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# Immunophenotyping of the Hepatocellular Carcinoma Patients

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vorgelegt von

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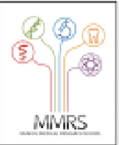

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# II. List of Abbreviations

| | |
|---|---|
| % | percentage |
| °C | degree celsius |
| µg | microgram |
| µl | microliter |
| µm | micrometer |
| AFB1 | aflatoxin B1 |
| aCTL | activated cytotoxic T cells |
| aTregs | activated regulatory T cells |
| aTh | activated helper T cells |
| BCLC | barcelona clinic liver cancer staging |
| BRC | biological resection criteria |
| Bregs | regulatory B cells |
| CD | cluster of differentiation |
| CHB | chronic hepatitis B |
| cmT cells | central memory T cells |
| cs-memory B cells | class-switched memory B cells |
| CTL | cytotoxic T lymphocytes |
| DC | dendritic cells |
| DFS | disease free survival |
| emT cells | effector memory T cells |
| eT cells | effector T cells |
| FACS | fluorescence–activated cell sorting |

| | |
|---|---|
| FCM | flow cytometry |
| FBC | full blood count |
| G-MDSC | granulocyte-like MDSC |
| HBV | hepatitis B virus |
| HBeAg | hepatitis B envelope antigen |
| HCC | hepatocellular carcinoma |
| HCV | hepatitis C virus |
| HD | healthy donors |
| IFN-γ | interferon-γ |
| Ig | immunoglobulin |
| IL | interleukin |
| LAT | local ablative treatment |
| LC | liver cirrhosis |
| LMR | lymphocyte-to-monocyte ratio |
| LR | liver resection |
| LT | liver transplantation |
| maTregs | memory-activated Tregs |
| mTregs | memory Tregs |
| MC | milan criteria |
| MCP-1 | monocyte chemoattractant protein 1 |
| MDSC | myeloid-derived suppressor cells |
| M/GLR | monocyte/granulocyte to lymphocyte ratio |
| min | minutes |

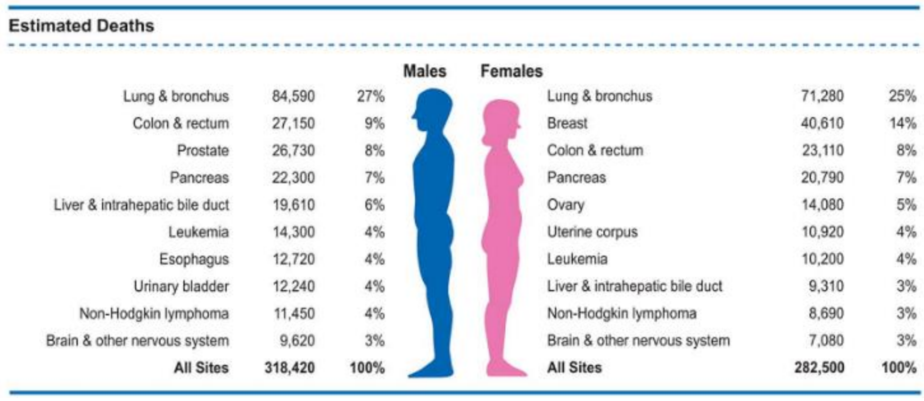
| | |
|---|---|
| ml | milliliter |
| mTregs | memory Tregs |
| ns-memory B cells | non class-switched memory B cells |
| nT cells | naive T cells |
| nTregs | naive Tregs |
| NLR | neutrophil to lymphocyte ratio |
| NK | natural killer |
| NKT | natural killer T |
| NMLR | neutrophil-monocyte to lymphocyte ratio |
| OS | overall survival |
| PB | peripheral blood |
| PBMC | peripheral blood mononuclear cells |
| Pre B cells | precursor B cells |
| Pro B cells | progenitor B cells |
| RFS | recurrence-free survival |
| Th | helper T cells |
| Th1 | type 1 helper T cells |
| Th2 | type 2 helper T cells |
| Th17 | type 17 helper T cells |
| Tregs | regulatory T cells |
| TILs | tumor-infiltrating leukocytes |
| TACE | transcatheter arterial chemoembolization |
| WB | whole blood |

# 1. Introduction

Primary hepatic carcinoma (PHC) is a malignant tumor that develops from either hepatocytes or intrahepatic biliary epithelial cells. Hepatocellular carcinoma (HCC) is the most common type of PHC. Even though medical technologies have been constantly improving, the overall 5-year survival rate remained stable and generally unfavorable.[1] Currently, it is believed that the progression and prognosis of various tumors, including HCC, are related to the immune status of the patient. Tumor-infiltrating leukocytes (TILs) have been shown to be predictive. These measurements however are only possible after the treatment. To truly predict outcome after surgical resection of HCC I established a comprehensive assessment of the immunophenotype of HCC patients.

## 1.1. Epidemiology of HCC

Hepatitis B virus (HBV) infections and hepatitis C virus (HCV) infections account for 75-80% and 10-20% of virus-associated HCC.[2] Cancer statistics estimated that 28920 people died of liver cancer and intrahepatic bile duct cancer worldwide in 2017. This globally accounts for 9% of the world's cancer deaths (Figure 1).[3] These statistics also emphazise that the incidence of liver cancer in the world increases by approximately 4% in men and 3% in women per year.[4]



**Estimated Deaths**

| Males | | | Females | | |
|---|---|---|---|---|---|
| Lung & bronchus | 84,590 | 27% | Lung & bronchus | 71,280 | 25% |
| Colon & rectum | 27,150 | 9% | Breast | 40,610 | 14% |
| Prostate | 26,730 | 8% | Colon & rectum | 23,110 | 8% |
| Pancreas | 22,300 | 7% | Pancreas | 20,790 | 7% |
| Liver & intrahepatic bile duct | 19,610 | 6% | Ovary | 14,080 | 5% |
| Leukemia | 14,300 | 4% | Uterine corpus | 10,920 | 4% |
| Esophagus | 12,720 | 4% | Leukemia | 10,200 | 4% |
| Urinary bladder | 12,240 | 4% | Liver & intrahepatic bile duct | 9,310 | 3% |
| Non-Hodgkin lymphoma | 11,450 | 4% | Non-Hodgkin lymphoma | 8,690 | 3% |
| Brain & other nervous system | 9,620 | 3% | Brain & other nervous system | 7,080 | 3% |
| **All Sites** | **318,420** | **100%** | **All Sites** | **282,500** | **100%** |

**Figure 1: Ten leading cancer types for new cancer deaths, 2017.** [3]

## 1.2. Treatment of HCC

At present, there are many therapeutic methods against HCC, mainly including surgery and non-surgical treatments. When treating HCC various factors, such as tumor volume, tumor number, metastasis, liver function reserve capacity, patient's age and complications should be considered.[5, 6]

Currently the standard treatment algorithm for HCC patients is Barcelona Clinic Liver Cancer (BCLC) staging system (Figure 2).[7] Surgical resection of early HCC in compensated cirrhosis or in non-cirrhosis is likely to result in long-term survival and can be considered curative. For advanced HCC the prognosis is poor. Liver transplantation (LT) is the most effective treatment for small liver cancer within cirrhosis that otherwise could not be resected (BCLC A). During LT, the tumor and the underlying liver cirrhosis, which can be considered a precancerosis, are removed. However, livers for transplantation are scarce and not every patient can receive the needed organ for the treatment of the disease. Therefore clinicians have opted to allocate these organs to patients who supposedly have the best survival.[8] At present, the most widely used criteria for selection are the Milan Criteria (MC). The MC was developed by Mazzaferro et al. in 1996, that is, one lesion with a diameter no more than 5 cm, or up to 3 lesions with the maximum diameter smaller than 3 cm, without vascular and lymph node invasion. [9] After the MC was proposed and implemented, the 5-year survival rate of HCC patients with LT increased from 25.3% to 61.1%.[10] Although HCC patients who meet the MC have a good prognosis after LT, however, with the progress of LT technology and the growing understanding of HCC, some researchers have found that some patients beyond MC can still achieve satisfactory results after LT.[11] This means the MC are regarded as overly strict and might exclude patients from transplantation

even though the benefit for the individual patient could be immense compared to interventional palliative treatment.[12] In addition, the MC pays less attention to the indicators affecting the prognosis of HCC, such as tumor differentiation, invasion, metastasis degree and liver function. These deficiencies prompt researchers to further explore a change in allocation of LT for HCC. Local ablative treatment (LAT) is the most important non-surgical treatment of HCC. Compared with surgical treatment, LAT is minimally invasive and can be utilized either as palliative treatment or bridging-to-transplant treatment.[13] Most chemotherapeutic drugs are less sensitive to primary liver cancer, such as adriamycin, gemcitabine, cisplatin, 5-fluorouracil.[14] The only officially approved targeted therapy drug for HCC is Sorafenib.

In summary, due to overly strict subsidiary selection of patients scheduled for LT and unclear prediction of postoperative course, treatment allocation of our patients needs to be optimized to achieve the best results.

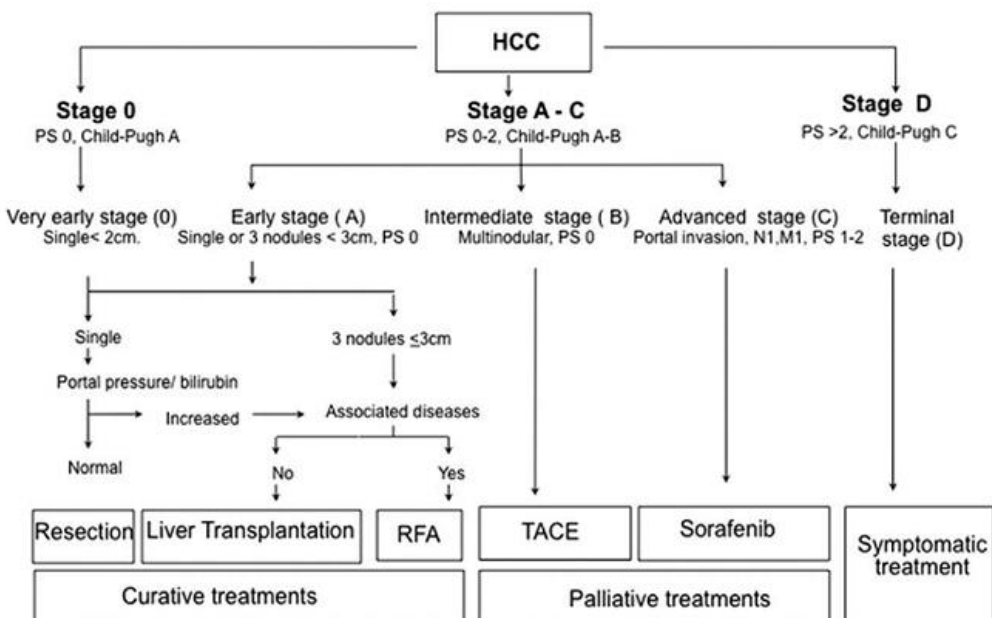



**Figure 2: The BCLC staging system.**

## 1.3. Predictive Markers for HCC after Surgery

A variety of factors may predict recurrence and overall survival (OS) after surgical treatment of HCC. I will concentrate on 9 key variables which I will describe in more detail below (Table 1).

The impact of patient's age and gender on its prognosis has been controversial. Most data indicate that the patient's age relates to prognosis. Surgical resection rate and postoperative prognosis of young patients were better than that of elderly patients, due to their better liver reserve function than elderly patients.[15, 16] However, there is also data showing that young patients tend to have tumors with a higher grading and poorer prognosis compared to elderly people.[17] It is also reported that the prognosis of female HCC patients was better than male. Because liver is a hormone-sensitive organ, this can be associated with sex hormone receptors. Additionally  male patients tend towards a decreased adherence in medical treatment.[18]

In comparison, tumor-free survival rate of multiple HCC nodules was generally lower than a single nodule. Laurent et al. showed that the 5-year disease-free survival (DFS) rates of single nodules and multiple nodules after radical resection were 37% and 6%, respectively.[19] Moreover they found that HCC with satellite foci had a poor prognosis. Also, they reported that small HCC had a better prognosis than large HCC. Arii et al. reported that the 5-year survival rate was 71.5% in stage III patients with single tumors <2 cm, compared to 42.8% in those with tumors between 2 to 5 cm.[20] Bismuth et al. believed that the size (>9 cm) of the main nodule was an important factor affecting postoperative survival time.[21] However, our previous study does not show any relevance of survival and tumor multiplicity.[22] Zhu et al. found that microvascular invasion is an important factor in the early postoperative recurrence of HCC.[23]

Hepatitis and cirrhosis are important factors affecting the prognosis of HCC patients. It is reported that among HCC patients who had received hepatectomy, those who had active HBV infection had a lower OS rate.[24] Cirrhosis is one of the most important risk factors for the prognosis of HCC since it is a precancerosis. Therefore, patients with a HCC in cirrhosis have a relatively poor prognosis.[25] Not only the hepatocarcinogenic effects of cirrhosis lead to worsened survival in the long-term but also immediate perioperative complications because of cirrhosis. HCC patients with cirrhosis are prone to bleeding during surgery and their functional liver remnant are reduced leading to postoperative liver failure.[26]

It is suggested that the immune system is suppressed in HCC patients, and is therefore conducive to the occurrence and development of HCC. Unitt E et al. indicated that TILs, especially CD4/CD8 ratio could even predict recurrence after LT.[27] Our previous study also showed that perivascular infiltration of $CD3^+$, $CD8^+$ cells had significance on predicting OS and DFS of HCC patients that were resected.[22]

Tumor markers are proteins and other substances that are abnormal in structure and quantity produced by tumor cells. AFP is a marker to assist diagnosis and possibly prognosis of HCC. It was reported that AFP>800 ng/mL was an independent factor influencing the recurrence of HCC.[23] Lubrano et al. believed that high serum concentrations of aspartate transaminase and alanine transaminase in HCC patients before surgery would reduce OS after LR.[28] Chen et al. found that in HCC patients without cirrhosis and low concentrations of albumin have reduced postoperative survival rates.[29]

As mentioned above, many factors influence survival of HCC patients and ideally combinations out of clinical and experimental data could be used to predict patients' survival. Schoenberg et al. proposed the concept of the biological resection criteria

(BRC), to establish criteria for safe and oncological satisfactory hepatectomy for early HCC patients. These criteria are a combination of liver function, age and tumor immunology in a simple score. Within the criteria LR patients, who in general have poorer survival rates than LT patients, achieved similar postoperative results.[30] However a better understanding of the tumor immunological factors are needed to get an accurate risk stratification between LR and LT.

**Table 1. Predictive markers for HCC patients after surgery.**

| Patient specific variables | Tumor specific variables |
|---|---|
| Age | The number of tumor nodule |
| Gender | The size of tumor nodule |
| Hepatitis | Microvascular invasion |
| Cirrhosis | Tumor-infiltrating leukocytes |
| AFP level | |

## 1.4. Components of the Immune System in the Context of HCC

In this part the relevant players of the immune response to HCC will be described.

### 1.4.1. Cells of the Innate Immune System

Neutrophils are the most abundant white blood cells in the circulatory system.[31] They play an important role in infection immunity. Li et al. found that HCC patients with neutrophil infiltration in tumor had worse survival.[32] Moreover, neutrophil to lymphocyte ratio (NLR) is often used to evaluate the balance of systemic inflammatory response and immune system function. It was reported that NLR may closely correlate with the survival of gastric cancer, colorectal cancer, and HCC patients. [33-35]

Monocytes are important innate immune cells that are produced by bone marrow and remain in peripheral blood (PB) for a short time.[36] Monocytes not only have functions such as opsonophagocytosis and antigen presentation, but also can produce a variety of cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α.[37] A meta-analysis showed that high levels of the lymphocyte-to-monocyte ratio (LMR) was related with better OS of HCC patients.[38] Macrophages are mainly found in lymph nodes, alveolar walls, liver and so on. They are highly plastic, can be polarized to tumor-associated macrophages (TAMs).[39] Atanasov G et al. found that elevated tumor infiltrating TAMs was positively correlated with OS of cholangiocarcinoma patients.[40] DC are the most functional antigen-presenting cells (APC), which can uptake, process and present antigens.[41] One explanation of tumor cells escape immune surveillance, leading to the formation and development of tumors, is the functional defects of DC initiated by tumors. For example, significantly reduced amounts of DC in cancer patients or DC in tumor patients that have an immature phenotype.[42, 43]

Myeloid-derived suppressor cells (MDSC) are heterogeneous cell populations. According to the different morphology of MDSC, they can be divided into two cell subpopulations: Monocyte-like MDSC (M-MDSC) and granulocyte-like MDSC (G-MDSC).[44] Elwan N et al. reported that HCC patients had significant higher counts of

MDSC in the PB than in HD.[45] MDSC can not only inhibit T cells mediated specific immunity, but also directly inhibit the immune functions of macrophages and DC.[46] MDSC can also promote the expansion of regulatory T cells (Tregs).[47, 48]

NK cells are non-specific immune cells, often associated with the first line of anti-tumor responses. The NKG2 family is an important class of receptors on NK cells, including NKG2A, NKG2B, NKG2C, NKG2D and other members.[49] NKG2 family members can be divided into two types: activating receptors and inhibitory receptors, which transmit positive and negative signals, respectively.[50] Tumor cells can inhibit the expression of activated receptors and inhibit the activation and function of NK cells.[51] More and more evidence showed that NKG2D expression was down-regulated in NK cells of patients with HCC, gastric cancer and so on.[51-54] Natural killer T (NKT) cells are a special group of cells that co-express NK cell receptors and TCR. NKT cells recognize specific glycolipid antigens presented by CD1d molecules, which is similar to MHC I.[55] After activation, NKT cells can secrete IL-2, IFN-γ, perforin, granzyme and so on.[56]

### 1.4.2. Cells of the Adaptive Immune System

T cells are the major player in cellular immunity. They have two subsets: Th and CTL. Th can assist B cells to secrete antibodies while CTL are the major cytotoxic effector cells.[57] Th differentiate into distinct cell subpopulations under the control of different specific transcription factors: Type 1 helper T cells (Th1), type 2 helper T cells (Th2), type 17 helper T cells (Th17) and Tregs.[58] Th1 cells mainly secrete IFN-γ, IL-2 and TNF-α, which can promote cellular immunity.[59] Th2 cells secrete IL-4 and IL-10 and mediate humoral immunity.[60] Th17 cells secrete high levels of IL-17, IL-21, IL-22, IL-6 and TNF-a.[61] A prospective study showed that HCC patients had higher amounts of Th17 in PB than in HD.[62] Tregs are an immunosuppressive cell subpopulation, which can maintain the tolerance of the immune system to its own components and maintain

the immune homeostasis.[63] It has immunoregulatory properties by releasing cytokines IL-10 and TGF-β.[64] Higher densities of Tregs were observed in paracancerous tissues of HCC, additionally, higher Tregs levels was associated with a worse prognosis.[65] According to the different differentiation stages, T cells can also be divided into: naive T cells, effector T cells and memory T cells.[66] After initial exposure to antigens, naive T cells can be activated, differentiate into effector T cells after recognizing the antigen presented by APC and migrate to the antigen site under chemotaxis to kill infected and transformed cells. When the antigen is cleared, most of the effector T cells will perform apoptosis. A portion of them however can differentiate into memory T cells.[66] Memory T cells can be further divided into central memory T cells (cmT cells) and effector memory T cells (emT cells) according to different homing characteristics and effector functions.[67] The emT cells exist in the non-lymphoid tissue of the infected site and perform immunological surveillance. When exposed to the antigen again, they can produce IFN-γ and IL-4 under T-cell receptor (TCR) stimulation. The cmT cells can rapidly expand and differentiate into effector T cells to aide in antigen clearance.[68]

B cells are an important part of humoral immunity. The differentiation of hematopoietic stem cells into mature B cells has gone through many stages: progenitor B (pro-B) cells, precursor B (pre-B) cells, immature B cells, transitional B cells and mature B cells.[69] In human PB, according to the different developmental stages and functions, B cells can be divided into naïve B cells, memory B cells and plasma cells. When naive B cells are sensitized by Th or by certain microbial antigens, part of them will rapidly proliferate into plasma cells.[70] These plasma cells can produce immunoglobulin (Ig) M, the remaining naive B cells will continue to develop and form the germinal center B cells.[71, 72] This continue to develop into memory B cells that eventually differentiate into plasmablasts.[73] Plasmablasts then evolve into plasma cells that secrete IgM, IgG and IgA.[74] At this stage they are called long-lived plasma cells. Memory B cells can be divided into non class-switched memory B cells (ns-memory B cells) and class-

switched memory B cells (cs-memory B cells). Both them play important roles in secondary immunity. The former mainly secretes IgM after stimulation and mediates protective immunity, while the latter mainly secretes IgG and IgA.[75] Regulatory B cells (Bregs) are a group of recently discovered B cells. Bregs can suppress the differentiation and proliferation of various cells by secreting IL-10 and TGF-β.[76, 77]

## 1.5. Aim of this Study

The purpose of this project was to establish an immunophenotyping protocol of relevant immune cells in non-HBV/non-HCV HCC patients prior surgery. Furthermore, we aim to investigate differential expression of immune cells between HCC patients and HD.

