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Cancer Stem-Like Cells in Hepatocellular Carcinoma

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I. Table of Contents

II. List of Abbreviations

%	Percentage
°C	Degree Celsius
µg	Microgram
µL	Microliter
µm	Micrometer
AFP	Alpha-Fetoprotein
AML	Acute Myeloid Leukemia
ASIR	Age-standardized Incidence Rate
ASMR	Age-standardized Mortality Rate
BCLC	Barcelona Clinic Liver Cancer Staging
Breg	Regulatory B Cell
BSA	Bovine Serum Albumin
CHB	Chronic Hepatitis B
CHC	Cholangiocarcinoma
CIC	Cancer Initiating Cell
CRC	Colorectal Cancer
CSC	Cancer Stem Cell
CTC	Circulating Tumor Cell
CTL	Cytotoxic T Cell
CXCL-1	Chemokines (C-X-C motif) Ligand-1
DC	Dendritic Cell

DFS	Disease Free Survival
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate-Buffered Saline
DSN	Digestive System Neoplasm
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-to-Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
eT cell	Effect T Cell
FACS	Fluorescence-activated Cell Sorting
FBS	Fetal Bovine Serum
FCM	Flow Cytometry
FMO	Fluorescence Minus One
GC	Gastric Cancer
G-MDSC	Granulocyte-like MDSC
GPC3	Glypican-3
HBV	Hepatitis B Virus
HBeAg	Hepatitis B Envelope Antigen
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HD	Healthy Donor
HE	Hematoxylin and Eosin Stain
IF	Immunofluorescence

IFN-γ	Interferon-γ
Ig	Immunoglobulin
IHC	Immunohistochemistry
IHF	Immunohistofluorescence
IL	Interleukin
LAT	Local Ablative Treatment
LC	Liver Cirrhosis
LDH	Lactate Dehydrogenase
LR	Liver Resection
LT	Liver Transplantation
LTT	Liver Tumor Tissue
MACS	Magnetic Activated Cell Sorting
maTreg	Memory-activated Treg
mTreg	Memory Treg
MC	Milan Criteria
MDSC	Myeloid-derived Suppressor Cell
MHC	Major Histocompatibility Complex
Min	Minute
Ml	Milliliter
M-MDSC	Monocyte-MDSC
MSC	Mesenchymal Stem Cell
nT cell	Naive T Cell
NASH	Non Alcoholic Steatohepatitis
NAFLD	Non Alcoholic Fatty Liver Disease

nTreg	Naive Treg
NK cell	Natural Killer Cell
NKT cell	Natural Killer T Cell
OS	Overall Survival
PB	Peripheral Blood
PC	Pancreatic Carcinoma
PBMC	Peripheral Blood Mononuclear Cell
PEI	Percutaneous Ethanol Injection
qRT-PCR	Quantitative Reverse Transcription-PCR
RFA	Radiofrequency Ablation
RFS	Recurrence-free Survival
SFT	Spheroid Formation Test
SP cell	Side Population Cell
TACE	Transcatheter Arterial Chemoembolization
TADC	Tumor-associated Dendritic Cell
TAM	Tumor Associated Microenvironment
TCGA	The Cancer Genome Atlas
Tcm cell	Central Memory T Cell
TCR	T-Cell Receptor
Tem cell	Effect Memory T Cell
TGF-β	Transforming Growth Factor-Beta
Th	Helper T Cell
Th1	Type 1 Helper T Cell
Th2	Type 2 Helper T Cell

Th17	Type 17 Helper T Cell
TIC	Tumor Initiating Cell
TIL	Tumor Infiltrating Lymphocyte
TMA	Tissue Microarray
TME	Tumor Microenvironment
Treg	Regulatory T Cell
UCSF	University of California San Francisco
WB	Whole Blood
WHO	World Health Organization

1. Introduction

As a subtype of tumor cells, cancer stem cells (CSCs) are also known as “tumor-initiating cells” or “tumorigenic cells”. Based on their altered immunogenicity, those special cells are in some cases responsible for tumor initiation, metastasis and recurrence. World Health Organization (WHO) data from 2018 states that hepatocellular carcinoma (HCC), the major type of liver cancer that accounts for around 80% of liver cancers, has the sixth highest incidence rate and is the third leading cause of cancer death globally[1]. Schoenberg et al. reported that approximately 70% of patients with HCC who underwent liver resection and 20% of patients who underwent liver transplantation developed recurrence within 5 years after surgery[2]. With the development of immunotherapy, more scientists are now focusing on tumor immunology and exploring a new approach for treating cancers. However, little research has been conducted to demonstrate the interactions between CSCs and the immune system, especially the immune effector cells, in human HCC. This study is aimed at measuring cancer stem-like cells in patients with HCC and analyzing their relationship with immune cells as well as some clinical parameters. In the following text, I would like to use CSCs to represent cancer stem-like cells for easy description. Moreover, based on the co-culture system established by our working group (AG Schoenberg, AVT Department, Grosshadern Hospital, LMU), the influence of immune effector cells on CSCs is investigated.

1.1. Cancer Stem Cell Concept

There is now a general consensus that Bonnet and Dick were the first scientists to find compelling evidence of the presence of CSCs through which the $CD34^{+}/CD38^{-}$ cells derived from acute myeloid leukemia (AML) could initiate hematopoietic malignancies in special immunodeficient mice in 1997[3]. Their pioneering work

inspired others, who demonstrated that this special cell subtype is present in different solid tumors, including but not limited to, breast cancer[4], pancreatic cancer[5], liver cancer[6], colon cancer[7], brain cancer[8], lung cancer[9] and melanoma[10], and with different marker. Similar to normal stem cells, the main properties of CSCs are those such as self-renewal and differentiation. However, unlike normal stem cells that can maintain the balance between self-renewal and differentiation to regulate cell expansion and differentiation through multiple pathways, CSCs have deregulated mechanisms of homeostasis and hence produce and expand increasingly aberrantly differentiated progeny[11].

To better observe and study CSCs, Singh et al. firstly isolated and cultured those special cancer stem cells as tumorospheres from the brain tumor patient with $CD133^+$ marker at the beginning of 21st century[12]. Those spheres owned the self-renewal ability and could differentiate into normal tumor cells whose biology behaved like in the patient. In the ensuring years, CSCs in different kinds of tumors like pancreatic cancer[13], colon cancer[14], lung cancer[15] etc. were also cultured with this method for study. Weiswald et al. summarized the spherical cancer model with 4 major types: the multicellular tumor spheroid model, tumorospheres, tissue-derived tumor spheres as well as organotypic multicellular spheroids and mentioned that tumorospheres have been proved to be a great model for CSCs enriching[16]. Thus I could use tumorosphere as the CSCs marker selection and identification.

In HCC, the following marker has been identified for detecting hepatic CSCs: epithelial cell adhesion molecule (EpCAM)[17], CD133[18], CD90[19], CD44[19], CD24[20], CD13[21], oval cell marker OV6[22], SP[23] and SALL 4[24] etc. (Table 1) For example, CD133/prominin-1, a pentaspan membrane glycoprotein, was proved that could play a key role in HCC tumorigenesis and chemo-resistance by activating Akt/protein kinase B and Bcl-2 survival pathways[25]. Researchers select these marker alone or in combination for detecting CSCs, and prior studies substantiate the belief that this

group of cancer cells represents a minor subpopulation of tumor cells with special properties such as multi-lineage differentiation, tumor initiation, and chemo- or radiotherapy resistance[26-28].

Table 1: Different Marker associated with CSCs in HCC

Marker	Functions in CSCs	Signaling Pathway	References
EpCAM	Tumorigenesis, self-renewal, chemo-resistance, invasion	Wnt/β-catenin	[17]
CD90	Tumorigenesis, self-renewal, multipotency	TGF-β	[19,27]
CD133	Tumorigenesis, self-renewal, chemo-resistance	Akt/protein kinase B and Bcl-2 survival pathways	[18, 25]
CD44	Tumorigenesis, self-renewal, multipotency, maintenance	NOTCH 3	[19,28]
CD24	Tumorigenesis, self-renewal, chemo-resistance	STAT-3 mediated NANOG regulation	[20]
CD13	Tumorigenesis, self-renewal, chemo- and radio resistance	ROS pathway (GCLM)	[21]
OV6	Tumorigenesis, self-renewal, invasion, metastasis	CXCR 4	[22]
SP	Tumorigenesis	Wnt pathway	[23]
SALL 4	Proliferation, differentiation,	Activation of Cyclin D and	[24]

chemo-resistance	EMT

CSCs are now widely and popularly accepted as the potential targets for treating HCC because of their key role in tumor initiation, recurrence, and metastasis. Hence, it is important to identify the mechanisms that could promote CSCs resistance to traditional and conventional cancer treatment modalities. Dawood et al. summarized several potential factors such as the tumor microenvironment, DNA repair function, the presence of multidrug-resistance membrane transporters, and also several self-renewal signaling pathways[29]. Oishi et al. summarized several molecular signaling pathways in liver CSCs such as Wnt/β-catenin, TGF-β family, Notch, Hedgehog, BMI1, Akt/PKB and Lin28/let-7 etc. which could be possible selected as potential therapeutic targets[30]. Smith et al. found that using AKT1 inhibitor significantly reduced the survival proteins expression in $CD133^+$ cells and in addition, the CD133 murine antibody conjugated to a cytotoxic drug could also suppress the proliferation of HCC cell line *in vitro*[31]. However, there remains much to be done before we fully understand the biology of CSCs in HCC.

1.2. Epidemiology and Treatment of Hepatocellular Carcinoma

As the major type of primary liver cancer, HCC is a global health problem, especially in endemic regions (Figure 1): East Asia and Mongolia have the highest age-standardized incidence rates (ASIRs), followed by parts of sub-Saharan Africa and Southeast Asia. Eastern Europe, South Central Asia, as well as Latin America and the Caribbean have the lowest ASIRs[1]. The age-standardized mortality rates (ASMRs) reflect the same tendency as the ASIRs, which prove that HCC is a deadly disease worldwide. The number of new cases and deaths caused by liver cancer are projected to increase from 841,080 and 781,631 in 2018 to 1,361,836 and 1,284,252 in 2040, representing increases of 62% and 64%, respectively[32]. Hepatitis B virus

(HBV) infection is the leading cause of liver cancers, and death caused by HBV-related liver cancer ranks first worldwide, especially in Asian countries. In Europe, alcohol is the greatest risk factor for liver cancer, accounting for nearly 50% of cases[33]. It was reported that over 20% of Europeans aged over 15 years old ingested at least 50 g alcohol once a week and indeed caused the liver cirrhosis, which could be finally developed into liver cancer[34]. Besides alcohol, the non-alcoholic steatohepatitis (NASH) also contributes to the growth of liver-related diseases in Europe. As a progressive form of non-alcoholic fatty liver disease (NAFLD), NASH affects around 1% of Europeans[35]. Considering the major pathogenic determinants to NASH, Type 2 diabetes and obesity also need to be attracted attention as liver cancer indicators[36, 37].

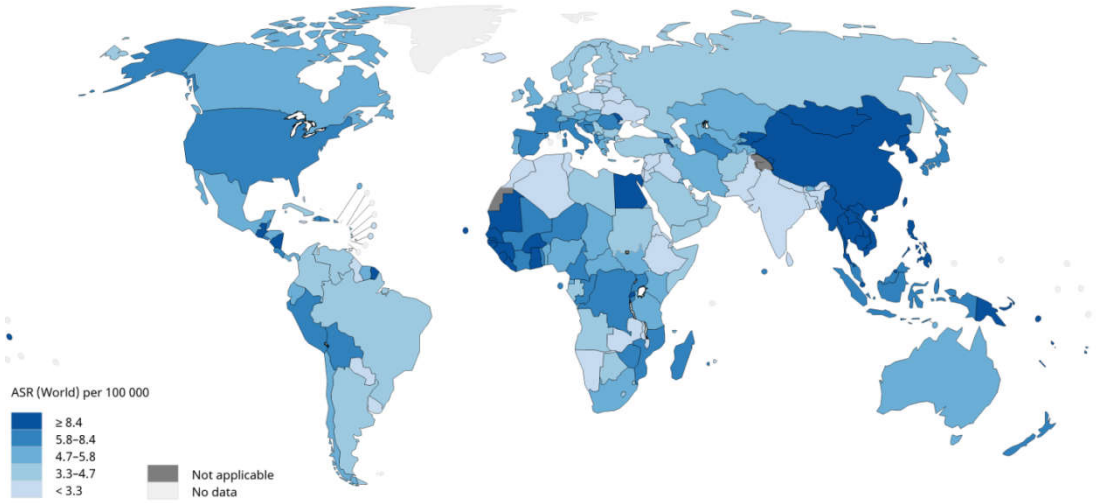


Figure 1: Estimated HCC ASIRs (world) in 2018. Data from WHO[1]

The traditional and conventional HCC treatment modalities include surgery, chemotherapy, radiotherapy, and biological therapy. Liver resection (LR) and liver transplantation (LT) are the major two types of surgery, LR in particular. In the beginning of liver surgery barely any patients survived, especially liver cirrhosis is still a limiting factor. As early as 1886, Dr. Luis performed the first LR, although the patient died 6 hours later due to bleeding[38]. However, over the last 100 years, the role of LR

in HCC has improved dramatically, with less patient morbidity, less mortality, and long-term survival[39]. It could be accomplished by the rapid development of technological advances like standardization, precision. In case of more severe liver cirrhosis with portal hypertension, LR is still no acceptable option for curative treatment. In this case, LT provides more options. However, due to the lack of organs and the need to transplant with acceptable results, the Milan Criteria (MC) has been developed. It states that patients with a single tumor ≤5 cm or no more than three tumors, each no larger than 3 cm, are suitable for LT[40]. But there are still some controversies remaining. Using a prospectively collected transplant database, Duffy et al. concluded that survival could be prolonged in patients beyond the Milan Criteria but within the University of California San Francisco (UCSF) criteria (single tumor <6.5 cm, maximum of three total tumors with none being >4.5 cm, and cumulative tumor size <8 cm)[41]. This underlines that size and number of tumors does not seem to be a good surrogate for tumor biology. In a recent work Schoenberg et al. could show that without upfront size restrictions patients with a good dynamically measured tumor biology can achieve similar results such as patients within MC[42]. As mentioned above however liver for transplantation are rare and not nearly enough to treat all patients. Therefore patients suitable for curative surgery should be stratified between LR and LT based on tumor biology and immunology, to allow for greater benefit for all patients.

Besides surgery, transarterial chemoembolization (TACE), radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), or chemotherapy drugs such as sorafenib[43], regorafenib[44] and lenvatinib[45] are also used for treating HCC as adjuvant therapies or palliative care. Figure 2 shows the most standard guideline for managing patients with HCC[46]. Nevertheless, the clinical treatment to HCC patients is comprehensive and different treatment interventions should be adopted under different conditions.

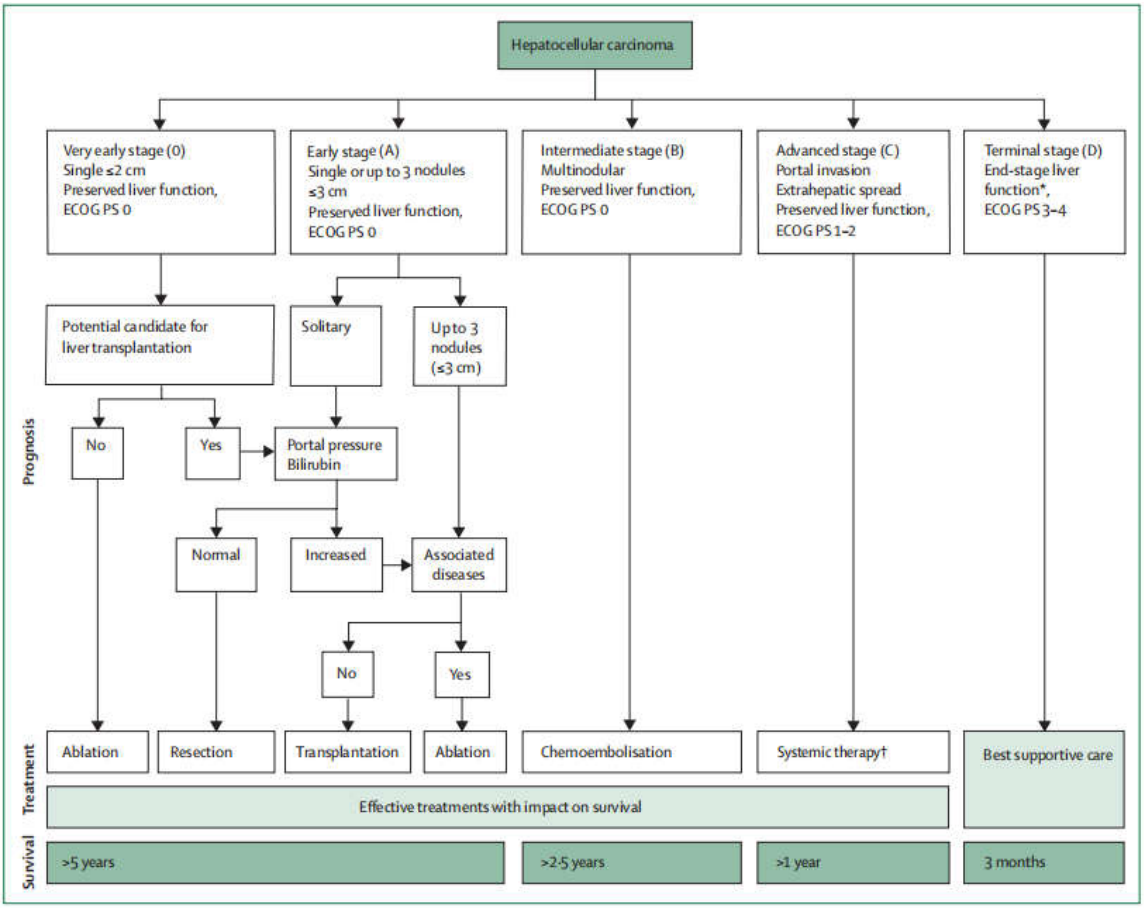


Figure 2: Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy[46] (The reproduction of this figure was kindly permitted by Elsevier and Copyright Clearance Center.)

1.3. Predictive Value of CSC Subsets in Patients with HCC

During the past few decades, scientists have developed different methods for isolating and detecting CSCs, which have provided more opportunities for obtaining pure target cells for examining their biological behavior and relationship with human diseases. Initial studies mostly used HCC cell lines to collect CSCs, which showed higher proliferative and tumorigenic capacity than negative cells. Suetsugu et al. revealed that when $CD133^+$ cells collected from the Huh7 cell line were subcutaneously

injected into severe combined immunodeficiency (SCID) mice, the resultant tumors were matured obviously compared to none or very few tumors formed in the $CD133^-$ control group[47]. Subsequent work by Boutler et al. showed that the percentage of $CD133^+$ in primary tumor tissue account for 1.3–13.6% of all tumor cell populations and that those cells retained the capacity for tumor initiation when inoculated into nude mice[48]. Yang et al. tested specimens from 28 HCC patients' tissue and 36 blood samples, and reported that $CD45^-/CD90^+$ cells account for 0.03–6.2% (0.25%, median percentage) compared to total cells, and that $CD45^-/CD133^+$ cells account for 0–0.41% (0.13%, median percentage) in tumor tissue; in blood, 0–6.9% (0.045%, median percentage) $CD90^+$ cells and 0–0.15% (0.02%,median percentage) $CD133^+$ cells were detected[19]. The proportion of CSCs in HCC cells varies in a certain range because of the use of different detection methods, target marker, and gating strategies.

Due to the capacity of CSCs for tumor initiation, progression, recurrence, and metastasis, some researchers have also investigated the relationship between CSCs and the survival rate of patients with HCC. Using the Cox regression model, Yang et al. included 387 patients with HCC after resection in their study, and confirmed that CD133, CD44, and nestin were independent predictors of OS (overall survival) and RFS (recurrence-free survival)[49]. Kakehashi et al. focused on HCV-infected patients with HCC, targeting $CD44^+$v9 (variant exons) as the CSCs marker, and demonstrated that both OS and RFS correlated negatively with $CD44^+$v9 marker expression[50]. The expression and predictive value of CSCs associated marker in blood or tissue of patients with HCC remains a largely underexplored domain that requires more clinical data for supporting. The above findings have provided preliminary proof that, although CSCs are as a small subgroup compared to normal liver cancer cells, they have a significant impact on prognosis.

1.4. Immunology in the Context of HCC and CSCs

The term “immunology” was proposed by Russian biologist Ilya Ilyich Mechnikov, who first observed the phenomenon of phagocytesis in starfish larvae[51]. The immune system, which consists of molecular and cellular components, is the major part of immunology, and is divided into the innate immune system and the adaptive or acquired immune system. The innate immunity response is rapid but does not have persistent function. Its major functions include activating the adaptive immune system through antigen presentation, recruiting cytokines to infective sites, activating the complement cascade, and mobilizing special white blood cells for identifying and removing foreign substances. Natural killer (NK) cells, neutrophils, macrophages, and dendritic cells (DCs) are the major components of the innate immune cells. In this context, the innate immune system forms the first line of defense for protection. The other major immune system, i.e., the adaptive immune system, leads to an enhanced immune response after an initial response to specific pathogens. It confers long-lasting protection because of immunological memory.

