsatellite instability or microsatellite stability and represent approximately 90% of all patients progressing toward CLM.^{34,35} Our results showed that the transcript levels of V δ 1 TILs in CRC tumor environment significantly correlate with the pro-inflammatory and antitumor *Cy-SI* (figure 4D). Importantly, higher *V δ 1-Cy-SI* predicts better disease-free survival in these patients. Moreover, we also found that patients with CRC ranked with a higher *V δ 1 (TRDV1)-Cy-SI* are associated with significantly longer OS at different cut-off values (figure 4E,F).

Distribution and transcriptional signatures of circulating and tumor-infiltrating $\gamma\delta$ T cells from patients with CLM

We then performed an integrated scRNA-seq analysis to compare the distribution and transcriptional signatures of $\gamma\delta$ T cells from matched PB and tumor specimens collected from patients undergone surgical resection of CLM. The differential expression of *TRDV* transcripts identified six clusters (c0–c5) of $\gamma\delta$ T cells showing different repertoires of V γ chains. We then excluded c4 from our further analyses, due to its low expression of δ and γ chains, respectively (figure 5A,B, online supplemental figure 3A).

The distribution of the different $\gamma\delta$ T cell clusters within PB and CLM tissue specimens shows that c1, c2 and c3 are present in both anatomic compartments, while c0 and c5 resemble respectively two V δ 1 and V δ 3 T cell subsets infiltrating the tumor mass and retained in situ (figure 5C). We observed that c1 comprises high frequencies of PB V δ 3 T cells, a subset of $\gamma\delta$ T cells that had been previously reported to be preferentially enriched in liver tissues under homeostatic conditions.¹⁸ Interestingly, the comparison of DEGs between PT-V δ 3 and PB-V δ 3 T cells in c1 revealed that the highest cytotoxic potential is held by the circulating ones displaying increased levels of *GNLY*, *GZMB*, *PRF1* and of the activating receptors *NKG7*, *NCR3* and *FCGR3A*. For more accurate immunophenotyping in CLM, we compared different $\gamma\delta$ T cell subsets in relation to their *CD27^{high/low}* transcriptional pattern. All V δ 3 T cells share *CD27^{low}* transcriptional profile of mature T cells (figure 5D). In addition, as a peculiarity of V δ 3 T cells, their *CD27^{low}* profile in the PB correlates with high expression of *CX3CR1*. The specific role of this marker in $\gamma\delta$ T cells is still unknown. However, it has been recently

