

SHORT REPORT

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# Chemotherapy accelerates immune-senescence and functional impairments of $V\delta 2^{POS}$ T cells in elderly patients affected by liver metastatic colorectal cancer

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## Abstract

Human (gamma delta)  $\gamma\delta$  T cells are unconventional innate-like lymphocytes displaying a broad array of anti-tumor activities with promising perspectives in cancer immunotherapy. In this context,  $V\delta 2^{POS}$  T cells represent the preferential target of several immunotherapy protocols against solid tumors. However, the impact of both aging and chemotherapy (CHT) on  $V\delta 2^{POS}$  T cells is still unknown. The present study evaluates with multi-parametric flow cytometry the frequencies, terminal differentiation, senescence and effector-functions of peripheral blood and tumor infiltrating  $V\delta 2^{POS}$  T cells purified from liver metastases (CLM) of patients affected by colorectal cancer (CRC) compared to those of sex- and age-matched healthy donors. The peripheral blood of CLM patients underwent CHT is characterized by decreased amounts of  $V\delta 2^{POS}$  T cells showing a relative increase of terminally-differentiated  $CD27^{neg}/CD45RA^{POS}$  ( $T_{EMRA}$ ) cells. The enrichment of this latter subset is associated with an increased expression of the senescent marker CD57. The acquisition of CD57 on  $T_{EMRA}$   $V\delta 2^{POS}$  T cells is also coupled with impairments in cytotoxicity and production of TNF- $\alpha$  and IFN- $\gamma$ . These features resemble the acquisition of an immune-senescent profile by  $V\delta 2^{POS}$  T cells from CLM patients that received CHT, a phenomenon that is also associated with the loss of the co-stimulatory marker CD28 and with the induced expression of CD16. The group of CLM patients underwent CHT and older than 60 years old showed higher frequencies of  $CD57^{POS}$  and  $T_{EMRA}$   $V\delta 2^{POS}$  T cells. Similar results were found for tumor infiltrating  $V\delta 2^{POS}$  T cell subset purified from CLM specimens of patients treated with CHT. The toxicity of CHT regimens also affects the homeostasis of  $V\delta 2^{POS}$  T cells by inducing higher frequencies of circulating  $CD57^{POS}$   $T_{EMRA}$  subset in CLM underwent CHT and younger than 60 years old. Taken together, our data demonstrate that the enrichment of senescent  $V\delta 2^{POS}$  T cells in CLM patients is not only induced by patients' aging but also by the toxicity of CHT that further accelerates the accumulation of  $CD57^{POS}$   $T_{EMRA}$  cells highly dysfunctional in their anti-tumor activities. These results are important to both predict the clinical outcome of CLM and to optimize those protocols of cell cancer immunotherapy employing unconventional  $V\delta 2^{POS}$  T cells.

**Keywords:**  $\gamma\delta$  T cells, Immune-senescence/Aging, Cancer, Chemotherapy

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## Introduction

Human  $\gamma\delta$  T lymphocytes are divided in the two main  $V\delta 1^{POS}$  and  $V\delta 2^{POS}$  subsets on the basis of their TCR $\delta$ -chain repertoire. While  $V\delta 1^{POS}$  cells preferentially localize in mucosal tissues and skin,  $V\delta 2^{POS}$  T cells are mainly enriched in peripheral blood (PB) where they represent about 5% of all circulating T cells. The activation of  $V\delta 2^{POS}$  T cells relies on the recognition of non-peptidic compounds (i.e. microbial or stress- or tumor-induced “phosphoantigens”) in association with butyrophilin 3A1 (BTN3A1 also known as CD277). Besides the TCR interactions with phosphoantigens/BTN3A1 complexes, several Natural Killer Receptors (NKR) are involved in triggering the anti-tumor functions of  $V\delta 2^{POS}$  T cells, with the C-lectin type NKG2D playing a major role [1, 2]. The differential expressions of CD27 and CD45RA surface markers identify different  $V\delta 2^{POS}$  T cell subsets:  $CD27^{POS}/CD45RA^{POS}$  naïve cells ( $T_{Naïve}$ ),  $CD27^{POS}/CD45RA^{neg}$  central memory ( $T_{CM}$ ),  $CD27^{neg}/CD45RA^{neg}$  effector-memory ( $T_{EM}$ ) and the terminally-differentiated ( $T_{EMRA}$ )  $CD27^{neg}/CD45RA^{POS}$  cells. These  $V\delta 2^{POS}$  T cell subsets diverge not only for their maturation/differentiation status, but also for proliferative capacities, effector functions and resistance to cell death in response to antigens and/or cytokine stimulations [3].

Growing evidences highlighted the high impact of  $V\delta 2^{POS}$  T cells in cancer immune-surveillance with promising perspectives in cancer immunotherapy [4, 5]. In this context, two main clinical approaches have been employed to boost anti-tumor activities of  $V\delta 2^{POS}$  T cells. The first one activates them through the in vivo administration of either IL-2 or synthetic nitrogen-containing bisphosphonates (NBPs) drugs that, in turn, induce intracellular accumulation of phosphoantigens. A second strategy relies on adoptive transfers of  $V\delta 2^{POS}$  T cells expanded in vitro with several methodologies such as the activation with zoledronate [5, 6]. However, these procedures showed both experimental and clinical limits and many efforts are currently being implemented to further improve the effector-functions and persistence in vivo of  $V\delta 2^{POS}$  T cells. In this context, cellular senescence is certainly one of the main issues to solve considering that age-related changes of T cells greatly impair their capacity to expand and proliferate, thus leading to dysfunctional immune responses against tumors and pathogens [7]. The shift to senescence and accumulation of mature T cells physiologically occur after 60 years old when both  $\alpha\beta$  and  $\gamma\delta$  T lymphocytes lose their costimulatory molecules (i.e. CD27 and CD28), acquire terminally-differentiated  $T_{EM}$  and  $T_{EMRA}$  phenotypic profiles, express high constitutive levels of the senescence marker CD57 and shorten their telomerase lengths [8–11]. However, it is still controversial whether

CD57 can be used as a single marker to identify senescent  $V\delta 2^{POS}$  T cells regardless of differential expression of CD27 and CD45 [3, 11, 12].