As with the innate immune system, the adaptive immune system includes both humoral immunity and cell-mediated immunity. And it is necessary to have a general introduction of major immune effect cells. T cells (common marker, CD3) which originate from the bone marrow and develop in the thymus gland, can differentiate into subsets based on their cell functions: cytotoxic T cells (CTLs), T helper (Th) cells, and regulatory T cells (Tregs). CTLs, which majorly express $CD3^+/CD8^+$, play a major role in tumor immunity by recognizing specific antigens via T cell receptors (TCRs) and releasing granule contents such as perforin and granzyme to trigger their cytolytic activities[52]. Th cells, also known as $CD4^+$ T cells, release cytokines that aid CTL activation and are essential for B cell antibody class-switching. Tregs commonly express CD4, CD25, and FOXP3. As typical immunosuppressive cells, they play an

important role in preventing autoimmune disease, but may also promote tumor progression. B cells, which can secrete antibodies and present antigens, mainly function in humoral immunity. Kaminski et al. reported that CD19, CD20, CD34, CD38, and CD45R are major marker of immature B cells without immunoglobulin M (IgM), while IgM and CD19 are key marker of mature B cells[53]. More evidence corroborates the fact that the intrahepatic follicle-like structure has the same function as lymph nodes for B cell activation, expansion, and maturation[54]. As considerable components, more studies have focused on the relationship between liver disease and B cells[55]. NK cells, also termed large granular lymphocytes, are enriched in healthy liver and play a critical role against tumor development[56]. In humans, NK cells are normally marked by $CD3^-/CD56^+$, and the main subset in human blood is defined as $CD16^+/CD56^{dim}$. Cai et al. found that patients with HCC had dramatically reduced peripheral $CD16^+/CD56^{dim}$ NK subsets as compared with healthy people, and that $CD16^+/CD56^{dim}$ NK subsets were also significantly reduced in tumor regions compared with non-tumor regions in the livers of patients with HCC[57]. The authors also noted that these NK cells had poor capacity for producing interferon gamma (IFN-γ) and were associated with Tregs[57].

As the largest digestive organ in the human body, the typical immune status of the liver is immunotolerant and anti-inflammatory, and the balance of immune response and immunotolerance is the key to liver normal function[58]. There is more evidence that the liver is the key and frontline immune organ in the human body. The liver contains immune cells such as T cells, B cells, NK cells, macrophages (Kupffer cells) and these cells tend to maintain a tolerogenic environment[59]. Racanelli concluded that, in healthy liver, lymphocytes account for about 5–10% of all liver cells, while T cells, NK cells, and B cells comprise around 63%, 31%, and 6%, respectively[54].

Accompanied by the initiation of HCC, some tumor-associated antigens like α-fetoprotein (AFP), glypican-3 (GPC3) etc. will express on the mutated hepatoma

cells[60]. As mentioned above, those presenting cells like DCs will then recognize and present them to T cells which could trigger the cytotoxic activity to eliminate those tumor cells. However, the tumor microenvironment (TME) which contains some special immunosuppressive factors, would in turn inhibit those T cells and facilitate cancer initiation and progression[61]. Guo et al. analyzed the phenotypes of the tumor infiltrating lymphocytes in HCC patients and found that $CD8^+$ T cells, NK cells and NKT cells have a lower density in tumor tissue compared to non-tumor tissue[62]. Besides that, Tregs, which could suppress effect T cells by secreting TGF-β and IL-10, also play an important role in regulating TME[63]. Macrophages which originate from blood monocyte and differentiate in different tissues, display varies function in their given tissue. In liver cancer, phenotypes of macrophages include M1 and M2 rely on the regional microenvironment[64]. M1 subtype is generally identified as classical activating macrophage and enable to initiate the immune response and tissue reconstruction[65]. On the contrary, M2-Macrophages, which are normally considered as tumor-associated macrophages, are capable of promoting HCC. Yeung et al. collected 95 HCC specimens and concluded that higher proportion of M2 correlated with more tumor nodules and venous invasion, and also indicated a poor prognosis[66]. Moreover, a latest research revealed that CD206, which is highly expressing in tumor-associated macrophages and used as a predictor for HCC occurrence, also could be a potential CSCs marker comparable with Oct-4, c-Myc, CD44 and Nanog[67]. Wang et al. also investigated the influence of tumor-associated macrophages (TAM) on CSCs in human HCC, and showed a promoting function of TAM on CSCs via interleukin-6 (IL-6) secretion and STAT3 signaling activation[68]. Besides, the connection between B cells, T cells and Macrophages was also revealed. Wei et al. found that the CXCL10, a cytokine produced by macrophages with $CD4^+$ T cells stimulation, could bind to the B cells and switch them to IgG producing plasma cells which in turn promoting macrophages to produce cytokines that inhibit anti-tumor

response[69]. Figure 3 shows a basic interaction network between major immune cells and tumor cells.

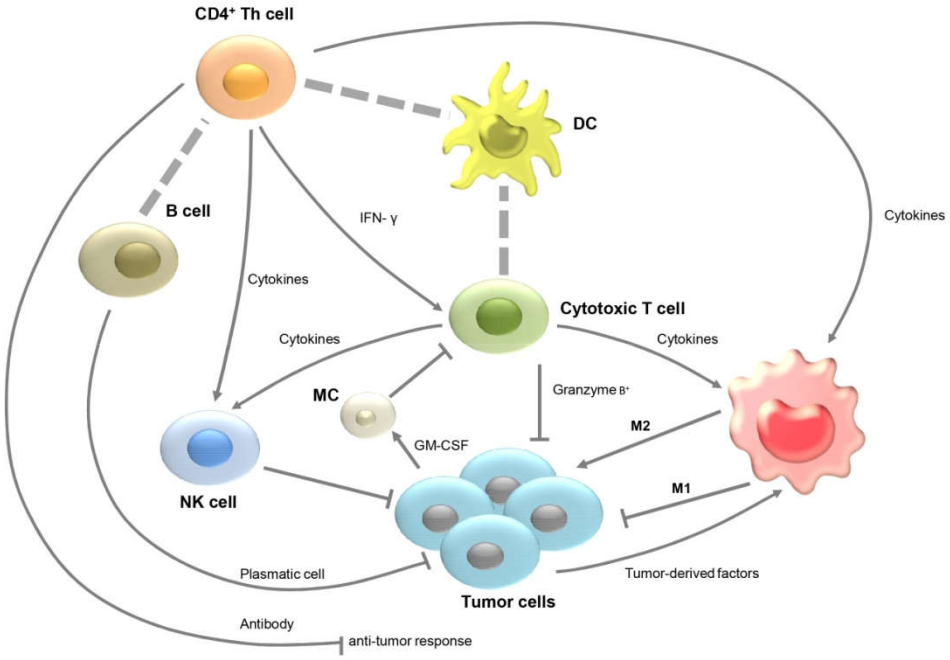


Figure 3: The interaction network between major immune cells and tumor cells

These immune cells have an important influence on the balance between liver immune status and HCC prognostics. However, the relationship between CSCs functionality and human HCC is still not clearly defined. Nevertheless, the immune response to HCC cells and CSCs in human liver remains unclear. Therefore there is an urgent need for more studies which would provide more choices for treating HCC.

1.5. Aim of the Study

This study is aimed at detecting CSCs in peripheral blood (PB) and liver tumor tissue (LTT) from patients with non-HBV/non-HCV HCC. Furthermore I aimed at the analysis of correlation between CSCs and paired immune cells as well as clinical parameters in selected patients. Thus more related data would be collected in understanding the CSC-related immunology in non-HBV/non-HCV HCC and more potential clinical

indicators could be explored. Additionally, based on the establishment of co-culture system by our working group, the influence of major immune effector cells on CSCs in HCC is investigated *in vitro* level.

2. Material and Methods

2.1. Material

2.1.1. Laboratory Equipment

Flow Cytometer	BD Biosciences, USA
Vortex	Labnet, Germany
Gentle MACS Octo Dissociator	Miltenyi Biotec, Germany
QuadroMACS Separator	Miltenyi Biotec, Germany
Microscope	Olympus, Japan
Centrifuge	Heraeus, Germany
CASY Cell Counter & Analyzer	OMNI Life Science, Switzerland
Laminar Flow	Thermo Scientific, USA
Multipette Plus	Eppendorf, Germany
MACS Multi Stand	Miltenyi Biotec, Germany
Pipettes	Sigma-Aldrich, USA
37°C Incubator	Binder, Germany
Water Bath	Köttermann, Germany
4°C Fridge	Liebherr, Germany

-20°C Fridge	Bosch, Germany
-80°C Fridge	Thermo Scientific, USA
Magnetic Mixer	GLW, Germany

2.1.2. Computer and Software

Computer Hardware	HP, USA
FACSDIVA™ Software	BD, USA
CASY 2.5 Ink Software	OMNI Life Science, Switzerland
Graphpad Prism 7	Graphpad Software, USA

2.1.3. Consumables

0.5-20 µL Ep T.I.P.S	Eppendorf, Germany
2-200 µL Ep T.I.P.S	Eppendorf, Germany
Gloves	ecoSHIELD, USA
7.5 mL Heparin Vacuum Blood Collection Tube	Sarstedt, USA
5mL Polystyrene Round-Bottom Tube	Falcon, USA
Gentle MACS C Tube	Miltenyi Biotec, Germany
15 mL Falcon	Falcon, USA

50 mL Falcon	Falcon, USA
5 mL Peptite	Greiner Bio-one, Austria-Germany
10 mL Peptite	Greiner Bio-one, Austria-Germany
25 mL Peptite	Greiner Bio-one, Austria-Germany
50 mL Peptite	Greiner Bio-one, Austria-Germany
1 mL CryoTube Vials	Thermo Fischer Scientific, USA
40 µm Cell Strainer	Corning Incorporated, USA
30 µm MACS SmartStrainer	Miltenyi Biotec, Germany
LS Column	Miltenyi Biotec, Germany

2.1.4. Chemical

Bovine Serum Albumin (BSA) Fraction V	Biomol, Germany
DPBS (1x)	PAN Biotech, Germany
RPMI Medium 1640 (1x)	Gibco, USA
DMEM:F12 (1:1)	Gibco, USA
Fetal Bovine Serum (FBS)	PAN Biotech, Germany
Penicillin-Streptomycin	PAN Biotech, Germany

FACS Lysing Solution (10x)	BD, USA
Ibidi Freezing Medium	Ibidi GmbH, Germany
IC Fixation Buffer (10x)	eBiosciences, Austria
Permeabilization Buffer (10x)	eBiosciences, Austria
Trypsin EDTA	Lonza, Switzerland
Trypan Blue	Sigma, Germany
Fixable Viability Stain (FVS) 510	BD, USA
B27	Thermo Fischer Scientific, USA
FGFb (100 µg/mL)	Immuno Tools, Germany
EGF (500 µg/mL)	Immuno Tools, Germany
Tumor Dissociation Kit	Miltenyi Biotec, Germany
CD45 (TILs) Micro Beads	Miltenyi Biotec, Germany
CD133 Micro Beads	Miltenyi Biotec, Germany

2.1.5. Buffers and Solutions

MACS Buffer	500 mL	DPBS (1x)
	292.25 mg	EDTA (2 m/M)

	2.5 g	BSA (0.5%)
Spheroid Medium	30 mL	DMEM/F12 (1:1)
	0.3 mL	Penicillin-Streptomycin
	600 µL	B27
	3 µL	FGFb (100 µg/mL)
	1.2 µL	EGF (500 µg/mL)
1x Lysing Solution	50 mL	10x Lysing Solution
	450 mL	Millipore H2O
1x Permeabilization Buffer	pH	7.3
	8 mL	10x Permeabilization buffer
	72 mL	Millipore H_2O
FACS Buffer	pH	7.3
	1 L	1x DPBS
	2 mL	Natriumacid
	5 g	BSA
Cell Culture Medium	445 mL	RPMI Medium 1640 (1:1)
	50 mL	FBS

	5 mL	Penicillin-Streptomycin
Co-Culture Medium	445 mL	DMEM:F12 (1:1)
	50 mL	FBS
	5 mL	Penicillin-Streptomycin

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-CD8	Mouse (BALB/c) IgG1, κ	APC-H7	Human
Anti-CD16	Mouse BALB/c IgG1, κ	FITC	Human
Anti-CD45	Mouse (BALB/c) IgG1, κ	BV650	Human
Anti-CD56	Mouse BALB/c IgG2b, κ	APC R700	Human
Anti-CD90	Mouse BALB/c IgG1,	APC	Human
Anti-CD107a	Mouse BALB/c IgG1, κ	PE-CF594	Human
Anti-CD133	Mouse IgG1, κ	PerCP-eFlour 710	Human

2.2. Methods

2.2.1. Literature Review

To investigate the interactions between cancer stem cells and major immune cells in digestive system neoplasms (DSNs), a systematic literature review was conducted by searching PubMed database and relevant references up to May 2020 to collect all related studies. The following search term (“Digestive System Neoplasms") [Mesh] AND ("Neoplastic Stem Cells"[Mesh] OR "Neoplastic Cells, Circulating"[Mesh]) AND ("Immune System"[Mesh] OR "Immune System Phenomena"[Mesh]) was used and 389 publications were left. Several exclusion criteria were applied for collecting the relevant literature: 1) Published before January 2000; 2) Not published in English; 3) Review, case report, letter, comment; 4) Clinical trials or study of therapy; 5) Not DSN or DSN metastatic cancer; 6) No CSCs; 7) No immune cells; 8) No interaction. Then the following data was extracted from available studies: authors’ name, objects of study, cell types, cell sources, analysis methods, interactions and mechanisms.

2.2.2 Patients and Clinical Data

In this study, 13 patients with primary HCC (No HBV/HCV infection) were recruited. All patients underwent curative liver resection from 2018 to 2019 at the Department of Surgery, Campus Grosshadern, Ludwig-Maximilians-University Munich (LMU) hospital. The collection of PBMC from healthy donors obtained the informed consent of the volunteers. Institutional review board approvement was obtained (#EK 54-16, 53-16, 261-16 UE).

Additionally, patients’ clinical characteristics including gender, age, hepatitis, the presence of cirrhosis, tumor lesions, micro-vascular invasion, tumor staging as well as biochemical indicators including serum alpha-fetoprotein, bilirubin, albumin, alanine

transaminase (ALT), aspartate transaminase (AST), activated partial thromboplastin time (APTT), creatinine, creactive protein (CRP), leukocytes count, and platelets count were all examined by laboratory medicine department and collected in our database for correlation analysis.

2.2.3. Cell Culture

The human HCC cell line HepG2 (HB-8065) was purchased from ATCC (Manassas, VA, USA). A routinely mycoplasma test and authentication were performed in our laboratory. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin (P-S) for proliferation. To increase the ratio of CSCs in HepG2, DMEM:F12 medium supplemented with 10% FBS，1% P-S was then used for cell culturing. Cells were incubated at 37°C in a 95% humidified atmosphere containing 5% CO_2 in our laboratory cell line room. Medium was routinely changed.

2.2.4. MACS Isolation of Cells

Cell line: Prepared single HepG2 cell suspension was stained with Trypan Blue and counted under microscope. Cells were centrifuge with 300×g for 10 mins and then the supernatant was completely discarded. 300 μL of MACS buffer was then added for re-suspension per 10^8 total cells for CD133 staining. 100 μL of FcR blocking reagent and 100 μL of CD133 microbeads per 10^8 total cells was added to those cells successively. The mixture was vortexed well and incubated for 30 mins in the dark place (refrigerator: 2-8°C). Cells were then washed with 1−2 mL of MACS buffer per 10^8 cells and centrifuged with 300×g for 10 mins. The supernatant was discarded completely and cells were re-suspended up to 10^8 cells in 500 μL of MACS buffer. LS column was then placed on the QuadroMACS Separator and rinsed with 3 mL of MACS buffer firstly. Cell suspension was applied carefully without bubble onto the

column and washed with 3 mL of MACS buffer 3 times. The effluent was collected together as unlabeled cells ($CD133^-$). Column was then moved out of the separator and placed onto a suitable collection tube. 5 mL of MACS buffer was pipetted into the column and the plunger was pushed into the column immediately to flush out cells. I collected those cells as target positive cells and then proceeded to next steps.

2.2.5. Spheroid Formation Assay

Previously divided HepG2 cells were centrifuged with 500×g, 5 mins separately and then the supernatant was discarded completely followed by cell counting with Trypan Blue. 50000 cells were seeded into a 12-well plate with 2.5 mL of spheroid medium per well. Cells were cultured in 37°C incubator for 7 days which provided CSCs enough time for spheroid formation and control group was set up with CD133 negative HepG2 cells in same condition. After 7 days, all groups were observed under microscope for spheroids counting and comparison.

2.2.6. Extraction of Single Cells from PB and LTT

Peripheral blood (PB) from patients or HD was diluted 1:1 with DPBS and mixed well. Maximum 20 mL of mixture was added onto 15 mL of Biocoll carefully. Then the mixture was centrifuged with 2000×g, 20 mins without brake and the interphase (Mononuclear cells phase) was collected carefully without breaking the surface of Biocoll. Those cells were then washed with 20 mL of DPBS and followed by centrifugation with 300×g, 10 mins with brake. 20 mL of DPBS was added in and those cells were centrifuged with 200×g, 10 mins for washing step. 10 mL of DPBS was pipetted inside for cell counting. For cell freezing, IBIDI freezing medium was used with 1.5 x 10^6 cells per cryotube and those cryotubes were stored in the isopropanolbath bath overnight at -80° and moved to the liquid nitrogen tank for long-term preservation.

Tumor infiltrating lymphocytes were mixed with tumor cells, fibroblasts, red blood cells, etc. Using tumor dissociated kit is better to remove those irrelevant cells and protect TILs and primary tumor cells. The kit consisting of three kinds of enzymes needed to be aliquoted before the experiment. Previously, 4.7 mL of RPMI 1640 medium was mixed with 200 µL of enzyme H, 20 µL of enzyme R and 25 µL of enzyme A which were suitable for 0.2-1.0 g liver tumor tissue (LTT). Tumor biopsies were cut into 2-4 mm pieces after removing fat, fibrous and necrotic areas and then transferred into a gentle MACS C tube with enzyme mixture. Program (Tough, 37C_h_TDK_3) was then run for cell dissociation. After termination of the program, the C tube was detached from the dissociator and samples were re-suspended. A 30 µm MACS Smart Strainer was placed on a 50 mL falcon tube to filter cell suspension and cells were washed with 20 mL of RPMI 1640 medium. A centrifugation was followed with 300×g, 7 mins and cell pellets were collected as the mixture of TILs and primary HCC. The storage steps were similar to PBMC.

2.2.7. Co-cultivation of HepG2 and PBMC from HD

1 x 10^6 HepG2 were seeded into a 6-well plate with 2 mL of co-culture medium per well as experimental group 6 hours before co-culture. Isolated PBMCs were then added into the well with the ratio of 25:1 (PBMC: HepG2). Cells were mixed well and incubated in the co-culturing incubator (37°C, 5% CO2) for 24h. Control groups including HepG2 and PBMC alone were also set up with same cell amounts per well to keep the consistent condition. For harvesting cells in experimental group, most of those suspended cells were PBMCs and attached cells were mainly HepG2 which needed to be digested by Trypsin/EDTA. FACS analysis was then applied for at 0h and 24h. The proportion of CSCs in total tumor cells was then measured.

2.2.8. Immunophenotyping Staining in PB

PB samples were harvested following operation manual. Related immunophenotyping panels include T cells, B cells, monocytes, neutrophils, DC, MDSC, NK cells, NKT cells were established by our group. Mature panels and staining methods are described in the dissertation of Tong Zhu which published on 16. Nov. 2020 (DOI: 10.5282/edoc.26929).

2.2.9. Staining Panel of FACS

The flow cytometry (FCM) analysis consists of CSCs marker expression both in PB and LTT of HCC patients, and also in co-culture system *in vitro*. Each panel includes unstained tubes which served as blank control, fluorescence minus one (FMO) control tubes and experimental tubes.

All samples were tested within 24 hours as soon as possible and conducted at room temperature. The specific steps are as follows:

Staining methods for CSCs in HCC patients' PB and LTT panel (extracellular staining): 200 µL of whole blood or cell suspension was added in each FACS tube, and then antibodies were added. All tubes were vortexed and incubated for 15-30 mins. 2 mL of 1xFACS lysing solution was added. Cells were then vortexed and incubated for another 10 mins. All tubes were then followed by centrifugation with 500×g for 5 mins and supernatant was discarded then. Cells were washed with 2 mL of FACS buffer and 200 µL of FACS buffer was added and all stained cells were measured. Table 2 shows the staining panel.

Table 2: FACS staining panel for CSCs in patients' PB and LTT

Tube	Antibody		
	BV650	APC	PerCP-eFlour 710
Unstained	--	--	--
FMO 1	CD45	--	CD133
FMO 2	CD45	CD90	--
FMO 3	--	CD90	CD133
Sample	CD45	CD90	CD133

Staining methods for CSCs in co-culture system: Cells were divided into 2 parts, 200 µL of cell suspension for unstained cells and the others were all used for FVS 510 staining. Cells were suspended to $1x10^6$ cells/mL and FVS 510 antibody was added with the ratio of 500:1 (1 mL cells with 2 µL FVS 510 antibodies). The mixture was incubated for 10-15 mins at room temperature or 30-60 minutes at 2-8°C protected from light and followed by 2 mL of FACS buffer washing step twice (500×g, 5 mins). After discarding supernatant, the mixture was gently vortexed and 1 mL of DPBS was added inside. The following FACS staining steps were same as PB and LTT measurement which described above. Table 3 shows the staining panel for co-culture system.

