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Establishment of a Co-Culture System to Study the Interactions between Major Immune Effector Cells and Hepatocellular Carcinoma

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II. List of Abbreviations

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microliter
µm	Micrometer
AFP	Alpha-Fetoprotein
AASLD	American Association for the Study of Liver Diseases
APC	Antigen-Presenting Cells
ACT	Adoptive Cell Transfer Therapy
BCLC	Barcelona Clinic Liver Cancer Staging
Bregs	Regulatory B cells
CHP	Cyclical Hydrostatic Pressure
CD	Cluster of Differentiation
CTL	Cytotoxic T Lymphocytes
CTLA-4	Cytotoxic T Lymphocyte-Associated Protein 4
DC	Dendritic Cells
DPBS	Dulbecco's Phosphate Buffered Saline
DFS	Disease Free Survival

DCC	Direct Contact Co-culture
EASL	European Association for the Study of the Liver
FVS510	Fixable Viability Stain 510
FACS	Fluorescence-Activated Cell Sorting
FCM	Flow Cytometry
FasL	Fas Ligand
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HD	Healthy Donors
IDO	Indoleamine 2, 3-dioxygenase
IFN-γ	Interferon-γ
IL	Interleukin
ICC	Indirect Contact Co-culture
KIRs	Killer Cell Immunoglobulin-like Receptors
LC	Liver Cirrhosis
LR	Liver Resection
LT	Liver Transplantation
LAG 3	Lymphocyte Activation Gene 3
MC	Milan Criteria

MDSC	Myeloid-derived Suppressor Cells
mTOR	Mammalian Target of Rapamycin
MMPs	Matrix Metalloproteinases
MWA	Microwave Ablation
min	Minutes
mL	Milliliter
NK	Natural Killer
OS	Overall Survival
PB	Peripheral Blood
PD-1	Programmed Cell Death-1
PHC	Primary Hepatic Carcinoma
PAMPs	Pathogen-associated Molecular Patterns
PRRs	Patterns Pattern Recognition Receptors
PBMC	Peripheral Blood Mononuclear Cells
RFA	Radiofrequency Ablation
ROS	Reactive Oxygen Species
RFS	Recurrence-free Survival
STAT3	Signal Transducer and Activator of Transcription 3
TGF-β	Transforming Growth Factor Beta
Tex cells	Exhausted T cells

Tem	Effector Memory T Cells
Tcm	Central Memory T Cell
Tscm	Stem-cell Memory T Cells
Th	Helper T Cells
Th1	Type 1 Helper T Cells
Th2	Type 2 Helper T Cells
Th17	Type 17 Helper T Cells
Tregs	Regulatory T Cells
TILs	Tumor-infiltrating Lymphocytes
TACE	Transcatheter Arterial Chemoembolization
TRAIL	TNF-related Apoptosis-inducing Ligand
TGN	Trans-Golgi Network
TNF-α	Tumor Necrosis Factor α
TRAIL	TNF Related Apoptosis Induced Ligand
TDCC	Three-dimensional Co-culture
T:E	Target:Effector
TNFR	Tumor Necrosis Factor Receptor
VEGF	Vascular Endothelial Growth Factor

1. Introduction

Immunology and oncology have interacted with each other for over 50 years[1]. The immune system plays an essential part in defending people against cancer, especially the major immune effector cells such as $CD8^+$ lymphocytes and natural killer cells (NK cells). Immunotherapy is an unique approach aimed at enhancing immune system activity to eliminate cancerous cells[2]. In recent years, immunotherapy has developed greatly, especially with regard to checkpoint blockade and cellular therapies[3]. As one of the most common primary hepatic carcinoma (PHC), Hepatocellular Carcinoma (HCC) has become the world's fourth leading cause of cancer death[4]. The immune system acts on HCC and HCC in turn impacts the immune system, but what truly happens within the interactions remains unclear. To date, there is insufficient data describing the direct cell-cell interactions with an appropriate model in a physiologically and clinically relevant manner. In this dissertation, a co-culture system was established to explore the cell-cell interactions between the major immune effector cells and HCC.

1.1. Hepatocellular Carcinoma

1.1.1. Epidemiology of HCC

In 2018, the sixth most frequently diagnosed cancer and the fourth leading cause of death from cancer worldwide was liver cancer. More precisely, the estimated number of new liver cancer cases and deaths was 841,080 and 781,631, respectively, while the number of new cases and deaths from all cancer combined was 18.1 million and 9.6 million, respectively.[4] Global cancer statistics from 2018 showed that liver cancer mortality in both sexes ranked fourth, while it ranked second in men and sixth in women (Figure 1)[4]. It is also estimated that the incidence cases of liver cancer will reach 1,361,836 and the deaths will number 1,284,252 by 2040 worldwide (Figure 2, 3), which is a tremendous increase despite new treatments which showed promising

results[4]. As the major types of liver cancer, HCC contributes significantly to cancer-related morbidity and mortality all over the world. Ninety percent of HCC occurs in cirrhotic or chronically inflamed livers mainly due to hepatitis B or C virus infection and alcohol abuse[5].

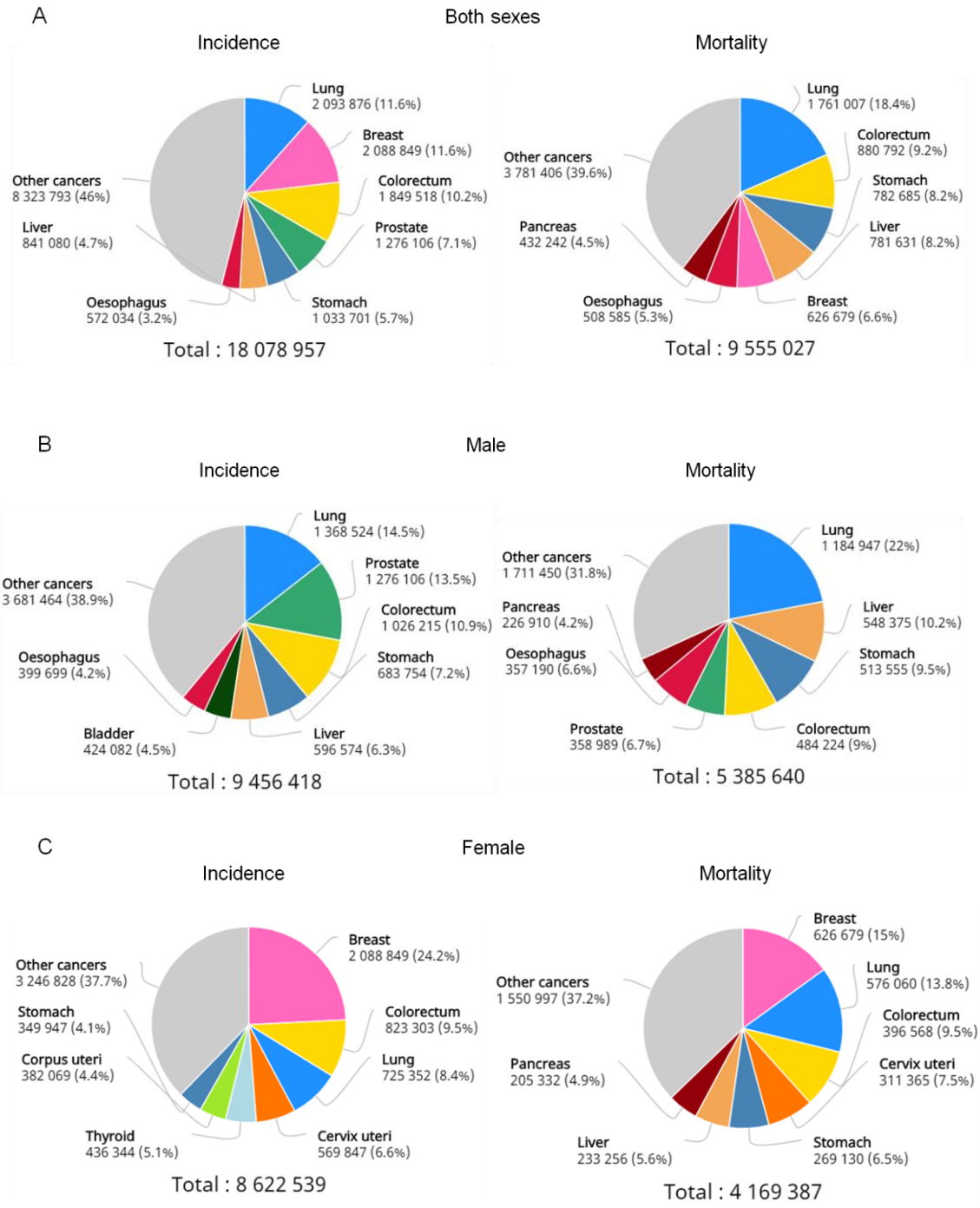

Figure 1. Pie charts presenting the distribution of cases and deaths for the 7 most common cancers in 2018. (A) both sexes; (B) males; (C) females. Data and Graphs from Global Cancer Observatory (GLOBOCAN) 2018. Accessed on June 12th, 2020, from http://gco.iarc.fr/

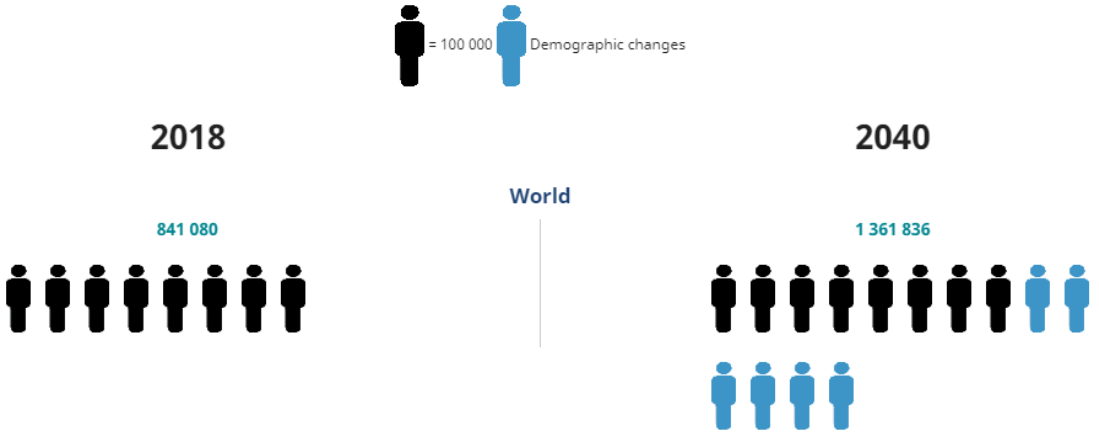

Figure 2. Estimated number of incident cases from 2018 to 2040 from liver cancer in both sexes and all ages. Data and Graph from GLOBOCAN 2018. Accessed on June 12th, 2020, from http://gco.iarc.fr/

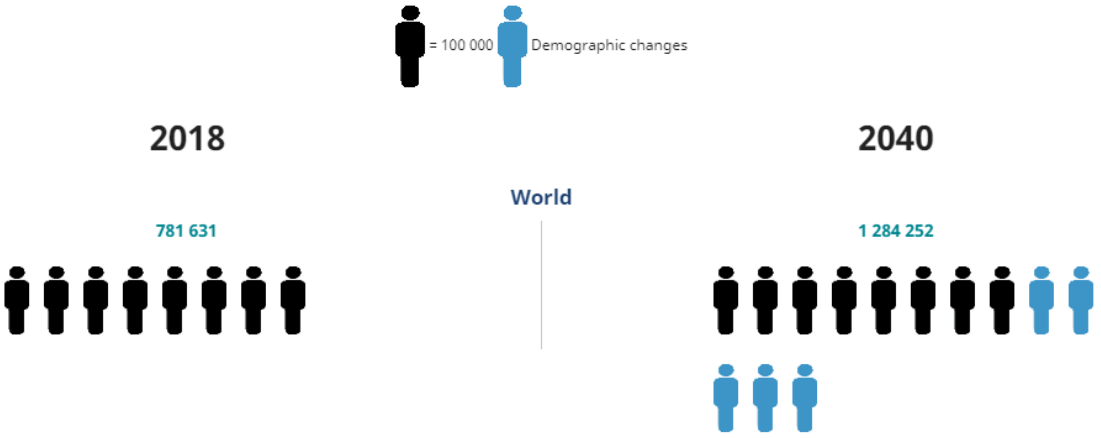

Figure 3. Estimated number of deaths from 2018 to 2040 from liver cancer in both sexes and all ages. Data and graph from GLOBOCAN, 2018. Accessed on June 12th, 2020, from http://gco.iarc.fr/

1.1.2. Treatment of HCC

Other than surgery, which remains the most effective method for treating HCC, other non-surgical therapeutic methods also have been developed in recent years, such as transarterial chemoembolization (TACE), radiofrequency ablation (RFA), and molecular targeted therapy. To make appropriate therapeutic decisions, it should be known that treatment varies with each patient, which makes clinical staging greatly important. Among the many staging systems, the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL) suggest the Barcelona Clinic Liver Cancer (BCLC) staging system[6] (Figure 4). It is the most widely used standard system for the treatment of HCC patients at present[7]. However, treatment of HCC requires a distinct knowledge of the interplay of liver function, tumor size and biology such as clinical state of the patient. Therefore especially regarding liver resection (LR) and liver transplantation (LT) acceptable results can be obtained by stratifying the treatments with modern and dynamic multi marker models[8-10].

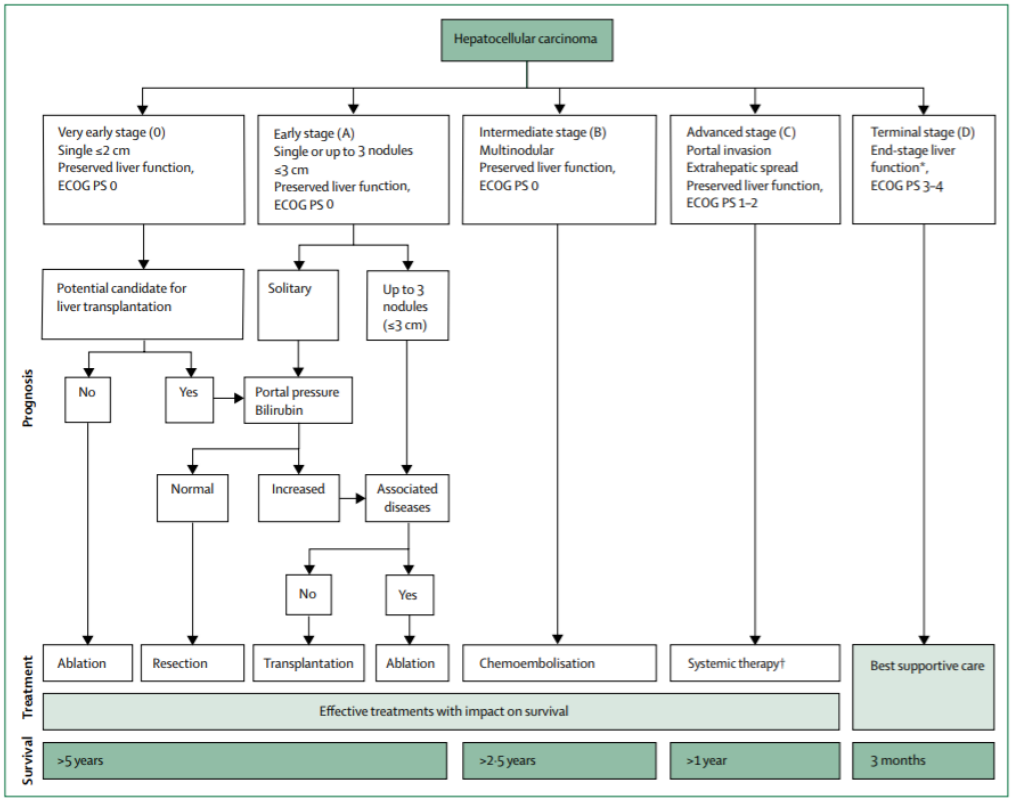

Figure 4. The BCLC staging system (Reproduction of this figure was kindly permitted by Elsevier and Copyright Clearance Center.)[6]

One of the most important surgery methods is LR, which is the first-line therapy for localized HCC without liver cirrhosis[11, 12]. However, portal hypertension is a contraindication to LR[11]. Patients undergoing LR should be fully assessed for both systemic and hepatic conditions. At present, Child-Pugh sometimes combined with indocyanine green (ICG) clearance test[13] is used for determining the liver reserve function. If the patient is undergoing a relatively large LR, in addition to evaluating liver function, the expected future liver remnant (FLR) should also be evaluated to determine its percentage of standard liver volume[14]. Expected FLR deficiency is a significant variable affecting the respectability of liver cancer. Generally, the necessary condition for massive LR is Child-Pugh A, ICG15 < 10%, FLR > 40% for liver cirrhosis patients and FLR > 30% for normal patients[15]. LR is a difficult operation due to the complicated anatomy of the liver and rich blood supply. However, with the improvement of surgical equipment and techniques such as ultrasonic scalpel, and bipolar coagulation, liver blood flow control technology, liver dissociation technology and liver hemostasis technology have developed greatly in recent years[16, 17]. Laparoscopic or robot-assisted minimally invasive hepatectomy has become increasingly popular, and indications for minimally invasive surgery have also expanded significantly due to the improved short term outcome[18], while the long-term tumor specific outcome remains to be validated[19, 20].

LT is a radical treatment for patients who cannot be treated with LR, especially those with severe cirrhosis. The Milan criteria (MC) are used worldwide for selecting patients who can undergo LT. For patients that meet the Milan criteria, the 4-year survival rate and recurrence-free survival (RFS) rate are 85% and 92%, respectively, and the recurrence rate is 8% after LT[21]. The Milan criteria are so strict that patients who may profit from LT are left out from waiting lists[22]. Given the shortage of liver sources, 25% of patients cannot receive LT due to tumor progression beyond the

Milan criteria if they have waited for >1 year[23]. The University of California has expanded the criteria to include diameter of single tumor < 6.5 cm, ≤ 3 tumors, maximum diameter < 4.5 cm, and total diameter < 8.0 cm; patients enrolled with these criteria achieve similar clinical outcomes to those enrolled with the Milan criteria[23]. However, more research is needed to determine the validity of this extended standard. In a recent work Schoenberg et al. showed that without upfront size restrictions patients with a good dynamically measured tumor biology can achieve similar results after LT as patients within MC[10]. While awaiting transplantation, patients can be treated with TACE, RFA and other auxiliary treatments to control tumor progression. For patients who exceed the indications of liver transplantation, TACE and RFA may reduce the tumor stage and improve the prognosis of some patients. It has been confirmed that RFA performed before LT can improve the overall survival (OS) and tumor-free survival (TFS) at 5 and 10 years following transplantation[24].

There has been significant development of local ablative therapy, including RFA and microwave ablation (MWA), for treating liver cancer. There are disputed differences in the local curative effect, complication occurrence rate, and long-term survival rate of RFA and MWA[25]. Ablative therapy has the advantages of fewer traumas, faster recovery, and less impact on liver function. Ablative therapy can be repeated for local recurrent lesions or new lesions in the liver after liver cancer treatment, which is of great significance for controlling tumor progression and prolonging survival.

TACE is one of the commonly used treatment methods for HCC that cannot be resected surgically. TACE can significantly delay tumor progression, especially for multiple tumors and tumors rich in arterial blood supply[26, 27]. However, TACE may retain residual cancer lesions, presenting the risk of stimulating the proliferation of residual cancer cells and vascular endothelial cells, and may activate hepatitis virus replication. These are all certain drawbacks of TACE, which not only limit its use, but also increase the potential risk to patients. Hence, TACE must be applied in combination with other therapies. TACE is easy to perform and causes little trauma,

which ensures that it is an effective means of palliative treatment, preoperative and postoperative adjuvant therapy for patients with liver cancer.

In recent years, HCC immunotherapy has become a hotspot both in basic research and clinical field[28]. The liver is a special immune-tolerant organ that can evade the immune response effectively. Immunotherapy can enhance the body's immune response, stimulate tumor-specific immunity, break immune tolerance, and reactivate immune cells to recognize and kill or inhibit tumor cells. HCC Immunotherapy includes treatment targeting the immune checkpoints, adoptive tumor immunotherapy, and tumor vaccines, which will be elaborated below.

Other than the above therapeutic methods, radiotherapy, chemotherapy, and molecular targeted therapy also play essential parts in HCC treatment. However, management of HCC should be individualized and combine all methods appropriate to each patient to yield the best result.

1.2. Tumor Immunology and Immunotherapy in the Context of HCC

The basic immunology research plays a major part in understanding the mechanisms of tumorigenesis as well as developing new and effective tumor therapies. Innate immunity and adaptive immunity work as the first-line and the second-line of defense, respectively, against pathogens or tumor cells. Tumor immunology is cyclic process starting from the release of tumor antigens and ending with the killing of tumor cells (Figure 5) [29].

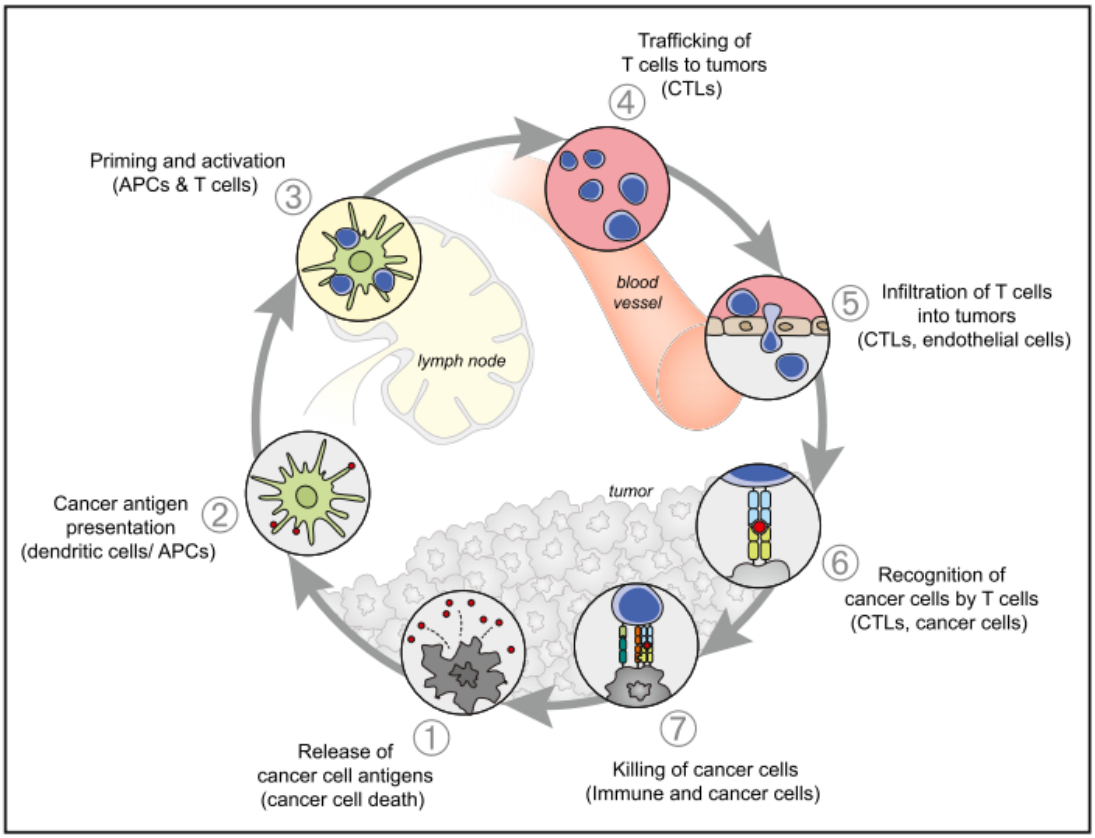

Figure 5. The cancer-immunity cycle[29] (Reproduction of this figure was kindly permitted by Elsevier and Copyright Clearance Center.)

1.2.1. Recognition and Regulation of Innate Immunity

Recognition is the very first step in immunological activity. When pathogens invade into the host, the pathogen-associated molecular patterns (PAMPs) produced by pathogens can be identified by pattern recognition receptors (PRRs) generated from innate immune cells such as macrophages, subsequently activating the innate immunity. During the initiation and occurrence of local immunity, physical or chemical factors in the immunological microenvironment can change significantly, such as temperature, pH value, osmotic pressure, and oxygen concentration. Recently, Solis et al[30]. found that cyclical hydrostatic pressure (CHP) which is a certain physical factor caused by inner fluid can trigger the inflammatory response by activating the PIEZO1 ion channel. Eukaryotic cells recognize intracellular pathogens by selective autophagy, which is termed xenophagy, an essential mechanism of the host's natural

immunological prevention. V-ATPase is the key protein for host cells to sense the membrane damage caused by bacterial infection, the downstream of which recruits the autophagic protein ATG16L1 and initiates xenophagy. The study revealed the molecular mechanism of autophagy recognition of intracellular pathogens [31]. NLRP3 inflammasomes can trigger the inflammatory response by recognizing a high diversity of excitation signals such as pathogen invasion and damage signal. Negative phospholipids such as PtdIns4P (phosphatidylinositol-4-phosphate) accumulating on the Trans-Golgi network (TGN) membrane can induce NLRP3 transport and aggregation to activate inflammasomes and subsequently activate downstream inflammatory signaling[32].