Aging is certainly a major burden for social health systems in the industrialized countries as the populations are longer exposed to several pro-tumorigenic risk factors. This leads to a significant higher incidence of cancer onsets in the 6th, 7th and 8th decades of life [13]. Hence, there are rising numbers of elderly patients undergoing anti-cancer conventional chemotherapies (CHT), whose high toxicities greatly hamper both duration and quality of life. In this regard, several lines of clinical and experimental evidence pointed out that these anti-neoplastic treatments further accelerate immune-cell senescence, thus representing negative prognostic factors in aging and worsening the overall clinical outcomes of cancer patients [14, 15].

Since the use of  $V\delta 2^{POS}$  T cells is currently considered one of the most promising tools in cancer immunotherapy [4, 5], understanding the exact impact of CHT on their immune-senescence is key to better predict the clinical outcomes of cancer in elderly and to optimize those therapeutic protocols targeting these highly cytotoxic unconventional T cell effectors. Colorectal cancer (CRC) represents the 3rd most frequent solid cancer and more than 50% of CRC patients undergo hepatic dissemination of the primary tumor. The gold-standard therapeutic approach of CRC patients with liver metastasis (CLM) is the surgical removal of hepatic secondary lesions after neoadjuvant combination CHT with or without biological therapy (BT) (Table 1) [16, 17]. Moreover, a higher infiltration of competent immune cells in tumor mass greatly improves the prognosis of CLM patients and increases their overall survival (OS) [18, 19]. Here, we analyze the impact of conventional CHT regimens on the homeostasis and effector-functions of  $V\delta 2^{POS}$  T cells in a cohort of CLM elderly patients.

## Methods

### Patients and specimen collections

Biological specimens from CLM patients underwent CHT ( $n = 58$ ), or from CHT naïve patients ( $n = 13$ ) and aged- and sex-matched healthy donors ( $n = 40$ ) (Table 1). Patients' recruitment was performed according to the Declaration of Helsinki and the protocol had been approved by the Institutional Review Board (IRB) of Humanitas Research Hospital (HRH) (Approval N.168/18). All enrolled patients signed the related consent forms. Liver specimens and peripheral blood mononuclear cells (PBMCs) were isolated and stored as we previously described [19, 20].

### Flow cytometry

Absolute  $\gamma\delta$  T cell counts were performed on 100  $\mu$ l of fresh PB stained with following anti-human monoclonal

**Table 1** Neoadjuvant combination chemotherapy (CHT) with or without biological therapy (BT) of enrolled CLM patients

	Patients (number)	Patients (%)	CHT cycles (mean number $\pm$ SD)
CHT/BT Regimens <sup>a</sup>	58	82	8.7 $\pm$ 5.3
Combination Therapy with Biologicals			
FOLFOX + VEGF-A mAb	12	21.5	7.7 $\pm$ 1.4
FOLFIRI + EGFR mAb	11	19.0	11.7 $\pm$ 4.3
FOLFIRI + VEGF-A mAb	10	17.2	7.5 $\pm$ 3.3
FOLFIRI + FOLFOX + VEGF-A mAb	7	12.0	13.0 $\pm$ 3.2
FOLFOX + EGFR mAb	6	10.3	11.0 $\pm$ 2.3
XELOX + VEGF-A mAb	4	6.9	8.5 $\pm$ 3.4
Combination Therapy without Biologicals			
FOLFOX	4	6.9	5.0 $\pm$ 1.6
XELOX	2	3.4	4.6 $\pm$ 1.2
FOLFIRI	2	3.4	7.0 $\pm$ 6.0
Naïve for CHT	13	18	0.0
Total Patients	71		

FOLFOX: 5-fluorouracil/oxaliplatin; XELOX: capecitabine/oxaliplatin; FOLFIRI: 5-fluorouracil/irinotecan

EGFR mAb Epidermal Growth Factor Receptor inhibitor monoclonal antibody

VEGF-A mAb Vascular Endothelial Growth Factor A monoclonal antibody

<sup>a</sup>Note:

a) All CLM patients completed their last CHT cycle at least 6 weeks before the blood draws used for our experiments and before surgical procedures

b) The table refers all therapies received by CLM patients before surgery

c) More than 91% of all CLM patients received one line therapy and all other patients received two lines (1<sup>st</sup> and 2<sup>nd</sup>) combination therapy: 3 patients received 1<sup>st</sup> FOLFOX and 2<sup>nd</sup> FOLFIRI + VEGF-A; 1 patient received 1<sup>st</sup> FOLFIRI + VEGF-A and 2<sup>nd</sup> FOLFOX + VEGF-A, and 1 patient received 1<sup>st</sup> FOLFIRI + VEGF-A and 2<sup>nd</sup> FOLFOX

antibodies (mAbs): CD3 (SK7; BV605) and CD45 (H130; AF700) (BioLegend) and V $\delta$ 2 (IMMU-389; FITC) (Beckman Coulter). We then used CountBright™ Absolute Counting Beads (Invitrogen) according to the manufacturer's instructions.