Table 3: FACS staining panel for CSCs in co-culture system

Tube	Antibody			
	BV510	BV650	APC	PerCP-eFlour 710

Unstained 1	--	--	--	--
Unstained 2	FVS 510	--	--	--
FMO 1	FVS 510	CD45	--	CD133
FMO 2	FVS 510	CD45	CD90	--
FMO 3	FVS 510	--	CD90	CD133
Sample	FVS 510	CD45	CD90	CD133

2.2.10. Gating Strategy of CSCs Marker

Using unstained cells and FMO control tubes, gating strategies are described as follows.

For MACS isolation, as shown in Figure 4, according to FSC/SSC scatter plot (Figure 4A) major cells were selected first and then the proportion of $CD133^+$ cells was compared to each other (Figure 4B, 4C, 4D).

In PB, as shown in Figure 5, according to FSC/SSC scatter plot (Figure 5A) and CD45/SSC plot (Figure 5B), all lymphocytes were selected and identified as $CD45^+$ cells. After inverting gate, major tumor cells were then selected as major $CD45^-$ cells. Then $CD90^+/CD45^-$ CSCs, $CD133^+/CD45^-$ and $CD90^+/CD133^+/CD45^-$ CSCs were gated based on the FMO control (Figure 5C, 5D, 5E).

Similar to the gating in PB, debris part was firstly moved out based on FSC/SSC scatter plot during LTT measurement (Figure 6A). Tumor infiltrating lymphocytes ($CD45^+$) and tumor cells ($CD45^-$) were then separated by FMO control (Figure 6B). As shown in Figure 6C, 6D and 6E, CSCs labeled with different kinds of marker were

then detected.

After selecting major cells (Figure 7A), CD45 marker was used to distinguish tumor cells and lymphocytes in experimental group (Figure 7B). FVS 510 was then selected for identifying dead cells in tumor cells and living tumor cells were gated by inverting (Figure 7C). Cell labeled with $CD90^+$, $CD133^+$ and $CD90^+/CD133^+$ were detected as shown in Figure 7D, 7E, 7F. In HepG2 control group, most of the gating steps were similar to the experimental group except $CD45^+$ step.

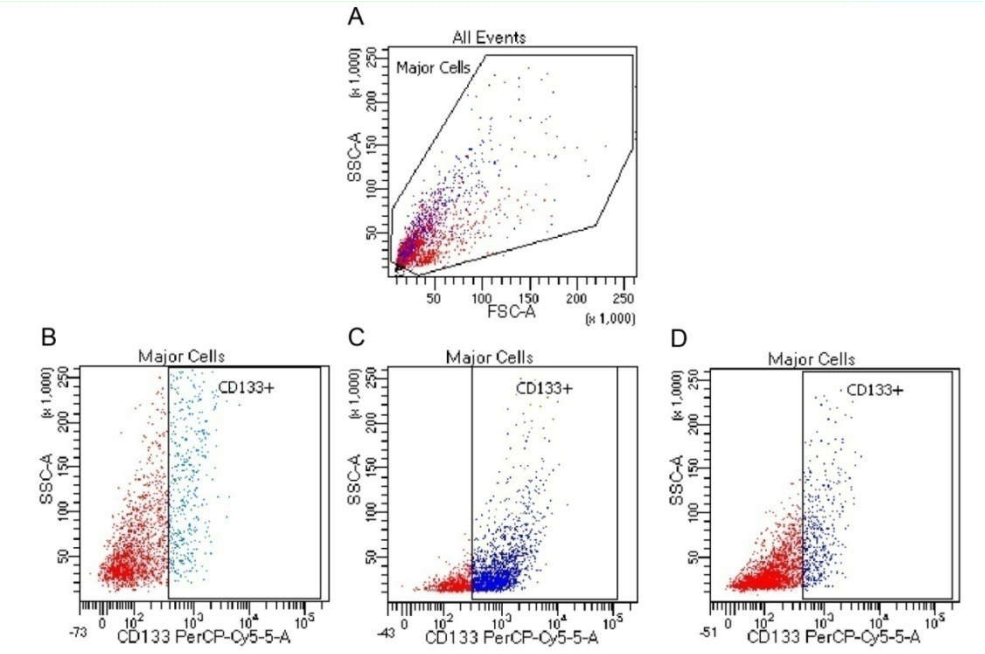


Figure 4: Gating strategy of CSCs after MACS isolation in HepG2 cell line. (A) Major cells from HepG2 cell line; (B) $CD133^+$ cells before MACS; (C) $CD133^+$ cells after MACS in positive group; (D) $CD133^+$ cells after MACS in negative group.

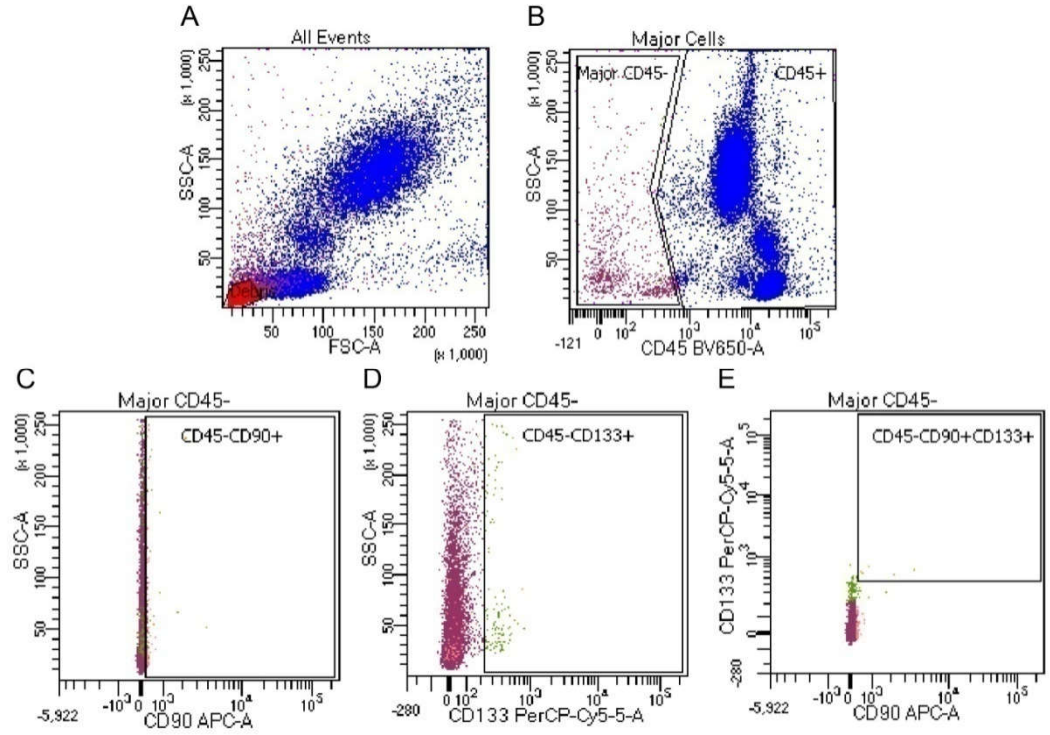


Figure 5: Gating strategy of CSCs in PB. (A) All cells from PB except red blood cells; (B) Leukocytes and major tumor cells; (C) $CD90^{+}/CD45^{-}$ CSCs; (D) $CD133^{+}/CD45^{-}$ CSCs; (E) $CD90^{+}/CD133^{+}/CD45^{-}$ CSCs.

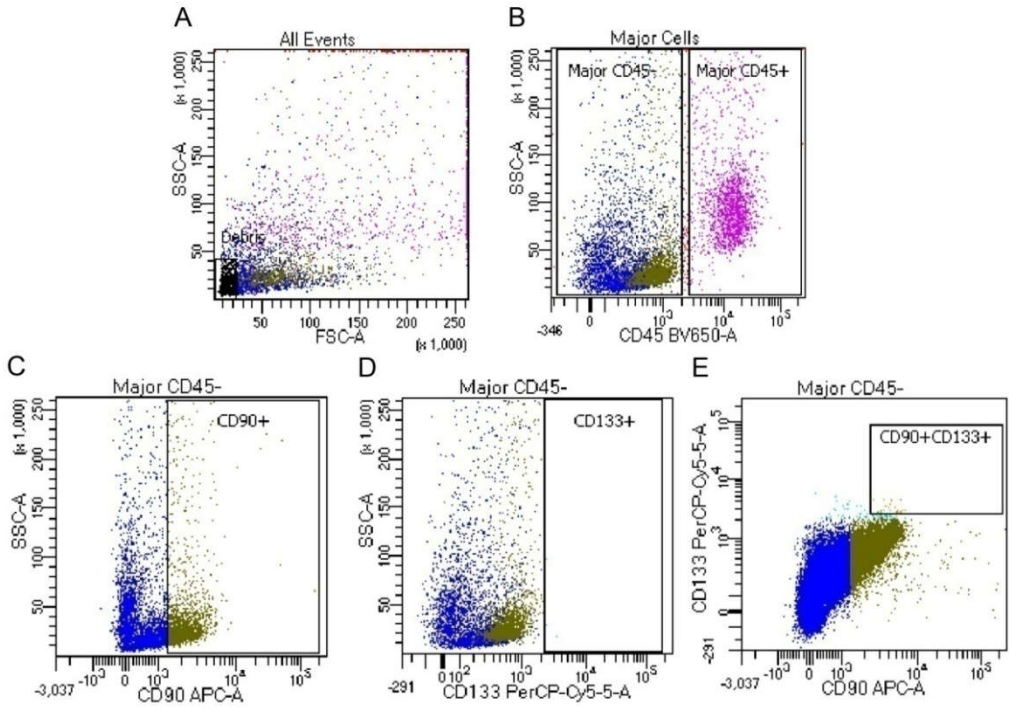


Figure 6: Gating Strategy of CSCs in LTT. (A) All cells from LTT including tumor cells and TILs; (B) Major leukocytes and tumor cells; (C) $CD90^+/CD45^-$ CSCs; (D) $CD133^+/CD45^-$ CSCs; (E) $CD90^+/CD133^+/CD45^-$ CSCs.

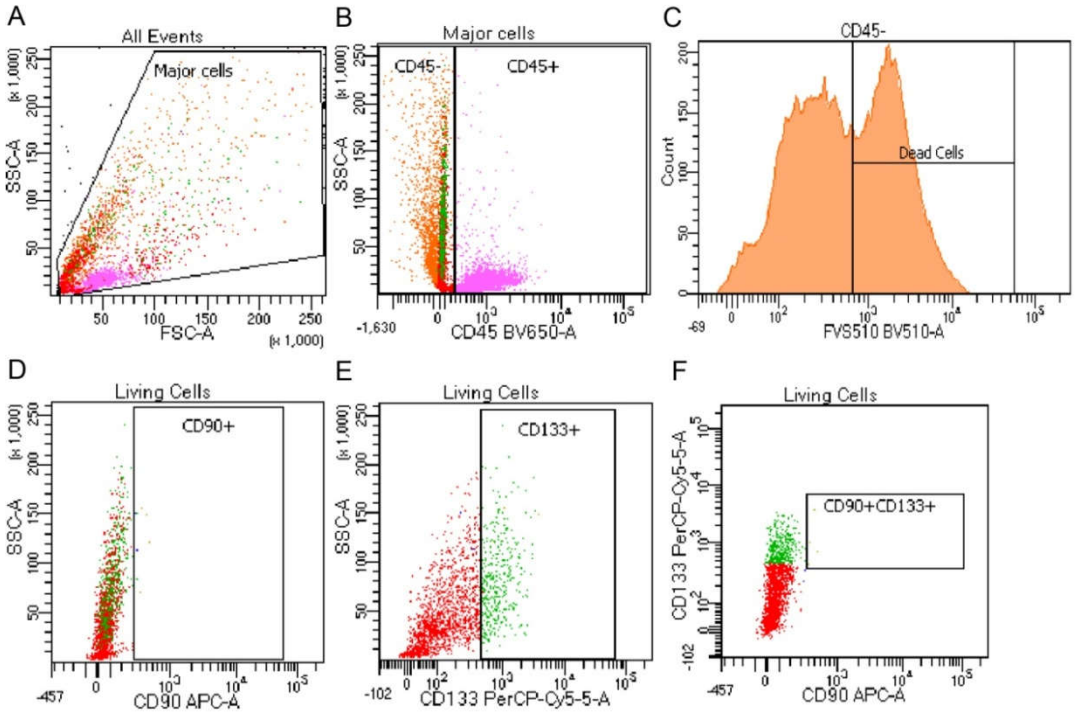


Figure 7: Gating strategy of CSCs in co-culture system. (A) All cells collected from experimental group; (B) Lymphocytes and major tumor cells; (C) Dead tumor cells; (D) Living $CD90^+/CD45^-$ CSCs; (E) Living $CD133^+/CD45^-$ CSCs; (F) Living $CD90^+/CD133^+/CD45^-$ CSCs. (In control group, there is no $CD45^+$ cells, thus the separation step of $CD45^+$ is canceled.)

2.2.11. Statistical Analysis

Graphpad Prism 7.0 software was used for statistical analysis. The Kolmogorov-Smirnov (K-S) method was used to test whether the measurement variables were subjected to normal distribution. Normal distributed variables were compared with parametric t test and corrected by Welch's t test if standard deviation (SD) was not equal. Two groups of non-normal distribution variables were compared with Mann-Whitney U test. One-way analysis of variance (One-way ANOVA) method

was used for the comparison of CSCs proportion in three groups after MACS isolation as well as spheroids formation assay. Pearson's Correlation Coefficient was applied for testing the correlation between two continuous data and showed as r and p values. Mean±SD (standard deviation) was calculated for presenting CSCs proportion. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Literature Review

This systematic literature review describes the interactions between CSCs and major immune cells in digestive system neoplasms (DSNs). Figure 8 shows that the above search terms yielded 389 publications, 147 of which were collected after their titles and abstracts had been assessed. Review of the full text led to 103 publications being excluded according to the exclusion criteria, and an eventual 44 articles were eligible and included in this literature review.

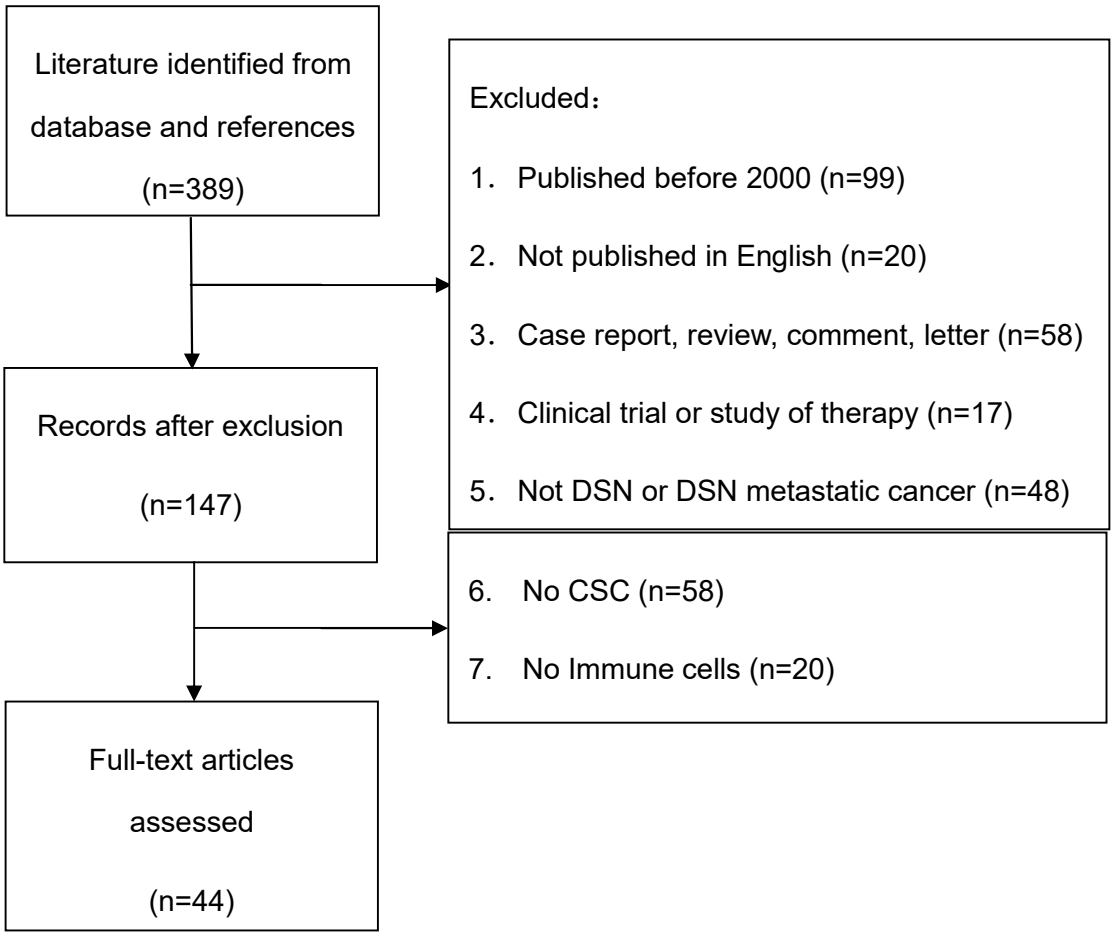


Figure 8: Flowchart of the literature exclusion criteria

I selected five major DSNs: liver cancer (LC), pancreatic cancer (PC), colorectal cancer (CRC), gastric cancer (GC), and cholangiocarcinoma (CCC) as my targets. Figure 9A shows their proportion in this review. In the past 20 years, more research has focused on CRC and LC, which separately account for 38.6% (n=17) and 29.5% (n=13) of DSNs, respectively. While only one publication referred to the interactions between CSCs and immune cells in cholangiocarcinoma.[68, 70-99]

Considering the difficulty of cell source acquisition and marker expression stability, cell lines are the most common source, especially for CSCs. Up to 61.36% (n=27) of publications use cell lines as a source of CSCs and/or immune cells, and only 11.36% of articles (n=5) select related patients as the cell source (Figure 9B).[68, 72-74, 76-80, 83, 85, 86, 88, 89, 92, 94-98, 100-106]

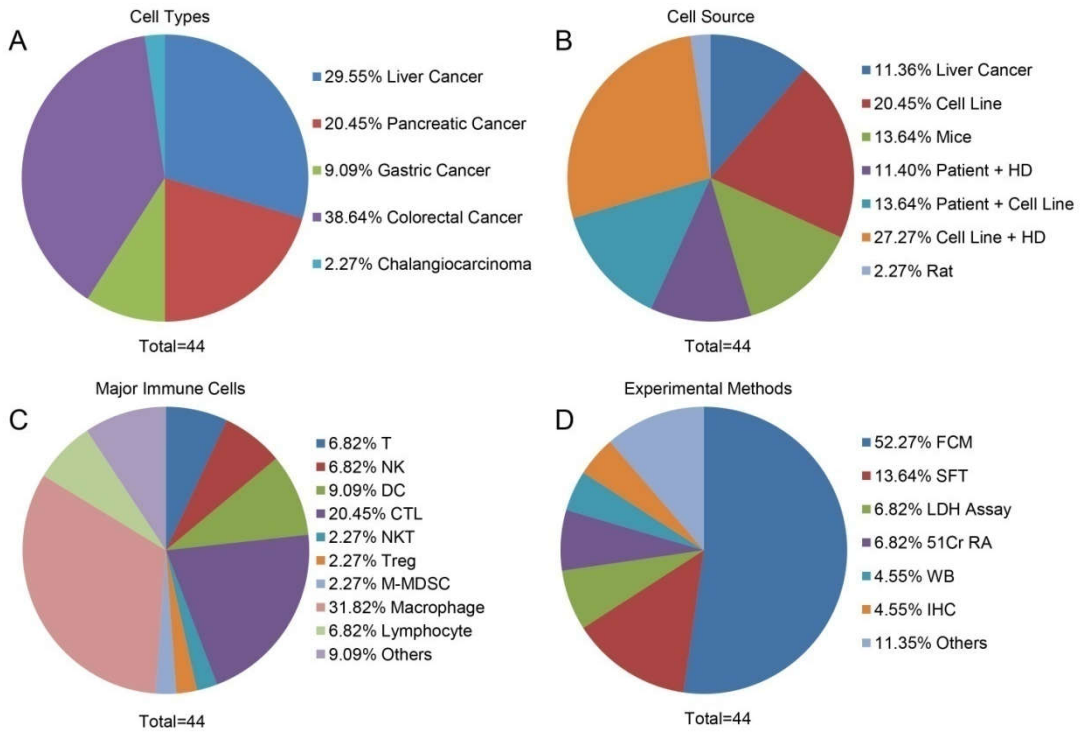


Figure 9: Characteristics of studies included in this review. (A) The cancer types involved in this study; (B) Source of the specimens; (C) Target major immune effector cells in the present study; (D) Experimental methods.

Immune effector cells are a major part of the immune system, and comprise several subsets. In this review, I focused on the major immune effector cells that play a key role in inhibiting or promoting CSCs. Figure 9C shows that macrophage (common marker,$CD14^+/CD68^+$) is the most commonly targeted immune cell (31.82%, n=14), followed by CTL ($CD3^+/CD8^+$, 20.45%, n=9). Four papers (9.09%) target DCs ($HLA\text{-}DR^+$ $lineage^-$), and three papers target T cells ($CD3^+$), NK cells ($CD3^-/CD56^+$), and lymphocytes ($CD45^+$) separately. By contrast, Tregs ($CD3^+/CD4^+/CD25^+/FOXP3^+$), M-MDSC ($CD11b^+/CD14^+/HLA\text{-}DR^{low}/CD15^-$), NKT cells ($NK1.1^+$) and Th cells ($CD3^+/CD4^+$) each only accounts for 2.27% (n=1) of papers in this review.[90, 96, 102, 107, 108]

The most common experimental method used in those studies is flow cytometry (FCM). Figure 9D shows that half of studies use FCM to examine interactions (52.27%, n=23).[68, 70, 71, 73, 74, 77, 79, 80, 84-87, 90, 93, 98, 101-104, 107, 109-111] Unlike other normal tumor cells, CSCs have the unique ability to form spheroids; hence, the sphere formation test was another commonly used method following FCM (13.64%, n=6)[75, 81, 82, 95, 99, 100]. Three publications use the lactate dehydrogenase (LDH) assay, which can detect low-level damage on the cell membrane[72, 77, 105]. Three CTL-related studies use the 51Cr release assay, which can assess T cell cytotoxicity precisely and accurately, particularly in the study of tumor cytolysis[89, 96, 97].