After a series of recognition signals, the innate immune cells secrete a large amount of interferon (IFN), triggering anti-viral or anti-tumor innate immunity. After activating an effective immune response, the body needs to terminate it in a timely manner and mediate the inflammation degradation to maintain immune homeostasis. The molecular mechanism of immune recognition is updated every year, and an increasing number of high-quality studies have been published in authoritative journals. Cadena et al.[33] reported that the E3 ligase is directly involved in oligomerization and ligand recognition of innate immunity and revealed a novel mechanism by which innate cells sense the length of foreign RNA. Shen et al.[34] reported that the DNA methylation oxidase TET2 can promote myeloid cell mobilization and differentiation and amplify the anti-pathogen effect; on the other hand, it can inhibit inflammatory cytokine production to avoid the tissue damage caused by natural immunity.

DCs recognize, process, and present tumor antigens, which is the premise of a powerful anti-tumor response. DCs work essentially in activating the body's immune response as well as maintaining its own immune tolerance. Research on the regulatory mechanism of DC function activation is a hotspot in immunology. Han et al.[35] found that modification of RNA*N*^{6}-methyladenosine (m^6A) was a key factor in

regulating the innate immune function of DC. Wang et al.[36] reported that RNA methylation transferase METTL3-mediated m^6A modification promoted functional DC activation by changing the mRNA translation levels. The tumor-initiated functional defects of DCs may lead to tumor cells evading immune surveillance[37, 38]. Compared with healthy people, patients with HCC have DCs that express less human leukocyte antigen (HLA)-class I molecules, and secrete less interleukin-12 (IL-12) and their swallowing function abates, which prompts DCs to present mature flaws when HCC develops[39].

Macrophages account for a large proportion of tumor-infiltrating lymphocytes (TILs), most of which are derived from peripheral mononuclear cells. Macrophages can be polarized to the M1 or M2 type in different ways in different tumor microenvironment. M1 macrophages have strong phagocytic, pro-inflammatory, and anti-tumor activity. However, M2 macrophages have anti-inflammatory and tumor-promoting activity[40]. Tumor-associated macrophages (TAM) are mainly M2 macrophages that are concentrated at the edge of the tumor, which can promote tumor cell survival and migration by activating nuclear factors and inducing IL-6[41]. By enlisting helper T cell 2 (Th2), TAM can inhibit Th1, deactivating naïve T cells to affect the regulatory immune response[41].

As important innate immune cells, NK cells play a key role in the anti-tumor and anti-infection process. NK cells account for 25–40% of lymphocytes in human liver. There are two subsets of NK cells based on the CD56 expression level: $CD56^{bright}$ and $CD56^{dim}$. The $CD56^{bright}$ subset can be amplified following IL-2 stimulation, and about 10% of them express killer cell immunoglobulin-like receptor (KIR). The $CD56^{dim}$ subset is not sensitive to IL-2 stimulation, and 85% of them are KIR^+, and secrete perforin and granzyme B[42]. Dong et al.[43] reported that the transcription factor XBP1 can directly activate the c-*MYC* gene to promote NK cell survival and function. What's more, as important receptors on NK cells, the NKG2 family, which includes NKG2A, NKG2B, NKG2C, and NKG2D, among other members[44] can be divided into two

types: activating receptors and inhibitory receptors, which transmit positive and negative signals, respectively[45]. As killer cells, NK cells have the potential to kill cancer cells; in turn, cancer cells can also inhibit activated receptors and inhibit NK cell activation and function[46]. In the presence of HCC, Rae1 presents on the HCC cells surface, and as a ligand of NKG2D, it can activate NK cells and promote their anti-tumor immunity[47]. However, the cytotoxic activity of NK cells is limited in HCC patients at the same time. Some studies reported that the peripheral blood of HCC patients have significantly lower $CD56^{dim}$ subsets than that of healthy controls[48], which means that cytotoxic NK cells is inhibited in HCC patients.

During tumor development, chronic inflammatory factors can recruit myeloid-derived suppressor cells (MDSCs) to tumor region. MDSCs own the ability of expressing a variety of angiogenic factors, such as vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs), to directly promote the formation of tumor blood vessels. MDSCs can also inhibit specific T cell–mediated anti-tumor immunity as well as NK cell and macrophage-mediated natural anti-tumor immunity via the high expression of ARG1, reactive oxygen species (ROS) and so on.[49]

1.2.2. Recognition and Regulation of Adaptive Immunity

As the main effector and regulatory cells in adaptive immunity, T cells play a pivotal role both in cellular and humoral immunity. T cells can differentiate into various subsets under the stimulus of all kinds of cytokines, antigen-presenting cells (APC), and other factors. Various T cell subsets show diversified functional features and action patterns, which determine the ultimate effect of the immune activity.

$CD4^+$ T cells proliferate and activate into Th cells under the influence of antigen signals, co-stimulatory signals, and cytokines. Different subsets of Th cell (e.g., Th1, Th2, Th17, Tfh, Th9, Th22) work together to affect the balance and effect of the immune response[50]. Patients with HCC have significantly fewer $CD4^+$ T cells than

healthy controls, suggesting their important role in HCC occurrence and progression[51].

Regulatory T cells (Treg cells) are functional subsets with immunosuppressive functions, which have a significant impact on immune tolerance and homeostasis maintenance[52]. The inhibition of Treg cell on TILs is an important cause of tumor immune escape and anti-tumor immune failure. A large number of experiments have shown that increased Treg cells are conducive to tumor immune escape[53]. The adaptive input of T cells without Treg cells can significantly improve the body's anti-tumor immune response. On the contrary, the proliferation or activation of $FOXP3^+/CD25^+/CD4^+$ Treg cells significantly inhibited tumor immunity.[54] Pro-inflammatory Tregs secreting IFN-γ are associated with autoimmune diseases and tumor development, while anti-inflammatory Tregs secreting IL-10 can alleviate the progression of chronic inflammation[55, 56]. It is not clear how Treg cells balance the secretion of IFN-γ and IL-10. The proportion of $CD8^+/FOXP3^+$ Tregs in the liver of advanced HCC patients was higher compared to that of early HCC patients. The increased Tregs infiltration in cancer nodules would lead to decreased $CD8^+$ T cells, which shows that there is a correlation between Tregs and HCC progression and prognosis[57].

Cytotoxic T cells (CTL, commonly expressing the marker CD8) are the key effector cells of anti-tumor and anti-virus infection. Infected and transformed cells will be killed by CTLs after they recognize the antigen presented by APC. However, continuous antigenic stimulation can lead to CTL differentiation into exhausted T cells (Tex cells). Tex cells display the reduced killing activity, reduced production of TNF and other effector molecules. What's more, Tex cells could express relatively high level of programmed cell death-1 (PD-1) which turns out to be an inhibitory receptor[58]. A few quality papers reported that the transcription factor thymocyte selection-associated high-mobility group box (TOX) is a key regulatory factor of Tex cell differentiation[59, 60].

At present, the reversal of T cell depletion or reinfusion of active T cells has shown good effects in T cell tumor immunotherapy.

Immune memory is an important characteristic of adaptive immunity, which means that the immune system can initiate a more rapid and efficient immune response to the exposed antigens. In this regard, memory T cells have an essential effect. Based on the expression of CCR7 and CD62L, memory T cells can be classified into central memory T (Tcm, $CD45RA^{-}/CCR7^{+}$) cells, effector memory T (Tem, $CD45RA^{-}/CCR7^{-}$) cells, and stem cell memory T (Tscm, $CD45RA^{+}/CCR7^{+}/CD95^{+}/CD122^{+}$) cells[61]. Tcm cells are characterized by rapid proliferation and differentiation, homing to secondary lymphoid organs. Tem cells show strong cytolytic function and can migrate to inflammatory tissues rapidly displaying their effector function.[62] Tscm cells have strong self-renewal ability and can exert long-term anti-tumor effects *in vivo*, which makes them the most promising cell subgroup in the field of immune cell therapy[63].

Specific humoral immune responses are mainly mediated by B cells and their production of antigen-specific antibodies. After being enabled, B cells can be divided into plasma cells and memory B cells[64]. Plasma cells can produce antibodies and participate in humoral immunity; their survival time is relatively short, generally 2–3 days. Memory B cells, on the other hand, exist for a longer time, usually around 3 months. When re-exposed to a specific antigen, memory B cells can quickly and massively produce high-affinity antibodies, which can activate the humoral immunity of the body effectively and play a protective role.[65] Regulatory B cells (Bregs) are a subgroup of B cells that can suppress the immune activity[66]. In the tumor microenvironment, Bregs are the major form of B cells with immunosuppressive effects on tumor[67], mainly through producing transforming growth factor beta (TGF-β), IL-10, IL-35 and other anti-inflammatory cytokines[68].

1.2.3. Tumor Immunotherapy (HCC Immunotherapy)

Tumor immunotherapy refers to the use of immunological methods and principles, aiming at the body's hyperactive or low immune state, to strengthen or weaken the immune response, target tumor cells, control tumor development or kill tumor cells. At present, tumor immunotherapy has recorded great achievements both at molecular and cell level.

1.2.3.1. Treatment Targeting the Immune Checkpoints

The basic principle of targeted immune checkpoint therapy is to block the signaling pathway through the use of co-inhibitory molecules or ligand antagonists or other drugs, to relieve the immunosuppression of patients with tumor, stimulate cytotoxic T cells activation, and enhance their ability to kill tumor cells[69]. The main checkpoints include PD-1 (CD279), cytotoxic T lymphocyte-associated protein 4 (CTLA-4, CD152) and indoleamine 2, 3-dioxygenase (IDO) [70]. PD-1 is mainly expressed on activated T/B cells and monocytes cells, DC, Tregs, and natural killer T(NKT) cells[71]. The combination of PD-1 and its ligand PD-L1/PD-L2 can suppress T cell proliferation, IL-2 and IFN-γ secretion, B cell proliferation and differentiation, and immunoglobin (Ig) secretion. PD-1/PD-L1 monoclonal antibodies can specifically block the binding of PD-1 and PD-L1, reactivate T cells, and restore its killing effect on tumors[72]. Antibodies against PD-1 and PD-L1 have been proven effective in those patients diagnosed with melanoma, lung cancer, bladder cancer, and other cancers, as well as in patients with advanced unresponsive tumors, significantly extending the survival[73]. As the first drug targeting PD-1 to treat melanoma, the monoclonal antibody nivolumab shows good safety and potential therapeutic effect for patients suffering advanced HCC[74]. CTLA-4 is an immunoregulatory receptor produced by activated T cells, and exerts an inhibitory effect, whose anti-tumor mechanism is similar to that of PD-1[75]. CTLA-4 blocker reactivates T cells and maintains T cell proliferation by blocking the combination of CTLA-4 and CD80 or CD86, so that reactivated T cells can attack cancer cells effectively[76]. As one of CTLA-4 blocker, Tremelimumab has been used for treating liver cancer, colorectal cancer, and metastatic melanoma[77].

Sangro et al.[78] treated 17 HCV-infected patients with HCC using tremelimumab, and got promising therapeutic effect.

1.2.3.2. Adoptive Tumor Immunotherapy

Adoptive immunotherapy is a process in which autologous or xenogenous immune cells or immune factors are amplified *in vitro* and given to patients with low immune function, such as patients with cancer, to help them obtain anti-tumor immunity. It mainly includes adoptive cytokine transfer therapy and adoptive cell transfer therapy (ACT). The cells used in ACT include TILs, cytokine-induced killer (CIK) cells, chimeric antigen receptor T (CAR-T) cells, and DCs, which have high specificity and low adverse effects[79, 80]. CARs and bispecific antibodies are two effective methods that have been studied in HCC recently. CARs can specifically recognize tumor-associated antigen (TAA), resulting in T cell activation and expansion[81]. CAR-T is the current hot topic for ACT for cancer treatment. There are many TAAs on HCC cells, and the key to effective immunotherapy lies in selecting appropriate target antigens. Thus far, GPC3 is the most commonly used CAR antigen in HCC[82]. Gao et al.[83] reported that GPC3-targeted CAR-T can effectively destroy HCC cells that are GPC3 positive. A phase 1 clinical trial showed that GPC3-targeted CAR-T displayed safe and efficient tumor lytic effects in patients suffering relapsed or refractory HCC[84]. Although some studies have reported good results of CAR-T cell therapy in patients with HCC, its effect on solid tumors, including HCC, remains limited. Notably, combined modification of T cells was needed in the management of HCC. Sun et al. demonstrated that NKG2D-based CAR-T cells could powerfully kill HCC cells that have high expression of NKG2DLs[85]. Another research work by Batra et al. revealed that GPC3-specific CAR-T co-expressing IL15 and IL21 exhibited strong anti-tumor activity against HCC cells[86]. It was also reported that the anti-tumor activity of GPC3-CAR-T was enhanced and the exhaustion of GPC3-CAR-T was reduced when PD-1 was disrupted[87]. Bispecific antibodies, on the other hand, can activate immune effector cells and promote their cytotoxicity to tumor cells[88]. Blinatumomab, a

CD19/CD3 bispecific initiator for T cells, was found to be effective in patients suffering from acute lymphoblastic leukemia, and it was the first FDA-approved bispecific antibody[89]. In a murine xenograft model of human HCC, Lin et al. found that a GPC3/CD3 bispecific antibody, which is a T cell-redirecting antibody, significantly inhibited HCC tumor growth[90]. NK cell-based immunotherapy has also been developed and is a promising treatment strategy. Researchers are trying to resolve the dysfunction of NK cells in tumor. Many strategies, including adoptive transfer, gene therapy, and cytokine therapy, can be applied. Autologous NK cells extracted from PBMCs can be expanded by thousands-fold *ex vivo*. Moreover, during expansion, the cytotoxicity of NK cells can also be reinforced by cytokines (e.g. IL-2, IL-15, IL-21)[91, 92]. However, the toxicity of systemic cytokine administration should be taken into consideration. Proliferation and cytotoxic effects can also be enhanced by cytokine gene modification. Several studies have reported that adoptive transfer of gene-modified NK cell lines greatly augmented their anti-tumor efficiency in HCC[93, 94]. Similar to CAR-T cells, CARs can be adapted to NK cells to enhance their efficacy. Since CAR-NK cells have a shorter lifespan than CAR-T cells, related risk of autoimmunity and tumor transformation may be reduced[95]. For now, the research and application of co-culture with DCs and CIK cells are the most widely used treatment. The therapeutic effect of DC-CIK for non–small cell lung carcinoma (NSCLC) was better than that of LAK cells[96]. Pan et al. revealed that CIK cell immune-assistive therapy greatly improved OS and RFS in HCC patients[97]. Zhang et al.[98] showed that DC-CIK cell therapy was able to prolong the median total survival time, and reduce alpha fetoprotein (AFP) levels in peripheral blood.

1.2.3.3. Tumor Vaccines

In recent years, research on non-infectious vaccines, especially tumor vaccines, has been emphasized and developed. The principle of tumor vaccines is that the tumor antigen is introduced into the body through tumor cells, tumor-associated protein, peptides, and nucleic acids that express tumor antigen. So that T cells and B cells are

activated and act on certain types of cancer cells, finally achieving the purpose of inhibiting tumor cell growth, metastasis, and recurrence[99]. Tumor vaccines are mainly divided into bacterial vaccines, genetically modified tumor cell vaccines, nucleic acid vaccines, recombinant virus vaccines, synthetic peptide vaccines, and DC vaccines[100]. However, tumor vaccines have very low immunogenicity; therefore, identifying a suitable combination of immune adjuvant for increasing the success rate of inducing the immune response is necessary. At present, the most commonly reported polypeptide vaccine for patients with HCC is the AFP polypeptide vaccine. Cany et al.[101] showed that the AFP vaccine can inhibit tumor growth effectively in mice, which provides an experimental basis for the clinical therapy of liver cancer. However, the AFP polypeptide vaccine also has some limitations, as it only targets the AFP-specific immune response, while clinically, one-third of patients with liver cancer show no significant increase in AFP levels.

Collectively, immunotherapy based on checkpoint inhibition, specific T cells or NK cells adoption and tumor vaccines are becoming promising new therapeutic strategies in HCC treatment. To develop immunotherapy of HCC, investigation of immune cells and HCC cells is necessary. One important direction is to study the cytotoxicity of major immune effector cells towards HCC cells. Co-cultivation would be a good way to mimic the inner microenvironment and explore the interactions between major immune effector cells and HCC cells. Therefore in the last part of my introduction, I would elaborate the present technique of co-culture.

1.3. Cell Co-Culture

Cell co-culture means two or more types of cells from the same specimen or different specimens cultured in same culturing system[102]. Cell co-culture technology has been attracting much attention since 1980s, which is mainly used in the research of inducing stem cell differentiation[103, 104], increasing metabolite production[105], improving cell viability, maintaining cell function and activity[106, 107], and *in vitro* tissue

construction[108]. Compared with mono cell culture technique, cell co-culture technique can simulate the *in vivo* environment to a large extent, so as to better observe the interactions between cells and cells, and between cells and the culture environment. Moreover, through detecting the interactions between different cytokines, the mechanism of drug action and possible targets of drugs can be explored. According to the normal morphology of co-cultured cells and the objective of investigators, methods of cell co-culture mainly include direct contact co-culture (DCC), indirect contact co-culture (ICC) and three-dimensional cell co-culture (TDCC).

DCC means two or more kinds of cells cultured together with certain proportion so that cells could contact with each other directly[109]. DCC can not only maintain cytokine delivery, but also keep cell-cell direct interactions working, which brings the cultured cells closer to the natural state of the body. Easy to operate and low requirement for culture condition are also advantages of DCC. This method is mainly applied to study the intercellular interactions and induce cell differentiation. Watanabe et al. found that the biological activity of human nucleus pulposus cells was clearly elevated in the co-culture of autologous human bone marrow mesenchymal stem cells[110]. Other studies showed that endothelial cells can promote the proliferation of neural stem cells when cultured directly together and differentiation into nerve cells under the action of vascular endothelial growth factor (VEGF) [111]. The difficulty of direct contact co-culture lies in the detection of indicators, due to the difficulty of completely separating different cell types. At present, morphological method, Immunohistochemistry (IHC), *in situ* hybridization and FACS could be applied to distinguish different cells.

ICC mainly consists of conditioned medium co-culture, “cell crawling” co-culture and transwell co-culture. In conditioned medium co-culture, the supernatant containing several cytokines which are secreted by one kind of cells is used to culture another kind of cells[112]. This method can focus on the influence of conditioned cell to target cells. In “cell crawling” co-culture, cells are seeded on a slide pretreated by

collagen Ibefore transferring to another cell culture dish and co-cultured with another type of cell[113]. Transwell co-culture system is the most widely used co-culture method at present. Different cell populations are separated by a membrane at the bottom of transwell chamber. Cells can interact with one another by cytokines penetrating the membrane. Cao et al. investigated the role of multi-walled carbon nanotube and palmitic acid in cardiovascular disease by co-culturing pretreated endothelial cells and epithelial cells in transwell co-culture system[114]. The advantage of indirect contact co-culture is that it is easy to separate two cell populations and gets better observation of their respective cellular state. However, the direct cell-cell interactions cannot be available.

In animals, cells actually exist in the three-dimensional growth environment, so the establishment of TDCC system is increasingly favored by researchers. In TDCC, different three-dimensional material was used as supporter and co-cultured with various cells. This co-culture system enables the cells to produce certain three-dimensional tissue-specific structure. There are studies stated that co-culture of nerve cells and fibroblasts in three-dimensional supporter showed good effect in repairing nerve injury[115]. Veiga et al. found that compared with monolayer culture, neural stem cells co-cultured with endothelial cells on three-dimensional scaffold is more conducive to proliferation and differentiation of neural stem cells[116]. Three kinds of cell co-culture models have their own benefits and drawbacks. We should select the most appropriate model according to our experimental purpose.

1.4. Aim of the Study

This study aimed to establish a HCC-TILs DCC system by establishing a HCC cell line (HepG2)–peripheral blood mononuclear cell (PBMC) co-culture initially, to investigate the cell-cell interactions between the major immune effector cells and HCC cells. To determine the activation and cytotoxicity of the major immune effector cells, the

degranulation marker of major immune effector cells and the viability of HCC cells were measured and analyzed using flow cytometry.

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
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
Pipette	Sigma-Aldrich, USA
37℃ Incubator	Binder, Germany
Water Bath	Köttermann, Germany
4℃ Fridge	Liebherr, Germany
-20℃ Fridge	Bosch, Germany
-80℃ 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	Sarstedt, USA
5 mL Polystyrene Round-Bottom Tube	Falcon, USA
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 Vial	Thermo Fischer Scientific, USA
40 µm Cell Strainer	Corning Incorporated, USA
30 µm MACS Smart Strainer	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 (P/S)	PAN Biotech, Germany
Trypsin EDTA	Lonza, Switzerland
Trypan Blue	Sigma, Germany
Ibidi Freezing Medium	Ibidi GmbH, Germany
Fixable Viability Stain (FVS) 510	BD, USA
Tumor Dissociation Kit	Miltenyi Biotec, Germany
Dead Cell Removal Kit	Miltenyi Biotec, Germany

CD45 (TILs) MicroBeads	Miltenyi Biotec, Germany
Dnase I Solution	Thermo Scientific, USA

2.1.5. Buffers and Solutions

MACS Buffer	500 mL	DPBS (1x)
	292.25 mg	EDTA (2m/M)
	2.5 g	BSA (0.5%)
FACS Buffer	pH	7.3
	1 L	1x DPBS
	2 mL	Natriumacid
	5 g	BSA
Cell Culture Medium	445 mL	RPMI Medium 1640 (1x)
	50 mL	FBS
	5 mL	P/S
Co-culture Medium	445 mL	DMEM:F12 (1:1)
	50 mL	FBS
	5 mL	P/S

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-CD107a	Mouse BALB/c IgG1, κ	PE-CF594	Human

2.2. Methods

2.2.1. Literature Review (Search Strategy and Study Selection)

Relevant studies were identified by searching PubMed database covering all papers published in recent five years up to January 2020 to make sure the data are updated. The following items ("Carcinoma, Hepatocellular"[Mesh]) AND ("CD8-Positive T-Lymphocytes"[Mesh]), ("Carcinoma, Hepatocellular"[Mesh]) AND ("Killer Cells, Natural"[Mesh]) were used respectively to select eligible publications assessing the interactions between HCC and CD8-Positive T-Lymphocytes or NK cells. Only studies with full text available in English were included. Eligible studies were then reviewed in detail to pick out those truly needed. Additionally, to reduce omissions, references of the selected publications were also scanned to identify additional related articles. Firm exclusion criteria were used in the review: 1) Published before 2014 ; 2) Not published in English; 3) Review, clinical trial, letter, case report, etc; 4) Non human subjects; 5) Not $CD8^+$ T cells or NK cells; 6) No interaction mentioned.

2.2.2. Cell Culture

HepG2 (HB-8065) was purchased from ATCC (Manassas, VA, USA). Mycoplamatest and authentication were routinely performed in our laboratory. Cells were cultured in DMEM:F12 medium supplemented with 10% FBS, 1% P/S and maintained in an incubator with 95% humidified atmosphere containing 5% CO_2 at 37°C. Medium was routinely changed and cells were routinely tested for mycoplasma contamination.

2.2.3. Patients and Healthy Donors (HDs)

In this study, three patients with primary HCC without HBV or HCV infection were recruited. All of the patients underwent curative liver resection from 2018 to 2019 at the Department of Surgery, Campus Grosshadern, Ludwig-Maximilians-University Munich (LMU) hospital. Six HDs were recruited to collect PBMCs and the informed

consent was obtained from volunteers. Institutional review board approvement was obtained (#EK 54-16, 53-16, 261-16 UE).

2.2.4. Preparation of PBMC

Peripheral blood from patients or HDs was mixed 1:1 with DPBS. A maximum 20 mL of mixture was carefully added onto 15 mL of Biocoll. This was centrifuged with 2000xg, 20 mins without brake. Then the interphase (mononuclear cells phase) was carefully collected without breaking the surface of Biocoll. The collected cells were washed with 20 mL of DPBS and centrifuged with 300xg, 10 mins with brake. Then the cells were washed again with same amount of DPBS at 200xg, 10 mins. 10 mL of DPBS was added for cell counting. Then cells were either applied into co-culture immediately or transferred into ibidi freezing medium with 1.5 x 10^6 cells per cryovial after centrifugation (500xg, 5 mins). The cryovial was put into the isopropanolbath in -80°C fridge overnight and then transferred into liquid nitrogen tank for long-term storage.

2.2.5. Preparation of TILs and Primary Tumor Cells

In resected tumor tissue, tumor infiltrating lymphocytes (TILs) were mixed with tumor cells, fibroblasts, red blood cells. To obtain TILs and primary tumor cells, tumor dissociated kit was applied to remove unwanted cells. Three kinds of enzymes (proprietary enzymes “H”, “R”, “A”) were included in the kit, which need aliquots before application. 200 µL of enzyme H, 20 µL of enzyme R and 25 µL of enzyme A mixed with 4.7 mL of RPMI 1640 medium were suitable for 0.2-1.0 g tumor tissue. Resected tumor tissue was weighed and matched enzymes were mixed well in advance. Tumor tissue was cut into 2-4 mm pieces after getting rid of fat tissue, fibrous and obviously necrotic parts. Then they were transferred into a gentle MACS C tube with enzyme mixture. The tube was applied on the gentle MACS octo dissociator with a heater. Program (Tough, 37C_h_TDK_3) was used to start cell dissociation. When the program was terminated, C tube was taken off from the

dissociator and the samples were resuspended. A 30 µm MACS SmartStrainer was used to filter the cell suspension. They were centrifuged with 300xg, 7 mins and the cell pellets were collected. Here we got the mixed single cell suspension including TILs and primary HCC cells. The storage steps were similar to PBMC.

