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Original Research / Basic Tumor Immunology

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

Running title: Impact of Vδ1 T cells in liver metastatic cancer.

Supplemental Material and Methods

Flow cytometry analysis

PBMCs were stained with Zombie Aqua™ fixable viability kit (BioLegend; San Diego, CA, USA) and classical protocol for staining was used.[1] All reagents and monoclonal antibodies clones are showed as supplemental data (**online supplemental table 1).** All samples were acquired by FACS Symphony A5 flow cytometer (BD Biosciences). Flow cytometry data were compensated by using single stained controls with BD Compbeads (BD Biosciences) conjugated to the specific fluorescent mAb, according to the guidelines for an accurate multicolor flow cytometry analysis.[1] All flow cytometry data, comprising the dimensionality reduction method with t-distributed stochastic neighbor embedding (*t-SNE*) algorithm, were analyzed by FlowJo software version 9.9.6 (FlowJo LLC; Ashland, OR, USA).

Immunohistochemistry

Immunohistochemistry (IHC) experiments were performed on 2 μm-thick paraffin-embedded sections of liver specimens. Slides were deparaffinized, rehydrated and antigen-retrieval technique was performed with a pH 9 EDTA solution at 98°. After rinsing in dH_2O, inhibition of endogenous peroxidase was performed with Peroxidase-Blocking Solution (Dako Agilent Technologies; Santa Clara, CA, USA). After washes in Phosphate-buffered saline (PBS; Lonza), slides were incubated with the specific mAb anti-γδTCR (clone B1; BD Biosciences; used 1:50) or

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anti-CD3 (Clone F7.2.38; Dako Agilent Technologies; used 1:100). Then the sections were washed and incubated with the secondary Ab- labeledlabelled polymer-HRP (EnVision; Dako; Agilent Technologies). Staining was visualized by 3,3'-diaminobenzidine solution (DAB) and counterstained with Hematoxylin both from Dako Agilent Technologies. Prepared slides were preserved by using the Eukitt mounting medium (Kindler; Freiburg, Germany). The percentage of positive cells was evaluated in a double-blind fashion by two expert pathologists. Images were acquired with Olympus BX51 microscope (Center Valley, PA, USA).

Single cell (sc)RNA-sequencing

Lymphoid cells isolated from blood and liver of 3 CLM patients were subjected to scRNA-seq analysis *(raw data are available from the corresponding author upon reasonable request)*. To avoid sample selection bias, we selected for scRNA-seq experiments 3 representative patients (two men and one female) who underwent limited liver resection for synchronous CLM disease. These 3 patients were treated with standard combination of Bevacizumab/Cetuximab na-CHT prior surgery. scRNA-seq analysis of γδ T cells was performed starting from total CD3+ T lymphocytes, as a part of larger study.

Freshly isolated $CD3^+$ flow cytometry-sorted cells were loaded into one channel of the Single Cell Chip A using the Chromium Single Cell 5' Library Construction Kit (10X Genomics; Pleasanton, CA, USA) for Gel bead Emulsion generation into the Chromium system. Following capture and lysis, cDNA was synthesized and amplified for 14 cycles following the manufacturer's protocol. 50 ng of the amplified cDNA were then used for each sample to construct Illumina sequencing libraries. Sequencing was performed on the NextSeq500 Illumina sequencing platform. 5' based sequencing data were aligned and quantified using the Cell Ranger Single-Cell Software Suite (version 3.0.2; 10X Genomics) against the GRCh38 human reference genome. For quality check and downstream clustering analysis, we used the Seurat Pipeline,[2] (version 3.1.1; R version 3.6.1). We used the *FindIntegrationAnchors* function to combine PT, DT and PB $CD3^+$ T cells from

3 CLM patients separately. In order to increase the statistical power (number of cells/clusters) of our dataset and to remove batch effects across different subjects, PT, DT and PB $CD3^+$ T cells from 3 CLM patients were integrated separately using an algorithmic approach based on Canonical Correlation Analysis (CCA). For each integrated data set, principal component analysis (PCA) was performed using the 2000 highly variable genes with the *RunPCA* function. Clusters were identified with the *FindClusters* function based on the first 30 PCs and Uniform Manifold Approximation and Projection (UMAP) were used for visualization. γδ T cell clusters were identified based on the average expression gamma-delta constant (*TRDC, TRGC1, TRGC2*) and variable (*TRDV1*, *TRDV2*, *TRDV3)* region-encoding segments. We then integrated and reclusterized the PT and DT γδ T cell clusters together. Differentially expressed genes were calculated using the Seurat FindAllMarkers function and the default Wilcoxon Rank Sum test, with thresholds of genes expressed by 25% of cells and with a log fold change of 0.25.

HCMV serology

Plasma from the study participants were thawed and analyzed for the presence of CMV IgG antibodies according to the manufacturer's instructions (Omega Diagnostics; Alva; UK).

Gene Ontology Enrichment analysis

Gene Ontology (biological process) enrichment analysis was performed by the R package clusterProfiler by using DEGs with adj. P value ≤ 0.05 and Log_2-FoldChange > 0. Only enriched Gene Ontology terms with adj. P value ≤ 0.05 and more than five genes were analyzed.

Clinical outcome GEPIA2 analysis

Gene Expression Profiling Interactive Analysis 2 (GEPIA2; http://gepia2.cancer-pku.cn/#index) is an open-access dataset for analyzing RNA sequencing expression data from tumors and normal samples from The Cancer Genome Atlas (TCGA; https://tcga-data.nci.nih.gov/tcga/). GEPIA2 was used to assess disease-free survival and OS of TCGA patients

with CRC. The cox proportional hazard ratio (HR) of the *Vd1-Cy-SI* high and low-expression cohort was calculated by GEPIA2, while 95% CI was calculated as "exp [ln(HR)± z*SE], with SE standard error.[3]

High-throughput analysis of TRG and TRD repertoires

CDR3 regions of either the γ-chain (TRG) or δ-chain (TRD) were amplified from flow cytometry-sorted (FACS Aria III cell sorter; BD Biosciences) total liver Vδ1 T cells and either PB $CD69^+$ or $CD69^-$ Vδ1 T cells via a previously described mRNA-based multiplex PCR amplification method.[4] PCR amplicons were indexed with the Illumina Nextera Index Kit, purified with Agencourt AMPure beads, equally pooled with 96-samples and sequenced at the Illumina MiSeq platform (paired-end, 500 cycles, high-output) as recommended by Illumina guidelines, while 20% PhIX was used as a spike-in control. The obtained fastq files of read1 were annotated with MiXCR software[5] and further analyzed with the R package TcR[6] and VDJtools.[7]

Statistical analysis

Analysis was performed using GraphPad Prism version 7. For the comparison of 2 groups of samples paired *Wilcoxon* test or unpaired *Mann-Whitney* or *Kolmogorov-Smirnov* tests was applied. Experiments with more than 2 groups were analyzed by *Kruskal-Wallis* test with *Dunn's* multiple comparisons test. To test the normality of our data distribution, we used *Shapiro–Wilk* test. When specified, Pearson's or Spearman's rank correlations analysis were used. The data are presented as mean value ±estimated standard error (±SEM). Statistically significant *P* values were represented with GraphPad (GP) style and summarized with following number of asterisks (*): *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$. Overall survival (OS) was examined from date of liver resection to date of death or last available follow-up. *Kaplan-Meier* survival curves were generated and compared using the log-rank test. The Cox proportional hazard model was applied for the identification of independent prognostic factors for OS.

References for Supplemental Material and Methods

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