Supplementary Information

Title: Immunological features of patients with advanced hepatocellular carcinoma before and during sorafenib or anti-PD-L1/PD-1 treatment

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Flow cytometry analyses:

Immediately after the liver biopsy procedure, tumor and non-tumor samples were transferred in RPMI medium and cells were recovered by mechanical disruption. Fresh peripheral blood and intrahepatic cell suspensions were immunostained without any stimulation (as pulished previously: [1, 2]), with the following anti-human antibodies of surface markers:

Tube 1 included anti-CD45-APC/Cy7 (clone HI30, BioLegend), anti-CD3-PerCP-Cy5.5 (clone UCHT1, BioLegend), anti-CD56-BV605 (clone HCD56, BioLegend), anti-CD16-AF700 (clone 3G8, BioLegend), anti-CD15-BV510 (clone W6D3, BioLegend), anti-CD8-PE/Cy7 (clone RPA-T8, BD Biosciences), anti-CD69-PE (clone FN50, BioLegend), anti-CTLA4-BV421 (clone BNI3, BioLegend), anti-PD-1-BV711 (clone EH12.2H7, BioLegend), anti-PD-L1-FITC (clone MIH1, BD Bioscience).

Tube 2 included anti-CD45-APC/Cy7 (clone HI30, BioLegend), anti-CD3-PerCP-Cy5.5 (clone UCHT1, BioLegend), anti-CD56-BV605 (clone HCD56, BioLegend), anti-CD16-AF700 (clone 3G8, BioLegend), anti-CD15-BV510 (clone W6D3, BioLegend), anti-CD8-PE/Cy7 (clone RPA-T8, BD Biosciences), anti-LAG3-PE (clone 3DS223H, eBioscience), anti-4-1BB-BV421 (clone 4B4-1, BioLegend), anti-TIM3-BV785 (clone F39-2E2, BioLegend), anti-OX40-FITC (clone Ber-ACT35, BioLegend).

Corresponding isotype controls were used as controls. Zombie UV™ Fixable Viability kit was used to exclude dead cells. FluoroFix™ Buffer (Biolegend) was used for fixation to stabilize tandem dyes. Data were acquired on BD-LSRII flow cytometer (BD Biosciences, Le Pont-De-Claix, France), collected with BD FACSDiva 6.3.1 software and analyzed using FCS Express 6 Flow software.

References:

1. Macek Jilkova Z, Afzal S, Marche H, Decaens T, Sturm N, Jouvin-Marche E, et al. Progression of fibrosis in patients with chronic viral hepatitis is associated with IL-17(+) neutrophils. Liver international : official journal of the International Association for the Study of the Liver 2016; 36:1116-24.
2. Macek Jilkova Z, Decaens T, Marlu A, Marche H, Jouvin-Marche E, Marche PN. Sex Differences in Spontaneous Degranulation Activity of Intrahepatic Natural Killer Cells during Chronic Hepatitis B: Association with Estradiol Levels. Mediators of inflammation 2017; 2017:3214917.

Supplementary Table 1 : Patient Characteristics

Advanced HCC patients, n=21	
Sex, male/female (n ; %)	17/4 ; 81%/19%
Age, y (±SE)	71.57±0.42
Bilirubin, mg/dL (±SE)	0.95±0.03
Albumin level (g/dL) (±SE)	39.38±0.28
INR (±SE)	1.09±0.1
Neutrophil/Lymphocyte ratio (±SE)	0.68±0.01
MELD score (±SE)	7.90±0.11
Child Pugh Score (median ; range)	5 ; 5-8
Liver fibrosis, METAVIR score ; F0-F2/F3/F4	5/5/11
Etiology of liver disease	
Alcohol	8
Alcohol+metabolic syndrome	3
NASH	3
HCV/HBV	3/1
No known liver disease	3
Alpha fetoprotein, ng/mL (median ; range)	32 ; 1-32.007
Number of HCC nodules/liver ; 1/2/3/>4	1/1/3/16
Mean largest size of HCC, mm (±SD)	86.26±2.46
Macrovascular invasion, Y/N (n ; %)	11/10 ; 52%/48%
Extrahepatic metastasis, Y/N (n ; %)	19/2 ; 91%/9%
BCLC stage, B/C/D (n ; %)	1/19/1 ; 5%/90%/5%
Obtained samples at baseline, Blood/Tumor/Non-tumoral liver biopsy	21/16/13
Treatment, sorafenib/ anti-PD-L1-TGF-β TRAP / anti-PD-1 antibody/ tepotinib / untreated	7/4/4/2/4

Non-alcoholic steatohepatitis (NASH); international normalized ratio (INR); neutrophil-to-lymphocyte ratio (NL ratio); model for end-stage liver disease (MELD); Barcelona Clinic Liver Cancer system (BCLC).