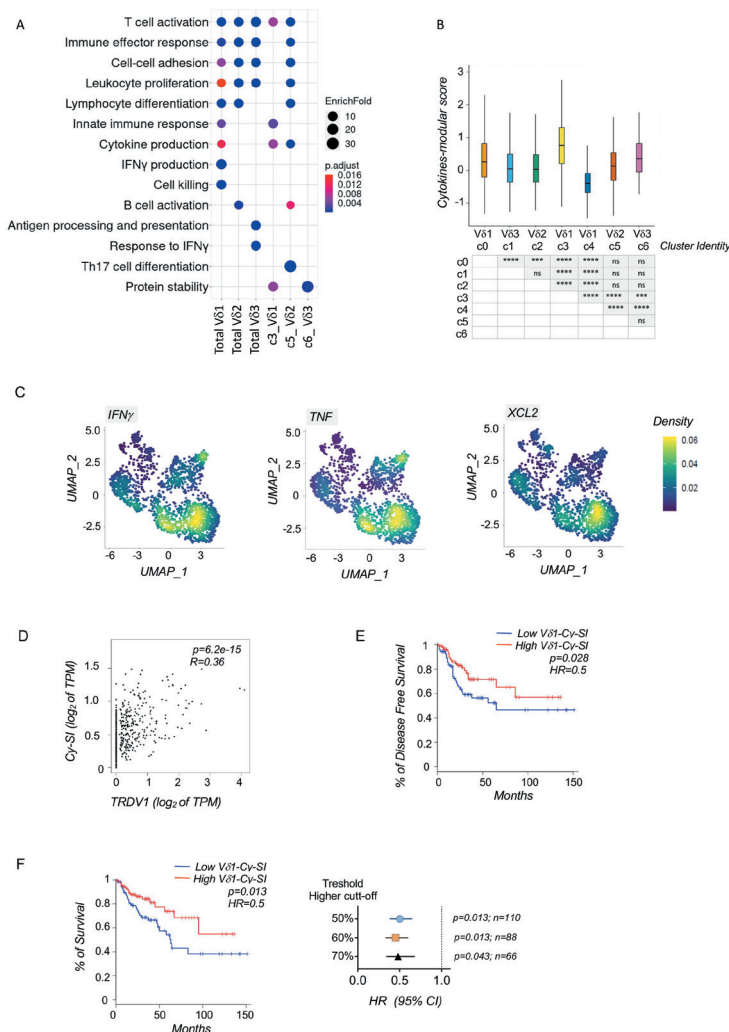


Figure 4 Functional annotations of gamma delta ($\gamma\delta$) tumor-infiltrating lymphocytes (TILs) in colon liver metastatic cancer (CLM). (A) Statistical dot plot graph showing the biological processes (BO) obtained by Gene Ontology (GO) enrichment analysis calculated for total $V\delta 1$, $V\delta 2$, $V\delta 3$ TILs and for the clusters c3 of $V\delta 1$, c5 of $V\delta 2$ and c6 of $V\delta 3$ TIL subsets. GO enrichment analyses are performed by using differentially expressed genes (DEGs) with adjusted $p \leq 0.05$ and log-foldchange > 0 . Only enriched GO terms with adjusted $p \leq 0.05$ and more than five genes are reported. (B) Statistical bar plot graph showing *interferon-gamma* (*IFN- γ*), *tumor necrosis factor* (*TNF*) and *X-C motif chemokine ligand 2* (*XCL2*) cytokine modular score calculated for each $\gamma\delta$ T cell clusters. (C) Uniform manifold approximation and projection (UMAP) density plot graphs showing *IFN- γ* , *TNF* and *XCL2* gene density distribution among $\gamma\delta$ TIL cell clusters. (D) Scatter dot plot distribution with Pearson's correlation between *TRDV1* and *V $\delta 1$ -Cy-SI* expression in The Cancer Genome Atlas (TCGA) and The Genotype-Tissue Expression (GTEx) cohorts of patients with CRC calculated by Gene Expression Profiling Interactive Analysis 2 (GEPIA2). (E) Kaplan-Meier curve showing postoperative disease-free survival (%) ranking in TCGA cohorts of low-frequency microsatellite instability (MSI-L) and microsatellite stability (MSS) patients with CLM stratified by the low (blue curve) or high (red curve) *V $\delta 1$ -Cy-SI* expression (HR 0.5; median high cut-off 50%–50%; $n=110$). (F) Kaplan-Meier curve (left panel) showing the OS (%) in TCGA cohorts of MSI-L and MSS patients with CLM stratified by the low (blue curve) or high (red curve) *V $\delta 1$ -Cy-SI* ranking (HR 0.5; median 50%–50% high cut-off; $n=110$). Forest plots (right panel) showing HR with 95% CI, p value (p) and number (n) of patients obtained for different high *V $\delta 1$ -Cy-SI* cut-off values in TCGA cohorts of MSI-L and MSS patients with CRC ($n=110$). Dashed line at HR=1 indicates the numerical distance from no survival benefit. The cox proportional HR of the *V $\delta 1$ -Cy-SI* high and low-expression cohort was calculated by GEPIA2, while 95% CI was calculated as 'exp [ln(HR) \pm z \times SE], with SE'.⁵¹ Statistically significant p values are represented with the following number of asterisks (*): * $p \leq 0.05$; *** $p \leq 0.001$; **** $p \leq 0.0001$; ns, not statistically significant.

proposed as a marker of differentiated and long-lived tissue resident T cells (figure 5D,E).³⁶

Previous studies reported that the release of $V\delta 3$ T cells in the bloodstream occurs preferentially in the presence of pathological conditions, including liver cancers.^{37,38}

In this context, our data confirmed this phenomenon by showing that terminally differentiated $V\delta 3$ T cells can egress from the liver to PB also in patients with CLM. Moreover, $V\delta 3$ T in c5 can be easily discriminated due to their high transcript level of *HSPs*, thus confirming