In the following paragraph, the results regarding the interactions between immune cells and CSCs are concluded and divided according to cancer types.

Table 4 shows that 10 studies report that CTLs can inhibit or kill CSCs: four studies (LC, n=3; GC, n=1) use DC-stimulated CTLs that could promote CTL cytotoxicity[72, 74, 76, 112]. Another five studies, which are all on CRC, target new antigens specifically expressed on CSCs, such as ASB4, CEP55, DNAJB8, and OR7C1, and that could be identified by CTLs, and then inhibited CSCs[88, 89, 92, 96, 97]. Xu et al. isolated TILs from

human LTT, and co-cultured them with the Hep12 cell line (primary cell line from a patient with recurrent HCC), and demonstrated that the TILs could recognize and kill CSCs, accompanied by tumor necrosis factor (TNF)-α and IFN-γ secretion[80].

Three publications (PC, n=1; GC, n=1; CRC, n=1) provide information about NK cells with CSCs, and all of them indicate that NK cells have the ability to inhibit or kill CSCs[87, 104, 105]. Xia et al. selected GC cell line BGC823 as the CSCs source and co-cultured them with NK cells from patient PB, and showed that NK cytotoxicity could be enhanced by $CD133^+$ CSCs and attenuated by DKK3[105].

Macrophages, especially TAM, typically have a similar phenotype toM2 macrophages, which play a pivotal part in regulating the inflammatory microenvironment in different solid tumors[113]. Table 4 shows that TAM in the LC, PC, CRC, and CCC groups all promote CSCs tumorigenesis, growth, metastasis, or angiogenesis through different signaling pathways[68, 70, 71, 75, 78, 81, 82, 85, 95, 99, 100, 110, 111]. However, Zhang et al. found that TAM has the opposite effect on gastric CSCs. They co-cultured TAM and GC-CSCs, both from cell lines, for 24h and 48h, separately. Cell viability was detected using the MTT colorimetric assay, which demonstrated that TAM suppress CSCs viability.[106] Guo et al. used a transgenic HCC mouse model and revealed that CSCs could recruit macrophages and in turn promote tumorigenesis[71].

As antigen-presenting cells, DCs are correlated with several immune responses against tumor cells. Two studies (LC, n=1; CRC, n=1) found that DCs could inhibit CSCs, and Zhong et al. indicated that CSCs could also in turn suppress DCs in a CRC mouse model[79, 86, 91]. Interestingly, Hsu et al. reported, on the contrary, that tumor-associated DCs increase the properties of cell mobility and epithelial-to-mesenchymal transition (EMT) of colon CSCs via CXCL-1 expression[83]. Amoletti et al. selected several immune cells that include DCs from patients with PC to culture with $CD44^+/CD147^+/EpCAM^+/CD45^-$ (common CSC marker) circulating

tumor cells (CTCs) and also observed the enhancement of CSCs evasion when DCs was pre-stimulated by CTCs[114].

Three studies involve lymphocytes (HCC, n=1; PC, n=2)[80, 101, 103]. A white blood cell subtype, lymphocytes have been revealed by many studies to play an important role in tumor suppression. Two papers report conclusions consistent with this, while Yin et al. showed that CSCs could also in turn inhibit lymphocyte proliferation[101].

Treg is an immunosuppressive T cell subpopulation that actively suppresses immune system activation as well as T cell proliferation. One study in the CRC group revealed that, consistent with normal tumor cells, Treg could promote CSCs[90].

Janakiram et al. used a PC mouse model and reported that NKT cells could inhibit PC-CSCs by modulating M2 macrophages[108]. Panni et al. proved that M-MDSC could promote CSCs stemness in human PC via a STAT3-dependent mechanism[102]. Xu et al. observed that Th cells could promote GC-MSC ($CD29^+/CD90^+/CD105^+$) by up regulating PD-L1 expression via STAT3 activation[107].

Taken together, the interactions between CSCs and the major immune effector cells in DSNs are controversial, and more data are required. For LC, especially HCC, more studies have concentrated on macrophages and DCs. CTLs, which comprise the major part of the adaptive immune system, were pre-treated with DCs or pre-stimulated by CSCs in most studies. For NK cells in HCC, only one study mentioned that DC and cytokine–induced killer (DC-CIK: NK and T) cells could inhibit CSCs. Thus, in the present study, thirty-nine kinds of immune cell subtypes are involved and analyzed for correlation with CSCs in non-HBV/non-HCV HCC patients. Besides, some clinical parameters are also selected for investigating related clinical significance. Furthermore, the interactions between CSCs and major immune effector cells like NK cells and cytotoxic T lymphocytes are studied in co-culture system.

Table 4: Summary of Included Studies. Abbreviations: LC: Liver cancer; GC: Gastric cancer; PC: Pancreatic cancer; CRC: Colorectal cancer; CCC: Cholangiocarcinoma; HD: Healthy donor; IF: Immunofluorescence; SFT: Sphere formation test; LDH: Lactate dehydrogenase; HE: Hematoxylin and eosin stain; WB: Western blot; IHC: Immunohistochemistry; IHF: Immunohistofluorescence; ELISA: Enzyme-linked immuno-sorbent assay; TMA: Tissue microarrays; MTT Assay: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR: Reverse transcription polymerase chain reaction; FCM: Flow cytometry; CCK-8: Cell counting kit-8; TIC: Tumor initiating cell; CSC: Cancer stem-like cell; CIC: Cancer initiating cell; CTL: Cytotoxic lymphocyte; Th: T helper; MDSC: Myeloid-derived suppressor cell; TAM: Tumor associated macrophage; TADC: Tumor-associated dendritic cell; Treg: Regulatory T cell; CTC: Circulating tumor cell; DC: Dendritic cell; CIK: Cytokine-induced killer; NK Cell: Nature killer cell; TIL: Tumor infiltrating cell; SP: Side population; TGF-beta1: Transforming growth factor beta 1; MHC: Major histocompatibility complex; TNF-α: Tumor necrosis factor-α; IFN-γ: Interferon gamma; IL22: Interleukin 22; N/A: Data not found.

LC Literature details

Reference	Cell			Interaction	Mechanism	Remark
	Cell Source	Cell Type (Marker)	Experimental Method	Promotion →		

	CSC	Immune Cell	CSC	Immune Cell		Inhibition ⊣		
Debebe A et al.2017[70]	Mice	Mice	TIC ($CD133^+/CD49f^+$)	Macrophage ($CD68^+$)	FCM	Macrophage → TICs	Wnt/β catenin pathway	Pten-null Mice
Guo X et al.2017[71]	Mice	Mice	TIC ($Ki67^+/K19^+$ / $HNF4\alpha^+$)	Macrophage ($CD11b^+/F4/80^+$)	FCM	Macrophage → TICs	YAP^+ Activation and Ccl2 /Csf1 expression	Transgenic Mice
Xiao L et al.2016[72]	Cell Line	HD Blood	CSC ($CD133^+/EpCAM^+$)	CTL ($CD8^+$)	LDH Releasing Assay	CTL ⊣ CSC	DC-SP cell-stimulated CTL	Cell line: MHCC97

Sun S et al.2016[73]	Cell Line	Cell Line	CSC ($CD44^+$/$CD133^+$/$ALDH1^+$)	TAM ($CD68^+$/$CD163^+$)	FCM	CSC → TAM	Macrophage trans-differentiation into TAM with M2-like phenotype properties	Cell lines: SMCC-7721/ THP-1
Pan Q Z et al.2015[74]	Cell Line	HD Blood	CSC ($CD133^+$)	CTL ($CD8^+$)	FCM	CTL ⊣ CSC	CTL induced by ANXA3-transfected DC	Cell line: Huh7
Wan S et al.2014[68]	Cell Line	Patient Blood	CSC ($CD44^+$)	TAM ($CD14^+$)	FCM	TAM → CSC	STAT3 and IL-6 signaling	Cell lines: HepG2/Hep3B. HCC Patients (n=5)
Fan Q M et al.2014[75]	Mice	Mice	CSC ($EpCAM^+$)	TAM (N/A)	SFT	TAM →	TAM secretes higher TGF-beta1 than other	CSC cell line: Hepa1-6

						CSC	phenotypes of macrophage	TAM cell line: RAW264.7
Sun J C et al.2010[76]	Cell Line	HD Blood	CSC ($CD133^+$)	CTL （$CD8^+$）	LDH Release Assay	CTL ⊣ CSC	DC loaded with CD133+ Huh7 cell RNA can induce specific CTL	CSC cell line: Huh7
Yang et al.2018[77]	Cell Line	Patient Blood	CSC ($CD90^+$/$CD133^+$)	DC ($CD14^+$/$CD83^+$/$CD86^+$) CIK:T/NK ($CD3^+$/$CD4^+$/$CD8^+$/$CD56^+$)	FCM	DC-CIK ⊣ CSC	Caspase-3	CSC cell lines: HepG2/SMMC7721. HCC Patients （n=7）
Chen et al.2019[78]	Cell Line	Cell Line	CSC ($CD133^+$)	M2-Macrophage	WB	M2-Macrophage	TNF-α Secretion and Wnt/β catenin pathway	CSC cell line: SMMC7721.

				(N/A)		→ CSC		M2 cell line: THP-1 Cell.
Li et al.2019[79]	Cell Line	Cell Line	CSC ($EpCAM^+$)	iDC (N/A)	FCM	DC ⊣ CSC	4-AAQB increases the expression of MHC-I and MHC II as well as CD80	CSC cell line: HepG2. iDC cell line: THP-1.
XU et al.2009[80]	Cell Line	Patient	CSC ($CK18^+$/CK 19^+/AFP^+)	Lymphocyte	FCM	TIL ⊣ CSC	TIL recognizes and kills CSC, especially pre-treated Hep12 cell line by TNF-α and IFN-γ.	CSC cell line: Hep12 from Recurrent HCC Patient. TIL from HCC Patient Tissue
Li et	Rat	Rat	CSC	Macrophage	SFT	Macrophage	TNFR1/Src/STAT3	CSC cell line: Rat

al.2017[81]		(EpCAM$^+$/ CD90$^+$)	(N/A)		⟶ CSC	pathway	LPC-like WB-F344

PC Literature details

Reference	Cell					Interaction	Mechanism	Remark
	Cell Source		Cell Type (Marker)		Experimental Method	Promotion ⟶ / Inhibition ⟞		
	CSC	Immune Cell	CSC	Immune Cell				
Arnoletti et al. 2018[114]	Patient Blood	Patient Blood	CTC (CD44$^+$/CD147$^+$/EpCA	T cell (CD3$^+$/CD45$^+$)	FCM	T ⟞	T cell could kill or inhibit CSC but itself was also	PDAC patients (n=11, 6 with preoperative

			$M^+/CD45^-$)	MDSC ($CD33^+/CD1$ $1b^+/CD14$ $^+/CD11c^-/MH$ CDR^-) DC ($CD11c^+/$ $MHCDR^+$)		CSC; MDSC ⟶ CSC; DC ⟶ CSC.	influenced; MDSC promotes CSC proliferation and apoptosis resistance; CSC-primed DC promotes CSC evasion.	chemotherapy); Patients with chemotherapy are more sensitive to T cell cytotoxicity.
Shi et al. 2019[100]	Cell Line	HD Blood	CSC ($CD90^+$)	Monocyte/Macrophage ($CD14^+/CD68^+$) T Cell（$CD4^+/CD8^+$）	SFT	Macrophage ⟶ CSC; CSC ⊣ T Cell	IL-8 mediates the promotion of monocyte/Macrophage; CD90+ CSC exhibits high PD-L1 which could drive T cell exhaustion and dysfunction.	CSC cell line: SW1990; Monocyte/Macrophage cell line: THP-1; T cell: HD Blood.

Janakiram et al. 2017[108]	Mice	Mice	CSC (DCLK1$^+$/ LCR5$^+$)	TAM ($CD68^+$/Stabilin$^+$) NKT (Nkp1.1$^+$)	IHC	NKT ⊣ CSC	NKT cell inhibits pancreatic tumorigenesis by modulating M2 macrophage and expression of mPGES-1/5-LOX$^-$.	KPT-CD1d mice (NKT deficient)
Yin et al. 2014[101]	Cell Line	HD Blood	CSC (N/A)	Lymphocyte (N/A)	FCM	CSC ⊣ Lymphocyte	CSC inhibits lymphocyte proliferation.	CSC cell line: Panc-1
Sainz et al. 2015[110]	PatientTissue	HD Blood	CSC ($CD133^+$)	TAM ($CD68^+$/CD16$^+$/$CD163^+$)	FCM	TAM → CSC	TAM secretes hCAP-18/LL-37.	-
Panni et al. 2014[102]	Cell Line	HD Blood	CSC	Mo-MDSC	FCM	Mo-MDSC	Mo-MDSC promotes stemness in human PC by	CSC cell lines: Panc-1/BxPC3;

			(CD45$^-$/EpCAM$^+$/ALDH1^{Bright},CD24$^+$/CD44$^+$)	(CD14$^+$)		⟶ CSC	a STAT3-dependent mechanism.	
Sainz et al. 2014[111]	Patient Tissue	HD Blood	CSC (CD133$^+$)	M2 Macrophage (CD68$^+$/CD163$^+$)	FCM	M2-Macrophage ⟶ CSC	Macrophage increases the expression and secretion of ISG15 and it promotes CSC phenotypes.	PDAC Patients (n=2)
Grotha et al. 2012[103]	Cell Line	HD Blood	CSC (EpCAM$^+$/CD24$^+$/CD44$^+$)	T cell (CD3$^+$/CD4$^+$/CD8$^+$)	FCM	TRAIL-lymphocyte ⟞ CSC	N/A	T cell was pretreated with TRAIL; CSC cell line: BxPC3.

Ames et al.2015[104]	Cell Line	HD Blood	CSC ($CD24^+/CD44^+/ALDH^{bright}$)	NK ($CD45^+/CD56^+/CD3^-$)	FCM	NK ⊣ CSC	N/A	CSC cell lines: Panc-1/BxPC3;

GC Literature details

Reference	**Cell**				**Interaction**	**Mechanism**	**Remark**
	Cell Source		Cell Type (Marker)		Experimental Method	Promotion → Inhibition ⊣		
	CSC	Immune Cell	CSC	Immune Cell				

Bagheri et al. 2019[112]	Patient Tissue	Patient Blood	CSC ($CD44^+$/$CD54^+$/$EpCAM^+$)	DC ($CD83^+$/$CD40^+$/$HLA\text{-}DR^+$) CTL ($CD8^+$)	Calcein Release Assay	CTL ⊣ CSC	CTL inhibits CSC through being stimulated by DC loaded with CSC	GC Patients (n=4)
Xu et al. 2018[107]	Patient Tissue	HD Blood	MSC ($CD29^+$/$CD90^+$/$CD105^+$)	Th ($CD4^+$)	FCM	Th → CSC	CD4+ T cell up-regulates PD- L1 expression in GC-MSC by activating p- STAT3	-
Xia P, et al. 2017[105]	Cell Line	Patient Blood	CSC ($CD133^+$)	NK (N/A)	LDH Release	NK ⊣	CD133+ CSC enhances NK cytotoxity and DKK3 could attenuate it.	CSC cell line: BGC823

					Assay	CSC		
Zhang C, et al.2015[106]	Cell Line	Cell Line	CSC (N/A)	TAM (N/A)	MTT	TAM ⊣ CSC	COX-2/PGE-2/TGF-β/VEGF signal pathways	CSC cell line: SGC-7901

CRC Literature details

Reference	Cell				Interaction	Mechanism	Remark
	Cell Source		Cell Type (Marker)		Experimental Method	Promotion → Inhibition ⊣		
	CSC	Immune	CSC	Immune Cell			

		Cell						
Fang M, et al.2017[82]	Patient Tissue	Patient Tissue	CSC (N/A)	Tumor infiltrating Macrophage ($CD3^-/CD19^-/CD14^+$)	SFT	Macrophage ⟶ CSC	IL33 promotes macrophage PGE2 production and in turn promotes colon cancer stemness	-
Hsu et al. 2018[83]	Cell Line	HD Blood	CSC ($CD44^+/CD133^+/CD326^+$)	TADC ($CD14^+$)	ALDH Assay	TADC ⟶ CSC	TADC-derived CXCL1 increases the stemness of CSC.	CSC cell line: SW620
Hwang et al. 2019[84]	Mice	Mice	CSC ($CD133^+/CD133^+/CD44^+$)	Neutrophils ($CD11b^+/Ly6G^{High}/Ly6C^{Low}$)	FCM	Neutrophils ⟶ CSC	CSC prolongs the viability of neutrophils and promotes a pro-tumoral phenotype and in turn promotes CSC	Balb/C mice; CSC Murine cell line:CT26;

							through secretion of IL-1β.	Neutrophils from mice bone marrow.
Huang et al. 2017[85]	Cell Line	Cell Line	CSC ($CD133^+$)	M2-TAM (N/A)	FCM	TAM → CSC	TAM is associated with YAP1 which could enhance the stemness of CSC.	CSC cell lines: HCT116/DLD-1; M2-TAM cell line: THP-1
Szaryńska et al.2018[86]	Cell Line	HD Blood	CSC ($CD133^+$/ $CD44^+$)	DC ($CD11c^+$/HLA-DR^+/$CD80^+$/ $CD83^+$)	FCM	DC ⊣ CSC	DC could inhibit CSC especially pre-stimulated by CSC.	CSC cell lines: HCT-119/HT29
Tallerico R, et al.2013[87]	Patient Tissue	Patient Blood	CIC ($CD166^+$/ $CD44^+$/C	NK ($CD56^+$/$CD57^+$/$CD3^-$/CD1	FCM	NK ⊣ CIC	N/A	-

			D24^{+}/CD133^{+}/Lgr5^{+}/EpCAM^{+}/CEA^{+})	6^{+}/CCR7^{+})				
Miyamoto et al. 2018[88]	Cell Line	HD Blood	CSC (N/A)	CTL (CD8^{+})	IHC	CTL ⊣ CSC	ASB4 is a specific target ligand expressing in CSC and can be identified by CTL.	CSC cell line: SW480
Gao et al.2015[89]	Cell Line	Cell Line	CSC (SP Cell)	CTL （CD8^{+}）	51Cr Release Assay	CTL ⊣ CSC	CEP55, a tumor-associated antigen can be identified by CTL.	CSC cell line: SW480; CTL from CTL clone-41.
Yang et al.2011[90]	HD Tissue	Patient Blood	CSC (CD133^{+}/	Treg (FoxP3^{+}/IL17	FCM	Treg →	N/A	-

			CD44^{+}/CD166^{+}/EpCAM^{+}/ALDH1^{+})	$^{+}$)		CSC		
Zhong et al.2018[91]	Mice	Mice	CSC (Sox2^{+}/Oct3/4^{+}/Nanog^{+}/c-Myc^{+}/β-catenin^{+}/ Klf4^{+})	DC (N/A)	WB	CSC ⊣ DC	DC can be suppressed by CSC and restored by anti-TGF-β1 and LMW-OPS.	C57BL/6 male mice ; CSC Murine cell lines: CMT93/CT26; DC derived from mice bone marrow.
Morita R, et al.2014[92]	Cell Line	HD Blood	CSC (CD133^{+})	CTL (CD8^{+})	RT-PCR	CTL ⊣	CTL clone specific for DNAJB8 recognized SP cells at a higher level than	CSC cell lines: SW480/HCT15/HT29

						CSC	that of MP cells and inhibits CSC.	
Volonté A, et al.2014[93]	Patient Tissue	Patient Blood	CIC ($Nanog^+$/ $OCT\text{-}4^+$/$Sox2^+$/$ALDH\text{-}1^+$/$Aldefluor^+$/ $CD133^+$/$CD44^+$/$CD24^+$/Ep-CAM$^+$/CEA^+)	T Cell ($CD3^+$/$CD4^+$/ $CD8^+$/$CD45RO^+$)	FCM	CIC ⟞ T Cell	CIC escapes from T cell–mediated immunosurveillance through Membrane-Bound IL-4.	CRC Patients (n=3)
Todaro M, et al.2013[94]	Cell Line	Cell Line	CIC ($CD133^+$/ ESA^+)	Vc9Vd2 T Cell (N/A)	Cytotoxicity Assay	Vc9Vd2 T Cell ⟞ CIC	N/A	CIC cell lines: DLD-1/SW620/ SW403/CDC#3 /

								CDC#4); T cell lines: COLD2-1/COLD2-2
Fan et al.2018[95]	Cell Line	Cell Line	CSC ($CD133^+$)	Macrophage （N/A）	SFT	Macrophage → CSC	LPS-activated Macrophage promotes CSC through the SHH signaling pathway.	CSC cell line: HCP-1; Macrophage cell line: U937
Inoda S, et al.2011[96]	Cell Line	Cell Line	CSC ($SOX2^+$/LGR5 $^+$/POU1F5 $^+$/ALDH1$^+$)	CTL (N/A)	51Cr Release Assay	CTL ⊣ CIC	CEP55 and HLA-Class1 expresses on CSC and could be recognized by CTL.	CSC cell lines: SW480/HT29/HCT15; CTL from CTL clone-41.