For both regular and intracellular staining,  $\gamma\delta$  T cells were first screened for viability with Zombie Aqua™ Fixable Viability kit (BioLegend) and then processed as previously described [20]. The following mAbs were used: CD28 (CD28.2; PE-Cy7) (BioLegend); V $\delta$ 2 (B6; BUV395), CD3 (UCHT1; BUV661), CD45RA (H100; BUV737), CD16 (348; BUV496) (BD); CD57 (REA769; PE-Vio615) (Miltenyi); CD27 (0322; APCeFluor780) (eBioscience). The intracellular amounts of TNF- $\alpha$  (Mab11; PE) and IFN- $\gamma$  (B27; Bv711) (BD) as well as the frequency of cytotoxic CD107a<sup>pos</sup> cells (H4A3, PE) (BD Biosciences) was evaluated after stimulating  $\gamma\delta$  T cells with Phorbol myristate acetate (PMA; 0.5  $\mu$ g/mL) and Ionomycin (0.1  $\mu$ g/mL) (Sigma Aldrich).

Flow cytometry experiments were performed on FACS Symphony™ (BD). All data and *t*-SNE algorithm were analyzed with FlowJo Software (version 9.6) (FlowJo LLC) using single stained controls BD CompBeads™ (BD).

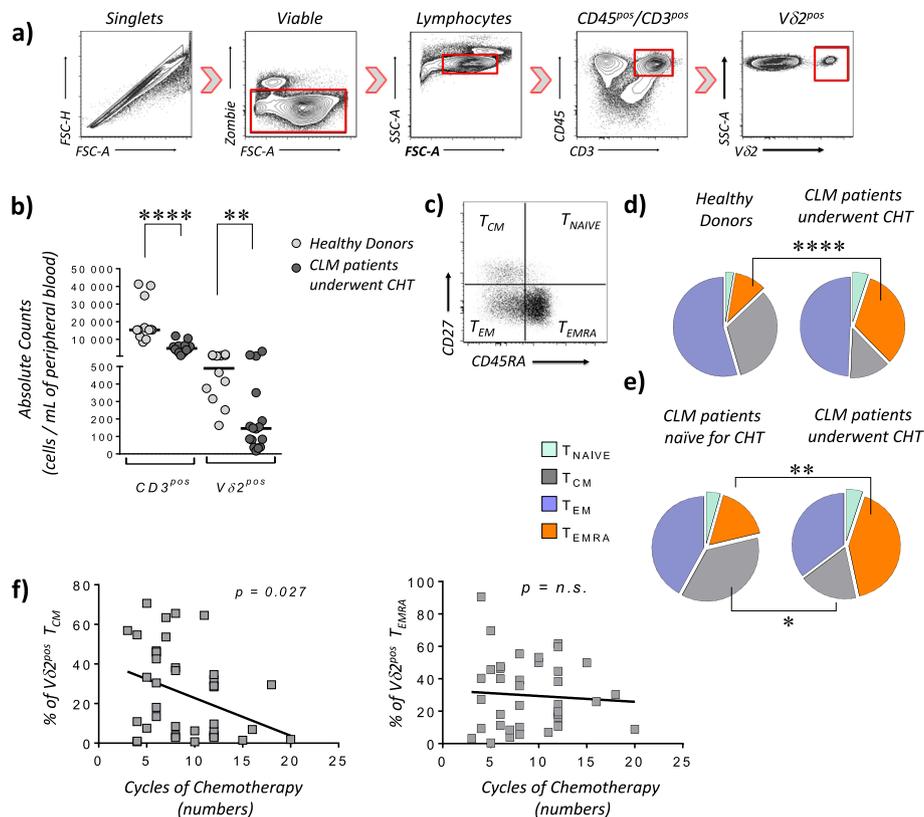
### Statistical analyses

The data were assessed by non-parametric *Mann-Whitney U* (unpaired) or *Wilcoxon* (matched-paired) tests by using *GraphPad Prism* version 7. For all correlation analysis Pearson's coefficient was applied. Statistically significant *p*

values were represented with GraphPad (GP) style and summarized with following number of asterisks (\*): \**P*  $\leq$  0.05; \*\**P*  $\leq$  0.01; \*\*\**P*  $\leq$  0.001; \*\*\*\**P*  $\leq$  0.0001.

### Results

V $\delta$ 2<sup>pos</sup> T cells were gated within viable CD3<sup>pos</sup>/CD45<sup>pos</sup> lymphocytes and their absolute counts are significantly lower in the PB of CLM patients underwent CHT compared to those of healthy donors (Fig. 1a-b). We then analyzed the surface expression of CD27 and CD45RA to track the differentiation and distribution of V $\delta$ 2<sup>pos</sup> T cell subsets. Our data showed a significant increase of V $\delta$ 2<sup>pos</sup> T<sub>EMRA</sub> in CLM patients underwent CHT (28.9  $\pm$  20.6%) compared to healthy controls (9.4  $\pm$  6.4%). This phenomenon is associated with the previous administration of CHT, as the frequency of circulating V $\delta$ 2<sup>pos</sup> T<sub>EMRA</sub> in those CLM patients naïve for CHT (16.7%  $\pm$  12.6) is similar to that of healthy donors and significantly lower to that of CLM patients underwent CHT (41.6%  $\pm$  19.6). The increased amounts of V $\delta$ 2<sup>pos</sup> T<sub>EMRA</sub> in CLM patients treated with CHT is counterbalanced by a significant decrease of V $\delta$ 2<sup>pos</sup> T<sub>CM</sub> in the same patients compared to their counterparts naïve for CHT (Fig. 1c-d-e). The great impact of neoadjuvant CHT in shaping the distribution of V $\delta$ 2<sup>pos</sup> T cell subsets in CLM patients is also confirmed by our findings showing that the number of CHT cycles (8.7  $\pm$  2.7) inversely correlates with the percentages of PB V $\delta$ 2<sup>pos</sup> T<sub>CM</sub>, while not affecting at