2.2.6. Establishment of HCC Cell Line-PBMC Co-Culture

In experimental group, 1 x 10^6 HepG2 cells were seeded into a 6-well plate 6 hours before co-culture with 3 mL of co-culture medium per well to allow them attaching to the bottom. Freshly isolated PBMCs were then added into the well with the ratio of 1: 10 or 1: 25 (HepG2: PBMC). They were incubated in the co-culture incubator (37°C, 5% CO_2) for 24h. Same amount of HepG2 cells and PBMCs were cultured alone with the same conditions as two control groups. 24 hours later, cells in the supernatant were collected as PBMC. Cells attached to the bottom including HepG2 cells and part of PBMCs can be detached by Trypsin/EDTA and distinguished by FACS. FACS analysis was applied at 0 h and 24 h.

2.2.7. Establishment of Primary HCC Cells-TILs Co-Culture

Thawing of cells and removal of dead cells

As mentioned above, frozen single cell suspension mixed with primary HCC cells and TILs were stored. After thawing, dead cell removal kit was used to remove most of the dead cells. Frozen cells were thawed quickly in the water bath and washed with 5 mL of culture medium to get rid of freezing medium. Then cells were centrifuged with 500xg, 5 mins and washed with 5 mL of DMEM again. Cell counting with trypan blue staining was followed to estimate the approximate number of living and dead cells. To make sure living cells can go through the “LS” column as much as possible, Dnase I solution was used to reduce aggregation of cell suspension after thawing. Cells were resuspended in 0.1 mg/mL of Dnase I solution and incubated at room temperature (15°C-25°C) for 15 mins. Cells were washed with 20 mL of sterile FACS buffer. 100 µL

of microbeads per 10^7 total cells were then added and the mixture was incubated at room temperature for 15 mins. The LS column was applied on the MACS separator and rinsed with 3 mL of 1 x binding buffer. 5 mL of same buffer was added into the cells. They were pipetted into the column and the living cells will pass through. The column was rinsed with 3 mL of buffer 4 times and the effluent was collected as living cells.

MACS Isolation

After the isolation of living cells, MACS isolation was then applied to separate $CD45^+$ cells (TILs) and $CD45^-$ cells (primary HCC cells). The isolated living cells were counted with trypan blue staining and the needed amount of MACS buffer and CD45 microbeads were calculated. After centrifugation (300xg, 5 mins), 80 µL of MACS buffer and 20 µL of CD45 microbeads per 10^7 cells was applied for incubation (15 mins, dark, 4℃). Certain amount of MACS buffer was added into the cell suspension to make the final volume 500 µL with up to 5 x 10^7 total cells. Then the LS column was put on the separator and rinsed with 3 mL of MACS buffer. The cell suspension was pipetted into the column and the column was washed with 1 mL of MACS buffer twice. Then the column was transferred on a 15 mL falcon. The $CD45^+$ cells were flushed out with 3 mL of MACS buffer by supporting plunger. The effluent was collected as $CD45^-$ cells.

Cell Co-Culture

The co-culture step was the same as HepG2-PBMC co-culture. In the experimental group, $CD45^-$ cells (primary HCC cells) were seeded into 6-well plate 6 hours before co-culture with 3 mL of co-culture medium per well allowing them to attach to the wall. $CD45^+$ cells (TILs) were then added into the well with the ratio of 1:10 (primary HCC cells: TILs). They were incubated in the co-culture incubator (37°C, 5% CO_2) for 24h. Same amount of TILs and primary HCC cells were cultured alone with the same condition as two control groups. 24 hours later, cells in the supernatant were collected

as TILs including small amount of primary HCC cells, because not all primary HCC cells can attach to the bottom. Cells attached to the bottom including most of primary HCC cells and part of TILs can be detached by Trypsin/EDTA. Similarly, TILs and primary HCC cells can be distinguished by FACS. FACS analysis was applied at 0h and 24h. The flow chart of co-culture is shown in Figure 6.

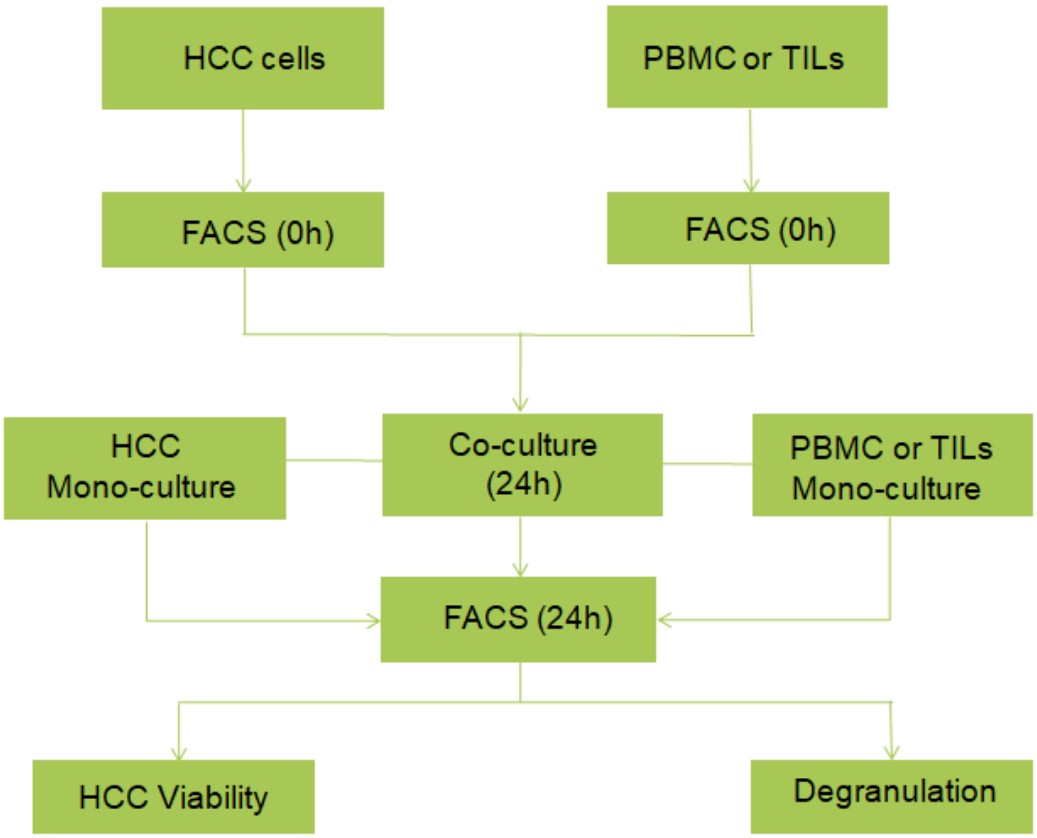

Figure 6: The flow chart of co-culture

2.2.8. Definition of Monitored Cell Subsets

Different immune cell subsets showed different phenotype and can be distinguished by different cluster of differentiation (CD) molecules. The definitions of involved immune cells are described below (Table 1).

Table 1. Definition of measured cell subsets

Cell Type	Marker
Lymphocytes	$CD45^+$
T cells, % of Lymphocytes	$CD3^+$, % of $CD45^+$
CTL, % of T cells	$CD8^+$, % of $CD45^+/CD3^+$
NK cells, % of Lymphocytes	$CD3^-/CD16^+/CD56^+$, % of $CD45^+$

2.2.9. Design of Cytotoxicity and Degranulation Panel

Flow cytometry (FCM) was used to measure the viability of HCC cells and degranulation level of major immune cells. Fixable Viability Stain 510 (FVS 510) was used to distinguish viable from non-viable mammalian cells. Intracellular amines and cell-surface could covalently bind to FVS 510, which means necrotic cells or dead cells with broken membrane could bind with more dye and show much stronger fluorescence. Lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) was believed to be a representative marker of degranulation for $CD8^+$ T lymphocytes and NK cells[117, 118]. Degranulation was related to cytokine secretion and cell-mediated lysis of target cell such as tumor cells, which means with the analysis of CD107a, we can measure the cytotoxic activity of $CD8^+$ T lymphocytes and NK cells. The panel designed includes unstained tube which served as blank control, fluorescence minus one (FMO) control tubes and experimental tubes. The added amount of each antibody was decided by titration to get optimal result.

The panel from table 2 was used for HepG2 at 0h and mono-cultured HepG2 at 24h, and the panel from table 3 was used for co-cultured HepG2, mono-/co-cultured primary HCC cells at 24h and primary HCC cells at 0h. Because in co-cultured HepG2 and all primary HCC cells groups, there was a small amount of PBMC or TILs ($CD45^+$)

left. We had to distinguish those $CD45^+$ immune cells from $CD45^-$ HCC cells with CD45 antibodies in FACS analysis. The panel from table 4 was used for all $CD45^+$ groups including PBMC at 0h, mono-/co-cultured PBMC at 24h, TILs at 0h, mono-/co-cultured TILs at 24h.

Table 2. Panel for mono-cultured HepG2

Tube	Antibody
	BV 510
Unstained	--
Sample	FVS 510

Table 3. Panel for co-cultured HepG2, mono-/co-cultured primary HCC cells

Tube	Antibody	
	BV 510	BV650
Unstained	--	--
FMO BV650	FVS 510	--
Sample	FVS 510	CD45

Table 4. Panel for cells in $CD45^+$ groups

Tube	Antibody					
	PerCP	APC-H7	APC-R	FITC	BV650	PE-CF
Unstained	--	--	--	--	--	--
FMO 1	CD3	CD8	CD56	CD16	CD45	--
FMO 2	CD3	--	CD56	--	CD45	CD107a
FMO 3	--	CD8	--	CD16	CD45	CD107a
Sample	CD3	CD8	CD56	CD16	CD45	CD107a

2.2.10. Staining Methods

2.2.10.1. FVS 510 Staining

Before staining, 10 mL of DPBS was used to wash cells. The cells were resuspended at the density of 1 x 10^6 cells/mL. Certain amount of cells were taken as unstained cells and the others were used for FVS 510 staining. FVS 510 antibody was added with the ratio of 500:1 (1 mL of cells with 2 µL of FVS 510 antibody). The mixture incubated for 15 mins at room temperature or 30-60 mins at 2-8°C protected from light. Then cells were washed twice with 2 mL of FACS buffer. The supernatant was decanted and cells were resuspended in appropriate amount of FACS buffer. Then the cells were either used for FACS analysis directly or used for other antibodies staining. FACS staining of other antibodies was described below.

2.2.10.2. FACS Staining of Other Antibodies

Because all the other antibodies used in this study were extracellular antibodies, the following staining steps were applied to extracellular antibodies staining. 200 µL of cell suspension was added into each FACS tube, then antibodies were added according to the panels showed previously. All the tubes were incubated for 15-30 mins after vortex at room temperature protected from light. Then they were centrifuged at 500xg for 5 mins and the supernatant was discarded gently. 300-500 µL of FACS buffer was added finally. Then all tubes were ready for FACS measurement.

2.2.11. Statistical Analysis

For statistical analysis, Graphpad Prism 7.0 software was applied. Data was collated in an Excel database (Microsoft Excel for Mac; version 15.19.1, Microsoft Corporation, Redmond, USA). Mean ± SD (standard deviation) was calculated for presenting the viability of HCC cells and degranulation level of immune effector cells. One-way analysis of variance (One-way ANOVA) method was used for the comparison in three repeated experiments of each co-culture procedure. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Literature Review

In this part, the interactions between HCC cells and $CD8^+$ T lymphocytes as well as NK cells will be elaborated according to the publications which were included in our systematic literature review.

3.1.1. Study Selection and Characteristics

HCC and $CD8^+$ T lymphocytes

As shown in Figure 7, a total of 435 publications related to HCC and $CD8^+$ T lymphocytes were identified initially following the searching strategy. According to the strict exclusion criteria, 357 publications were excluded, and 78 articles with full texts were scrutinized. While 27 of them were not related to human HCC, 22 articles didn't mention $CD8^+$ T lymphocytes and 20 articles didn't show the interactions between HCC and $CD8^+$ T lymphocytes. Finally 9 articles were included in the review[119-127].

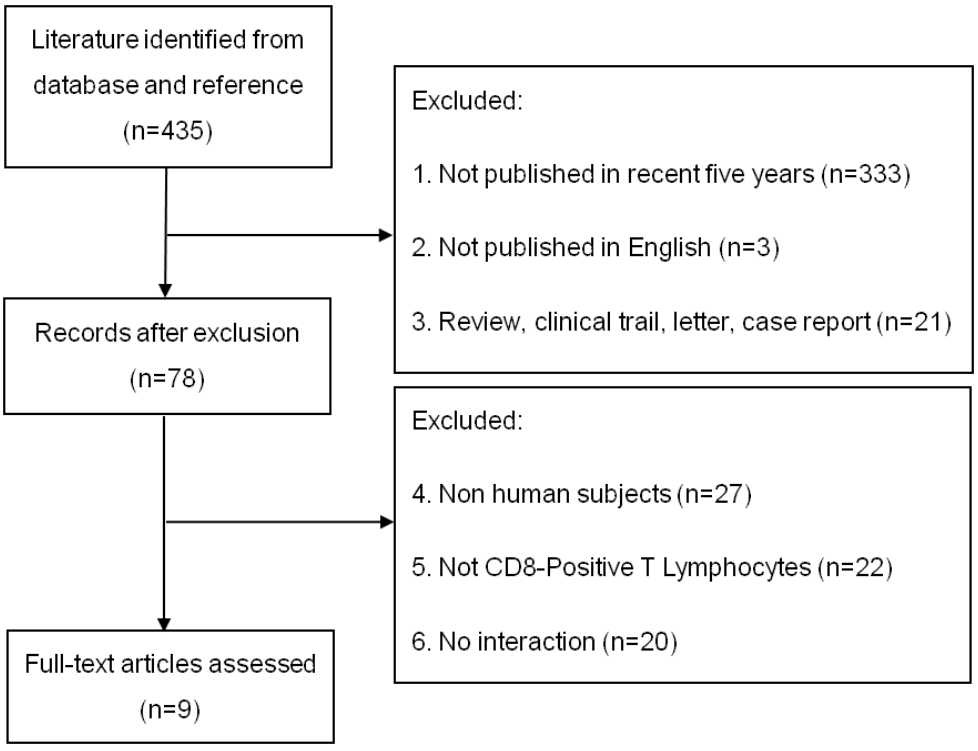

Figure 7: Flow diagram of the study selection with key words ("Carcinoma, Hepatocellular"[Mesh]) AND "CD8-Positive T-Lymphocytes"[Mesh]

Among them, 7 (78%) studies were conducted in East Asia, especially in China (n=6, 67%)[120-126] and 2 (22%) in Europe[119, 127] (Figure 8A). As for the cancer cell source, tumor tissue was used in half of the studies and others were HCC cell lines (Figure 8B). But none of them used cancer cells from tumor tissue in co-culture. In most of the studies, immune cells were obtained from PBMC which was isolated from fresh blood (n=6, 67%) and others were obtained from TILs (n=2, 33%) (Figure 8C). To investigate the interactions between HCC cells and $CD8^+$ T lymphocytes, flow cytometry was used in most of the studies (n=8, 80%), cell cytotoxicity assay and ^{51}Cr-release assay were applied in four studies, which can be used directly to analyze the cytotoxic activity of CTLs towards HCC cells (Figure 8D).

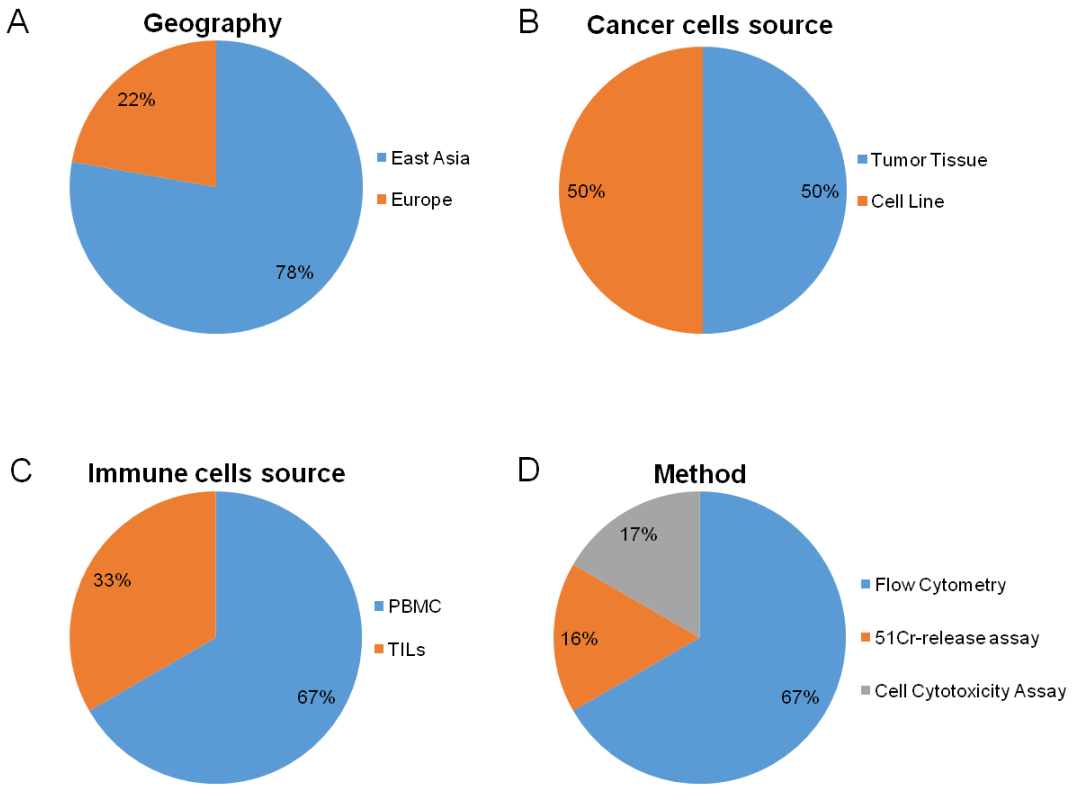

Figure 8: Characteristics of studies about HCC and $CD8^+$ T lymphocytes. (A) Geographic distribution of publications; (B) Cancer cell source; (C) Immune cell source; (D) Detection methods.

HCC and NK cells

375 publications related to HCC and NK cells were initially retrieved following the searching strategy mentioned above (Figure 9). 322 publications were excluded according to the exclusion criteria. Then the left 53 articles were scrutinized, 31 unrelated studies were rejected and 22 studies were finally included in the review. Our search and selection processes were performed strictly adhere to the exclusion criteria.

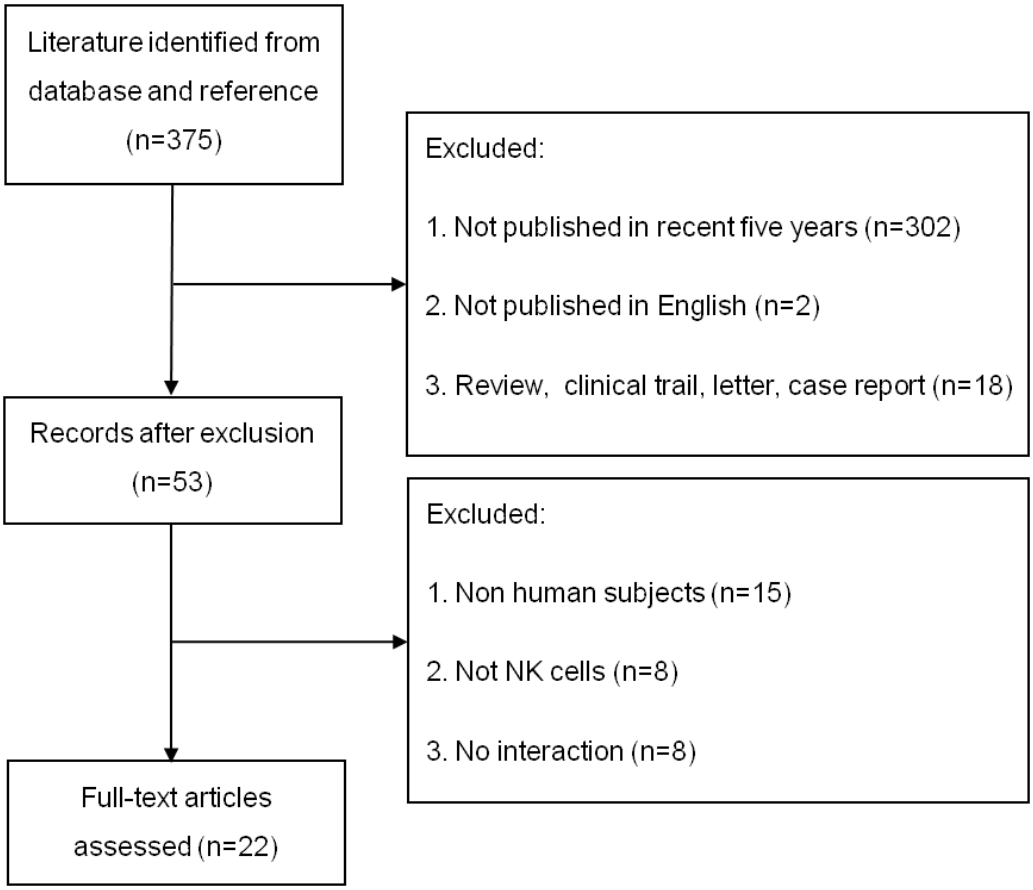

Figure 9: Flow diagram of study selection with key words ("Carcinoma, Hepatocellular"[Mesh]) AND "Killer Cells, Natural"[Mesh]

As shown in Figure 10A, most of the studies were carried out in East Asia (n=17, 77%), including 15 in China[128-142], one in Korea[143] and one in Singapore[144]. Only two were conducted in Europe[145, 146], two in Africa[147, 148] and one in North America[149]. As

for the source of cancer cells, cell lines were used in 21 (84%) studies and tumor tissue was used in four studies (16%) (Figure 10B). None of the studies investigated cancer cells from tumor tissue in co-culture. More than half of the studies selected PBMC which obtained from fresh blood as immune cells source (n=15, 54%). The second largest source of immune cells was cell line (n=10, 36%), and others were from TILs, hepatic sinusoid or liver perfusates (Figure 10C). Similar to T cell part, flow cytometry was mostly used to analyze the interactions between HCC cells and NK cells (n=16, 40%). Notably, methods applied for detecting the cytotoxicity of NK cells were also performed in most of the studies, such as LDH Assay (n=10, 25%), Cell Cytotoxicity Assay (n=9, 23%), MTT Assay (n=4, 10%) and CFSE Proliferation Assay (n=1, 2%) (Figure 10D).

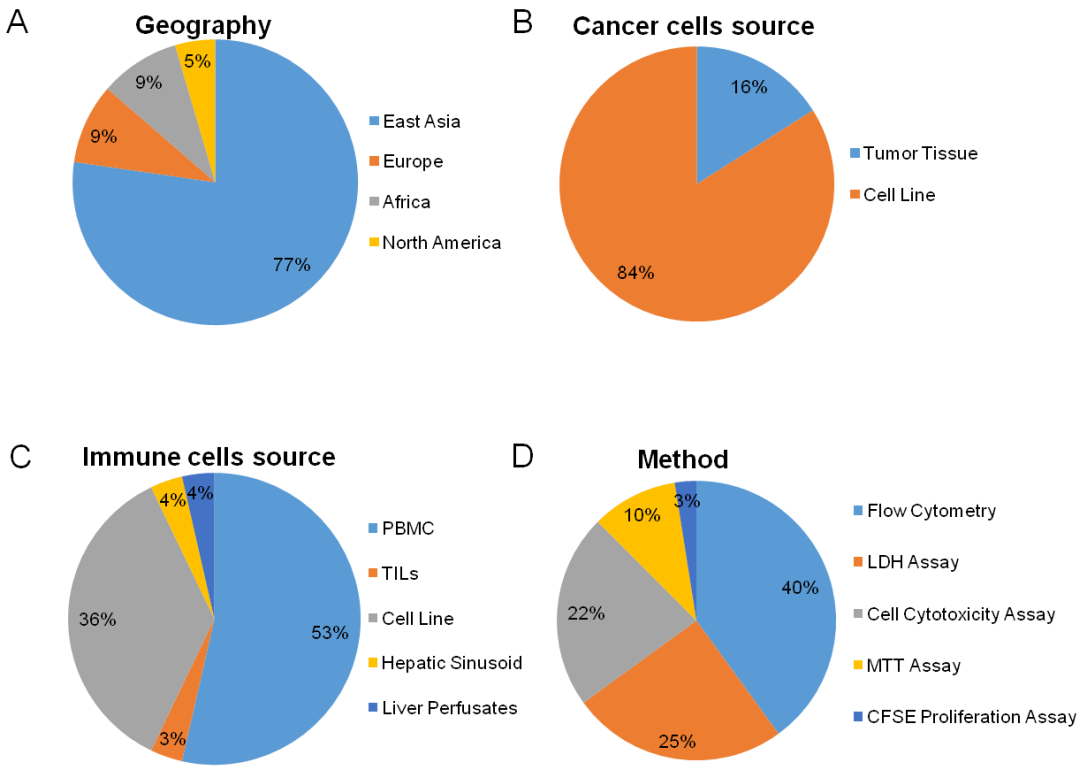

Figure 10: Characteristics of studies about HCC and NK cells. (A) Geographic distribution of publications; (B) Cancer cell source; (C) Immune cell source; (D) Detection methods.