# 2. Material and Methods

## 2.1. Materials

### 2.1.1. Laboratory Equipment

| | |
|---|---|
| Centrifuge | Heraeus, Germany |
| Cell culture incubator | Binder, Germany |
| Combitips Plus | Sigma-Aldrich, USA |
| Flow Cytometer | BD Biosciences, USA |
| Multipette Plus | Eppendorf, Germany |
| Pipettes | Sigma-Aldrich, USA |
| Vortex | Labnet, Germany |
| Water bath | Köttermann, Germany |
| 4℃ fridge | Liebherr, Germany |

### 2.1.2. Computer and Software

| | |
|---|---|
| Computer hardware | HP, USA |
| FACSDIVA™ SOFTWARE | BD, USA |
| Prism | Version 7.0, USA |
| SPSS | Version 21, USA |

### 2.1.3. Consumables

| | |
|---|---|
| 0.5-20µl Ep T.I.P.S | Eppendorf, Germany |
| 2-200µl Ep T.I.P.S | Eppendorf, Germany |
| Gloves | ecoSHIELD, USA |
| 7.5ml Heparin vacuum blood collection tube | Sarstedt, USA |
| 5ml Polystyrene Round-Bottom Tube | Falcon, USA |
| 15ml Falcon | Falcon, USA |
| 50ml Falcon | Falcon, USA |
| 40µm cell strainer | BD, USA |
| 100µm cell strainer | BD, USA |

### 2.1.4. Chemical

| | |
|---|---|
| Ammonium Chloride | Fluka, USA |
| Bovine Serum Albumin(BSA)Fraction V | Biomol,Germany |
| Calcium bicarbonate | Fluka, USA |
| Collagenase NB 8 | SERVA, Germany |
| EDTA | Calbiochem,Germany |
| FACS Lysing Solution | BD, USA |
| Golgi Stop | BD, USA |

| | |
|---|---|
| leukocytes activation kit | BD, USA |
| IC Fixation Buffer(10x) | eBiosciences, Austria |
| Permeabilization buffer(10x) | eBiosciences, Austria |
| Trypsin EDTA | Lonza,Switzerland |
| trypan blue | Sigma,Germany |

### 2.1.5. Buffers and Solutions

| | | |
|---|---|---|
| ACK Lysing Buffer | pH | 7.3 |
| | 8.3g/l | Ammonium Chloride |
| | 1g/l | Calcium bicarbonate |
| | 0,0372g/l | EDTA |

| | | |
|---|---|---|
| 2.55U/ml Collagenase NB 8 | | |
| | 250mg | Collagenase NB 8 |
| | 100ml | 1x DPBS |

| | | |
|---|---|---|
| 1x Lysing Solution | | |
| | 50ml | 10x Lysing Solution |
| | 450ml | Millipore $H_2O$ |

| | | |
|---|---|---|
| 1xPermeabilization buffer | pH | 7.3 |
| | 8ml | 10 x Permeabilization |
| | 72ml | Millipore H2O |
| | | |
| FACS buffer | pH | 7.3 |
| | 1 L | 1 x DPBS |
| | 2ml | Natriumacid |
| | 5g | BSA |

### 2.1.6. Antibodies

| Antibody | Isotype | Flourochrom | Reactivity |
|---|---|---|---|
| Anti-CD3 | Mouse (BALB/c) IgG1, κ | PerCP Cy5.5 | Human |
| Anti-CD4 | Mouse (BALB/c) IgG1, κ | BUV395 | Human |
| Anti-CD5 | Mouse (BALB/c) IgG1, κ | BV421 | Human |
| Anti-CD8 | Mouse (BALB/c) IgG1, κ | APC-H7 | Human |
| Anti-CD10 | Mouse (BALB/c) IgG1, κ | PE | Human |
| Anti-CD14 | Mouse BALB/c IgG2b, κ | BV510 | Human |
| Anti-CD15 | Mouse IgG1, κ | PECF594 | Human |
| Anti-CD16 | Mouse BALB/c IgG1, κ | FITC | Human |
| Anti-CD19 | Mouse (BALB/c) IgG1, κ | FITC | Human |
| Anti-CD20 | Mouse BALB/c IgG2a, κ | APC-H7 | Human |
| Anti-CD24 | Mouse BALB/c IgG2a, κ | PE-CF594 | Human |

| | | | |
|---|---|---|---|
| Anti-CD25 | Mouse (BALB/c) IgG1, κ | BB515 | Human |
| Anti-CD27 | Mouse (BALB/c) IgG1, κ | BV786 | Human |
| Anti-CD33 | Mouse BALB/c IgG1, κ | BV786 | Human |
| Anti-CD38 | Mouse (BALB/c) IgG1, κ | BV605 | Human |
| Anti-CD45 | Mouse (BALB/c) IgG1, κ | BV650 | Human |
| Anti-CD56 | Mouse BALB/c IgG2b, κ | APC R700 | Human |
| Anti-CD68 | Mouse BALB/c IgG2b, κ | BV711 | Human |
| Anti-CD69 | Mouse IgG1, κ | BUV395 | Human |
| Anti-CD127 | Mouse IgG1, κ | PE-CF594 | Human |
| Anti-CD194 | Mouse C57BL/6 IgG1, κ | BV510 | Human |
| Anti-CD196 | Mouse IgG1, κ | PE | Human |
| Anti-CD197 | Mouse IgG2a | BV421 | Human |
| Anti-CD1d | Mouse (BALB/c) IgG1, κ | APC | Human |
| Anti-CD11b | Mouse IgG1, κ | PECy7 | Human |
| Anti-CD11c | Mouse (BALB/c) IgG1, κ | PE | Human |
| Anti-CD45RO | Mouse (BALB/c) IgG2a, κ | PE-Cy7 | Human |
| Anti-CD66b | Mouse BALB/c IgM, κ | Alexa 647 | Human |
| Anti-IgD | Mouse BALB/c IgG2a, κ | PE-Cy7 | Human |
| Anti-IgM | Mouse (BALB/c) IgG1, κ | BV510 | Human |
| Anti-HLA-DR | Mouse IgG2a, κ | APC | Human |
| Anti-HLA-DR | Mouse IgG2a, κ | BV421 | Human |
| Anti-IFN-γ | Mouse IgG1, κ | FITC | Human |

## 2.2. Methods

### 2.2.1. Literature Review

A systematic literature review was conducted to investigate the available literature of circulating immune cells in HCC patients. The following search terms ("Liver Neoplasms/blood"[Mesh]) AND ("Leukocytes"[Mesh]) were used to search the PubMed and Medline database to assess the differences of circulating immune cells between HD and HCC patients. The last time point for the search was April 2018. The retrieval strategy was to browse the titles and abstracts of the literature to select relevant publications. When a relevant article was identified, the full text was retrieved and checked. Inclusion criteria: (1) research type: Clinical research; (2) research object: Human; (3) research content: Circulating immune cells; (4) literature languages: English. Exclusion criteria: (1) animal research or non-HCC; (2) clinical trials or studies of therapy; (3) basic researches on genes, proteins, etc.; (4) case reports, meta-analysis or reviews; (5) articles published before 2000 or not in English.

### 2.2.2. HD and Patients

In this study, 10 patients with primary HCC were recruited. None of them had HBV or HCV infection. All the patients underwent surgery from 2016 to 2017 at the Ludwig-Maximilians-University Munich (LMU) hospital. Among them: 7 males and 3 females; average age 58. 10 HD were used as healthy controls. Among them: 6 males and 4 females; average age 61; the collection of specimens obtained the informed consent of the volunteers. Institutional review board approvement was obtained (#EK 54-16, 53-16).

### 2.2.3. Definition of Monitored Cell Subsets

Immune cells can be separated into various subsets by their diverse nature, distinct activation stages, as well as differential cytokine production profiles. Specific subsets can be selected by different cluster of differentiation (CD) molecules. The definitions for immune cells involved in this study are described below. (Table 2)

**Table 2. Definition of measured cell subsets.** Abbreviations: Bregs: Regulatory B cells; CTL: Cytotoxic T cells; MDSC: Myeloid-derived suppressor cells; M-MDSC: Monocyte-like MDSC; G-MDSC: Granulocyte-like MDSC; NK: Natural killer cells; NKT: Natural killer T; pro B cells: Progenitor B cells; pre B cells: Precursor B cells; Tregs: Regulatory T cells; Th: Helper T cells; Th1: Type 1 helper T cells; Th2: Type 2 helper T cells; Th17: Type 17 helper T cells.

| Cell type | Marker |
|---|---|
| T cells, % of Leukocytes | $CD3^+$, % of $CD45^+$ |
| Th, % of T cells | $CD4^+/CD8^-$, % of $CD45^+/CD3^+$ |
| CTL, % of T cells | $CD8^+/CD4^-$, % of $CD45^+/CD3^+$ |
| Th1, % of Th | $CD4^+/CD8^-/CCR4^-/CCR6^-$, % of $CD45^+/CD3^+$ |
| Th2, % of Th | $CD4^+/CD8^-/CCR4^+/CCR6^-$, % of $CD45^+/CD3^+$ |

| | |
|---|---|
| Th17, % of Th | $CD4^+/CD8^-/CCR4^+/CCR6^+$[78], % of $CD45^+/CD3^+$ |
| Effector memory T cells, % of T cells | $CCR7^-/CD45RO^+$, % of $CD45^+/CD3^+$ |
| Central memory T cells, % of T cells | $CCR7^+/CD45RO^+$, % of$CD45^+/CD3^+$ |
| Effector T cells, % of T cells | $CCR7^-/CD45RO^-$, % of $CD45^+/CD3^+$ |
| Naïve T cells, % of T cells | $CCR7^+/CD45RO^-$[79], % of $CD45^+/CD3^+$ |
| Activated T cells, % of T cells | $HLA\text{-}DR^+/CD38^+$[80] , % of $CD45^+/CD3^+$ |
| Regulatory T cell, % of Th | $CD4^+/CD8^-/ CD25^+/CD127^-$, % of $CD45^+/CD3^+$ |
| Memory Tregs, % of Tregs | $HLA\text{-}DR^-/CD45RO^+$, % of $CD45^+/CD3^+/CD4^+/CD8^-/ CD25^+/CD127^-$ |
| Naive Tregs, % of Tregs | $HLA\text{-}DR^-/CD45RO^-$, % of $CD45^+/CD3^+/CD4^+/CD8^-/ CD25^+/CD127^-$ |
| Activated Tregs, % of Tregs | $HLA\text{-}DR^+/CD45RO^-$, % of $CD45^+/CD3^+/CD4^+/CD8^-/ CD25^+/CD127^-$ |

| | |
|---|---|
| Memory-activated Tregs, % of Tregs | HLA-DR$^+$/CD45RO$^+$[81-83], % of CD45$^+$/CD3$^+$/CD4$^+$/CD8$^-$/ CD25$^+$/CD127$^-$ |
| B cells, % of Leukocytes | CD3$^-$/CD19$^+$, % of CD45$^+$ |
| Memory B cells, % of B cells | CD27$^+$, % of CD45$^+$/CD19$^+$/CD3$^-$ |
| Class-switched memory B cells, % of B cells | CD27$^+$/IgD$^-$/IgM$^-$/CD20$^+$/CD38$^+$[84], % of CD45$^+$/CD19$^+$/CD3$^-$ |
| Plasmablast, % of B cells | CD27$^+$/IgD$^-$/IgM$^-$/CD20$^-$/CD38$^{hi}$[85], % of CD45$^+$/CD19$^+$/CD3$^-$ |
| Bregs-1 B cells, % of B cells | CD27$^+$/IgD$^-$/IgM$^-$/CD20$^-$/CD38$^{hi}$/CD5$^+$ [76], % of CD45$^+$/CD19$^+$/CD3$^-$ |
| Non class-switched memory B cells, % of B cells | CD27$^+$/ IgD$^+$[86], % of CD45$^+$/CD19$^+$/CD3$^-$ |
| Naïve B cells, % of B cells | CD27$^-$/IgD$^+$ [87], % of CD45$^+$/CD19$^+$/CD3$^-$ |
| Transitional B cells, % of B cells | CD24$^{hi}$/CD38$^{hi}$ [88], % of CD45$^+$/CD19$^+$/CD3$^-$ |

| | |
|---|---|
| Bregs-2 B cells, % of B cells | $CD24^{hi}/CD38^{hi}/CD1d^{+}/CD5^{+}$[89], % of $CD45^{+}/CD19^{+}/CD3^{-}$ |
| Pro B cells, % of B cells | $CD24^{hi}/CD38^{hi}/CD10^{+}/IgM^{-}$, % of $CD45^{+}/CD19^{+}/CD3^{-}$ |
| Pre B cells, % of B cells | $CD24^{hi}/CD38^{hi}/CD10^{+}/IgM^{-}/CD20^{+}$, % of $CD45^{+}/CD19^{+}/CD3^{-}$ |
| Plasma cells, % of B cells | $CD10^{-}/IgD^{-}/IgM^{-}/CD27^{hi}/CD38^{hi}$[90], % of $CD45^{+}/CD19^{+}/CD3^{-}$ |
| Neutrophils, % of Leukocytes | $CD66b^{+}/CD15^{+}$[91-93], % of $CD45^{+}$ |
| Monocytes, % of Leukocytes | $CD14^{+}/CD33^{+}$[94, 95], % of $CD45^{+}$ |
| Macrophages, % of Leukocytes | $CD33^{+}/CD11b^{+}/CD11c^{+}$/CD68+[96], % of $CD45^{+}$ |
| DC, % of Leukocytes | $CD33^{+}/HLA\text{-}DR^{+}/CD11b^{-}/CD11c^{+}$[97, 98], % of $CD45^{+}$ |
| MDSC, % of Leukocytes | $HLA\text{-}DR^{-}/ CD11b^{+}/CD33^{+}$[99, 100], % of $CD45^{+}$ |

| G-MDSC, % of Leukocytes | HLA-$DR^-$/ $CD11b^+$/$CD33^+$/$CD14^-$/$CD15^+$[101, 102], % of $CD45^+$ |
|---|---|
| M-MDSC, % of Leukocytes | HLA-$DR^-$/ $CD11b^+$/$CD33^+$/$CD14^+$/$CD15^-$[103, 104], % of $CD45^+$ |
| NK cells, % of Leukocytes | $CD3^-$/$CD16^+$/$CD56^+$/$CD8^{+-}$[105, 106], % of $CD45^+$ |
| NKT cells, % of Leukocytes | $CD3^+$/$CD16^+$/$CD56^+$/$CD8^{+-}$[107, 108], % of $CD45^+$ |

### 2.2.4. Staining Panels

The flow cytometry (FCM) analysis was designed in a modular system comprising 4 different panels which examine T cells and its subsets (Supplement Table 1); B cells and its subsets (Supplement Table 2); monocytes, neutrophils, DC, MDSC, NK cells and NKT cells (Supplement Table 3); IFN-γ (Supplement Table 4). Each panel included unstained tubes which served as blank control, fluorescence minus one (FMO) control tubes and experimental tubes.

### 2.2.5. Immunophenotyping Staining of PB

5ml PB samples were collected from HD and HCC patients before operation. All specimens were tested as soon as possible and never after longer than 24h after

collection. All experiments were conducted at room temperature. Whole blood was measured directly. The specific steps were as follows:

Staining methods for T cell panel (extracellular staining): 200μl whole blood was added in each FACS tube, then antibodies were added (Supplement Table 1). All tubes were vortexed and incubated for 15-30 minutes. 2ml 1x FACS lysing solution was added. Vortex and then incubated for 15-30 minutes. All tubes were centrifuged 500xg for 5 minutes and supernatant was discarded. 2 ml FACS buffer was added. Vortex and centrifuge were done as described before. 200μl FACS buffer was added and all stained cells were measured.

Staining methods for B cell panel, monocytes, neutrophils, DC, MDSC, NK cells and NKT cells: 200μl whole blood was added in each FACS tube, then antibodies were added (Supplement Table 2-3, except CD20 and CD68). All tubes were vortexed and incubated for 15-30 minutes. 2ml 1x FACS lysing solution was added. Vortex and then incubated for 15-30 minutes. All tubes were centrifuged 500xg for 5 minutes and supernatant was discarded. 100μl IC fixation buffer was added. Vortex and incubated for 20 minutes. 2ml 1x perm buffer was added. Vortex and centrifuge were done as described before (2 times). Antibodies CD20 and CD68 were added according to table4 and table5. All tubes were vortexed and incubated for 15-30 minutes. 2ml 1x perm buffer was added. Vortex and centrifuge were done as described before. 2 ml FACS buffer was added. Vortex and centrifuging were done as described before. 300μl FACS buffer was added and all stained cells were measured.

### 2.2.6. Immunophenotyping Staining of Tumor Infiltrating Cells

Fresh tumor tissues of HCC patients after surgery was collected and cut into 1-3mm small pieces. 10 ml collagenase was added in slices of tumor tissues. Tumor tissues were shaked for 20 minutes in a shaking water bath at 37 °C. 5 ml trypsin EDTA was

added in the above mixture sand the mixture was pressed through 100μm filter. The collected mixture was centrifuged 500xg for 5 minutes and the supernatant was discarded. 10ml ACK buffer was added and the mixture was pressed through 40μm filter. Centrifuge was done as before. 10ml PBS was added and the mixture was pressed through 40μm filter. Centrifuge was done as before (2 times). A portion of collected tumor infiltrating cells was measured directly after cell counting. Other cells were used to detect the production of IFN-γ. These cells were divided into two groups, stimulated cells group and unstimulated cell group. BD leukocytes activation kit (BLK) and Golgi stop were added separately into the two groups. These cells were placed in a 37 °C, 5% $CO_2$ incubator for 4 hours before staining. The specific staining operations were as follows:

Staining methods for T cells panel of tumor infiltrating cells (extracellular staining): Tumor infiltrating cell suspension 200μl was added in each FACS tube, then antibodies were added (Supplement Table 1). All tubes were vortexed and incubated for 15-30 minutes. All tubes were centrifuged 500xg for 5 minutes and supernatant was discarded. 2 ml FACS buffer was added. Vortex and centrifuge were done as before. 200μl FACS buffer was added and all stained cells were measured staining methods for B cell panel, monocytes, neutrophils, DC, MDSC, NK cells and NKT cells and IFN-γ of tumor infiltrating cells: Tumor infiltrating cell suspension 200μl was added in each FACS tube, then antibodies were added (Supplement Table 2-4, except CD20, CD68 and IFN-γ). All tubes were vortexed and incubated for 15-30 minutes. 100μl IC fixation buffer was added. All tubes were vortexed and incubated for 20 minutes. 2ml 1x perm buffer was added. Vortex and centrifuge were done as before (2 times). Antibodies CD20 and CD68 were added according to Supplement Table 2 and Supplement Table 3. Vortexing was done as described before and then incubated for 15-30 minutes. 2ml 1x perm buffer was added. Vortex and centrifuge were done as described before. 2 ml FACS buffer was added. Vortex and centrifuge were done as before. 300μl FACS

buffer was added and all stained cells were measured.

### 2.2.7. Gating Strategy

The gating was done by using FMO and unstained cells. The specific gating strategy was as follows:

**T cell panel**

As shown in Figure 3, first, on the basis of FSC/SSC scatter plot (Figure 3A) and CD45/SSC plot (Figure 3B), all leukocytes were selected. On the basis of CD3$^{+}$ T cells, Th and CTL were selected (Figure 3D). This assay was continued by detecting the expression of CD45RO and CD197 in Th and CTL (Figure 3H, 3F).[79] Both Th and CTL include naïve, effector, effector memory, and central memory subsets.[109, 110] Activated Th and CTL are definite as CD38$^{hi}$/HLA-DR$^{hi}$ (Figure 3I, 3G). For Th, Th1(CD194$^{-}$ CD196$^{-}$), Th2 (CD194$^{+}$ CD196$^{-}$), Th17 (CD194$^{+}$ CD196$^{+}$) were also examined (Figure 3E).[78] CD127$^{-}$ CD25$^{+}$ cells were considered as Tregs (Figure 3J) and its subsets include naïve, memory-activated, memory, and activated (Figure 3J) were also measured.[81, 83]

**B cell panel**

As shown in Figure 4, on the basis of CD45/SSC plot (Figure 4A), all leukocytes were selected. For the definition of B cells, the negative expression of CD3 and positive expression of CD19 were used (Figure 4B). The B cells were further differentiated into transitional B cells (Figure 4H), CD27$^{-}$ and CD27$^{+}$ memory populations (Figure 4C).[88] From the CD27$^{+}$ memory populations, IgD$^{-}$ IgM$^{-}$ subsets and non-class switched memory subsets were gated (Figure 4E).[87] From the IgD$^{-}$ IgM$^{-}$ subsets, plasmablasts and class switched memory B cells were achieved (Figure 4F). From plasmablasts,

Bregs-2 were analyzed (Figure 4G).[84, 85] From transitional B cells, Bregs-1(Figure 4K) and pro B cells (Figure 4I) were analyzed. Additionally, pre B cells were analyzed from pro B cells (Figure 4J). Plasma cells (Figure 4N) were gated from $CD10^-$ (Figure 4L) and then $IgD^-$ $IgM^-$ subsets (Figure 4M).[90]

**Monocytes, neutrophils, DC, MDSC, NK cells and NKT cells panel**

As shown in Figure 5, firstly, all leukocytes were selected (Figure 5A). The neutrophils expressed CD66b and CD15 (Figure 5B), monocytes expressed CD14 and CD33 (Figure 5C), and they were gated from leukocytes.[93, 94] The $CD68^+$ granulocytes (Figure 5D) were defined as $CD33^+$ $CD11b^+$ $CD11c^+$ $CD68^+$, and they were gated from monocytes. The DC (Figure 5F) were defined as CD33, HLA-DR, CD11b and CD11c.[96] It is well described that MDSC (Figure 5H) are CD11b and CD33 positive. They can then further be subdivided in to G-MDSC and M-MDSC by their expression of CD14 and CD15 (Figure 5I).[99, 102, 103] NK cells lack of CD3, but express CD56 and CD16. The NKT cells are identified by their CD3 expression that simultaneously express CD56 and CD16. Thus, I investigated the expression of CD56, CD16, CD3 to record NK cells (Figure 5K) and NKT cells (Figure 5L).[106, 107]

**IFN-γ panel**

The gating strategy of IFN-γ is shown in Figure 6. First, CD45 negative subsets and CD45 positive leukocytes were selected (Figure 6A). Then the $CD4^+$ and $CD8^+$ populations from T cells were selected (Figure 6F). IFN-γ of $CD45^-$ subsets (Figure 6B), $CD45^+$ subsets (Figure 6C), $CD3^+$ subsets (Figure 6D), $CD4^+$ subsets (Figure 6G) and $CD8^+$ subsets (Figure 6H) were respectively selected.

### 2.2.8. Statistical Analysis

SPSS 21.0 software package was used for statistical analysis. The Kolmogorov-Smirnov method was used to test whether the measurement variables were subjected to normal distribution. Two groups of normal distribution variables were compared with independent t test. The two groups of non-normal distribution variables were compared with Mann-Whitney U test. Paired t test was used to compare the PB and tumor tissues of the same patient. Paired t test was used to compare IFN-γ with and without stimulation of the same patient. A p value less than 0.05 was considered statistically significant.