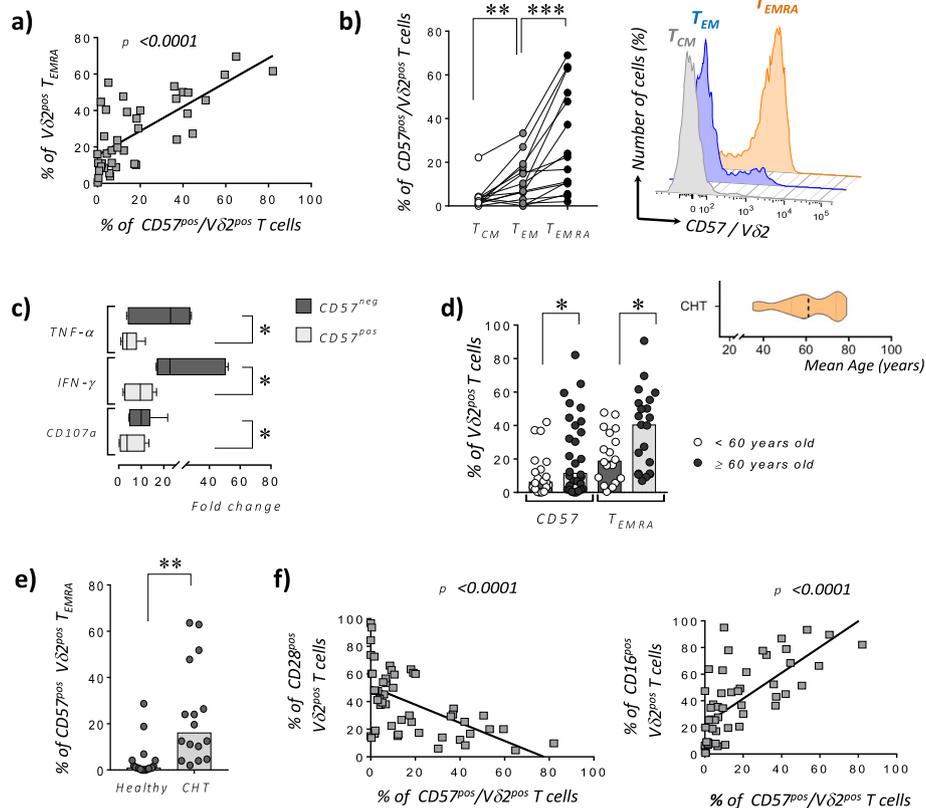
**Fig. 1** Frequency and distributions of peripheral blood  $V\delta 2^{POS}$  T cell subsets in patients affected by liver metastasis of colorectal cancer and underwent chemotherapy. **a** Representative dot plot flow cytometric graphs showing the gating strategy of viable  $CD45^{POS}/CD3^{POS}/V\delta 2^{POS}$  T lymphocytes. **b** Statistical dot plot graph showing the absolute number of  $CD3^{POS}$  (left) and  $V\delta 2^{POS}$  (right) T cells per 1 mL of blood in healthy donors ( $n = 12$ ; mean age:  $49.3 \pm 9.5$ ) and CLM patients underwent CHT regimens ( $n = 16$ ; mean age:  $51.5 \pm 8.1$ ). **c-e** Representative dot plot graph flow cytometric graph (**c**) and pie charts (**d** and **e**) showing respectively the distribution and the percentages of  $CD27^{POS}/CD45RA^{POS}$   $T_{Naive}$  (upper right in dot plot graph and light green in pie charts),  $CD27^{POS}/CD45RA^{NEG}$  central memory ( $T_{CM}$ ) (upper left in dot plot graph and gray in pie charts),  $CD27^{NEG}/CD45RA^{NEG}$  effector-memory ( $T_{EM}$ ) (lower left in dot plot graph and purple in pie charts) and terminally-differentiated  $CD27^{NEG}/CD45RA^{POS}$  ( $T_{EMRA}$ ) (lower right in dot plot graph and orange in pie charts)  $V\delta 2^{POS}$  T cell subsets. Pie charts compare the frequencies of  $V\delta 2^{POS}$  T cell subsets between healthy donors ( $n = 34$ ; mean age:  $51.7 \pm 10.8$ ) with age-matched CLM patient underwent CHT ( $n = 33$ ; mean age:  $51.5 \pm 8.1$ ) **d** as well as between CLM patients naïve for CHT ( $n = 13$ ; mean age:  $69.5 \pm 8.1$ ) and age-matched CLM patients underwent CHT ( $n = 41$ ; mean age:  $70.1 \pm 6.5$ ) **e**. **f** Statistical analysis showing the Pearson correlations between the frequency (%) of either  $T_{CM}$  (left) or  $T_{EMRA}$  (right)  $V\delta 2^{POS}$  T cells with the number of CHT cycles (mean number:  $8.7 \pm 6.5$ ) administered to patients affected by CLM ( $n = 40$ )

all the overall frequencies of PB  $V\delta 2^{POS}$   $T_{EMRA}$  (Fig. 1f). This latter dichotomy reflects the different homeostatic status of  $V\delta 2^{POS}$   $T_{CM}$  compared to that of  $V\delta 2^{POS}$   $T_{EMRA}$ , as the first subset is composed of proliferating lymphocytes high susceptible to the toxicity of those chemotherapy compounds that kills all dividing cells without any specificity against tumor blasts. Instead,  $T_{EMRA}$   $V\delta 2^{POS}$  cells are terminally differentiated and not proliferating effectors resistant to CHT, thus explaining their high frequency even after several cycles of neoadjuvant anti-tumor chemotherapies.