3.1.2. Cytotoxicity of CD8+ T Lymphocytes against HCC Cells

CD8+ T lymphocytes work importantly in the pathogenesis of HCC for their recognition and cytotoxicity of infected/tumoural cells, especially HCC with HBV or HCV infection[70, 150]. The mechanisms of CD8+ T lymphocytes playing cytotoxic activity have been investigated extensively and the cytotoxicity can be regulated by certain factors. In this review, six studies were found to report anti-tumor effect and direct cytotoxicity test was performed in four studies (Table 5). The study of Otano et al.[119] pointed out that knockdown of PD-1 improved the cytotoxic effect of CD8+ T lymphocytes, but drove the senescence of T cell. Chen et al.[124] proved that the tumor inhibiting effectiveness of CD8+/NKG2D+ T cells was enhanced towards MV-Edm-infected HCC cells. Similarly, the work of Zhou et al.[126] demonstrated that HCC cells transducted by rAAV/AFPp-IFN-γ resulted in an increased CTL anti-tumor response. In two of the studies, direct cytotoxicity of CD8+ T lymphocytes was not tested but indicated by degranulation. Jin et al.[122] reported that CXCR5+/CD8+ T cells released higher granzyme B suggesting more potent cytotoxicity. And Brunner et al.[127] revealed IL-33+/CD8+ T cells have higher degranulation level indicating better cytotoxic effect. Co-culture was applied in four studies, but none of them used primary HCC cells and autologous CD8+ T lymphocytes to investigate the anti-tumor activity of CD8+ T lymphocytes on HCC cells.

3.1.3. CD8+ T Lymphocytes Dysfunction in HCC

Three studies in this review demonstrated that the anti-tumor effect of CD8+ T lymphocytes was impaired in HCC. It has been shown that the HBV-related HCC microenvironment is more immunosuppressive and exhausted because of more PD-1 expressing[120]. Huang et al.[123] also showed that tumor PD-L1 expression inhibited the anti-tumor activity of CD8+ T cells through binding to PD-1. On the other side, the study of Liu et al.[121] indicated that the proliferation of CD8+ TILs was promoted as well as the secretion of IFN-γ and TNF-a when Tim-3 and PD-1 was blocked. This

verified from the opposite side that the dysfunction of $CD8^+$ T lymphocytes in HCC was partially caused by tumor PD-L1 expression. It was also reported that TGF-β expressed by HCC suppressed the cytotoxicity of $CD8^+$ T lymphocytes by reducing the secretion of IFN-γ[125]. TGF-β is a key molecule associated with tumor initiation and progression which could regulate the cancer-associated immune cells such as $CD8^+$ T lymphocytes[151]. Accumulating evidence proved that TGF-β is involved in $CD8^+$ T lymphocytes dysfunction in HCC[152].

3.1.4. Cytotoxicity of NK cells towards HCC cells

It has been shown that NK cells can recognize malignant cells with general specificity and show extensive anti-tumor effect. However, clinical trial results showed that NK cell treatment has different effects on different tumors. As shown in table 6, 20 studies presented that NK cells could inhibit HCC cells and the cytotoxic activity of NK cells could be enhanced or down-regulated by certain cytokines or drugs. Su et al.[128] reported that MiR-506 can promote the anti-tumor effect of NK cell. Zhuang et al.[145] showed that a cytokine cocktail consists of IL-12/15/18 + IL-2 can activate human NK cells and promote their anti-tumor effect against HCC cells. The research result of Huang YX et al. proved that sunitinib sensitized HepG2 cells to NK cells mediated cytotoxicity[131]. The function of NK cells mainly rely on the interactions between various functional receptors expressed by NK cells and corresponding ligands. The well-known immune checkpoint PD-1 was proven to be highly expressed on the surface of NK cells from patients with liver cancers, and the cytotoxicity of NK cells can be increased by blockade of PD-1[153]. NKG2D, NKp44, NKp30, NKp80, and NKp46 were the well-known activating receptors of NK cells[154]. Synergy of these activating receptors can induce NK cells to release more anti-tumor cytokines or directly lyse tumor cells.

In most of these studies, HCC cells were co-cultured with NK cells to research the cytotoxicity of NK cells against HCC, but none of them applied primary HCC cells and

autologous NK cells into co-culture.

3.1.5. NK Cell Dysfunction in HCC

NK cells account for 30% to 50% of the intrahepatic lymphocytes in human liver, and show major impact on the surveillance of HCC[155]. However, in the microenvironment of HCC, the anti-tumor activity of NK cells is impaired in cytotoxicity and cytokine secretion. Easom et al.[146] found that HCC impacted NK cells by down-regulating NKG2D and IL-15 could restore HCC-induced NK cell dysfunction. The study of Vujanovic et al.[149] declared that tumor-derived AFP directly drove the NK cells activation, but negatively impacted NK cell viability. Shi et al.[136] investigated the impact of androgen receptor (AR) on NK cells in liver cancer and proved that AR decreased NK cells cytotoxicity by suppressing IL-12A. Interestingly, there is one study reported that $CD11b^{-}/CD27^{-}$ (DN) NK subsets infiltrated in liver displayed a poor cytotoxic capacity against HCC[133]. The existing of DN NK subsets proved to relate to NK cells dysfunction and promote tumor progression. As mentioned above, activating receptors on NK cells play important roles in regulating NK cells function. It is believed that HCC cells could express a large amount of ligand binding to those activating receptors resulted in NK cells dysfunction[156, 157].

Based on the literature review we concluded that $CD8^{+}$ T cells and NK cells have the capacity of inhibiting or killing tumor cells in HCC. While in turn, tumor cells in some way could also result in the dysfunction of those effector cells through different mechanisms. Most of research selected tumor cell line and PBMC as cell resource to establish the co-culture systems which have their own limitations. In this study I tried to establish a direct contact co-culture (DCC) system using primary HCC cells and tumor infiltrating immune cells which may reflect the interactions between them in a physiologically and clinically relevant way.

Table 5. Summary of included studies (HCC and $CD8^+$ T lymphocytes)

Abbreviations: PBMC: Peripheral blood mononuclear cells; Trm: resident memory T cells; Tim-3: T cell Ig- and mucin-domain-containing molecule-3; PD-1: programmed cell death 1; OS: Overall survival; RFS: Recurrence-free survival; MV-Edm: measles virus vaccine strain Edmonston; TGF-β: transforming growth factor β; IFN-γ: interferon-γ; rAAV: recombinant adeno-associated virus; AFPp: α-fetoprotein promoter.

Reference	Study Object		Cells				Interaction		Remarks
	Object Source	Region	Cell Type		Cell Source		Analysis Method	Promotion → / Inhibition ⊣	
			Cancer cells (Marker)	Immune cells (Marker)	Cancer cells	Immune cells			
Otano, et al. 2018[119]	HCC Patients	UK	HCC cells	T cell ($CD3^+$/	Tumor Tissue,	PBMC	Flow Cytometry,	$CD8^+$ T cell	Knockdown of PD-1

	(N=38)			CD8$^+$/ CD4$^-$)	Cell line		Cytotoxicity Assay	⊣ HCC cells	
Lim, et al. 2019[120]	HCC Patients (N=23)	Singapore	HCC cells	Trm (CD8$^+$/ CD103$^+$/ CD45RO$^+$)	Tumor Tissue	TILs	Flow Cytometry	Trm ⊣ HCC cells	
Liu, et al. 2018[121]	HCC Patients (N=90)	China	HCC cells	T cell (CD3$^+$/ CD8$^+$)	Tumor Tissue	TILs	Flow Cytometry	HCC cells ⊣ CD8$^+$ T cells	Blockade of Tim-3 and PD-1
Jin, et al. 2017[122]	HCC Patients (N=7)	China	HCC cells	T cell (CD3$^+$/ CD8$^+$/	Tumor Tissue	PBMC	Flow Cytometry, ^{51}Cr-release	CXCR5$^+$/ CD8$^+$ T cells	Granzyme B

				CXCR5^{+})			Assay	⊣ HCC cells	
Huang, et al. 2017[123]	cell line	China	HCC cells	CTL (CD3^{+}/ CD8^{+})	Cell line： Bel7402 HepG2	PBMC	Flow Cytometry	HCC cells ⊣ CTLs	PD-L1
Chen, et al. 2017[124]	cell line	China	HCC cells	T cell (CD8^{+}/ NKG2D^{+})	Cell line: LM3, 97H	PBMC	Flow Cytometry, Cytotoxicity Assay	CD8^{+}/ NKG2D^{+} T cells ⊣ HCC cells	MV-Edm-infected HCC cells
Huang, et al. 2017[125]	cell line	China	HCC cells	CTL (CD3^{+}/	Cell line: HepG2,	PBMC	Flow Cytometry	HCC cells ⊣	TGF-β

				CD8$^+$)	Huh7			CTLs	
Zhou, et al. 2016[126]	cell line	China	HCC cells	CTL (CD3$^+$/ CD8$^+$)	Cell line: Hep3B	PBMC	^{51}Cr-release Assay	CTL ⊣ HCC cells	rAAV/AFPp-IFN-γ transducted HCC cells
Brunner,et al. 2015[127]	HCC Patients (N=119)	Germany	HCC cells	T cells (CD8$^+$/ IL-33$^+$)	Tumor Tissue	TILs	Flow Cytometry	CD8$^+$/ IL-33$^+$ T cells ⊣ HCC cells	IL-33$^+$/ CD8$^+$ T cells with higher degranulation level

Table 6. Summary of included studies (HCC and NK cells)

Abbreviation: NK cells: natural killer cells; DCs: dendritic cells; PBMC: peripheral blood mononuclear cell; STAT3: signal transducer and activator of transcription 3; IL: interleukin; INF-α: interferon-α; AFP: alpha fetoprotein; TGF-βR II: Transforming growth factor-β receptor II; IGF-1: insulin-like growth factor-1; CARs: chimeric antigen receptors; GPC3: Glypian-3; PESV: polypeptides extracted from scorpion venom; LDH assay: lactate dehydrogenase assay; AR: androgen receptor; NKG2D: NK group 2, member D; MICA: MHC class I-related chain A;TLR: Toll-like receptor; GEP: Granulin–epithelin precursor; sGEP: serum Granulin–epithelin precursor; sMICA: serum MHC class I-related chain A

Reference	Study Object		Cells		Interaction	Remarks
	Object Source	Region	Cell Type	Cell Source	Analysis Method	Promotion → / Inhibition ⊣

			Cancer cells (Marker)	Immune cells (Marker)	Cancer cells	Immune cells			
Shin, et al. 2019[143]	Healthy donor, (N=69) Cell line	Korea	HCC cells	NK cells ($CD56^+$)	Cell lines: Huh7, SNU398	Hepatic Sinusoid	Flow Cytometry, LDH Assay	NK cells ⊣ HCC cells	
Su, et al. 2019[128]	HCC patients, (N=15) Cell line	China	HCC cells	NK cells	Cell lines: SMMC7721, HepG2 cells	PBMC Cell line: NK-92, 293T cells	Flow Cytometry, CFSE/7AAD Cytotoxicity Assay, LDH Assay	NK cells ⊣ HCC cells	MiR-506; targeting STAT3.

Zhuang, et al. 2019[145]	HCC patients, (N=20) Cell line	UK	HCC cells	NK cells ($CD56^+$)	Cell lines: SNU387, SNU398, SNU423, SNU475, Huh7, HepG2	PBMC	CFSE Proliferation Assay, Flow Cytometry,	NK cells ⊣ HCC cells	IL-12/15/18 + IL-2 cytokine cocktail
Easom, et al.2018[146]	HCC patients, (N=10) Cell line	UK	HCC cells	NK cells ($CD56^+$)	Tumor Tissue, Cell lines: PLC/PRF/5	PBMC, TILs, Liver Perfusates	Flow Cytometry	HCC cells ⊣ NK cells	NKG2D; IL-15
Yu, et al. 2018[129]	Healthy donor, Cell line	China	HCC cell (GPC3)	NK cells	Cell lines: HepG2, Hep3B,	PBMC, Cell lines: NK-92,	Flow Cytometry, LDH Assay,	NK cells ⊣ HCC cells	CAR (9.28.z); $GPC3^+$ HCC cells.

					PLC/PRF/5, SK-HEP-1, Huh-7, SMMC-7721	NK-92/9.28.z			
Wan, et al. 2017[130]	Cell line	China	HCC cells	NK cells	Cell lines: HepG2, Huh7	Cell lines: NK92	MTT Assay, LDH Assay	NK cells ⊣ HCC cells	TT-1 (a mutant of melittin); INF-α
Vujanovic ,et al. 2017[149]	HCC patients, (N=10) Cell line	USA	HCC cells	NK cells ($CD56^+$/ $CD16^+$)	Cell lines: HepG2	PBMC	Flow Cytometry, MTT Assay	NK cells ⊣ HCC cells	Tumor-derived AFP
Huang, et al. 2017[131]	Cell line, Healthy	China	HCC cells	NK cells	Cell lines: HepG2	PBMC	Cytotoxic Sensitivity	NK cells ⊣	Sunitinib

	donor						Assay	HCC cells	
Wang, et al. 2017[132]	Cell line	China	HCC cells	NK cells ($CD56^+$)	Cell lines: SMMC7721, SK-HEP-1	Cell lines: NK-92	Flow Cytometry, LDH Assay	NK cells ⊣ HCC cells	TGF-βR II; NKG2D
Youness, et al. 2016[147]	HCC patients, (N=135) Cell line	Egypt	HCC cells	NK cells ($CD3^-$/ $CD16^+$/ $CD56^+$)	Cell lines: Huh7	PBMC	Flow Cytometry, LDH Assay, MTT assay	NK cells ⊣ HCC cells	miR-486-5p; IGF-1
Zhang, et al. 2017[133]	HCC patients, (N=30)	China	HCC cells	NK cells ($CD27^-$/ $CD11b^-$)	Timor tissue	TILs, PBMC	Flow Cytometry	$CD11b^-$/ $CD27^-$NK subsets → HCC cells	

Abdelrahman, et al. 2016[148]	HCC patients, (N=72) Cell line	Egypt	HCC cells	NK cells	Cell lines: Huh7	PBMC	LDH Assay	NK cells ⊣ HCC cells	miR-182
Kamiya, et al. 2016[144]	Healthy donor, (N=23) Cell line	Singapore	HCC cells	NK cells	Cell lines: Hep3B, SNU-398, HepG2, SNU-449, PLC/PRF/5	PBMC	Cytotoxicity Assays	NK cells ⊣ HCC cells	Expanded activated NK cells
Chen, et al. 2016[134]	Healthy donor, Cell line	China	HCC cells	NK cells	Cell lines: HepG2	PBMC	Flow Cytometry, LDH Cytotoxicity	NK cells ⊣ HCC cells	PESV; MICA.

							Assay		
Xu, et al. 2016[135]	HCC patients, (N=62) Cell line	China	HCC cells	NK cells	Cell lines: HepG2	PBMC Cell lines: NK-92	Flow Cytometry, CFSE/7AAD Cytotoxicity Assay, LDH Assay	NK cells ⊣ HCC cells	miR-146a; STAT1 signaling
Shi, et al. 2016[136]	Cell line	China	HCC cells	NK cells	Cell lines: HepG2, SK-Hep1, SKAR3	Cell lines: NK-92MI	MTT Cell Viability Assay, LDH Assay	NK cells ⊣ HCC cells	AR; IL-12A.
Shi, et al. 2016[137]	Cell line	China	HCC cells	NK cells	Cell lines: SK-Hep1,	Cell lines: NK-92MI	LDH Assay	NK cells ⊣ HCC cells	Cisplatin

					SNU423				
Wang, et al. 2016[138]	Cell line	China	HCC cells	NK cells	Cell lines: Huh-7,BEL-7402	Cell lines: NK-92	Flow Cytometry, Cytotoxicity Assay	NK cells ⊣ HCC cells	rG7S-MICA; NKG2D
Bian, et al. 2016[139]	Cell line	China	HCC cells	NK cells	Cell lines: HepG2, HepG2.2.15	Cell lines: NKL	Flow Cytometry, Cytotoxicity Assay	NK cells ⊣ HCC cells	miR-152
Zhou, et al. 2015[140]	Healthy donor, Cell line	China	HCC cells	NK cells	Cell lines: HepG2, H7402, PLC/PRF/5	PBMC, Cell lines: NKL	Flow Cytometry, Cytotoxicity Assays	NK cells ⊣ HCC cells	TLR7/8 agonists

Cheung, et al. 2014[141]	HCC patients, (N=80) Healthy donor, (N =28) Cell line	China(HK)	HCC cells	NK cells	Tumor tissue, Cell lines: Hep3B, HepG2	PBMC	Flow Cytometry, Cytotoxicity Assay	NK cells ⊣ HCC cells	GEP blockade; MICA.
Gong, et al. 2014[142]	HCC patients, (N=96) Healthy donor, Cell line	China	HCC cells	NK cells	Cell lines: SMMC7721, HepG2	PBMC	Flow Cytometry, Cytotoxicity Assay	NK cells ⊣ HCC cells	UPR; CD155

3.2. Establishment of Gating Strategy for Co-Cultivation

To analyze the viability of HCC cells and the degranulation of major immune effector cells, I established the gating strategy for co-cultivation. FMOs and unstained tubes were used as control in the gating process. Detailed gating strategy is described below.

3.2.1. Gating of Mono-Cultured HepG2

As shown in Figure 11A, the first step is to select the major group on FSC/SSC scatter plot to minimum the influence of cell debris and some scattered cells on results. The second step is to gate dead cells as shown in Figure 11B. The gate of dead cells is settled base on the combination of unstained cells and peak of the wave. After calculating the proportion of dead cells, the viability of HCC cells can be calculated.

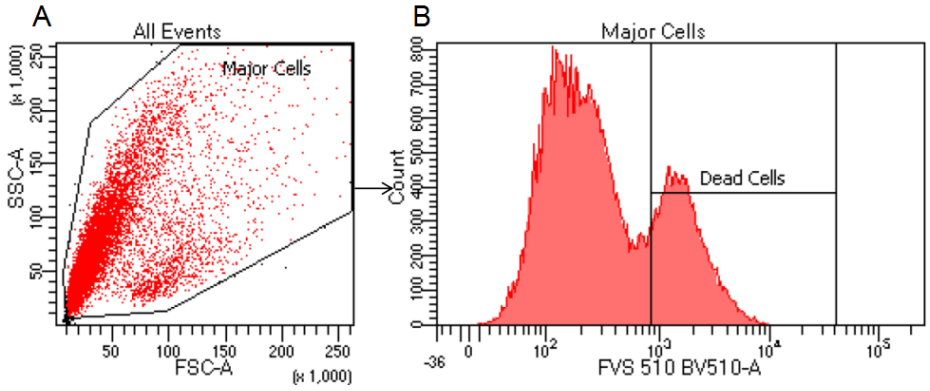

Figure 11: Gating strategy for the viability of mono-cultured HepG2. (A) Major Cells from mono-cultured HepG2; (B) According to unstained cells and peak of the wave, dead cells were selected.

3.2.2. Gating of Co-Cultured HepG2, Mono-/Co-Cultured Primary HCC Cells

As mentioned previously, there are small amount of $CD45^+$ cells like PBMC and TILs were mixed in HCC cells. To get the exact viability of HCC cells, $CD45^+$ cells must be

excluded. First, major cells are selected on FSC/SSC scatter plot (Figure 12A). Then $CD45^+$ and $CD45^-$ cells are gated respectively according to unstained cells, FMO and cell clusters (Figure 12B). Last, dead cells from $CD45^-$ cells are gated base on the combination of unstained cells and peak of the wave (Figure 12C). The viability of HCC cells is easily calculated with the proportion of dead cells.

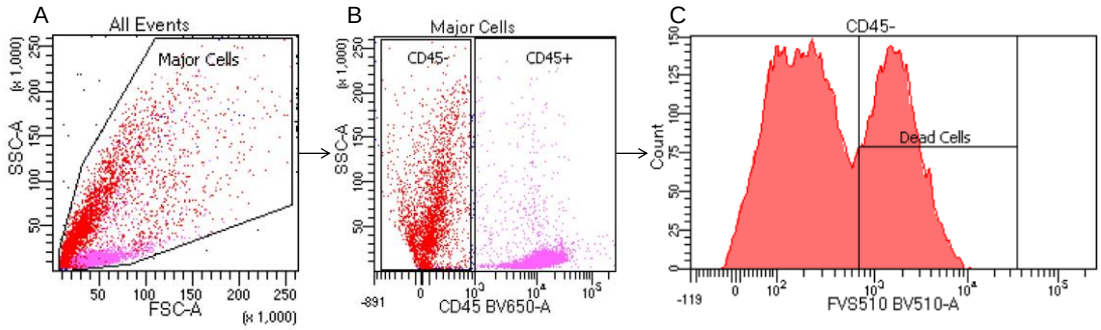

Figure 12: Gating strategy for the viability of co-cultured HepG2, mono-/co-cultured primary HCC Cells. (A) Major cells selected from all recorded cells; (B) $CD45^+$ and $CD45^-$ cells gating; (C) Dead cells in $CD45^-$ cells.

3.2.3. Gating of cells in $CD45^+$ groups including mono-/co-cultured PBMC, mono-/co-cultured TILs

Three FMOs are used in this gating. FMO1 is settled for CD107a, FMO2 is settled for CD8 and CD16, and FMO3 is settled for CD3 and CD56. The first step is to select lymphocytes according to the cell cluster (Figure 13B). In the population of lymphocytes, $CD3^+$ T lymphocytes form distinct cell cluster on CD3/SSC plot. So the second step is to select $CD3^+$ T lymphocytes by cluster and double check it with FMO 3 (Figure 13C). As the major part of $CD3^+$ lymphocytes, $CD8^+$ T lymphocytes also form distinct cell cluster on CD8/SSC plot. Then the third step is to select $CD8^+$ T lymphocytes by cluster and FMO 2 (Figure 13D). As shown in Figure 13E, the fourth step is to select $CD107a^+$ T lymphocytes from $CD8^+$ T lymphocytes controlled by FMO1. $CD107a^+/CD8^+$ T lymphocytes represent degranulated $CD8^+$ T lymphocytes. NK cells, which express $CD3^-/CD16^+/CD56^+$ marker, are also gated from lymphocytes

as shown in Figure 13F, 13G. The degranulation level of NK cells can be gated according to FMO1 (Figure 13H).

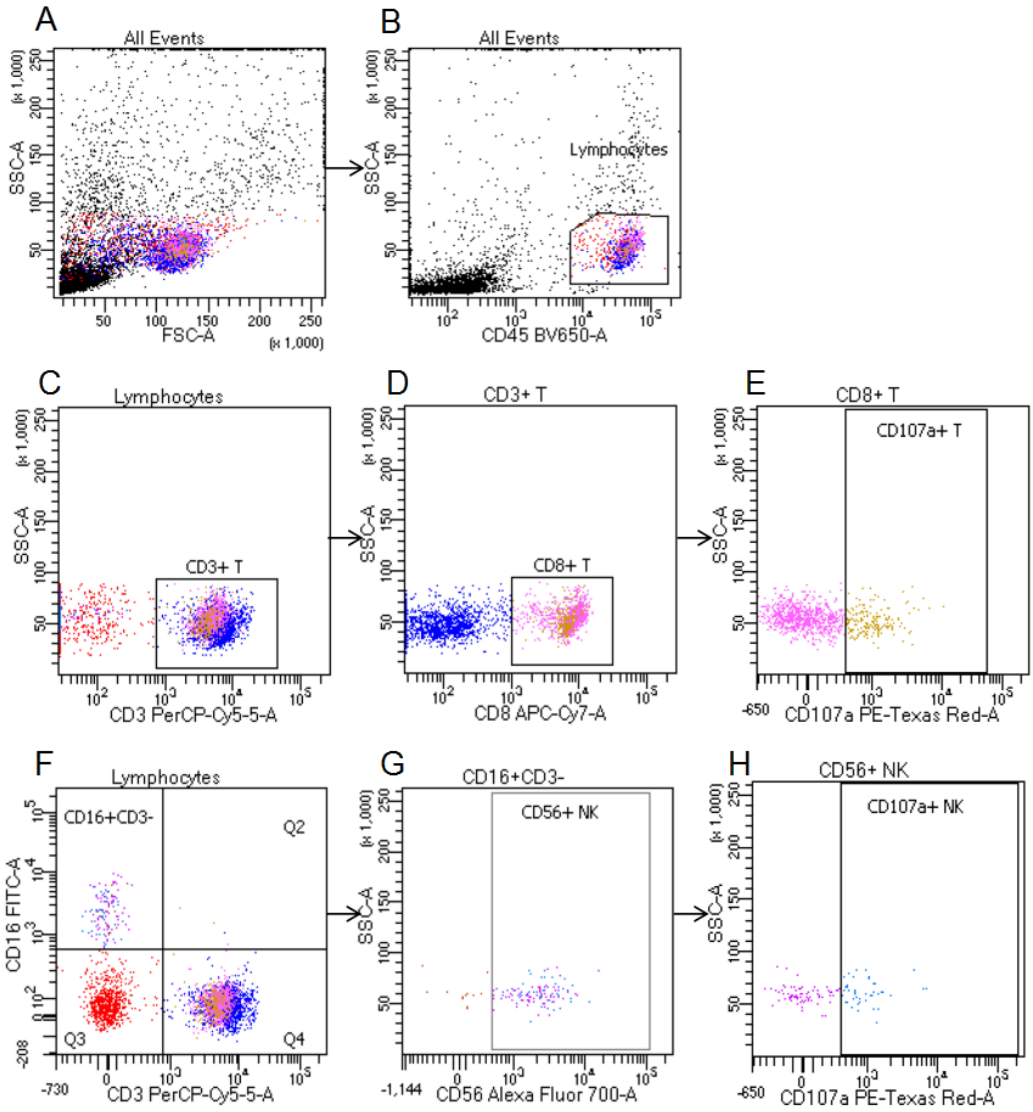

Figure 13: Gating strategy for cells in CD45⁺ groups. (A) All recorded cells in experimental tube; (B) Cell cluster of lymphocytes; (C) Cell cluster of $CD3^+$ T lymphocytes; (D) $CD8^+$ T lymphocytes; (E) $CD107a^+/CD8^+$ T lymphocytes; (F) $CD3^-/CD16^+$ cells; (G) $CD3^-/CD16^+/CD56^+$ NK cells; (H) $CD107a^+/CD56^+$ NK cells.