Morita et al.2016[97]	Cell Line	Patient	CIC ($SOX2^+$/LGR5$^+$/$POU1F5^+$)	CTL ($CD8^+$)	51Cr Release Assay	CTL ⊣ CIC	OR7C1 is a novel marker of CIC and can be targeted by OR7C1-specific CTL clone.	CIC cell lines: SW480/HCT15-B2M /HT29); CTL from 3 patients and 3 healthy donors' PBMC and induced to CTL clone.
Ilona K, et al.2014[98]	Cell Line	Patient Tissue	CSC ($ALDH1^+$/$EpCAM^+$)	T Cell ($CD3^+$/$CD4^+$/$IL22^+$)	FCM	$CD4^+$/$IL22^+$ T Cell → CSC	CD4+ T expresses IL22 which promotes stemness of CSC	CSC cell lines: DLD-1/HT29

CCC Literature details

Reference	Cell				Experimental Method	Interaction	Mechanism	Remark
	Cell Source		Cell Type (Marker)			Promotion ⟶ / Inhibition ⊣		
	CSC	Immune Cell	CSC	Immune Cell				
Raggi C, et al.2016[99]	Patient Tissue	HD Blood	CSC (N/A)	Macrophages ($CD14^+$)	SFT	Macrophage ⟶ CSC	CSC shapes macrophage to TAM subset and promotes tumorigenesis.	-

3.2. CSC Marker Selection and Identification

To detect CSCs and analyze their relationship with immune cells as well as clinical parameters, it is essential to select suitable marker for identification. As described in the introduction section, CD133 and CD90 are both identified as special marker for CSCs according to literature. Thus, in this part I prefer to verify those two kinds of marker with experimental methods. For this purpose, MACS isolation is applied for the enrichment of CSCs with potential marker and followed by spheroid formation assay for verification.

3.2.1. MACS Isolation

CD133 microbeads could specifically bind to CD133 positive cells. These cells are left in the magnetic column for collection after aforementioned washing steps. As shown in Figure 10A-D, $CD133^+$ cells are collected and measured by FACS as well as gated by FMO control. The proportion of $CD133^+$ cells in native group (without MACS) is 20.13±0.07% (Figure 10B). After MACS, a higher proportion of $CD133^+$ cells are left in positive group with 65.13±14.57% (Figure 10C). While only 15.80±2.55% $CD133^+$ cells are left in negative group (Figure 10D). Figure 10E shows that there is an obvious increase of $CD133^+$ cells in positive group compared to negative and native groups after MACS isolation (Pos. vs. Neg., p=0.019; Pos. vs. Nat., p=0.037).

Those results indicate an efficiency of MACS isolation targeted $CD133^+$ cells in HepG2 cell line and those enriched cells are proceed to spheroid formation assay.

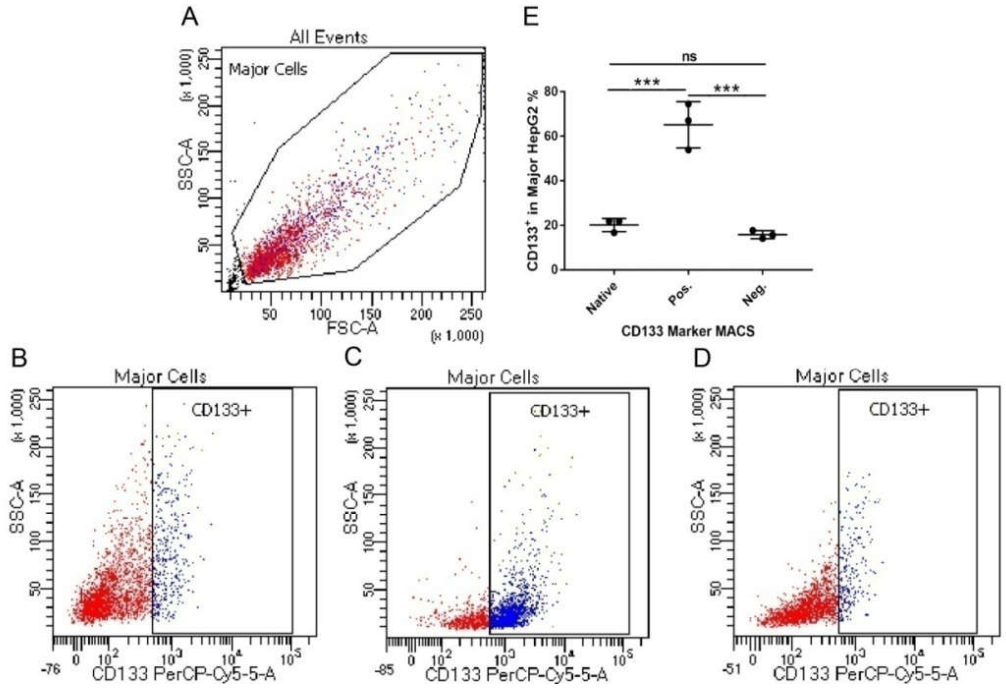


Figure 10: Typical FCM pictures of CD133$^+$ cells in HepG2 cell line with MACS isolation and statistical analysis. (A) Major tumor cells selected from HepG2; (B) CD133$^+$ cells before MACS; (C) CD133$^+$ cells in positive group after MACS; (D) CD133$^+$ cells in negative group after MACS; (E) One-way ANOVA analysis, *** $p<0.001$. (Three times repeated, Pos.: Positive; Neg.: Negative; ns: No significance.)

3.2.2. Spheroid Formation Assay

Those enriched CD133$^+$ cells were then seeded into 12-well plate with special medium for spheroids formation assay. After 7 days co-cultivation, spheroids amounts were counted under microscope as shown in Figure 11. The results show that the positive group, which has the highest amounts of CD133$^+$ cells, forma total amount of 71 spheroids in three times repeated experiments than other groups. On the contrary, the negative group, which has the lowest proportion of CD133$^+$ cells, only 19 spheroids form finally. The native group (without MACS isolation) also form 24 spheroids totally. Figure 11D indicates the difference in spheroid formation between the native, positive and negative selection groups.

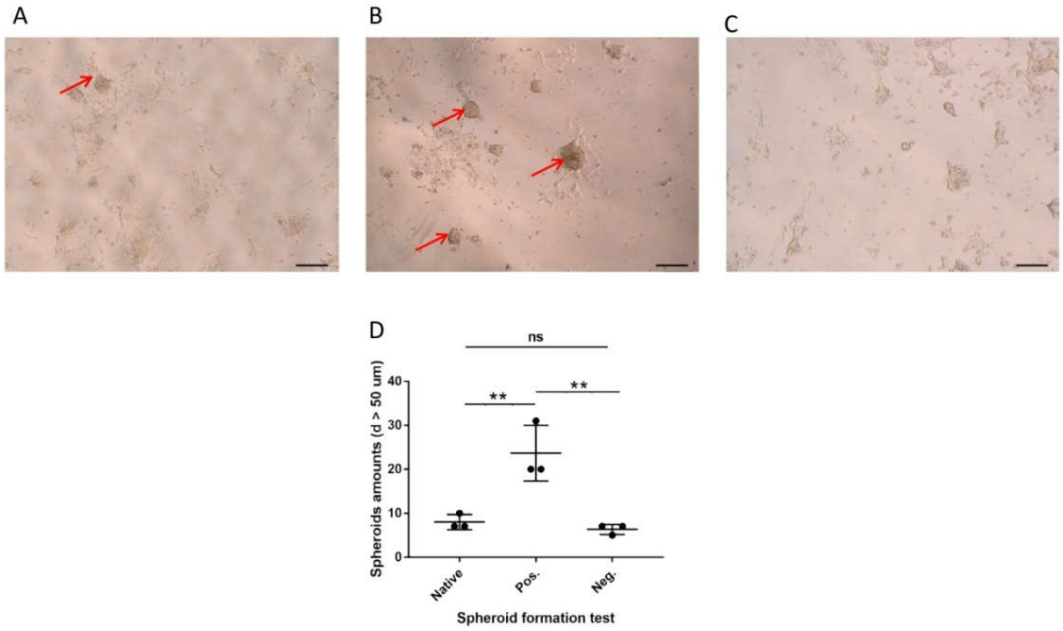


Figure 11: Representative spheroids pictures in different groups (50x magnifications, scale bar: 200 μm) and statistical analysis. (A) Native group; (B) Positive group; (C) Negative group; (D) One-way ANOVA analysis, ** $p<0.01$. (Three times repeated, red arrows point to spheroids. Pos.: Positive; Neg.: Negative; ns: No significance)

Considering the complexity of CSCs origin, one marker is not enough to define their heterogeneity and plasticity. Thus CD90 marker, another antigen which also commonly used as liver CSC marker is also selected as an option for CSCs detection in HCC patients and co-culture system. Unfortunately, the MACS isolation of $CD90^+$ cells from HepG2 was not satisfyingly achieved. There were not enough targeted cells collected after isolation. Figure 12A-D show the typical FCM pictures of $CD90^+$ marker after MACS. The proportion of $CD90^+$ cells in positive group is only 0.83±0.28% compared to 0.23±0.14% in negative group. Cells with low expression of $CD90^+$ marker can't proceed to spheroid formation assay even though a statistical significance is observed in figure 12E ($p=0.012$). While Yang et al. reported that $CD90^+$ cells in human liver cell line displayed the tumorigenic capacity[19]. Sukowati et al. investigated $CD90^+$ cells in HCC and observed a higher proliferation capacity

compared to $CD90^-$ cells in 3D clonogenic assay as well as a higher proportion in liver tumor tissue[115]. An *in vitro* study from Zhang et al. displayed an increased viability, migration and invasive ability of $CD90^+$ cells isolated from SK-Hep-1 cell line than $CD90^-$ cells[116]. Convinced by those related literature, CD90 is also selected as an opinion for CSCs identification in our following research.

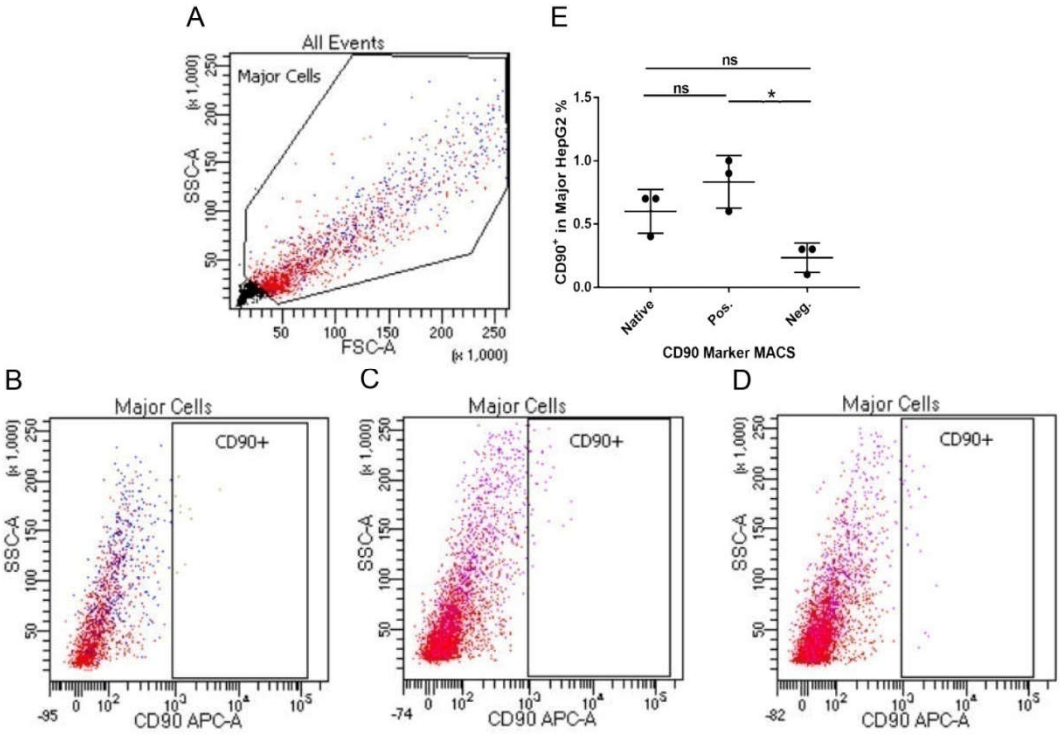


Figure12: Typical FCM pictures of $CD90^+$ cells in HepG2 cell line with MACS isolation and statistical analysis. (A) Major tumor cells selected from HepG2; (B) $CD90^+$ cells before MACS; (C) $CD90^+$ cells in positive group after MACS; (D) $CD90^+$ cells in negative group after MACS; (E) One-way ANOVA analysis, * $p<0.05$. (Three times repeated, Pos.: Positive; Neg.: Negative; ns: No significance.)

Increased spheroids formation in higher $CD133^+$ group represents a higher self-renewal ability of $CD133^+$ cells. This kind of marker has the potential to identify CSCs in HCC. Combined with CD90 marker which one has been proved by previous studies, I am able to detect CSCs in HCC patients.

3.3. Measurement of CSCs in HCC Patients' Peripheral Blood and Liver Tumor Tissue

For this purpose, FACS measurement was performed with HCC patients' peripheral blood (PB) and liver tumor tissue (LTT). Marker of CD90 and CD133 was applied for identifying CSCs from $CD45^-$ cells. There were 11 HCC blood samples and 5 tissue samples finally selected for the CSCs detection. In PB, the proportion of $CD90^+/CD45^-$ CSCs is around 0.76±0.62% compare to the major circulating tumor cells and $CD133^+/CD45^-$ CSCs account for 1.02±0.69%. The double positive CSCs ($CD90^+/CD133^+/CD45^-$) only account for a small proportion with 0.07±0.08% (n=11). While in LTT (n=5), higher amount of $CD90^+/CD45^-$ CSCs are detected at 16.1±13.96% (LTT vs. PB, p=0.002) whereas no difference for $CD133^+/CD45^-$ CSCs which account for 1.16±1.12% (LTT vs. PB, p=0.759). $CD90^+/CD133^+/CD45^-$ CSCs in LTT account for 0.44±0.55% in major tumor cells and no difference is found compared to that in PB (LTT vs. PB, p=0.214). Figure 13 shows the statistical analysis. Thus, the existence of CSCs labelled with these two marker in HCC is proved and they only account for a small proportion in total tumor cells. Besides, an increased proportion of $CD90^+/CD45^-$ cells are observed in LTT than in PB. While there is no statistical significance observed between PB and LTT with $CD133^+/CD45^-$ cells as well as $CD90^+/CD133^+/CD45^-$ cells.

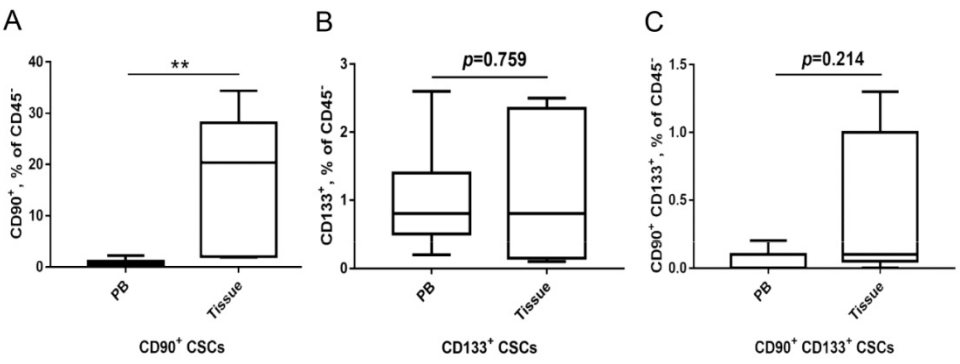
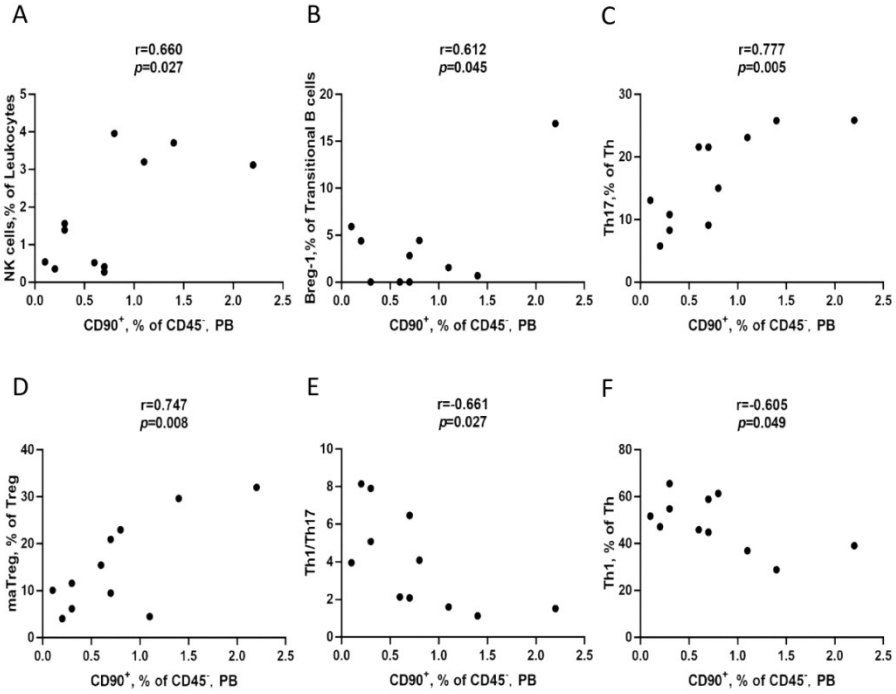


Figure 13: The proportion of CSCs in HCC patients' PB and LTT. (Unpaired t test, ** $p<0.01$)

3.4. Correlation between CSCs and Immune Cells

The analysis of correlated relationship between CSCs in HCC with paired immune cells might provide potential immunotherapy targets in the future. The data of CSCs in non-HBV/non-HCV HCC patients has been obtained now. For immune cells, another project conducted by Tong Zhu and Yongsheng Han in our group acquired abundant data for analysis. As mentioned in 2.2.8 section, details of immunophenotyping method and part of results are described in the published dissertation by Tong Zhu. Some other related data will be displayed in the dissertation of Yongsheng Han. Thus, the correlation between CSCs and paired major immune cells in PB and LTT is analyzed in this section. As shown in Figure 14, there is a positive correlation between $CD90^+$ CSCs in PB with Breg-1/Transitional B cell, ma-Treg/Treg, Th17/Th as well as NK/Leukocyte (r=0.612, p=0.045, Breg-1/Transitional B cell; r=0.747, p=0.008, ma-Treg/Treg; r=0.777, p=0.005, Th17/Th; r=0.660, p=0.027, NK/Leukocyte). In the contrast, the ratio of Th1/Th and Th1/Th17 show a negative correlation with $CD90^+$ CSCs in PB (r=-0.605, p=0.049, Th1/Th; r=-0.661, p=0.027, Th1/Th17). While in LTT, only class-switched B cell/B cell (C-S B cell/B cell) shows a positive correlation with $CD90^+$ CSCs (r=0.963, p=0.008). For $CD133^+$ CSCs in PB, the ratio of DC/Leukocyte has a positive correlation with it whereas aCTL/CTL and $CD69^+$ NKT/NKT show a negative correlation (r=0.653, p=0.03, DC/Leukocyte; r=-0.615, p=0.044, aCTL/CTL; r=-0.608, p=0.047, $CD69^+$ NKT/NKT). As for $CD133^+$ CSCs in LTT, there is no obvious correlation found yet. Likewise, the ratio of aCTL/CTL also has a negative correlation with $CD90^+/CD133^+$ CSCs in PB (r=-0.643, p=0.033, aCTL/CTL). A positive correlation is found between the ratio of Th1/Th17, Th1/Th2, eTh/Th, plasmablast/B cells and $CD90^+/CD133^+$ CSCs in LTT (r=0.907, p=0.034, Th1/Th17; r=0.900,

p=0.037, Th1/Th2; r=0.957, p=0.011, eTh/Th; r=0.918, p=0.028, plasmablast/B cells). Data of related HCC patients' immune cell in PB and the correlation of CSCs with all kind of immune subsets are shown in supplement table 1, 2. The correlation analysis shows that several kinds of immune subtypes like NK cells, Breg-1 cells, Th17 cell, ma Treg cells, etc. have a positive correlation with $CD90^+$ CSCs in PB. On the contrast, the ratio of Th1/Th17 and the proportion of Th1 cells in Th cells show a negative correlated relationship. For $CD133^+$ CSCs in PB, DC cells have a positive correlation with them. While $CD69^+$ NKT cells and aCTL show a negative tendency. The same tendency is also observed between aCTL and $CD90^+/CD133^+$ CSCs in PB. Some other correlated data is also found between both $CD90^+$ and double positive CSCs in LTT with different kinds of immune subtypes.



