Characterization of γδ T cells infiltrating colorectal cancer

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We have read with great interest the paper by de Vries and colleagues (1) ~~which provides~~ reporting on the immune landscape of colorectal cancer (CRC) by high-dimensional mass cytometry, flow cytometry and single cell RNA sequencing. Amongst clusters of immune cells infiltrating CRC, authors have identified two populations of γδ T cells: one PD-1$^+$ population is almost exclusively found in MMR-deficient (d) tumours, constitutes up to 8.4% of CD45$^+$ cells and has an activated phenotype, and a PD-1$^-$ counterpart with a resting phenotype, which is also found in colorectal ~~healthy~~ normal mucosa and MMR-proficient (p) tumours.

Using deconvolution of transcriptomic datasets and single cell RNA sequencing, we have detected γδ cells in many different human tumors, including CRC (2,3), but it is important to know also their subset distribution, maturation ~~/activation/exhaustion~~ states and functional profiles. In fact, there are two subsets of γδ T cells, those expressing Vδ1 which reside at mucosal sites and those expressing Vδ2 which are found in the blood but are recruited to the tumor site (4).
Our previous study (5) in ~~a large number (n=70) of CRC~~ tissue specimens of n=70 CRC patients using flow cytometry has shown that γδ T cells account for ~~approximately~~ 4.5% of CD45$^+$ cells both in normal and in CRC mucosa, and these data have been confirmed by data-mining of an independent cohort of CRC transcriptomes (n=585).
We confirm here in a new cohort of 16 CRC patients (Supplementary Table 1) that Vδ1 T cells are more represented than Vδ2 T cell both in normal and in CRC mucosa (Figure 1A). The identification of two different and separate clusters of Vδ1 and Vδ2 T cells in CRC and normal mucosa is confirmed and better visualized by the t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis ~~CD45$^+$/CD3$^+$ cells from CRC specimens were also analyzed by a dimensionality reduction method that used the t-distributed stochastic neighbor embedding (t-SNE) algorithm~~ (6)~~, that identified two different and separate clusters of Vδ1 and Vδ2 T cells on the basis of population boundaries distinguishable on the polychromatic flow cytometry density plots~~ (Figure 1B).
~~Phenotypical a~~Analysis of the differentiation status by CD27 and CD45RA expression clearly showed the predominance of effector memory phenotypes in tumor infiltrating Vδ~~d~~1 and Vδ2 T cells, compared to normal and PBMC~~.~~ (Figure 1C). ~~(nei d1 non si evince)~~

~~Moreover~~Accordingly, we previously reported that upon stimulation *in vitro* with ionomycin

and PMA, Vδ1 and Vδ2 T cells from normal mucosa express IFN-γ, ~~but not IL-17,~~ which was significantly reduced in CRC tissue. (5). ~~Thus, our results confirm and extend the findings of de Vries and colleagues who showed that γδ T cells from either MMR-d or MMR-p tumors express IFN-γ on stimulation with PMA/ionomycin.~~

~~Moreover~~Linking our data to the clinic, ~~and~~ differently than the data reported by de Vries ~~and colleagues~~, leucocyte deconvolution of the CRC dataset demonstrated that the abundance of CRC-infiltrating γδ cells was significantly higher in MMR-p than MMR-d tumors (5). In addition, ~~comparing the~~ abundance of CRC-infiltrating γδ cells correlated with ~~some~~ mutational status generally related to a ~~worse~~short survival ~~prognosis of CRC, but with a better response to PD-1 inhibitors~~ (~~?~~7), as we observed that these cells are significantly higher in BRAF- and in TP53-mutated tumors, ~~in~~ tumors positive for the CPG island methylator phenotype (CIMP) ~~(CIMP)~~ and in tumors negative for the chromosomal instability phenotype (CIN) ~~(CIN)~~.
~~Moreover~~Finally, CRC patients with more abundant tumor-infiltrating γδ T cells had a better disease-free survival (DFS) and absence of lymph node invasion (5).
~~, supporting their antitumor role.~~