Supplementary Table 2 : Absolute number of T cells analysed by flow cytometry per tube

	T cells ($CD45^+CD3^+CD56^-$)	$CD4^+$ T cells	$CD8^+$ T cells
Blood	35463±3223	26448±2452	9016±890
Tumor	4843±614	2504±329	2339±313
Non-tumor	5773±765	2798±444	2975±358

Supplementary Table 3 : Correlations of secreted IL-10 levels by PBMC with circulating $CD4^+/CD8^+$T cells ratio

	(pg/mL)	Spearman coefficient	p-value
Blood $CD4^+/CD8^+$T cells ratio	IL10 non-stimulated	0.3609	*0.1289*
	IL10 PMA/Iono stimulated	0.4072	*0.0835*
Tumor $CD4^+/CD8^+$T cells ratio	IL10 non-stimulated	0.5627	***0.0391***
	IL10 PMA/Iono stimulated	0.5459	***0.0461***
Non-tumor $CD4^+/CD8^+$T cells ratio	IL10 non-stimulated	0.6552	*0.3320*
	IL10 PMA/Iono stimulated	0.6568	***0.0319***

Supplementary Table 4 : Correlations of immune checkpoint-positive T cells

Correlation	Blood vs Tumor		Blood vs Non-Tumor	
	Spearman coefficient	p-value	Spearman coefficient	p-value
$PD\text{-}1^+$ T cells	0.4857	*0.0505*	0.4286	*0.1440*
$LAG3^+$ T cells	0.4685	*0.1245*	0.5667	*0.1116*
$TIM3^+$ T cells	0.4505	*0.1223*	0.4667	*0.1739*
$CTLA4^+$ T cells	0.2476	*0.6796*	0.2249	*0.5321*
$4\text{-}1BB^+$ T cells	0.6143	***0.0255***	0.4545	*0.1869*
$OX40^+$ T cells	0.1871	*0.5406*	0.4255	*0.2202*

Supplementary Table 5: Correlations of immune checkpoint-positive cells with circulating Alpha-fetoprotein levels

(%)	(ug/L)	Spearman coefficient	p-value
Intra-tumoral $PD\text{-}1^+$ T cells	Alpha-fetoprotein	0.5022	***0.0474***
Intra-tumoral $LAG3^+$ T cells	Alpha-fetoprotein	0.8182	***0.0011***
Intra-tumoral $LAG3^+$ $CD8^+$T cells	Alpha-fetoprotein	0.7343	***0.0065***
Intra-tumoral $OX40^+$ T cells	Alpha-fetoprotein	0.7125	***0.0063***
Intra-tumoral $OX40^+$ $CD4^+$T cells	Alpha-fetoprotein	0.7373	***0.0040***
Intra-tumoral $CD69^+CD4^+$T cells	Alpha-fetoprotein	0.6259	***0.0095***