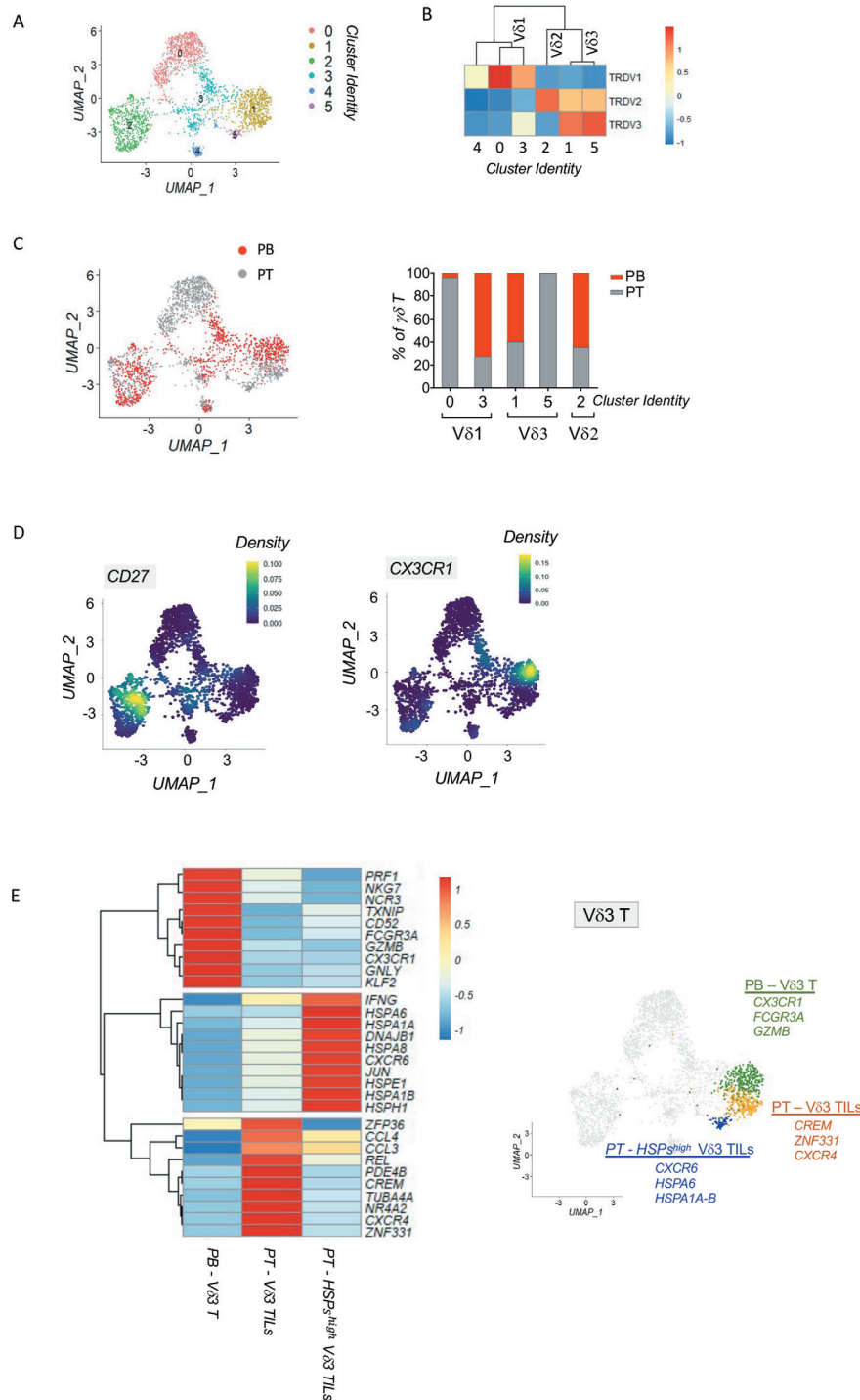


Figure 5 Single cell RNA-sequencing (scRNA-seq) integrated analysis comparing the distribution of circulating and tumor-infiltrating gamma delta ($\gamma\delta$) T cells and transcriptional profiles of V δ 3 T cells from patients with colon liver metastatic cancer (CLM). (A) Uniform manifold approximation and projection (UMAP) graph showing the $\gamma\delta$ T clusters from cells purified from matched peritumoral (PT) and peripheral blood (PB) samples of three patients with CLM undergoing surgical resection of tumors. (B) Heatmap showing the average of T cell receptor (TCR) δ chain expression along the six identified $\gamma\delta$ T cell clusters showed in panel A. (C) UMAP graph (left panel) and bar plot (right panel) showing the distribution (%) of the $\gamma\delta$ T cell clusters within PT (gray) and PB (red) anatomic compartments. (D) UMAP density plot graphs showing *CD27* and *CX3CR1* gene density distribution among $\gamma\delta$ T cell clusters. (E) Heatmap showing the top 30 differently expressed genes (DEGs) (adjusted $p < 0.05$) between PB and PT V δ 3 T cells in patients with CLM (left panel). Representative UMAP graph showing the distinct molecular signatures of PB and PT V δ 3 T cells (right panel).

their liver-identity in the context of an immune response within the CLM microenvironment.

We then analyzed the transcriptional signatures of V δ 1 and V δ 2 TILs that are characterized by higher degrees of activation (eg, *FOS*, *FOSB*, *JUN*, *JUNB-D*, *KLF6*) and increased transcription of chemokine/cytokine transcripts compared with their PB counterparts. Moreover, as already shown in several cancer settings,³⁹ V δ 1 and V δ 2 TILs express decreased levels of mitochondrial (mt) DNA-encoded genes (*MT-CYB*, *MT-ATP6-8*, *MT-ND1-6*, *MT-COI-3*) compared with their circulating homologs (online supplemental figure 3B,C). A more in-depth analysis revealed that among the V δ 2 compartment in c2, there is a population of approximately 40% of cells able to infiltrate CLM (figure 5C). c2 is composed of both less mature *CD27^{high}* and more differentiated *CD27^{low}* V δ 2 T cells. These latter two populations show distinct transcriptional signatures, are mainly enriched in the PB compartment and share a similar capacity to infiltrate the tumor. The third subset comprised in c2 is composed by type 3 V δ 2 cells, a population that had been previously reported of being able to polarize into Th-17.³³ These latter lymphocytes are mainly enriched within the PT compartments of CLM and express a distinct transcriptional signature, thus being tightly associated with tumor-associated activation within the CLM microenvironment (figure 5D, figure 6A,B).³⁶