The relative increased frequency of PB  $T_{EMRA}$   $V\delta 2^{POS}$  in CLM patients underwent CHT correlates with their higher expression of CD57. Notably, the expression of this latter marker of immune senescence follows the terminal differentiation of  $V\delta 2^{POS}$  T cells. Indeed, the frequency of

PB  $CD57^{POS}$   $T_{EMRA}$   $V\delta 2^{POS}$  T cells resulted significantly higher compared to that of  $CD57^{POS}$   $T_{EM}$   $V\delta 2^{POS}$  T cells that, in turn, showed significantly higher amounts of CD57 when compared to  $T_{CM}$   $V\delta 2^{POS}$  T cells (Fig. 2a-b). The acquisition of CD57 by terminal-differentiated  $V\delta 2^{POS}$  T cells is also associated with significantly impaired effector-functions in term of anti-tumor cytokines production (i.e. IFN- $\gamma$  and TNF- $\alpha$ ) and ability to degranulate (i.e. decreased amounts of cytotoxic  $CD107a^{POS}$  cells) when compared to  $CD57^{NEG}/V\delta 2^{POS}$  T cells (Fig. 2c). Taken together, these data indicate that the PB of CLM patients underwent CHT is highly enriched of senescent  $CD57^{POS}/T_{EMRA}$   $V\delta 2^{POS}$  T cells dysfunctional in their anti-tumor effector functions.

To assess the impact of patients' aging in the higher frequencies of  $CD57^{POS}$  and  $T_{EMRA}$   $V\delta 2^{POS}$  T cells in

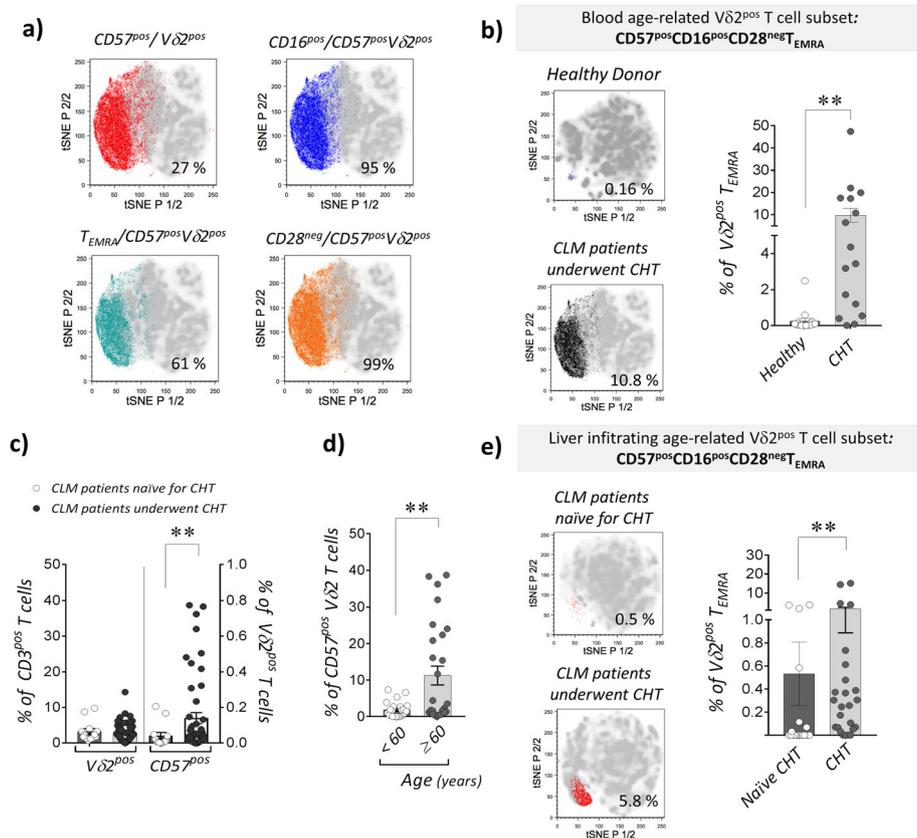


**Fig. 2** Senescence of peripheral blood  $V\delta 2^{POS}$  T cell in patients affected by liver metastasis of colorectal cancer and underwent chemotherapy. **a** Statistical analysis showing the correlations between the frequencies (%) of  $V\delta 2^{POS}$   $T_{EMRA}$  and  $CD57^{POS}/V\delta 2^{POS}$  T and in CLM patients underwent CHT ( $n = 40$ ). **b** Statistical dot plot (left) and representative histogram (right) graphs showing the expressions (%) of CD57 on matching  $T_{CM}$ ,  $T_{EM}$  and  $T_{EMRA}$   $V\delta 2^{POS}$  T cell subsets in CLM patients underwent CHT ( $n = 15$ ). **c** Statistical bar graphs showing the fold change increases of CD107a expression as well as of intracellular amounts of IFN- $\gamma$  and TNF- $\alpha$  by  $CD57^{NEG}$  and  $CD57^{POS}$   $V\delta 2$  T cell effector subsets (i.e.  $T_{EMRA}$  and  $T_{EM}$ ) from CLM patients underwent CHT and following in vitro stimulation with PMA and Ionomycin ( $n = 6$ ). **d** Statistical dot plot analysis showing the expressions (%) of CD57 and the frequencies (%) of  $T_{EMRA}$  within  $V\delta 2^{POS}$  T cell compartments in CLM patients underwent CHT and divided in two groups of respectively < (white circles;  $n = 18$ ) and  $\geq$  (black circles;  $n = 21$ ) of 60 years old. The mean age of the entire cohort of CLM patients underwent CHT is of  $61 \pm 10.7$  years old as shown in statistical graph on right upper side. **e** Statistical dot plot analysis showing the expressions (%) of CD57 on  $V\delta 2^{POS}$   $T_{EMRA}$  cells from CLM patients underwent CHT and under 60 years old ( $n = 16$ ) compared to age-matched healthy donors ( $n = 16$ ). **f** Statistical analysis showing the correlations between the surface levels (%) of CD57 and CD28 ( $n = 51$ ) (left graph) or CD16 ( $n = 51$ ) (right graph) on  $V\delta 2^{POS}$  T cells in CLM patients underwent CHT