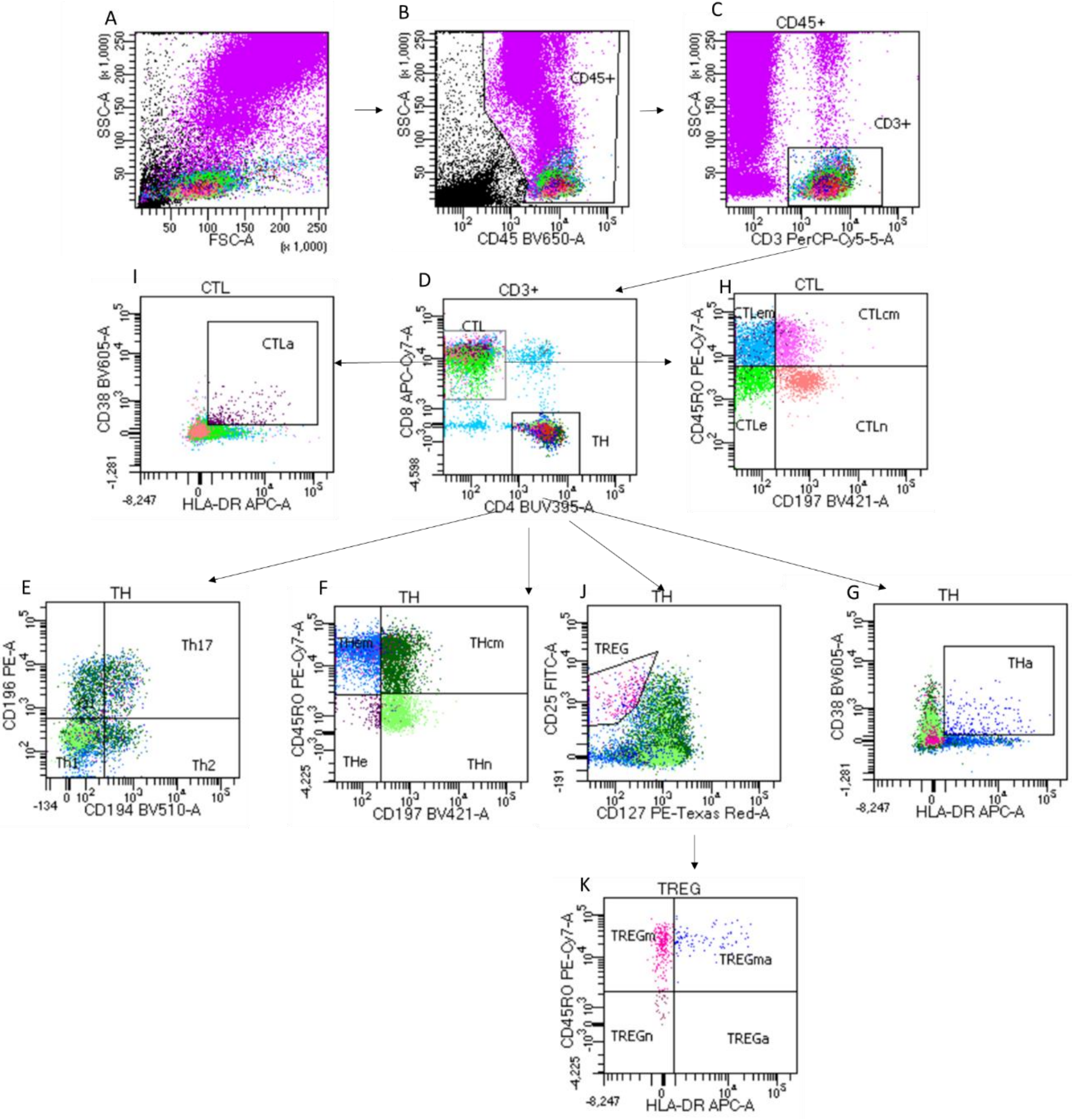



**Figure 3: Gating strategy for the T cell subsets.** (A) All cells from PB except red blood cells; (B) Leukocytes; (C) CD3⁺ T cells; (D) According to differential CD4 and CD8 expression, Th and CTL were identified; (E) By CD194 and CD196 expressions, the Th were distinguished into Th1, Th2, and Th17; (F) According to differential CD197 and CD45RO expression, the Th were further distinguished into eTh, nTh, emTh, cmTh subsets; (G) CD38⁺/HLA-DR⁺ aTh; (H) According to differential CD197 and CD45RO expression, the CTL were further distinguished into eCTL, nCTL, emCTL, cmCTL; (I) CD38⁺/HLA-DR⁺ aCTL; (J) Tregs were identified by CD25⁺/CD127⁻ expression; (K) By the CD45RO and HLA-DR expression, Tregs can be defined as naïve, memory, memory activated and activated Tregs.

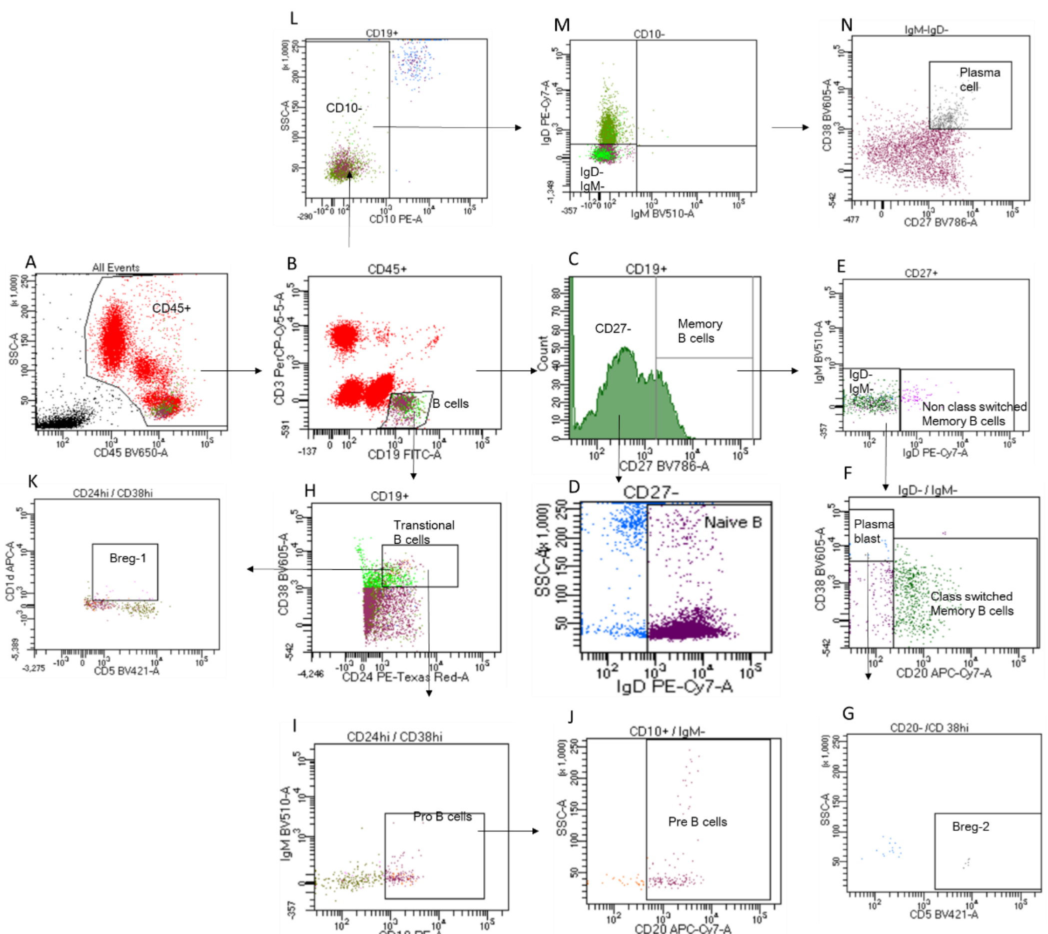


**Figure 4: Gating strategy for B cell subsets.** (A) Leukocytes; (B) CD3- CD19+ B cells; (C) B cells were differentiated into CD27- populations and CD27+ memory populations; (D) Naïve B cells; (E) IgD- IgM- populations and IgD+ IgM- non class switched memory B cells; (F) Plasmablast and class switched memory B cells; (G) Bregs-2; (H) Transitional B cells; (I) Pro B cells from transitional B cells; (J) Pre B cells from pro B cells; (K) Regulatory B cells-1; (L) CD10- populations from B cells; (M) IgD- IgM- populations from CD10- populations of B cells; (N) Plasma cells.

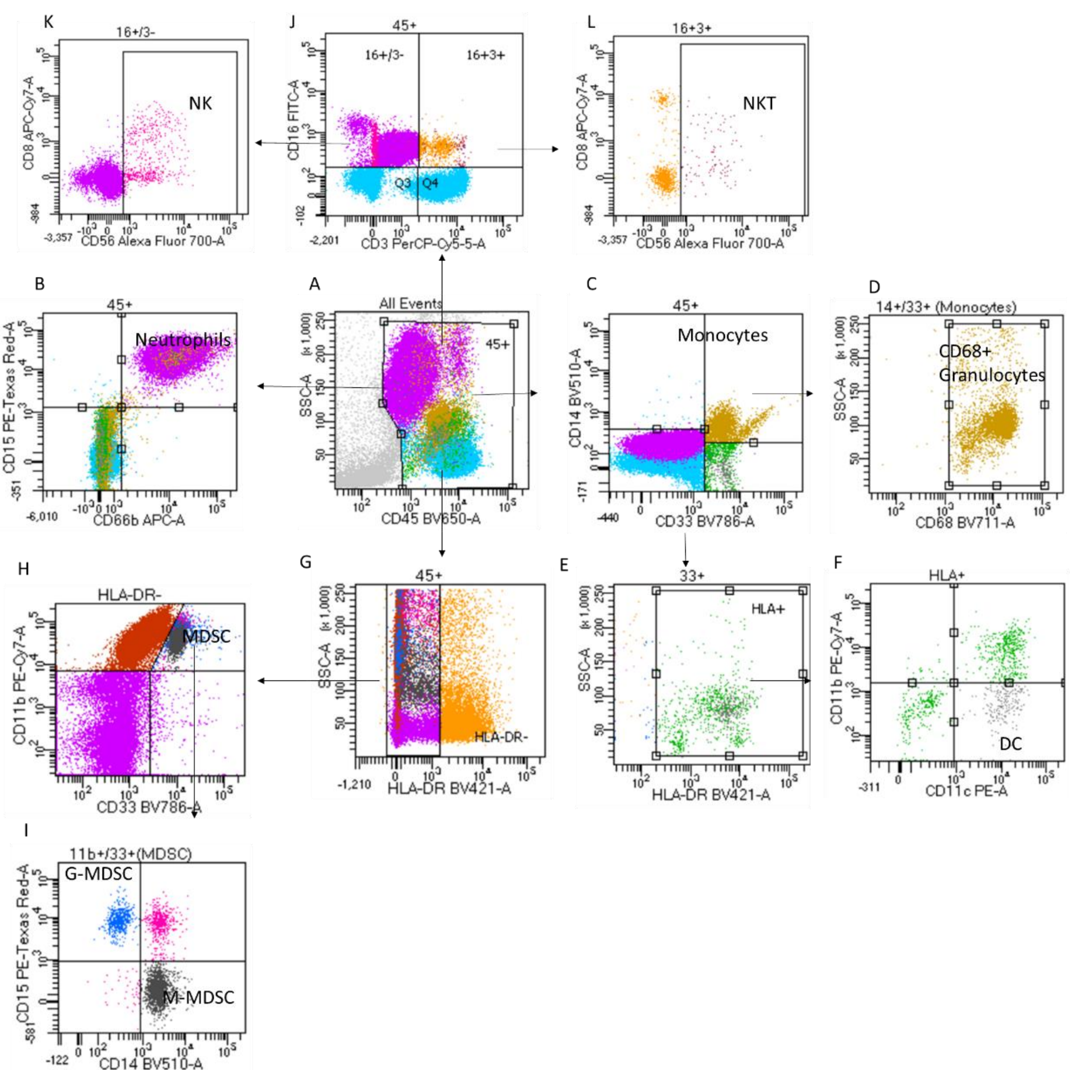



**Figure 5: Gating strategy for neutrophils, monocytes, macrophages and DC, MDSC.** (A) Leukocytes; (B) Neutrophils; (C) Monocytes; (D) Macrophages; (E) HLA-DR$^{+}$ subsets; (F) DC; (G) HLA-DR$^{-}$ subsets; (H) MDSC; (**I**) G-MDSC and M-MDSC; (J) CD3$^{-}$ CD16$^{+}$ and CD3$^{+}$ CD16$^{+}$ subsets; (K) NK cells gated from CD3$^{-}$ CD16$^{+}$ subsets; (L) NKT cells gated from CD3$^{+}$ CD16$^{+}$ subsets.

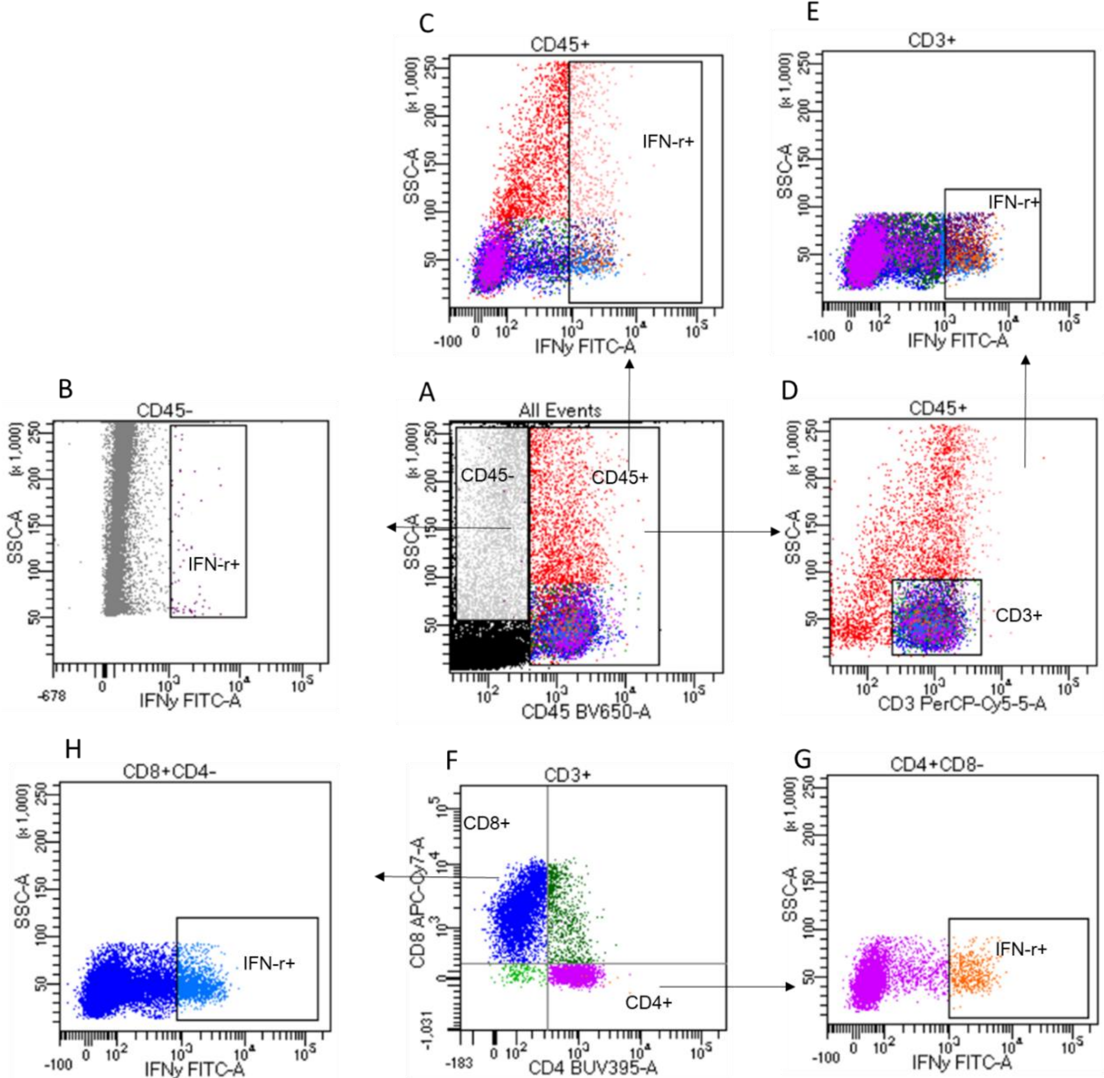



**Figure 6: Gating strategy for measuring IFN-γ in HCC tumor tissue.** (A) Leukocytes and $CD45^-$ populations; (B) IFN-γ of $CD45^-$ populations; (C) IFN-γ of $CD45^+$ leukocytes; (D) $CD3^+$ T cells; (E) IFN-γ of $CD3^+$ T cells; (F) $CD4^+$ and $CD8^+$ populations from T cells; (G) IFN-γ of $CD4^+$ subsets; (H) IFN-γ of $CD8^+$ subsets.

# 3. Results

## 3.1. Literature Review

In this systematic review the relationship between circulating immune cells and clinicopathological characteristics or prognosis of HCC patients will be described.

### 3.1.1 Characteristics of Studies included in the Review

After the systematic search of the literature, 258 potentially related publications were identified. According to the exclusion criteria mentioned above, 226 articles were excluded. One full-text was not available through institutional subscriptions, therefore this article was also excluded.[111] Finally, 31 studies were included in this review (Figure 7).

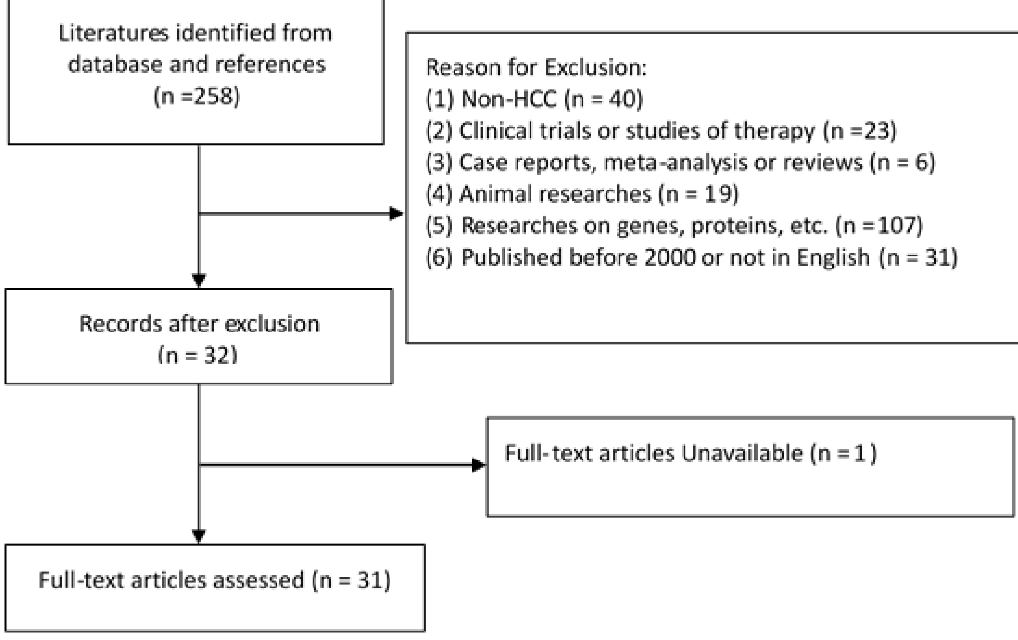



**Figure 7: Flow diagram of study selection**

In these studies, circulating immune cells were analyzed from 31 publications including 7447 HCC patients and 925 HD or hepatitis patients. As can be seen from Figure 8A, most of the studies (n=27, 87%) were conducted in East Asia.[112-138] Only four studies were conducted in Europe (n=3, 10%) and North America (n=1, 3%).[139-142] The most common treatment of the included studies was resection (n=16, 52%).[113, 115-117, 120, 126-128, 130, 132-134, 137, 138, 141, 142] Some other studies offered information on patients undergoing multimodal treatments (6%), such as combined ablative therapies.[122, 136] LT was only performed in one study (3%) (Figure 8B).[118]

As shown in Figure 8C, PBMC isolated from fresh blood were measured in 15 (48%) studies.[125, 127-134, 136-141] Immune cells direct from fresh whole blood samples were used in 4 (13%) studies.[116, 124, 126, 135] FCM analysis were done in more than half of the studies (18, 58%) (Figure 8D).[124-141]

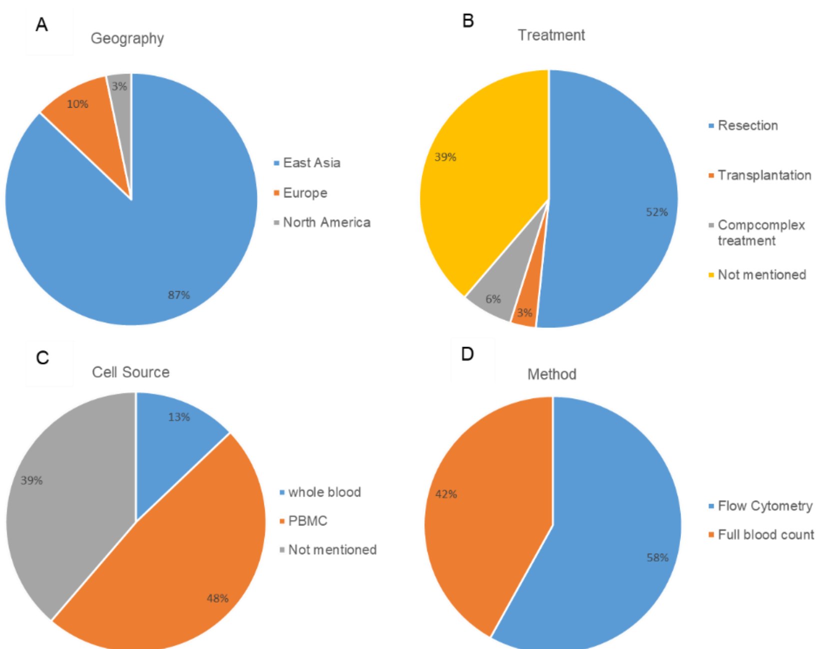



**Figure 8: Characteristics of studies included in the review.** (A) Geographic distribution of publications; (B) Surgical methods; (C) Source of the specimen; (D) Detection methods. (Abbreviations: PBMC: Peripheral blood mononuclear cells)

In the following paragraphs, the results regarding differences of immune cells between HCC patients and HD in PB as well as their correlation with clinical characteristics and outcomes are described.

### 3.1.2 Immune Cell Counts and their Ratios

Evidence has accumulated that immune cell counts and ratios have prognostic value in HCC. They can be easily obtained without expensive measurement costs. Li J et al. showed that the frequency of peripheral leukocytes, neutrophils and monocytes in HCC patients were elevated compared with HD. But HCC patients had lower lymphocytes amounts than HD.[112]

As shown in Table 3, elevated preoperative monocyte counts in HCC patients indicated worse prognosis in three studies.[113, 114, 142] Another two studies showed that higher ratios of peripheral neutrophil-monocyte/lymphocyte and monocyte-granulocyte / lymphocyte also correlated with worse outcomes.[121, 122] Similarly, another study showed that higher lymphocyte / monocyte ratio indicated lower cirrhosis grading, lower levels of bilirubin and better outcome.[123] Six publications presented data of NLR. All of them demonstrated that the low NLR HCC patients group had better OS and recurrence free survival (RFS).[115-120] Additional to the results obtained in resection, one of these studies claimed that a preoperative NLR ≥ 4 can even be an independent predictive factor to predict tumor recurrence after LT.[118]

These results already indicate that the differences of relevant circulating immune cells have predictive value of HCC. In the following these broadly analyzed circulating immune cells were further subspecified to possibly identify the relevant effector cells responsible for the observed effects.