V δ 1 T cells are contained within c0 and c3 and all have in common an effector *CD27^{low}* signature. While V δ 1 T cells in c0 show a high tissue-affinity resembling their liver residency, V δ 1 T cells in c3 are mainly enriched within the PB compartment (figure 5C,D). Our in-depth integrated analyses identified two subsets of circulating V δ 1 T cells showing different transcription levels of CD69. In particular, PB *CD69^{high}* V δ 1 T cells express a 'liver transcriptional signature' characterized by higher expressions of *CXCR6*, *CREM*, *RGS1*, *ZNF331*, *SRRT* and *GZMK* compared with PB *CD69^{low}* V δ 1 T cells. In addition, PB *CD69^{high}* V δ 1 T cells show an activated profile (eg, increased levels of *XCL2*, *JUNB*, *ZFP36*, *DUSP4* and *FYN*) and lower expression of (mt)DNA-encoded genes (*MT-ND4-6*, *MT-CO2*, *MT-ATP6*) compared with PB *CD69^{low}* V δ 1 T cells (figure 6C,D), thus further confirming their liver tropism. Taken together, our integrated analyses strongly suggest that the subset of *CD69⁺* V δ 1 T cells in CLM has the capacity to egress the liver and migrate to PB.

Circulating *CD69⁺* T_{EMRA} V δ 1 cells share similar phenotypic and TCR repertoires with their intrahepatic counterparts

Considering that V δ 1 TILs represent the main $\gamma\delta$ T cell subset enriched in CLM specimens (figure 1C) and given the similarities in the transcriptomic signatures of *CD69⁺* V δ 1 T cells in PB and tumor mass (figure 6C,D), we proceeded to assess the frequencies, phenotypes and TCR repertoires of circulating V δ 1 T cells in patients with CLM. Multiparametric flow cytometry results showed that the frequencies of PB V δ 1 T cells increase in patients with CLM compared with HDs. In particular,

we found significantly higher percentages of T_{EMRA} V δ 1 cells in the PB of patients with CLM compared with HDs (figure 7A,B). Interestingly, circulating V δ 1 T cells from patients with CLM express significantly higher surface levels of CD69 compared with their counterparts in HDs. This phenomenon is mainly due to the expansion of PB *CD69⁺* T_{EMRA} V δ 1 T cells that account up to 70% of total V δ 1 T_{EMRA} cells in CLM (figure 7C,D). Moreover, the high frequencies of PB *CD69⁺* T_{EMRA} V δ 1 T cells significantly correlate with the enrichment of their cellular homologs in the tumor mass of the same patients with CLM (figure 7E).

We previously reported that toxicity of na-CHT administered prior surgery to elder patients with CLM increases circulating V δ 2 T cells with highly differentiated T_{EMRA} phenotype (online supplemental figure 4A).²⁴ To assess whether a similar phenomenon occurs also for PB V δ 1 T cells, we divided our cohort in two subgroups either undergone or not to conventional na-CHT and immunotherapies (table 1). Surprisingly, we did not observe any significant differences, in terms of frequencies and phenotype of circulating V δ 1 T cells between the two subgroups of patients with CLM (figure 7B and online supplemental figure 4B). Likewise, different biological agents used in combination with na-CHT (table 1) showed no effect on the frequencies of PB *CD69⁺* T_{EMRA} V δ 1 cells (figure 7F). Importantly, our data also showed that the frequencies of *CD69⁺* T_{EMRA} V δ 1 cells are independent from age, sex and human cytomegalovirus status of the enrolled patients with CLM (online supplemental figure 4D,E).^{24 37 40} Even more interestingly, similar to *CD69⁺* V δ 1 TILs in the liver, the expression of CD69 in PB V δ 1 T cells of patients with CLM displays phenotype which correlates with the expression of CD56 and CD161 and inversely associates with CD28 and CD57 in the entire patients with CLM (figure 7G). Indeed, *t*-distributed Stochastic Neighbor Embedding (*t*-SNE) analysis shows striking phenotyping overlap of PB *CD69⁺* V δ 1 T_{EMRA} phenotype with the expression of CD56 and CD161 and low expression of CD28 (online supplemental figure 4F,G).

We then compared the TCR repertoires of FACS-sorted *CD69⁻* and *CD69⁺* V δ 1 TILs with the ones of their PB homologs from the same patients with CLM. To this end, we performed high-throughput sequencing of V(D)J regions that paired the V δ 1 chain with different γ chains (online supplemental figure 4H,I). Although analyses of cell clonality revealed high interchanges of V δ 1 T cell clones between PB and CLM, PB *CD69⁺* V δ 1 T cells share a significantly higher number of V δ 1 clones with their CLM counterparts when compared with PB *CD69⁻* V δ 1 T cells (figure 7H). These data demonstrate that PB *CD69⁺* V δ 1 T cells present in the blood of patients with CLM include V δ 1 TIL clones egressing from tumor and keeping their high expression of the tissue-residency marker CD69 within the circulating T_{EMRA} compartment.