3.3. Co-Culture of HepG2 with PBMC from HDs (1:10)

As mentioned previously, we co-cultured HepG2 with PBMC from healthy donors with

the ratio of 1:10 as a start to build the co-culture system. Then we investigated the interactions between HCC cells and major immune effector cells by measuring the viability of HepG2 and degranulation of major immune effector cells at 0h and 24h after co-culture.

The viability of HepG2 at 0h (H-0h) is 65.87 ± 9.60% (n=3, Figure 14B). The viability of mono-cultured HepG2 at 24h (mono-H-24h) is 77.20 ± 16.13% (n=3, Figure 14D) and the viability of co-cultured HepG2 at 24h (co-H-24h) is 76.97 ± 13.84% (n=3, Figure 14G). There is no obvious difference among three groups regarding the viability of HepG2. This can be proven by the statistical analysis of three repeated experiments. (Figure 18A, H-0h vs. mono-H-24h: p=0.354; H-0h vs. co-H-24h: p=0.201; mono-H-24h vs. co-H-24h: p=0.997)

Compared with the degranulation of $CD8^+$ T lymphocytes at 0h (CTL-0h) (1.27 ± 1.36%, n=3, Figure 15E) and the degranulation of mono-cultured $CD8^+$ T lymphocytes at 24h (mono-CTL-24h) (1.10 ± 0.95%, n=3, Figure 16E), the degranulation of co-cultured $CD8^+$ T lymphocytes at 24h (co-CTL-24h) (7.03 ± 3.47%, n=3, Figure 17E) increase to some extent. Similarly, compared with the degranulation of NK cells at 0h (NK-0h) (0.90 ± 0.75%, n=3, Figure 15H) and the degranulation of mono-cultured NK cells at 24h (mono-NK-24h) (0.90 ± 0.89%, n=3, Figure 16H), the degranulation of co-cultured NK cells at 24h (co-NK-24h) (9.83 ± 6.73%, n=3, Figure 17H) also rise. However, in the statistical analysis of three repeated experiments no significant difference could be shown. (Figure 18B, CTL-0h vs. mono-CTL-24h: p=0.990; CTL-0h vs. co-CTL-24h: p=0.286; mono-CTL-24h vs. co-CTL-24h: p=0.101) (Figure 18C, NK-0h vs. mono-NK-24h: p>0.999; NK-0h vs. co-NK-24h: p=0.268; mono-NK-24h vs. co-NK-24h: p=0.259)

These results indicate that with the ratio of 1:10 (HepG2: PBMC), major immune effector cells can not kill or inhibit HCC cells effectively after 24h co-cultivation. However, degranulation of major immune effector cells is observed after 24h

co-cultivation although without statistical significance.

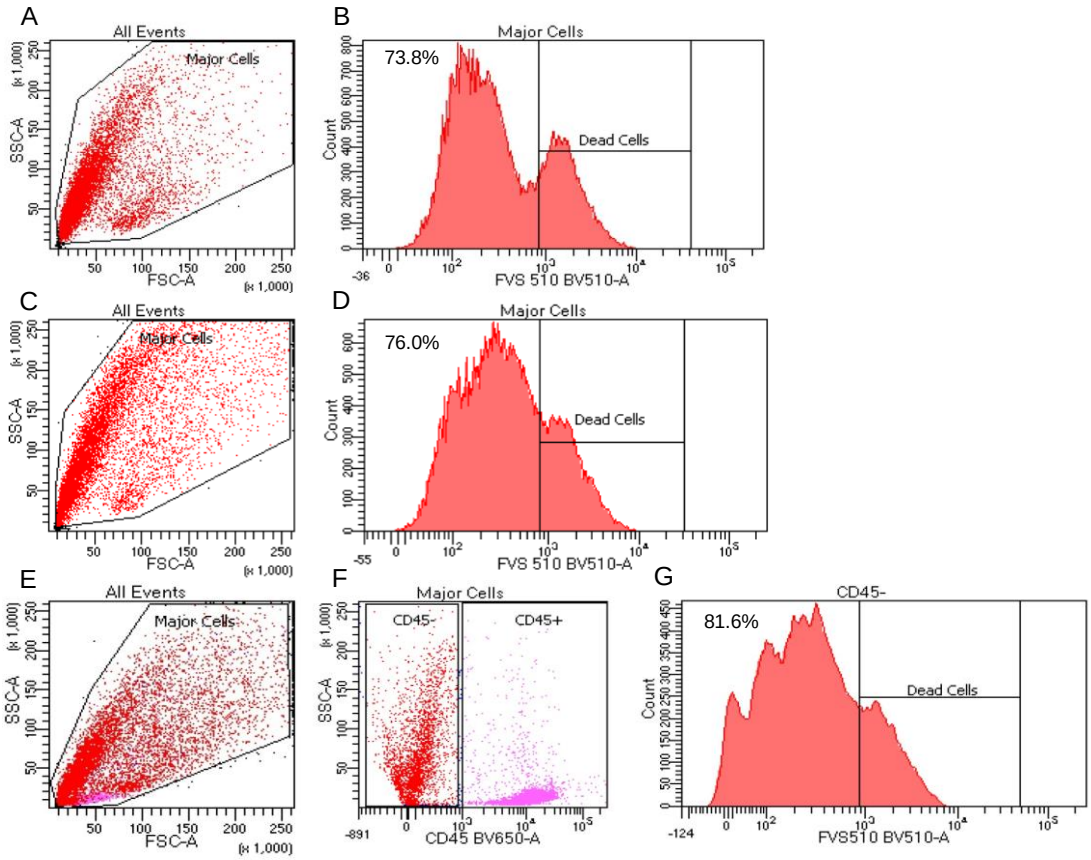

Figure 14: Typical FCM pictures of HepG2 at 0h and 24h (Co-culture of HepG2 with PBMC, 1:10). (A) Major cells of H-0h; (B) Viability of H-0h; (C) Major cells of mono-H-24h; (D) Viability of mono-H-24h; (E) Major cells of co-H-24h; (F) $CD45^-$ and $CD45^+$ cells in major cells; (G) Viability of co-H-24h.

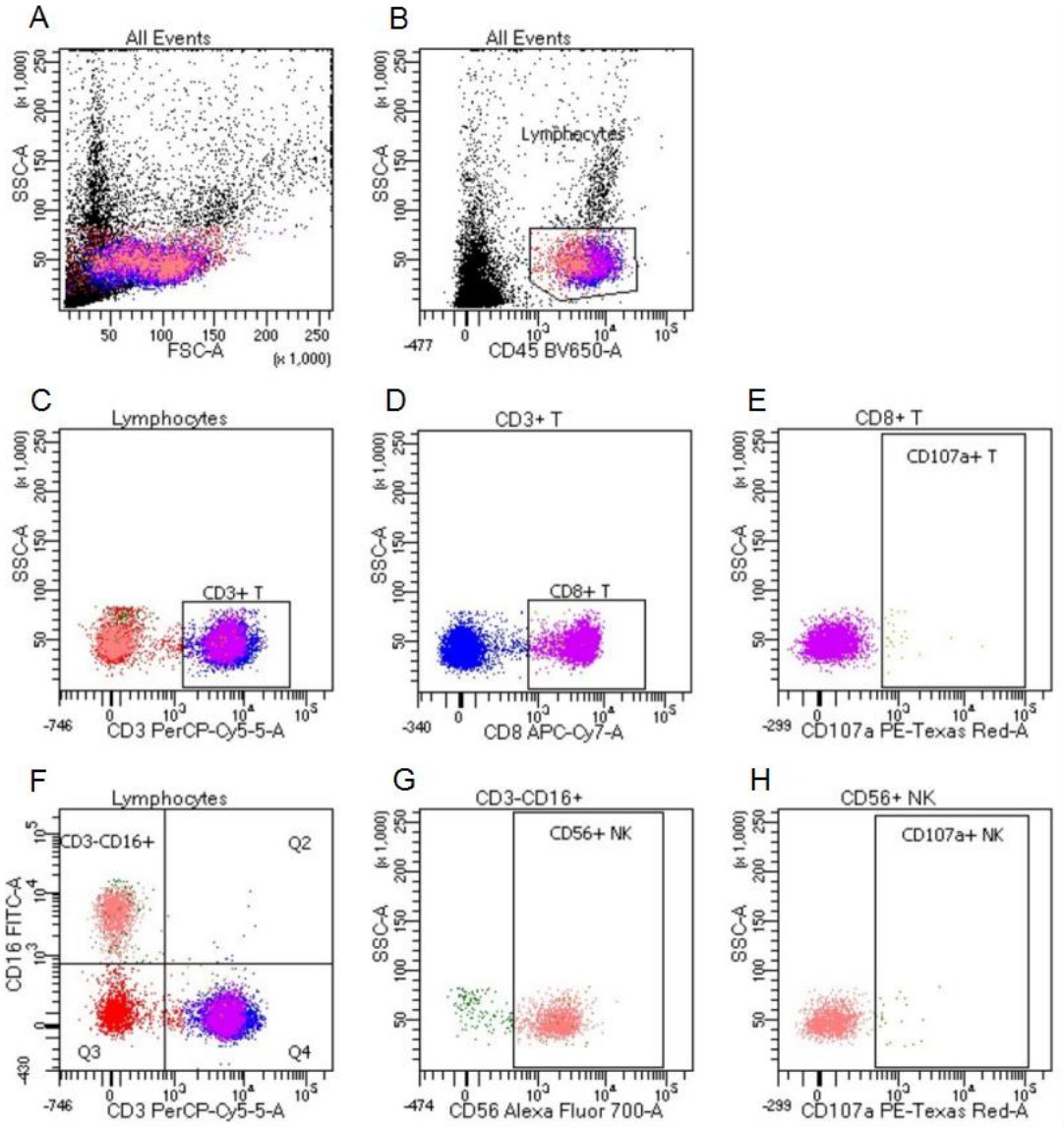

Figure 15: Typical FCM pictures of PBMC at 0h (Co-culture of HepG2 with PBMC, 1:10).

(A) All cells from 0h PBMC; (B) Lymphocytes; (C) $CD3^+$ T lymphocytes; (D) $CD8^+$ T lymphocytes; (E) $CD107a^+/CD8^+$ T lymphocytes; (F) $CD3^-/CD16^+$ cells; (G) $CD3^-/CD16^+/CD56^+$ NK cells; (H) $CD107a^+$ /$CD56^+$ NK cells.

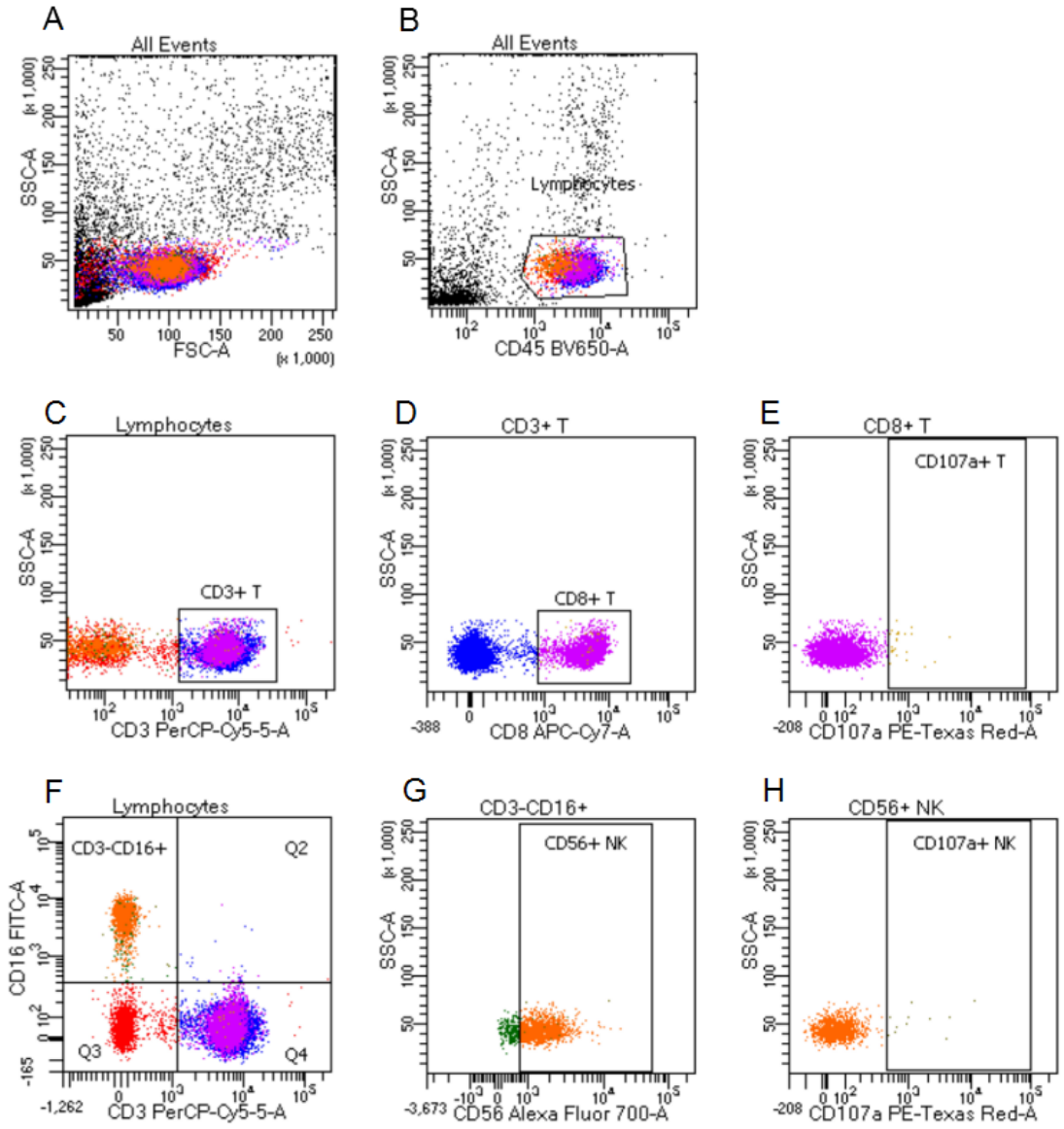

Figure 16: Typical FCM pictures of mono-cultured PBMC at 24h (Co-culture of HepG2 with PBMC, 1:10). (A) All cells from mono-cultured PBMC; (B) Lymphocytes; (C) $CD3^+$ T lymphocytes; (D) $CD8^+$ T lymphocytes; (E) $CD107a^+/CD8^+$ T lymphocytes; (F) $CD3^-/CD16^+$ cells; (G) $CD3^-/CD16^+/CD56^+$ NK cells; (H) $CD107a^+$ /$CD56^+$ NK cells.

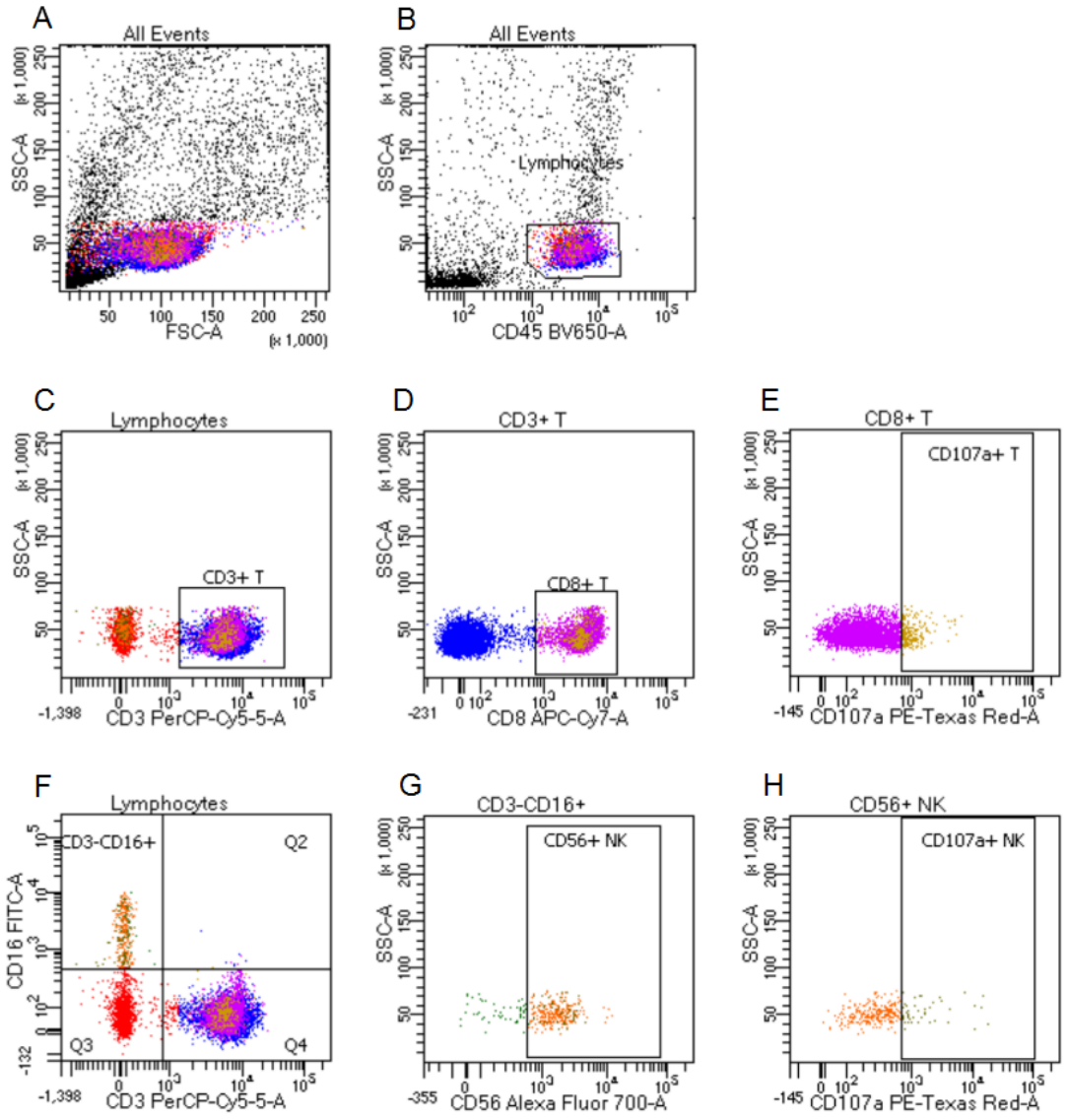

Figure 17: Typical FCM pictures of co-cultured PBMC at 24h (Co-culture of HepG2 with PBMC, 1:10). (A) All cells from co-cultured PBMC; (B) Lymphocytes; (C) $CD3^+$ T lymphocytes; (D) $CD8^+$ T lymphocytes; (E) $CD107a^+/CD8^+$ T lymphocytes; (F) CD3-/CD16+ cells; (G) $CD3^-/CD16^+/CD56^+$ NK cells; (H) $CD107a^+/CD56^+$ NK cells.

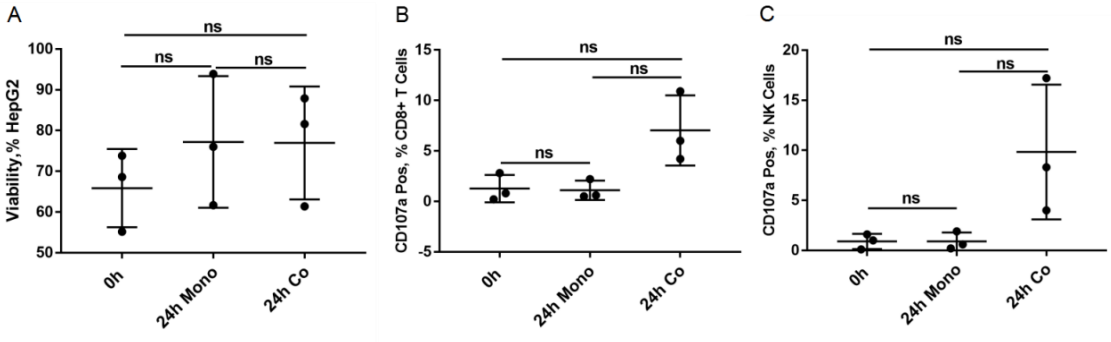

Figure 18: Statistical analysis of three repeated experiments (Co-culture and

Mono-culture of HepG2 with PBMC, 1:10). (A) Viability of HCC cells (HepG2); (B) Degranulation of $CD8^+$ T lymphocytes in PBMC; (C) Degranulation of NK cells in PBMC. (One-Way ANOVA, ns: no significance.)

3.4. Co-Culture of HepG2 with PBMC from HDs (1:25)

Aforementioned results show that the ratio of 1:10 (HepG2: PBMC) is not enough for major immune cells to show cytotoxicity against HCC cells in the co-culture system. Even though none of the differences in degranulation are statistical significant, an increasing tendency of degranulation of $CD8^+$ T lymphocytes as well as NK cells was observed. Thus we decided to increase the ratio to 1:25 (HepG2: PBMC) to observe whether more PBMC could influence the viability of HepG2 or not. Viability of HepG2 and degranulation of major immune effector cells at 0h and 24h after co-culture were recorded as before.

The viability of H-0h is 92.80 ± 3.00% (n=3, Figure 19B). The viability of mono-H-24h and co-H-24h is 74.70 ± 10.28%, (n=3, Figure 19D) and 65.10 ± 10.61%, (n=3, Figure 19G), respectively. Compared with the viability of H-0h and mono-H-24h, the viability of co-H-24h decrease significantly (Figure 23A, H-0h vs. co-H-24h: p=0.044; mono-H-24h vs. co-H-24h: p=0.020). Compared with the viability of H-0h, the viability of mono-H-24h also decrease, but without statistical significance (Figure 23A, H-0h vs. mono-H-24h: p=0.091).

Compared with the degranulation of CTL-0h (1.53 ± 0.59%, n=3, Figure 20E) and the degranulation of mono-CTL-24h (3.50 ± 1.50%, n=3, Figure 21E), the degranulation of co-CTL-24h (19.80 ± 0.44%, n=3, Figure 22E) increase obviously. Similarly, compared with the degranulation of NK-0h (1.33 ± 1.12%, n=3, Figure 20H) and the degranulation of mono-NK-24h (5.53 ± 6.21%, n=3, Figure 21H), the degranulation of co-NK-24h (27.40 ± 3.66%, n=3, Figure 22H) also increase distinctly. All the differences mentioned above are statistically significant. (Figure 23B, CTL-0h vs.

co-CTL-24h: p<0.0001; mono-CTL-24h vs. co-CTL-24h: p=0.005) (Figure 23C, NK-0h vs. co-NK-24h: p=0.009; mono-NK-24h vs. co-NK-24h: p=0.042) Compared with the degranulation of CTL-0h (Figure 20E), the degranulation of mono-CTL-24h (Figure 21E) increase a little, but without statistical significance. (Figure 23B, CTL-0h vs. mono-CTL-24h: p=0.146) The same tendency happened with NK cells. (Figure 23C, NK-0h vs. mono-NK-24h: p=0.474)

From these results, we can conclude that with the ratio of 1:25 (HepG2: PBMC), HCC cells stimulate major immune effector cells into degranulation state after 24h co-cultivation. In turn, stimulated major immune effector cells can obviously show cytotoxic effect to HCC cells.