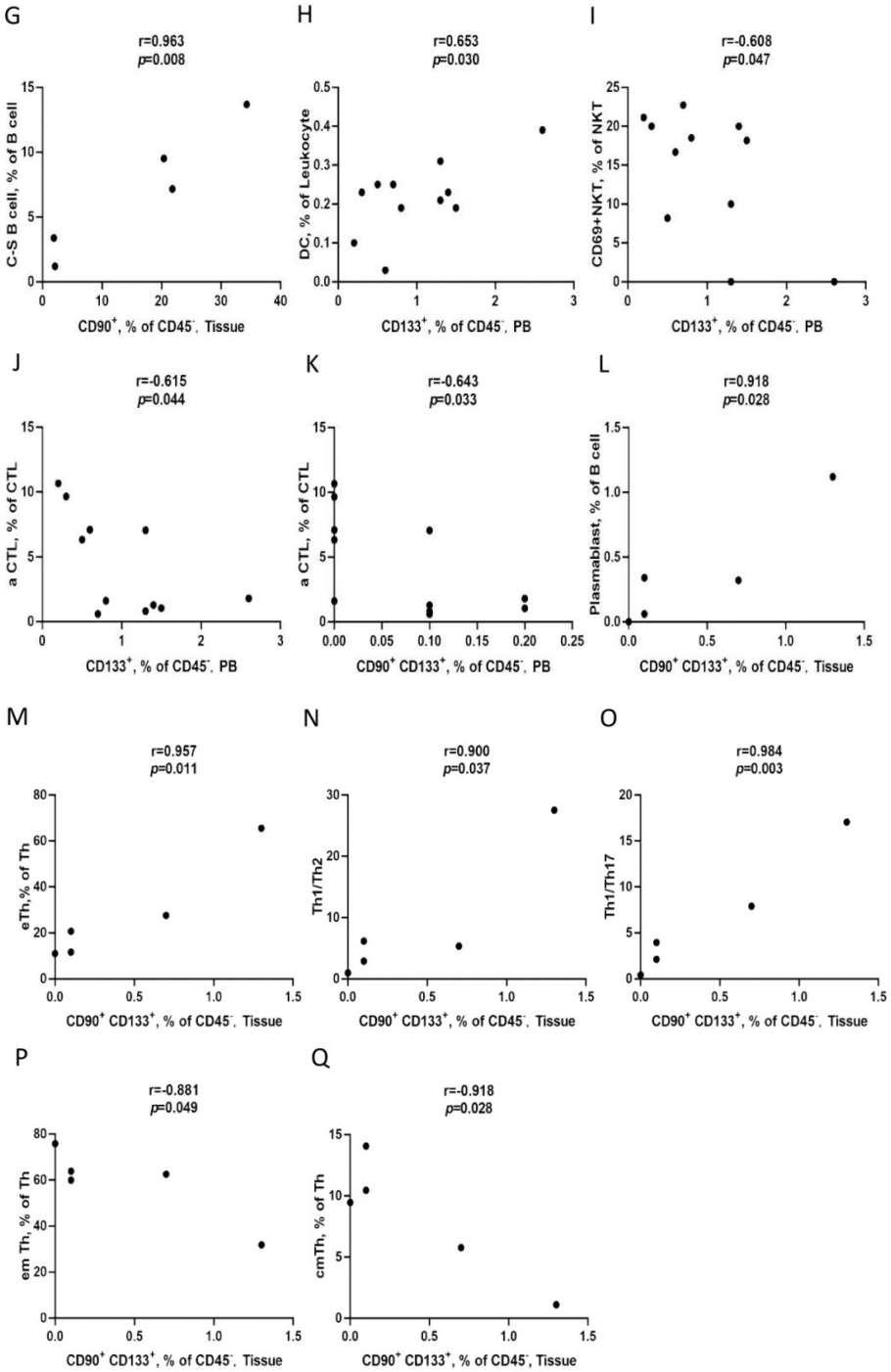


Figure 14: Significant correlation analysis results of CSCs with different immune subsets. A-F: $CD90^+$ CSCs in PB; G: $CD90^+$ CSCs in LTT; H-J: $CD133^+$ CSCs in PB; K: $CD90^+/CD133^+$ CSCs in PB; L-Q: $CD90^+/CD133^+$ CSCs in LTT. (Pearson's Correlation Coefficient)

3.5. Correlation between CSCs and Clinical Parameters

Besides measuring immune cells in HCC patients, several clinical parameters are also collected for correlation analysis. These parameters could reflect patients' condition or predict disease outcome to some extent. The correlation between CSCs and them may provide more indicators in the prediction of HCC patients. Supplement table 3 lists the data of related HCC patients' demographic and clinicopathological parameters. And supplement table 4 summarizes all correlation analysis between CSCs and clinical data.

3.5.1. Demographic and Clinicopathological Characteristics of Patients

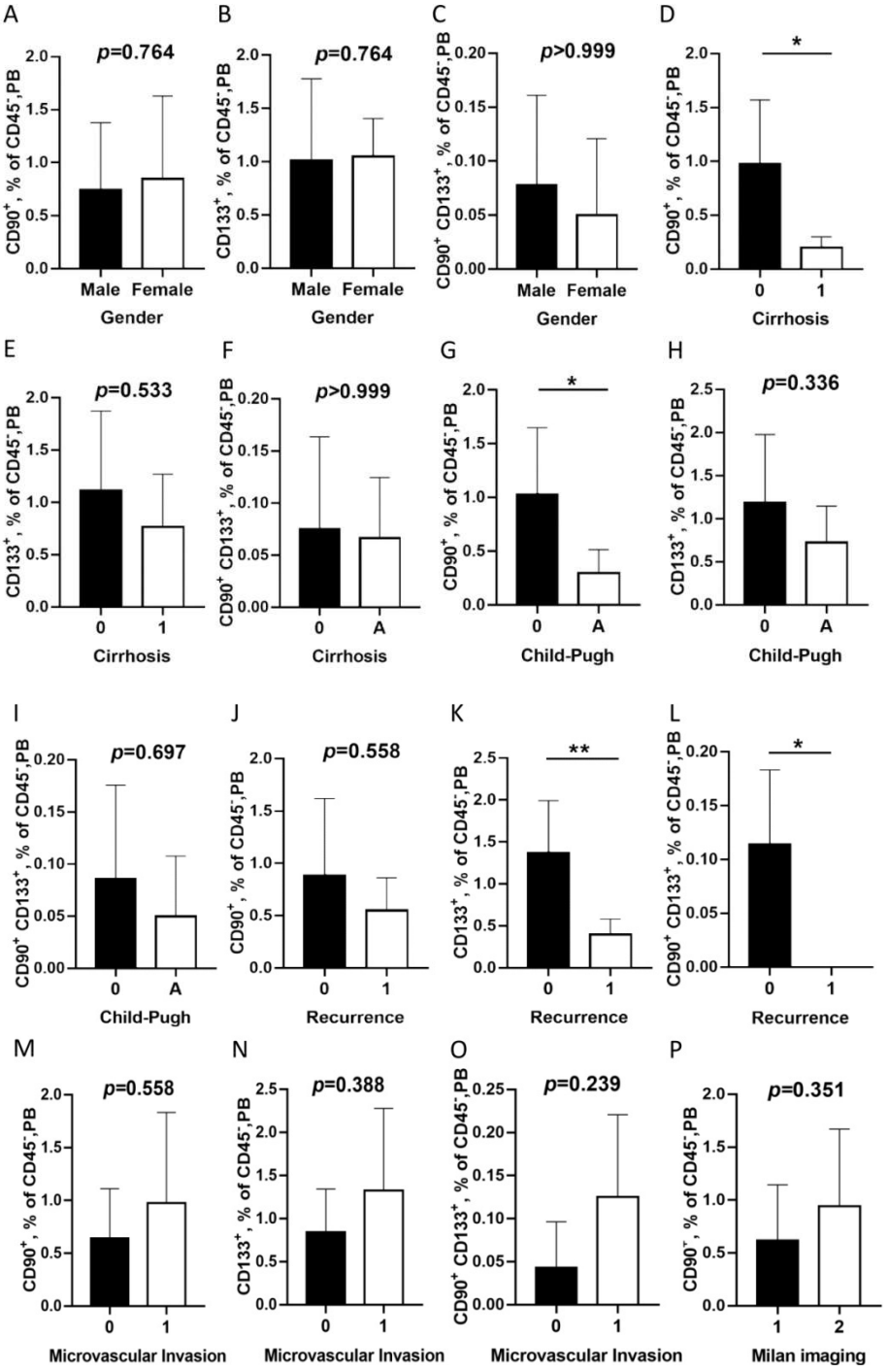
As mentioned before, 11 HCC patients were selected for measuring CSCs in PB and 5 for LTT measurement. In PB, most of patients are male (nine cases, 81.82%) and only two are female (18.18%). The median age is 75 years old. None of them are HBV or HCV infected and only one patient suffers from alcoholic hepatitis (9.09%). Three of those patients' livers develop into the clinical cirrhosis stage (27.27%) as well as four patients are classified as Child-Pugh A grade (36.36%). Luckily, no patient suffers with ascite. The imaging examination reported that all of those 11 patients are single tumor lesion while two of them (18.18%) have macro-vascular invasion as well as four (36.36%) have microvascular invasion. In addition, four patients (36.36%) are reported to have the recurrence in the subsequent follow-up.

Besides, more than half of those patients (n=6, 54.55%) and 5 cases (45.45%) are inside the Milan imaging and Milan pathology criteria, respectively. Seven cases (63.64%) are classified to within UCSF criteria. According to WHO grading criteria, three cases (27.27%) are included in stage 1, six cases (54.55%) in stage 2 and one (9.09%) in stage 3. Using UICC staging criteria, stages1-4 are divided and each stage includes six, one, two and one cases, respectively. In BCLC grouping criteria, 45.46%

of patients are within group A (n=5), 36.36% are group B (n=4) and 18.18% in group C (n=2). Supplement table 3 summarizes the demographic parameters of all patients in PB. Limiting to the amount of tissue samples, five HCC patients with LTT data are not described in this part while the correlation between CSCs in LTT and clinical biochemical indicators was still analyzed then.

3.5.2. Comparison of CSCs in PB between Demographic Groups

As shown in Figure 15D, patients suffered from liver cirrhosis express lower $CD90^+$ marker than those without clinical cirrhosis (0.2±0.1% vs. 0.98±0.59%, $p<0.05$). For $CD133^+$ and $CD90^+/CD133^+$ CSCs, there is no difference between groups. The same tendency is also observed in Child-Pugh A grade group, which has the lower proportion of $CD90^+$ cells compared to 0 stage patients (0.30±0.22% vs. 1.03±0.62%, $p<0.05$). Interestingly, no such trend could be identified when analyzing CD133 and clinical cirrhosis. Four patients were diagnosed with recurrence and their CSCs which marked with $CD133^+$ (Fig.15K) and $CD90^+/CD133^+$ (Fig. 15L) both show a decreased tendency (Fig. 15K: 1.37±0.62% vs. 0.4±0.18%, $p<0.01$; Fig. 15L: 0.11±0.07% vs. 0±0%, $p<0.05$). While for $CD90^+$ CSCs, there is no significant difference with or without recurrence. The rest of demographic parameters like gender, Milan imaging criteria, BCLC grouping criteria etc. were also analyzed and no difference was found.


A
p=0.764
CD90+, % of CD45-,PB
Male Female
Gender
B
p=0.764
CD133+, % of CD45-,PB
Male Female
Gender
C
p>0.999
CD90+ CD133+, % of CD45-,PB
Male Female
Gender
D
*
CD90+, % of CD45-,PB
0 1
Cirrhosis
E
p=0.533
CD133+, % of CD45-,PB
0 1
Cirrhosis
F
p>0.999
CD90+ CD133+, % of CD45-,PB
0 A
Cirrhosis
G
*
CD90+, % of CD45-,PB
0 A
Child-Pugh
H
p=0.336
CD133+, % of CD45-,PB
0 A
Child-Pugh
I
p=0.697
CD90+ CD133+, % of CD45-,PB
0 A
Child-Pugh
J
p=0.558
CD90+, % of CD45-,PB
0 1
Recurrence
K
**
CD133+, % of CD45-,PB
0 1
Recurrence
L
*
CD90+ CD133+, % of CD45-,PB
0 1
Recurrence
M
p=0.558
CD90+, % of CD45-,PB
0 1
Microvascular Invasion
N
p=0.388
CD133+, % of CD45-,PB
0 1
Microvascular Invasion
O
p=0.239
CD90+ CD133+, % of CD45-,PB
0 1
Microvascular Invasion
P
p=0.351
CD90-, % of CD45-,PB
1 2
Milan imaging

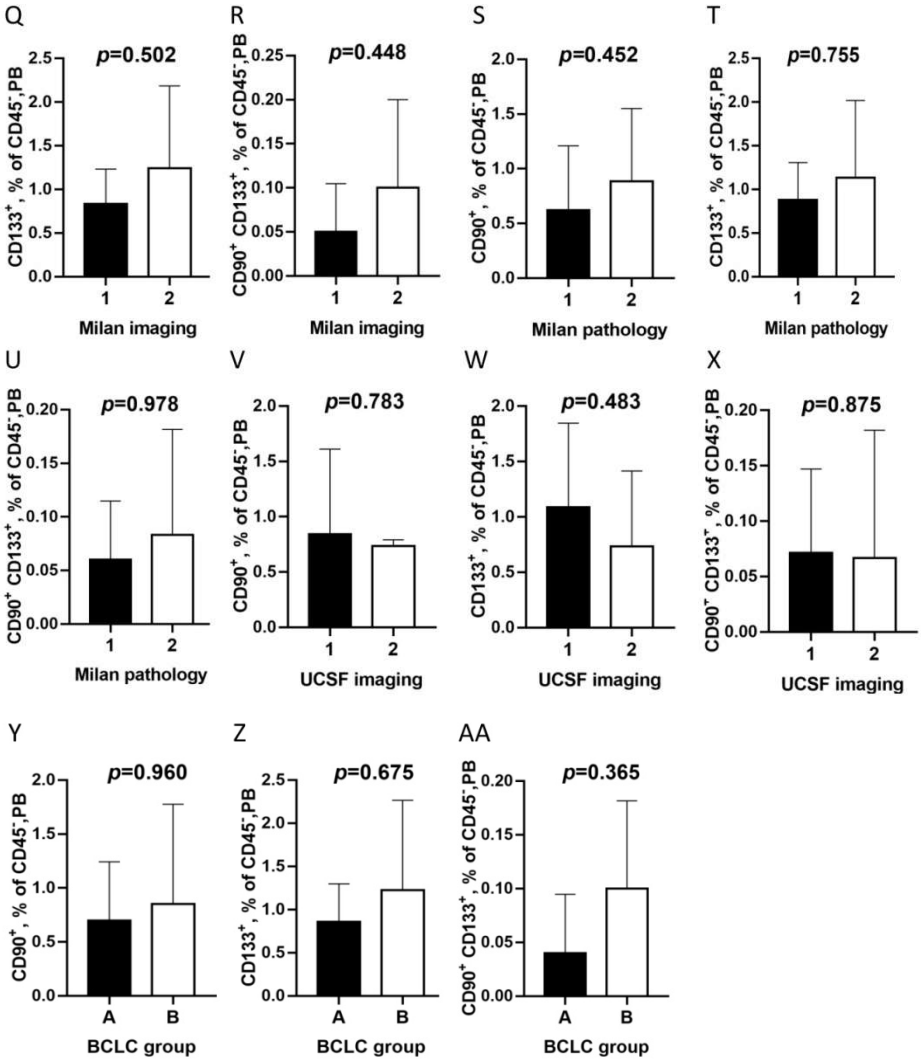


Figure 15: Comparison of CSCs in PB between different demographic groups. A-C: Gender group; D-F: Cirrhosis group; G-I: Child-Pugh group; J-L: Recurrence group; M-O: Microvascular invasion group; P-R: Milan imaging criteria group; S-U: Milan pathology group; V-X: UCSF grading group; Y-AA: BCLC group. (Mann-Whitney U test, * $p<0.05$ and ** $p<0.01$)

3.5.3. Correlation Analysis of CSCs in PB and LTT with Clinical Characteristics

The data of clinicopathological parameters is summarized in supplement table 3. The measurement of CSCs both in PB and LTT is included for correlation analysis. As shown in figure 16A, a positive correlation could be found between circulating $CD133^+$ CSCs and the frequency of leukocytes in PB. Also a positive correlation is found

between $CD90^+/CD133^+$ CSCs and circulating leukocytes (Figure 16B). In LTT, no correlation is found between circulating leukocytes and CSCs. The level of ALT in HCC patients is related to the proportion of $CD133^+$ as well as $CD90^+/CD133^+$ in LTT (Figure 16C, D). While in PB, there was no correlation between them. Some other parameters such as bilirubin, AFP, AST etc. don't exhibit any correlated relationship with CSCs (Supplement table 4).

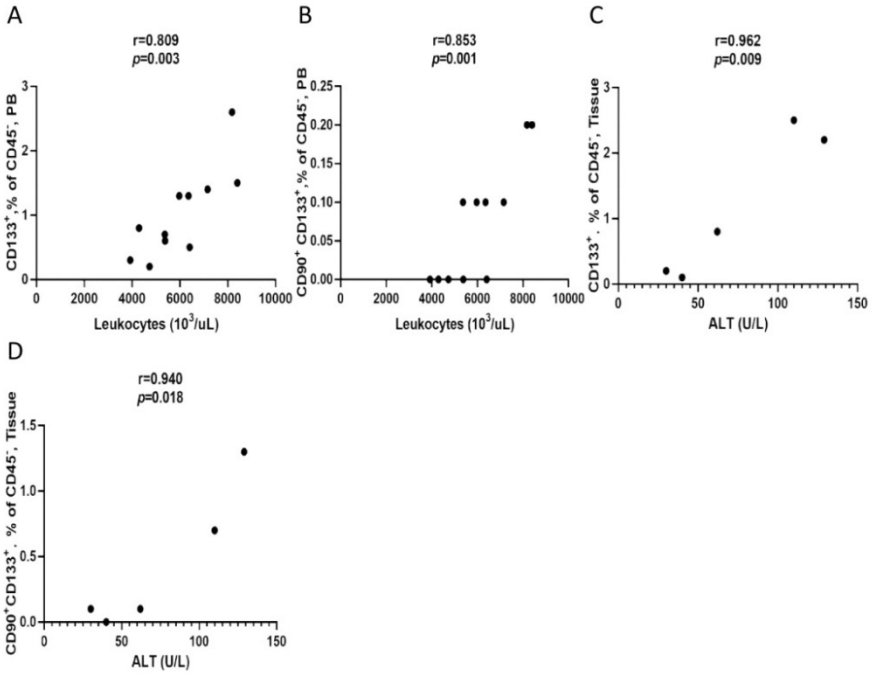


Figure 16: Significant correlation analysis results between CSCs and clinical characteristics. A: $CD133^+$ in PB with Leukocytes; B: $CD90^+/CD133^+$ in PB with Leukocytes; C: $CD133^+$ in LTT with ALT; D: $CD90^+/CD133^+$ in LTT with ALT. **(**A-D: Pearson's Correlation Coefficient)

3.6. Changes of CSCs in co-cultivation with Immune Effector Cells *in vitro*

As part of the project in our group, the establishment of co-culture system *in vitro* and *ex vivo* was conducted by Xiaokang Li. Tumor cells from HepG2 cell line were

co-cultivated with immune cells from healthy donors with a ratio of 1:25. Following the co-culture method mentioned before, both PBMCs and tumor cells were harvested after 24h co-culture. Based on their different growth characteristics, more immune cells were suspended in the medium and tumor cell preferred to be attached to the bottom. FACS was used to distinguish tumor cells as well as CSCs from PBMCs. Following the gating strategy of CSCs in co-culture system, CSCs labeled with $CD90^{+}/CD45^{-}$, $CD133^{+}/CD45^{-}$ and $CD90^{+}/CD133+/CD45^{-}$ were separated from living $CD45^{-}$ cells. Compared to control group, the proportion of living CSCs identified with $CD90^{+}$ decrease from 1.90±0.70% to 0.57±0.29% ($p<0.05$). For $CD133^{+}$ cells, the proportion decreases from 26.73±2.34% to 14.70±3.21% ($p<0.01$). CSCs labeled with $CD90^{+}/CD133^{+}$ in major $CD45^{-}$ tumor cells also show the same tendency which decreased from 0.87±0.29% to 0.10±0% ($p<0.05$). (Figure 17A-C) Measurement of CSCs marker on dead cells seems to have no sense; therefore we could not directly assess the $CD133^{+}$ or $CD90^{+}$ or both marker in the gate of dead cells. The decreased tendency still shows a potential influence of immune effector cells on CSCs.

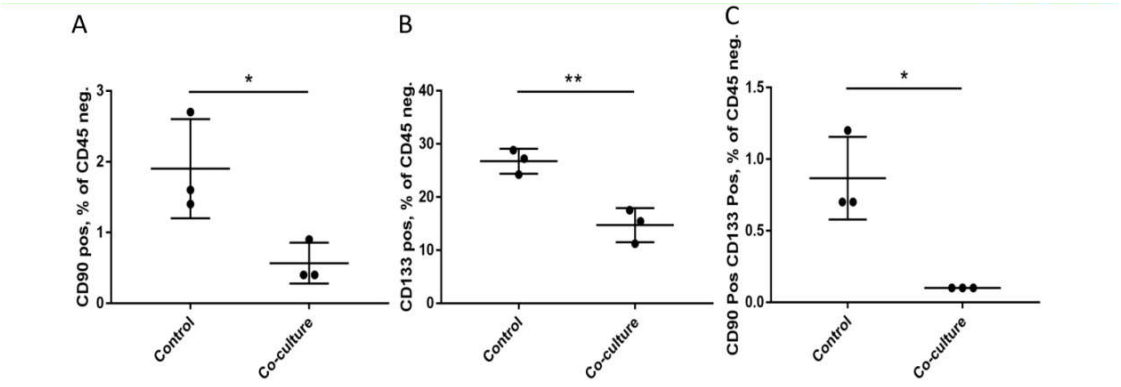


Figure 17: The comparison of CSCs between experimental and control groups in co-culture system. (Three times repeated, unpaired T test, * $p<0.05$; ** $p<0.01$)

4. Discussion

Cancer stem cells (CSCs) have been identified using various marker in multiple types of cancers. CSCs represent a small subset of tumor cells, and the detection of CSCs in patients' blood and tumor tissues can vary based on the marker selection and detection methods used. The interactions between CSCs and other types of immune cells are complicated due to the unique characteristics of CSCs. Considering the fact that most HCCs develop in conjunction with hepatitis, all published results concentrate on either HBV pos. or HCV pos. patients. This has no doubt an influence on the immunological state of the patients. Therefore the immunological reaction towards HCC cells and most certainly their CSCs might be altered[117, 118]. Our study is the first that concentrated on non-HBV/non-HCV HCC patients. In addition, in the literature correlation between CSCs and immune cells was focused on only one or a few selected immune cell types. Contrary to that, this study correlated CSCs in PB and LTT with results from a comprehensible immunophenotyping protocol. Additionally, the clinicopathological characteristics of the patients were also collected for correlation analysis. The establishment of co-culture system by our working group allows us to study the interactions between CSCs and immune cells. In this study, CSCs from HepG2 cell line were co-cultivated with PBMCs from healthy donors to observe their changes.