### 3.1.3 Cells of the Adaptive Immune System

One study reported that PB from HCC patients, contains lower amounts of T cells compared to HD.[124] In contrast to that, another study reported no difference in T cells between HCC patients and HD.[125] In a large study with 715 HCC patients a higher frequency of CTL could be demonstrated.[126] None of these studies reported on survival. Another experimental analysis reported that the amount of Th in HCC patients showed no difference from HD. However, they identified higher frequencies of Th17 cells, lower frequencies of Th1 cells and a higher Th17/Th1 ratio in HCC patients when compared to HD.[127]

Six articles provided information on Tregs. Four of these studies demonstrated that HCC patients had higher preoperative frequency of Tregs in their PB than HD.[128, 129, 139, 140] Furthermore, they found that higher Tregs levels positively correlated with tumor burden, disease progression and poorer OS.[129, 140] However, Chen et al. found that preoperative circulating Tregs frequency in HCC patients was lower when compared to HD.[130] One study concentrated on further phenotypes of Tregs. Takata et al reported that CD45RO antigen (commonly expressed on memory cells) was elevated on Tregs as compared to HD. Also this higher frequency of memory Tregs indicated a larger tumor burden.[131]

One study providing information about $CD4^+$ cytotoxic T cells ($CD4^+$ CTL). It was showed that HCC patients had higher amounts of $CD4^+$ CTL when compared to HD in PB.[132] In a singular report Duan et al. showed that amounts of Th1-like and Th17-like subsets of T follicular helper cells (Tfh) ($CXCR5^+$ $CD4^+$ $CD45RA^-$) were significantly decreased in HCC patients, while the Th2-like subset were increased compared to HD.[133] Two articles provided information regarding NKT cells, both of them demonstrated that there was no difference between the two groups.[125, 134]

Only three studies that investigated B cells were identified.[125, 126, 135] Two of them reported lower amounts of B cells in PB of HCC patients HD.[125, 126] In contrast to that one study did not confirm these results. However, they showed that the amount of memory B cells was lower in HCC patients. [135] Chen et al. additionally looked at the newly defined group of Bregs ($CD19^{+}$ $IL\text{-}10^{+}$). In their work they report that HCC patients had lower amounts of Bregs than in HD.[130]

### 3.1.4. Cells of the Innate Immune System

Attallah et al. demonstrated that HCC patients had an increased amounts of NK in blood.[124] However, Cai et al. showed that NK cells were significantly decreased in HCC patients.[136] Another two studies did not find any difference of NK cells amounts between HCC patients and HD.[125, 134] $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells are two different NK cell subsets, the former mainly produces cytokines, while the latter exerts direct cytotoxic effects.[143] Two studies showed higher amounts of $CD56^{bright}$ NK cells and lower amounts of $CD56^{dim}$ NK cells in the PB of HCC patients compared to HD.[136, 141] Also, Cariani et al. revealed that higher amounts of $CD56^{bright}$ NK cells and lower amounts of $CD56^{dim}$ NK cells correlated with worse OS. [141] As shown in Table 3, one study found that the amount of MDSC positively correlated with a more advanced disease, larger tumors and worse Child-Pugh stage.[137] This was confirmed by Li and colleagues who found that MDSC were increased in HCC patients.[138]

**Table 3. Summary of included studies.** Abbreviations: Bregs: Regulatory B cells; CHB: Chronic hepatitis B; CTL: Cytotoxic T Lymphocytes; DFS: Disease-free survival; DC: Dendritic cells; FCM: Flow cytometry; FBC: Full blood count; FBCA: Full blood count analysis; HBeAg: hepatitis B envelope antigen; LT: Liver transplantation; LC: Liver cirrhosis; M/GLR: Monocyte/granulocyte to lymphocyte ratio; NK: Natural killer cells; HD: Healthy donors; NLR: Neutrophil-to-lymphocyte ratio; NMLR: Neutrophil-monocyte/lymphocyte ratio; N/A: Data not found; N.S.: Data found but have no significance; LMR: Lymphocyte-to-monocyte ratio; MDSC: Myeloid-derived suppressor cells; OS: Overall survival; RFS: Recurrence-free survival; PBMC: Peripheral blood mononuclear cells; TTR: Time to disease recurrence; Tregs: Regulatory T cells; TIL: Tumor-infiltrating leukocytes; Tfh: T follicular helper cells; WB: Whole blood.

| **Reference** | **Study Population** | | | | **Study Cell** | | | | **Changing Tendency in HCC** | **Higher Amount Association** | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | HCC Patients | Controls | Region | Treatment | Cell Source | Method | Cell Marker | Cell Type | | Clinicopathological Characteristics | Survival |
| Li J1 et al.2016[112] | 175 | 69 HD | China | N/A | N/A | FBCA | N/A | Leukocytes | Higher in non-HBV HCC patients | N/A | N/A |
| | | | | | | | | Lymphocytes | Lower | | |
| | | | | | | | | Monocytes | Higher | | |
| | | | | | | | | Neutrophils | Higher | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Shen SL et al.2014[113] | 351 | N/A | China | Resection | N/A | FBCA | N/A | Monocytes | N/A | N/A | Monocytes ≥ 545/$mm^3$; Worse prognosis |
| Sasaki A et al.2014[142] | 198 | N/A | Japan | Resection | N/A | FBCA | N/A | Monocytes | N/A | Microvascular invasion, large tumor, increased platelet count | Monocytes >300/$mm^3$; Worse DFS |
| Lee, S.D et al.2014[114] | 603 | N/A | Korea | N/A | N/A | FBCA | N/A | Monocytes | N/A | N/A | Monocytes >7%; Worse DFS |
| Yang HJ et al.[115] | 526 | N/A | China | Resection | N/A | FBCA | N/A | NLR | N/A | N/A | NLR ≥ 2.81; Worse OS, DFS |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Mano Y et al.2013[116] | 958 | N/A | Japen | Resection | WB | FBCA | N/A | NLR | N/A | N/A | NLR ≥ 2.81; Worse OS and RFS |
| Peng W et al.2014[117] | 189 | N/A | China | Resection | N/A | FBCA | N/A | NLR | N/A | N/A | Worse OS and RFS |
| Xiao, GQ et al.2013[118] | 280 | N/A | China | LT | N/A | FBCA | N/A | NLR | N/A | More recurrence | NLR ≥ 4; Worse OS and RFS |
| Okamura, Y. et al.2015[119] | 256 | N/A | China | N/A | N/A | FBCA | N/A | NLR | N/A | N/A | Worse OS and RFS |

| Liao R et al.2015[120] | 222 | N/A | China | Resection | N/A | FBCA | N/A | NLR | N/A | Higher bilirubin, white blood cell counts and HBsAg | Worse OS and TTR |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Liao, R. et al.2016[121] | 387 | N/A | China | N/A | N/A | FBCA | N/A | NMLR | N/A | NMLR > 1.2; Increased ALT, tumor number, tumor size and BCLC stage | NMLR > 1.2; Worse OS and RFS |
| Zhou D et al.2015[122] | 1061 | N/A | China | ResectionTACE | N/A | FBCA | N/A | M/GLR | N/A | N/A | Worse OS and RFS |
| Lin, Z.X. et al.2015[123] | 210 | N/A | China | N/A | N/A | FBCA | N/A | LMR | N/A | LMR > 3.23; Lower presence of cirrhosis, lower levels of bilirubin | LMR > 3.23; Better OS and RFS in LC-HCC |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A.M. Attallah. et al.2003[124] | 40 | 42 HD | Egypt | N/A | WB | FCM | $CD3^+$ | T cells | Lower | N/A | N/A |
| | | | | | | | $CD4^+$ | Th | Lower | | |
| | | | | | | | $CD8^+$ | CTL | N.S. | | |
| | | | | | | | $CD57^+$ | NK cells | Higher | | |
| Lin, J.C. et al.2010[125] | 45 | 46 HD | China | N/A | PBMC | FCM | $CD3^+$ | T cells | N.S. | B cells: Worse TNM stage, more tumor numbers | N/A |
| | | | | | | | $CD3^-$ $CD19^+$ | B cells | Lower | | |
| | | | | | | | $CD3^+$ $CD4^+$ | Th | Lower | | |
| | | | | | | | $CD3^+$ $CD8^+$ | CTL | N.S. | | |
| | | | | | | | $CD3^-$ $CD16^+$ $CD56^+$ | NK cells | N.S. | | |
| | | | | | | | $CD3^+$ $CD16^+$ $CD56^+$ | NKT cells | N.S. | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Liu, H.Z. et al.2016[126] | 715 | 100 | China | Resection | WB | FCM | N/A | NK cells | Lower | N/A | N/A |
| | | | | | | | $CD19^+$ | B cells | Lower | | |
| | | | | | | | $CD8^+$ | CTL | Higher | | |
| Yan, J. et al.2014[127] | 150 | 50 HD | China | Resection | PBMC | FCM | N/A | Th | N.S. | N/A | Th1: Better OS and DFS |
| | | | | | | | | Th1 | Lower | | |
| | | | | | | | | Th17 | Higher | | Th17 and Th17/Th1 : Worse OS and DFS |
| | | | | | | | | Th17/Th1 | Higher | | |
| Ormandy, L.A. et al.2005[139] | 84 | 74 HD, HBV patients | Germany | N/A | PBMC | FCM | $CD4^+$ $CD25^+$ | Tregs | Higher | N/A | N/A |
| Cao, M. et al. 2007[140] | 105 | 20 HD | USA | N/A | PBMC | FCM | $CD4^+$ $CD25^+$ | Tregs | Higher | Larger tumor burden | N/A |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Feng X et al.2011[128] | 42 | 15 HD | China | Resection | PBMC | FCM | $CD4^+FoxP3^+$ $CD25^{high}$ | Tregs | Higher | N/A | N/A |
| Fu J et al.2007[129] | 123 | 47 HD | China | N/A | PBMC | FCM | $CD4^+$ $FoxP3^+$ $CD25^+$ | Tregs | Higher | N/A | Worse OS |
| Chen, T. et al.2012[130] | 36 | 10 HD | China | Resection | PBMC | FCM | $CD4^+$ $CD25^+$ $CD127^-$ | Tregs | Lower before surgery, increased after surgery. | Advanced clinical stages | N/A |
| | | | | | | | $CD19^+$ IL-$10^+$ | Bregs | | HBeAg and HBV DNA number | |
| Takata, Y. et al. 2011 [131] | 62 | 41HD | Japan | N/A | PBMC | FCM | $CD45RO^+$ $CD4^+$ $CD25^{high}$ | Memory Tregs | Higher | Larger tumor burden | N/A |
| Fu, J. et al.2013[132] | 232 | 44CHB 86 LC 88 HD | China | Resection | PBMC | FCM | $CD4^+$ granzyme $^+$ $perforin^+$ | $CD4^+$ CTL | Early stage higher, progressive stage lower | N/A | Better OS |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Duan Z et al.2015[133] | 21 | 11 HD | China | Resection | PBMC | FCM | CXCR5$^+$ CD4$^+$ CD45RA$^-$ | Tfh | Lower Th1-like and Th17-like Tfh cells, higher Th2-like Tfh cells, lower ratios of Th1-/Th2-like and Th17-/Th2-like Tfh cells | N/A | N/A |
| Li, X.F. et al.2017 [134] | 11 | 11HD | China | Resection | PBMC | FCM | CD3$^+$ CD56$^+$ | NKT cells | N.S. | N/A | N/A |
| | | | | | | | CD3$^-$ CD56$^+$ | NK cells | N.S. | N/A | N/A |
| Wang, X.D. et al.2012[135] | 38 | 30HD | China | N/A | WB | FCM | CD19$^+$ | B cells | N.S. | B cells: Lower ALT | N/A |
| | | | | | | | CD29$^+$ CD27$^-$ | Naïve B cells | N.S. | Memory B cells: Better BCLC scores | |
| | | | | | | | CD19$^+$ CD27$^+$ | Memory B cells | Lower | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cai L. et al.2008[136] | 110 | 69 HD | China | ETCT<br>TACE<br>Resection<br>LT | PBMC | FCM | $CD3^{-}$ $CD56^{+}$ | NK cells | Lower | N/A | N/A |
| | | | | | | | $CD56^{br}$ $CD16^{neg}$ | NK cells | Higher on stage III | | |
| | | | | | | | $CD56^{dim}$ $CD16^{pos}$ | NK cells | Lower | | |
| Cariani, E. et al.2016 [141] | 70 | 18 HD | Italy | Resection | PBMC | FCM | $CD3^{-}$ $CD56^{br}$ | $CD56^{br}$ NK cells | Higher | N/A | Worse OS ,TTR |
| | | 12 HCV-related cirrhosis | | | | | $CD3^{-}CD56^{dim}$ | $CD56^{dim}$ NK cells | Lower | | Better OS,TTR |
| Wang, D. et al.2016[137] | 92 | 22HD | China | Resection | PBMC | FCM | $CD14^{+}$ HLA-$DR^{-/low}$ | MDSC | Higher | More advanced disease, bigger tumor size, worse Child-Pugh stage | Worse OS |
| Li, X. et al.2017[138] | 55 | 20HD | China | Resection | PBMC | FCM | N/A<br>HLA-$DR^{-/low}$ | MDSC | Higher | N/A | N/A |

| | | | |
|---|---|---|---|
| | $CD11b^{+}CD33^{+}CD14^{+}$ HLA-DR$^{-/low}$ | M-MDSC | Higher |
| | $CD11b^{+}CD33^{+}CD15^{+}CD66b^{+}$ | G-MDSC | Higher |

As shown in the review above, many publications have reported on circulating immune cells in HCC patients. These studies were limited to investigate one or only selected immune cells. Naturally many results are contradictory and not comparable across the different methods and patients' etiology of HCC. Therefore, in this translational study, I aimed to comprehensively assess the immune signature of HCC patients and compare it to the immune signature of age and gender matched HD. In the following, I will concentrate on results that have showed significant differences between the groups. The detailed cell counts and comparisons are listed in Supplement Table 5-7.

## 3.2. FCM Measurements of PB Immune Cells in HCC Patients and HD

First, I compared the difference of immune cells in PB between HD and HCC patients.

### 3.2.1. HCC Patients have lower Frequency of Macrophages and DC but Higher Amounts of MDSC than HD

HCC patients had lower amounts of macrophages when compared to HD (0.32±0.24% vs. 0.84±0.47%, p=0.01) (Figure 9G). Similarly, the amount of DC in HCC patients was lower than in HD (0.12±0.14% vs. 0.39±0.24%, p=0.01) (Figure 9H). On the contrary, HCC patients showed a higher frequency of MDSC in the PB (HCC: 2.01±2.02% vs. HD: 0.56±0.44%, p=0.04) (Figure 9I). No differences were found between the two groups in neutrophils, monocytes, M-MDSC and G-MDSC (p=0.25, p=0.28, p=0.35, p=0.29, respectively) (Supplement Table 5, Figure 9M, 9N, 9K, 9L).

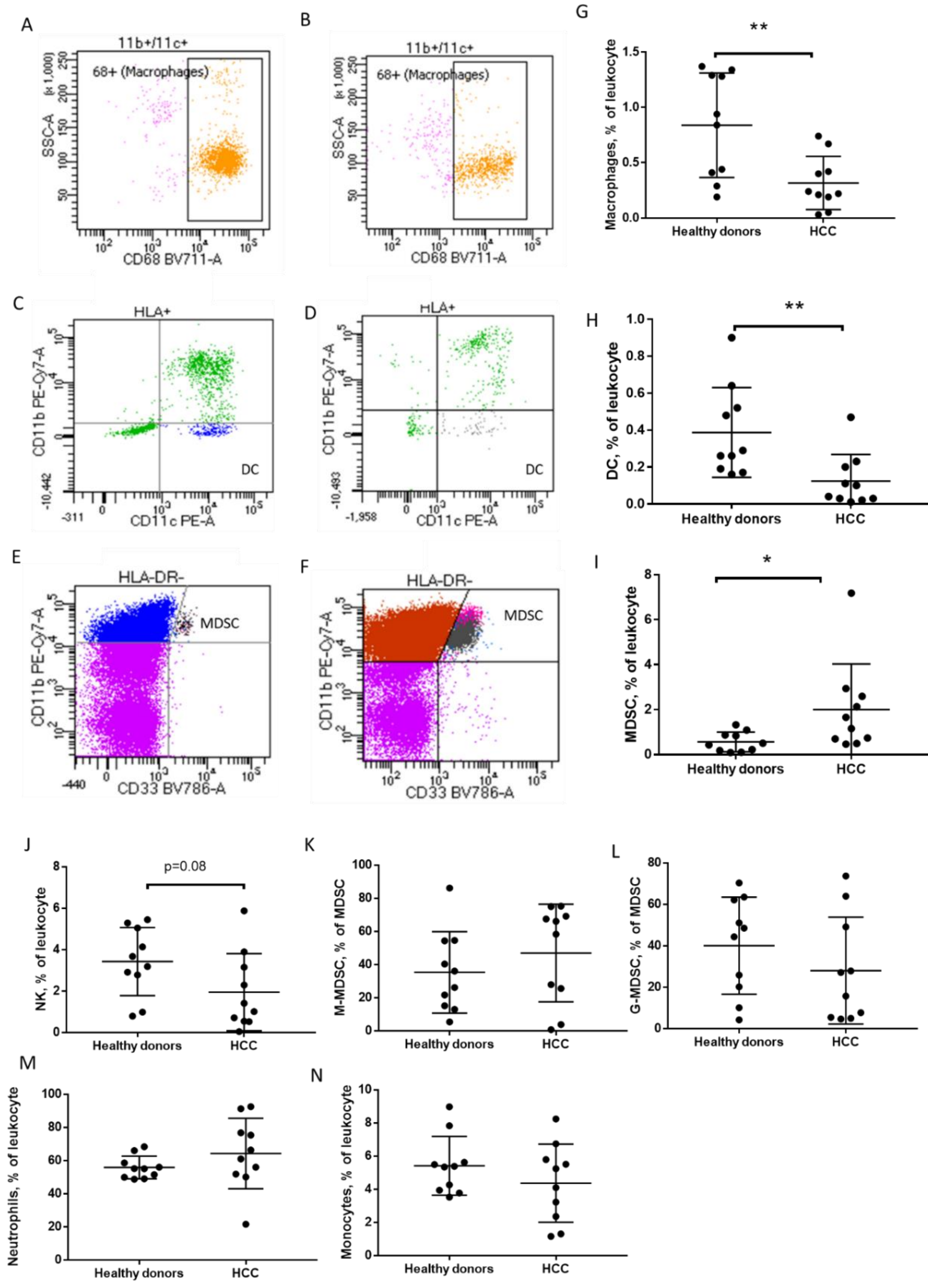



**Figure 9: Typical FCM pictures of significant differentially expressed macrophages, DC and MDSC in HD and HCC patients.** (A: Macrophages in HD; B: Macrophages in HCC; C: DC

in HD; D: DC in HCC; E: MDSC in HD; F: MDSC in HCC). Dot plots illustrating cell subsets in HD and HCC patients. (G: Lower amounts of macrophages in HCC; H: Lower amounts of DC in HCC; I: Higher amounts of MDSC in HCC). No differences were found in the following cell groups between HD and HCC patients (J: NK cells; K: M-MDSC; L: G-MDSC; M: Neutrophils; N: Monocytes) (Unpaired t test, * $p<0.05$ and ** $p<0.01$).

### 3.2.2. HCC Patients have lower Amounts of T cells than HD

T cells in HCC patients were less frequent when compared to HD (19.15±12.55% vs. 30.93±5.14%, p=0.01) (Figure 10I). Similarly, HCC patients also had lower amounts of NKT cells (HCC: 0.06±0.04% vs. HD: 0.24±0.27%, p<0.001) (Figure 10L). The frequency of cmTh in HCC patients was lower than in HD (10.45±7.38% vs. 21.85±9.94%, p=0.01) (Figure 10N). Also, the amount of nTh in HCC patients was significantly lower (HCC: 9.35±9.62% vs. HD: 26.99±13.35%, p<0.01) (Figure 10N). On the contrary, the frequency of emTh in HCC patients was significantly higher than HD (55.92±14.77% vs. 34.54±13.48%, p<0.01) (Figure 10J). Similarly to emTh, Tregs had a higher frequency in HCC patients (HCC: 10.2±4.8% vs. HD: 6.49±2.11%, p=0.04) (Figure 10K). The frequency of Th, CTL, CD4/CD8 ratio, Th1, Th2, Th17, eTh, aTh, emCTL, cmCTL, eCTL, nCTL, aCTL, mTregs, nTregs, aTregs and maTregs were not significantly different between the two groups (p=0.87, p=0.7, p=0.38, p=0.46, p=0.99, p=0.62, p=0.3, p=0.1, p=0.61, p=0.41, p=0.34, p=0.73, p=0.07, p=0.24, p=0.26, p=0.76, p=0.96, respectively) (Supplement Table 5, Supplement Figure 1).