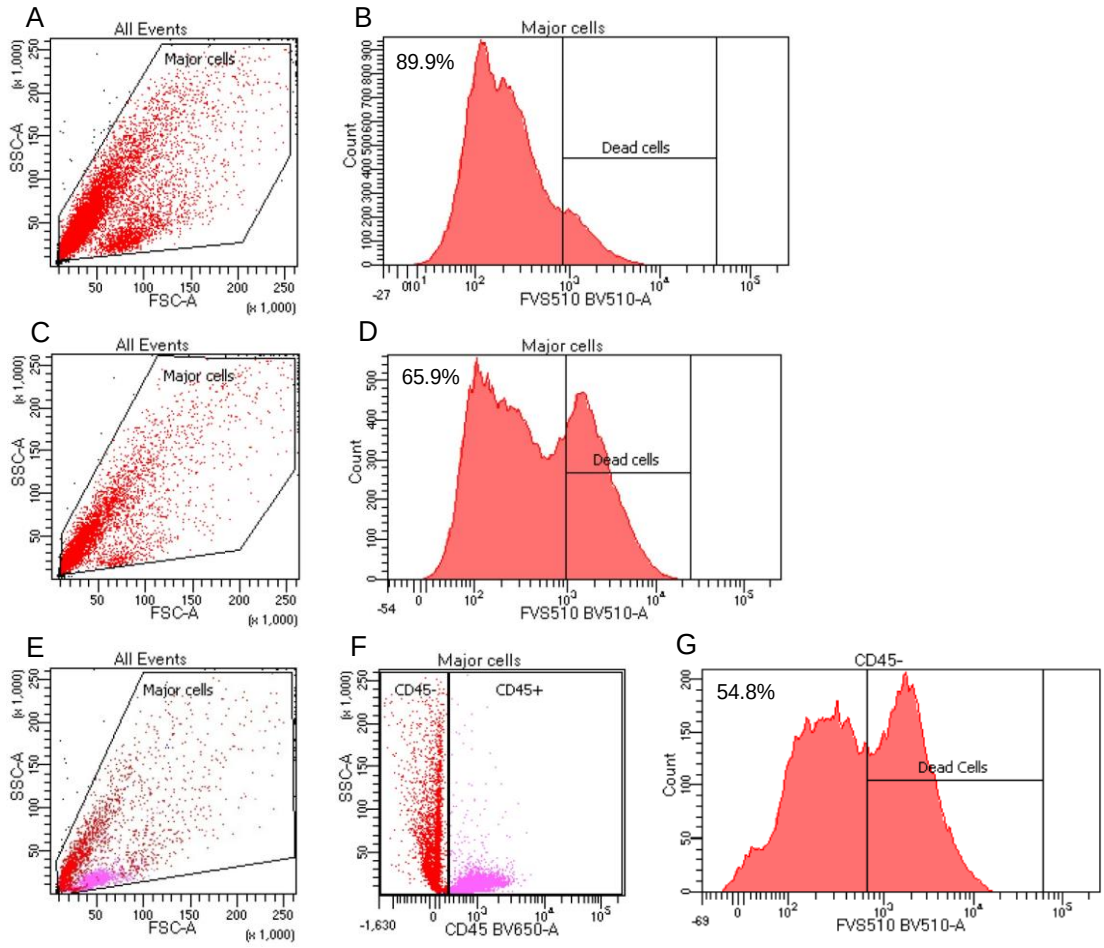

Figure 19: Typical FCM pictures of HepG2 at 0h and 24h (Co-culture of HepG2 with PBMC, 1:25). (A) Major cells of H-0h; (B) Viability of H-0h; (C) Major cells of mono-H-24h; (D)

Viability of mono-H-24h; (E) Major cells of co-H-24h; (F) $CD45^-$ and $CD45^+$ cells in major cells; (G) Viability of co-H-24h.

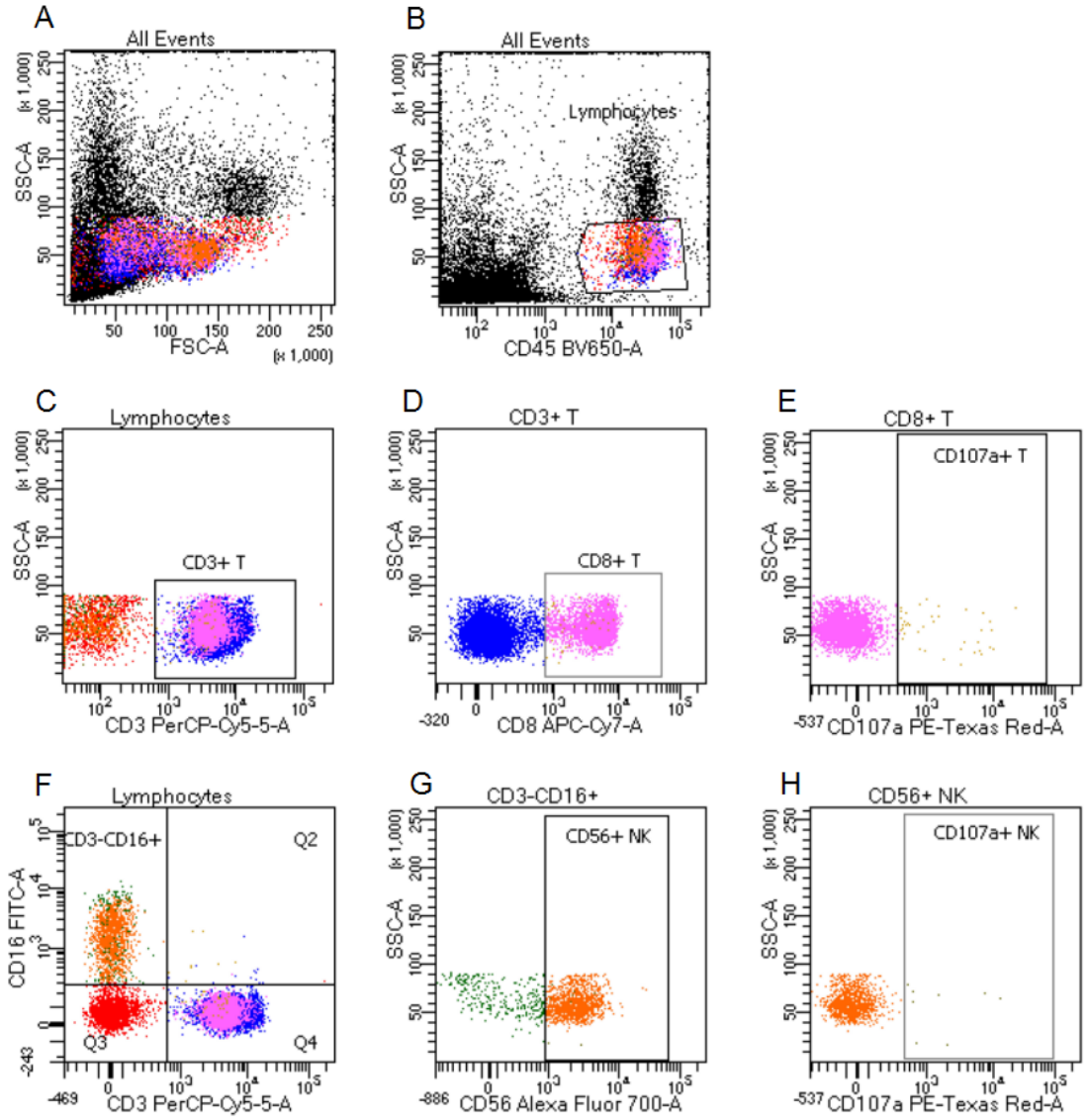

Figure 20: Typical FCM pictures of PBMC at 0h (Co-culture of HepG2 with PBMC, 1:25).

(A) All cells from 0h PBMC; (B) Cell cluster of lymphocytes; (C) CD3+ T lymphocytes; (D) $CD8^+$ T lymphocytes; (E) $CD107a^+/CD8^+$ T lymphocytes; (F) $CD3^-/CD16^+$ cells; (G) $CD3^-/CD16^+/CD56^+$ NK cells; (H) $CD107a^+/CD56^+$ NK cells.

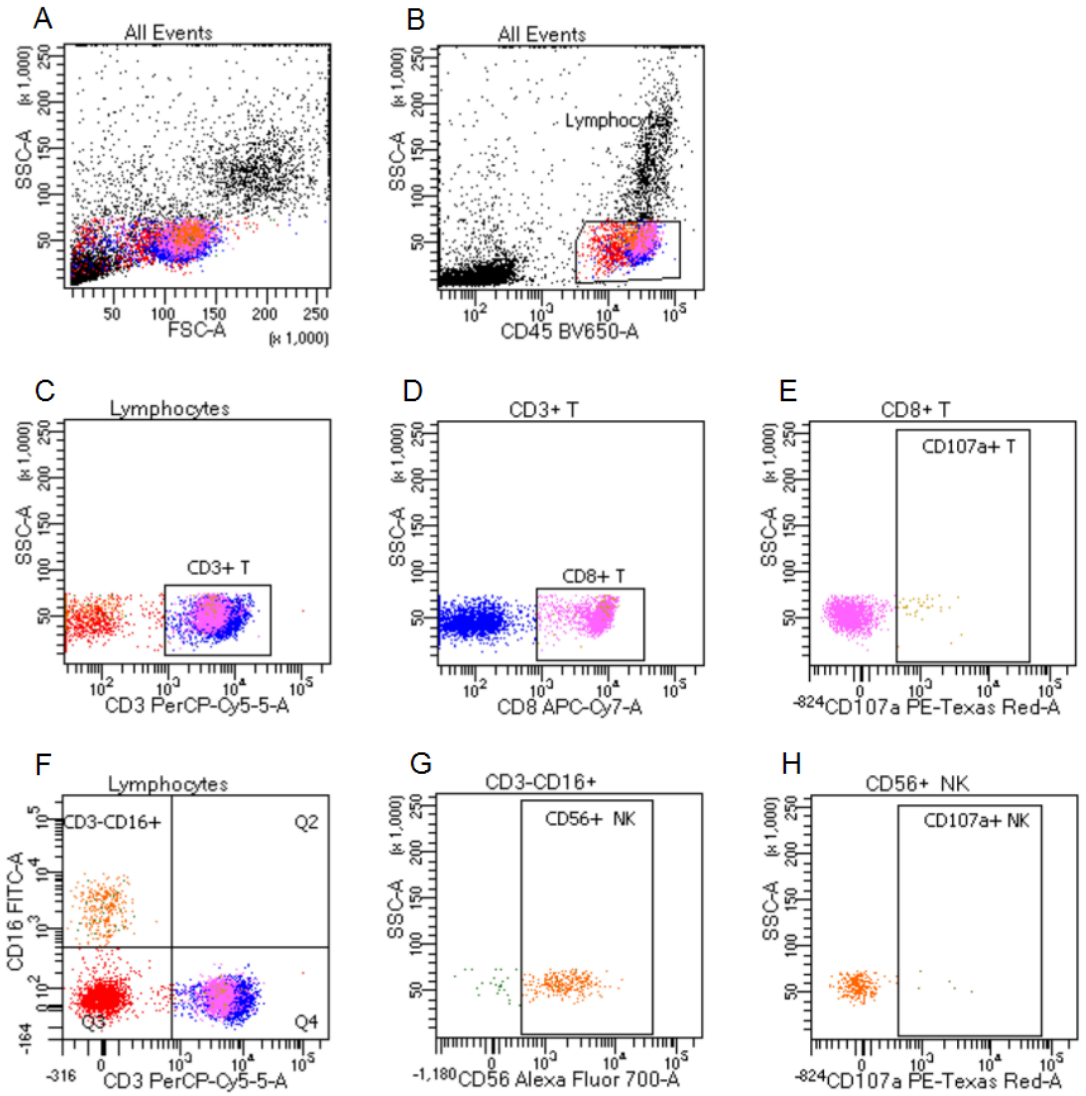

Figure 21: Typical FCM pictures of mono-cultured PBMC at 24h (Co-culture of HepG2 with PBMC, 1:25). (A) All cells from mono-cultured PBMC; (B) Cell cluster of lymphocytes; (C) $CD3^+$ T lymphocytes; (D) $CD8^+$ T lymphocytes; (E) $CD107a^+/CD8^+$ T lymphocytes; (F) $CD3^-/CD16^+$ cells; (G) $CD3^-/CD16^+/CD56^+$ NK cells; (H) $CD107a^+/CD56^+$ NK cells.

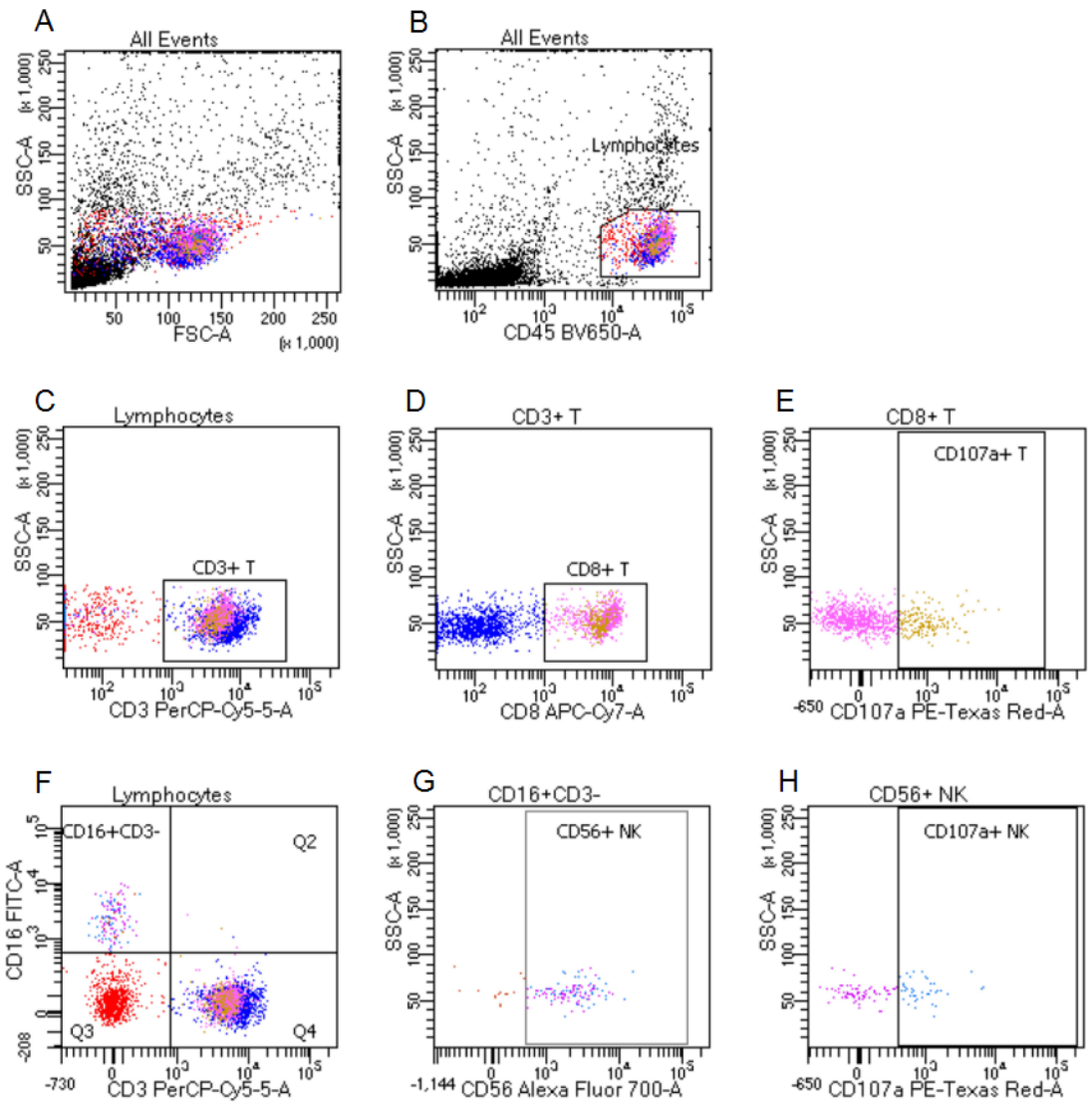

Figure 22: Typical FCM pictures of co-cultured PBMC at 24h (Co-culture of HepG2 with PBMC, 1:25). (A) All cells from co-cultured PBMC; (B) Cell cluster of lymphocytes; (C) $CD3^+$ T lymphocytes; (D) $CD8^+$ T lymphocytes; (E) $CD107a^+/CD8^+$ T lymphocytes; (F) $CD3^-/CD16^+$ cells; (G) $CD3^-/CD16^+/CD56^+$ NK cells; (H) $CD107a^+/CD56^+$ NK cells.

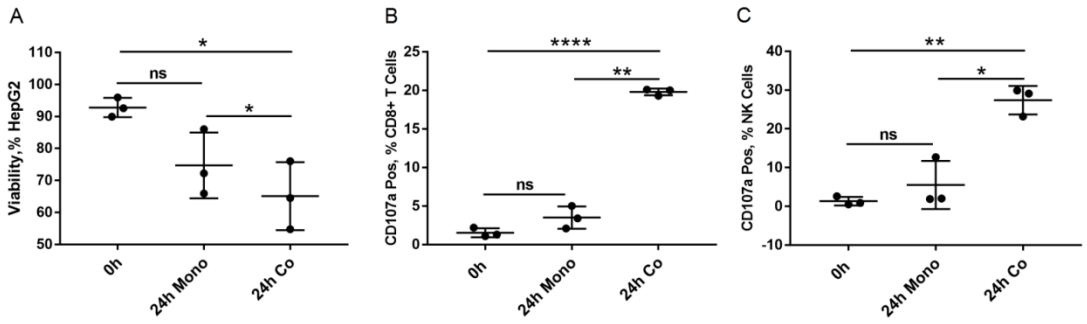

Figure 23： Statistical analysis of three repeated experiments (Co-culture and Mono-culture of HepG2 with PBMC, 1:25). (A) Viability of HCC cells (HepG2); (B) Degranulation of $CD8^+$ T lymphocytes in PBMC; (C) Degranulation of NK cells in PBMC. (One-Way ANOVA, ns: no significance, * $p<0.05$, ** $p<0.01$ and **** $p<0.0001$)

3.5. Establishment of primary HCC cells and TILs separation procedure

Followed by the establishment of co-culture system with HepG2 and PBMC, I aimed to establish a DCC system using primary HCC cells and TILs which may reflect the interactions between them in a physiologically and clinically relevant way.

3.5.1. Dead cell removal

At the beginning, CD45 MACS isolation was used directly to separate primary HCC cells and TILs. However, the MACS column would be blocked and MACS isolation was not successful. With trypan blue staining, I found that more than half of the cells were dead cells (50.4%). Because of dissociation, frozen and thawing process, too much dead cells appeared in the mixture of primary HCC cells and TILs. Some of the dead cells would stay in the column and block it, resulting in $CD45^-$ cells can't go through the column. Then CD45 MACS isolation can't work successfully. It is impossible to proceed to next experimental step with so many dead cells. To acquire enough primary HCC cells and TILs with high viability, dead cell removal must be the first step. And dead cell removal kit is applied in this step. As shown in Figure 24B, the viability of single cell suspension is 49.6% before dead cell removal. However, the

viability increases to 82.9% after dead cell removal (Figure 24D) which is applicable for co-culture.

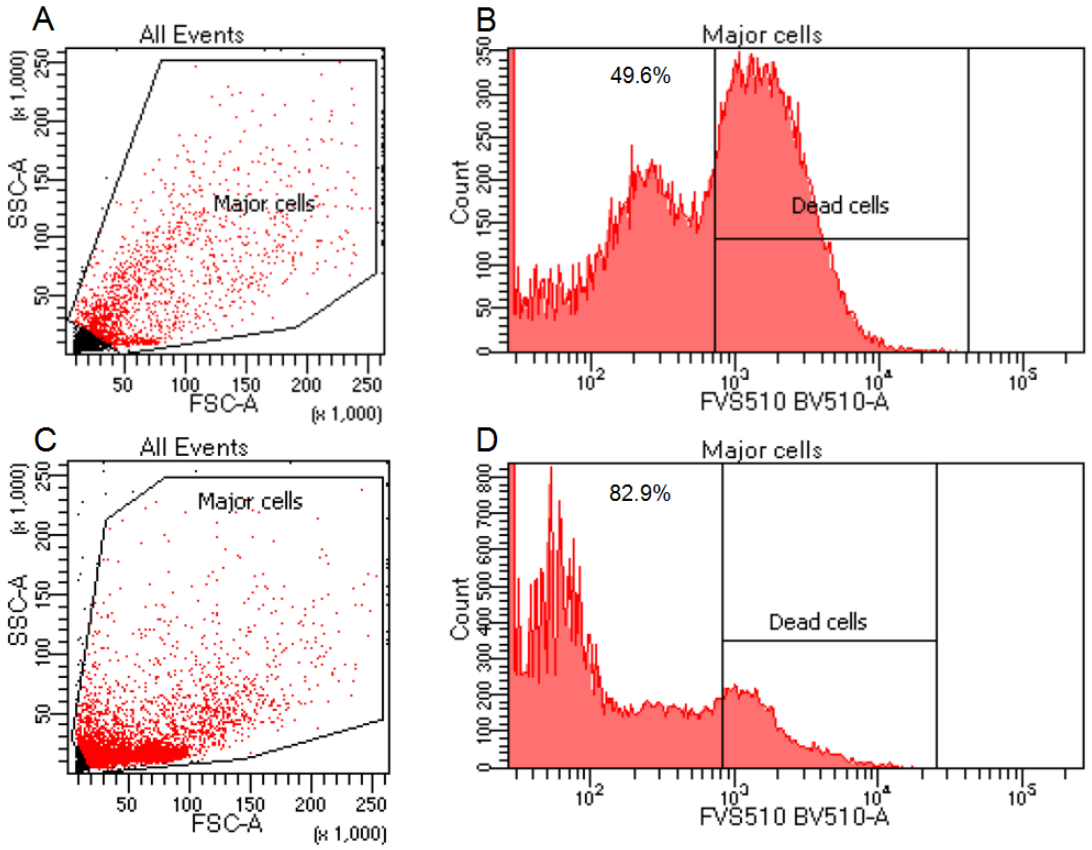

Figure 24: Typical FCM pictures of single cell suspension (mixture of primary HCC cells and TILs) before and after dead cell removal. (A) Major cells before removing dead cells; (B) Viability of major cells before removing dead cells; (C) Major cells after removing dead cells; (B) Viability of major cells after removing dead cells.

3.5.2. MACS Isolation of Primary HCC Cells and TILs

After dead cell removal, the next step is to separate primary HCC cells and TILs. CD45 microbeads are used in this step to separate primary HCC cells (mainly $CD45^-$ cells) and TILs (mainly $CD45^+$ cells). From the data showed in Figure 25, it is apparent that CD45 MACS isolation works well. Before isolation, the proportion of $CD45^-$ cells and $CD45^+$ cells is 69.8% and 28.8%, respectively (Figure 25B). After isolation, in $CD45^-$ group, most of the cells are $CD45^-$ cells (98.1%, Figure 25D). In $CD45^+$ group,

most of the cells left are CD45$^+$ cells (79.8%, Figure 25E). The results indicate that relative pure primary HCC cells and TILs can be harvested with CD45 MACS isolation.

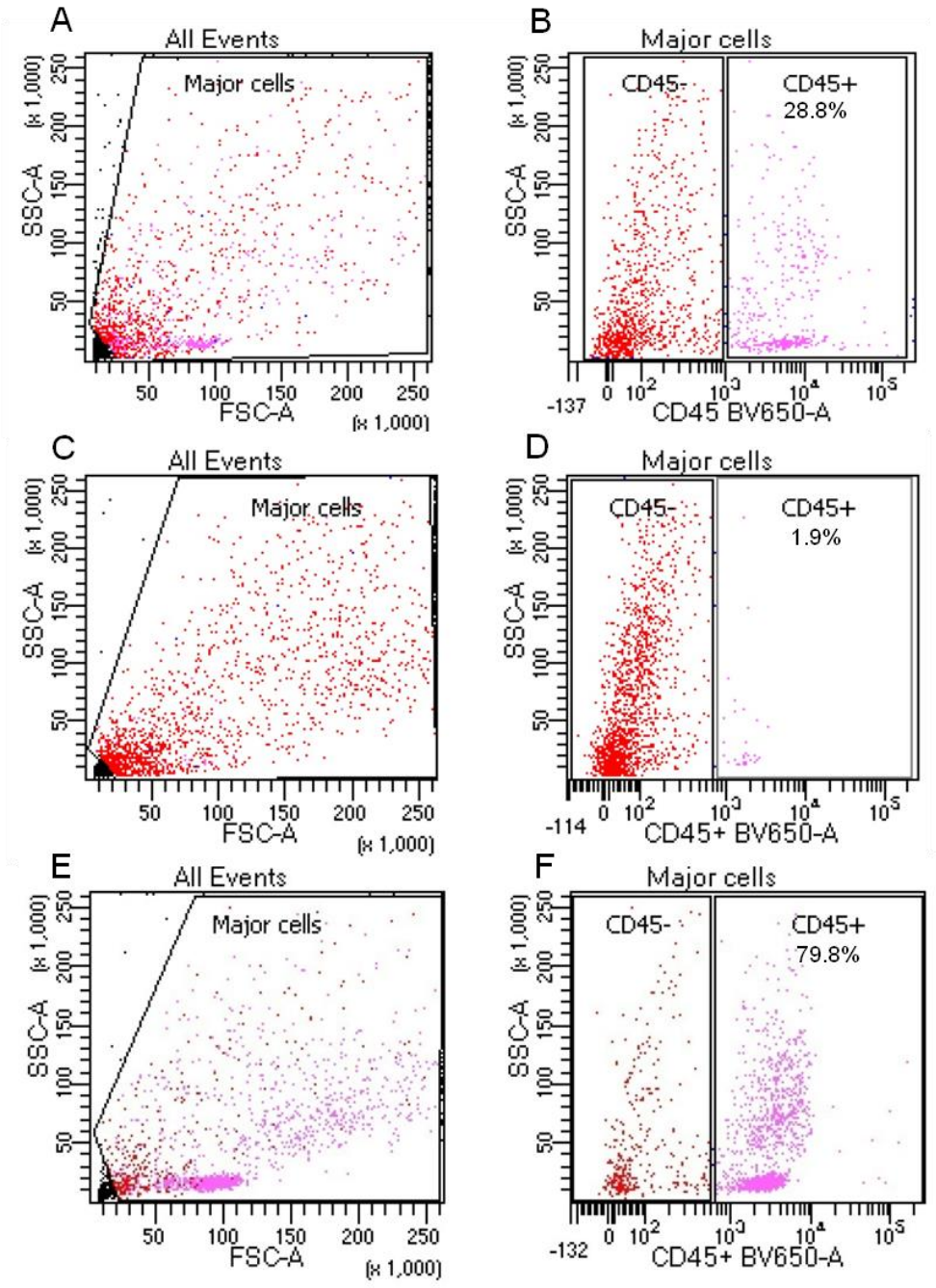

Figure 25: Typical FCM pictures of single cell suspension (mixture of primary HCC cells and TILs) before and after CD45 MACS isolation. (A) Major cells of single cell suspension before CD45 MACS isolation; (B) CD45$^-$ and CD45$^+$ cells before CD45 MACS isolation; (C)

Major cells in $CD45^-$ group after CD45 MACS isolation; (D) $CD45^-$ and $CD45^+$ cells in $CD45^-$ group after CD45 MACS isolation; (E) Major cells in $CD45^+$ group after CD45 MACS isolation; (F) $CD45^-$ and $CD45^+$ cells in $CD45^+$ group after CD45 MACS isolation.