In a more clinical context, we then observed that higher frequencies of PB *CD69⁺* *CD28⁻* V δ 1 T cells are associated

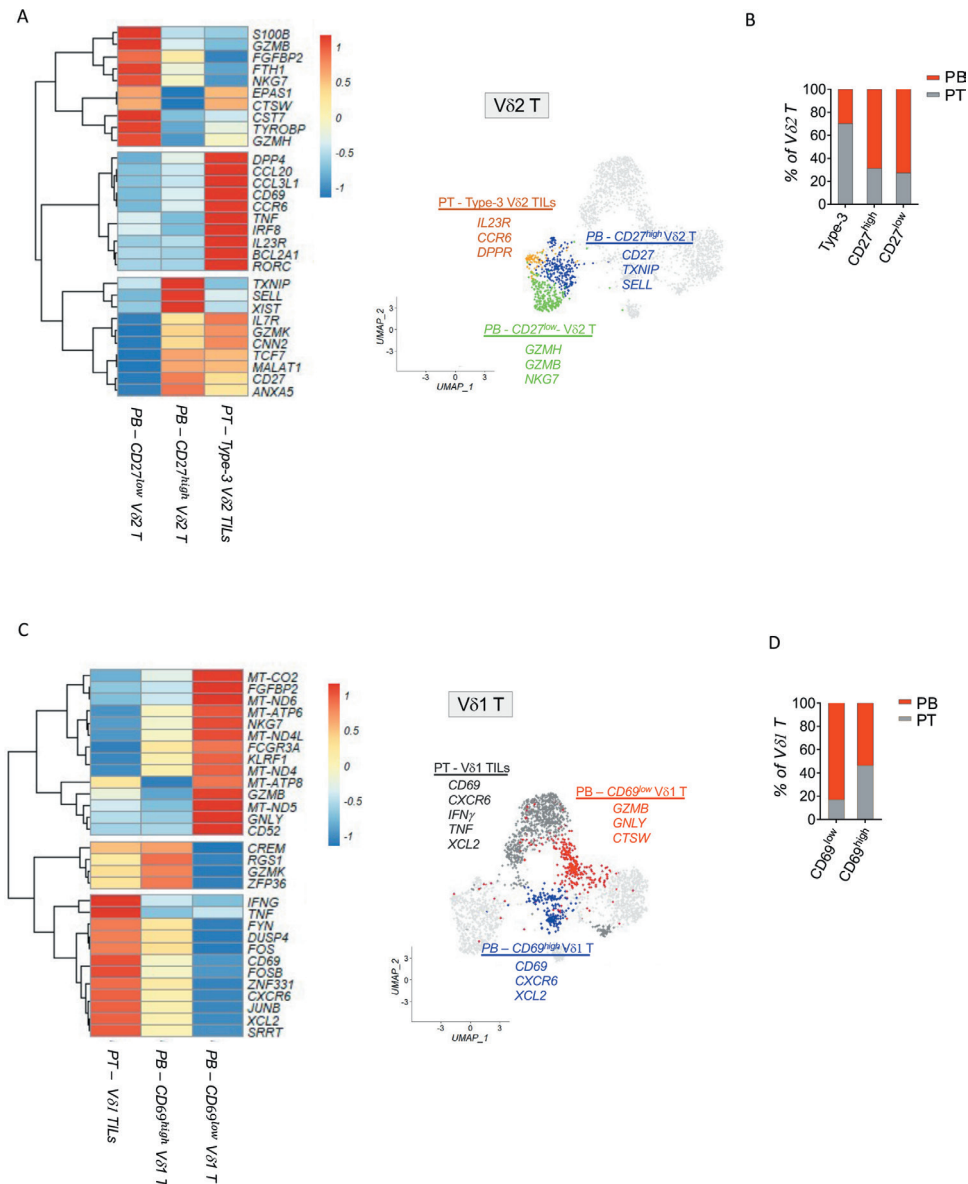


Figure 6 Single cell RNA-sequencing (scRNA-seq) integrated analysis comparing the transcriptional profiles of circulating and tumor infiltrating Vδ1 and Vδ2 T cells from patients with colon liver metastatic cancer (CLM). (A) Heatmap showing the top 30 differently expressed genes (DEGs) (adjusted $p < 0.05$) between peripheral blood (PB) and peritumoral (PT) Vδ2 T cells from patients with CLM (left panel). Representative uniform manifold approximation and projection (UMAP) graph showing the distinct molecular signatures of PT-enriched type 3, PB CD27^{high} and CD27^{low} Vδ2 T cells (right panel). (B) Bar plot graph showing the distribution (%) of type 3, CD27^{high} and CD27^{low} Vδ2 T cells in PB (red) and PT (gray) anatomic compartments from patients with CLM. (C) Heatmap showing the top 30 DEGs (adjusted $p < 0.05$) between PT and PB Vδ1 T cells from patients with CLM (left panel). Representative UMAP graph showing the distinct molecular signatures of the PT and PB CD69^{high} and CD69^{low} Vδ1 T cells subsets in patients with CLM (right panel). (D) Bar plot graph showing the distribution (%) of the CD69^{high} and CD69^{low} Vδ1 T cells in PB (red) and PT (gray) anatomic compartments from patients with CLM.

with longer OS of patients with CLM (figure 7I). These latter findings mirror the positive prognostic impact on OS of higher frequencies of CD69⁺CD28⁻ Vδ1 TILs in CLM specimens detected at the time of surgery (figure 1H).