During this discussion section, the results will be discussed and placed into the context of the existing literature.

4.1. Interactions between CSCs and immune cells in Digestive System Neoplasms

Five major digestive system neoplasms (DSNs) such as liver cancer (LC), gastric

cancer(GC), pancreatic cancer (PC), colorectal cancer (CRC) and cholangiocarcinoma (CCC) were selected as the study objects to summarize the interactions between CSCs and related immune cells. Collectively, I could conclude that major immune effector cells like CTLs, NK cells, DCs and NKT cells display an inhibiting ability to CSCs in LC, PC, GC or CRC. However, macrophages (especially TAMs), Tregs have the promoted function on CSCs. As the typical immunosuppressive cells, those cells take effect on the CSCs through different pathways or mechanisms. In turn, CSCs could also impact some immune cells like TAMs, T cells, or DCs.

For HCC, it is more complicated to explore the interactions between tumor cells and immune cells due to the existence of HBV and/or HCV infection. Through the literature review, however, most of the subjects are HBV/HCV related HCC. Besides, the majority of studies only focused on one or a few subtypes of immune cells. Based on those previous studies, I prefer to select non-HBV/non-HCV HCC patients as experimental subject. Moreover, the establishment of immunophenotyping method enable me to analyze up to thirty-nine kinds of immune cell subtypes in this study.

4.2. Expression of CSCs Marker in non-HBV/non-HCV HCC Patients

The expression level of CSCs marker varies within a certain range due to different detection methods but normally still in a low level. For CD133, Song et al. examined tissue from 63 HCC patients (hepatitis viral infection status unknown) using the IHC method and found that approximately 1.32%±0.81% cells are $CD133^+$ compared with a lack of detectable CD133 expression in control liver tissues from normal adults[119]. Sasaki et al. examined 30 HCC specimens (some of these patients were HBV- or HCV-infected) with IHC and reported that the proportion of $CD133^+$ cells is

0%-9.4%[120]. In this study, $CD133^+$ CSCs in non-HBV/non-HCV HCC patients were examined both in PB and LTT samples using FACS. In PB, 1.02%±0.69% of total circulating tumor cells express $CD133^+$, whereas in LTT samples, $CD133^+$ CSCs account for 1.16%±1.12%. Those results are consistent with most of previous studies which show a low level of CSCs in tumor cells. Notably, my results provide new data in HCC patients without virus infection.

For CD90, Yang et al. used FCM to detect $CD90^+$ cells in HCC patients (the majority of patients were HBV infected) and found that 0.3% of PB cells were $CD90^+$, whereas 2.51% of LTT cells were $CD90^+$[121]. In comparison, the $CD90^+$ cells in non-HBV/non-HCV HCC patients examined in this study account for 0.76%±0.62% of total $CD45^-$ tumor cells in the PB and represent 16.1%±13.96% of $CD45^-$ cells in the LTT. The proportion of CSC in my experiment is markedly higher than in the literature. After analyzing the literature regarding DSNs I could not find any literature that supports my finding. With the limitation of patient's quantity in this study, more patients should be involved in the future.

As mentioned above single-marker identification of CSCs might not be sufficient to accurately identify CSCs. CD90/OCT4[122], $CD44^+/CD133^+$[123], and $CD90^+/CD133^+$[124] are some of the various combinations that have been selected as potential CSCs marker. To our knowledge, data regarding the expression of $CD90^+/CD133^+$ CSCs marker in non-HBV/non-HCV HCC patients is lacking. My results show that the percentage of $CD90^+/CD133^+$ cells in total $CD45^-$ cells is 0.07%±0.08% in PB and 0.44%±0.55% in LTT.

Besides, a higher expression level of CD90 is found in LLT than in PB ($p<0.01$). Yang et al. also tested $CD45^-/CD90^+$ marker expression both in PB and LTT which show a higher expression in LTT than in PB.[121] It should be noted that in this study the majority of patients was HBV-positive. Limited to the lack of related literature, there is no more data to support my results. One possible reason might be the small size of

cases and the statistical analysis could not be performed well to evaluate the comparison and correlation between PB and LTT. Another reason might the TME recruitment formed in LTT. More circulating CSCs may be recruited to the local tumor tissue and detected.

Taken together, my results show that CSCs also represent a small subpopulation of tumor cells in non-HBV/non-HCV HCC patients, which broadly supports the work reported by other researches. My results also add to the available data by using different marker and provide new data for non-HBV/non-HCV HCC patients.

4.3. Correlation between CSCs and Several Immune Cells both in PB and LTT

As important components of the immune system, immune cells play a key role in interactions with CSCs. As the field of immunology research continues to develop, more immune cells have been recognized and selected as potential targets for immunotherapy. In addition, the functions played by CSCs during tumorigenesis, progression, metastasis, and recurrence have been supported by increasing evidence. Thus, exploring the interactions between CSCs and various types of immune cells during HCC development and progression is important.

In PB, $CD133^+$ CSCs are positively correlated with the DC/leukocyte ratio. As the professional antigen-presenting cells, DCs are able to recognize and capture special tumor antigens and induce the activation of $CD4^+$ and $CD8^+$ T cells to suppress tumor progression. Broz et al. examined the TCGA database to perform a survival analysis on 12 cancer types and found that patients with a higher density of DC-2 cells (a subtype of DCs) show improved survival[125]. The existence of tumor cells, including CSCs, could stimulate the accumulation of DCs, and consistent with the literature, my findings support the occurrence of this interaction.

In contrast, the aCTL/CTL ratio shows negative correlation with $CD133^+$ CSCs in PB. The ratio of aCTL/CTL represents the proportion of activated CTL in total CTLs. An increased ratio could indicate an enhanced immune-activating response. In turn, a decreased ratio may represent an immunosuppressive activity occurring. As the activated form of CTLs, aCTLs are generated from native T-cells through several signaling pathways, including the recognition of Peptide-MHC class I complex by TCR (with the help of APCs, especially DCs), co-stimulatory signals, such as B7 and CD28, and IL-2 receptor activation[126]. Combined with an increase in the presence of DCs among leukocytes, this result may indicate the possible existence of a suppressive interaction between CSCs and CTLs.

NKT cells, a special heterogeneous T cell subgroup that express both NK and T cell surface marker, are abundant in the liver[127]. NKT cells have been shown to play important roles in the regulation of innate and adaptive immunity by producing large quantities of cytokines and chemokines, such as IFN-γ, IL-2, IL-4, and GM-CSF, in addition to promoting B-cell activation[128]. CD69 is typically expressed on T cells as an early co-stimulation and activation marker[129]. The ratio of $CD69^+$ NKT/NKT could represent the activating status of NKT cells. The negative correlation result shows that higher proportion of $CD133^+$ CSCs in PB correlates with lower percentage of activated NKT cells in total or increased percentage of activated NKT cells is correlated with decreased proportion of$CD133^+$ CSCs in PB. Additional studies have focused on NKT immunotherapy, but little is known regarding the interaction between $CD69^+$ NKT cells and CSCs in HCC. My results indicate that CSCs might inhibit the activation of NKT cells and, hence, prevent immune cells from killing cancer cells. Or an increased percentage of activated NKT cells may in turn inhibit $CD133^+$ CSCs in PB.

Some correlated relationships have also been identified with $CD90^+$ CSCs. A positive correlation between $CD90^+$ CSCs and the mTreg/Treg ratio was calculated during this analysis. Tregs have been studied as an important immunosuppressive T cell

subgroup and were recently selected as a potential immunotherapeutic target[130-132]. As a long-term existing T-cell, mTregs generally express behavior consistent with low or no immunosuppressive activity until they are exposed to a specific antigen. Though the potential function of memory mechanisms of mTregs is not intuitively obvious like conventional memory T cells, this subpopulation still play an important role in expanding and responding to secondary activation for immunosuppressive activity[133]. An increased ratio of mTreg/Treg may represent the potential strengthen of immunosuppressive activity. Considering the role of CSCs in immune suppression, a positive correlated relationship between $CD90^+$ CSCs and the ratio of mTreg/Treg seems reasonable. Because of the lack of relevant literature, no more details could be found to explore the real mechanisms in this issue. And the collection of additional data remains necessary to verify my results.

The NK/leukocyte ratio is also positively correlated with $CD90^+$ CSCs in PB. A higher ratio of NK/leukocyte means more NK cells are mobilized for immune response. As an important component of the innate immune system, NK cells respond rapidly by attacking tumor cells that lack MHC class I presenting antigens and activation[134]. The exposure of CD90 antigen may trigger an increase in NK cell differentiation and activation to increase tumor inhibition.

Th17 cells, which have been identified to play an immunosuppressive role in HCC patients[135], were positively correlated with $CD90^+$ CSCs. Zhang et al. investigated the percentage of Th17 in HCC patient tissue and revealed that the density of Th17 cells is positively correlated with microvessel invasion and is an independent prognostic indicator of OS and DFS[136]. During the formation of the tumor immune microenvironment, CSCs may promote the proliferation of immunosuppressive cells. An IL17A (the production of Th17) blocker was shown to prevent NASH and HCC in high-risk patients[137]. A related review summarized the interaction between CSCs and Th17 cells in different types of cancers and concluded that CSCs could promote the

differentiation to Th17 cells and in turn, Th17 cells are able to enhance the function of CSCs through secreting several cytokines[138]. Thus, combined with the immunosuppressive function of CSCs to immune effector cells like Th1, the negative correlation between $CD90^+$ CSCs and Th1/Th17 and Th1/Th is reasonable.

Taken together, my results reveal that most of the immuno-activating cells, including Th1, aCTL, cmTh, emTh, and $CD69^+$ NKT cells, display a negative correlation with CSCs. A possible mutual inhibition exists in their connection process and those cells have the potential to become immunotherapy weapons like CAR-T. Some other immuno-suppressive cells, including Th17 and mTregs, have the positive correlation with CSCs in HCC patients which may indicate a mutual improvement between them. The inhibition of those cells may lead to the suppression of CSCs.

4.4. Correlation of CSCs and Clinical Parameters

The results show that patients who suffered from clinical cirrhosis have a low expression level of CD90 marker in PB. As a major precancerous, liver cirrhosis has been characterized by the replacement of normal liver tissue with fibrosis[139]. Several factors, including hepatitis virus infection, alcohol, and NAFLD, may cause liver cirrhosis[139, 140]. One study reported that the expression of $CD90^+$ marker in liver tumor tissue had no correlation with liver cirrhosis but was correlated with age, HBV infection, and histological grade[141]. Considering the lack of related data, my results provide new findings regarding the correlation between clinical cirrhosis and circulating $CD90^+$ CSCs in non-HBV/non-HCV HCC patients (lower expression of $CD90^+$ marker in clinical cirrhosis patients).

Consistently, differences in the levels of circulating $CD90^+$ CSCs are also observed according to the Child-Pugh grade assignment. Since first introduced by Child in 1964, this grading method has been widely used to evaluate liver reserve function and to

estimate prognosis in liver cirrhosis patients. All involved patients were scored from 1–3 points for five clinical indicators, including serum albumin, PT/INR, total bilirubin, ascites, and hepatic encephalopathy, and classified as A-C, based on the total score[142]. In my study, those patients scored as Child-Pugh grade A express lower percentage of circulating $CD90^+$ CSCs compared with patients who scored less than 5 points. The tendency is consistent with the results observed for the clinical cirrhosis group, indicating that worse liver reserve function and prognosis are associated with higher levels of CSCs. However, interactions between CSCs and liver cirrhosis status remain unclear.

Interestingly, the reduction of circulating $CD133^+$ and $CD90^+/CD133^+$ cells was observed in patients with recurrence. CSCs have been shown to play an important role in the recurrence of many solid tumors, including HCC[119, 143, 144]. Song et al. collected 60 HCC LTT specimens and revealed that patients with increased $CD133^+$ CSCs exhibited a higher recurrence rate than patients with low $CD133^+$ marker expression[119]. Due to the sample size is insufficient, the result is limited.

Moreover, the results of some clinical chemistry panels were also examined for correlation with circulating CSCs. Leukocyte ($10^3/\mu L$) counts are positively correlated with $CD133^+$ and $CD90^+/CD133^+$ CSCs in PB. Complicated interactions between immunosuppressive and immuno-activating cells and cytokines occur when tumor-specific antigens expose to leukocytes, which consist of different types of functional immune cell clusters. Aforementioned results display complex relationships such as positive correlation between NK cells, Th17 as well as maTreg and $CD90^+$ in PB; a negative correlation between $CD69^+$ NKT, aCTL and $CD133^+$ in PB; a positive correlation between DC and $CD133^+$ in PB, etc. Thus, the positive correlation between leukocyte counts and $CD133^+$ as well as $CD90^+/CD133^+$ CSCs in PB is under those combined effect.

Alanine transaminase (ALT), a common liver functional indicator which can reflect

liver damage, is positively correlated with $CD133^+$ and $CD90^+/CD133^+$ CSCs in tumor tissue. ALT, AST, and the AST/ALT ratio may be potential predictors of HBV-related HCC patient prognosis[145]. However, the data regarding the relationship between these liver functional indicators and liver CSCs remains scarce. Although the number of tissue specimens involved in this analysis was quite small, a clear tendency was observed, indicating that ALT levels may influence the existence of CSCs.

4.5. Changes of CSCs in an *in Vitro* Co-Culture System

As mentioned previously, an *in vitro* co-culture system was established using an HCC cell line and PBMCs from healthy donors. Compared with CSCs isolated from HCC patients, CSCs are easier to isolate from the HCC cell line due to higher proportion of $CD133^+$ CSCs in HepG2 compared with patients' PB. Based on experiences in previous studies and our own pre-test results, the final ratio of HCC tumor cells to immune effector cells used was 1:25. After 24 hours of direct contact in a special culturing medium, FACS was applied for measuring the proportion of CSCs. A decreased tendency of living CSCs proportion in co-culture group was observed after 24 hours co-cultivation compared to control group. This was true for $CD90^+$, $CD133^+$ and $CD90^+/CD133^+$. These differences compared to the control group were statistically significant. In my first part of results I describe the study by Sun et al., who induced specific $CD8^+$ T cells using $CD133^+$ RNA-loaded DCs and found the inhibition of HCC tumor cells, both *in vitro* and *in vivo*[76]. Yang et al. also indicated that DC-CIK cells could inhibit LCSCs *in vitro*[77]. Faezeh et al. co-cultured NK cells with HepG2 cell with 1:10 ratio and observed the inhibition of proliferation to HepG2 cells[146]. Duan et al. summarized the basic mechanisms of CTL ($CD8^+$ T lymphocytes) against tumor cells including the direct lysis through granzyme, perforin, etc.; the apoptotic signal regulated by CD95/CD95L and also the secretion of cytokines such as TNF-α and IFN-γ[147]. As the major effector immune cells, NK cells and $CD8^+$ cytotoxic T

lymphocytes play an important inhibiting role in CSCs. However most of those studies prefer to stimulate effector cells before co-cultivation. In this study, the direct cytotoxity of those cells to CSCs are not observed. While the decreased tendency of living CSCs provide the possibility which means the un-stimulated effector cells could also applied in co-culture system.

4.6. Limitations of the Study

Generally the sample size of this study is insufficient. To investigate the interactions of a supposedly healthy immune system with the tumor CSCs, I limited the patients to non-HBV/non-HCV HCC patients and only 11 patients' PB samples and 5 LTT samples finally met the inclusion criteria. Dividing of CSCs in different demographic groups suffered the influence of small quantity and no comparison was made in LTT specimens. Besides, some positive results refer to the correlation between CSCs in LTT and immune cells as well as clinical parameters still need more data to verify.

Generally calculation of correlations can point towards a relationship of variables. In my study correlations between circulating CSCs with Child-Pugh grade, clinical cirrhosis as well as recurrence might seem interesting and worthy of further exploration. There is however no proven causation. For this traditionally larger datasets and more precise measurements of the mechanisms need to be employed. Alternatively novel network analyses can triangulate effects leading to a fuller and possibly unbiased picture.

Besides, the identification of CD90 as one of the CSCs marker, I failed to collect enough positive cells with the MACS. However since this marker is established and based on the literature I decided to still select this marker as one of my target CSCs marker.

Due to the lower amount of CSCs in co-culture system, I was not able to measure the

viability of CSCs and detect the cytotoxity of major immune effector cells to them. An enrichment of CSCs before co-cultivation is necessary for better study.

5. Conclusion

Relying on the establishment of CSCs detection method and immunephenotyping protocol, I am able to detect CSCs and also analyze the correlated relationship with immune cells as well as clinicopathological data in non-HBV/non-HCV HCC patients.

My results display a low percentage of CSCs both in tumor tissue and peripheral blood except $CD90^+$ CSCs in tumor tissue. It enhances our understanding of cancer stem cells which play an important role in tumor biology. Moreover, several immune cells like Breg-1, maTreg, NK, aCTL, Th, DC, class-switched B cell and $CD69^+$ NKT are correlated with CSCs which may serve as the potential immunotherapy targets in the future. Besides, some demographic factors may have an impact on CSCs like liver cirrhosis, recurrence as well as Child-Pugh grade and these findings add to a growing body of literature on related HCC patients. Some clinicopathological indicators such as ALT level and the count of leukocytes are also correlated with CSCs and may add toward a future prediction of results. The co-culture system results provide a possibility that some major immune effector cells without pre-stimulation could also contribute to the inhibition of CSCs.However, due to the small sample size caution must be applied as the findings might not be transferable to all HCC patients. The low expression level of CSCs marker also increased the difficulty of detection.

6. Summary

Hepatocellular carcinoma (HCC) is accused of their high recurrence rate after surgery. In recent years, immunotherapy is developing rapidly and some small molecular inhibitors as well as some anti-surface marker antibodies have been demonstrated the effect on targeting tumor cells in pre- and clinical approaches. Notably, cancer stem cells (CSCs) are accused of triggering the tumor initiation, progression, metastasis, recurrence and chemo-/radiotherapy resistance. There are a large proportion of HCC patients with hepatitis virus infection and thus the interactions between immune cells and liver CSCs is complicated. In this study, HCC patients without hepatitis virus infection were selected and the purpose is to investigate their CSCs both in peripheral blood (PB) and liver tumor tissue (LTT). Based on the establishment of immunophenotyping measurement by our group, I am also able to analyze the relationship between CSCs and paired immune cells as well as clinical parameters. Those results may contribute to find more immunotherapy targets and also indicators for patients' clinical outcome.

CD133 and CD90 were finally identified as CSCs marker which has been proved by the spheroid formation assay or previous studies. In patients' peripheral blood (PB), $CD90^{+}/CD45^{-}$ CSCs account for around 0.76±0.62% compared to the major circulating tumor cells and $CD133^{+}/CD45^{-}$ CSCs account for 1.02±0.69%. A smaller proportion of double positive CSCs are also detected (0.07±0.08%, Mean±SD, n=11). In liver tumor tissue (LTT), cells labelled with $CD133^{+}/CD45^{-}$ and $CD90^{+}/CD133^{+}/CD45^{-}$ account for 1.16±1.12% and 0.44±0.55%, separately. While $CD90^{+}/CD45^{-}$ CSCs have a higher proportion than in PB (16.10±13.96% vs. 0.76±0.62%, p=0.002).

Immune cells can be divided into two parts according to their function in immune system, immune-activating cells and immune-suppressive cells. The established

immunephenotyping protocol allows us to detect 39 kinds of immune cell subtypes and their related ratio. The correlation analyses demonstrate most of the immune-activating cells like aCTL, Th1, $CD69^{+}$ NKT, emTh and cmTh have a negative correlation with CSCs in PB or LTT, except NK cells. On the contrary, some immune-suppressive cells like maTreg and Th17 show a positive correlation with CSCs in PB. Some other immune cells like DC, Breg-1 and plasmablast also have the correlation with CSCs while they remain the controversy due to the complicated functions or other unknown reasons. Besides, some clinical data such as Child-Pugh grade, recurrence, liver cirrhosis, ALT level as well as the count of leukocytes are also observed the correlation with CSCs and part of the data is new findings in related field.

The co-culture system allows us to explore the possible interactions between major immune effector cells and CSCs *in vitro*. A decreased tendency is observed in all kinds of living CSCs after co-cultivation. With the improvement of this system, we will be able to detect more targeted immune cells and explore more potential mechanisms of interacting with CSCs in the future.

Taken together, this study provides new data of CSCs marker expression level in non-HBV/non-HCV HCC patients and reveals that CSCs may have an impact on immune cells. Those cells might be the potential immunotherapy targets in the future. Besides, some clinical indicators can also be used in patients' prognostic evaluation. Furthermore, a co-culture system should be improved to enable us explore interactions between CSCs and immune cells in different experimental levels.