3.6. Co-Culture of Primary HCC cells and TILs (1:10)

Co-culture of primary HCC cells and TILs is one of the best ways to reflect the interactions between HCC cells and major immune effector cells[158]. Figure 26 displayed the whole process of primary HCC cells and TILs obtaining. The co-culture procedure was similar with co-culture of HepG2 and PBMC. Due to the limitation of HCC tumor tissue we could obtain and the minimum cell amount for effective FACS measurement, 1:10 (primary HCC cells vs. TILs) is the maximum ratio we could achieve.

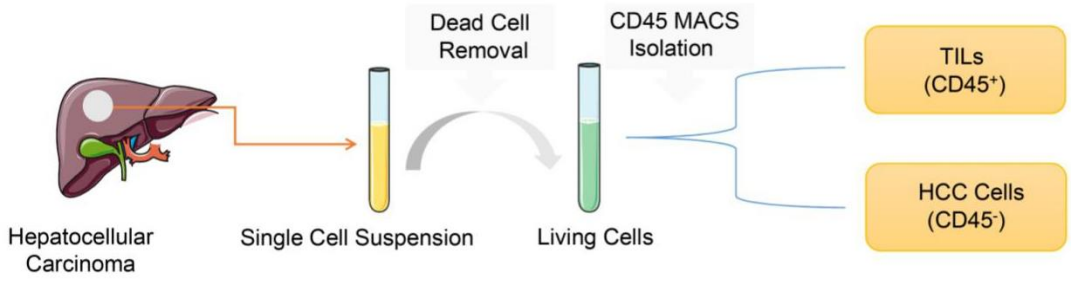

Figure 26: The isolation of primary HCC cells and TILs from HCC tumor tissue.

Viability of primary HCC cells and degranulation of major immune effector cells in TILs were measured at 0h and 24h after co-culture. As mentioned in the gating strategy, $CD45^-$ cells mainly represent primary HCC cells and $CD45^+$ cells mainly represent TILs. However, in both mono-cultured and co-cultured $CD45^-$ cells, there are small amount of $CD45^+$ cells left after separation as mentioned above. The first step of gating is to select $CD45^-$ cells with CD45 antibody. Then we can analyze the viability of primary HCC cells ($CD45^-$). The viability of primary HCC cells ($CD45^-$) at 0h (HCC-0h) is 84.60 ± 11.11% (n=3, Figure 27C). The viability of mono-cultured primary

HCC cells ($CD45^-$) at 24h (mono-HCC-24h) is 94.40 ± 5.20% (n=3, Figure 27F) and the viability of co-cultured primary HCC cells ($CD45^-$) at 24h (co-HCC-24h) is 95.47 ± 0.74% (n=3, Figure 27I). It is clear that there is no difference among three groups regarding the viability of primary HCC cells ($CD45^-$). This is true with the statistical analysis of three repeated experiments (Figure 31A, HCC-0h vs. mono-HCC-24h: p=0.196; HCC-0h vs. co-HCC-24h: p=0.371; mono-HCC-24h vs. co-HCC-24h: p=0.930).

Compared with the data in 0h group and mono-culture group at 24h, the degranulation of $CD8^+$ T lymphocytes and NK cells in co-culture group at 24h show a slight upward trend (Degranulation of $CD8^+$ T lymphocytes: CTL-0h, 8.27 ± 5.19%; mono-CTL-24h, 10.43 ± 5.73%; co-CTL-24h, 12.13 ± 2.99%; degranulation of NK cells: NK-0h, 15.00 ± 11.19%; mono-NK-24h, 39.10 ± 28.14%; co-NK-24h, 55.80 ± 14.05%). However, the statistical analysis of three repeated experiments demonstrates no significant difference (Figure 31B, CTL-0h vs. mono-CTL-24h: p=0.445; CTL-0h vs. co-CTL-24h: p=0.168; mono-CTL-24h vs. co-CTL-24h: p=0.569) (Figure 31C, NK-0h vs. mono-NK-24h: p=0.826; NK-0h vs. co-NK-24h: p=0.506; mono-NK-24h vs. co-NK-24h: p=0.934).

Collectively, after 24h co-cultivation of primary HCC cells and TILs with the ratio of 1:10, cytotoxicity and degranulation of major immune effector cells against HCC cells are not conclusive *ex vivo*.

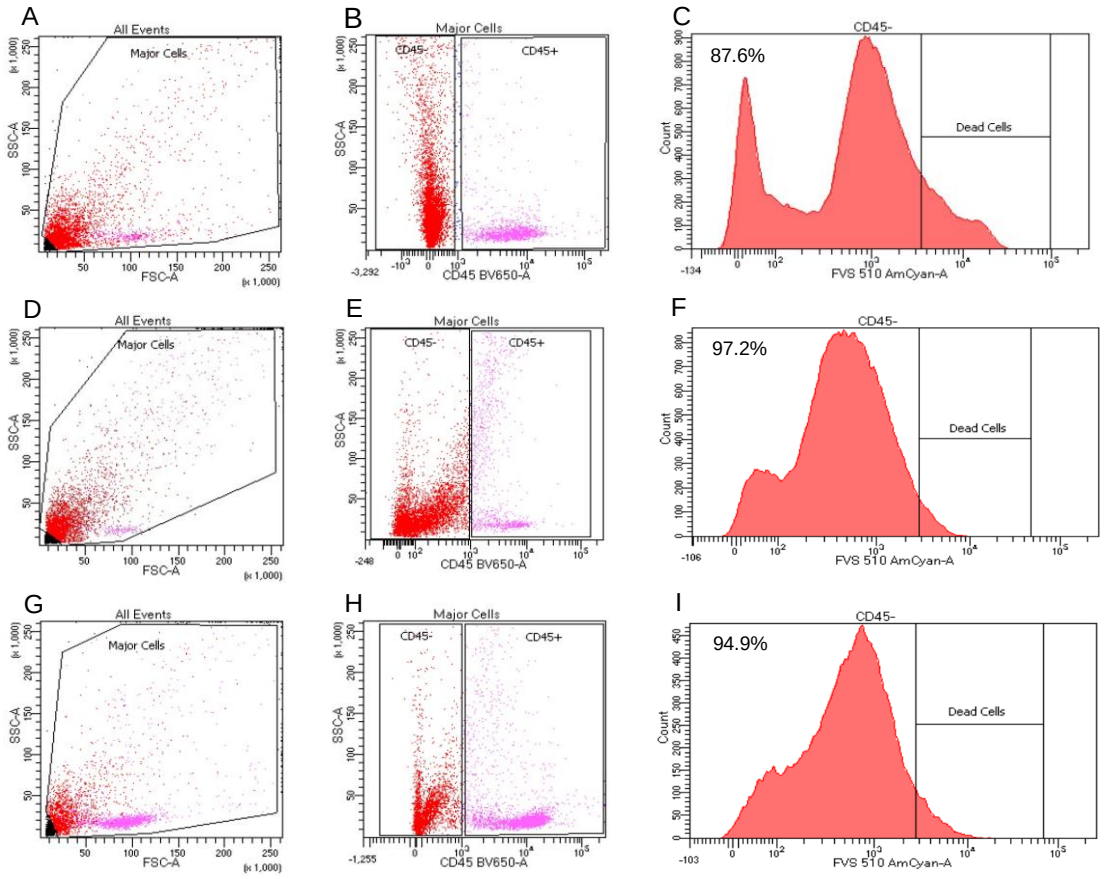

Figure 27: Typical FCM pictures of primary HCC cells at 0h and 24h (Co-culture of primary HCC cells with TILs, 1:10). (A) Major cells of HCC-0h; (B) $CD45^-$ and $C45^+$ cells in major cells at 0h; (C) Viability of HCC-0h; (D) Major cells of mono-HCC-24h; (E) $CD45^-$ and $CD45^+$ cells in mono-HCC-24h; (F) Viability of mono-HCC-24h; (G) Major cells of co-HCC-24h; (H) $CD45^-$ and $C45^+$ cells in co-HCC-24h; (I) Viability of co-HCC-24h.

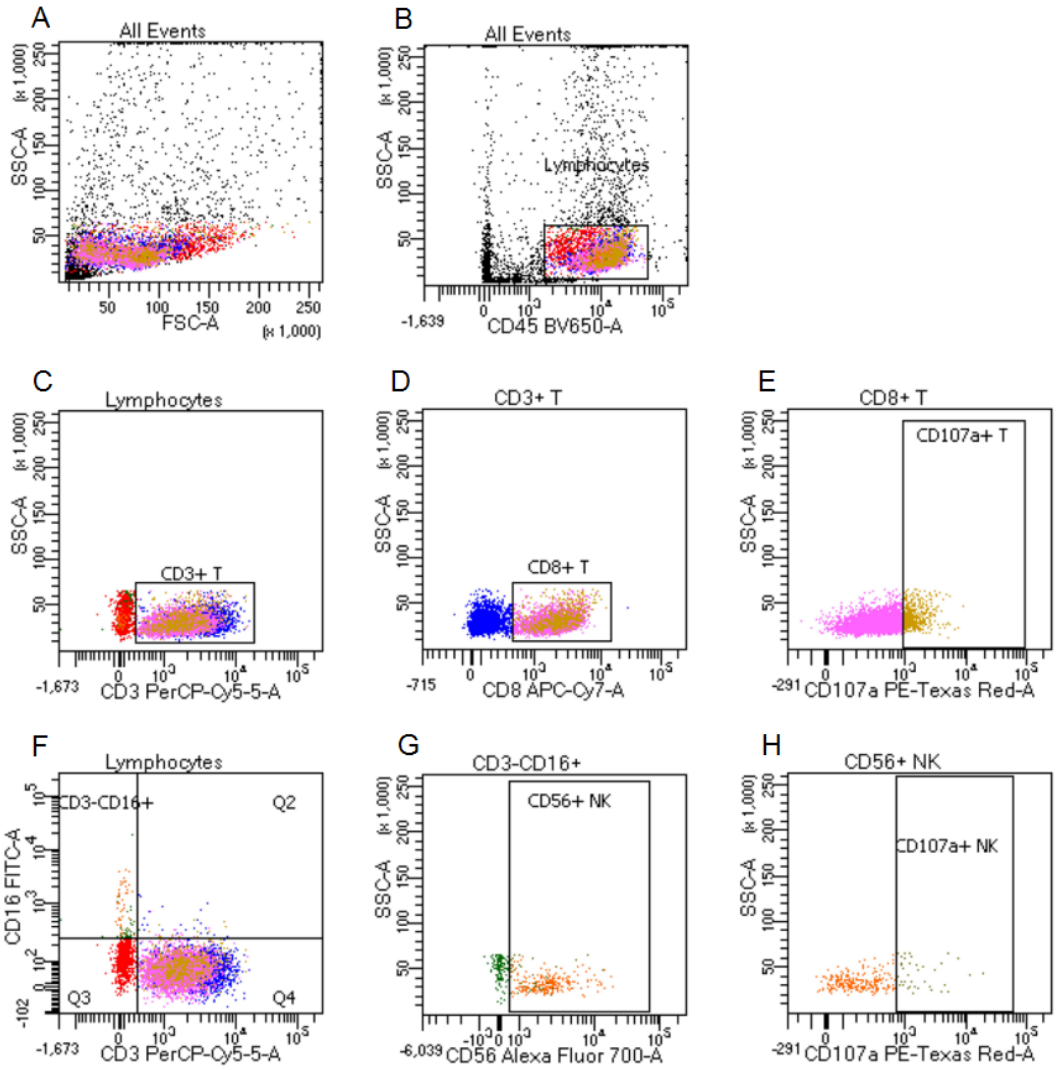

Figure 28: Typical FCM pictures of TILs at 0 h (Co-culture of primary HCC cells with TILs, 1:10). (A) All cells from 0h TILs; (B) Cell cluster of lymphocytes; (C) $CD3^+$ T lymphocytes; (D) $CD8^+$ T lymphocytes; (E) $CD107a^+/CD8^+$T lymphocytes; (F) $CD3^-/CD16^+$ cells; (G) $CD3^-/CD16^+/CD56^+$ NK cells; (H) $CD107a^+/CD56^+$ NK cells.

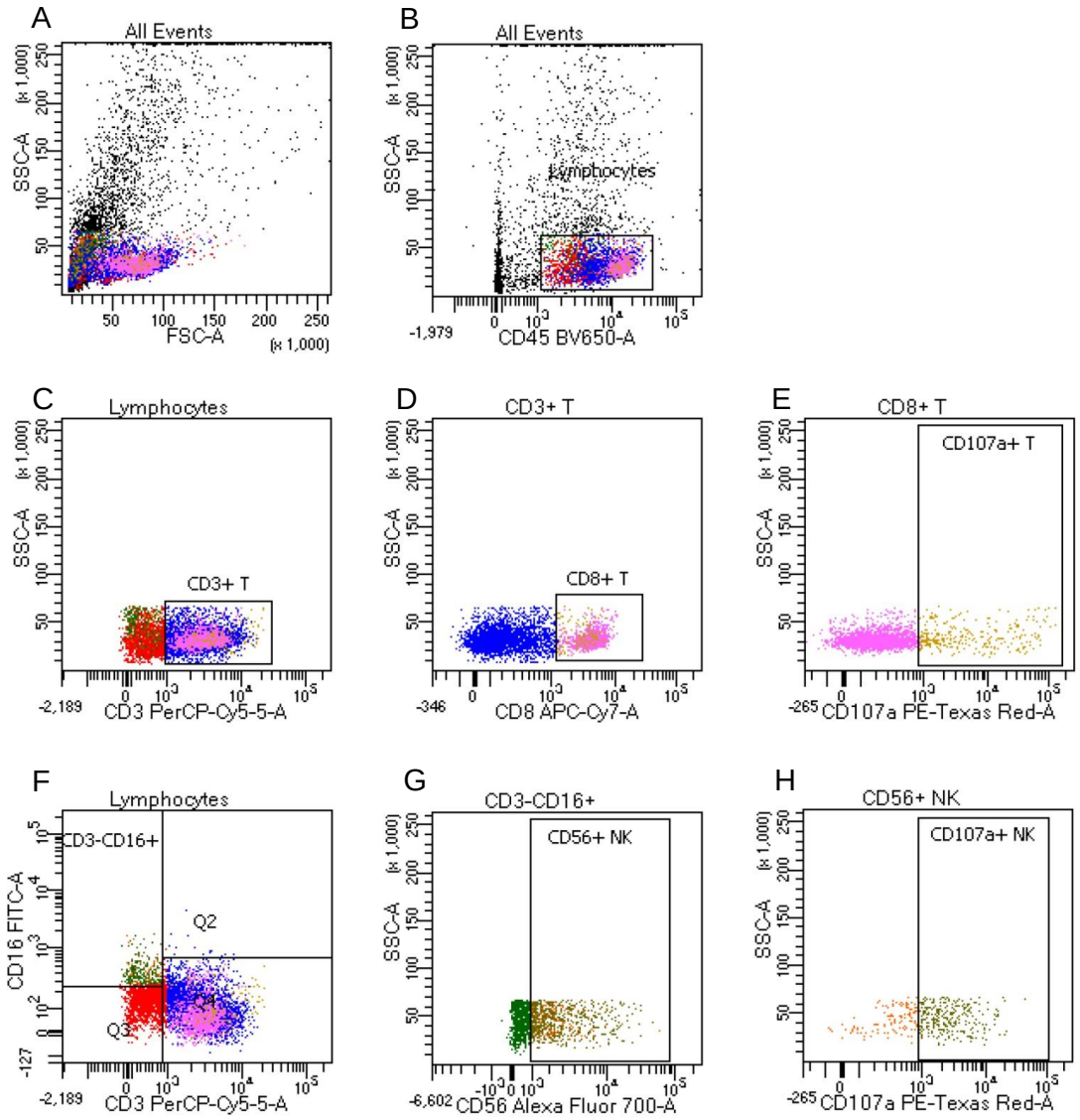

Figure 29: Typical FCM pictures of mono-cultured TILs at 24h (Co-culture of primary HCC cells with TILs, 1:10). (A) All cells from 0h TILs; (B) Cell cluster of lymphocytes; (C) $CD3^+$ T lymphocytes; (D) $CD8^+$ T lymphocytes; (E) $CD107a^+/CD8^+$T lymphocytes; (F) $CD3^-/CD16^+$ cells; (G) $CD3^-/CD16^+/CD56^+$ NK cells; (H) $CD107a^+/CD56^+$ NK cells.

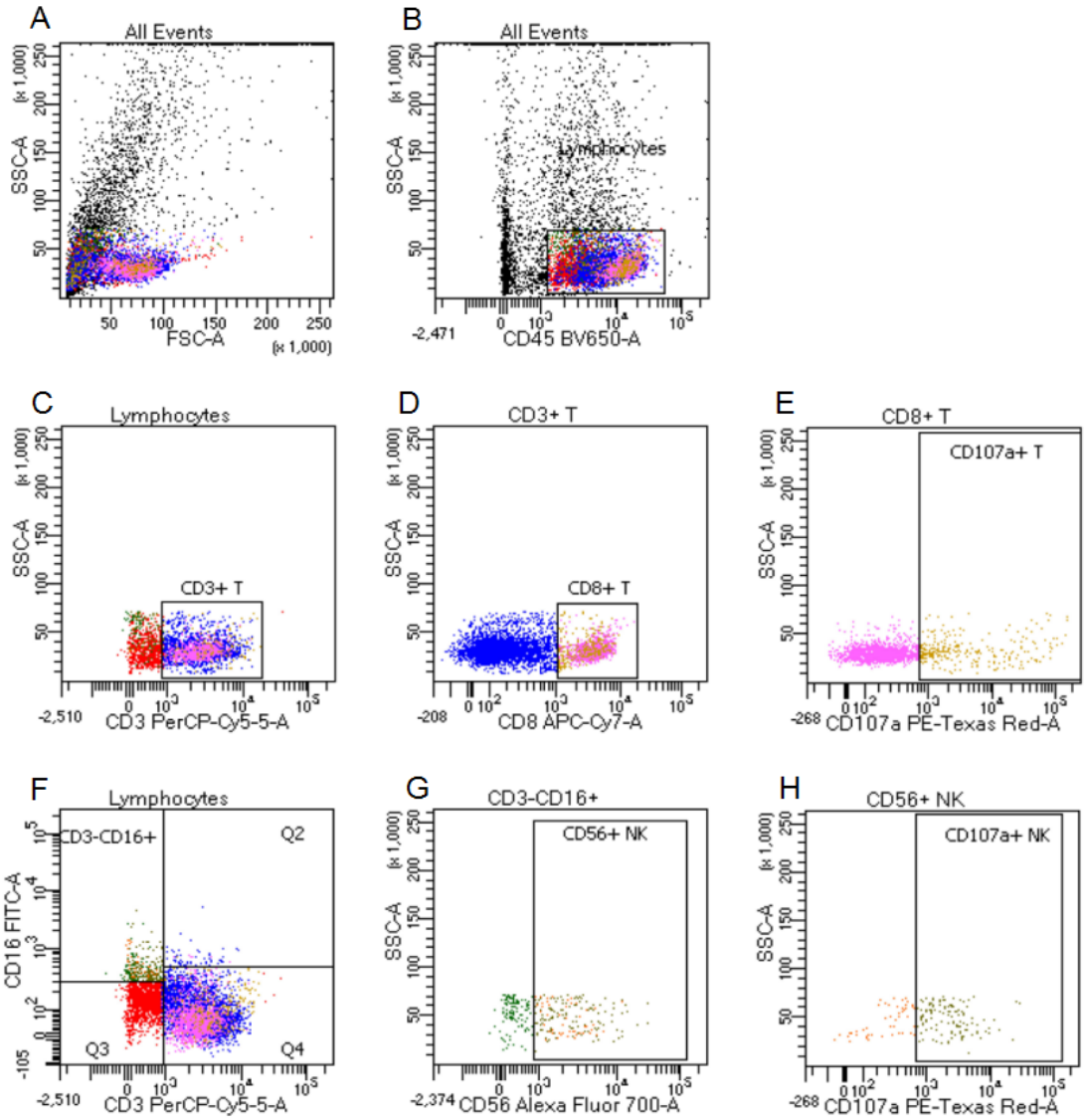

Figure 30: Typical FCM pictures of co-cultured TILs at 24h (Co-culture of primary HCC cells with TILs, 1:10). (A) All cells from 0h TILs; (B) Cell cluster of lymphocytes; (C) $CD3^+$ T lymphocytes; (D) $CD8^+$ T lymphocytes; (E) $CD107a^+/CD8^+$T lymphocytes; (F) $CD3^-/CD16^+$ cells; (G) $CD3^-/CD16^+/CD56^+$ NK cells; (H) $CD107a^+/CD56^+$ NK cells.

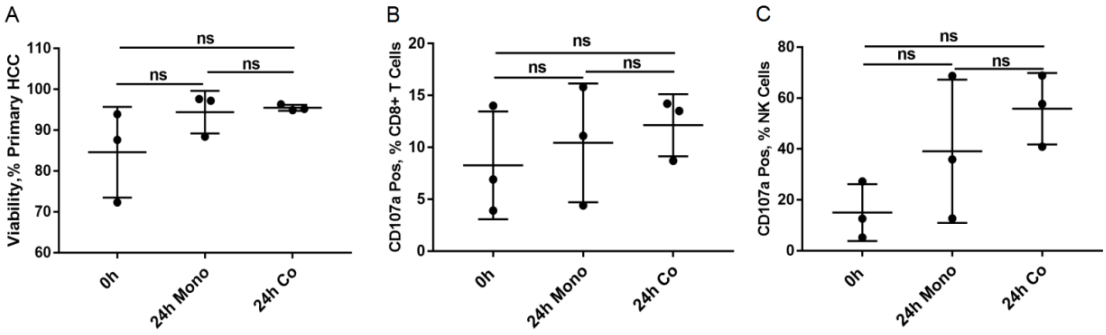

Figure 31: Statistical analysis of three repeated experiments (Co-culture and Mono-culture of primary HCC cells with TILs, 1:10). (A) Viability of primary HCC cells; (B) Degranulation of $CD8^+$ T lymphocytes in TILs; (C) Degranulation of NK cells in TILs. (One-Way ANOVA, ns: no significance)

4. Discussion

As mentioned in the literature review, few studies investigate the interactions between the major immune effector cells and HCC cells via co-culture of primary HCC cells and autologous TILs, which is an ideal model that resembles the physiological conditions in HCC patients. Due to the limitation of conducting experiments with human tissue, most data on HCC immunity were acquired from animal models or cell lines. Here we successfully set up a detailed direct contact co-culture (DCC) system with primary HCC cells and TILs by initially establishing a DCC of HepG2 and PBMCs. Regarding the DCC system, we give a detailed description for the preparation of tumor single-cell suspension, dead cell removal after cell thawing, MACS isolation of primary HCC cells and autologous TILs, cell seeding and harvesting of each groups. Using this DCC system, we analyzed the degranulation of $CD8^+$ T lymphocytes and NK cells, and assessed the viability of HCC cells by FACS in order to study the interactions between them. In the following sections, the results and related questions will be discussed.