DISCUSSION

The present study reveals the heterogeneous distribution, phenotypes, functional relevance and prognostic values of $\gamma\delta$ T cells either infiltrating CLM or re-circulating

in PB. Our results showed that CLM tissue specimens comprise all main subsets of $\gamma\delta$ TILs including Vδ3 T cells, a subset naturally resident in healthy human liver without egressing the organ under physiological conditions.^{17,18} scRNA-seq results identified seven $\gamma\delta$ TIL clusters enriched in CLM specimens and expressing distinct transcriptional signatures and functional annotations. In particular, we found that CLM microenvironment is enriched in type 3 Vδ2 T cells, a subset known for

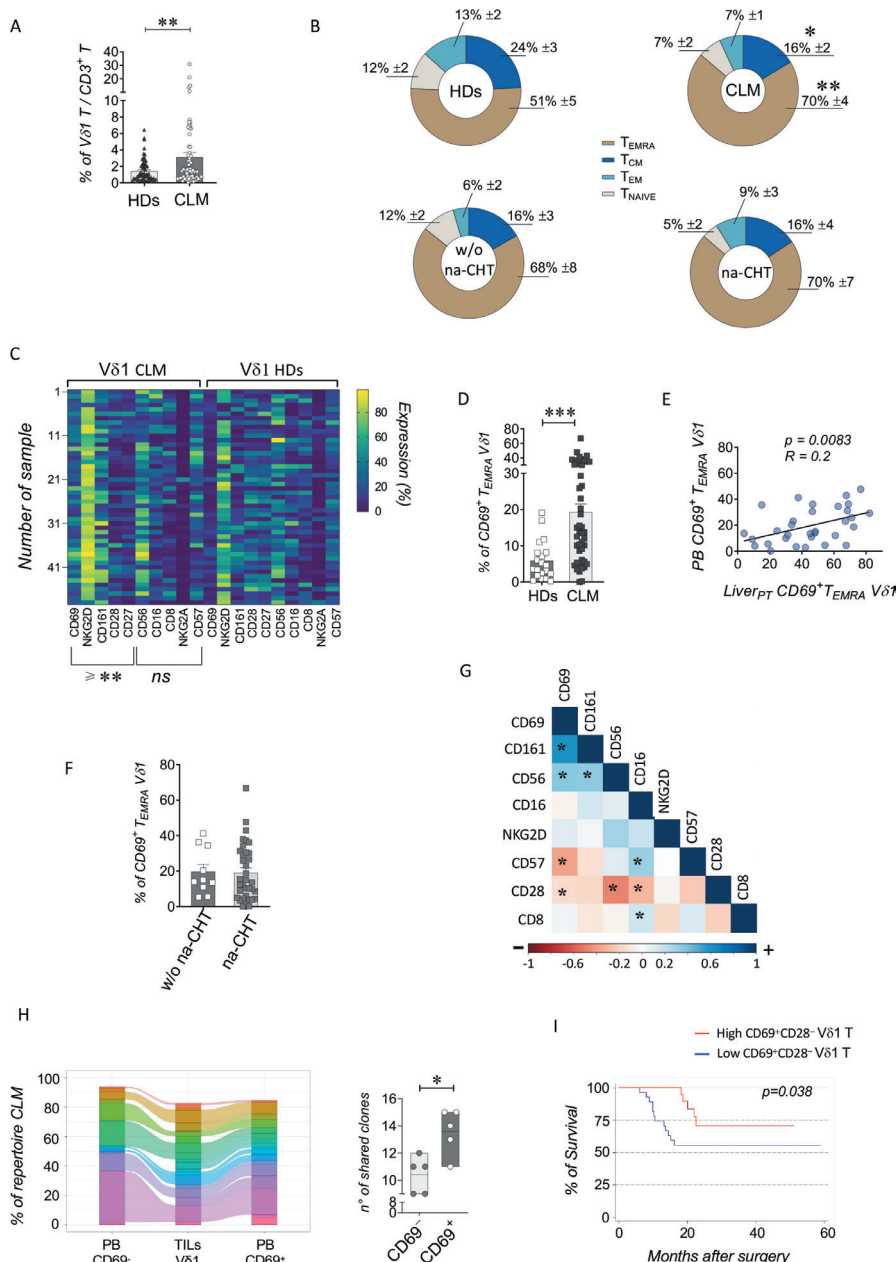


Figure 7 Identification of intrahepatic V δ 1 tumor-infiltrating lymphocytes (TILs) egressing tumor and re-circulating in peripheral blood (PB) of patients with colon liver metastatic cancer (CLM). (A) Statistical bar graph showing the mean (\pm SEM) frequency (%) of PB V δ 1 T cells among CD3⁺ T lymphocytes in health donors (HDs) (n=66) and patients with CLM (n=75). (B) Pie charts showing the mean frequency distribution (%) of PB naive T (T_{NAIVE}), T central memory (T_{CM}), T effector memory (T_{EM}) and terminally differentiated (T_{EMRA}) V δ 1 T cells in HDs (n=33) and in patients with CLM (n=50) either in the absence (n=13) (lower left) or in the presence (n=38) (lower right) of neoadjuvant conventional/biological chemotherapies (na-CHT) (lower panel). (C) Heatmaps of multiparametric flow cytometry data showing the expression of several surface markers between PB V δ 1 T cells isolated from patients with CLM (left) and HDs (n=49) (right). Group of markers which statistically differ between two groups are annotated under the heatmap as “ \geq ” and indicates the range of significant p values; ‘ns’ indicates a group of markers not statistically significant. (D) Statistical bar graph showing the mean (\pm SEM) frequency (%) of PB CD69⁺ T_{EMRA} V δ 1 cell subset in HDs (n=22) and patients with CLM (n=45). (E) Pearson's correlation of flow cytometry percentages (%) between matched PB CD69⁺ T_{EMRA} and PT CD69⁺ T_{EMRA} V δ 1 TILs (n=45). (F) Statistical bar graph showing the mean (\pm SEM) frequency (%) of PB CD69⁺ T_{EMRA} V δ 1 cells from patients with CLM either in the absence (n=11) or in the presence (n=45) of na-CHT. (G) Heatmap of flow cytometry data showing Spearman's rank between the mean of different cell markers expression (%) in PB V δ 1 T cells from patients with CLM (n=49). (H) Overlap analysis of T cell receptor (TCR) repertoires from FACS-sorted PB CD69⁺ and CD69⁻ and liver PT V δ 1 T cells in patients with CLM (n=5). Shared top 20 TRG clones of one representative patient with CLM are represented as colored bands between columns (left panel) and included in the summary statistic (right plot). (I) Kaplan-Meier curve showing postoperative OS (%) of patients with CLM stratified on the basis of the median frequency (%) of the PB CD69⁺CD28⁻ V δ 1 T cells in patients with CLM (cut-off \leq 10%; n=53). Statistically significant p values are represented with the following number of asterisks (*): * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns, not statistically significant.