Supplementary Figure 1

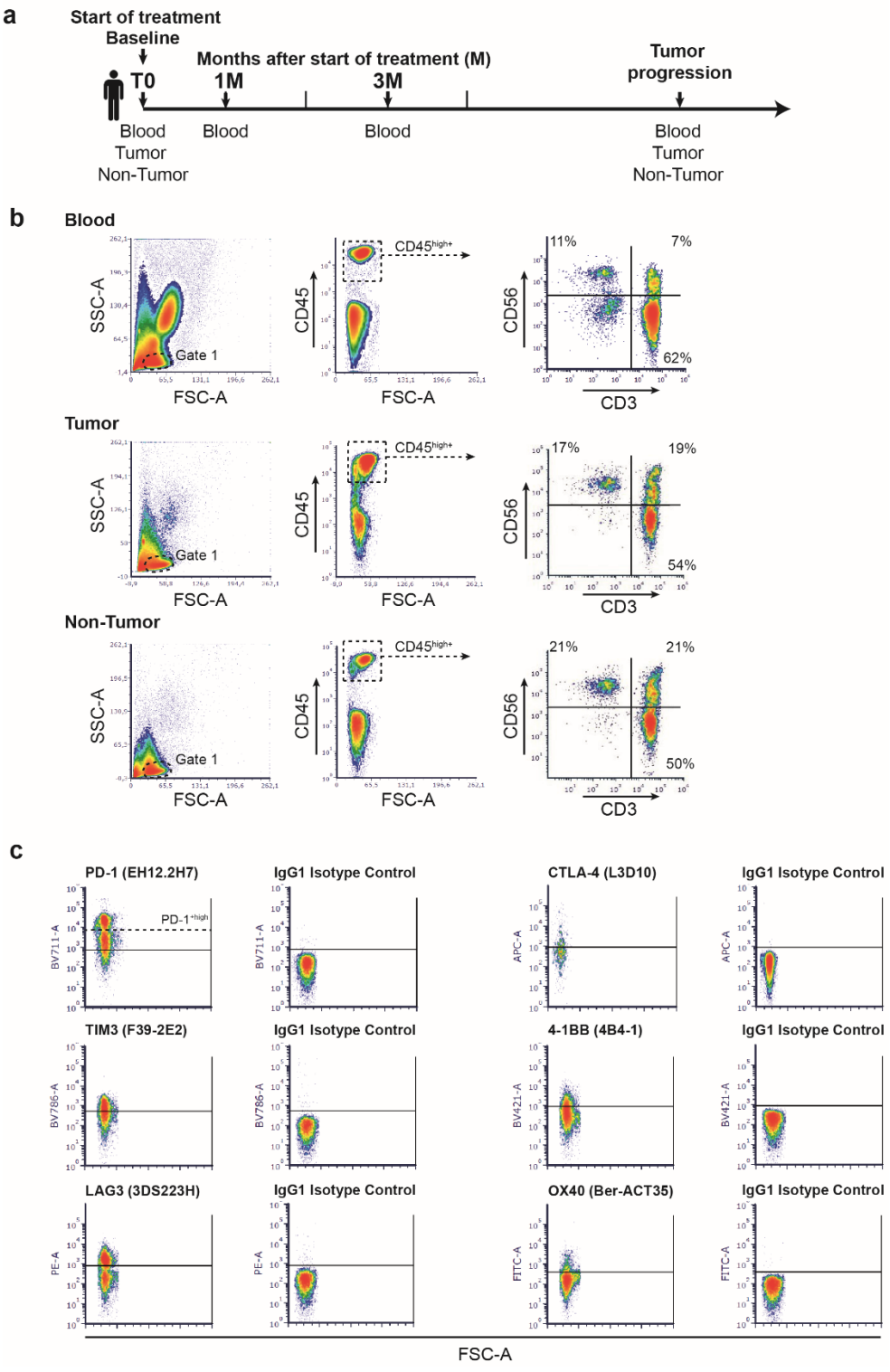

Figure 1 : Sampling plan of patients with HCC and gating strategy for flow cytometry analysis

A) Sampling plan of patients with HCC. Samples were collected only when the condition of the patient allowed it. B) Gating strategy for flow cytometry analysis. Fresh samples of blood, tumor and non-tumoral liver tissues were stained for FACS analyses. After exclusion of dead cells and doublets, lymphocytes were gated based on SSC and FSC and then based on CD45 expression. We identified CD3-CD56+ NK cells, CD3+CD56+ cells (NKT and CD3brightCD56+T cells) and CD3+CD56- classical T cells. C) Representative pictures of staining on intra-tumoral T cells. Fluorescence-labeling of immune checkpoint molecules and their isotype controls were used to set limits for the discrimination of positive vs. negative events.