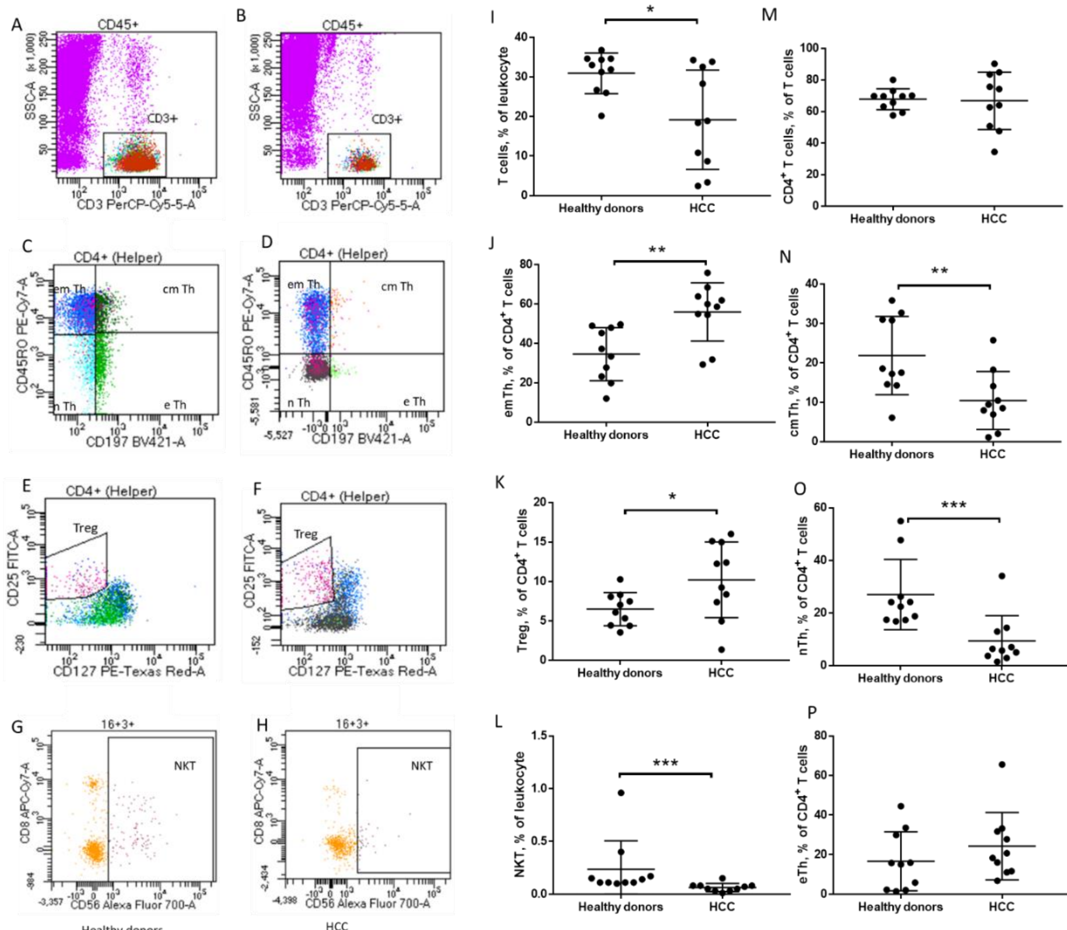



**Figure 10: Typical FCM pictures of significant differentially expressed T cells, emTh, cmTh, nTh, Tregs and NKT cells in HD and HCC patients.** (A: T cells in HD; B: T cells in HCC; C: Subsets of Th in HD; D: Subsets of Th in HCC; E: Tregs in HD; F: Tregs in HCC; G: NKT cells in HD; H: NKT cells in HCC). Dot plots illustrating cell subsets in HD and HCC patients. (I: Lower amounts of T cells in HCC; J: Higher amounts of emTh in HCC; K: Higher amounts of Tregs in HCC; L: Lower amounts of NKT cells in HCC; M: No differences of Th; N: Lower amounts of cmTh in HCC; O: Lower amounts of nTh in HCC; P: No differences of eTh) (Unpaired t test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

### 3.2.3. HCC Patients have lower Amounts of Memory B Cells, NS-Memory B Cells and Bregs-2 than HD

HCC patients had lower amounts of memory B cells and ns-memory B cells than HD (memory B cells: 11.6±8.53% vs. 31.57±28.34%, $p<0.05$; ns-memory B cells: 3.59±4.03% vs. 8.44±5.06%, $p=0.01$, respectively) (Figure 11G, 11H). Similarly, the amount of Bregs-2 in HCC patients was lower than HD (0.32±0.81% vs. 1.65±1.57%, $p=0.01$) (Figure 11I). The frequency of B cells, naïve B cells, transitional B cells, pro B cells, pre B cells, plasma cells and plasmablasts were not significantly different between the two groups ($p=0.2$, $p=0.34$, $p=0.06$, $p=0.07$, $p=0.23$, $p=0.14$, $p=0.11$, respectively) (Supplement Table 5, Figure 11).

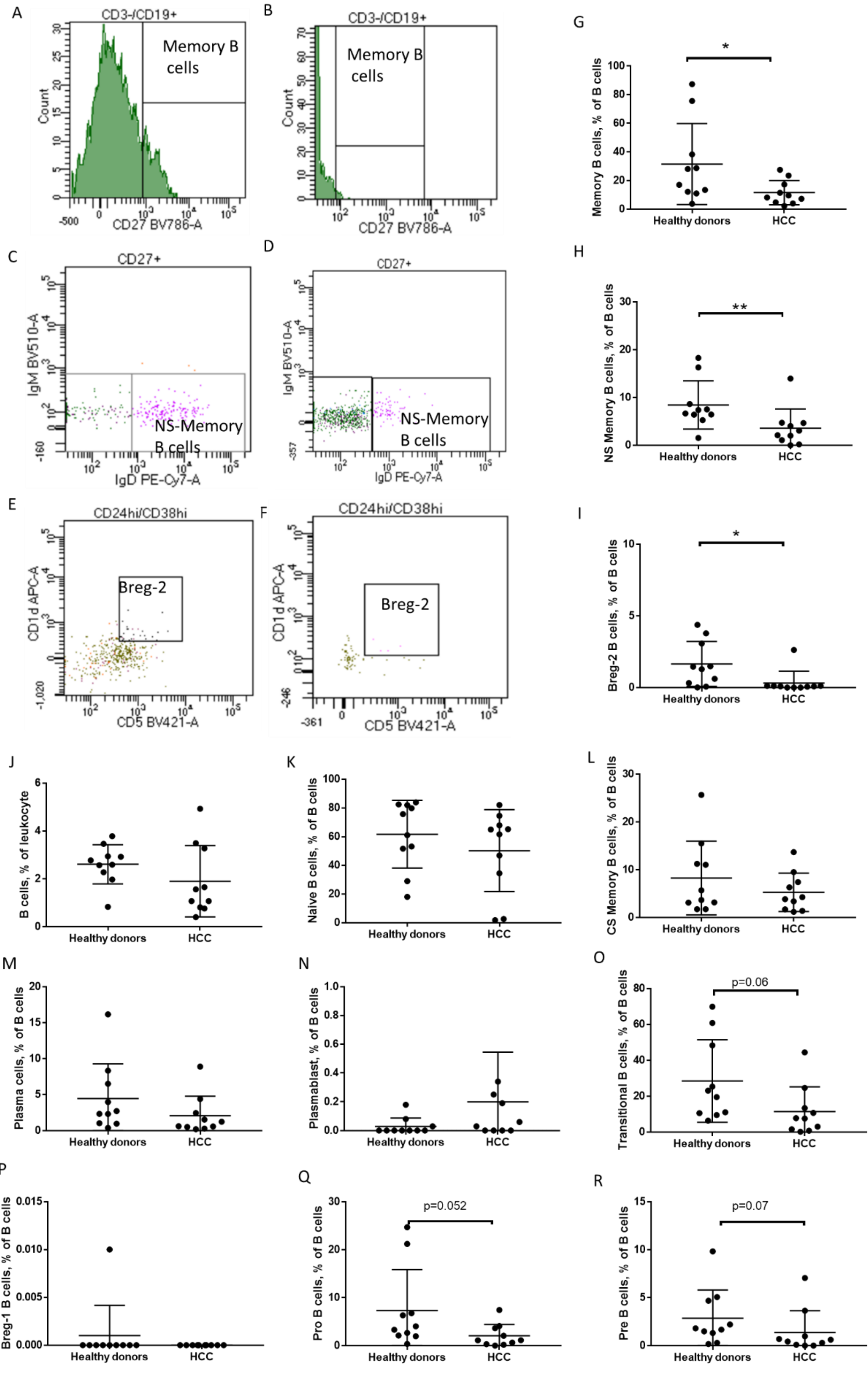

A
CD3-/CD19+
Memory B cells
Count
CD27 BV786-A
B
CD3-/CD19+
Memory B cells
Count
CD27 BV786-A
G
*
Memory B cells, % of B cells
Healthy donors
HCC
C
CD27+
IgM BV510-A
NS-Memory B cells
IgD PE-Cy7-A
D
CD27+
IgM BV510-A
NS-Memory B cells
IgD PE-Cy7-A
H
**
NS Memory B cells, % of B cells
Healthy donors
HCC
E
CD24hi/CD38hi
CD1d APC-A
Breg-2
CD5 BV421-A
F
CD24hi/CD38hi
CD1d APC-A
Breg-2
CD5 BV421-A
I
*
Breg-2 B cells, % of B cells
Healthy donors
HCC
J
B cells, % of leukocyte
Healthy donors
HCC
K
Naive B cells, % of B cells
Healthy donors
HCC
L
CS Memory B cells, % of B cells
Healthy donors
HCC
M
Plasma cells, % of B cells
Healthy donors
HCC
N
Plasmablast, % of B cells
Healthy donors
HCC
O
p=0.06
Transitional B cells, % of B cells
Healthy donors
HCC
P
Breg-1 B cells, % of B cells
Healthy donors
HCC
Q
p=0.052
Pro B cells, % of B cells
Healthy donors
HCC
R
p=0.07
Pre B cells, % of B cells
Healthy donors
HCC

**Figure 11: Typical FCM pictures of significant differentially expressed memory B cells, ns-memory B cells and Bregs-2 in HD and HCC patients.** (A: Memory B cells in HD; B: Memory B cells in HCC; C: ns-memory B cells in HD; D: ns-memory B cells in HCC; E: Bregs-2 in HD; F: Bregs-2 in HD). Dot plots illustrating cell subsets in HD and HCC patients. (G: Lower amounts of memory B cells in HCC; H: Lower amounts of ns-memory B cells in HCC; I: Lower amounts of Bregs-2 in HCC). No differences were found in the following cell groups between HD and HCC patients. (J: B cells; K: Naive B cells; L: cs-memory B cells; M: Plasma cells; N: Plasmablasts; O: Transitional B cells; P: Bregs-2; Q: Pro B cells; R: Pre B cells) (Unpaired t test, * $p<0.05$, ** $p<0.01$).

## 3.3. FCM Measurements of Tumor Infiltrating Immune Cells

To further understand the local immune status of HCC, I isolated tumor infiltrating immune cells and compared them with circulating immune cells in the same patients.

### 3.3.1. Tumor Tissues have lower rate of Accumulation of Neutrophils and Monocytes compared to PB

In HCC tumor tissues neutrophils had a significantly lower rate of accumulation than PB (0.85±0.56% vs. 55.92±22.52%, $p=0.01$) (Figure 12H). Similarly, monocytes were less frequent in HCC tumor tissues when compared to PB (1.97±1.38% vs. 5.12±2.64%, $p=0.03$) (Figure 12I). The frequency of macrophages, DC, MDSC, G-MDSC and M-MDSC showed no difference between tumor tissues and PB ($p=0.27$, $p=0.84$, $p=0.76$, $p=0.08$, $p=0.03$, respectively) (Supplement Table 5, Figure 12).

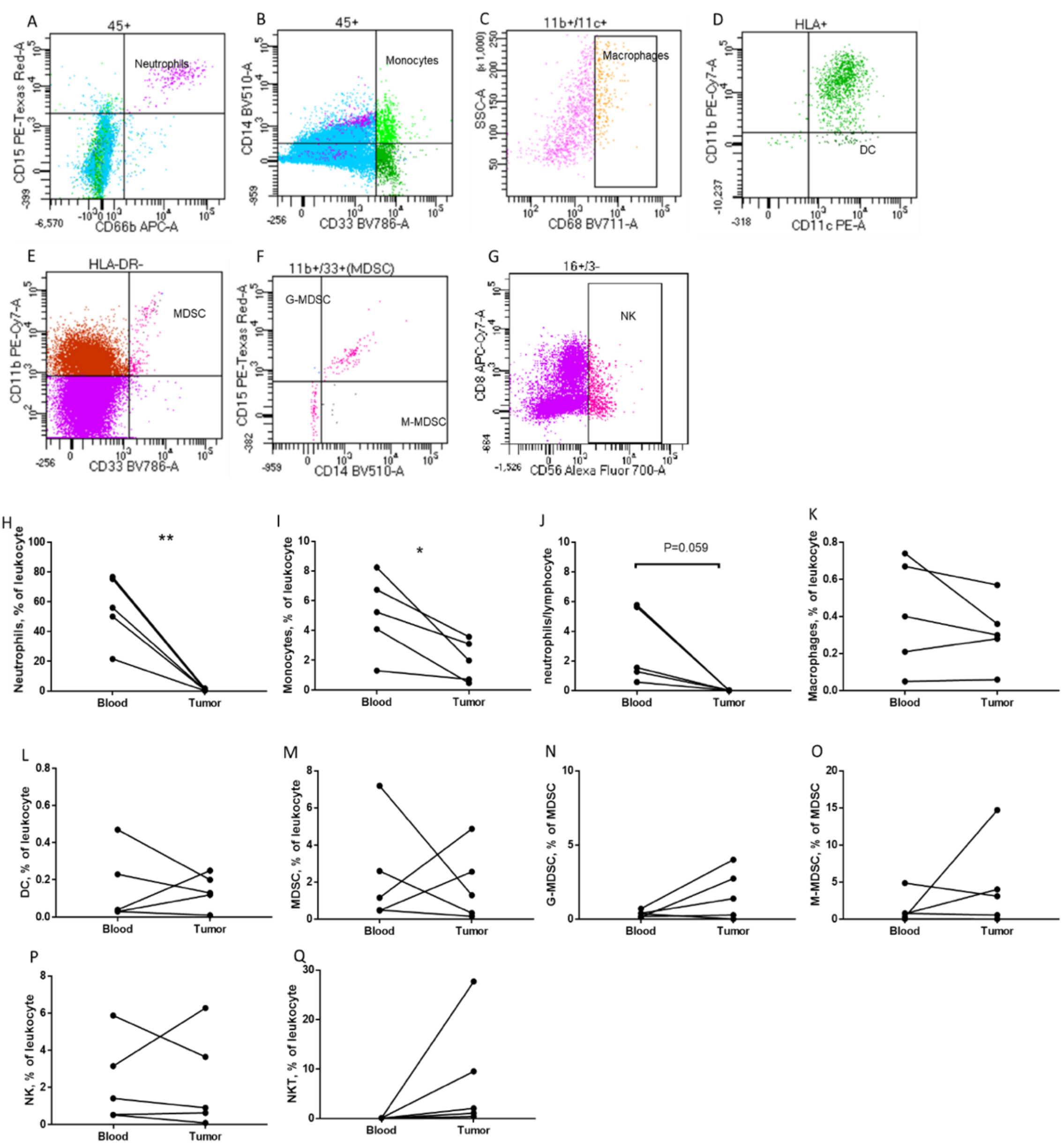



**Figure 12: Typical FCM pictures of immune cells in freshly harvested HCC tissues.** (A: Neutrophils; B: Monocytes; C: Macrophages; D: DC; E: MDSC; F: G-MDSC and M-MDSC; G: NK cells). Dot plots illustrating cell subsets in PB and HCC tissues. (H: Lower amounts of neutrophils in HCC tissues; I: Lower amounts of monocytes in HCC tissues; J: No differences of the neutrophil to lymphocyte ratio; K: No differences of macrophages; L: No differences of DC; M: No differences of MDSC; N: No differences of G-MDSC; O: No differences of M-MDSC; P: No differences of NK cells) (Paired t test, * p<0.05 and ** p<0.01).

### 3.3.2. Tumor Tissues have a higher rate of Accumulation of T cells than PB

In HCC tumor tissues T cells accumulated (Tissues: 58.17±15.95% vs. PB: 22.84±12.15%, $p=0.02$) (Figure 13K). Th had a lower rate of accumulation in HCC tumor tissues when compared to PB (42.61±14.99% vs. 73.82±10.43%, $p<0.01$) (Figure 13L). Similarly, the ratio of CD4/CD8 in HCC tissues was lower than PB (2.19±1.14% vs. 4.26±2.17%, $p=0.02$) (Figure 13M). HCC tumor tissues had a higher accumulation degree of Th1, lower accumulation degree of Th2 and higher ratio of Th1/Th2 when compared to PB (Th1: 77±22.1% vs. 48.23±21.05%, $p=0.04$; Th2: 3.54±0.71% vs. 12.03±6.36%, $p<0.05$; Th1/Th2 ratio: 21.54±3.4% vs. 8.18±10.98%, $p=0.04$) (Figure 13N, 13O, 13Q). In HCC tumor tissues emTh were more frequent (78.43±10.67% vs. 57.28±16.18%, $p=0.04$), while cmTh and mTregs were less frequent (cmTh: 0.09±0.12% vs. 8.41±4.82%, $p=0.02$; mTregs: 33.25±20.1% vs. 62.53±10.71%, $p=0.02$, respectively) than PB (Figure 13R, 13S, 13V). The frequency of Th17, eTh, aTh, nTh, CTL, emCTL, cmCTL, eCTL, nCTL, aCTL, Tregs, nTregs, aTregs and maTregs were not different between tumor tissues and PB ($p=0.06$, $p=0.47$, $p=0.38$, $p=0.06$, $p=0.74$, $p=0.95$, $p=0.17$, $p=0.16$, $p=0.05$, $p=0.87$, $p=0.69$, $p=0.81$, $p=0.23$, $p=0.73$, respectively) (Supplement Table 6, Supplement Figure 2).

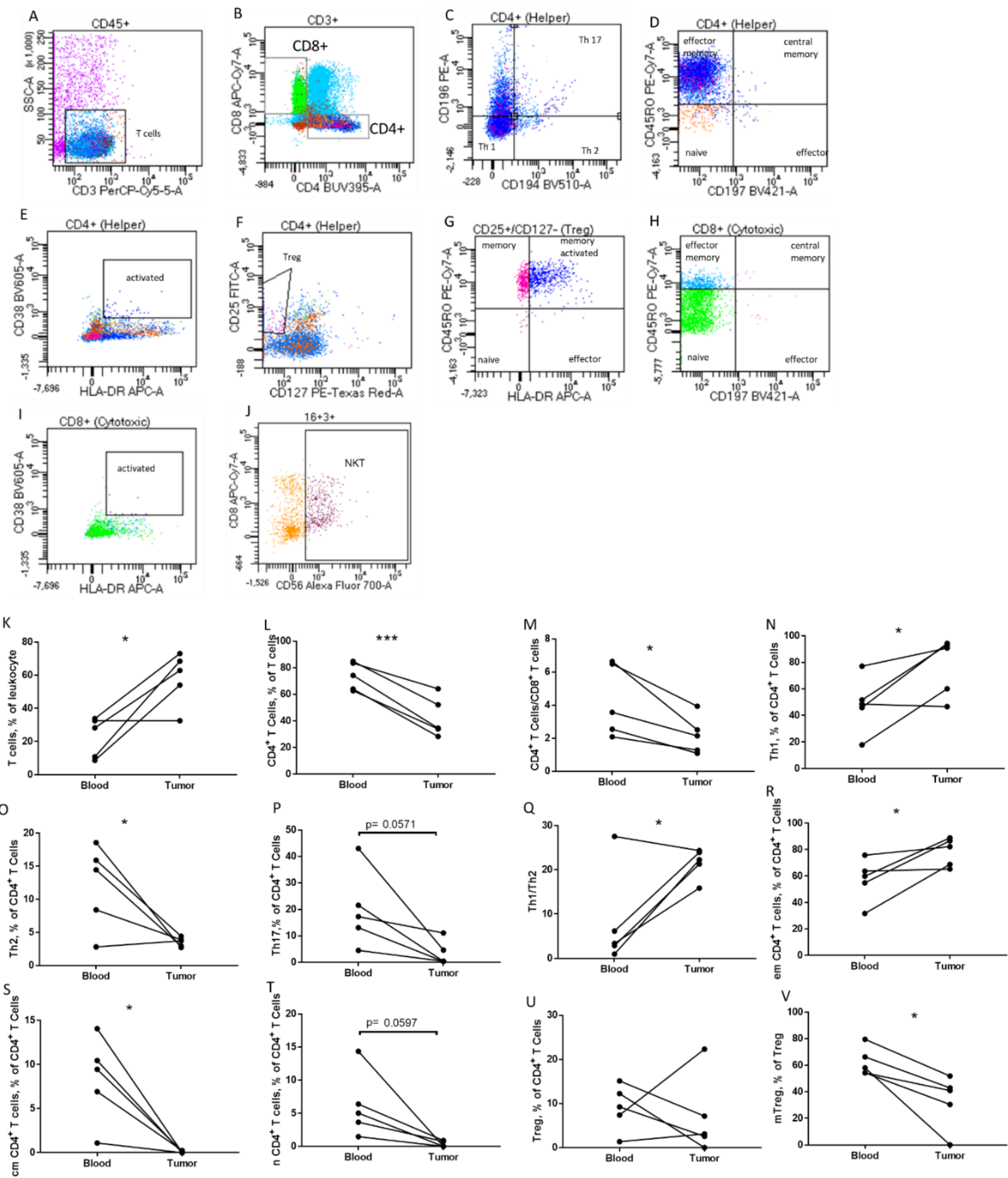



**Figure 13: Typical FCM pictures of T cells and its subsets in freshly harvested HCC tissues.** (A: T cells; B: Th and CTL; C: Th1; Th2 and Th17; D: emTh, cmTh, eTh and nTh; E: aTh; F: Tregs; G: mTregs, maTregs, nTregs and aTregs; H: emCTL, cmCTL, eCTL and nCTL; I: aCTL; J: NKT cells). Dot plots illustrating T cells and its subsets in PB and HCC tissues. (K: Higher amounts of T cells in HCC tissues; L: Lower amounts of Th in HCC tissues; M: Lower amounts of $CD4^+$ T cells to $CD8^+$ T cells ratio in HCC tissues; N: Higher amounts of Th1 in HCC tissues; O: Lower amounts of Th2 in HCC tissues; P: No differences of Th17; Q: Higher amounts of Th1 to Th2 ratio in HCC tissues; R: Higher amounts of emTh in HCC tissues; S: Lower

amounts of cmTh in HCC tissues; T: No differences of nTh; U: No differences of Tregs; V: Lower amounts of mTregs) (Paired t test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

### 3.3.3. There are no Differences between B cells and its Subsets in HCC Tissues and PB.

The frequency of B cells, naïve B cells, transitional B cells, pro B cells, pre B cells, memory B cells, ns-memory B cells and cs-memory B cells were not different between the two groups (p=0.87, p=0.73, p=0.46, p=0.2, p=0.24, p=0.16, p=0.26, p=0.15, respectively) (Supplement Table 6, Figure 14). Plasmablasts, plasma cells, Bregs-1, and Bregs-2 were almost undetectable in HCC tissues (Supplement Table 6, Figure 14).