Low ~~levels~~ and transient PD-1 expression has been found only on circulating Vδ2 T cells upon activation (~~7~~8). As shown in Figure 2A, ~~around~~ there was a significant increase in PD-1 expression on Vδ1 T cells in CRC mucosa, as compared to normal mucosa and peripheral blood, according to either MFI or percentage of marker positive cell. Conversely, Vδ2 T cells upregulated PD-1 expression both in normal and in CRC mucosa, as compared to peripheral blood, although PD-1 expression was significantly lower than that detected in CRC-infiltrating Vδ1 T cells
~~30%±2,96% of the Vδ1 T cells in CRC specimen expressed PD-1, while 20%±3,05 Vδ2 T cells expressed PD-1 in the normal mucosa, but not in CRC. However, intensity PD-1 expression, as calculated by MFI analysis, was increased in CRC infiltrating Vδ1 and Vδ2 T cells (Figure 2B), as compared to normal tissue and peripheral blood.~~

In conclusion, our data integrate the study by de Vries *et al.*, providing information on both Vδ1 and Vδ2 subsets of γδ T cells ~~present~~ in CRC tissues. Given the capacity of γδ T cells to impact positively to cancer outcome, fully understanding of their subset composition and functions in CRC may allow improvement in immunotherapeutic strategies.

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Legend to Figure

Figure 1. Frequency of infiltrating and circulating γδ T cells expressing either Vδ1 or Vδ2 TCR δ chains in CRC patients. A) Cumulative data showing percentages of Vδ1 and Vδ2 T cells in normal (blue) and tumor (green) mucosa and in PBMC (red). Vδ1 and Vδ2 T cells are indicated as percentage of the total number of $CD45^+$ cells in each sample. *$p \leq 0.05$ by non parametric unpaired and two tailed Mann-Whitney test, with 95% confidential interval. B) Cells were acquired by FACS Canto II and were processed using principal component analysis, clustered and visualized by t-distributed stochastic neighbour embedding (t-SNE) using FlowJo software. The scaled heat map shows the relative expression of CD45, CD3, Vδ1 and Vδ2 markers within all clusters. C) Flow cytometry panels of a representative dot plot showing phenotypical analysis of Vδ1 and Vδ2 T cells among normal and tumor mucosa, and PBMC of CRC patients, upon staining with mAbs to CD45RA and CD27, and gating on $CD3^+V\delta1^+$ or $CD3^+V\delta2^+$ T cells.

Figure 2. PD1 expression on Vδ1 and Vδ2 T cells in blood and tissue of CRC patients. A) Representative histogram plots showing the percentage of PD-1^+ Vδ1 or Vδ2 T cells in normal (blu) and tumor (green) mucosa and PBMC (red) of CRC patients. The isotype control is represented in gray histogram. Gating strategy is shown in Supplementary Figure 1. B) Cumulative analysis of PD1 expression on Vδ1 and Vδ2 T cells (percentage and MFI of PD-1^+ cells) in normal and tumor mucosa, and PBMC. *$p<0.05$ and **$p<0.01$ by non parametric unpaired and two tailed Mann-Whitney test, with 95% confidential interval. Spider chart plots shows PD-1 intensity (MFI) and percentages of PD-1^+ Vδ1 and

Vδ2 T cells in normal and tumor mucosa, and PBMC. The axes arrange radially and the value scale is unique for all variables. Value scale is proportional to the magnitude of the variable. A line is drawn connecting the data values for each spoke (red from PBMC to normal mucosa, blue from normal to tumor mucosa and green from tumor mucosa to PBMC).