their high plasticity and potential to polarize into pro-inflammatory $\gamma\delta$ Th-17 cells.^{33,40} Although $\gamma\delta$ Th-17 cells have been reported to promote cancer development,⁴¹ secretion of IL-17A by human V δ 2 T cells is still a matter of discussion. In our study, we did not observe any *IL-17A* production at transcriptional level that, instead, revealed a preferential production of TNF- α by type 3 V δ 2 TILs. Hence, additional investigations are required to disclose the impact of type 3 V δ 2 T cells in the natural history of CLM. Our findings also disclosed the molecular signature of mature cytotoxic V δ 3 T cells, whose activation and response to CLM is associated with a strong stress-*HSP*-induced transcriptional profile. The integrated analyses of scRNA-seq data from matched PB and CLM specimens also revealed the capacity of intrahepatic V δ 1 and V δ 3 TIL clusters to egress tumor and re-circulate in PB.

V δ 1 T cells represent the largest fraction of $\gamma\delta$ TILs enriched in CLM and highly impact the clinical outcomes of these patients. Indeed, we demonstrate here that liver CD69⁺ V δ 1 T cells highly infiltrate CLM and limit the metastatic progression of CRC to liver. In specific, our data show an inverse correlation between the percentage of T_{EF} CD69⁺ V δ 1 TILs and the number of metastatic lesions in the liver that, in turn, predicts longer patients' OS after surgery. These findings indicate the existence of local CD69⁺ V δ 1 TIL-mediated antitumor response at tissue sites. In line with recently reported data during liver inflammatory diseases,¹⁸ these anticancer activities in patients with CLM are exerted by two subsets of the liver CD69⁺ T_{EF} V δ 1 cell: the T_{EM} V δ 1 cells that reside permanently in the liver tissue and the T_{EMRA} V δ 1 cells that can re-circulate toward PB.

The ability of V δ 1 TILs to control CRC progression is further confirmed by their strong pro-inflammatory *Cy-SI* characterized by high expression of *IFN- γ* , *TNF* and *XCL2* compared with other $\gamma\delta$ TIL subsets. Certainly, future studies are necessary to evaluate whether these properties of V δ 1 TILs can be translated to the CLM clinic, nevertheless, our data support a model in which V δ 1 TILs create large cytokine field able to penetrate tumor and whose concentrations depend on the collective V δ 1 T cell activation. In this context, recently it was observed that while outset of IFN- γ secretion requires local T cell response, prolonged activation diffuses IFN- γ to distant areas and extensively alters the tumor phenotype.⁴²