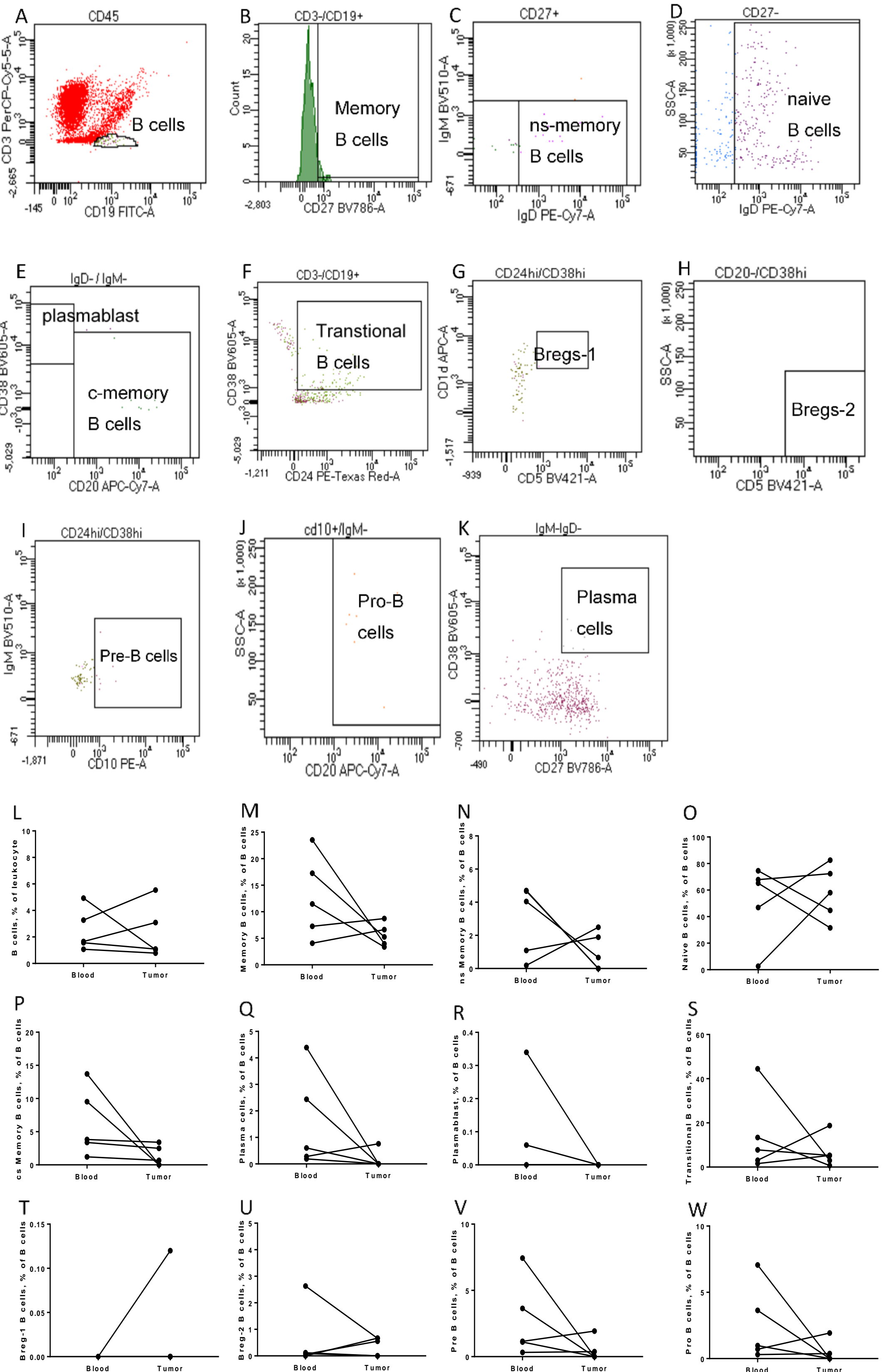

A
CD45
B cells
CD3 PerCP-Cy5-5-A
CD19 FITC-A
B
CD3-/CD19+
Memory
B cells
Count
CD27 BV786-A
C
CD27+
ns-memory
B cells
IgM BV510-A
IgD PE-Cy7-A
D
CD27-
naive
B cells
SSC-A
IgD PE-Cy7-A
E
IgD- / IgM-
plasmablast
c-memory
B cells
CD38 BV605-A
CD20 APC-Cy7-A
F
CD3-/CD19+
Transtional
B cells
CD38 BV605-A
CD24 PE-Texas Red-A
G
CD24hi/CD38hi
Bregs-1
CD1d APC-A
CD5 BV421-A
H
CD20-/CD38hi
Bregs-2
SSC-A
CD5 BV421-A
I
CD24hi/CD38hi
Pre-B cells
IgM BV510-A
CD10 PE-A
J
cd10+/IgM-
Pro-B
cells
SSC-A
CD20 APC-Cy7-A
K
IgM-IgD-
Plasma
cells
CD38 BV605-A
CD27 BV786-A
L
B cells, % of leukocyte
M
Memory B cells, % of B cells
N
ns Memory B cells, % of B cells
O
Naive B cells, % of B cells
P
cs Memory B cells, % of B cells
Q
Plasma cells, % of B cells
R
Plasmablast, % of B cells
S
Transitional B cells, % of B cells
T
Breg-1 B cells, % of B cells
U
Breg-2 B cells, % of B cells
V
Pre B cells, % of B cells
W
Pro B cells, % of B cells
Blood
Tumor

**Figure 14: Typical FCM pictures of B cells and its subsets in freshly harvested HCC tissues.** (A: B cells; B: Memory B cells; C: ns-memory B cells; D: Naïve B cells; E: Plasmablast and cs-memory B cells; F: Transitional B cells; G: Bregs-1; H: Bregs-2; I: Pre B cells; J: Pro B cells; K: Plasma cells. Dot plots illustrating B cells and its subsets in PB and HCC tissues. (L: No differences of B cells; M: No differences of memory B cells; N: No differences of ns-memory B cells; O: No differences of naïve B cells; P: No differences of cs-memory B cells; Q: No differences of plasma cells; R: No differences of plasmablasts; S: No differences of transitional B cells; T: No differences of Bregs-1; U: No differences of Bregs-2; V: No differences of pro B cells; W: No differences of pre B cells) (Paired t test).

## 3.4. TILs from HCC Tissues Produce IFN-γ

From the above results, it can be seen that there is immune cell infiltration in HCC tissues. Some cell groups accumulate to mount the antitumor defense. However, to investigate the functional state, I further analyzed IFN-γ production to understand whether TILs were stimulated and functionally active.

As mentioned above we created a stimulated and an unstimulated group. For this investigation we analyzed only effector cells of the antitumor immune response. As shown in Figure 15, the production of IFN-γ from $CD45^+$ and $CD3^+$ cells in the stimulated group were significantly higher when compared to unstimulated group ($CD45^+$ cells: 13.75±7.7% vs. 0.49±0.29%, p=0.02; $CD3^+$ cells: 13.76±8.17% vs. 0.26±0.16%, p=0.02, respectively) (Supplement Table 7, Figure 15L, 15M). The IFN-γ production of $CD3^+$ cells was driven by the effector subsets of T cells: $CD4^+$ and $CD8^+$ cells. In stimulated group the production of IFN-γ from $CD4^+$ cells and $CD8^+$ cells were significantly higher when compared to the unstimulated group ($CD4^+$ cells: 18.9±10.6% vs. 0.75±0.12%, p=0.02; $CD8^+$ cells: 42.93±25.07% vs. 2.03±2.94%, p=0.02, respectively) (Supplement Table 7, Figure 15O, 15N). The production of IFN-γ from $CD45^-$ cells (which were used as control) was not different between the two groups (p=0.11).

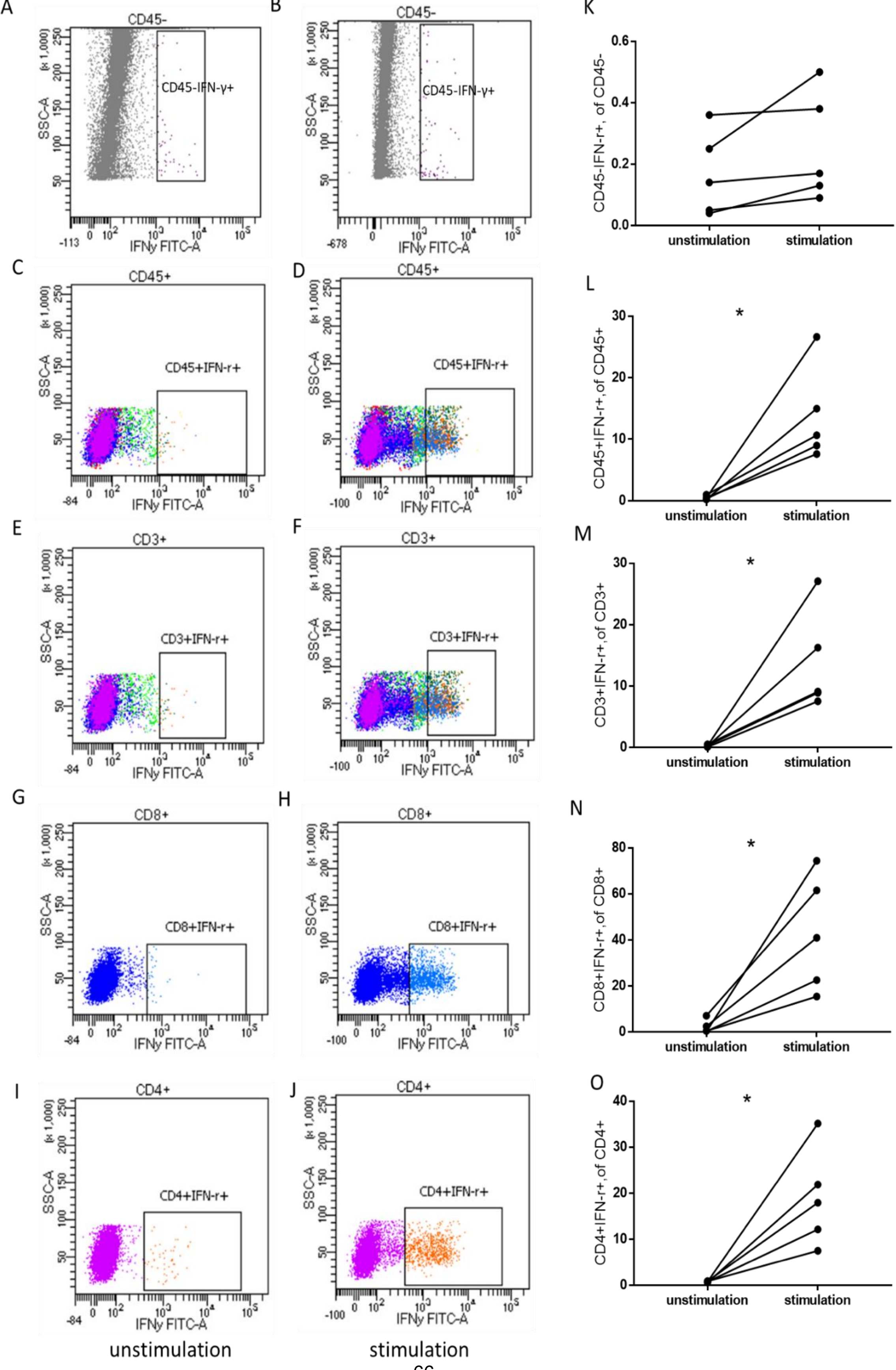

A
CD45-
CD45-IFN-γ+
SSC-A
IFNγ FITC-A
B
CD45-
CD45-IFN-γ+
K
CD45-IFN-r+, of CD45-
unstimulation
stimulation
C
CD45+
CD45+IFN-r+
D
CD45+
CD45+IFN-r+
L
CD45+IFN-r+,of CD45+
*
E
CD3+
CD3+IFN-r+
F
CD3+
CD3+IFN-r+
M
CD3+IFN-r+,of CD3+
*
G
CD8+
CD8+IFN-r+
H
CD8+
CD8+IFN-r+
N
CD8+IFN-r+,of CD8+
*
I
CD4+
CD4+IFN-r+
J
CD4+
CD4+IFN-r+
O
CD4+IFN-r+,of CD4+
*
unstimulation
stimulation

**Figure 15: Typical FCM pictures of IFN-γ in unstimulated group and stimulated group.** (A: IFN-γ of $CD45^-$ cell populations in unstimulated group; B: IFN-γ of $CD45^-$ cell populations in stimulated group; C: IFN-γ of $CD45^+$ cells in unstimulated group; D: IFN-γ of $CD45^+$ cells in stimulated group; E: IFN-γ of $CD3^+$ cells in unstimulated group; F: IFN-γ of $CD3^+$ cells in stimulated group; G: IFN-γ of CD8+ cells in unstimulated group; H: IFN-γ of $CD8^+$ cells in stimulated group; I: IFN-γ of $CD4^+$ cells in unstimulated group; J: IFN-γ of $CD4^+$ cells in stimulated group. Dot plots illustrating IFN-γ in unstimulated group and stimulated group (K: No differences of IFN-γ of $CD45^-$ cell populations; L: Higher amounts of IFN-γ of $CD45^+$ cell populations in stimulated group; M: Higher amounts of IFN-γ of $CD3^+$ cell populations in stimulated group; N: Higher amounts of IFN-γ of $CD8^+$ cell populations in stimulated group; O: Higher amounts of IFN-γ of $CD4^+$ cell populations in stimulated group) (Paired t test, * $p<0.05$).

# 4. Discussion

In reviewing the literature, there are differential expression of multiple immune cells between HCC patients and HD. The differential expression of these cells is not only related to clinicopathological characteristics but can even predict the prognosis of HCC patients after treatment.[128, 129, 139, 140] However, these studies were limited to investigate one or only selected immune cells. Naturally many results are contradictory and not comparable across the different methods and patients' etiology of HCC. This study established an immunophenotyping protocol for detecting more than 40 immune cells in PB and tumor tissues of HCC patients. Furthermore, to our knowledge, this is the first study to comprehensively assess the immune signature of nonHBV/nonHCV HCC patients.

In this discussion I will systematically analyze my results and put these into context with the literature. To allow for a structured understanding of the many subsets of measured immune cells in this study, the discussion will be partly divided into immunosuppressive and immune stimulating cells. A separate paragraph will be devoted to cell groups from the innate immune cells which have dual (immune stimulating and immunosuppressive) function.

Tregs are a small subset of T cells with an important immune regulatory function. They repress the immune response through intercellular contact with other T cells.[144] Additionally, they can also exert immunoregulatory effects by producing IL-10 and TGF-β.[145] I found that the frequency of Tregs in PB was higher in HCC patients when compared to HD. This is in accordance with previous studies (FCM was used), which have also demonstrated that Tregs amount was higher in the PB of HCC patients

compared to HD.[128, 130] One explanation is that HCC exerts an immunosuppressive environment, which expands the amount of circulating Tregs and their immunosuppressive function.[128] Another explanation might be that this immunosuppressive environment inhibits other cell compartments on favor of expanding Tregs. As reported Tregs can inhibit the function of other immune cells, such as T cells, NK cells, NKT cells and B cells. [159] My results indicated that the count of overall T cells, NKT cells were significantly reduced in PB of HCC patients. Feng et al found that HCC patients had a higher amounts of tumor-infiltrating Tregs than that in PB.[128] Contrary to that, my results, also obtained with FCM, illustrated that there was no difference in relative Tregs quantity between tumor tissues and PB. These results may be different because the progression of chronic hepatitis is corelated with an increased amounts of Tregs.[146] Our cohort however was comprised of nonHBV/nonHCV HCC patients. Theories for the infiltration of Tregs in tumor tissue are still not unified. One previous study has reported that tumor cells and macrophages in malignant ascites can secrete a large number of chemokines and cause Tregs in PB to aggregate locally into tumors.[147]

Bregs mainly play a negative regulatory role by secreting IL-10 (similarly to Tregs). This plays an important protective role in autoimmune diseases and chronic inflammatory diseases.[148] However, such a negative regulatory role may also promote the progress of cancer.[149] Studies have indicated that Bregs also can induce Treg activation and thus accelerate the metastasis of cancer cells.[150] In addition, IL-10, IL-35 and IL-33 had the effect of promoting Bregs, thus promoting tumor metastasis and accelerating disease progression.[151] With FCM Chen et al. found that Bregs increased significantly in patients with HCC.[130] However, my results revealed that the frequency

of Bregs in PB of HCC patients was lower than that of HD. This may also be related to nonHBV/nonHCV HCC patients in this study. This might be underlined by the fact that the above mentioned study by Chen et al. also showed a positive correlation between the frequency of Bregs and HBV DNA copy number.[130] Another study of Li et al., which used FCM, also reported an increased amount of Bregs in chronic hepatitis patients in China.[152] In general, therefore, it seems that the inflammatory state caused by hepatitis rather than HCC may play a major role on the frequency of circulating Bregs. Previous studies have demonstrated that intertumoral Bregs correlated with impaired anti-tumor immunity.[153, 154] Wang et al. revealed that Bregs were significantly elevated in tumor tissues compared to PB in gastric cancer patients (FCM).[155] Moreover, they demonstrated that Bregs exert immune suppressive capacity by producing IL-10 and TGF-β. However, little is known about the Bregs in HCC tissues. I found that Bregs were almost undetectable in HCC tissues. A possible explanation for this might be the heterogeneity between different tumors. Another possibility may be related to nonHBV/nonHCV HCC patients in this study. As mentioned above, the inflammatory state caused by hepatitis may play a major role on the frequency of Bregs.

MDSC are heterogeneous cell populations and mainly play an immunosuppressive role. [156] MDSC can not only inhibit T cells mediated specific immunity, but also directly inhibit the immune functions of macrophages, NK cells and DC.[46] Hoechst et al. found that MDSC count was significantly increased in PB of HCC patients. FCM was applied in this study. Moreover they found that MDSCs can significantly inhibit the production of IFN-γ and T cells proliferation.[157] Similarly, with FCM Arihara et al. showed that the frequency of MDSC in HCC patients was significantly higher compared to HD.[158] In agreement with previous findings, in this study the results also demonstrated that HCC patients had higher amounts of MDSC in PB than that of HD. One explanation for the expansion of MDSC is that under pathological conditions, the immature myeloid cells differentiation process is blocked, resulting in an increase of MDSC. Another

explanation is that tumor cells release immunosuppressive molecules, which can recruit Tregs and MDSC. These inhibiting cells can secrete inhibitors to further promote the growth of tumors, and they can inhibit T cells, B cells, NK cells and so on.[159] My results showed that the T cells amount was significantly reduced while Tregs amounts were increased in PB of HCC patients. Taken together, it suggests that MDSCs can affect the expansion of Tregs and work together with Tregs to exert an immunosuppressive state. Only a few publications regarding HCC have evaluated MDSCs in tumor tissues. In colorectal cancer, it was demonstrated that the frequency of MDSC in the tumor tissues was higher than paracancerous tissues (FCM was used).[160] This finding is consistent with that of Sun et al. who also found that the frequency of MDSC was markedly increased in tumor tissues of colorectal cancer patients.[161] In this study FCM was employed to measure MDSC. In addition, they presented that intertumoral MDSCs was correlated with metastasis and tumor stage. In my results, MDSCs were observed in HCC tissues. But no difference was found in MDSCs between tumor tissues and PB. The reasons for the MDSC infiltration may include the following aspects: COX-2 is highly expressed in cancer cells, which can produce prostaglandin E2 (PGE2). PGE2-COX2 pathway can induce MDSC production. Tumor cells can secrete negative regulatory factors such as IL-1β and IL-6, which effectively prevent the differentiation and maturation of bone marrow precursor cells.[162]

Supporting the notion that HCC may exert an immunosuppressive environment, it was found in this study that HCC patients had a lower frequency of T cells in PB than HD. This is in accordance with previous studies (FCM was employed).[123, 124] As mentioned above, the decrease in circulating T cells may be caused by higher amounts of MDSCs and Tregs. MDSC can significantly inhibit T cells proliferation and Tregs can cause T cell death through perforin/granzyme pathway.[163] Another possibility explanation is that circulating T cells migrate to the tumor tissues. Since I found that the amount of

tumor infiltrating T cells were higher than that in PB. TILs were firstly reported in 1986 and the phenotype of TILs are heterogeneous. In general, the majority of TILs are $CD3^+$ cells.[164] This study also indicated that T cells were the main infiltrating cells in HCC tissues, which was consistent with previous reports. Increased T cell infiltration, suggesting that the body can produce a local cellular immune response to HCC.

T cells have two major subsets. Studies found that in HCC patients the frequency of $CD4^+$ T cells in PB were significantly reduced, while $CD8^+$ T cells were not different from that of HD.[124, 125] In these publications FCM was employed. However, in a large study with 715 HCC patients a higher amounts of $CD8^+$ T cells could be demonstrated (FCM).[126] Another study with the same experimental method found that the amount of $CD4^+$ T cells in HCC patients did not differ significantly from HD.[127] Our results indicated that the frequency of both $CD4^+$ T cells and $CD8^+$ T cells were not different from that of HD. This inconsistency may be due to the limited patient number. Another possible explanation might be that although the overall T cells are significantly reduced, the major two compositions of T cells are still stable. This is because under most circumstances, $CD4^+$ T cells and $CD8^+$ T cells are in a relatively stable balance to maintain the immune function of the body.[165] But in HCC tissues, this balance is altered. This study revealed that tumor tissues had lower amount of $CD4^+$ T cells and $CD4^+/CD8^+$ ratio than that in PB. With FCM Elisabetta et al. displayed that HCC tumor tissues had higher amounts of $CD4^+$ T cells while $CD8^+$ T cells were more represented in paracancerous tissues .[166] My study did not compare to paracancerous tissues. The abnormalities of $CD4^+$ and $CD8^+$ T cells may be due to the immunosuppressive factors in tumor microenvironment, such as TGF-β, VEGF and IL-10.[167] The decrease of $CD4^+/CD8^+$ ratio indicates that the immune regulation function of the tumor microenvironments is abnormal, showing an immunosuppressive state.[168]

In 1986, Mosmann et al. divided $CD4^+$ T cells into Th1 and Th2 according to their

different cytokine profile.[169] In 2005, Th17 were described.[60] A study by Yan et al., which used FCM, reported that HBV-related HCC patients had higher frequencies of Th17, lower frequencies of Th1 and a higher Th17/Th1 ratio in PB compared to non-HBV-related HCC and HD. My results revealed that frequencies of Th1, Th2 and Th17 were not different from that of HD. This inconsistency may be due to nonHBV/nonHCV HCC patients in this study. In the previous study reported by Yan et al. a decreased amount of Th1 was also not found in non-HBV-related HCC patients (FCM was used). They indicated that HBV infection may correlate with abnormalities of Th1, TH17.[127] A probable explanation is that chronically inflammation induced by HBV infection can affect $CD4^+$ T cells response.[170] However, the distribution of Th1, Th2 and Th17 in HCC tissues displayed a dramatically difference. My results indicated that the frequency of Th1 was elevated in HCC tissues compared to PB, while Th2 were significantly decreased and Th17 showed a decreased trend. Yan et al. revealed that amounts of tumor infiltrating Th17 and Th1 were significantly higher in HBV-related HCC and non-HBV-related HCC patients than a paracancerous tissues. However, this study used IHC to measure and count immune cells. Moreover, they found that patients with higher infiltrating Th1 densities had besser OS and DFS, while patients with higher infiltrating Th17 densities had worse OS and DFS.[127] Lorvik et al. found that when Th1 were activated in PB, they could migrate to the local microenvironment of tumors.[171] My results also indicated that tumor infiltrating Th1 was dramatically higher than circulating Th1. Higher amounts of Th1 infiltration in this study, suggest that the body can produce a strong antitumor response to HCC. As we found IFN-γ (mainly secreted by Th1) from TILs. Th2 in HCC tissues was rarely reported. My results revealed that amounts of tumor infiltrating Th2 was significantly lower than that in PB. The lower amounts of Th2 cells may partly explain the humoral immunodeficiency in HCC microenvironment. Since the frequency of infiltrating B cells had no difference compared to PB in HCC patients, there was also no difference regarding circulating B

cells between HCC patients and HD. Moreover, most tumor infiltrating B cell subsets were undetectable. The role of Th17 cells in the tumor microenvironment remains controversial. Studies have found that Th17 cells amounts were higher in HCC tissues than corresponding paracancerous tissues.[127, 172] However, IHC was applied to measure Th17 cells in this research. Th17 in paracancerous tissues were not measured in this study. However, my result showed a decreased trend of infiltrating Th17 compared to that in PB. An explanation may be that the majority of Th cells differentiate into Th1 cells to exert anti-tumor effects, resulting in a relative decreased proportion of Th17.