~~**Figure 1**: Frequency of infiltrating and circulating γδ T cells expressing either Vδ1 or Vδ2 TCR δ chains in CRC patients. A) Cumulative data showing percentages of Vδ1 and Vδ2 T cells in normal (colorectal healthy mucosa, blue color) and tumor (colorectal tissue, green colour), and in PBMC (peripheral blood, red colour). Vd1 and Vd2 T cells are indicated as percentage of the total number of CD45+ cells in each sample. *p≤0.05 by non parametric Mann-Whitney test, unpaired and two tailed, with 95% confidential interval. B) Cells were acquired by FACS Canto II and were processed using principal component analysis, clustered and visualized by t-distributed stochastic neighbour embedding (t-SNE) using FlowJo software (TreeStar Inc.). The scaled heat map shows the relative expression of CD45, CD3, Vδ1 and Vδ2 markers within all clusters. C) Flow cytometry panels of a representative dot plot showing phenotypical analysis of Vδ1 and Vδ2 T cells among normal, tumor and PBMC of CRC patients, upon staining with mAbs to CD45RA and CD27, and gating on CD3⁺ Vδ1⁺ or CD3⁺ Vδ2⁺ T cells.~~

~~**Figure 2**: PD1 expression on Vδ1 and Vδ2 T cells in blood and tissue of CRC patients. A) Representative histogram plots showing the percentage of PD-1⁺ Vδ1 or Vδ2 T cells in normal (blu color), tumor (green colour) and PBMC (red colour). The isotype control is represented in gray histogram. Gating strategy is shown in Supplementary Figure 1. B) Cumulative analysis of PD1 expression on Vδ1 and Vδ2 T cells (percentage and intensity as MFI analysis) in normal, tumor and PBMC. *p<0.05 and **p<0.01 performed by nonparametric Mann-Whitney test, unpaired and 2-tailed with confidential interval 95% Spider chart plots shows PD-1 intensity (MFI) and percentages of PD-1⁺ Vδ1 and Vδ2 T cells in PBMC, CRC tissue and healthy mucosa. The axes arrange radially and the value scale is unique for all variables. Value scale is proportional to the magnitude of the variable. A line is drawn connecting the data values for each spoke (red from PBMC to mucosa, blue from mucosa to CRC and green from CRC to PBMC).~~

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Contributors. FD and SM designed and supervised the research and wrote the paper. ELP performed the experiments, analysed the data and wrote the manuscript. AMC and MDS selected and processed the samples and performed the experiments.

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SUPPLEMENTAL INFORMATION

Materials and Methods

Patients and cells

Tissue samples (healthy and cancer colon) and peripheral blood were obtained from 16 CRC patients undergoing therapeutic intervention at the Department of Surgery at the University Hospital of Palermo. All patients gave consent in according to the Declaration of Helsinki. Cells suspension was obtained as previously described [5].

Flow cytometry analyses and monoclonal antibodies

The frequency of Vδ1 and Vδ2 T cells and PD1 expression were evaluated on cell suspensions by flow cytometry, upon staining cells with the following mAbs: Vδ1-PE; CD3-PerCp; Vδ2-Pe-Cy7; PD1-APC; CD45-APC-H7; The phenotype of Vδ1 and Vδ2 T cells was evaluated with the following mAbs: Vδ1- FITC; Vδ2-PE; CD3-PerCp; CD45RA-Pe-Cy7; CD27-APC; CD45-APC-H7, , ~~**AGGIUNGERE CD27 E CD45RA MABS**~~ all purchased from BD Biosciences. Zombie Violet Fixable Viability Kit for live/dead staining was purchased from Biolegend. All samples were acquired on a BD FACS Canto II flow cytometer and- analysed using FlowJo software (TreeStar Inc.).

Statistical analyses

Statistical analyses were performed with GraphPad Prism 7 software (La Jolla, CA). Data were analyzed for statistical significance using Mann-Whitney test for 2 groups. Differences between groups with a probability of <0.05 were regarded as significant.

Where not indicated, data were not statistically significant.

Supplementary Table 1

Supplementary Figure 1.

Representative gating strategy using FlowJo software (TreeStar Inc.) to identify Vδ1 and Vδ2 subsets of γδ T cells in (A) peripheral blood (red counter plot), (B) CRC samples (green counter plot) and (C) healthy mucosa samples (blue counter plot). Arrows indicate the flow chart of the analysis.