CD69 is a C-type lectin receptor widely used to track immune cell activation and tissue-resident T lymphocytes that persist throughout adult life. Indeed, after entering tissues, all T lymphocytes upregulate expression of CD69 which, in turn, promotes cell retention into peripheral organs.²⁹ The expression of CD69 on tissue T lymphocytes is constitutive and not associated with degrees of activation as its surface levels do not correlate with the ones of activation markers such as CD25 or CD38.⁴³ However, the functional relevance of CD69 on immune cells at tissue sites is still being discussed.²⁹ We characterize here a distinct subset of CD69⁺ T_{EMRA} V δ 1 TILs that can egress

from CLM to re-circulate in the bloodstream where it retains a phenotypic/transcriptional profiles resembling its liver tropism and origin. In this regard, all $\gamma\delta$ T cells residing in tissues constitutively express CD69 together with specific repertoires of homing receptors different from those of their circulating counterparts.^{18,44,45} The re-circulation of V δ 1 TILs in PB of the same patients with CLM is further confirmed by TCR sequencing experiments showing that these two anatomic compartments share high frequencies of identical CD69⁺ V δ 1 T clones. In addition, scRNA-seq analysis identified in the PB of patients with CLM the existence of two CD27^{low} V δ 1 T cells with distinct transcriptional profiles that include high expression levels of CD69. Hence, the ability of intrahepatic CD69⁺ V δ 1 TILs to migrate toward PB defines de facto a novel liver-blood barrier in CLM. The mechanisms regulating the re-circulation of intrahepatic CD69⁺ T_{EMRA} TILs in patients with CLM are unclear. Moreover, the extent of clonal exchange between PB-PT compartments should be further investigated for V δ 3 T cells that were detected at high frequencies in the PB of patients with CLM. The inducible expression of CX3CR1 might contribute to explain the egressing of V δ 3 TILs in CLM considering that CX3CR1⁺CD8⁺ T cells were recently identified as tissue peripheral memory cells with unique migratory and self-renewal properties.³⁶ In addition, expression of CX3CR1 was recently proposed as a blood-based T-cell biomarker in response to cancer treatment.⁴⁶ Interestingly, it has been reported that skin-resident CD4⁺ T cells with a distinct memory tissue-signature can egress to PB even under homeostatic conditions.⁴⁷ Therefore, further investigations are required to better understand the homing of long-term persistent liver V δ 1 and V δ 3 T cells.

Higher frequencies of both PB and TI CD69⁺ T_{EMRA} V δ 1 cells predict a longer OS of patients with CLM. This phenomenon represents a potent and reliable prognostic marker immediately transferable to everyday clinical practice through the implementation of liquid biopsies without the need to always analyze liver tissue. Our gained knowledge can remarkably improve our capacity to predict tumor progression or response to therapies. Indeed, the identification of novel prognostic biomarkers in CLM is hampered by the fact that the majority of patients receive na-CHT. In this regard, the toxic side effects of these therapeutic regimes induce cell death and induce proliferative-senescence, as we observed in V δ 2 T cells.²⁴ This is not the case of V δ 1 T cells. Indeed, despite the many na-CHT options available in CLM, the frequency and the phenotype of mature PB V δ 1 cells are not altered or affected by drug toxicity. This implies that circulating CD28⁻CD69⁺ V δ 1 cells are valuable prognostic markers predicting CLM clinical outcome regardless of the administration of na-CHT.

Considering the adaptive nature of human V δ 1 T cells,⁴⁸ the identification of specific clones of circulating antitumor V δ 1 T cells is also key for developing new protocols of adoptive cell transfer therapies. Up to date, the

vast majority of currently active clinical trials relies on the transfer of *in vitro* expanded V δ 2 T cells or polyclonal $\gamma\delta$ T cells.^{10,11} CD69⁺ T_{EF} V δ 1 cells represent the perfect target for setting novel protocols of cellular immunotherapy in CLM, as this subset of lymphocytes egresses from tumor sites, is endowed with high antitumor potential, is present at high frequencies both in PB of patients with CLM and is expandable *in vitro*.^{49,50}

CONCLUSION

The tumor microenvironment of CLM is highly enriched in a heterogeneous infiltrate of $\gamma\delta$ TIL subsets with potent antitumor potentials. The present study identifies a specific subset of liver CD69⁺ V δ 1 T_{EMRA} cell able to egress and re-circulate in the PB. The higher are its frequencies both at tissue site and in PB, the longer is patients' OS regardless of na-CHT administered to patients with CLM. Taken together, these findings demonstrate that CD69⁺ V δ 1 T_{EF} cells in CLM specimens limit the metastatic progression of CRC to liver and can also serve as a reliable prognostic marker in 'liquid biopsies' to identify those patients with better clinical outcomes.

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Contributors EB performed experiments, analyzed data and wrote the manuscript; MMC recruited patients, provided reagents/specimen and analyzed clinical data; MD and GT recruited patients and provided reagents/specimen; RC, ST and RP performed scRNA-seq analysis; SR and IP designed and performed $\gamma\delta$ TCR-seq analysis; VC and PM performed experiments; PK, ST and CG provided assistance with the scRNA-seq experiments; CS and BF performed immunohistochemistry study; FSC provided assistance with flow cytometry and cell sorting; CG and AM provided reagents and assistance in the manuscript's preparation; JM and DM directed and designed the study, analyzed data and wrote the manuscript. J.M and D.M are the guarantors of this study.

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Competing interests None declared.

Patient consent for publication Consent obtained directly from patient(s)

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