Memory T cells react rapidly and strongly after re-contact with antigens, producing a large number of effector cells and releasing a large number of cytokines.[173] Memory T cells survive in the host for many years in the form of functional silence and slow cell cycle.[174] According to different homing characteristics and effector functions, memory T cells can be divided into two subgroups: emT cells and cmT cells. The results of my study revealed that naive $CD4^+$ T cells decreased significantly in PB of HCC patients. This may suggest that circulating naive T cells are stimulated by tumor antigens and transformed into memory T cells, thus leading to a decline in naive T cell reserve. Beckhove et al. isolated the initial T cells and memory T cells derived from bone marrow, stimulated them with DC, and then imported them into breast cancer bearing mice. It was found that initial T cells did not infiltrate tumors, whereas memory T cells selectively infiltrated tumor tissues.[175] The results of this study indicated that the amount of both circulating and tumor infiltrating $emCD4^+$ T cells was significantly higher, while both circulating and tumor infiltrating $cmCD4^+$ T cells was significantly lower than the control groups. This has not been described previously. This may indicate that more $cmCD4^+$ T cells differentiate to $emCD4^+$ T cells, to exert strong killing function.

B cells play an important role in humoral immunity, even in anti-tumor immune

response. Previous researches, which used FCM, have reported that HCC patients had lower amounts of B cells in PB than HD.[125, 126] However, my results revealed that there was no difference of B cell amounts in PB between HCC patients and HD. This may be related to nonHBV/nonHCV HCC patients in this study. Since it was reported that viral infections result in quantitative and qualitative changes in circulating B cell developmental systems.[176] In the previously reported study by Liu et al., they found that HBV and HCV infections altered the proportions of the lymphocyte subsets, including B cells. Moreover, they suggested that the reduced amounts of T cells and B cells in HCC patients indicated a decreased antiviral ability of the body.[177] Studies have found that the abnormal distribution of memory B cells exists in a variety of autoimmune diseases.[178-180] Consistent with the literature, this research found that memory B cells amounts in PB of HCC patients was significantly lower than that of HD.[135] Similarly, the ns-memory B cells amounts of HCC patients was significantly reduced. The reduction of memory B cells in the PB of HCC patients may due to the development and differentiation of memory B cells into plasma cells after recruitment to secondary lymphoid tissues. Since I found no difference in the frequency of circulating B cells and most of their subsets between HCC patients and HD, and no difference in B cells and their subsets between tumor tissues and PB, I speculate that humoral immunity does not play a major role in nonHBV/nonHCV HCC patients.

As the first line of defense against bacterial invasion, neutrophils play an important role in infection immunity. The NLR is often used to evaluate the balance of systemic inflammatory response and immune system function. Studies found that the OS and RFS of resected patients were significantly poorer in the high NLR HCC patients group.[115-120] My results presented that circulating NLR had an elevated tendency in HCC patients compared to HD. One explanation for an elevated NLR with worse outcome is that many patients with elevated NLR had low amounts of lymphocytes, which may lead to a weakened immune response.[181] As I found no difference of

circulating neutrophils between HCC patients and HD, while lymphocytes especially T cells were significantly reduced. Intertumoral neutrophils are independent prognostic factors for a variety of malignancies.[182, 183] Li et al. found that the density of neutrophils was lower in HCC than the paracancerous tissues. (IHC was used). In addition, increased tumor-infiltrating neutrophils can predict worse outcome of HCC patients after resection.[32] To my knowledge, this is the first study to show that the frequency of tumor-infiltrating neutrophils was dramatically lower than that in PB. It seems that the distribution of cell subsets from the innate immune system, especially neutrophils, was significantly different between tumor tissues and PB.

Monocytes are precursor cells of DCs and macrophages. Pre-mononuclear cells, mononuclear cells, macrophages and cells at all stages of their development are generally referred to as mononuclear macrophage systems. My results firstly presented that monocytes had a significantly lower rate of accumulation in HCC tissues than PB. A possible explanation for this is that monocytes in tissues mature and differentiate into DCs and macrophages. Activated macrophages include two types: Classically activated macrophage (M1) and alternative activated macrophage (M2).[184] Tumor-associated macrophages (TAM), which may have M2 phenotype, are closely related to tumor angiogenesis and lymphangiogenesis.[185,186] Some studies demonstrated that the frequency of macrophages in HCC tissues was lower than that in normal control tissues.[187, 188] However, IHC and real time PCR were used in these two studies, respectively. Macrophages in normal liver tissues were not measured in this study. However, macrophages showed no significant difference between tumor tissues and PB. DC are less than 1% of mononuclear cells in PB, but it has strong antigen-presenting ability.[189] My results illustrated that the frequency of DC in PB of patients with HCC was significantly lower than that of HD, which was consistent with previous reports (performed by FCM).[190, 191] One explanation is that immature DC in PB of HCC patients had maturation disorders, mainly manifested by low expression of

MHC-I molecules.[192] Another possible explanation for this might be higher amounts of MDSC in PB of HCC patients. As mentioned above MDSC can directly inhibit the immune functions of DC. [58] In this study tumor infiltrating DC were also obtained, but they showed no difference from that in PB. The role of DC in tumor microenvironment is controversial. On the one hand, as APC DC can induce antitumor immune responses. On the other hand, under tumor microenvironments DC may polarize into immunosuppressive DC, which can promote appearance of Tregs and MDSC.[193]

NKT cells exert direct anti-tumor effects through the perforin pathway, and can also kill tumor cells via the Fas/FasL pathway and the TNF-α pathway.[194] One research, which used FCM, found that the number of NKT cells in PB of cancer patients was lower than HD, and this reduction was not related to the type or load of tumors.[195] My results also indicate that compared to HD, NKT cells significantly decreased in the PB of HCC patients. A possible explanation for this may be NKT cell death or impaired NKT cell proliferation in cancer patients.[195] Another possible explanation for this might be higher amounts of Tregs in PB of HCC patients. As mentioned above, Tregs can directly inhibit the immune functions of NKT cells. Whether NKT cells are accumulated in tumor tissues are not clear. Some researchers have found that only a small amount of NKT cells exist in the tumor-bearing livers.[196] On the contrary, Motohashi et al. reported an increased amounts of NKT cells in lung tumors.[197] However, these two studies were performed by IHC and did not compare tumor infiltrating NKT cells with circulating NKT cells. My results revealed that the frequency of NKT cells had no difference between tumor tissues and PB.

IFN-γ is a glycoprotein mainly produced by Th and CTL after activated by various physical and chemical factors.[198] In addition, NKT cells, DC and macrophages can also produce a small amounts of IFN-γ.[199] IFN-γ has antiviral, anti-tumor and immune regulation effects. Kortylewski et al. reported that IFN-γ can significantly inhibit the

growth of human melanoma cell lines.[200] Similarly, Majewski et al. found that by directly injecting IFN-γ into tumor-bearing hosts can inhibit tumor growth.[201] In the liver, IFN-γ can regulate hepatocyte apoptosis and cell cycle progression.[202] Jian et al. illustrated that IFN-γ-producing cells were found in HCC tumor tissues and its frequency were higher than adjacent liver tissues. Moreover, this higher frequency of intertumoral IFN-γ-producing cells can predict better OS and DFS.[127] This study confirms that TILs from HCC tissues can produce IFN-γ. Moreover, after stimulating the production of IFN-γ was significantly increased. It can thus be suggested that tumor tissues can induce specific T cell responses with the production of IFN-γ and TILs can play an important role in anti-tumor immunity.

This study has limitations. The sample size of the current study cohort was small. However, nonHBV/nonHCV HCCs represent a select but etiologically homogenous group of patients. All patients with primary HCC resection from 02.2016 to 04.2017 in our center were screened. To obtain this homogenous group we had to exclude patients suffering from hepatitis or with a history of hepatitis. Additionally, ethical concerns only allowed for 10 HD. However, to create balanced groups between HD and HCC patients we chose the sample size of n=10 per group. However, despite the limited sample size we were able to show distinctive immune patterns between those groups. Experimentally, FCM is the appropriate and most effective way to measure the immunophenotype in PB and tumor tissue. However, measurement with FCM in general includes a certain degree of subjectivity when setting gates. By rigorous standardization of our gating strategy and the fact that gating was double checked we were able to prevent human variability in gate setting.

# 5. Conclusion

In conclusion, we have established the technique to measure the immune patterns of circulating immune cells in patients suffering from non-HBV/non-HCV HCC. Compared to age and gender matched HD, we showed that non-HBV/non-HCV HCC patients exhibit distinct differences in the measured immune pattern. Further we demonstrated an immunosuppressive gradient from tumor to peripheral blood. This immunosuppressive state in the PB is actively caused by high amounts of Tregs and MDSC. Moreover, the lower frequency of T cells, NK cells, NKT cells and DC in PB contribute to the weakened anti-tumor response of HCC patients. In contrast, we found the tumor to activate a specific T-cell response. This led to an active secretion of IFN-γ, illustrating the functional state of the involved cell groups.

In the future we plan to use this, combined with machine learning algorithms, to accurately predict survival of resected and transplanted patients. This holds the potential to guide therapy to those patients that truly benefit from surgery.

# 6. Summary

Hepatocellular Carcinoma (HCC) is one of the most lethal tumors in the world. Even though medical technologies have been constantly improving, the overall 5-year survival rate remained unfavorable. Liver transplantation (LT) is the most effective treatment for HCC within cirrhosis and confined to the liver. The Milan Criteria for LT selection are regarded as overly strict. They pay no attention to tumor differentiation, metastasis degree and immune state. These deficiencies prompted people to further explore the allocation of treatment for HCC. Currently, it is believed that the progression and prognosis of various tumors, including HCC, are also related to the

immune status of the patient. Tumor infiltrating leukocytes (TILs) have been revealed to be predictive for outcome of HCC patients after both resection and transplantation. These measurements however are only possible after the treatment. To truly predict outcome before surgery of HCC patients I established a comprehensive assessment of the immunophenotype of nonHBV/nonHCV HCC patients.

In this study, firstly, I conducted a systematic review to assess the differences of circulating immune cells between HD and HCC patients. I found that these studies were limited to investigate one or only selected immune cells. Naturally many results are contradictory and not comparable across the different methods and patients' etiology of HCC. For example, most studies focused on HCC patients with hepatitis. As we know, chronic hepatitis infection can also affect the immune status of HCC patients. In order to truly reflect the impact of HCC on immune status, we selected nonHBV/nonHCV HCC patients. I aimed to comprehensively assess the immune signature of HCC patients and compare it to the immune signature of age and gender matched HD. To further understand the local immune status of HCC, I isolated TILs from HCC patients and compared them with circulating immune cells in the same patients. Lastly, to investigate the functional state, I analyzed IFN-γ production to understand whether TILs were stimulated and functionally active.

Our results indicated that HCC patients had lower amounts of T cells, NKT cells and DC in PB than that in HD. Similarly, the frequency of circulating cmTh and nTh in HCC patients was significantly lower than in HD. On the contrary, HCC patients showed a higher frequency of emTh, Tregs and MDSC in the PB. HCC patients had lower amounts of memory B cells and ns-memory B cells when compared to HD. In HCC tumor tissues neutrophils and monocytes were less abundant when compared to the PB. HCC tumor tissues had a higher accumulation degree of T cells and Th1. Contrary to that, Th and Th2 were less accumulated in HCC tumor tissues when compared to

PB. In HCC tumor tissues emTh were more frequent, while cmTh were less frequent when compared to PB. Moreover, the infiltrating T cells, $CD4^{+}$T cells and $CD8^{+}$T cells in 5 HCC patients can produce IFN-γ.

In conclusion, a comprehensive FCM analysis method was established to assess the immune signature of nonHBV/nonHCV HCC patients. The distribution of circulating immune cells in HCC patients is abnormal, showing an immunosuppressive tendency. With regard to HCC tumor tissue, effector T cells can induce specific anti-tumor response.

With this we can measure the immune patterns of circulating immune cells in HCC patients. In the future we plan to use this to predict survival of resected and transplanted patients to guide therapy to those patients that truly benefit from surgery.

# 7. Zusammenfassung

Das hepatozelluläre Karzinom (HCC) ist einer der tödlichsten Tumoren der Welt. Obwohl sich die medizinischen Technologien ständig verbessert haben, blieb die 5-Jahres-Überlebensrate insgesamt ungünstig. Lebertransplantation (LT) ist die wirksamste Behandlung für HCC bei Leberzirrhose. Die Mailänder Kriterien für die LT-Auswahl gelten als zu streng. Sie achten nicht auf Tumordifferenzierung, Metastasierungsgrad und Immunzustand. Diese Mängel veranlassten die Menschen dazu, die Verteilung der Behandlung für HCC weiter zu untersuchen. Derzeit wird angenommen, dass das Fortschreiten und die Prognose verschiedener Tumore, einschließlich des HCC, auch mit dem Immunstatus des Patienten zusammenhängen. Es wurde festgestellt, dass tumorinfiltrierende Leukozyten (TILs) sowohl nach Resektion als auch nach Transplantation prädiktiv für das Ergebnis von HCC-Patienten sind. Diese Messungen sind jedoch erst nach der Behandlung möglich. Um

das Ergebnis vor der Operation von HCC-Patienten wirklich vorhersagen zu können, habe ich eine umfassende Bewertung des Immunphänotyps von Nicht-HBV / Nicht-HCV-HCC-Patienten durchgeführt.

In dieser Studie führte ich zunächst eine systematische Literatur Review durch, um die Unterschiede der zirkulierenden Immunzellen zwischen gesunden Menschen und HCC-Patienten zu bewerten. Ich fand heraus, dass sich diese Studien darauf beschränkten, eine oder nur ausgewählte Immunzellen zu untersuchen. Natürlich sind viele Ergebnisse widersprüchlich und nicht vergleichbar. Zum Beispiel konzentrierten sich die meisten Studien auf HCC-Patienten mit Hepatitis. Wie wir wissen, kann eine chronische Hepatitis-Infektion auch den Immunstatus von HCC-Patienten beeinflussen. Um den Einfluss von HCC auf den Immunstatus wirklich widerzuspiegeln, haben wir Nicht-HBV/Nicht-HCV-HCC-Patienten ausgewählt. Ich wollte die Immunsignatur von HCC-Patienten umfassend bewerten und mit der Immunsignatur von alters- und geschlechtsangepasster gesunden Menschen vergleichen. Um den lokalen Immunstatus von HCC besser zu verstehen, isolierte ich TILs von HCC-Patienten und verglich sie mit zirkulierenden Immunzellen bei denselben Patienten. Um den Funktionszustand zu untersuchen, analysierte ich schließlich die IFN-γ Produktion, um zu verstehen, ob TILs stimuliert und funktionell aktiv waren.

Unsere Ergebnisse zeigten, dass HCC-Patienten im Blut geringere Mengen an T-Zellen, NKT-Zellen und DC aufwiesen als in gesunden Menschen. In ähnlicher Weise war die Häufigkeit der Zirkulation von cmTh und nTh bei HCC-Patienten signifikant niedriger als bei gesunden Menschen. Im Gegensatz dazu zeigten HCC-Patienten eine höhere Häufigkeit von emTh, Tregs und MDSC in der PB. HCC-Patienten hatten im Vergleich zur gesunden Menschen eine geringere Menge an Speicher-B-Zellen und ns-Speicher-B-Zellen. In HCC-Tumorgeweben waren Neutrophile und Monozyten im Vergleich zum Blut niedriger. HCC-Tumorgewebe hatten einen höheren

Akkumulationsgrad von T-Zellen und Th1. Im Gegensatz dazu waren Th und Th2 im Vergleich zum Blut in HCC-Tumorgeweben weniger akkumuliert. In HCC-Tumorgeweben war emTh häufiger, während cmTh im Vergleich zum Blut weniger häufig waren. Darüber hinaus können die infiltrierenden T-Zellen, $CD4^+$ T-Zellen und $CD8^+$ T-Zellen bei 5 HCC-Patienten IFN-γ produzieren.

Zusammenfassend wurde eine umfassende FCM-Analysemethode etabliert, um die Immunsignatur von Nicht-HBV/Nicht-HCV-HCC-Patienten zu bewerten. Die Verteilung der zirkulierenden Immunzellen bei HCC-Patienten ist abnormal und zeigt eine immunsuppressive Tendenz. In Bezug auf das HCC-Tumorgewebe können Effektor-T-Zellen eine spezifische Antitumorantwort induzieren.

In Zukunft planen wir, dies zu nutzen, um das Überleben von resezierten und transplantierten Patienten vorhersagen zu können, um die Therapie zu den Patienten zu leiten, die wirklich von einer Operation profitieren.

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**Supplement Table 1: Overview of the 6 staining tubes to dedicate T cell and its subsets.** (Tube 1, served as blank control; Tube 2-5, served as FMO control; --, no antibody was added). Tube 1 had no antibodies; Tube 2 included all the antibodies except antibody CD197, CD25, CD196 and antibody HLA-DR; Tube 3 included all the antibodies except antibody CD194, CD127; Tube 4 included all the antibodies except antibody CD38, CD45RO; Tube 5 included all the antibodies except antibody CD27; Tube 6 was the sample.

| Tube | Antibody | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tube 1 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Tube 2 | CD4 | -- | CD194 | CD38 | CD45 | CD27 | -- | CD3 | -- | CD127 | CD45RO | -- | CD8 |
| Tube 3 | CD4 | CD197 | -- | CD38 | CD45 | CD27 | CD25 | CD3 | CD196 | -- | CD45RO | HLA-DR | CD8 |
| Tube 4 | CD4 | CD197 | CD194 | -- | CD45 | CD27 | CD25 | CD3 | CD196 | CD127 | -- | HLA-DR | CD8 |
| Tube 5 | CD4 | CD197 | CD194 | CD38 | CD45 | -- | CD25 | CD3 | CD196 | CD127 | CD45RO | HLA-DR | CD8 |
| Tube 6 | CD4 | CD197 | CD194 | CD38 | CD45 | CD27 | CD25 | CD3 | CD196 | CD127 | CD45RO | HLA-DR | CD8 |

**Supplement Table 2. Overview of the 6 staining tubes to dedicate B cell and its subsets.** (Tube7, served as blank control; Tube8-11, served as FMO control; --, no antibody was added; *, Intracellular antibody). Tube 7, had no antibodies. Tube 8, included all the antibodies except antibody CD5, CD10, CD14; Tube 9, included all the antibodies except antibody CD24, CD20 and antibody IgM; Tube 10, included all the antibodies except antibody CD38 and antibody IgD; Tube 11, included all the antibodies except antibody CD27; Tube 12 was the sample.

| Tube | Antibody | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tube 7 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Tube 8 | -- | IgM | CD38 | CD45 | CD27 | CD19 | CD3 | -- | CD24 | IgD | -- | CD20* |
| Tube 9 | CD5 | -- | CD38 | CD45 | CD27 | CD19 | CD3 | CD10 | -- | IgD | CD1d | -- |
| Tube 10 | CD5 | IgM | -- | CD45 | CD27 | CD19 | CD3 | CD10 | CD24 | -- | CD1d | CD20* |
| Tube 11 | CD5 | IgM | CD38 | CD45 | -- | CD19 | CD3 | CD10 | CD24 | IgD | CD1d | CD20* |
| Tube 12 | CD5 | IgM | CD38 | CD45 | CD27 | CD19 | CD3 | CD10 | CD24 | IgD | CD1d | CD20* |

**Supplement Table 3. Overview of the 6 staining tubes to dedicate monocytes, neutrophils, DC, MDSC, NK and NKT cells.** (Tube13, served as blank control; Tube14-17, served as FMO control; --, no antibody was added; *, intracellular antibody). Tube 13, had no antibodies. Tube 14, included all the antibodies except antibody CD69, CD68, CD16, CD11c and CD66b; Tube 15, included all the antibodies except antibody HLA-DR, CD15 and CD96; Tube 16, included all the antibodies except antibody CD14, CD11b; Tube 17, included all the antibodies except antibody CD33; Tube 18, was the sample.

| **Tube** | | | | | | | **Antibody** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tube 13 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Tube 14 | -- | -- | HLA-DR | CD14 | CD45 | CD33 | -- | CD3 | -- | CD15 | CD11b | -- | CD56 | CD8 |
| Tube 15 | CD69 | CD68* | -- | CD14 | CD45 | CD33 | CD16 | CD3 | CD11c | -- | CD11b | CD66b | -- | CD8 |
| Tube 16 | CD69 | CD68* | HLA-DR | -- | CD45 | CD33 | CD16 | CD3 | CD11c | CD15 | -- | CD66b | CD56 | CD8 |
| Tube 17 | CD69 | CD68* | HLA-DR | CD14 | CD45 | -- | CD16 | CD3 | CD11c | CD15 | CD11b | CD66b | CD56 | CD8 |
| Tube 18 | CD69 | CD68* | HLA-DR | CD14 | CD45 | CD33 | CD16 | CD3 | CD11c | CD15 | CD11b | CD66b | CD56 | CD8 |

**Supplement Table 4. Overview of the 4 staining tubes to dedicate IFN-γ.** (Tube 19, served as blank control; Tube 20, served as FMO control; --, no antibody was added; *, intracellular antibody; Tube 22 were added with non-stimulated cell group, while all other tubes were added with stimulated cell group). Tube 19, had no antibodies; Tube 20, included all the antibodies except antibody IFN-γ; Tube 21, included all the antibodies; Tube 22, was the sample.