CLM patients underwent CHT, we divided this cohort in subjects younger or older than 60 years old. Our data confirmed that the age-induced immune-senescence significantly increases the percentages of both  $CD57^{POS}$  and  $T_{EMRA}$   $V\delta 2^{POS}$  T cells in those patients > 60 years old (Fig. 2d). We also showed that CHT alone induces immune-senescence regardless of patients' age. Indeed, the percentage of  $CD57^{POS}$   $T_{EMRA}$   $V\delta 2^{POS}$  cells resulted significantly higher in those CLM underwent CHT and younger than 60 years old compared to that of age-matched healthy donors (Fig. 2e). These data clearly indicate that both CHT and aging play synergic roles in the regulation of  $V\delta 2^{POS}$  T cell homeostasis in CLM patients with the final result of greatly accelerating their terminal differentiation towards a senescent  $CD57^{POS}/T_{EMRA}$  subset highly impaired in its anti-tumor effector-

functions. We also demonstrate here that the acquisition of CD57 inversely correlates with the surface expression of CD28 while being associated with increased surface amounts of CD16 (Fig. 2f), the Fc $\gamma$ RIII receptor known to define highly differentiated human  $V\delta 2^{POS}$   $T_{EMRA}$  cells [21]. The clustering of  $CD57^{POS}/V\delta 2^{POS}$   $T_{EMRA}$  co-expressing CD16 and lacking CD28 in CLM patients underwent CHT is confirmed and better visualized by the t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis (Fig. 3a). This analytic approach also allowed us to compare the impact of CHT in inducing high frequencies of PB  $CD57^{POS}/CD16^{POS}/CD28^{NEG}/V\delta 2^{POS}$   $T_{EMRA}$  cells in CLM patients compared to those of age-matched healthy donors (Fig. 3b).

Although the overall frequency of tumor infiltrating  $V\delta 2^{POS}$  T cells purified from CLM specimens is not



**Fig. 3** Clustering of peripheral blood and tissue infiltrating senescent  $CD57^{pos}/CD28^{neg}/CD16^{pos} T_{EMRA} V\delta2^{pos}$  T cells in patients affected by liver metastasis of colorectal cancer and underwent chemotherapy. **a** t-SNE analysis plots in CLM patients underwent CHT ( $n = 16$ ) showing the cluster of PB  $CD57^{pos}/V\delta2^{pos}$  T cells (red, upper left plot) co-expressing CD16 (blue, upper right plot), CD45RA but not CD27 ( $T_{EMRA}$  in green, lower left plot) and negative for CD28 (black, lower right plot). **b** t-SNE analysis plots (left) and statistical dot plot graph (right) showing the frequency (%) of senescent PB  $CD57^{pos}/CD28^{neg}/CD16^{pos} T_{EMRA} V\delta2^{pos}$  T cells in healthy donors (upper plot;  $n = 12$ ; mean age:  $51.7 \pm 10.8$ ) and CLM underwent CHT (lower plot;  $n = 16$ ; mean age:  $61 \pm 10.7$ ). **c** Summary dot plot analysis showing the frequencies (%) of liver tumor-associated  $V\delta2^{pos}$  T cells within total  $CD3^{pos}$  T lymphocytes or  $CD57^{pos}/V\delta2^{pos}$  T cells in CLM patients receiving CHT regimen (black circles;  $n = 58$ ) and naïve for CHT (white circles;  $n = 13$ ). **d** Statistical dot plot analysis showing the frequencies (%) of  $CD57^{pos}$  cells on liver tumor infiltrating  $V\delta2^{pos}$  T cells in CLM patients underwent CHT regimen and sub-divided in two groups of respectively  $< 60$  (white circles;  $n = 22$ ) and  $\geq 60$  (black circles;  $n = 27$ ) of 60 years old. **e** t-SNE analysis plots (left plots) and statistical chart (right graph) of the CHT-mediated changes in the frequency (%) of the age-related, liver tumor infiltrating  $CD57^{pos}/CD28^{neg}/CD16^{pos} T_{EMRA} V\delta2^{pos}$  T cell cluster in CHT treated CLM patients (lower plot,  $n = 25$ ; mean age:  $61 \pm 10.7$ ) and naïve for CHT patients (upper plot,  $n = 13$ ; mean age:  $69.5 \pm 8.1$ )

affected by the administration of CHT, we found a significant increase of CD57 expression on those  $V\delta2^{pos}$  T cells from patients underwent CHT compared to naïve ones (Fig. 3c). Similar to their PB counterparts, the frequency of  $CD57^{pos}/V\delta2^{pos}$  T cells is significantly higher in elderly CHT patients  $\geq 60$  years old (Fig. 3d). Consistently with these data, t-SNE analysis showed also in CLM specimens of patient administered with CHT an increased frequency of age-related tumor infiltrating  $CD57^{pos}/CD28^{neg}/CD16^{pos} T_{EMRA} V\delta2^{pos}$  T lymphocytes (Fig. 3e).