7. Zusammenfassung

Das Hepatozelluläre Karzinom (HCC) rezidiviert nach der Resektion häufig früh. Dennoch bleibt die Resektion, die kurative Therapie der Wahl in Patienten mit einer kompensierten Leberzirrhose. Da die Leberzirrhose als Präkanzerose bei der Leberesektion nicht entfernt wird, wäre eine adjuvante Chemotherapie in HCC-Patienten sinnvoll. Klassische Chemotherapeutika konnten bisher keinen Nutzen als Adjuvanz nachweisen. In den letzten Jahren entwickelte sich die Immuntherapie rasant, und einige kleinmolekulare Inhibitoren sowie einige Antikörper konnten in prä- und klinischen Studien eine gezielte Wirkung auf das HCC nachweisen. Ob Immuntherapeutika jedoch einen perioperativen Nutzen haben hängt zu einem signifikanten Teil davon ab ob sie auch eine Wirkung gegen Krebsstammzellen (CSCs) haben. CSCs werden beschuldigt, die Tumorinitiation, -progression, -metastasierung, -rezidiv und -resistenz gegen Chemo- und Strahlentherapie auszulösen. In dieser Studie wurden HCC-Patienten ohne Hepatitis-Virus-Infektion ausgewählt um ihre CSCs sowohl im peripheren Blut (PB) als auch im Lebertumorgewebe (LTT) zu untersuchen. Basierend auf einer in unserer Gruppe etablierten Immunphänotypisierunghaben wir die Korrelation zwischen CSCs und den Immunzellen sowie klinischen Parametern analysiert.

CD133 und CD90 wurden als CSCs-Marker durch einen Sphäroidbildungsassay und einer systematischen Literatursuche identifiziert. Im peripheren Blut (PB) der Patienten machten $CD90^{+}/CD45^{-}$ CSCs etwa 0,76±0,62% (Mittelwert±SD) aus. $CD133^{+}/CD45^{-}$ CSCs machten 1,02±0,69% aus. Es wurde auch ein geringerer Anteil doppelt positiver CSCs festgestellt (0,07±0,08%, n=11). Im Lebertumorgewebe (LTT) machen die mit $CD133^{+}/CD45^{-}$ und $CD90^{+}/CD133^{+}/CD45^{-}$ markierten Zellen 1,16±1,12% und 0,44±0,55% aus. Während $CD90^{+}/CD45^{-}$ CSCs einen höheren Anteil als im PB haben (16,10±13,96% vs. 0,76±0,62%, p=0,002).

Immunzellen lassen sich nach ihrer Funktion im Immunsystem in zwei Teile unterteilen: Immunaktivierende Zellen und immunsuppressive Zellen. Das in unserem Labor etablierte Immunphänotypisierungsprotokoll erlaubt es uns, 39 Arten von Immunzell-Subtypen und ihr jeweiliges Verhältnis zu messen. Die Korrelationsanalysen zeigten, dass die meisten der immunaktivierenden Zellen wie aCTL, Th1, $CD69^+$ NKT, emTh und cmTh eine negative Korrelation mit CSCs in PB oder LTT haben. Hiervon sind NK-Zellen ausgenommen. Im Gegensatz dazu zeigen einige immun-suppressive Zellen wie maTreg und Th17 eine positive Korrelation mit CSCs in PB. Andere Immunzellen wie DC, Breg-1 und Plasmablasten weisen ebenfalls eine positive Korrelation mit CSCs auf. Wobei ihre Funktion in der Tumorimmunologie noch ungeklärt bleibt. Darüber hinaus beobachteten wir, dass klinische Daten wie Child-Pugh-Grad, Vorhandensein eines Rezidivs, Leberzirrhose, ALT-Level sowie die Leukozytenzahl ebenfalls mit der Anzahl der CSCs korrelierten.

In einem Co-Kultivierungs systemist es möglich die Interaktionen zwischen wichtigen Immuneffektorzellen und CSCs *in vitro* zu erforschen. Die Viabilität der CSCs war nach Co-Kultivierung mit Immunzellen signifikant niedriger. Mit diesem Systems sind wir in der Lage, in Zukunft die Interaktion von Immunzellen und CSCs zu untersuchen.

Zusammenfassend konnte in dieser Studie gezeigt werden, dass die Anzahl der CSCs von Nicht-HBV/Nicht-HCV-HCC-Patienten mit Immunzellen korreliert. Diese Zellen könnten in der Zukunft potentielle Ziele für eine Immuntherapie sein. Zudem wurde in dieser Studie gezeigt, dass die CSC ebenfalls mit prädiktiv relevanten klinischen Parametern korrelierte. Mit dem Co-Kultivierungs system kann in Zukunft die Interaktion zwischen CSCs und Immunzellen auf verschiedenen experimentellen Ebenen untersuchtwerden um eine adjuvante Immuntherapie gegen HCC CSCs zu finden.

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Supplement Table1: Major immune cells in related 11 HCC patients' peripheral blood. Abbreviations: DC: Dendritic cell; MDSC: Myeloid-derived suppressor cell; G-MDSC: Granulocyte-like MDSC; M-MDSC: Monocyte-like MDSC; NK: Natural killer cell; NKT: Natural killer T cell; N-S B cell: Non-class switched B cell; C-S B cell: Class-switched B cell; Breg cell: Regulatory B cell ; Pro B cell: Progenitor B cell; Pre B cell: Precursor B cell; Th cell: Helper T cell; emTh: Effector memory helper T cell; cmTh: Central memory helper T cell; eTh: Effector helper T cell; nTh: Naïve helper T cell; aTh: Activated helper T cell; CTL: Cytotoxic T lymphocyte; emCTL: Effector memory CTL; cmCTL: Central memory CTL; eCTL: Effector CTL; nCTL: Naïve CTL; aCTL: Activated CTL; Treg: Regulatory T cell; mTreg: Memory Treg; maTreg: Memory-activated Treg; nTreg: Naïve Treg; aTreg: Activated Treg.

Cell Type	Percentage (Mean±SD), %
Neutrophils, % of Leukocyte	57.48±14.80
Monocyte, % of Leukocyte	5.81±2.19
Macrophage, % of Leukocyte	0.39±0.25
DC, % of Leukocyte	0.22±0.10
MDSC, % of Leukocyte	1.43±1.00
G-MDSC, % of MDSC	40.93±30.77
M-MDSC, % of MDSC	30.91±24.92
G-MDSC, % of Leukocyte	0.52±0.67
M-MDSC, % of Leukocyte	0.47±0.52
NK, % of Leukocyte	1.73±1.48

NKT, % of Leukocyte	0.07±0.10
$CD69^+$ NK, % of NK	2.54±2.38
$CD69^+$ NKT, % of NKT	14.13±8.28
B cell, % of Leukocyte	2.81±0.95
N-S B cell, % of B cell	3.25±3.18
Naive B cell, % of B cell	44.26±23.30
C-S B cell, % of B cell	2.89±4.16
Plasma cell, % of B cell	0.75±1.01
Plasma cell 1, % of B cell	2.42±2.05
Plasmablast, % of B cell	0.24±0.33
Transitional B cell, % of B cell	11.75±11.75
Breg-1, % of Transitional B cell	3.34±4.98
Pro B cell, % of Transitional B cell	27.36±17.17
Pre B cell, % of Pro B cell	58.22±37.56
CD3, % of CD45	23.92±7.27
CD4, % of CD3	75.40 ±10.12
Th17, % of Th	16.37±7.43
Th1, % of Th	48.66±11.16

Th2, % of Th	18.40 ±11.40
em Th, % of Th	54.51±11.78
cm Th, % of Th	18.16±13.34
e Th, % of Th	14.80 ±10.90
n Th, % of Th	12.53±5.98
a Th, % of Th	1.36±0.73
CD8, % of CD3	21.10 ±9.61
em CTL, % of CTL	46.87±12.96
cm CTL, % of CTL	9.84±10.86
e CTL, % of CTL	36.26±20.11
n CTL, % of CTL	7.04±6.15
a CTL, % of CTL	4.36±3.85
Treg/Th	7.61±5.00
m Treg, % of Treg	67.92±8.12
ma Treg, % of Treg	15.16±9.88
n Treg, % of Treg	16.70 ±11.82
a Treg, % of Treg	0.21±0.11
CD4/CD8	4.71±3.09

Th1/Th2	3.52±2.09
Th1/Th17	4.01±2.59
Neutrophils/lymphocyte	2.32±1.18

Supplement Table 2: Correlation analysis between CSCs in PB & LTT with major immune cells

Cell Type	$CD90^+$ CSCs in PB r value/p value	$CD90^+$ CSCs in Tissue r value/p value	$CD133^+$ CSCs in PB r value/p value	$CD133^+$ CSCs in Tissue r value/p value	$CD90^+/CD133^+$ CSCs in PB r value/p value	$CD90^+/CD133^+$ CSCs in Tissue r value/p value
Neutrophils, % of Leukocyte	0.279/0.406	0.227/0.713	0.309/0.355	0.104/0.868	0.397/0.227	0.413/0.490
Monocyte, % of Leukocyte	0.404/0.218	0.291/0.634	0.486/0.130	-0.231/0.709	0.352/0.288	-0.068/0.914
Macrophage, % of Leukocyte	0.317/0.342	0.364/0.547	0.539/0.087	-0.583/0.302	0.498/0.119	-0.451/0.446
DC, % of Leukocyte	0.464/0.150	0.801/0.103	0.623/0.030	0.178/0.774	0.571/0.066	-0.031/0.961
MDSC, % of	-0.0121/0.971	-0.346/0.569	-0.364/0.271	-0.679/0.208	-0.256/0.448	-0.495/0.396

Leukocyte						
G-MDSC, % of MDSC	-0.245/0.468	-0.258/0.675	-0.449/0.167	-0.015/0.981	-0.355/0.285	-0.265/0.667
M-MDSC, % of MDSC	0.157/0.645	0.037/0.954	0.518/0.103	0.276/0.653	0.461/0.153	0.481/0.413
G-MDSC, % of Leukocyte	-0.148/0.664	0.256/0.678	-0.517/0.103	-0.862/0.060	-0.396/0.228	-0.676/0.210
M-MDSC, % of Leukocyte	0.151/0.657	-0.462/0.433	0.181/0.595	-0.537/0.351	0.192/0.572	-0.378/0.531
NK, % of Leukocyte	0.660/0.027	-0.472/0.422	0.295/0.378	-0.149/0.811	-0.048/0.888	-0.073/0.908
NKT, % of Leukocyte	0.101/0.769	0.648/0.237	-0.253/0.453	-0.071/0.910	-0.353/0.287	0.357/0.555

$CD69^+$ NK, % of NK	-0.211/0.534	-0.335/0.582	-0.158/0.644	0.037/0.953	-0.152/0.655	-0.192/0.757
$CD69^+$ NKT, % of NKT	-0.492/0.124	0.637/0.248	-0.608/0.047	0.379/0.530	-0.368/0.266	0.170/0.784
B cell, % of Leukocyte	-0.033/0.923	-0.490/0.403	0.218/0.519	-0.214/0.730	0.452/0.163	-0.518/0.371
N-S B cell, % of B cell	-0.389/0.237	0.340/0.505	-0.035/0.919	0.791/0.111	-0.107/0.753	0.438/0.461
Naive B cell, % of B cell	-0.424/0.193	0.306/0.617	0.102/0.765	0.520/0.369	0.330/0.321	0.588/0.297
C-S B cell, % of B cell	-0.504/0.114	0.963/0.008	-0.194/0.567	0.105/0.866	-0.205/0.545	0.318/0.602
Plasma cell, % of B cell	-0.526/0.097	0.574/0.311	-0.110/0.747	0.349/0.565	-0.101/0.768	0.731/0.161

Plasma cell 1, % of B cell	-0.459/0.156	0.097/0.877	-0.354/0.286	0.095/0.879	-0.352/0.288	0.225/0.716
Plasmablast, % of B cell	-0.302/0.367	0.119/0.849	0.099/0.771	0.704/0.185	0.042/0.903	0.918/0.028
Transitional B cell, % of B cell	-0.397/0.227	0.688/0.200	-0.552/0.078	-0.450/0.447	-0.476/0.139	-0.338/0.578
Breg-1, % of Transitional B cell	0.612/0.045	0.611/0.274	0.485/0.131	-0.605/0.280	0.333/0.317	-0.350/0.564
Pro B cell, % of Transitional B cell	0.322/0.335	0.172/0.783	0.520/0.101	0.687/0.200	0.487/0.128	0.674/0.212
Pre B cell, % of Pro B cell	0.425/0.192	0.019/0.976	-0.017/0.960	-0.434/0.465	-0.122/0.720	-0.031/0.961
CD3, % of CD45	-0.508/0.111	0.355/0.557	-0.567/0.069	-0.105/0.867	-0.539/0.087	-0.375/0.534

CD4, % of CD3	0.138/0.685	-0.065/0.918	0.075/0.827	-0.637/0.248	-0.171/0.615	-0.849/0.069
Th17, % of Th	0.777/0.005	-0.711/0.179	0.443/0.172	-0.728/0.163	0.204/0.548	-0.726/0.165
Th1, % of Th	-0.605/0.049	0.598/0.287	-0.301/0.369	0.822/0.088	-0.192/0.572	0.849/0.069
Th2, % of Th	-0.078/0.819	-0.723/0.167	0.134/0.695	-0.505/0.386	0.336/0.312	-0.784/0.117
em Th, % of Th	-0.004/0.990	-0.425/0.476	-0.017/0.961	-0.601/0.284	-0.094/0.782	-0.881/0.049
cm Th, % of Th	0.410/0.210	0.047/0.940	-0.007/0.984	-0.831/0.082	-0.197/0.561	-0.918/0.028
e Th, % of Th	-0.602/0.050	0.163/0.793	-0.138/0.686	0.742/0.152	0.115/0.737	0.957/0.011
n Th, % of Th	0.190/0.577	0.602/0.283	0.300/0.370	-0.557/0.330	0.417/0.203	-0.529/0.359
a Th, % of Th	-0.146/0.668	-0.257/0.677	-0.109/0.750	-0.228/0.713	-0.092/0.788	-0.583/0.302
CD8, % of CD3	-0.128/0.708	0.065/0.917	-0.059/0.863	0.733/0.158	0.158/0.643	0.862/0.06
em CTL, % of CTL	0.572/0.066	0.188/0.763	0.366/0.268	-0.2500.685	0.037/0.914	-0.581/0.304

cm CTL, % of CTL	-0.011/0.975	-0.232/0.707	-0.226/0.505	-0.448/0.449	-0.320/0.338	-0.568/0.318
e CTL, % of CTL	-0.325/0.329	0.023/0.971	-0.097/0.777	0.522/0.367	0.096/0.778	0.804/0.101
n CTL, % of CTL	-0.124/0.717	0.019/0.976	-0.055/0.872	-0.445/0.453	0.172/0.612	-0.444/0.454
a CTL, % of CTL	-0.355/0.284	0.281/0.647	-0.615/0.044	-0.632/0.253	-0.643/0.033	-0.591/0.294
Treg/Th	0.204/0.548	-0.524/0.365	0.506/0.112	-0.103/0.870	0.573/0.066	0.086/0.891
m Treg, % of Treg	-0.078/0.820	0.610/0.274	-0.097/0.777	-0.184/0.767	0.008/0.981	-0.362/0.549
ma Treg, % of Treg	0.747/0.008	-0.719/0.171	0.177/0.603	-0.627/0.258	-0.155/0.648	-0.763/0.133
n Treg, % of Treg	-0.565/0.070	-0.125/0.841	-0.079/0.817	0.476/0.417	0.125/0.714	0.695/0.193
a Treg, % of Treg	-0.533/0.091	-0.036/0.954	-0.258/0.444	0.539/0.349	-0.091/0.791	0.332/0.585
CD4/CD8	0.174/0.609	0.006/0.992	0.010/0.977	-0.657/0.228	-0.148/0.663	-0.747/0.147
Th1/Th2	-0.341/0.305	0.343/0.572	-0.469/0.146	0.574/0.312	-0.471/0.144	0.900/0.037

Th1/Th17	-0.661/0.027	0.417/0.485	-0.316/0.344	0.783/0.117	-0.105/0.759	0.984/0.003
Neutrophils/ lymphocyte	0.557/0.075	0.224/0.776	0.550/0.080	0.540/0.460	0.503/0.115	0.919/0.082

Supplement Table 3: Demographic and clinicopathological parameters of study population. Abbreviations: IQR: Interquartile range; HBV: Hepatitis B virus; HCV: Hepatitis C virus; UCSF: University of California at San Francisco; UICC: Union for International Cancer Control；WHO: World Health Organization; BCLC: Barcelona Clinic Liver Cancer; AFP: Alpha-fetoprotein; ALT: Alanine transaminase; AST: Aspartate transaminase; APTT: Activated partial thromboplastin time; CRP: C-reactive protein; INR: International normalized ratio.

Variables	Results
Age (Years) (Median (IQR))	75.0 (9.5)
Gender (Female/Male)	2 (18.18%)/9 (81.82%)
Hepatitis (HBV/HCV/Alcoholic)	0 (0%)/0 (0%)/1 (9.09%)
Clinical Cirrhosis (Yes/No)	3 (27.27%)/8 (72.73%)
Ascite (Yes/No)	0 (0%)/11 (100%)
Child-Pugh Grade (0/A/B/C)	7 (63.64%)/4 (36.36%)/0 (0%)/0 (0%)
Number of Lesions (1/>1)	11 (100%)/0 (0%)
Macrovascular Invasion (Yes/No)	2 (18.18%)/9 (81.82%)
Micovascular Invasion (Yes/No)	4 (36.36%)/7 (63.64%)
Milan Imaging Criteria (Inside/Outside)	6 (54.55%)/5 (45.45%)
Milan Pathology Criteria (Inside/Outside)	5 (45.45%)/6 (54.55%)
UCSF Imaging Criteria (Inside/Outside)	7 (63.64%)/3 (27.27%)
UICC Staging Criteria (Ⅰ/Ⅱ/Ⅲ/Ⅳ)	6 (54.55%)/1 (9.09%)/2 (18.18%)/1 (9.09%)

WHO Grading Criteria (1/2/3)	3 (27.27%)/6 (54.55%)/1 (9.09%)
Recurrence (Yes/No)	4 (36.36%)/7 (63.64%)
Albumin (mg/dL) (Median (IQR))	39.0 (5.8)
AFP (ng/mL) (Median (IQR))	15.3 (15.0)
ALT (U/L) (Median (IQR))	54.0 (17.0)
AST (U/L) (Median (IQR))	51.0 (24.5)
APTT (s) (Median (IQR))	24.0 (3.0)
Bilirubin (mg/dL) (Median (IQR))	0.8 (0.6)
Creatinine (mg/dL) (Median (IQR))	1.0 (0.2)
CRP (mg/L) (Median (IQR))	2.0 (6.5)
Leukocytes (10^3/μL) (Median (IQR))	5970.0 (330.0)
INR (Median (IQR))	1.0 (0.2)
Platelets (10^3/μL) (Median (IQR))	173.0 (75.5)
BCLC Grouping Criteria (0/A/B/C/D)	0 (0%)/5 (45.45%)/4 (36.36%)/2 (18.18%)/0 (0%)

Supplement Table 4: Correlation analysis between CSCs in PB & LTT with major clinical parameters

Cell Type	$CD90^+$ CSCs in Blood r value/p value	$CD90^+$ CSCs in Tissue r value/p value	$CD133^+$ CSCs in Blood r value/p value	$CD133^+$ CSCs in Tissue r value/p value	$CD90^+$ $CD133^+$ CSCs in Blood r value/p value	$CD90^+$ $CD133^+$ CSCs in Tissue r value/p value
Albumin (mg/dL)	0.161/0.656	0.677/0.527	-0.030/0.934	0.749/0.462	-0.373/0.289	0.156/0.901
Age (Year)	0.551/0.079	-0.192/0.758	0.397/0.227	-0.401/0.504	0.111/0.746	-0.783/0.118
AFP (ng/mL)	0.267/0.610	0.390/0.610	-0.395/0.438	0.165/0.835	-0.387/0.449	-0.270/0.730
ALT (U/L)	0.106/0.757	0.120/0.847	0.271/0.420	0.962/0.009	0.183/0.591	0.940/0.018
AST (U/L)	-0.114/0.739	-0.545/0.342	-0.061/0.859	0.032/0.959	0.099/0.771	0.011/0.987
APTT (s)	0.051/0.882	-0.064/0.919	-0.030/0.930	0.009/0.989	0.041/0.904	-0.018/0.977
Bilirubin (mg/dL)	0.585/0.059	-0.137/0.826	0.137/0.689	-0.723/0.167	-0.042/0.902	-0.7450.148

CRP (mg/L)	-0.176/0.605	-0.819/0.090	-0.235/0.486	-0.247/0.689	-0.253/0.453	-0.089/0.886
INR	-0.274/0.415	0.571/0.314	-0.251/0.457	-0.330/0.588	-0.210/0.535	-0.086/0.891
Platelets (10^3/μL)	0.554/0.077	-0.143/0.818	0.572/0.066	-0.199/0.749	0.428/0.189	0.153/0.806
Leukocytes (10^3/μL)	0.376/0.255	-0.541/0.346	0.809/0.003	0.152/0.807	0.853/0.0009	0.222/0.720
Creatinine (mg/dL)	-0.074/0.830	-0.440/0.458	0.473/0.142	-0.522/0.367	0.567/0.069	-0.605/0.280

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