| Tube | Antibody | | | | |
|---|---|---|---|---|---|
| Tube 19 | -- | -- | -- | -- | -- |
| Tube 20 | CD4 | CD45 | CD3 | -- | CD8 |
| Tube 21 | CD4 | CD45 | CD3 | IFN-γ* | CD8 |
| Tube 22 | CD4 | CD45 | CD3 | IFN-γ* | CD8 |

**Supplement Table 5: Statistical calculations for all detected subsets between HD and HCC patients.** Abbreviations: aT cells: Activated T cells; aTregs: Activated Tregs; Bregs: Regulatory B cells; cmT cells: Central memory T cells; CTL: Cytotoxic T cells; cs-memory B cells: Class-switched memory B cells; DC: Dendritic cells; eT cells: Effector T cells; emT cells: Effector memory T cells; G-MDSC: Granulocyte-like MDSC; pro-B: Progenitor B cells; pre-B: Precursor B cells; MDSC: Myeloid-derived suppressor cells; M-MDSC: Monocyte-like MDSC; mTregs: Memory Tregs; maTregs: Memory-activated Tregs; nT cells: Naïve T cells; nTregs: Naive Tregs; NK: Natural killer; NKT: Natural killer T; Tregs: Regulatory T cells; Th: Helper T cells; Th1: Type 1 helper T cells; Th2: Type 2 helper T cells; Th17: Type 17 helper T cells.

| Cell Type | HD (Mean ± SD, Number) | HCC (Mean ± SD, Number) | p value |
|---|---|---|---|
| Neutrophils, % of Leukocytes | 55.79±6.83, n=10 | 64.23±21.26, n=10 | 0.25 |
| Monocytes, % of Leukocytes | 5.41±1.77, n=10 | 4.36±2.35, n=10 | 0.28 |
| Macrophages, % of Leukocytes | 0.84±0.47, n=10 | 0.32±0.24, n=10 | 0.01 |
| DC, % of Leukocytes | 0.39±0.24, n=10 | 0.12±0.14, n=10 | 0.01 |

| | | | |
|---|---|---|---|
| MDSC, % of Leukocytes | 0.56±0.44, n=10 | 2.01±2.02, n=10 | 0.04 |
| G-MDSC, % of MDSC | 40±23.43, n=10 | 28±25.75, n=10 | 0.29 |
| M-MDSC, % of MDSC | 35.26±24.52, n=10 | 46.9±29.47, n=10 | 0.35 |
| NK cells, % of Leukocytes | 3.42±1.64, n=10 | 1.94±1.86, n=10 | 0.08 |
| NKT cells, % of Leukocytes | 0.24±0.27, n=10 | 0.06±0.04, n=10 | <0.001 |
| B cells, % of Leukocytes | 2.61±0.82, n=10 | 1.9±1.49, n=10 | 0.2 |
| ns-memory B cells, %of B cells | 8.44±5.06, n=10 | 3.59±4.03, n=10 | 0.01 |
| Naïve B cells, % of B cells | 61.67±23.6, n=10 | 50.2±28.59, n=10 | 0.34 |
| cs-memory B cells, % of B cells | 8.25±7.73, n=10 | 5.28±4.02, n=10 | 0.29 |
| Plasma cells, % of B cells | 4.46±4.82, n=10 | 2.06±2.72, n=10 | 0.14 |
| Plasmablasts, % of B cells | 0.03±0.06, n=10 | 0.2±0.35, n=10 | 0.11 |

| Transitional B cells, % of B cells | 28.46±22.98, n=10 | 11.4±13.76, n=10 | 0.06 |
|---|---|---|---|
| Bregs-2, % of B cells | 1.65±1.57, n=10 | 0.32±0.81, n=10 | 0.01 |
| Pro B cells, % of B cells | 7.35±8.49, n=10 | 2.06±2.36, n=10 | 0.07 |
| Pre B cells, % of B cells | 2.86±2.94, n=10 | 1.38±2.26, n=10 | 0.23 |
| Memory B cells, % of B cells | 31.57±28.34, n=10 | 11.6±8.53, n=10 | <0.05 |
| Bregs-1, % of B cells | 0±0, n=10 | 0±0, n=10 | 0.33 |
| T cells, % of Leukocytes | 30.93±5.14, n=10 | 19.15±12.55, n=10 | 0.01 |
| Th, % of T cells | 67.76±6.66, n=10 | 66.75±18.22, n=10 | 0.87 |
| Th17, % of Th | 15.68±9.83, n=10 | 18.07±11.18, n=10 | 0.62 |
| Th1, % of Th | 54.3±16.44, n=10 | 48.61±17.07, n=10 | 0.46 |
| Th2, % of Th | 13.08±7.71, n=10 | 13.14±8.19, n=10 | 0.99 |

| | | | |
|---|---|---|---|
| emTh, % of Th | 34.54±13.48, n=10 | 55.92±14.77, n=10 | <0.01 |
| cmTh, % of Th | 21.85±9.94, n=10 | 10.45±7.38, n=10 | 0.01 |
| eTh, % of Th | 16.62±14.93, n=10 | 24.28±17.04, n=10 | 0.3 |
| nTh, % of Th | 26.99±13.35, n=10 | 9.35±9.62, n=10 | <0.01 |
| aTh, % of Th | 0.75±0.57, n=10 | 1.21±0.61, n=10 | 0.1 |
| CTL, % of T cells | 25.59±6.8, n=10 | 27.83±17.06, n=10 | 0.7 |
| emCTL, % of CTL | 38.5±17.3, n=10 | 34.78±14.48, n=10 | 0.61 |
| cmCTL, % of CTL | 11.05±4.8, n=10 | 7.85±10.98, n=10 | 0.41 |
| eCTL, % of CTL | 33.4±13.64, n=10 | 42.9±27.54, n=10 | 0.34 |
| nCTL, % of CTL | 17.06±14.11, n=10 | 14.46±18.22, n=10 | 0.73 |
| aCTL, % of CTL | 1.73±1.18, n=10 | 4.4±4.19, n=10 | 0.07 |

| | | | |
|---|---|---|---|
| Tregs, % of Th | 6.49±2.11, n=10 | 10.2±4.8, n=10 | 0.04 |
| mTregs, % of Tregs | 59.36±6.56, n=10 | 64.45±11.6, n=10 | 0.24 |
| maTregs, % of Tregs | 14.53±7.39, n=10 | 14.36±8.5, n=10 | 0.96 |
| nTregs, % of Tregs | 25.82±4.9, n=10 | 20.98±12.08, n=10 | 0.26 |
| aTregs, % of Tregs | 0.19±0.18, n=10 | 0.22±0.18, n=10 | 0.76 |
| Th/CTL | 2.92±1.19, n=10 | 3.91±3.31, n=10 | 0.38 |
| Th1/Th2 | 8.26±9.61, n=10 | 7.58±8.74, n=10 | 0.87 |
| Th1/Th17 | 6.48±7.51, n=10 | 4.7±4.95, n=10 | 0.54 |
| neutrophils/lymphocytes | 1.55±0.58, n=10 | 6.51±8.14, n=10 | 0.07 |

**Supplement Table 6: Statistical calculations for all detected subsets between PB and tumor tissues of HCC patients.** Abbreviations: aT cells: Activated T cells; aTregs: Activated Tregs; Bregs: Regulatory B cells; cmT cells: Central memory T cells; CTL: Cytotoxic T cells; cs-memory B cells: Class-switched memory B cells; DC: Dendritic cells; eT cells: Effector T cells; emT cells: Effector memory T cells; G-MDSC: Granulocyte-like MDSC; pro-B: Progenitor B cells; pre-B: Precursor B cells; MDSC: Myeloid-derived suppressor cells; M-MDSC: Monocyte-like MDSC; mTregs: Memory Tregs; maTregs: Memory-activated Tregs; nT cells: Naïve T cells; nTregs: Naive Tregs; NK: Natural killer; NKT: Natural killer T; Tregs: Regulatory T cells; Th: Helper T cells; Th1: Type 1 helper T cells; Th2: Type 2 helper T cells; Th17: Type 17 helper T cells.



| Cell Type | Blood ( Mean ± SD, Number) | Tumor (Mean ± SD, Number) | p value |
|---|---|---|---|
| Neutrophils, % of Leukocytes | 55.92±22.52, n=5 | 0.85±0.56, n=5 | 0.01 |
| Monocytes, % of Leukocytes | 5.12±2.64, n=5 | 1.97±1.38, n=5 | 0.03 |
| Macrophages, % of Leukocytes | 0.41±0.29, n=5 | 0.31±0.18, n=5 | 0.27 |
| DC, % of Leukocytes | 0.16±0.19, n=5 | 0.14±0.09, n=5 | 0.84 |

| | | | |
|---|---|---|---|
| MDSC, % of Leukocytes | 2.38±2.82, n=5 | 1.83±1.94, n=5 | 0.76 |
| G-MDSC, % of MDSC | 29.34±26.11, n=5 | 1.68±1.69, n=5 | 0.08 |
| M-MDSC, % of MDSC | 44.68±27.91, n=5 | 4.468±5.96, n=5 | 0.03 |
| NK cells, % of Leukocytes | 2.3±2.27, n=5 | 2.31±2.61, n=5 | 0.99 |
| NKT cells, % of Leukocytes | 0.06±0.03, n=5 | 8.13±11.52, n=5 | 0.19 |
| B cells, % of Leukocytes | 2.5±1.59, n=5 | 2.31±2.03, n=5 | 0.87 |
| ns-memory B cells, %of B cells | 2.94±2.27, n=5 | 1.01±1.13, n=5 | 0.26 |
| Naïve B cells, % of B cells | 51.41±29.13, n=5 | 57.83±20.51, n=5 | 0.73 |
| cs-memory B cells, % of B cells | 6.33±5.14, n=5 | 1.31±1.55, n=5 | 0.15 |
| Plasma cells, % of B cells | 1.58±1.82, n=5 | 0.15±0.34, n=5 | 0.18 |
| Plasmablasts, % of B cells | 0.30±0.48, n=5 | 0±0, n=5 | 0.23 |

| | | | |
|---|---|---|---|
| Transitional B cells, % of B cells | 14.03±17.62, n=5 | 6.6±7.07, n=5 | 0.46 |
| Bregs-2, % of B cells | 0.58±1.15, n=5 | 0.38±0.35, n=5 | 0.69 |
| Pro B cells, % of B cells | 2.73±2.91, n=5 | 0.46±0.84, n=5 | 0.2 |
| Pre B cells, % of B cells | 2.54±2.85, n=5 | 0.46±0.84, n=5 | 0.24 |
| Memory B cells, % of B cells | 12.72±7.81, n=5 | 5.58±2.15, n=5 | 0.16 |
| Bregs-1, % of B cells | 0±0, n=5 | 0±0, n=5 | |
| T cells, % of Leukocytes | 22.84±12.15, n=5 | 58.17±15.95, n=5 | 0.02 |
| Th, % of T cells | 73.82±10.43, n=5 | 42.61±14.99, n=5 | ＜0.01 |
| Th17, % of Th | 19.89±14.38, n=5 | 3.40±4.69, n=5 | 0.06 |
| Th1, % of Th | 48.23±21.05, n=5 | 77±22.1, n=5 | 0.04 |
| Th2, % of Th | 12.03±6.36, n=5 | 3.54±0.71, n=5 | ＜0.05 |

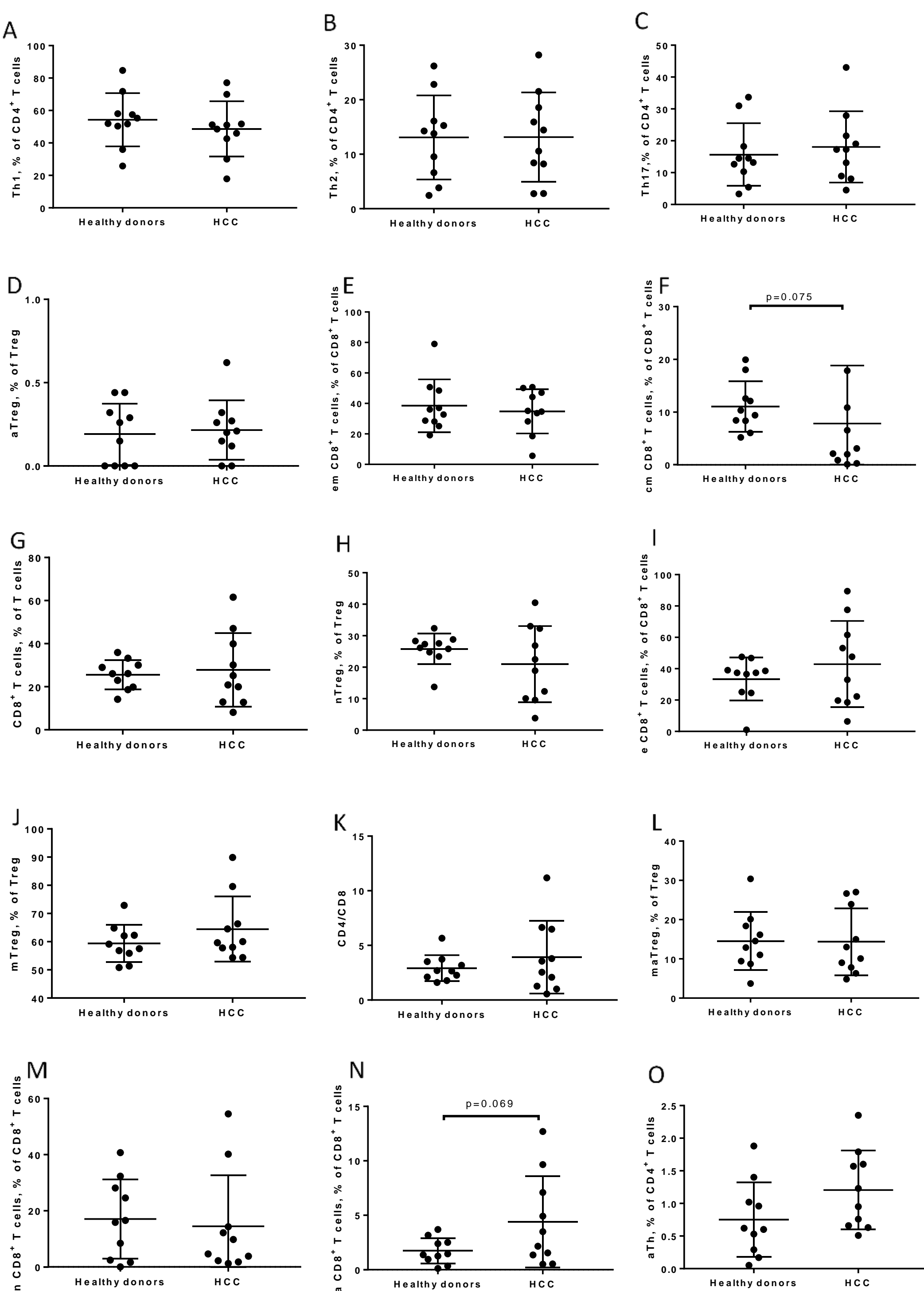
| | | | |
|---|---|---|---|
| emTh, % of Th | 57.28±16.18, n=5 | 78.43±10.67, n=5 | 0.04 |
| cmTh, % of Th | 8.41±4.82, n=5 | 0.09±0.12, n=5 | 0.02 |
| eTh, % of Th | 28.13±22.56, n=5 | 21.15±11, n=5 | 0.47 |
| nTh, % of Th | 6.18±4.91, n=5 | 0.33±0.45, n=5 | 0.06 |
| aTh, % of Th | 1.05±0.48, n=5 | 2.71±3.49, n=5 | 0.38 |
| CTL, % of T cells | 20.35±7.61, n=5 | 21.18±5.04, n=5 | 0.74 |
| emCTL, % of CTL | 38.9±12.78, n=5 | 38.24±21.96, n=5 | 0.95 |
| cmCTL, % of CTL | 11.33±14.92, n=5 | 0.05±0.05, n=5 | 0.17 |
| eCTL, % of CTL | 43.82±24.18, n=5 | 61.62±21.84, n=5 | 0.16 |
| nCTL, % of CTL | 5.95±4.72, n=5 | 0.09±0.11, n=5 | 0.05 |
| aCTL, % of CTL | 4.19±3.97, n=5 | 3.44±7.16, n=5 | 0.87 |

| | | | |
|---|---|---|---|
| Tregs, % of Th | 9.06±5.22, n=5 | 7.02±8.93, n=5 | 0.69 |
| mTregs, % of Tregs | 62.53±10.71, n=5 | 33.25±20.1, n=5 | 0.02 |
| maTregs, % of Tregs | 16.08±9.18, n=5 | 18.74±21.73, n=5 | 0.73 |
| nTregs, % of Tregs | 21.17±12.93, n=5 | 23.89±27.76, n=5 | 0.81 |
| aTregs, % of Tregs | 0.21±0.083, n=5 | 4.12±6.13, n=5 | 0.23 |
| Th/CTL | 4.26±2.17, n=5 | 2.19±1.14, n=5 | 0.02 |
| Th1/Th2 | 8.18±10.98, n=5 | 21.54±3.4, n=5 | 0.04 |
| Th1/Th17 | 5.27±6.71, n=5 | 139.02±120.43, n=5 | 0.06 |
| neutrophils/lymphocyte | 2.96±2.52, n=5 | 0.01±0.01, n=5 | 0.06 |

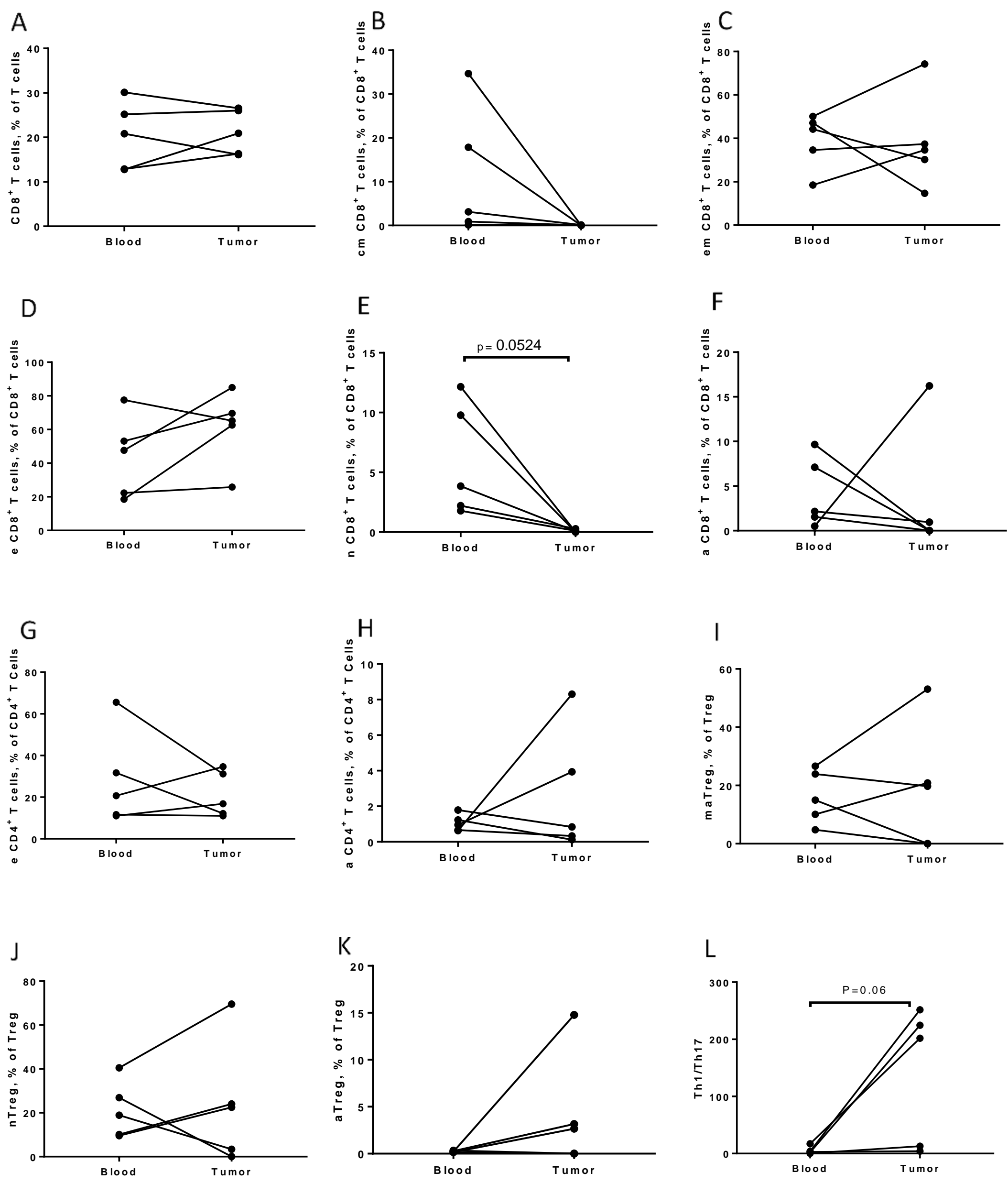
**Supplement Table 7: Statistical calculations for IFN-γ between unstimulated group and stimulated group from tumor tissues of HCC patients.**

Abbreviations: IFN-γ: interferon-γ.

| Cell type | Unstimulated group (Mean ± SD, Number) | Stimulated group (Mean ± SD, Number) | p value |
|---|---|---|---|
| $CD45^{-}$ IFN-$\gamma^{+}$, % of $CD45^{-}$ | 0.17±0.14, n=5 | 0.25±0.18, n=5 | 0.11 |
| $CD45^{+}$ IFN-$\gamma^{+}$, % of $CD45^{+}$ | 0.49±0.29, n=5 | 13.75±7.7, n=5 | 0.02 |
| $CD3^{+}$ IFN-$\gamma^{+}$ ,% of $CD3^{+}$ | 0. 26±0.16, n=5 | 13.76±8.17, n=5 | 0.02 |
| $CD8^{+}$ IFN-$\gamma^{+}$, % of $CD8^{+}$ | 2.03±2.94, n=5 | 42.93±25.07, n=5 | 0.02 |
| $CD4^{+}$ IFN-$\gamma^{+}$, % of $CD4^{+}$ | 0.75±0.12, n=5 | 18.9±10.6, n=5 | 0.02 |




Supplement Figure 1: Dot plots illustrating Th1, Th2 and other subsets of T cells in HD and HCC patients. (Unpaired t test).




Supplement Figure 2: Dot plots illustrating CD8⁺ T cells and its subsets, and other subsets of T cells in blood and HCC tissues (Paired t test).

# IV. Acknowledgment

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