4.1. Co-Culture model selection and target:effector ratio selection

To study the cell-cell interactions between major immune effector cells and HCC cells, it is important to choose an appropriate co-culture model. It has been previously introduced that there are three main types of cell co-culture model based on the normal morphology of co-cultured cells and the objective of the investigators, including DCC, indirect contact co-culture (ICC) and three-dimensional cell co-culture (TDCC). Each model has its own benefits and drawbacks. DCC is mainly used to study intercellular interactions and to induce cell differentiation. It is easy to operate and the culture conditions required are simple, but the detection of indicators is challenging due to the difficulty of separating cells[159, 160]. ICC is mainly used for cells

that communicate through secretion of molecules. Cells can be separated completely in this co-culture model; however, direct cell-cell interactions do not occur[161, 162]. The TDCC system can better simulate the internal environment, but the selection of supporting material is critical and the repeatability needs to be improved[163, 164]. The aim of this research was to investigate the interactions between major immune effector cells and HCC cells. These interactions consist of both cytokine delivery and direct cell-cell interactions. Thus, direct contact was necessary for this study and DCC was selected as the co-culture model.

Besides the co-culture model, another essential part assuring the success of the co-culture is target:effector (T:E) ratio which has been investigated in my work. To select the best T:E ratio, we referred to related publications. Jeroen Melief et al. reported that, 1:2 was the most commonly applied T:E ratio in the co-culture of tumor cells and autologous T cells[158]. However, this work, which was published in 2019, only described the co-culture method and did not mention any results. In the study conducted by Faezeh et al., HepG2 cells and NK cells were co-cultured at T:E ratios of 10:1, 1:1, and 1:10, and the proliferation of HepG2 cells was most inhibited at the ratio of 1:10[165]. Zhao et al. demonstrated that, when HepG2 cells were co-cultured with PBMCs at a ratio of 1:3, the secretion of IFN-γ and TNF-α was up-regulated compared to that when L02 cells (a normal liver cell line) were co-cultured with PBMCs[166]. In a co-culture study of primary HCC cells with autologous PBMCs, Doumba et al. reported that at a T:E ratio of 1:5, primary HCC cells could promote apoptosis and necrosis of $CD8^+$ T cells[167]. An animal study by Wu et al. observed a protective effect of JBP485 (a dipeptide isolated from Laennec) when primary rat hepatocytes were co-cultured with autologous lymphocytes at a ratio of 1:10[168]. Based on these previous studies, the T:E ratio of 1:10 was selected to study the cytotoxicity of immune effector cells toward HCC cells. However, in the co-culture system of HepG2 and PBMCs, we found a trend toward increasing cytotoxicity and degranulation, but this finding was not statistically significant at a T:E ratio of 1:10. Since the PBMCs used in this study were not stimulated in advance before co-culture,

more PBMCs were needed to show effective cytotoxicity. We then increased the ratio to 1:25 in an attempt to achieve statistically significant co-culture results. Then statistically significant cytotoxicity and degranulation of $CD8^+$ T lymphocytes and NK cells were observed. While co-culturing primary HCC cells and autologous TILs, a T:E ratio of 1:10 is the maximum ratio we were able to achieve due to the restricted amount of patient tumor tissue we could obtain. The related results will be discussed later.

The aim of our study is to investigate the interactions between major immune effector cells and HCC cells. Therefore, factors that might influence the results should be excluded as much as possible. Virus infection is a major element that can stimulate immune effector cells, which may make it difficult to discern between the activation of immune effector cells caused by HCC cells and activation caused by virus infection. Thus, only primary HCC patients without HBV or HCV infection were included in this study. However, this presented a challenge for the study, as the numbers of primary HCC patients without virus infection are limited in western world. Here, we collected samples from three non-HBV/non-HCV primary HCC patients.

4.2. $CD8^+$ T Lymphocytes Show Cytotoxicity to HCC Cells but maybe dysfunctional in HCC patients

$CD8^+$ T lymphocytes can inhibit or kill virus-infected cells and tumor cells in an activated state. Thus, activated $CD8^+$ T lymphocytes are also called cytotoxic T cells (CTL). [169, 170] As previously mentioned, in the co-culture system, FVS 510 was applied to test the viability of HCC cells and CD107a was applied to test the degranulation of major immune effector cells. By assessing the viability changes in different groups, the cytotoxicity of major immune effector cells can be analyzed. Degranulation is a functional state in which certain types of cells release cytotoxic granules or other molecules to kill target cells, such as infected cells or tumor cells[171]. Our results reveal that, in the co-culture of HepG2 with PBMCs from healthy donors

(1:25), the viability of co-H-24h is dramatically declined compared to H-0h and mono-H-24h in three repeated experiments. At the same time, the degranulation level of co-CTL-24h is markedly increased compared with CTL-0h and mono-CTL-24h. With these direct co-culture results, it can be speculated that $CD8^+$ T lymphocytes can be activated into a degranulation state by HCC cells and exhibit cytotoxicity toward HCC cells in the cell line and PBMC co-culture. This finding is consistent with those of the studies in our previous literature review[119, 122, 126]. The cytotoxic effect of CTLs is mainly fulfilled via three mechanisms. First, CTLs can secret cytokines (e.g. TNF-α and IFN-γ)[170]. TNF-α combines with tumor necrosis factor receptor (TNFR) of target cells and induces apoptosis of target cells by initiating the intracellular caspase pathway. IFN-γ can not only promote the production of MHC molecules on target cells and enhances antigen presentation, but also promote the phagocytosis of macrophages and dendritic cells. Second, CD95L on the surface of CTLs binds to CD95 on the tumor cells surface and initiates apoptotic signals[170]. Third, CTLs lyse target cells by releasing perforin, granzymes, and other cytotoxic molecules[170]. The co-culture system can be used to substantiate all of these mechanisms.

However, in the co-culture of primary HCC cells and TILs, no significant differences were found regarding cytotoxicity and degranulation between experimental group and control group. Previous work of our group showed that tumor infiltrating $CD8^+$ T lymphocytes can secret INF-γ in HCC patients[172]. So it's true that $CD8^+$ T lymphocytes can be activated by HCC cells, but there are some other factors that don't allow them to kill or inhibit HCC cells in our primary HCC cells/ TILs co-culture system. Various possible reasons maybe involved in this. First of all, the functional state of immune effector cells may be impaired after the freezing, thawing, and separating process prior to co-culture. Second, the T:E ratio of 1:10 may not be enough, since we did not find obvious cytotoxicity in the co-culture of HepG2 and PBMCs with this ratio either, but a increasing trend of degranulation. Third, the most convincing explanation may be the dysfunction of $CD8^+$ T lymphocytes in HCC microenvironment. Inhibition of $CD8^+$ T lymphocytes in the tumor microenvironment

has been widely reported. $CD8^+$ T lymphocyte dysfunction and depletion is characterized by up-regulation of immunosuppressive molecules, for example, PD-1[173], CTLA-4[174], TIM3[175], and lymphocyte activation gene 3 (LAG 3) [176], which inhibit $CD8^+$ T lymphocyte activation. The persistence of inhibition in the tumor microenvironment may lead to infiltrating $CD8^+$ T lymphocytes unable to kill tumor cells, and eventually cause tumor immune escape[177]. Several studies discussed in the literature review demonstrate that PD-1/PD-L1 expression inhibit the anti-tumor effect of $CD8^+$ T lymphocytes and blockade of PD-1 can enhance $CD8^+$ T lymphocyte proliferation as well as IFN-γ and TNF-α secretion[119, 121, 123]. In addition, TGF-β is a key molecule associated with tumor initiation and progression and could regulate cancer-associated immune cells, such as $CD8^+$ T lymphocytes[151]. Accumulating evidence has demonstrated that TGF-β is involved in $CD8^+$ T lymphocyte dysfunction in HCC[152]. Other publications have revealed that the dysfunction of $CD8^+$ T cells is closely related to metabolic abnormality[178, 179]. In a mouse sarcoma model, for example, they found that T cells' nutrition was consumed by tumor cells, leading to reduced activity of mammalian target of rapamycin (mTOR) as well as glycolysis and decreased secretion of the effector IFN-γ, which promote tumor progression[180]. The mechanisms of $CD8^+$ T lymphocyte dysfunction in HCC patients are not yet completely understood. The relevant cytokines, molecular signals, metabolic factors, etc. can be studied with our co-culture system.

4.3. Cytotoxicity of NK Cells toward HCC Cells maybe dysfunctional in HCC patients

NK cells are produced in lymphoid organs and perform a variety of important immune functions. As an essential component of the intrinsic immune system, NK cells can directly kill tumor cells with perforin and granzymes to inhibit the process of tumor cell genesis and progression. What's more, NK cells can secret various cytokines to control the functions of other immune cells.[181, 182] In human liver, NK cells are

identified to protect hepatic cells from hepatitis virus attack and malignant transformation. In our results, the viability of HepG2 is decreased significantly when co-cultured with PBMCs compared with the control groups in three repeated experiments. Moreover, the degranulation of NK cells is notably enhanced than that of the control groups. These findings reveal that NK cells are activated into a degranulation state by HCC cells and demonstrate cytotoxic effects on HCC cells in the co-culture of HepG2 and PBMCs. It is in accordance with the results discussed in the previous literature review[128, 143]. Kamiya et al. reported that NK cells showed notably cytotoxic effect against HCC cells when they were expanded and activated[144]. Other researchers have demonstrated that the cytotoxicity of NK cells toward HCC cells can be promoted in different ways. For example, miR-506 promotes the anti-tumor effect of NK cell by regulating STAT3[128]. And IL-12/15/18 + IL-2 cytokine cocktail[145]; TT-1 (a mutant of melittin) and INF-α[130]; miR-486-5p and IGF-1[147]; miR-182[148]; miR-152[139]; and TLR7/8 agonists[140] can augment the cytotoxic effect of NK cells against HCC cells through various mechanisms. Other studies reported ways that NK cell cytotoxicity can be inhibited. For example, miR-146a negatively regulates NK cell cytotoxicity toward HCC cells via STAT1 signaling[135], while Androgen Receptor (AR) decreases NK cell cytotoxicity in HCC by suppressing IL-12A[136].

The main characteristic of NK cells is that they can directly lyse target cells without previous stimulation of antigens. The specific mechanism of their natural cytotoxicity has not yet been fully elucidated. It is known, however, that cell surface receptors are needed to recognize target cells and transmit cytolytic signals. For example, CD11a/CD18, CD2, CD44, CD54, CD58, and CD69 help NK cells establish stable connections with target cells and form immunological synapses[183]. When NK cells are activated by target cells, they can release perforin and granzyme by a degranulation process, mediating cytolysis and apoptosis, respectively[184]. Moreover, NK cells can express Fas ligand (FasL)[185] and TNF-related apoptosis-inducing ligand (TRAIL)[186], which can combine with their respective receptors on target cells and induce cell apoptosis. In addition to direct killing, NK cells can produce cytokines (e.g.

IFN-γ, TNF-α, IL-3) to modulate the anti-tumor activity of other immune cells[187]. Thus, NK cells are also thought to be regulatory cells in the immune system. The cytotoxicity of NK cells against tumors is regulated not only by cytokines such as IL-2, but also by various activating and inhibitory receptors. It has been shown that when MHC class I[188] and NKG2D[189] were up-regulated, tumor cells were more sensitive to NK cell-mediated killing. Killer cell immunoglobulin-like receptors (KIRs)[190] and NKG2A[191] can disrupt NK cells from triggering. Thus, the combination of activating and inhibitory receptors influences the cytotoxicity of NK cells. However, most of these mechanisms were uncovered in cell lines or animal experiments. Here, we can study the mechanisms of NK cytotoxicity toward HCC using our human co-culture system in the future.

Same as the dysfunction of $CD8^+$ T lymphocytes, dysfunction of NK cells may also be a reason for the lack of cytotoxicity or degranulation in the co-culture of primary HCC cells with autologous TILs. Increasing evidence has come up that the cytotoxicity of NK cells is impaired in HCC patients, especially in advanced tumor sites. Several studies have demonstrated that the cytotoxicity and cytokine secretion of NK cells were decreased in patients suffering HCC and that dysfunction of NK cells might be related to the progression and invasion of HCC[192-194].

As major immune effector cells, NK cells are indispensable in the surveillance and management of HCC. Thus, it is important to elucidate the mechanisms related to the impairment of NK cells function in HCC patients, as this is essential to the HCC immunotherapy based on NK cell. Although they remain unclear, several possible mechanisms have been put forward. For example, researches have reported that the proportion of tumor infiltrating NK cells was less than that of non-tumor NK cells in HCC[146, 192]. Research by Wu, et al. showed that NK cells in tumor regions secreted more deficient IFN-γ and TNF-α compared to that in regions without tumor in HCC patients[195]. Thus, decreased frequency of NK cells and defective cytokine secretion may be reasons for NK cell dysfunction in HCC. As mentioned previously, the

combination of activating and inhibitory receptors influences the cytotoxicity of NK cells. Down-regulation of the activating receptor NKG2D and its ligand MICA in HCC patients has been reported by several researchers[196, 197]. Hence, decreased tumor recognition due to those receptors may be another reason for NK cell function impairment in HCC. In the HCC microenvironment, NK cell function is also regulated by immunoregulatory cells and immunosuppressive cytokines. Tumor-infiltrating Tregs can decrease the activity of NK cells by secreting TGF-β[198]. Intratumoral macrophages in HCC can impair NK cell responses via CD48/2B4 interactions[195]. In addition, immunosuppressive cytokines such as TGF-β, IL-6, and IL-10 were implicated in NK cell dysfunction mediated by DC[199, 200].

4.4. Limitations of the Study

Certain limitations arise in the implementation of this study. The incidence of primary HCC in western world is relatively low, and that of non-HBV/non-HCV primary HCC is even lower. As a result, only a few patients met the inclusion criteria. Limited to the quantity of tumor specimens we could obtain from surgery and the cell number required for valid FACS results, a 1:10 ratio of primary HCC cells to TILs is the maximum we could attain, which may not be enough to achieve cytotoxicity. Moreover, the functional state of immune effector cells may be impaired after the freezing, thawing, or separating processes prior to co-culture. Thus, it would be better to use fresh tissue in the future. Finally, the viability of HCC cells and the degranulation of major immune effector cells were measured by FCM, the results of which might be influenced by subjectivity when setting the gate. However, standardized training was conducted before we started the experiment, and we believe that the validity and authenticity of experimental results can be guaranteed.

4.5. Future Plans

Extensive research can be performed using the functional co-culture system.

Supernatant could be collected and stored for further study, and relevant cytokines (such as IFN-γ, TNF-α, and IL-2) can be measured by ELISA or other preferred experimental methods. Functional marker (such as PD1/PD-L1, NKG2D/NKG2DL, and MHC molecules) can be detected by FACS, and activating or inhibitory signals occurring in the crosstalk of tumor cells and immune cells can be analyzed. Moreover, by manipulating co-cultured cells prior to co-cultivation, additional questions can be elaborated. For instance, TILs can be sorted into pure T cells, NK cells, and DC cells to study their properties in more detail. Since the target cells and effector cells were all retrieved from HCC patients, all of the cytokines, functional marker, and signals investigated by the co-culture system authentically reflect the target/effector cell interactions occurring in HCC patients.

5. Conclusion

This dissertation described the establishment of a HCC-TILs co-culture system by establishing a HCC cell line-PBMC co-culture initially, to study the cell-cell interactions between the major immune effector cells and HCC cells. HCC-TILs co-culture system applying primary HCC cells with autologous tumor infiltrating immune cells which mimics the tumor microenvironment of HCC patients, may reflect the interactions between them in a physiologically and clinically relevant way. To determine the activation and cytotoxicity of the major immune effector cells, I measured and analyzed degranulation level of major immune effector cells and viability of HCC cells using flow cytometry. From the results of HCC cell line-PBMC co-culture, we can conclude that HCC cells could activate major immune effector cells into degranulation state, and activated major immune effector cells could in turn kill or inhibit the viability of HCC cells. In the co-culture of primary HCC and TILs, no difference was found regarding cytotoxicity and degranulation between groups. The most important reason may lie in the dysfunction of major immune effector cells resulted from tumor microenvironment in HCC patients. Nevertheless, the co-culture system we established is a powerful tool to explore more deep mechanisms related to the interactions between major immune effector cells and HCC cells, and find more evidence for HCC immune interactions. This warrants a larger study with more patients to further investigate the immune interactions of HCC and TILs.

6. Summary

The immune system plays an essential role in protecting humans from cancer, especially the major immune effector cells such as $CD8^+$ T lymphocytes and NK cells. In recent years, immunotherapy has developed greatly, especially with regard to checkpoint blockade and cellular therapies. As the fourth leading cause of cancer death, hepatocellular carcinoma (HCC) is of public health concern. Surgery remains the most effective method for treating HCC, but immunotherapy has become a hotspot in the management of refractory and recurrent HCC and might therefore serve as adjuvant treatment in the future. However, the interactions between major immune effector cells and HCC cells and related mechanisms remain unclear which is quite essential to the study of immunotherapy for HCC. To date, there are insufficient studies describing the direct cell-cell interactions with an appropriate model in a physiologically and clinically relevant manner. In our research work, a detailed co-culture system was set up with primary HCC cells and autologous tumor infiltrating lymphocytes (TILs) by establishing co-culture of HCC cell line and PBMC initially, to study the direct interactions between major immune effector cells and HCC cells.

First, a systematic review was conducted to assess the interactions between HCC cells and $CD8^+$ T lymphocytes as well as NK cells. It was found that most of these studies use cell line and PBMC as co-cultured cell source. There's no study conducting co-culture with primary HCC cells and TILs to investigate the interactions between HCC cells and major immune effector cells. The co-culture system we established with primary HCC cells and TILs holds the potential to give a more complete picture of the immune response against HCC.

In the co-culture of HepG2 and PBMC with target: effect (T:E) ratio of 1:10, no obvious difference is detected between mono-cultured group and co-cultured group regarding the viability of HCC cells (mono-H-24h vs. co-H-24h: 77.20 ± 16.13% vs. 76.97 ± 13.84%, p=0.997). Compared to mono-cultured group, the degranulation of $CD8^+$ T

lymphocytes and NK cells in co-cultured group increased to some extent but without statistical significance (mono-CTL-24h vs. co-CTL-24h: 1.10 ± 0.95% vs. 7.03 ± 3.47%, p=0.101; mono-NK-24h vs. co-NK-24h: 0.90 ± 0.89% vs. 9.83 ± 6.73%, p=0.259). In the co-culture of HepG2 and PBMC with T:E ratio of 1:25, compared to mono-cultured group, the viability of HCC cells in co-cultured group declines obviously (mono-H-24h vs. co-H-24h: 74.70 ± 10.28% vs. 65.10 ± 10.61%, p=0.020). The degranulation of $CD8^+$ T lymphocytes and NK cells in co-cultured group increased significantly (mono-CTL-24h vs. co-CTL-24h: 3.50 ± 1.50% vs. 19.80 ± 0.44%, p=0.005; mono-NK-24h vs. co-NK-24h: 5.53 ± 6.21% vs. 27.40 ± 3.66%, p=0.042). From the results mentioned above, we can conclude that HCC cells could activate major immune effector cells into degranulation state, and activated major immune effector cells could in turn kill or inhibit the viability of HCC cells. The T: E ratio must be bigger enough to got positive results. In the co-culture of primary HCC cells and TILs, no difference was found regarding cytotoxicity and degranulation (mono-HCC-24h vs. co-HCC-24h: 94.40 ± 5.20% vs. 95.47 ± 0.74%, p=0.930; mono-CTL-24h vs. co-CTL-24h: 10.43 ± 5.73% vs. 12.13 ± 2.99%, p=0.569; mono-NK-24h vs. co-NK-24h: 39.10 ± 28.14% vs. 55.80 ± 14.05%, p=0.934). Several possible reasons maybe involved, but the most obvious reason may lie in the dysfunction of major immune effector cells resulted from tumor microenvironment in HCC patients.

Taken together, a novel direct contact co-culture (DCC) system with primary HCC cells and TILs has been established in this study. The DCC system could study the direct interactions between major immune effector cells and HCC cells in a physiologically and clinically relevant manner. It can be used as a powerful tool to study the mechanisms related to the target/effector cell interactions and provide more evidence for HCC relevant immunotherapy.

7. Zusammenfassung

Das Immunsystem spielt eine relevante Rolle bei der Elimination von Tumorzellen. Immuneffektorzellen wie $CD8^+$ T-Lymphozyten und NK-Zellen haben eine übergeordnete Rolle im Hinblick auf neu entwickelte Immuntherapien wie die Checkpoint-Blockade und zellbasierte Therapien. Das hepatozelluläre Karzinom (HCC) stellt die vierthäufigste Krebstodesursache dar. Die Resektion (entweder vollständig als Transplantation oder als Leberteilresektion) ist nach wie vor die wirksamste Methode zur Behandlung des HCC. Diese wird jedoch in Zukunft möglicherweise mit neuen Immuntherapien als adjuvante Behandlung kombiniert werden.

Bisher ist die Interaktion zwischen den wichtigsten Immuneffektorzellen und den HCC-Zellen sowie die damit verbundenen Mechanismen nach wie vor unklar, was für die Untersuchung der Immuntherapie des HCC von entscheidender Bedeutung ist. Hierfür bietet sich eine Untersuchung der direkten Zell-Zell-Interaktion an. Bisher gibt es nur unzureichende Studien, die die direkte Zell-Zell-Interaktion mit einem geeigneten Modell in physiologisch und klinisch relevanter Weise beschreiben. In dieser Dissertation wurde ein detailliertes Co-Kultivierungs system mit primären HCC-Zellen und autologen tumorinfiltrierenden Lymphozyten (TILs) entwickelt.

Zunächst wurde eine systematische Übersichtsarbeit, um die Interaktion zwischen HCC-Zellen und $CD8^+$ T-Lymphozyten sowie NK-Zellen zu bewerten, erstellt. Es konnte festgestellt werden, dass die meisten dieser Studien Zelllinien und PBMC als Zellquellen verwendeten. Es gibt keine publizierte Studie, die eine Co-Kultur mit primären HCC-Zellen und TILs etabliert hat, um die Interaktion zwischen HCC-Zellen und Immuneffektorzellen zu untersuchen.

In der Co-Kultur von HepG2 und PBMC mit einem Ziel-Wirkungs-Verhältnis (T:E) von 1:10 konnte kein signifikanter Unterschied zwischen der co-kultivierten Gruppe und

der Kontrollgruppe bezüglich der Viabilität der HCC-Zellen festgestellt werden (Mono-H-24h vs. Co-H-24h: 77,20 ± 16,13% vs. 76,97 ± 13,84%, p=0,997). Im Vergleich zur monokultivierten Gruppe nahm die Degranulation von $CD8^+$ T-Lymphozyten und NK-Zellen in der co-kultivierten Gruppe bis zu einem gewissen Grad zu, jedoch ohne statistische Signifikanz (Mono-CTL-24h vs. Co-CTL-24h: 1,10 ± 0,95% vs. 7,03 ± 3,47%, p=0,101; Mono-NK-24h vs. Co-NK-24h; Mono-NK-24h vs. Co-NK-24h: 0,90 ± 0,89% vs. 9,83 ± 6,73%, p=0,259). In der Co-Kultur von HepG2 und PBMC mit einem T:E-Verhältnis von 1:25 nahm die Viabilität der HCC-Zellen in der co-kultivierten Gruppe im Vergleich zur Kontrollgruppe signifikant ab (Mono-H-24h vs. Co-H-24h: 74,70 ± 10,28% vs. 65,10 ± 10,61%, p=0,020), und die Degranulation von $CD8^+$ T-Lymphozyten und NK-Zellen in der co-kultivierten Gruppe nahm signifikant zu (Mono-CTL-24h vs. Co-CTL-24h: 3,50 ± 1,50% vs. 19,80 ± 0,44%, p=0,005; Mono-NK-24h vs. Co-NK-24h: 5,53 ± 6,21% vs. 27,40 ± 3,66%, p=0,042). Aus den oben erwähnten Ergebnissen können wir schlussfolgern, dass HCC-Zellen Immuneffektorzellen in den Degranulationszustand aktivieren können. Aktivierte Immuneffektorzellen können ihrerseits HCC-Zellen abtöten oder hemmen. In der Co-Kultur von primären HCC-Zellen und TILs wurden weder Zytotoxizität noch Degranulation gefunden (Mono-HCC-24h vs. Co-HCC-24h: 94,40 ± 5,20% vs. 95,47 ± 0,74%, p=0,930; Mono-CTL-24h vs. Co-CTL-24h: 10,43 ± 5,73% vs. 12,13 ± 2,99%, p=0,569; Mono-NK-24h vs. Co-NK-24h; Mono-NK-24h vs. Co-NK-24h: 39,10 ± 28,14% vs. 55,80 ± 14,05%, p=0,934). Einer der möglichen Gründe könnte eine durch das sogenannten Microenvironmnent des Tumors bei HCC-Patienten hervorgerufene Funktionsstörung der Immuneffektorzellensein.

Zusammenfassend wurde in dieser Studie ein neuartiges direct contact co-culture (DCC) system mit primären HCC-Zellen und TILs etabliert. Das DCC-System kann die direkte Interaktion zwischen den Immuneffektorzellen und den HCC-Zellen auf physiologisch und klinisch relevante Weise untersuchen. Es kann als leistungsstarkes Instrument zur Untersuchung der Mechanismen im Zusammenhang mit der Ziel-/Effektorzell-Interaktion eingesetzt werden und mehr Informationen für eine

HCC-relevante Immuntherapie liefern.

III. Reference

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