Supplementary Figure 2

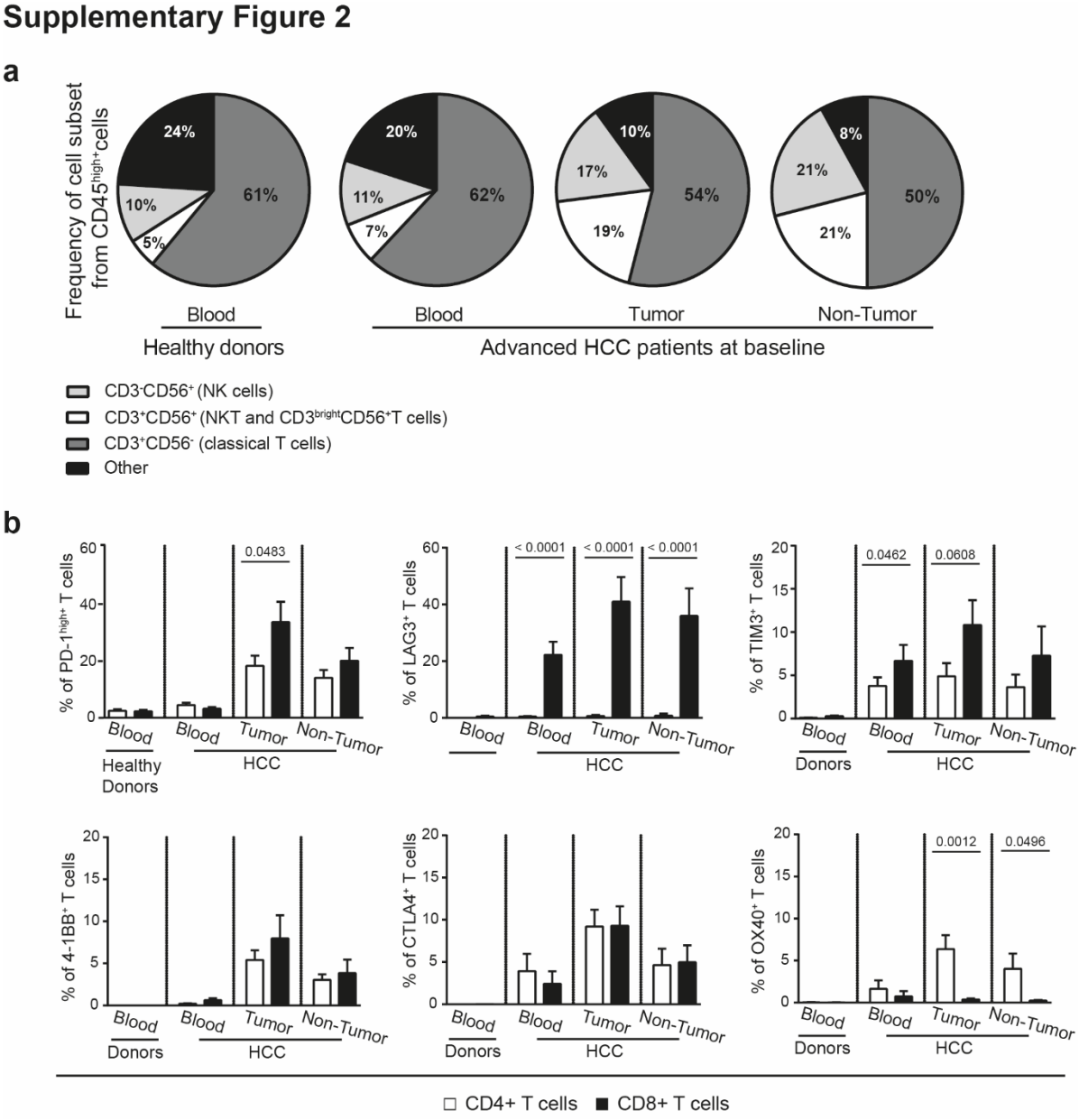

Figure 2: ***Distribution of lymphocyte subsets in healthy donors and in patients with advanced HCC***

A) Frequency of immune cell populations gated from CD45high+ lymphocytes in blood of healthy donors (n=7), blood of HCC patients (n=21), tumor (n=16), non-tumoral tissue (n=13). We identified CD3-CD56+ NK cells, CD3+CD56+ cells (NKT and CD3brightCD56+T cells) and CD3+CD56- classical T cells. B) The percentage of immune checkpoint-positive cells among CD4+ T cells or CD8+ T cells in in blood of healthy donors (n=7), blood of HCC patients (n=21), tumor (n=16), non-tumoral tissue (n=13).