## Discussion

The present study is aimed to measure the true impact of conventional CHT regimens on unconventional T cell

senescence in elderly cancer patients, since the toxicity of conventional anti-tumor therapies greatly impairs their ability to clearance malignant cells [7, 12, 14, 15]. In particular, we focused our investigations on circulating  $V\delta2^{pos}$  cells that are endowed with great anti-tumor potentials currently being targeted by several protocols of cancer immunotherapies [4–6]. Our data showed that CLM patients underwent CHT, although showing lower absolute counts of circulating  $V\delta2^{pos}$  cells, retain high relative frequencies of terminally differentiated and senescent  $CD57^{pos}/CD28^{neg}/CD16^{pos} T_{EMRA} V\delta2^{pos}$  cells greatly impaired in their effector-functions. This latter subset is resistant to the toxicity exerted by repeated CHT cycles administering DNA-damaging drugs that, in contrast, are highly toxic against less differentiated and still proliferating  $T_{CM} V\delta2^{pos}$  cells.

The preferential accumulation in PB of senescent CD57<sup>POS</sup> T<sub>EMRA</sub> Vδ2<sup>POS</sup> cells in CLM patients underwent CHT is associated with two major mechanisms. The first one is linked to natural immune-senescence of people aging as the incidence of many cancers is higher in patients  $\geq$  of 60 years old. In this context, liver metastatic CRC is one of the most common causes of cancer deaths worldwide with a higher incidence in elderly [16, 17]. Indeed, our cohort of recruited CLM subjects had a mean age of  $61 \pm 10.7$  years old and both the frequencies of CD57<sup>POS</sup> and T<sub>EMRA</sub> Vδ2 T cell subsets resulted higher in that fraction of patients older than 60 years. The second mechanism is associated with a direct toxicity of CHT on immune cells, as also highlighted by several studies both in pediatric and geriatric cancer patients [14, 15, 22]. As a matter of fact, we show here that the expression of CD57 on T<sub>EMRA</sub> Vδ2<sup>POS</sup> cells is much higher on those CLM patients underwent CHT and younger than 60 years old compared to age-matched healthy donors. This demonstrates that neoadjuvant CHT induces immune senescence also on unconventional T cells regardless of CLM patients' age. Notably, high frequencies of impaired CD57<sup>POS</sup>/T<sub>EMRA</sub> Vδ2<sup>POS</sup> cells were able to persist in PB of CLM patients even after 6 weeks from the completion of the last CHT cycle and before surgical removal of liver metastases. Further prospective studies are required to assess how long senescent and functional impaired Vδ2<sup>POS</sup> T cells survive after CHT and what clinical impact they have on the OS of CLM patients. In this regard, it has been already reported that the enrichment of circulating subsets of CD57<sup>POS</sup> αβ T cells represents a negative prognostic factor in the clinical outcome of gastrointestinal cancers [23].

Our study also contributes to better characterize immune-senescence of Vδ2<sup>POS</sup> T cells, since it has been recently reported that expression of CD57 can define alone their senescent status without the need of also evaluating the expression of both CD27 and CD45RA [11]. This represents a key point that is currently being debated both in physiological and pathological conditions. We found that, at least in a human cancer setting, the expression of CD57 on senescent Vδ2<sup>POS</sup> T cell parallels their terminal differentiation towards T<sub>EMRA</sub> (CD27<sup>NEG</sup>/CD45RA<sup>POS</sup>), a phenomenon associated with the loss of CD28 and the acquired expression of CD16. These results are in line with a previous study showing that Vδ2<sup>POS</sup> T<sub>EMRA</sub> are refractory to phosphoantigen stimulation, but rather respond to activation via FcγRIII [21].

The majority of cancer patients are older than 65 years old in line with population aging [14]. In this context, several clinical trials in the elderly are currently being implemented to optimize the anti-tumor activities of unconventional T cells. These therapeutic protocols are mostly aimed to expand Vδ2<sup>POS</sup> T cells both in vivo and

in vitro [6]. Hence, a better understanding of the mechanisms accelerating immune-senescence in aging is fundamental to boost the effector-functions of these cytotoxic and cytokine-producer T lymphocytes. We show here that, neoadjuvant CHT regimens, although absolutely required to reduce tumor mass in CLM patients before surgery, greatly speed the senescence of Vδ2<sup>POS</sup> T cells in synergy with aging of cancer patients. This knowledge will allow us to better optimize immune-therapies against cancers in elderly. Indeed, senescence process can be reversed through the inhibition of p38 mitogen-activated protein kinase (MAPK) signaling [24]. This methodology could be then approached to develop new protocols implementing pre-treatment with MAPK inhibitors in elderly patients with CRC [25]. Alternatively, new methodology can be implemented in vitro to select and expand CD57<sup>NEG</sup>/Vδ2<sup>POS</sup> T cells that better resist to the terminal differentiations and senescence induced by CHT. Further studies are also required to better identify those CHT associated accumulation of impaired and senescent circulating Vδ2<sup>POS</sup> T cells.

#### Abbreviations

CHT: Chemotherapy; CLM: Colorectal liver metastatic cancer; CRC: Colorectal cancer; NBPs: Nitrogen-containing bisphosphonates; T<sub>CM</sub>: Central memory T; T<sub>EM</sub>: Effector memory T; T<sub>EMRA</sub>: Terminally differentiated T; T<sub>Naive</sub>: Naive T; t-SNE: t-Distributed Stochastic Neighbor Embedding; γδ T: Gamma delta T

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#### Authors' contributions

JM and DM developed the study. EB, VC, GS and GL performed the experiments and statistical analyses. MC, MD and GT recruited patients and collected biological specimens. JM, EB, FD and DM contributed to the interpretation of the data and wrote article. All authors read and approved the final manuscript.

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#### Availability of data and materials

The dataset generated and analyzed in the current study are available from the corresponding authors on reasonable request.

#### Ethics approval and consent to participate

The collection of human samples for research purposes was ethically approved by the Institutional Review Board (IRB) of Humanitas Research Hospital (HRH) (Approval 168/18).

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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**References**

- Vantourout P, Hayday A. Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nat Rev Immunol*. 2013;13(2):88–100.
- Willcox BE, Willcox CR. gammadelta TCR ligands: the quest to solve a 500-million-year-old mystery. *Nat Immunol*. 2019;20(2):121–8.
- Caccamo N, Meraviglia S, Ferlazzo V, Angelini D, Borsellino G, Poccia F, et al. Differential requirements for antigen or homeostatic cytokines for proliferation and differentiation of human Vgamma9Vdelta2 naive, memory and effector T cell subsets. *Eur J Immunol*. 2005;35(6):1764–72.
- Zou C, Zhao P, Xiao Z, Han X, Fu F, Fu L. gammadelta T cells in cancer immunotherapy. *Oncotarget*. 2017;8(5):8900–9.
- Silva-Santos B, Serre K, Norell H. gammadelta T cells in cancer. *Nat Rev Immunol*. 2015;15(11):683–91.
- Godfrey DJ, Le Nours J, Andrews DM, Uldrich AP, Rossjohn J. Unconventional T cell targets for cancer immunotherapy. *Immunity*. 2018; 48(3):453–73.
- Chou JP, Effros RB. T cell replicative senescence in human aging. *Curr Pharm Des*. 2013;19(9):1680–98.
- Larbi A, Fulop T. From “truly naive” to “exhausted senescent” T cells: when markers predict functionality. *Cytometry A*. 2014;85(1):25–35.
- Bernadotte A, Mikhelson VM, Spivak IM. Markers of cellular senescence. Telomere shortening as a marker of cellular senescence. *Aging (Albany NY)*. 2016;8(1):3–11.
- Mou D, Espinosa J, Lo DJ, Kirk AD. CD28 negative T cells: is their loss our gain? *Am J Transplant*. 2014;14(11):2460–6.
- Xu W, Monaco G, Wong EH, Tan WLW, Kared H, Simoni Y, et al. Mapping of gamma/delta T cells reveals Vdelta2+ T cells resistance to senescence. *EBioMedicine*. 2019;39:44–58.
- Xu W, Larbi A. Markers of T cell senescence in humans. *Int J Mol Sci*. 2017; 18(8):1742.
- Smetana K Jr, Lacina L, Szabo P, Dvorankova B, Broz P, Sedo A. Ageing as an important risk factor for cancer. *Anticancer Res*. 2016;36(10):5009–17.
- Hurria A, Jones L, Muss HB. Cancer treatment as an accelerated aging process: assessment, biomarkers, and interventions. *Am Soc Clin Oncol Educ Book*. 2016;35:e516–22.
- Onyema OO, Decoster L, Njemini R, Forti LN, Bautmans I, De Waele M, et al. Chemotherapy-induced changes and immunosenescence of CD8+ T-cells in patients with breast cancer. *Anticancer Res*. 2015;35(3):1481–9.
- Konopke R, Roth J, Volk A, Pistorius S, Folprecht G, Zophel K, et al. Colorectal liver metastases: an update on palliative treatment options. *J Gastrointest Liver Dis*. 2012;21(1):83–91.
- Kolligs FT. Diagnostics and epidemiology of colorectal cancer. *Visc Med*. 2016;32(3):158–64.
- Rubbia-Brandt L, Giostra E, Brezault C, Roth AD, Andres A, Audard V, et al. Importance of histological tumor response assessment in predicting the outcome in patients with colorectal liver metastases treated with neo-adjuvant chemotherapy followed by liver surgery. *Ann Oncol*. 2007;18(2):299–304.
- Donadon M, Hudspeth K, Cimino M, Di Tommaso L, Preti M, Tentorio P, et al. Increased infiltration of natural killer and T cells in colorectal liver metastases improves patient overall survival. *J Gastrointest Surg*. 2017;21: 1226–36.
- Roberto A, Di Vito C, Zaghi E, Mazza EMC, Capucetti A, Calvi M, et al. The early expansion of anergic NKG2A(pos)/CD56(dim)/CD16(neg) natural killer represents a therapeutic target in haploidentical hematopoietic stem cell transplantation. *Haematologica*. 2018;103(8):1390–402.
- Angelini DF, Borsellino G, Poupot M, Diamantini A, Poupot R, Bernardi G, et al. FcgammaRIII discriminates between 2 subsets of Vgamma9Vdelta2 effector cells with different responses and activation pathways. *Blood*. 2004; 104(6):1801–7.
- Henderson TO, Ness KK, Cohen HJ. Accelerated aging among cancer survivors: from pediatrics to geriatrics. *Am Soc Clin Oncol Educ Book*. 2014: e423–30. [https://doi.org/10.14694/EdBook\\_AM.2014.34.e423](https://doi.org/10.14694/EdBook_AM.2014.34.e423).
- Akagi J, Baba H. Prognostic value of CD57(+) T lymphocytes in the peripheral blood of patients with advanced gastric cancer. *Int J Clin Oncol*. 2008;13(6):528–35.
- Lanna A, Henson SM, Escors D, Akbar AN. The kinase p38 activated by the metabolic regulator AMPK and scaffold TAB1 drives the senescence of human T cells. *Nat Immunol*. 2014;15(10):965–72.
- Pancione M, Giordano G, Parcesepo P, Cerulo L, Coppola L, Curatolo AD, et al. Emerging insight into MAPK inhibitors and immunotherapy in colorectal cancer. *Curr Med Chem*. 2017;24(14):1383–402.

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